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Characterization of Multiple
Conductance States in a
Nicotinic Acetylcholine
Receptor/Channel Preparation

by

Madeleine R. Montpetit

A thesis presented to the University of Ottawa in
partial fulfillment of the requirements for the
degree of Masters of Science in the
Department of Biology

Ottawa, Ontario, 1986

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ABSTRACT

The acetylcholine-activated channel of vertebrate skeletal muscle, as manifested in cultured developing cells, is able to adopt more than one conductance state. This thesis reviews the evidence for such multiple conductance channels and presents single channel data suggesting that subconductances represent discrete, allosterically-activated channel conformations. Since the amplitude of subconductance openings does not depend on agonist size or valence, the possibility that subconductances occur during partial occlusion of the channel by agonist molecules was ruled out. The conductances found for each event type are as follows; M Type = 40pS, S1 = 13pS and S2 = 7pS. While agonists do not determine conductance of the AChR, different agonists have differential abilities at stabilizing the sublevel openings. The kinetic analysis of open time histograms for the various conductance states reveals that the full channel openings are made up of two distinct kinetic open states; one of which is agonist-dependent and the other agonist-independent. In general, the subconductances S1 and S2 both displayed openings with a single agonist-independent time constant. Bursting behavior in the G8 AChRs was observed when channels were activated by strong agonists.

RÉSUMÉ

Plusieurs études sur les canaux activés par l'acétylcholine de certaines cellules squelettiques musculaires, en voie de développement, ont démontrées que ces pores ioniques pouvaient adopter plus d'un état de conductance. Dans cette thèse, nous avons démontré que ces multiples états de conductance représentent des conformations discrètes des canaux activés allostériquement par des molécules d'agonistes. Les complexes récepteurs-canaux adoptaient dans nos cellules G8 des états de conductance de 40pS, 13pS, et 7pS. Parce que l'amplitude des courants traversant les canaux ouverts en état de sous-conductance ne dépendait pas de la dimension et/ou de la valence des molécules d'agoniste, nous pouvons exclure la possibilité que les sous-conductances se produisent comme résultat de l'occlusion partielle du canal par une molécule d'agoniste. L'analyse cinétique des périodes d'activation des canaux a démontré qu'il existe deux états cinétiques distincts pour les canaux ouverts à conductance maximale (M); un de ceux-ci variait avec l'agoniste d'activation et l'autre en était indépendant. En général, les ouvertures de canaux en état de sous-conductance (S1 et S2) démontraient seulement un état cinétique chacun qui était indépendant de l'agoniste d'activation. Des périodes d'oscillation rapides entre les configurations ouvertes (M, S1, et S2) et les configurations fermées se produisaient surtout lors de l'activation par des agonistes efficaces.

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INTRODUCTION

Ionic channels are macromolecular pores found in all types of cell membranes. They warrant electrophysiological investigation because of their role as fundamental excitable elements in the membranes of excitable cells.

It has been said that these channels bear the same relation to electrical signaling in nerve, muscle and synapse as enzymes bear to metabolism (Hille, 1984). While the diversity of channels is less broad than that of enzymes, there are nevertheless various types of these pores working in concert, opening and closing in complex patterns which determine overall signaling in the neuromuscular system.

Two major channel categories can be distinguished on the basis of the stimuli which cause them to open. A large group of channel types (i.e. Na^+ , K^+ , Cl^- , Ca^{++} channels, Ca^{++} -activated K^+ channels) are electrically excitable, having voltage-gated pores, which open or close at rates responsive to membrane potential. A second group of channels (i.e. ACh, glutamate, GABA etc.) specialized for mediating chemical synaptic transmission or transducing sensory stimuli, respond to non-electrical stimuli in the form of chemical transmitters. As in the case of electrically excitable channels, the electrically inexcitable channels also gate ion movement and generate electrical signals. While the voltage-dependent varieties of channels are usually widely distributed over the surface membranes of excitable

cells, the electrically inexcitable channels are generally restricted to specialized areas such as nerve terminals and postsynaptic membranes where they produce graded potential changes and may trigger or suppress firing of action potentials (Hille, 1984).

The best studied non-electrically stimulated channel is the nicotinic acetylcholine (ACh)-activated channel found in vertebrate neuromuscular junction. This channel is ligand-gated so that the binding of an agonist is needed to open the ionic pore. Messages are often propagated from one excitable cell to another via extracellular messengers. When the message comes from a nerve terminal and is to be delivered to an adjacent cell, the messenger is referred to as a neurotransmitter; the neurotransmitter, acetylcholine, is the natural agonist at the nicotinic synapses. The ACh-activated channels of the neuromuscular junction are classified as nicotinic because the alkaloid nicotine imitates the effect of ACh on these channels (Langley, 1907). Related ACh-activated channels are found in the membranes of embryonic myotubes, denervated muscle cells and fish electric organs. While all the subtypes of the ACh receptor (AChR) behave similarly, several pharmacological and electrophysiological differences separate them into distinct categories. For example, the AChRs of developing embryological muscle display open state conductances inferior to and mean open times longer than that shown by adult end-plate nicotinic cholinergic channels (Leonard et. al, 1984).

There exists another class of ACh-activated receptors and channels which are not activated by nicotine but are gated by the alkaloid muscarine; they are appropriately named muscarinic AChRs. These ligand-gated pores are dramatically different in their pharmacological and electrophysiological behavior, from those found at the neuromuscular junction. Further characterization of the muscarinic AChR may be found in the following reviews; Gerschenfeld, (1973); Kehoe and Marty, (1980); Hartzell, (1984). The research to be presented here is limited to the nicotinic cholinergic receptor channel complex of a clonal line of myotubes.

Nicotinic Acetylcholine-activated receptor/channel complex

AChR/channel: function.

The role of the AChR/channel within the neuromuscular junction is to depolarize the postsynaptic membrane of the muscle when the presynaptic nerve terminal releases its neurotransmitter, acetylcholine. If two molecules of ACh manage to diffuse across the synaptic cleft and bind to the ACh-binding sites of the AChR/channel complex, the channel opens into a wide pore, permeable to several cations (Adams, 1975; Sine and Taylor, 1980). The current associated with the movement of these ions through the pore initiates a depolarization of the membrane which if it exceeds threshold may stimulate the activation of voltage dependent ionic channels (i.e., Na^+ , K^+) thereby resulting in an action potential within the postsynaptic muscle cell. Acetylcholine analogues are also able to activate the opening of the AChR/channel complex. In general, the potency of these compounds depends on their affinity for the ACh binding sites of the receptor and their ability to destabilize the closed state of the channel.

AChR/channel: structure

The nicotinic cholinergic receptor channel complex is one of the most-studied macromolecular proteins in biology. Information on structure and function of this ionic pore has been

gleaned from research in many disciplines (i.e. protein biochemistry, pharmacology, molecular genetics and electrophysiology).

A major stumbling block to the study of composition of any protein is the difficulty of obtaining it in large quantities. This obstacle, however was overcome with the discovery of very rich sources of AChR molecules; namely, the electric organ plasma membranes of certain teleosts and elasmobranchs. These electric organs are muscle cell derivatives composed of stacks of hundreds of cells in series, each generating a pulse of current through a vast array of AChR channels in response to cholinergic stimulation from presynaptic axons. Though some characteristics of the nicotinic AChR may be tissue- or species-specific, studies on the electric organ membrane AChRs provide a basis with which to compare the cholinergic receptors of less amenable systems. Recent advancements in protein biochemistry have allowed the AChR/channel complexes to be solubilized from their constituent membranes, isolated, purified and characterized structurally all without the loss of functional integrity (as shown electrophysiologically). Thorough reviews of the isolation techniques and structural characterization of the nicotinic AChR/channel are available (Conti-Tronconi and Raftery, 1982; Karlin, 1980; Changeux, 1981; Anholt et al., 1984). Refinement of receptor membrane reconstitution techniques have led to the discovery that the AChR/channel complex is a self-contained

entity since all those elements essential to its functioning are present upon isolation of the macromolecule. Experiments with purified receptors in reconstituted vesicles exhibit the two salient features of the postsynaptic membrane, namely cholinergic analogue-induced activation and desensitization (Suarez-Isla, 1983; Labarca et al., 1983; Hanke and Breer, 1986).

AChR/channel protein; primary structure.

In the past few years, studies with purified protein and reconstituted AChR vesicles have led to elucidation of the peptide composition of the ionic channels. The AChR/channel protein is constructed of 5 peptide subunits. Gel electrophoresis indicates that there are four different glycoproteins; α (39 KD), β (48 KD), γ (58 KD), δ (64 KD) (Weill et al., 1974) which exist in a pentameric stoichiometry ($\alpha_2, \beta, \gamma, \delta$) in the AChR/channel complex (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980; Brisson and Unwin, 1985). The total molecular mass of the complex is 268 kilodaltons, the majority of which is comprised of 2,380 amino acids (Noda et al., 1983a). Four to seven percent of the native receptor/channel complex is glycosylated; oligosaccharide chains are attached to the extracellular side of each of the peptide subunits (Lindstrom et al., 1979). Several sites of phosphorylation have also been observed in this complex (Huganir and Greengard, 1983; Huganir, 1986).

The AChR/channel is a transmembrane protein in which each of the α , β , γ , δ subunits contributes hydrophilic amino acid residues on both extracellular and cytoplasmic sides of the membrane, and hydrophobic residues within the lipid bilayer (Noda et al, 1983a). The amino acid sequences forming the primary structure of the four different peptide subunits have been obtained using techniques combining protein biochemistry and molecular genetics. Studies to elucidate the channels' amino acid composition involved sequencing of the mRNAs for the entire AChR/channel, forming cDNA transcripts to these, and developing subunit clones by insertion of cDNA into plasmids. The four different subunit chains show 40% homology in their amino acid sequences (Noda et al., 1983a). Similar sequencing of AChR subunits from elasmobranchs, teleosts and mammals as well as studies with immunological probes for specific immunogenic regions have shown extensive sequence homology in the animal kingdom. Evidently, the four different subunit peptides are coded for by related genes that arose early by gene duplication from a single ancestral gene (Raftery et al, 1980; Noda et al., 1983b; Gullick and Lindstrom, 1982; Ballivet et al., 1983; Lunt, 1986).

For a number of years, there has been discussion as to whether functional differences seen between embryonic and adult forms of muscle AChR resulted from developmental modification (eg. phosphorylation) of pre-existing fetal type receptors or

whether differences resulted from synthesis and insertion into the membrane of new adult-type molecules. Using nucleotide sequencing techniques, Japanese researchers (Mishina et al., 1986) have very recently discovered differences in the peptide subunits between fetal and adult AChRs in bovine muscle. The adult bovine AChR contains, in place of the fetal γ subunit, an ϵ subunit which has only a 53% homology with the γ subunit and even less with α , β , δ subunits. Mishina et al., (1986) compared the developmental changes in the mRNA encoding for γ and ϵ with the developmental shift in conductance and gating properties of the AChR as the predominant receptor type switched from fetal to adult. They suggest that replacement of the γ subunit by the ϵ subunit is responsible for the developmental changes in conductance and kinetics of the AChR. Embryological myotubes, denervated muscle fibers and tissue cultured muscle cells, all of which lack normal motor neuron innervation, display similar conductance and gating behavior which differ from that of AChRs at adult neuromuscular junctions (Brehm et al., 1984; Leonard et al., 1984; Mishina et al., 1986). It has been suggested that unknown signals from the motor nerve (Siegel, 1983; Brenner et al., 1983) switch on expression of the gene encoding ϵ subunit while stopping expression of the gene encoding the γ subunit (Colquhoun, 1986). Cells in which adult and embryological receptors are present concurrently are referred to frequently in the AChR literature (eg. Siegelbaum et al., 1984). The simultaneous occurrence of these two populations could represent a

period in the cell's development where the production of one receptor type is waning and the other is being turned on.

AChR/channel protein; tertiary and quaternary structure

The tertiary and quaternary structures of the AChR are directly responsible for the general shape of the receptor/channel complex. The basic outline of this transmembranous pore has been determined by electron microscopy and low-angle diffraction of membranes with purified AChR complexes (Kistler et al., 1983; Kistler and Stroud, 1982). Membranes treated with negative stains when viewed en face displayed rosettes of AChR with diameters of $85\text{\AA}$, and $20\text{\AA}$ stain-filled central pits. The overall AChR/channel complex length is $110\text{\AA}$ with $20\text{\AA}$ extending out on the cytoplasmic side and a $55\text{\AA}$ protrusion on the extracellular face of the membrane (Ross et al, 1977). This complex has been likened to a lopsided dumbbell with the transmembrane bar resembling a funnel, widest at the synaptic side ($20\text{\AA}$) and narrowing near the cytoplasmic side (approx. $6.5\text{\AA}$) (Klymkowsky and Stroud, 1979). At its narrowest point, the diameter of the channel's aqueous pore has been calculated to between $6.2\text{-}7.2\text{\AA}$ depending on the methods of investigation used (Kistler et al., 1972; Maeno et al, 1977).

As mentioned previously, the arrangement of the polar and apolar amino acids in the primary structure gives rise to distinct hydrophilic and hydrophobic regions in the AChR which stabilize it as a transmembrane protein. Hydrophobic regions of

all five AChR subunits, contain four extended runs each of non-polar amino acid residues (Noda et al. 1983b) which are arranged into 20 helices perpendicular to the membrane and which define the channel pore. For the AChR channel to be cation permeable, the pore formed by these hydrophobic helices must be modified to permit ion conduction. Recently a fifth helix which displays an amphipathic nature, has been defined for each AChR subunit; one side of these helices is hydrophobic and thereby can interact with the lipid bilayer and the other side is hydrophilic and forms the cation-selective pore (Finer-Stroud, 1984; Guy, 1984; Stevens, 1985). This amphipathic region of amino acids interacts with the other helices of the subunits by twisting into a helix between the others. One would have anticipated that a cation-selective channel might have a negatively charged lining to make it hydrophilic but the positive and negative side chains of the amphipathic helices produce a pore with bands of positive and negative charge. Most workers would have thought that a cation-selective pore would not have positive charges; these findings, therefore, were a surprise (Stevens, 1985; Bash et al., 1985) and will require further explanation.

From studies on the higher orders of ACh structure it has been determined that there exist two ACh binding sites on the extracellular surface of the ionic channel which modulate its activation. Binding studies with neurotoxins and immunological probes both specific for the ACh-activating site indicate that one binding site is found on each of the α -subunits of the

receptor (Reitler et al., 1972; Klymkowsky and Stroud, 1979). Channel openings are more probable when both sites are occupied by agonist but can still occur, albeit rarely, when one or none of the binding sites is occupied (Jackson, 1984; 1986).

Evidence has recently been provided to show that changes in certain critical areas of the primary amino acid sequences of the AChR can alter the channel's functional behavior substantially. Site-directed mutagenesis has permitted researchers to alter the primary sequences of a given AChR subunit, then monitor the effect of these changes at the electrophysiological level (Mishina, et al., 1985). Such studies have provided information on the exact location of the ACh binding sites on the α -subunits and have implicated the δ -subunit in the rate of closing of an open channel (Sakmann et al., 1985; Hall, 1986).

The pentameric structure of the AChR/channel complex is apparently not fully symmetrical. An asymmetry in the reactivity of the two binding sites on the α -subunits has been described on the basis of binding of curariform agents (Sine and Taylor, 1980). The asymmetry does not, however, extend to binding of agonists, like ACh, for which dissociation constants on both sites are identical (Dionne, 1985). While ACh and most competitive inhibitors act primarily at the ACh binding site, there exists a diverse collection of non-competitive inhibitors of the AChR/channel complex (including some local anesthetics, aromatic tertiary amines, detergents, etc) which act elsewhere on the

receptor. The most salient features shared by these agents are their amphipathicity and non-specific behavior towards any channel types (i.e. not just AChR channel specific). The effects of some of these substances on the kinetics of end-plate and single channel currents can sometimes lead to information about other binding sites on the AChR macromolecule. Some noncompetitive inhibitors appear to bind to regions within the open channel pore thereby displaying their inhibitory behavior or antagonism by blocking further flow of current through the channel (Neher and Steinbach, 1978). Some antagonists may bind to a region exposed when the receptor is in the closed state and decrease the opening rate of the channels (Adams, 1977). Finally some non-competitive inhibitors by binding may promote the transition of the receptors to the high affinity desensitized state (Sine and Taylor, 1982). Despite the complexity of their actions, non-competitive inhibitors provide useful probes for functionally important sites other than the ACh binding site.

AChR/channel: Ion selectivity and conductance

AChR selectivity

Ionic channels are highly permeable to some but not all ions. Thus Na^+ channels show high permeability to Na^+ ions while showing little permeability to K^+ . The converse is true for K^+ channels. Neither type of channel is permeable to anions. Without some selectivity, ionic channels would not be

able to make use of the electromotive force of ion gradients, which are critical for electrical signaling. Charge separations and concentration gradients of various ions are responsible for the transmembrane potential difference; the driving force on any ion is a combination then, of the chemical and electrical potential differences across the cell membrane. For an ionic channel which is perfectly selective for a single ion, current flow will follow channel opening when the chemical driving force in one direction is not offset by the electrical force in the other direction. When these forces balance out, there is no net flow (i.e. no current) though the channel is open and ions are moving back and forth through it. The potential at which this equilibrium is achieved is the so-called Nernst potential and is defined as follows;

R=gas constant

$$E_x = \frac{RT}{Fz} \ln \frac{[X]_I}{[X]_{II}} \quad T=\text{absolute temperature}$$

$$F=\text{Faraday constant}$$

z=valence of permeant ion

However, many channels, such as the AChR/channel, are permeable to more than one ionic species. The potential at which no net current flows through such a channel, is determined by the concentration gradients of all the permeant ions making up the current and by their relative permeabilities. In this case, there is no equilibrium potential, but the zero-current potential can be defined by the Goldman-Hodgkin-Katz (GHK) equation (Hodgkin, 1951).

$$V_{rev} = \frac{RT}{F} \ln \frac{P_X[X]_I + P_Y[Y]_I + P_Z[Z]_I + \dots}{P_X[X]_{II} + P_Y[Y]_{II} + P_Z[Z]_{II} + \dots}$$

The concentration gradients are usually expressed as the extracellular ion concentration over the cytoplasmic ion concentration if the permeant ion is a cation. The gradient of permeant anions, however, is inversed. The zero-current or reversal potential (V_{rev}) of the channel, thus, differs fundamentally from a Nernst potential in that it is a steady-state voltage where the relative permeabilities and magnitude of the ion gradients are important.

At voltages other than the reversal potential, a net current will flow through the channel in one direction or the other depending on which force (chemical or electrical) is dominating.

When ion concentrations are known, the GHK equation tells us that the reversal potential can be used to characterize the selectivity of an ionic channel. With the salines normally used for electrophysiological recordings (i.e. approximately equal and opposite Na^+ and K^+ gradients) the AChR/channel has been shown to have a reversal potential of 0mV. Studies of reversal potentials under various ionic conditions indicate that Na^+ , K^+ , Ca^{++} , Mg^{++} are the major permeant ions through the AChR channels in normal salines (Takeuchi and Takeuchi, 1960; Ritchie and Fambrough, 1975; Lewis, 1979). The permeability ratio of these ions relative to sodium follows (Huang et al., 1978):

$$P_{K^+}(1.47): P_{Na^+}(1.0): P_{Ca^{++}}(0.22) \cong P_{Mg^{++}}$$

While Na^+ and K^+ make up the bulk of the ionic current through an open ACh-activated channel, other ionic species may also pass through. Naeno et al., (1977) showed that a variety of inorganic and organic cations can cross the channel. Huang et al., (1978), demonstrated with measurements of initial influx rates for radioactive compounds, that the ACh-activated channels transport alkali cations of small hydration radii and high mobility; molecules with positive charge and/or hydrogen-bond donating moieties are more permeable than ones without (Lewis and Stevens, 1983). Finally, several nonelectrolytes (i.e. glycol, urea) do have small but measurable permeabilities through the channels. The channel selectivity, as measured by reversal potential is independent of the agonist used to activate the receptor and of temperature (Stevens and Steinbach, 1976; Hoffman and Dionne, 1983).

AChR conductance

The notion of permeability is closely related to that of conductance. Conductance, the inverse of resistance, is defined as a measure of the ease with which a conductor carries an electric current. Although an individual channel is a much better conductor of current flow than an equivalent volume of lipid bilayer, some channels are relatively poor conductors compared with an equivalent volume of a free solution of ions. The narrowness and charge structure of ionic pores restrict

movement of ions. The random collision of ions with the walls of the channel cause them to lose energy; the greater the frequency of collisions and the stronger the electrostatic interactions between ion and channel, the greater the resistance to ion movement and the lower the channel conductance.

The conductance of a specific channel can easily be determined by applying Ohm's Law to single channel I/V data obtained with the patch-clamp technique (Neher and Sakmann, 1976)

$$I = g \times V_m$$

For the AChR channels, measurement of the current amplitude (I) of the elementary channel openings can be plotted against driving force (V_m) in an I/V graph and the slope of the resulting line gives the value of the channel conductance (the constant 'g' in Ohm's law). Single channel data has shown that the unit conductance of the AChR/channel (20-50pS) is several times larger than that of the more selective channels responsible for action potentials (review; Colquhoun, 1979). The conductance of the ACh-activated channel has been shown to increase with temperature (Sakmann, 1978; Hoffman and Dionne, 1983) with Q_{10} values in the range 1.3-1.6. These low Q_{10} values support the view that the AChR channel is essentially a water-filled pore (Lewis and Stevens, 1983) with the movement of ions through it being a diffusion-limited process. The conductance of the AChR channel also varies with the ionic composition of the environment (Horn and Patlak, 1986) in accordance with the selectivity of the channel. Under physiological saline conditions, an average of

over 10^7 cations per second cross through the open AChR channel (Hess et al., 1983). Having alluded to variability in conductance measurements stemming from the physical parameters of the experiment, I will now turn to the more interesting variability in conductance values derived from biologically-induced differences in the current flow through the AChR/channel.

The term acetylcholine-activated receptor-channel complex encompasses many versions of the nicotinic channels. The adult neuromuscular form of the AChR shows two distinct subtypes; junctional and extrajunctional channels (Dreyer et al., 1976). Though these two types are activated by ACh and its analogues and share similar selectivities, they differ in their membrane density, toxin binding kinetics, immunological reactivity, gating kinetics and conductances (Brehm et al., 1983; Hall et al., 1983; Moody-Corbett et al., 1983). The mean open time of the junctional receptors is shorter while their conductance values are usually greater than that of extrajunctional receptors (Fischbach and Schuetze, 1980; Brehm et al., 1984; Colquhoun and Sakmann, 1985). These two basic conductance states, seen in adult muscle preparations, have been observed in the AChRs of developing muscle; embryonic cultured amphibian, avian and mammalian muscle cells all demonstrate two discrete AChR populations distinguished by their unitary current amplitudes and kinetics (Hamill and Sakmann, 1981; Auerbach and Sachs, 1984; Morris et al. 1983; Leonard et al., 1984; Siegelbaum et al., 1984). For example, Brehm et al. (1984) report 64pS and 46pS

events for Xenopus with the lower amplitude events having a mean open time (~ 2 ms at -85 mV) two to three times longer than the higher amplitude events. Similarly, ACh-activated channels in rat muscle have been shown to have opening populations of 54pS (2.1ms open time) and 35pS (8.5ms open time at -70 mV) (Siegelbaum et al., 1984). In almost all embryonic preparations, the proportion of the large fast channels, which share similarities with the mature neuromuscular junction channels, increases with the days in culture. It had been thought that the distinction in channel types may simply be a manifestation of different developmental states of the receptor/channel complex (Brehm et al., 1982, 1984; Kullberg et al., 1981). However, with the recent discovery of differences in the subunit composition between fetal and adult AChR/channel complexes (Mishina et al., 1985), it would appear that the small conductance slow channels, similar to the extrajunctional channels in adult are not precursors of the adult-junctional like large fast channels but represent separate populations of molecules.

AChR subconductances

The second way in which AChRs manifest an ability to adopt different conductance levels does not involve different populations. The conductance profiles of some individual channels show discrete openings to more than one current level. This was first reported by Hamill and Sakmann (1981). Recording at low temperature ($5-8^{\circ}\text{C}$), they observed that the AChRs of tissue-cultured rat

muscle could assume three (and possibly more), distinct long-lived conducting states. In this work, they demonstrated the presence of subconductance openings which were a fraction of the current flow of the more classical AChR openings.

Transitions to sublevels were not absent at room temperature but were too short-lived to be adequately resolved. In normal saline with cell-free configuration, these ACh activated states had conductances of 10, 25 and 35pS; rarely, 18pS events were observed. The 35pS openings accounted for 36% of all openings while the 25pS events made up 63% of the total current jumps. Rarely, the 10pS openings occurred in isolation; usually they followed as a transition from one of the larger conductance states (mostly 25pS events). The 25pS and 35pS events were assumed to arise from distinct channel types since their probability of overlap could be explained by assuming independence, however, the 10pS events were clearly due to a channel substate. The ionic selectivity of each state and channel type, as determined by reversal potentials were indistinguishable ($P_{Cs} > P_{Na} > P_{Li}$ for all levels).

Hamill and Sakmann postulated that the ability of the AChRs to adopt subconductance states indicates a rearrangement of the five subunits forming the channel. If this is correct it would seem that these conformational degrees of freedom are lost as the AChR matures (Morris and Montpetit, 1986). Several single channel studies in the synaptic region of adult muscle cells fail to exhibit subconductances. For example, no sublevel

openings were reported in studies on the perisynaptic AChR of frog cutaneous pectoris muscle (Colquhoun and Sakmann, 1981) and the junctional AChRs of garter snake costocutaneous muscle (Dionne and Leibowitz, 1982). However, recently Colquhoun and Sakmann (1985) observed very short-lived subconductance states in gaps within bursts of acetylcholine, suberyldicholine and carbachol activated junctional nicotinic receptors of adult frog muscle fibers. Improvements in the bandwidth of the system used by Colquhoun and Sakmann permitted resolution of the multiple conductance levels in the adult endplate preparation. It is possible that previous studies of adult neuromuscular junction tissue failed to demonstrate the subconductance openings due to the brevity of these channel events (which may be due to development) and limited recording bandwidth.

Variability of occurrence of subconductance levels is not just apparent between tissue preparations, it also manifests itself in the same tissue. Trautmann (1982) patch-clamped rat myotubes, the same tissue used by Hamill and Sakmann. He did not, however, pretreat the cells with colchicine as they had done. Trautmann, like Hamill and Sakmann, found that at room temperature, subconductance openings with ACh were not very evident but found that curare could activate sublevel current openings (50pS, 35pS, 12pS) with great frequency. Morris et al., (1983), again studying cultured rat myotubes observed curare-induced AChR activation, but there were no resolvable subconductance events in their data. It is very difficult to assess at

this stage whether the occurrence of subconductance events is a general property of the channel or whether it is essentially an abnormality induced by some aspect of the tissue culture regime or the recording technique.

Regardless of whether subconductances are a normal phenomenon of AChRs, their occurrence is not limited to mammalian preparations. Multiple conducting states have also been demonstrated in chick muscle cultures (Auerbach and Sachs, 1982; 1984). Openings of 50pS, 35pS and subconductances of approximately 12% of the main 35pS openings were observed. These AChRs normally display a tendency towards agonist-induced bursting (Colquhoun and Sakmann, 1981; Siegelbaum et al., 1984). Auerbach and Sachs (1984) were able to show that many of the flickers in bursts were not transitions to zero-conductance states but in fact jumps to a state having a conductance of approximately 12% of the main level. This represents a current flow substantially lower than those seen in Hamill and Sakmann's subconductances (1981) which were 25% of full openings. However, Auerbach and Sachs (1983) note that the presence of dimethyl sulphoxide (DMSO) in the growth medium modulated subconductance properties: without DMSO in the bath, substates showed conductances of 33% the amplitude of full openings.

In myotubes of the amphibian Xenopus, Brehm et al., (1984) also observed ACh-activated subconductances, but apparently at very low frequency. Multiple conductance states of the AChR have also been shown in the clonal muscle cell lines L6

(Sachs, 1983) and BC3H-1 (Chabala et al., 1982). Subconductances have been observed (albeit at very low incidence) in AChRs reconstituted from the electric organ of the fish Torpedo (Tank et al., 1983) and from insect neuronal acetylcholine receptor protein reconstituted in planar lipid bilayers (Hanke and Breer; 1986). The rare sublevel events observed in reconstituted AChRs may represent the activity of a fraction of AChRs which are not fully mature. Whatever their provenance, they indicate that the ability of an AChR to assume sublevels is inherent in the protein itself; the occurrence of subconductance openings is not dependent on any cytoskeletal attachments or special lipid environment of the native AChR/channel complex (Morris and Montpetit, 1986). Conversely, the fact that most AChRs isolated do not exhibit subconductance activity deserves emphasis since it indicates that experimental insult imposed on the channel protein during isolation, purification and reconstitution techniques does not promote the occurrence of substates. If, as suggested by Hamill and Sakmann (1981), developing AChRs are in some way loose in the pentameric structure, the process responsible for tightening them is not easily reversed.

Multiple conductance states are not restricted to the acetylcholine receptor channel complex. Subconductance states have been shown in single chloride channels in molluscan neurons (Geletyuk and Kazachenko, 1985), in calcium-activated potassium channels in rat myotubes (Barrett et al., 1982), serotonin-modulated potassium channels in Aplysia neurons (Siegelbaum et

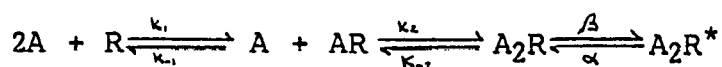
al., 1982), rabbit cation-selective sarcoplasmic reticulum channels (Fox, 1985) and an anion selective channel from rat pulmonary alveolar cells (Krause et al., 1986). Evidently, the ability of ion channels to assume different conductance states is a characteristic common to most channels. Models of the mechanisms of subconductance openings (eg. Takeda and Trautmann, 1984) have either assumed that the subconductance state constitutes a discrete channel conformation of the receptor or that it results from the partial block by an agonist or ion of the fully opened channel. Further investigations are needed to resolve this question; in this thesis one of the questions I address is whether AChR subconductances are more likely to represent discrete allosteric conformations of the activated channel or fully-open states partially blocked by agonist molecules.

AChR/channel: activation

The opening of the AChR/channel complex has been studied using a gamut of experimental techniques, all in attempt to characterize those steps involved with activation and conformational change of the receptor.

As mentioned previously, two molecules of an agonist normally bind to the AChR activation sites of the α -subunits to cause the opening of the AChR/channel complex (Adams, 1975;

Dionne et al., 1978). The classical model describing this activation first proposed by Magleby and Stevens, (1972), follows;

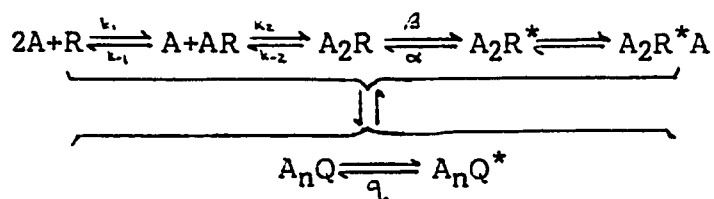


A = agonist R = closed receptor R* = open receptor

This scheme suggests that the receptor R, experiences sequential binding of two agonist molecules to the binding site. The binding of the two molecules does not in itself cause the conformational change within the channel to the open state. It does, however tend to destabilize the closed state and favor the open state. The reverse appears true for inhibitors of channel opening; for example α -neurotoxins stabilize the closed state when bound. Several modifications to this model are necessary for it to predict and explain recently acquired activation data. The Magleby-Stevens model assumes that the channel has several closed states but only one open state. Recent studies on various experimental preparations have shown deviations from this assumption. Open time histograms of single channel data have in the past been fit by a single exponential function whose rate constant when inversed is taken to represent the mean open time of the channels. Recently, several researchers have found that the open time distributions of their data were best fit by the sum of two exponential functions (Sine and Steinbach, 1986; Sakmann et al., 1984). They have assumed therefore that their

data represents a minimum of two types of openings, or open states, each with characteristic mean open times. While these open states may share the same conductance profile, they can be distinguished by their kinetic behavior; it is of course, only possible to distinguish two open states of the same conductance if one state is more long-lived than the other.

It was originally thought that the fast openings were in fact short-lived openings of the mono-liganded receptor and the slower events reflected conformational changes in a doubly-liganded receptor. However, Sine and Steinbach (1984) demonstrated that the fast openings were not concentration dependent; in conditions of high agonist concentration where one could expect most receptors to be doubly-liganded, the proportion of fast to slow openings was similar to that found in low agonist concentration challenges. One can therefore assume that two distinct kinetic bi-liganded open states are commonly found in the AChR/channel complex repertoire of some tissues. Sine and Steinbach (1984;1986) proposed a modified kinetic model that accounts for the fast and slow open times observed in their and others data (Colquhoun and Sakmann,1981; Morris et al.,1983; Gardner and Barnard,1985).



A_2R^* in this model represents the longer-lived openings whereas A_nQ^* reflects the short events. For this model to fit the data appropriately, one must assume that q , the closing rate for A_nQ^* state is much larger than that of α , the closing rate for A_2R^* . Brief openings in this particular model are thought to arise from a separate closed state of the receptor, in relatively rapid equilibrium with the normal activatable state which gives rise to long openings. It is not known how the A_nQ or A_nQ^* states arise from the R state, hence the open brackets. It is possible that brief openings could arise from several closed states of the receptor. For example, at low agonist concentration, the brief openings may represent the occurrence of AR^* (a mono-liganded opening) whereas at high agonist concentrations, they could reflect the opening of channels associated with a desensitized state of the receptor. However, because brief openings appear kinetically identical at low and high agonist concentrations, Sine and Steinbach simplified their model by assuming that all brief openings result from a single state of the receptor.

This model as well as others can also explain the phenomenon of bursting as seen in the AChR activation of some preparations with a variety of agonists (Nelson and Sachs, 1979; Auerbach and Sachs, 1984). Colquhoun and Sakmann (1981) demonstrated that the strong agonist, suberyldicholine, at low concentrations, activated channel openings in frog skeletal muscle endplate that were interrupted by very brief channel closings. Such closings or gaps may be so brief that they go unnoticed in single channel

data when bandwidth limitations do not permit them to be fully resolved. This open-shut oscillation, which they termed Nachschlge, may persist for several milliseconds before the channel assumes a long-lived closed state. Flickering has also been shown in numerous other AChR/channel preparations with a variety of agonists (Dionne and Leibowitz, 1982; Colquhoun and Sakmann, 1983, 1985; Jackson et al., 1983; Sine and Steinbach, 1984, 1986) as well as in other agonist-gated channel types (glutamate-activated channels in locust muscle fibers, Cull-Candy et al., 1981; Ca^{++} -g(K^+) channels, Barrett, et al., 1982)

As would be expected for channels which show flickering, multiple components are found in the closed time histograms for all these channels, indicating the existence of a number of different kinetic zero-conductance states. The very brief components of these distributions almost certainly represent the short-lived closings seen in the burst. Nachschlgen are seen with many strong agonists, however the mean number of closings per burst and the time constant of the closed time attributed to intraburst closings are agonist dependent. The longer components of the closed-time histograms (the interburst interval) can be attributed to AChRs in a partially or fully liganded state. In the Sine and Steinbach model, Nachschlge may be represented by brief transitions between the closed A_2R state and the long-lived A_2R^* open state (Colquhoun and Sakmann, 1983). In cases of bursting behavior, one must assume that channel opening rate β must be very fast compared to the closing rate α and

β should also be comparable to k_{-2} . The liganded channel is then able to open rather quickly before the agonist can dissociate from the receptor. Once the channel is open, it can switch back and forth between states A_2R and A_2R^* several times. The channel then enters the long-closed state upon dissociation of the agonist.

Finally, a third phenomenon that should appear in activation models is the block of the open channel following activation. This step has been added to Sine and Steinbach's model as A_2R^*A . Blocking appears to occur when a molecule or ion binds to a site inside the channel and prevents permeant ions from passing through. A number of compounds can interact with the receptor to produce a blocked state, for example, local anesthetics (Adams, 1981), curare (Shaker et al., 1982; Trautmann, 1982), agonist (Neher and Steinbach, 1978) and ions (Colquhoun, 1980), also see review Steinbach (1980). Blocking is voltage sensitive for cationic agents since the greater the hyperpolarization of the membrane the greater their attraction for the inside of the cell and the more the blocking of the channels.

In summary, therefore, given the proper conditions most experimental preparations can display very complex kinetic behaviors which are not easily modeled. While some activation schemes seem to closely predict the steps towards opening of AChRs given certain parameters, others are better at defining the events associated with openings given different conditions.

As yet, an all-encompassing model for channel activation and gating has not yet been developed.

Models such as those of Sine and Steinbach, whether or not they represent the true picture of channel activation are useful in trying to understand the effects of factors, such as agonist concentration and membrane voltage, on the ionic channel. Several studies have shown the association and disassociation constants (k_1 , k_2 , and k_{-1} , k_{-2}) for agonist binding to the activation sites of the α -subunits to be dependent on the nature and concentration of the drug (Adams, 1976; Sheridan and Lester, 1977; Dionne et al., 1978). By contrast, these are not strongly voltage-dependent, which has been taken to mean that the binding sites are located outside the membrane electric field. The rate of channel closings, α , is however, influenced by the membrane voltage as manifested by an e-fold increase in the mean open time of the channel per 90 mV hyperpolarization (Colquhoun et al., 1979; Morris et al., 1983; Takeda and Trautmann, 1984). A weak voltage sensitivity in β , the rate of channel opening, has been detected in a few preparations (Steinbach and Sine, 1986). Models predict, therefore, that drug-dependent kinetic behavior of the AChR will result from the differential effect of compounds on the various transition rate constants in the reaction scheme. By way of illustrating how a channel might show various kinetic behaviors with different compounds, let us consider the scheme proposed by Lecar et al. (1983). For simplicity of explanation, this model assumes that the binding of a single agonist molecule

can lead to channel opening. The scheme is introduced here to present ideas which should hold true for more complicated schemes involving two binding sites (see figure A). In this three-state model for drug-induced channel activation and competitive inhibition, the receptor-channel complex R only displays two conformations open (o) and closed (c). It is assumed that the binding rate (K_a) for the drug (A) is not the rate-limiting step, and the concentration of A is assumed to be low enough so that the probability of a channel being bound is low. Therefore, transitions (whether one or several in succession) are attributed to an individual channel during a single binding episode (Lecar et al. 1983). Differential kinetic behavior with various compounds may be dissected as follows in Figure A.

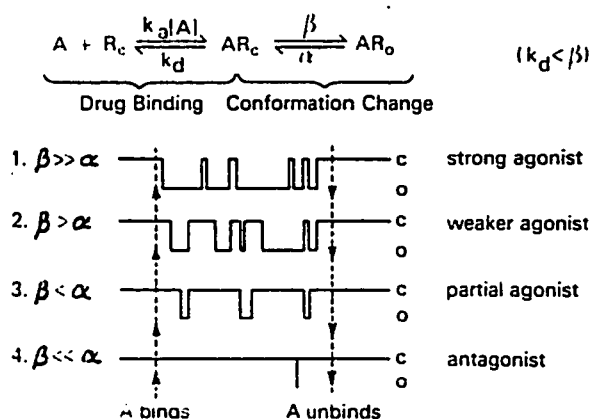


Figure A: A unified model for partial agonist action (Lecar et al., 1983).

CASE 1: STRONG AGONIST

The channel is open the majority of the time but intermittent flickers to the closed state occur. This may be representative of one burst of activity if this channel has a tendency for bursting behavior with strong agonists (eg. ACh)

CASE 2: WEAKER AGONIST

The channel spends a smaller fraction of its time in the open state, however the channel is open sufficiently long to carry enough current to discharge the membrane voltage (eg. Carbachol)

CASE 3: PARTIAL AGONIST

During a single binding episode the channel is usually closed except for infrequent and brief excursions to the open state. The drug in this case would produce weak agonism however, it could show competitive antagonism for the actions of stronger agonists in combined drug trials due to its preference for the non-conducting state (eg. Curare).

CASE 4: ANTAGONIST

Binding of the drug results in a strong stabilizing of the closed state throughout the entire binding episode. If the channel does open, it closes again so rapidly that any current developed is insignificant for detection (eg. α -bungarotoxin).

This summary allows us to appreciate the complexity possible in a system with only two liganded distinct states (opened and closed). Let us not forget, however, that real biological channels frequently exhibit a variety of open states varying in conductance and/or kinetic behaviour. Consequently, adding the equivalent of α and β for each open state (with their respective influences) can lead to kinetic schemes of great and probably intractable complexity.

In closing this section, it is worth mentioning that an understanding of channel activation seems most likely to come from studies combining site-directed mutagenesis and single channel recording (Michina et al., 1986). These types of investigation permit localization of certain functions to various areas of the subunits; for example, Hall (1986) has reported an involvement of the δ -subunit in regulating the rate of channel closing and Mishina et al., (1986) have precisely localized the ACh binding sites on the α -receptors. The combination of electrophysiological and molecular genetic studies will undoubtedly in the future provide significant information on which more realistic activation models may be based.

Curare; portrait of a partial agonist

In the past it has often been convenient to use the terms agonist and antagonist as if they described two mutually exclusive classes of drug/receptor interaction. However, there now

appear to be several cases where these terms refer only to extremes on a continuum. The actions of curariform agents on nicotinic acetylcholine receptors are a case in point. These drugs have been shown to activate the AChR/channel complex, at a very low frequency, to competitively antagonize activation by other agonists and finally to noncompetitively antagonize by blocking previously opened channels (Ziskind and Dennis, 1978; Colquhoun et al., 1979; Katz and Miledi, 1978).

There are several binding sites for curariform agents on the receptor channel complex, the most evident of which are the ACh binding/activation sites on each of the two α -subunits. Whereas ACh binding sites show identical affinity for binding of ACh molecules, their affinity for curare and its analogues is not functionally equivalent (Taylor and Sine, 1982; Sine and Steinbach, 1986). Binding of curare to these activation sites results in the classical competitive antagonist action of the drug. The inhibitory effect of curare on the nicotinic AChR of postsynaptic membranes is attributed to a reversible attachment to endplate receptors, thereby blocking access to ACh and preventing ACh's depolarizing action. One can test a drug's ability for competitive-inhibition for the AChR by verifying whether the ACh dose-response curve in double log coordinates presents a parallel shift in the presence of the inhibitor and whether the relation between dose-ratio and inhibitor concentration is linear and independent of the amplitude of the control response (Jenkinson,

1960). Studies of this kind on curare, however, do not show pure competitive-type inhibition. There is evidence that acetylcholine antagonists for AChRs such as tubocurarine, can act by blocking the ionic channels opened by acetylcholine as well as by competing with ACh for receptor sites (Sine and Steinbach, 1986; Shaker et al., 1982, Trautmann, 1982). This type of noncompetitive antagonism can be distinguished from competitive antagonism in various ways; 1) the degree of block increases rather than decreases if the agonist concentration is increased 2) if the blocking agent is cationic, the degree of block increases dramatically with hyperpolarization of the cell and the kinetic behavior (opening and closing) of the channel is significantly affected (Rang, 1951; Katz and Miledi, 1978; Colquhoun et al., 1979; Marty et al., 1976; Ascher et al., 1979). The noncompetitive mode of action of curare inhibition on endplate ionic channels appears to resemble closely the mode of action of local anaesthetics (Neher and Steinbach, 1978).

A third mode of action of curare which is not classical was first shown by Ziskind and Dennis (1978). In embryonic and neonatal rat muscle, they showed that curare not only diminished the depolarizing effect of acetylcholine, but also produced on its own, an α -bungarotoxin-sensitive depolarization of the cells. Trautmann (1982, 1983), studying rat myotubes and adult muscle cells with single channel recording, directly confirmed these paradoxical actions of curare on the same receptor molecule.

Trautmann observed curare activated channel openings of 50pS (adult-like), 35pS (embryonic) and 15pS (subconductance). The mean open times of these channels were much shorter than that of ACh-activated openings. Increasing the curare concentration resulted in a decrease in mean open time as would be expected in a case of activation with subsequent blocking of the opened channel with another curare molecule. Conversely, compared to simple cholinergic agonist activation, in which τ is increased with hyperpolarization (Magleby and Stevens, 1972), curare-activated channels close sooner at more negative membrane potentials. Curare molecules bind to a site within the membrane: increasing the negativity of the internal face of the membrane enhances the rate of plugging of the open channel thereby reducing the τ . Thus Trautmann's results profile curare as both agonist and blocker, the term partial agonist therefore appears appropriate for this drug on this preparation.

Ziskind and Dennis (1978) reported the loss of curare's depolarizing abilities on rat embryonic muscle within a week of parturition. Trautmann (1983) however, observed curare-activated channel openings in adult muscle fibres; albeit these openings displayed τ of 20% the embryonic mean open times and activation frequencies of 1/20th the embryonic level. The timing of the loss of curare's depolarizing effect seen by Ziskind and Dennis parallels 4-fold decreases in mean open time of ACh-activated channels in rat muscle in the week after birth (Sakmann and

Brenner, 1978). It may be the case that AChRs do not lose sensitivity to curare activated depolarizations with age; moderate quantitative kinetic differences between the AChR/channel complex of embryonic and adult muscle fibres may make the partial agonist effect of curare on adult muscle difficult to observe. Morris et al., (1983) demonstrated, at the population and single channel level, curare's ability to activate embryonic tissue cultured muscle in the same way it activates excised tissue AChRs. Saturation of curare depolarization was shown to occur at -50mV membrane potential. Since the resting membrane potential of cultured myotubes is approximately -55mV, this could explain why the agonistic abilities of curare on this preparation had been overlooked previously.

As well as activating AChR/channel complexes in rat myotubes, curare has been shown to open nicotinic cholinergic channels in human myotubes (Jackson et al., 1982), in adult rat muscle endplate (Sakmann and Steinbach, unpublished observation), and in a clonal line of murine myotubes, G8 (Morris and Montpetit, 1986). A peculiarity seen in curare-activated channels is the high frequency of subconductance type openings. Takeda and Trautmann (1984) showed that as well as having different conductance levels, these sublevel openings showed different kinetic behavior from the full openings. The mean open time of the substate, unlike the full openings, is independent of curare concentration suggesting an inability to be blocked. As with

other known agonists, the activation frequencies of both curare-activated full and subconductance openings are concentration dependent.

Because of curare's ability to open channels to subconductance states more frequently than other stronger agonists, this drug may be useful in elucidating steps occurring during activation and conformational transitions that were previously masked. Such information is essential when attempting to model the process of activation.

THESIS TOPIC:

Given this brief review of the salient aspects of the nicotinic acetylcholine receptor-channel complex structure, function and behavior, one can appreciate the present limits and vast gaps in our knowledge of this biologically important macromolecular protein. To further characterize the behavior of the nicotinic AChR under various conditions, electrophysiological studies on the G8 murine muscle cell line were initiated. The clonal line, G8, was obtained from M114, an uncloned myogenic cell line which arose spontaneously in a culture of cells disassociated from mouse hindlimb muscle (Christian et al., 1977). When confluent, most G8 cells form parallel arrays of spindle-shaped mononucleated cells which fuse to form multinucleated myotubes up to 500um in length. Well differentiated G8 myotubes display striations that closely resemble normal mouse

myotubes. They have been observed to contract spontaneously and to form functional primitive neuromuscular junctions when co-cultured with nerve (Christian et al., 1977). They differ from normal murine muscle cells in that their resting membrane potentials are lower (-49mV , a reduction of 10mV), however, like their normal counterparts, they are highly sensitive to acetylcholine. Iontophoretic measurements indicate that their responsiveness to agonist is uniformly high over the surface of the cell and that ACh evokes a characteristic depolarization due to an increase in permeability to sodium and potassium ions.

There are several advantages to using G8s for our studies. Because murine muscle cell lines are easier to maintain than primary tissue cultures and can be kept in steady supply, they are well suited to electrophysiological studies. The cells can be plated at densities which permit differentiation but also allow sufficient isolation of each myotube so that easy access by recording electrodes is achieved. The lack of connective tissue in cultured cells permits direct access of patch electrodes on to the membrane. By contrast, patch clamp recording on excised tissue must be preceded by enzymatic treatment of the cells to remove adventitious coatings (Jackson et al., 1983).

The focus of these investigations lay essentially in the characterization of the multiple conductance states demonstrated by the AChR/channel complex of this particular experimental

preparation. Of major interest in this study was the role played by an agonist in determining the current levels, probability of occurrence and other kinetic behavior of various conductance states. These parameters were best studied using single channel recording techniques.

Agonists of varying molecular weight were used to activate channel openings in an attempt to determine if the size of the binding compound influences the conductance values obtained for the various open states. Essentially, the question asked was--are the sublevel openings of distinct conductance regardless of the drug used to activate the opening or do they represent channels whose current flow is reduced due to partial occlusion produced by an agonist molecule in the ionic pore. One would expect that in the case of the latter explanation, agonists of varying molecular weight would lead to sublevel openings with different agonist-dependent conductances.

The kinetic behaviors of the various conductance states activated by the different agonists were scrutinized for any agonist-dependent trends or for any kinetically distinct open states within the multiple conductance states. The drugs used in these studies ranged in potency from strong agonist to weak partial agonist; therefore, some differences in the open times of the channels activated by the various compounds were expected. However, it was unknown whether these differences occurred and showed similar trends for the subconductance as well as the full-type openings of our G8 AChRs.

Because curare-induced subconductances provide a particularly good opportunity to observe transitions between openings of different current level, studies were initiated to determine if the various states represent an equilibrium condition for the channel. If not, then the dissipation of energy related to current flow through the channel might be determining the occurrence of substate conductances. In essence, we determined if all transition sequences between full openings and subconductance states were equally probable in both directions as expected for equilibrium. Because the pattern of subconductances gave the appearance of occurring in a cyclic manner, microscopic reversibility was also examined.

Before single channel studies were initiated, it was necessary to determine via intracellular recording whether the behavior of the G8 myotubes challenged with a known agonist, such as suberyldicholine, paralleled that seen in classical nicotinic cholinergic preparations. As well, since the literature presents only two cases so far in which embryonic myotubes (Morris et al., 1983; Ziskind and Dennis, 1978) respond by depolarizing when challenged with the classical antagonist curare, G8 myotubes were studied for their response to this partial agonist.

Once the macroscopic studies were completed, the single channel investigations previously described could be initiated in attempts to characterize the microscopic behavior of these nicotinic murine AChR/channel complexes.

MATERIALS AND METHODS

Experiments were conducted on mouse myotubes from the clonal line, G8. (Christian et al., 1977). This skeletal muscle cell line was originally obtained from M114, an uncloned myogenic cell line which arose spontaneously in a culture of cells dissociated from Swiss Webster mouse hind limb muscle.

The G8 cell line stock was obtained from the American Type Culture Collection (ATCC CRL-1456, batch #F-1853, Rockland, Md.). The stock was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 10% horse serum (designated 10/10 medium) on collagen coated 100mm culture dishes incubated at 8% CO₂, at 37 C. The stock was passaged every 4 days before it reached confluency.

To produce differentiated myotubes, cells were seeded at approximately 10⁶ cells per 35mm culture dishes in 2ml 10/10 medium. When cells become confluent, their medium was switched to DMEM containing 2% fetal calf serum and 2% horse serum supplemented with penicillin/streptomycin (Flow). After a further 3-5 days, the 2/2 medium was replenished and 5-fluoro-2'-deoxyuridine, an antimitotic drug (FUDR - 40 uM final concentration; Hoffman LaRoche) was added for 2-4 days to prevent further proliferation and promote differentiation. The cells were maintained in 2/2 medium in DMEM without FUDR until they were used. Experiments were performed on these cells at about 10-18 days after first plating on the differentiation regime.

The G8 stock was never passaged more than 30 times after which new stock cultures of the cell line were initiated from stores kept at -70°C . Cells were frozen down slowly in 10% DMSO, a cryoprotectant, in fetal calf serum and kept frozen indefinitely. Thawing of a G8 stock vial, unlike freezing, was performed rapidly. Once thawed, the stock solution was resuspended in DMEM. The DMSO was diluted out and removed by centrifugation. The remaining pellet of cells was then resuspended in 10/10 medium at 10^6 cells/ml and plated on collagen coated 100mm culture dishes. On some occasions, the growth medium of the stock was supplemented with penicillin/streptomycin for the first passage. The horse and fetal calf sera (Gibco Inc) required heat inactivation at 56°C for 30 minutes before being used.

The DMEM (Gibco Inc.) specifically contained glutamine and glucose and was without bicarbonate and pyruvate. A trypsin (0.025%)/EDTA (1mM) solution in phosphate buffered saline was used to detach cells from the culture dishes when repassing the stocks.

Collagen solutions used to thinly cover culture dishes were made up of calf skin collagen (Calbiochem) with distilled water and glacial acetic acid.

When confluent, G8 cells form parallel arrays of spindle-shaped mononucleated cells which fuse to form multinucleated myotubes of up to $500\mu\text{m}$ in length. Striations in the well-differentiated G8 myotubes were observed infrequently while spontaneously contracting cells were commonplace. Tetrodotoxin (Sigma)

was used on the myotubes at $10^{-7}M$ as necessary during the electrophysiological recording to prevent contraction.

Experiments using a given agonist were carried out on cells differentiated from at least 2 different passages. As well, different agonists were examined for a single passage, to avoid any possibility that phenomena peculiar to a given cell batch would be misinterpreted as different agonist effects.

Salines

For intracellular microelectrode recording, Hepes buffered Eagle medium (Gibco) was used as bathing medium for the cells, as well as for diluting the drug solutions being used (i.e. d-tubocurarine and suberyldicholine). For single channel recording, a simpler solution was used both in the bath and in the recording pipette with or without the agonists. This solution is that used by Hamill and Sakmann (1981) on rat myoballs and is composed of;

150mM	NaCl	
1mM	MgCl ₂	
1mM	CaCl ₂	
3mM	KCl	adjusted to pH=7.2
10mM	Hepes	with NaOH

Drugs

A summary of all agonists used can be found in Table 1. Agonists are presented in increasing order of size based on

TABLE 1: Agonists used

Name	Molecular weight	Concentration used (M)	Formulation and supplier
Chol	104	10-5	Choline chloride, Fisher
RCh	146	10-6	Acetylcholine chloride, Sigma
Carb	147	10-6	Carbamylcholine chloride, Sigma
Nic	162	10-6	Nicotine hydrogen (+)-tartrate, BDH, Poole, Eng.
SucCh	290	10-6	Succinylcholine (di) chloride, Sigma
SubCh	346	10-7	Suberylcholine dichloride, synthesized by M. Jackson
Cur	625	10-5	d-tubocurarine (di) chloride, Sigma

Note: Chol, choline; RCh, acetylcholine; Carb, carbachol; Nic, nicotine; SucCh, succinylcholine; SubCh, suberylcholine; Cur, curare.

molecular weight. Concentrations, formulations as well as suppliers of the drugs have also been included. Compounds were originally prepared as 10^{-3}M frozen stock solutions in PBS (phosphate buffered saline);

40 g NaCl
5.75 g Na_2HPO_4
1 g KCL
1 g KH_2PO_4
in 500ml distilled water

As required on the day of use, these stocks were thawed and diluted to the desired concentration with the appropriate saline for the electrophysiological recording technique to be used. All solutions used were filtered ($0.2\ \mu\text{m}$ pores) just prior to the experiments.

Drug concentrations were chosen according to several criteria:

- a) concentrations similar to those previously found in the literature
- b) concentrations which were not prone to elicit dramatic concentration-dependent kinetic behavior in the AChRs (i.e. desensitization) of G8 cells.
- c) concentrations which produced activation frequencies that fell between unacceptably low ($<1\ \text{s}^{-1}$) and unacceptably high levels (i.e. frequent occurrence of simultaneous openings of more than one channel).

Using lower concentrations of drug would not only have made the number of events per unit time too small, it would have increased the ratio of singly-liganded/doubly-liganded channels. Since we know that unliganded channels can open spontaneously, we can

expect that singly-liganded channels could also open and thereby complicate the kinetic analysis.

Intracellular recording

Intracellular recording is a technique that permits one to monitor the transmembrane potential of a cell. To evaluate the whole cell effect of d-tubocurarine and suberyldicholine, G8 cells were impaled with a 4M K^+ -acetate filled microelectrode with a resistance of approximately $25M\Omega$. The glass electrodes were prepared using glass capillary tubes (Kwik-fil, W.P.I. 1B120F) and a vertical pipette puller (David Kopf Instruments, Model 700C). The microelectrode was connected to a shielded unity gain impedance convertor whose output could subsequently be amplified as necessary. The fluid/electronics interface was achieved using Ag-Ag/Cl reversible half cells. One of the other half cells is used to provide the reference or ground from the bath surrounding the cells under study. The amplifier system used (Microprobe model KS-700, W.P.I.) provides an active bridge circuit which makes it possible to inject hyperpolarizing or depolarizing current while simultaneously recording the potential through the same microelectrode. In general, cells were hyperpolarized to potentials below -50mV. Upon initial impalement, the measured potential of G8 cells ranged from -35 to -55mV. Data were recorded using a Gould 220 strip recorder.

Drug application on the impaled cell consisted of drug containing saline being pressure ejected from a blunt-tipped

pipette positioned 20-30 μ m from the target. The pipette was connected to a Picospritzer II (General Valve Corp.) which permitted control of duration (usually 100ms) and force (low, to prevent jarring of the cell) of each drug pulse. Just prior to drug application, the microscope was focused on the tip of the drug pipette rather than the impaled cell. This simplified repositioning the pipette between repeated drug applications; care was taken to remove the pipette between pulses to prevent possible leak of drug from the pipette.

Single channel recording

The activity of individual ion channels can be observed directly with the extracellular patch clamp technique (Neher and Sakmann, 1976). This technique permits one to electrically isolate a patch of membrane of approximately 1 μ m in diameter with a constricted, blunted glass micropipette pressed against the cell surface. If the isolated patch is sufficiently small, the experimenter can reduce the background electrical current to such an extent that current jumps in the picoampere range caused by the activation of individual molecular channels can be detected.

Patch pipettes were constructed using the vertical pipette puller modified to follow a two step regime. A thick walled pyrex capillary tube (1.5mm O.D., 0.85mm I.D.) is first constricted using relatively low heat and the pull is interrupted after the glass has been stretched less than 1cm. The glass is

then repositioned so that the thinnest part of the constriction is in the centre of the heating filament and the second pull at altered heat breaks the glass leaving a 1-3 μm diameter tip. The shank of the electrode is then Sylgard coated (180 silicone elastomer, Dow Corning) to decrease the capacitance of the glass near the tip. The glass tip is heat polished using a thin platinum/iridium heating filament to produce tips with a smooth and even surface. Such tips can be pressed firmly against the cell membrane without rupturing it. Great care must be taken to keep the electrode tip clean so that adhesive interactions between glass and membrane can be obtained at the time of patching. Electrodes can only be used once because contact with cellular material makes them unable to adhere to a second cell. Filtering of all solutions is also critical in maintaining a clean tip. The glass electrode or pipette is connected via a Ag/AgCl bridge to a low-noise high gain current-to-voltage converter (List EPC-5 amplifier). A schematic diagram including the basic elements of the single channel recording circuit is displayed in figure B. Currents flowing across the patch were amplified 50mV/pA by the current-to-voltage converter.

FIGURE B

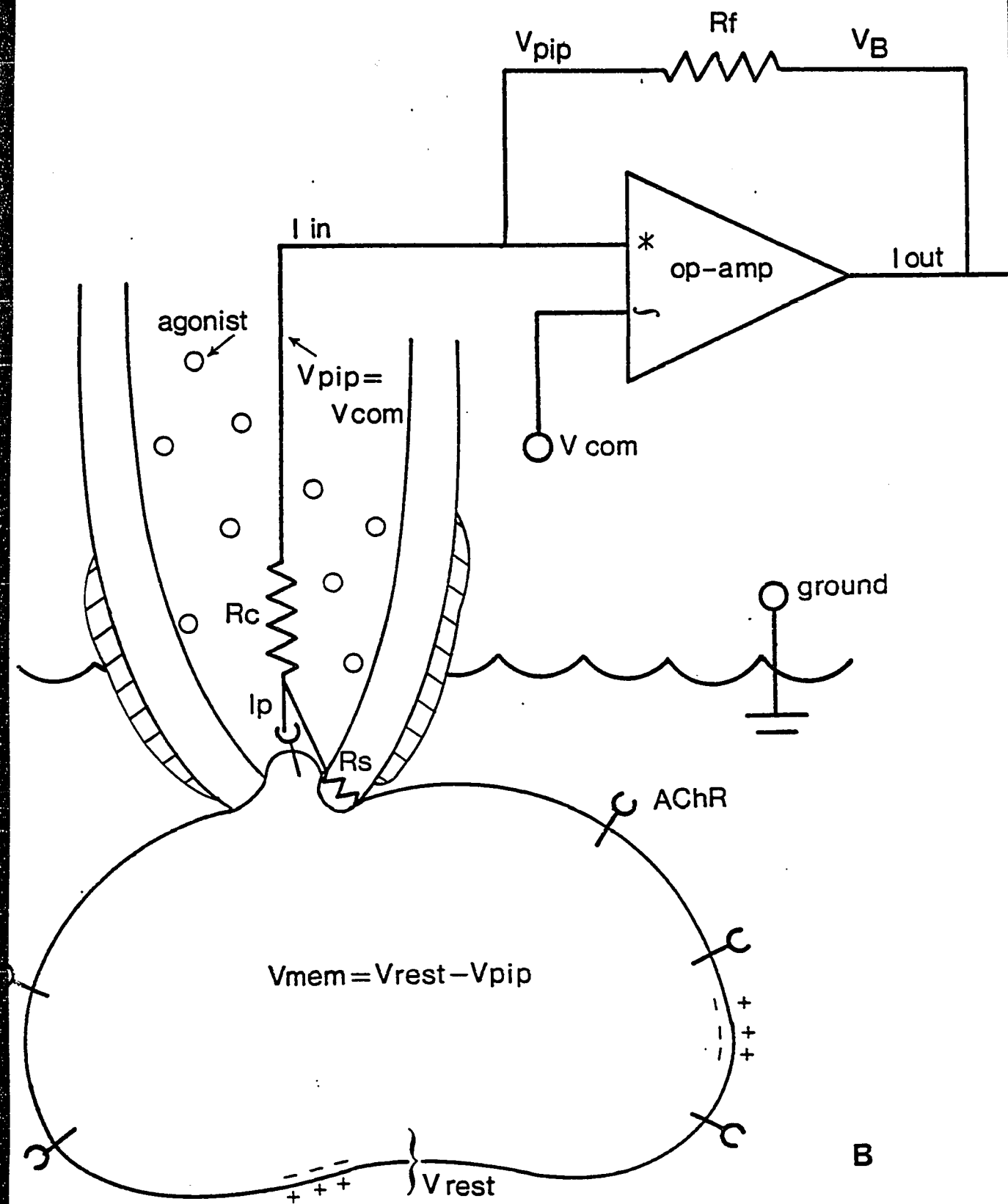
The current-to-voltage converter used in single-channel studies permits one to measure membrane currents while clamping an isolated patch of membrane to a specified voltage. The system works as follows (figure B).

As a channel opens in the patch, an ionic current (I_p) produced by the movement of Na^+ and K^+ ions flows up the pipette to the I/V converter (I_{in}). The I_{in} current will tend to change the voltage (i.e. $I_{in} \times R_f$) found at the point (*) of the operational-amplifier of the I/V converter. The op-amp continuously compares (*) with the virtual ground (which is at the command voltage specified by the experimenter) and compensates for any discrepancies by sending out enough current (I_{out}) to return voltage (*) to voltage ($\sim$). Thus, it is the output current that clamps the pipette voltage. In a virtual ground I/V convertor, I_{out} is made to flow across the large feedback resistor and in accordance with Ohm's law $(V_B - V_{pip}) = -I_{out} \times R_f$ produces a large voltage drop between V_B and V_{pip} . Thus the minute current through the single channel has been converted to a measurable voltage. The current-to-voltage converter (List EPC-5) for most of the studies, amplified the signal to 50mV/pA.

The current through the membrane patch (I_p) is divided between the path through the seal (R_s) to the bath ground and the path through the electrode (R_e) to the amplifier virtual ground (V_{com}). This leads to the relationship;

$$I_{in} = \frac{I_p R_s}{R_e + R_s}$$

so that I_{in} approaches I_p as R_s becomes very much larger than R_e . In effect, the high signal-to-noise ratio (I_{in} / I_p) of the single channel recordings is achieved with high R_s of giga-ohm range.



The G8 cells have clean cell membranes which do not possess adventitious coatings, thus, they readily permit formation of giga-ohm seals (Sigworth and Neher, 1980; Hamill et. al., 1981). Electrodes with resistances of approximately $3-4 \text{ M}\Omega$, upon touching the cell membrane generally sealed to the surface with a resistance jump of $100 \text{ M}\Omega$. When gentle suction was applied to the internal solution of the patch electrode via a side arm on the microelectrode holder, seals were produced which jumped to about $10-100 \text{ G}\Omega$ ($10-100 \times 10^9$ ohms) in resistance. This greatly reduces the background noise producing excellent signal to noise conditions. Following giga seal formation, the trans-patch voltage was altered to various known voltages through control of the pipette voltage (Horn and Patlak, 1980). Essentially, the exterior face of the membrane was voltage clamped at specific potentials. The pipette voltages were held at different values alternating between high and low potentials rather than increasing them monotonically. Several minutes worth of data was recorded at each standard voltage used (i.e. $V_{\text{pip}} = 0 \text{ mV}$, $+40 \text{ mV}$, $+80 \text{ mV}$, $+120 \text{ mV}$). Outward currents were recorded in patches where V_{pip} was clamped at -50 mV , -80 mV and -100 mV (attempts made with most patches). Due to very high noise levels, (probably the result of activation of voltage-dependent channels), this data was difficult to analyze and thus appears infrequently in the analysis.

Values for intracellular resting potentials were not obtained from the cells used for patch clamp recordings. From

previous intracellular recordings, however, (our own and Christian et. al. (1977)), the resting potential of the cells studied was assumed to be -50mV . Hence, the transpatch voltage (V_m) is calculated according to $(V_{\text{rest}}) - (V_{\text{pipette}})$.

The single channel data recorded through the List was low pass filtered at 4KHz by a 4 pole Bessel filter (Frequency Devices) and stored on FM tape, using a Racal Instrumentation recorder (15 ips, 1 volt peak). Data were not obtained from more than one patch per cell. All experiments were carried out at room temperature ($19-23^\circ\text{C}$). Data appearing in this thesis are from cell-attached patches, however, both outside-out and inside-out patch configurations were obtained on occasion.

Analysis

For data processing, the records stored on tape were low pass filtered at 2KHz and digitized at $50\mu\text{s}$ intervals into an LSI 11/23 computer. Analysis of the data required several programs all of which have been included (in order of use) in the appendix. Only brief explanations of the fundamentals of each shall be presented at this time. Data reduction was performed by operator inspected (i.e. interactive) computer programs.

CONVERSION:

1. KSAMPL: program which controls the analogue to digital conversion of data.

Records are stored on the hard disk of the mini computer.

This program uses the real time clock of the LSI 11/23 to sample 896 blocks x 4096 points of $I(t)$ data with sampling intervals of $50\mu s$. Data from one patch is thus continuously sampled in sections of up to 11.5 secs.

ANALYSIS: conductance

2. CHKIN₂: captures events and, by use of cursors gives amplitudes (pA) of events.

With this program, the digitized record may be scanned visually and the current amplitude of well resolved events (typically $>1ms$) may be measured using computer-driven cursors. Amplitudes in this analysis represent the difference between the midline of the local background noise and the midline of the open channel. Inspection of the raw data demonstrates that events of more than one amplitude are present. Several voltages were tested on each patch, and for most patches, it was possible to measure 20 events of each distinct amplitude category at each voltage (exceptions; mostly with S_1 openings where insufficient numbers of this type of event were present at each voltage). Due to the small amplitude of the two subconductance opening categories, it was difficult to measure open channel currents above background noise at low transpatch voltages. Consequently, not all patches contained enough information to

obtain a slope conductance value and the smaller conductance openings were expressed as a percentage of the main open event.

The current measurements were plotted on a current / voltage ($V_{\text{pipette}} + V_{\text{membrane}} = V_{\text{rest}}$ = driving force, assuming a reversal potential of 0mV for the channel) curve and corresponding conductance values for each category of openings for individual patches were determined by the slope of a linear regression line through the data. Average conductances were calculated from the means of a number of patches.

In isolating the giga-ohm seals, we cannot be certain that all the current jumps observed were due to the activation of the AChR/channel complex. As a result, we did not feel justified in accepting various subconductance-type openings unless they occurred occasionally as a transition from the main AChR/channel open state.

ANALYSIS: kinetics

3. HITE 3: displays records of digitized raw data organized into amplitude histograms from which rough averages of currents may be gleaned.

Before any automated analysis may be initiated a rough average of the amplitude of each distinct conductance state must be determined. This program displays the raw digitized data record by record and after each set of 4096 points it plots it into amplitude histograms. From

these, one can measure the average current going through the channels when they are opened to a particular conductance state. These values may then be used on the same data as the thresholds for classification of different types of events using automated analysis programs.

4. 5CAT: assigns channel openings into various specified conductance categories and then measures the open time, current flow and time since last event for individual channel openings.

The operator indicates what values have been found for the mean conductance for each of the four open states (adult, main, subconductance 1, subconductance 2) as well as the baseline. The program then scans the data automatically comparing the position of each point ($50\mu s$) to these threshold values as well as to each other. In addition to categorizing openings into conductance types, this program measures the time that the channel current resides within the threshold boundaries for a particular conductance state (i.e. open times of individual channel events)

Problems arise with this program when the signal-to-noise ratio is low. Because subconductance open states 1 and 2 are not as well defined in data with high background noise, two types of contamination may occur;

(a) points past the threshold for subconductance states may in fact be due to high peaks of noise thereby giving erroneous indication of a substate opening.

(b) when elevating thresholds slightly above their real values (in attempts to eliminate false openings), one reduces the resolution of the system in measuring channel open times in each specific state. Consequently, once the event has crossed the elevated baseline threshold to finally reach the threshold for a subconductance, it has been open longer than that indicated by the record. Because of the need to elevate the baseline somewhat, the current values measured by the 5CAT program are also artificially reduced which explains the necessity of using CHKIN₂ for conductance measurements.

Finally, another source of contamination that is anticipated is the classification of unresolved full openings into substate categories. These unresolved openings are automatically assigned to the conductance state for which the criteria has been satisfied.

5. CLEAN: eliminates events (both openings and closings) of less than a specified length of time, from the 5CAT output files.

To compensate for the short-lived false events mentioned previously, a program was designed which enabled the operator to automatically delete channel events from 5CAT output files which are below a specified cut off time. Most records were cleaned with a 0.150ms cut-off.

However, on occasion with the more noisy patches, channel events of less than 0.200ms were discarded. The program concatenates open times of the channel events eliminated with the current level preceeding the deletion. "Cleaned files" were archived on floppy disks.

6. ZFBHST: arranges data from "cleaned files" into open time histogram tables.

Open time histogram tables were constructed according to open time in a specific channel configuration (i.e. adult, main, substate 1, substate 2). This program deals with only one state at a time and does not lump data from several open time bins.

7. REBINRT: arranges data in open time histogram tables into bins with a minimum of 5 channel events each.

This program uses the output of ZFBHST. It runs on a COMPAC 286/DESKPRO microcomputer. Histogram files for several patches were concatenated into larger files according to their specific open state and the agonist used for activation. These combined files were then rearranged for fitting by REBINRT, which automatically lumps bins in histogram files so that they will contain a minimum of 5 events. This program also calculates a bin factor for the modified bins so that they will be properly weighted in subsequent fitting routines.

8. NFIT287: attempts to fit the open time histogram data for each open state with various agonists to two exponentials (COMPAC 286; also NFIT187 and NFIT387 for one and three exponentials respectively).

Time constants for open times of the various channel events were extracted from the data by regression of a sum of exponential probability density functions using a modified Marquardt non-linear least squares algorithm (Schreiner et al., 1985). The fitting procedure involved comparison of the data to the integral over each bin of the appropriate function (be it the sum of one, two or three exponentials) (Guharay and Sachs, 1984). The squared residuals were weighted inversely with the expected variance (see eq. 39 Colquhoun and Sigworth, 1983; Sigurdson et al., in press). The fitting program appears in the appendix as NFIT287 and is specific for two exponentials (one and three exponential routines were not included but were tried on all histograms). The criteria for choosing the number of exponential terms to best fit the data was established on the basis of a minimum chi-square value derived from the fitting routine (Colquhoun and Sigworth, 1983; Sigurdson et al., in press). The mean open times of the channels events were calculated from the inverse of the rate constants of each exponential.

ANALYSIS: probabilities

9. TALLY: is an automated program which scans the "cleaned files" (i.e. output of clean) to sum the number of occurrences of each transition type and calculate the relative probabilities of each state. To compensate for contamination in the cleaned files (i.e. unresolved openings, noise, etc.) a cut-off value for the fast components of the open time distribution was used (usually 0.300ms).

ANALYSIS: reversibility

10. MIRROR: compares the number of occurrences of one sequence of transitions (eg. main to subconductance 1) to its reverse (eg. subconductance 1 to main). The results of such a study give information concerning the reversibility of excursions of the channel from one state to another, or for cyclic schemes, it can test for microscopic reversibility.

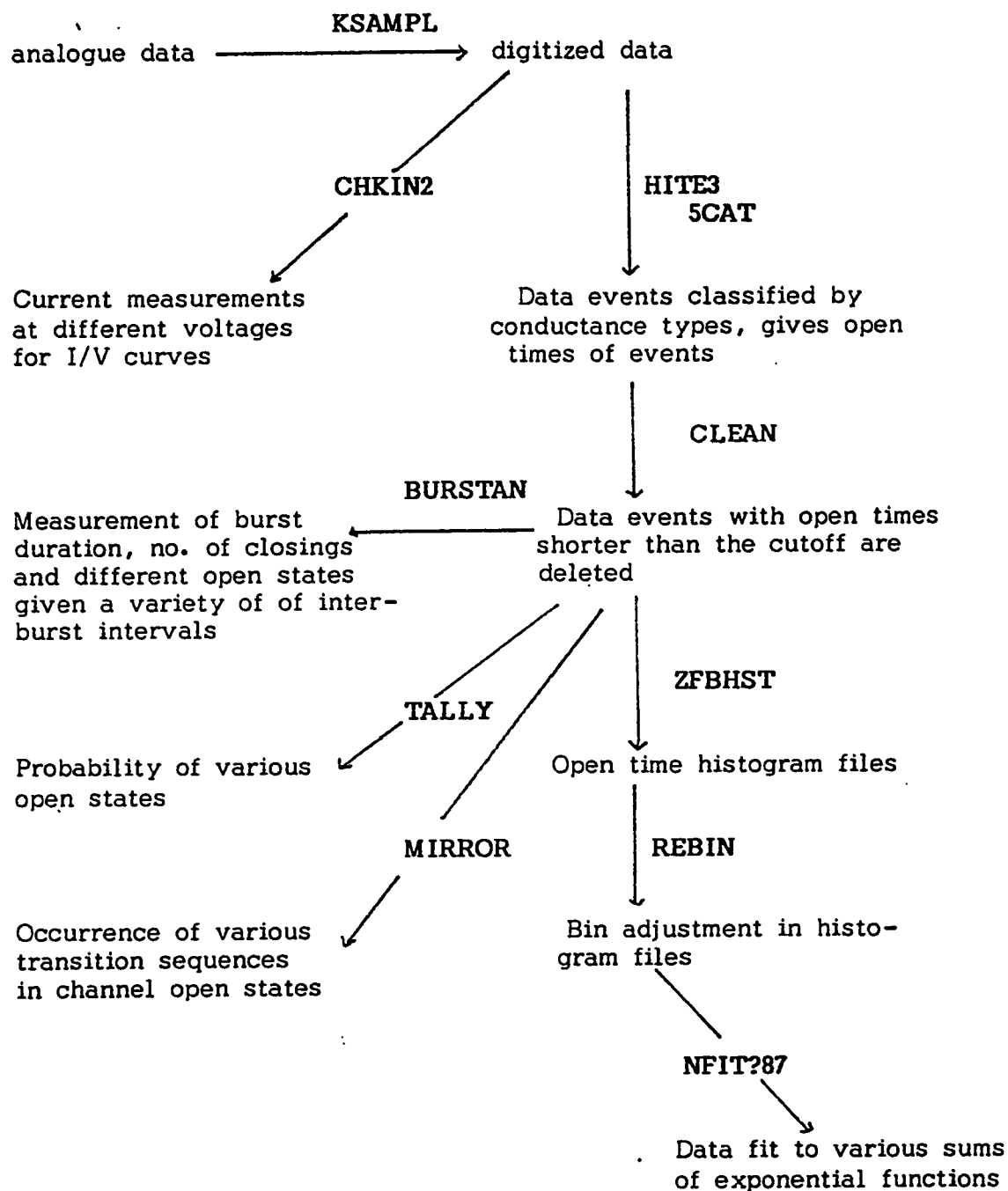
ANALYSIS: bursting behavior

11. BRSTAN: provides a rough average of burst duration and calculates the number of closings or specific types of openings per burst.

Since channel openings with some agonists occur in distinct groups separated by periods of inactivity, burst analysis was performed. For those agonists which elicited well-separated bursts of openings, estimates of average burst duration were obtained. Graphs of rough

average durations versus interburst interval (i.e. minimum duration for a closed event which is deemed to represent a period between bursts) were constructed. The average burst duration was subsequently estimated from the Y axis position of an arbitrarily chosen midpoint, in the area of the curve insensitive to interburst intervals (see also Colquhoun and Hawkes, 1982). Results for specific opening-types from several patches with the same agonists, were averaged. For agonists which elicited bursts closely spaced in the record or did not demonstrate bursting behavior, the question of whether a given closed event is a between burst or a within-burst even cannot be resolved, and an estimation of burst length is impossible.

Flow Chart of Data Reduction Programs



RESULTS

Initially some of the whole cell responses of the cell line, G8, to various agonists were characterized.

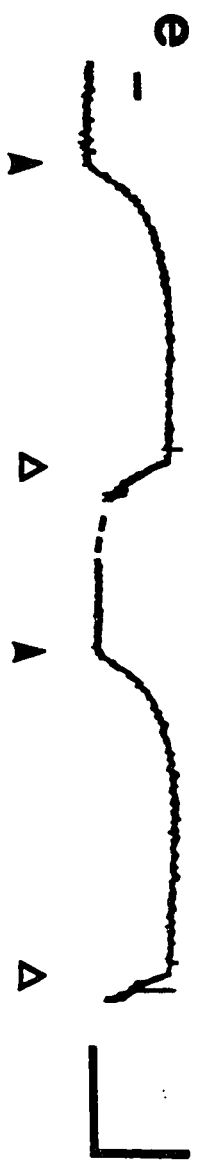
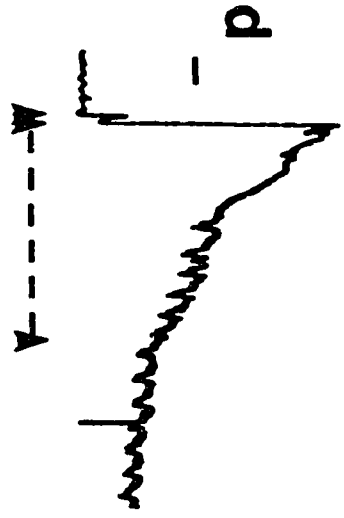
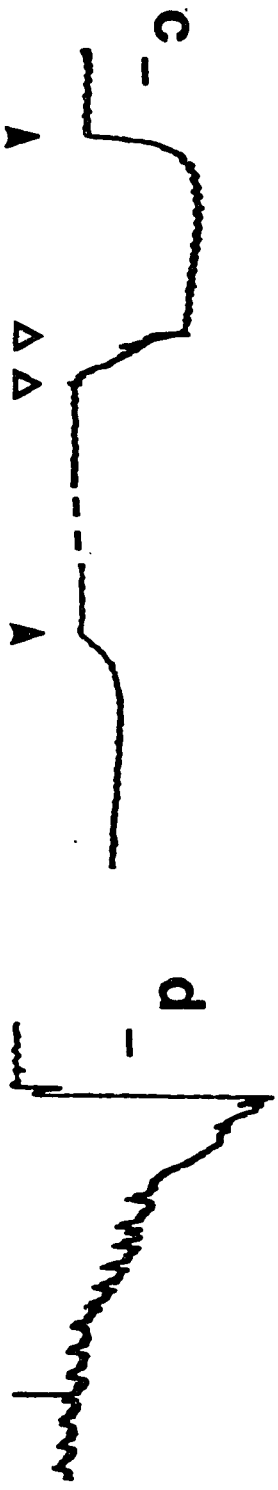
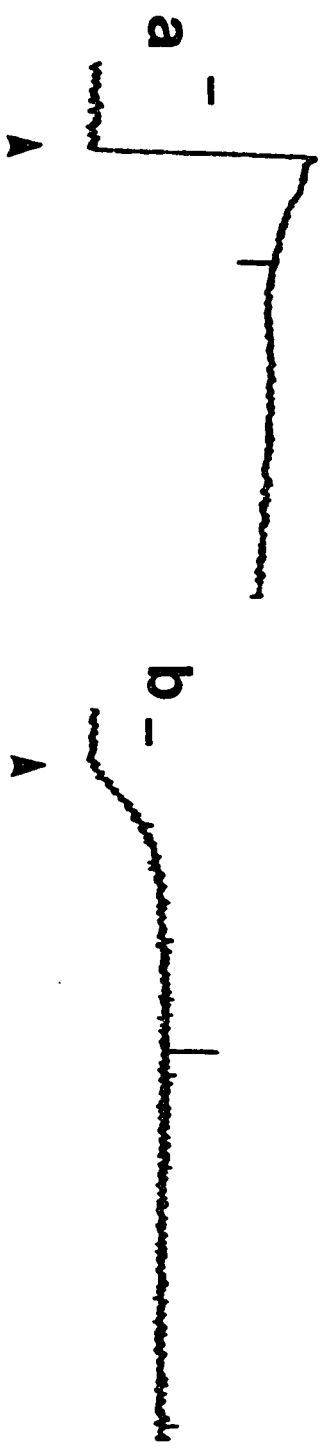
A/Intracellular recording data

The resting potentials of the G8 cells impaled with K⁺-acetate electrodes ranged between -35 and -55mV with an average of -50mV $\pm$ 4.8mV, (n=36) which is in agreement with the findings of Christian et al., (1977) with KCl electrodes.

When exposed to a 100msec pulse of suberyldicholine, (10^{-5} M), a strong agonist for most adult and embryonic-type AChRs, the G8 cells responded with a depolarization which reached its peak within approximately 0.2sec. The response was prolonged and slow to decay (>10sec, sometimes minutes). Usually the resting membrane potential reattained its original level within two minutes after drug application (see figure 1 a and b). The traces illustrated show that although the detailed response patterns of each cell to the agonist vary somewhat, the initial responses and second application responses for any given cell have the same general characteristics (figure 1e). All cells challenged with suberyldicholine responded by depolarizing (n=22).

FIGURE 1

Responses of G8 cells to suberyldicholine. Traces a) and b) illustrate the range of responses observed in these cells for 10^{-5}M suberyldicholine applications; G8 responses are very prolonged. Desensitization and/or the activation of additional conductances (eg. $\text{Ca}^{++}\text{-gK}^{+}$) may explain why cells exposed to pulses of suberyldicholine (one minute apart) do not respond with as great a depolarization on the second trial as indicated in c) (Note that the chart speed was slowed 60-fold when indicated by open triangles and that the traces here and in e) have been interrupted as indicated by dashed line). An example of a second drug challenge on the same cell after a 2 min interval is shown in e). Trace d) illustrates the effect of prolonged application of 10^{-9}M suberyldicholine (application was continuous between first and third arrowheads except for a transient interruption at the onset of perfusion. For this trace the horizontal scale corresponds to 2 min; the decline in response indicates desensitization. The short bars to the left of each tracing indicate -60mV . Scales: horizontal, 2 sec (except where adjusted as above); vertical, 20mV .



Studies with paired suberyldicholine pulses (10^{-5}M , 100msec) applied to the same impaled cell with variable intervals between application, demonstrated that the second response was smaller than the first when the interpulse interval was less than 2 minutes. We attribute this to desensitization and/or the activation of Ca^{++} -gK⁺ channels with the influx of Ca^{++} through the suberyldicholine-activated AChR/channels (Barrett et al., 1982). Examples of this are illustrated in figure 1 c and d (where continuous drug perfusion was used).

Freshly dissected and cultured embryonic rat myotubes depolarized in response to curare (Ziskind and Dennis, 1978; Morris et al., 1983); we tested for this response in G8 cells.

Trials with 10^{-5}M curare on the clonal line G8 produced no obvious depolarizing response when measured at the whole cell level with intracellular recording techniques. To demonstrate that this negative response was not due to immature cells, (vis à vis development of AChR) several cells from each of the experimental preparations were pretested for sensitivity to suberyldicholine (100msec pulses with 10^{-5}M SubCh). Once responsiveness to a known agonist had been established, cells from the same culture plates were challenged with 100msec pulses of 10^{-5}M curare (trials with 10^{-4}M and 10^{-6}M were also tested - see Table 2). Following several trials with curare on a series of randomly chosen cells, the bath solution for the preparations was replaced and cells were rechallenged with suberyldicholine.

FIGURE 2

Effects of suberyldicholine and curare application to G8 cells. All traces shown here are taken from cells in the same plate. Pressure ejection tests (a and b) were done to demonstrate the responsiveness of the cells to a known cholinergic agonist (10^{-5}M suberyldicholine). Subsequently (c and d) cells in the same plate were challenged with curare (10^{-5}M); often, as illustrated, the pressure ejections of curare were repeated at intervals and the preparation was observed to verify proper drug flow. Finally, to ensure that the lack of response to curare was not due to deteriorations, the cells were again tested with 10^{-5}M suberyldicholine (e and f). These traces are representative of the data summarized in Table 2. In some cases, the drug pipette was leaky, as indicated by the dashed lined before the arrowheads. Short bars to left of each trace indicate -60mV . Scales: horizontal, 2 sec (except for 60-fold decrease following the open triangles); vertical, 20mV .

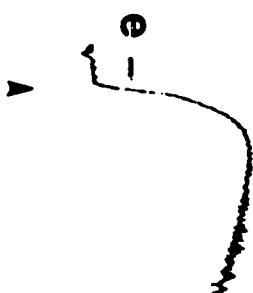
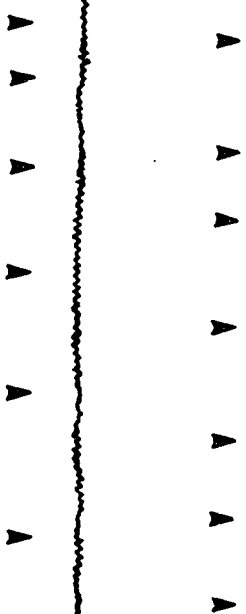
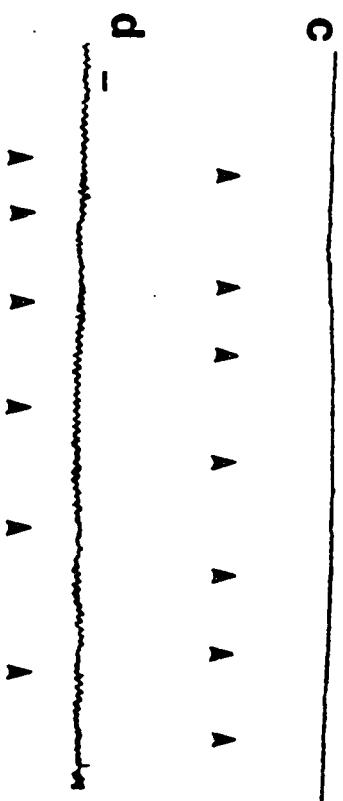
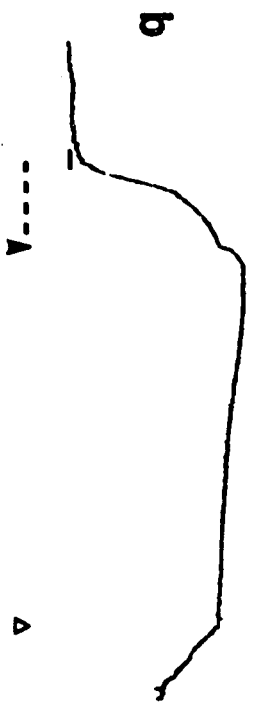


TABLE 2: Effect of pressure-ejected suberyldicholine
and curare on G8 cell membrane potentials*.

Suberyldicholine	Curare
a) 10-5M 28.2±6.5mV, n=32 (cells from plates 1-5) [potentials prior to drug challenge: >80mV, n=4 70-79mV, n=12 60-69mV, n=11 <59mV, n=5]	a) 10-5M 0±0mV, n=27 (from plates 1-5) [>80mV, n=8 70-79mV, n=16 60-69mV, n=3]
b) 10-7M 36.6±3.3mV, n=7 (from plate 6) [>70mV, n=4 <70mV, n=3]	b) 10-4M 0±0mV, n=6 (from plate 6) [>70mV, n=6]
c) 10-5M 35.0±11.4mV, n=8 (from plate 7) [>83mV, n=8]	c) 10-6M 0±0mV, n=6 (from plate 7) [>89mV, n=6]

*Data represent mean ± SE of depolarizations induced by 100ms pulses of the indicated drug, as explained in the text.

Multiple pulses of curare were used to ensure that the responses observed were valid (figure 2 c and d) and that prolonged exposure would not produce effects undetectable with brief challenges. Table 2 and Figure 2 summarize data obtained from these experiments; values for suberyldicholine-induced depolarizations before and after curare trials were lumped.

From studies on rat myotubes (Morris et al., 1983, Ziskind and Dennis, 1978) it has been shown that curare fails to depolarize these cells beyond about -50mV. As a precaution, the G8 cells challenged with curare were hyperpolarized to levels beyond -50mV (see Table 2 and 3 for specifics). In no case did we detect curare-induced depolarizations, whether 10^{-6}M , 10^{-5}M , or 10^{-4}M curare was used.

These tests establish that curare is unable to depolarize G8 cells, in contrast to its depolarizing action on rat myotubes (Morris et al., 1983). It thus became necessary to determine if curare has any activity on the AChRs of this clonal cell line. As a simple check, we tested whether curare can interfere with the depolarizing action of the AChR agonist, suberyldicholine. Curare, when included in the bath medium (10^{-5}M final concentration), antagonized the depolarizing action of the 10^{-5}M pulses of suberyldicholine (figure 3c and d) on cells from a population previously shown to be sensitive to this strong agonist (figure 3a and b). Data from these experiments is outlined in Table 3.

In summarizing the intracellular recording data obtained, it would appear that curare does indeed bind to the AChR of G8

FIGURE 3

Effects of curare as an antagonist to suberyldicholine-induced depolarization in G8 cells. Each trace represents data from a separate patch in a different cell. Suberyldicholine (10^{-5}M) applications before (a and b), during (c and d) and after (e and f) inclusion of 10^{-5}M curare in the bathing medium indicate that curare antagonizes the action of this cholinergic agonist. Following exposure to curare, the plates were given 2×10 min washes. In c) (the largest response of any G8 cell in the presence of curare) the record is interrupted for 7.5 sec where indicated by dashed lines. The data from which these traces were taken are summarized in Table 3. Short bars to the left of each trace indicate -70mV . Scales; horizontal, 2 sec (except for 60-fold decrease following the open triangles); vertical, 20mV .

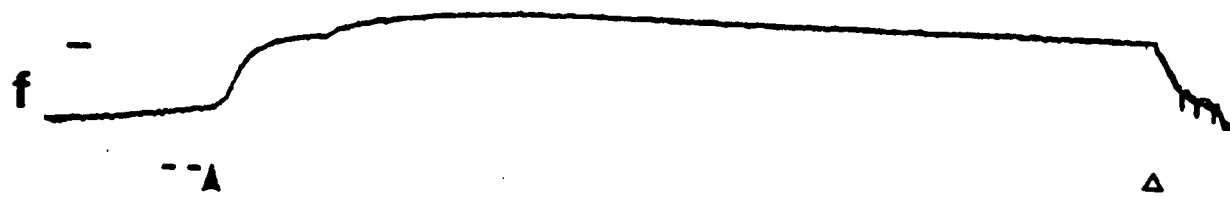
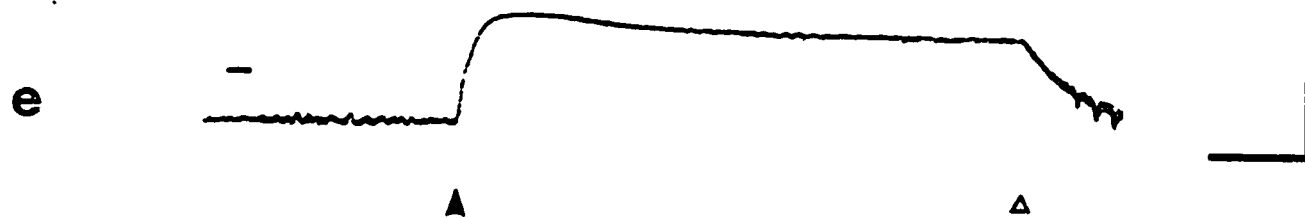
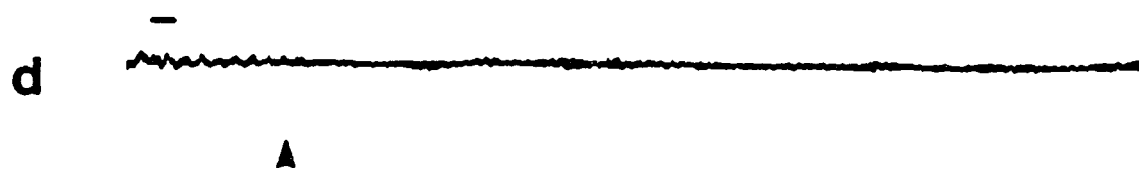
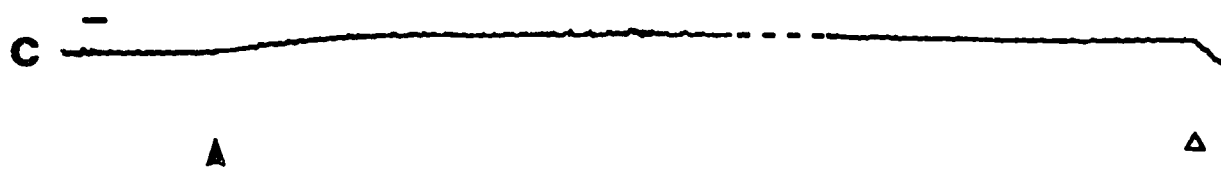
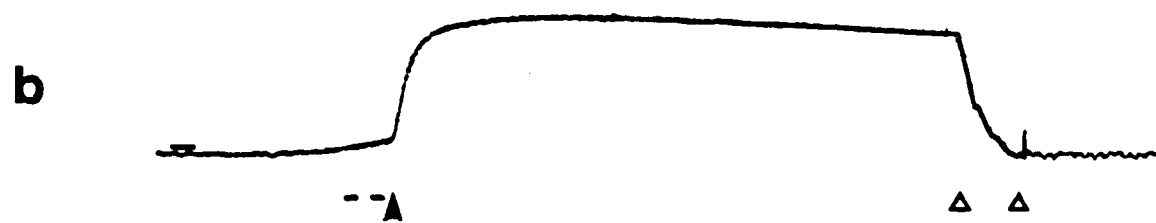
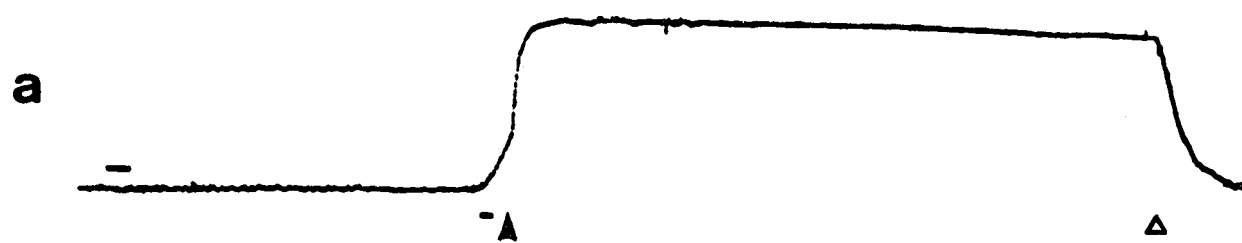


TABLE 3: Cholinergic antagonist action of curare on G8.

	Control	10-5M Curare in Bath
G8 cells	23.4±4.6mV, n=21	0.5±0.5mV, n=12
	(cells from 2 plates)	(cells from same 2
	[potentials prior	plates as control)
	to suberyldicholine	[>80mV, n=3
	challenge:	70-79mV, n=4
	>80mV, n=2	60-69mV, n=5]
	70-79mV, n=6	
	60-69mV, n=13]	

*Data represent depolarizations induced by 100ms pulses of 10-5M suberyldicholine. Control challenges were made before and after challenges in the presence of 10-5M curare, as indicated in the text. The mean depolarizations are significantly different between control and experimental conditions.

cells. Although this drug does not induce measurable depolarizations at the whole cell level in these cells (as seen in embryonic rat myotubes), there seems little doubt that it does play its more traditional role as an antagonist.

Initial Single Channel Data

To further characterize the behavior of the AChRs of G8 cells to agonist activation at the microscopic level, preliminary single channel recordings were undertaken with the same drugs as were used at the macroscopic level (i.e. SubCh and Cur). Initial single channel recordings showing inward currents with suberyldicholine (10^{-7}M ; figure 4) and curare (10^{-5}M ; figure 5) indicate that both drugs are able to activate the AChR/channel complex of this clonal cell line. However, even upon cursory inspection, the data indicate that curare-induced currents are briefer than those provoked by suberyldicholine. Both drugs, however, displayed openings of two current levels. The larger amplitude openings were by far the most frequent while a second smaller opening level (approximately one third of the main type) occurred more rarely. Curare displayed a higher ratio of the subconductance events to main-events than did suberyldicholine (figure 6). Channel openings with both drugs were observed in all patch configurations (i.e. inside out, outside out and the most frequently used configuration, cell-attached). In accordance with previous electrophysiological work on AChR, no channel activity was displayed when the membrane potential was

FIGURE 4

Suberyldicholine-activated single channel currents in a G8 cell. The recording is made in the cell-attached configuration following the formation of a gigohm seal by a pipette containing $10^{-7}M$ suberyldicholine. The outer face of the membrane was clamped to +100mV. Since the resting membrane voltage is assumed to be about -50mV, the overall transmembrane voltage should be about -150mV. The traces shown are a representative sample of channels since events (recorded directly on a storage oscilloscope) were photographed without any selection process beyond rejecting traces with no events. In addition, a series of about 30 superimposed sweeps at one-fifth the speed is included in the lower right hand trace. For these recordings the filter was set at 2KHz. Two conductance levels seem to be present in these traces. The larger amplitude channel openings are of approximately 8pA while the smaller openings are of 2.5pA. Scales: horizontal, 50 msec or 250 msec; vertical, 8pA.

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FIGURE 5

Curare-activated single-channel currents in a G8 cell. The recording pipette contained 10^{-5}M curare and the currents were filtered at 1 KHz, otherwise the experimental conditions were the same as in Figure 4. The illustrated recordings are not accurately representative of the channel durations as they are in Fig. 4, however, since many of the traces containing very brief attenuated channel recordings were not included. Thus, the illustration underemphasizes the briefness of the events. The longer-lasting events are over-represented here to better illustrate the channel conductances. The larger conductance openings are represented by current jumps of 6pA while the smaller conductance events are closer to 2pA in value. Very infrequently, larger currents which were probably not fully resolved were seen. Scales: horizontal, 50 msec; vertical, 8pA.

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FIGURE 6

Curare-activated channels from the same patch and under the same conditions as in Figure 5 but recorded at one-fifth the sweep speed and with about 20 superimposed sweeps in each trace. The traces give a clearer idea of the distribution of conductances and durations. Notice that the larger events are more abundant and that the smaller ones are characterized by longer open times. Scales: horizontal, 250 msec; vertical, 8pA.

held at $0\text{mV} \pm 5\text{mV}$ which is the reversal potential for most AChR species using similar salines (Hamill and Sakmann, 1981). Inward currents were readily detected at the membrane resting potential and at more negative transpatch voltages up to a maximum of -200mV (which is close to the breakdown voltage for the membrane). Less frequently it was possible to observe outward current jumps in these patches when the membrane potential was held at values more depolarized than the reversal potentials of the channels.

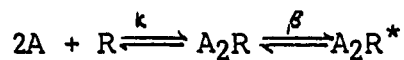
From these initial studies, it appeared as if activation of curare-induced openings was also somewhat voltage-dependent (figure 7). As would be expected, the amplitude of the channel events was increased with increased transpatch potential, however the increased number of channel openings with greater V_m was especially interesting since preliminary results from rat myotubes (Morris et al., 1983) showed the same trend. Further investigation of this phenomenon, which appeared specific for curare, was initiated.

The frequency of activation of agonist-induced events is not generally expected to vary with membrane voltage since the agonist-binding site is presumed to be external to the membrane field (Klymkowsky and Stroud, 1979). Results from rat myotubes (Morris et al., 1983) indicated that the opening frequency of curare-activated events declined by a factor of e-fold per 50mV depolarization and that this voltage-dependence was consistent

FIGURE 7

The voltage dependence of curare activation frequency. Each trace represents 10 superimposed sweeps taken in rapid succession (same conditions as Figure 6). The pipette voltage was varied as follows: (in mV) +50, +100, +150, +50, +100, +150; the whole process was carried out in less than 5 min. Assuming that the resting membrane voltage -50mV, the upper two traces correspond to a transmembrane voltage of -100mV, the next two, -150mV, and the bottom two, -200mV. Scales: horizontal, 200 msec; vertical, 10pA.

with the macroscopic effects (i.e. whole cell depolarizations) with the following binding sequence as a model;



*=open A=agonist R=receptor

These preliminary studies would suggest that for curare, the processes of receptor binding (k) and channel activation (β) do not occur in rapid succession and that hyperpolarization could produce an increase in the closed/liganded (A_2R) $\longrightarrow$ open/liganded (A_2R^*) transition rate (re. β). This could explain how the same ligand/receptor interaction could simultaneously behave as an antagonist at the macroscopic level and a weak agonist at the single channel level. Curare could in fact at low V_m favour the long-lived closed/liganded states with occasional brief excursions to the open/liganded states.

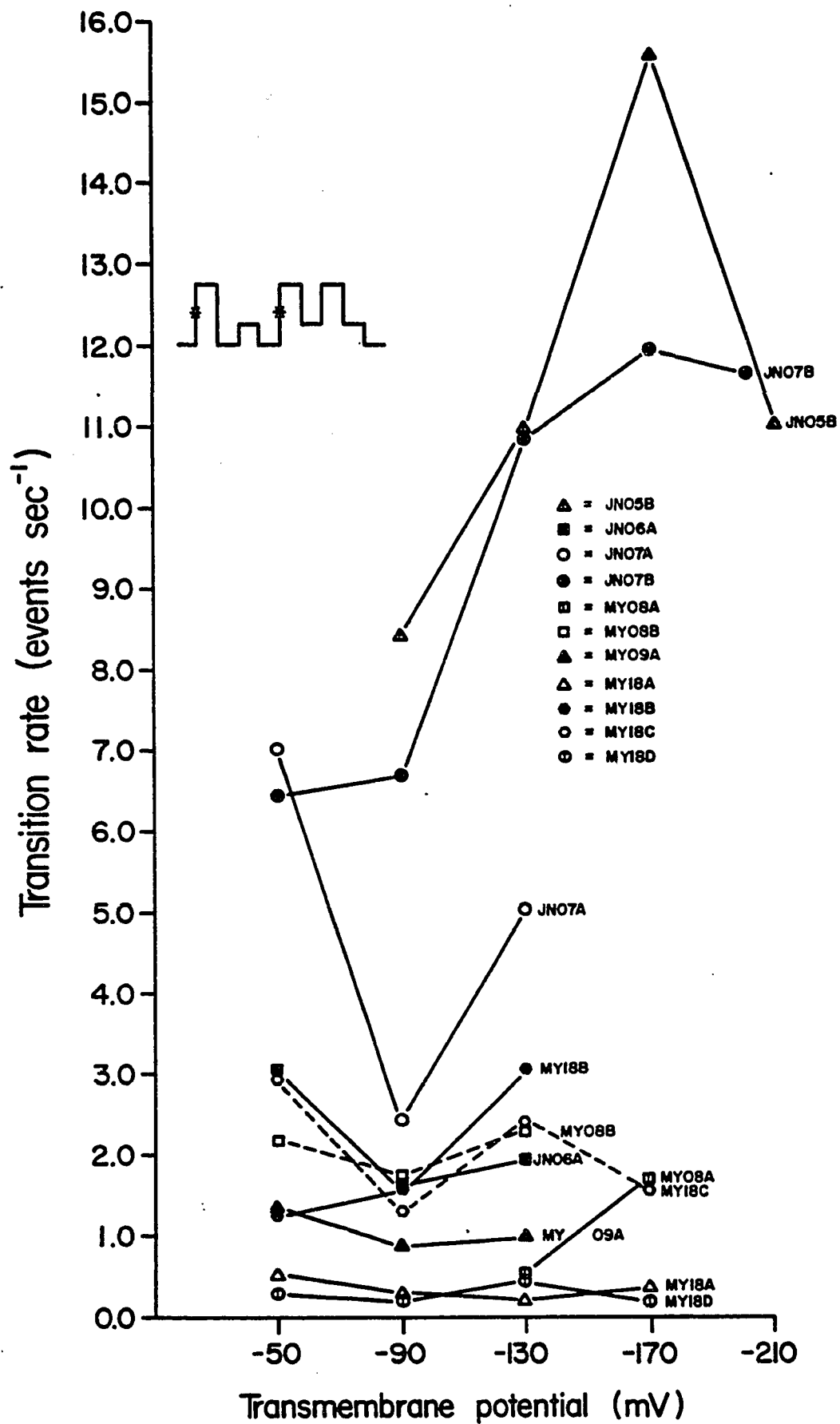
To follow up on the question of voltage dependence of curare-induced activation, 11 patches were probed and the activation frequencies of two of the open states (M and S1) were measured as a function of voltages ($V_m = -50\text{mV}$, -130mV , -90mV and -170mV). Unfortunately, no consistent positive correlation between activation frequency and voltage were found for transitions between the various states. Figures 8a to f display the results for the various transition types; for some patches, frequencies were completely unaffected by voltage, others showed

FIGURE 8

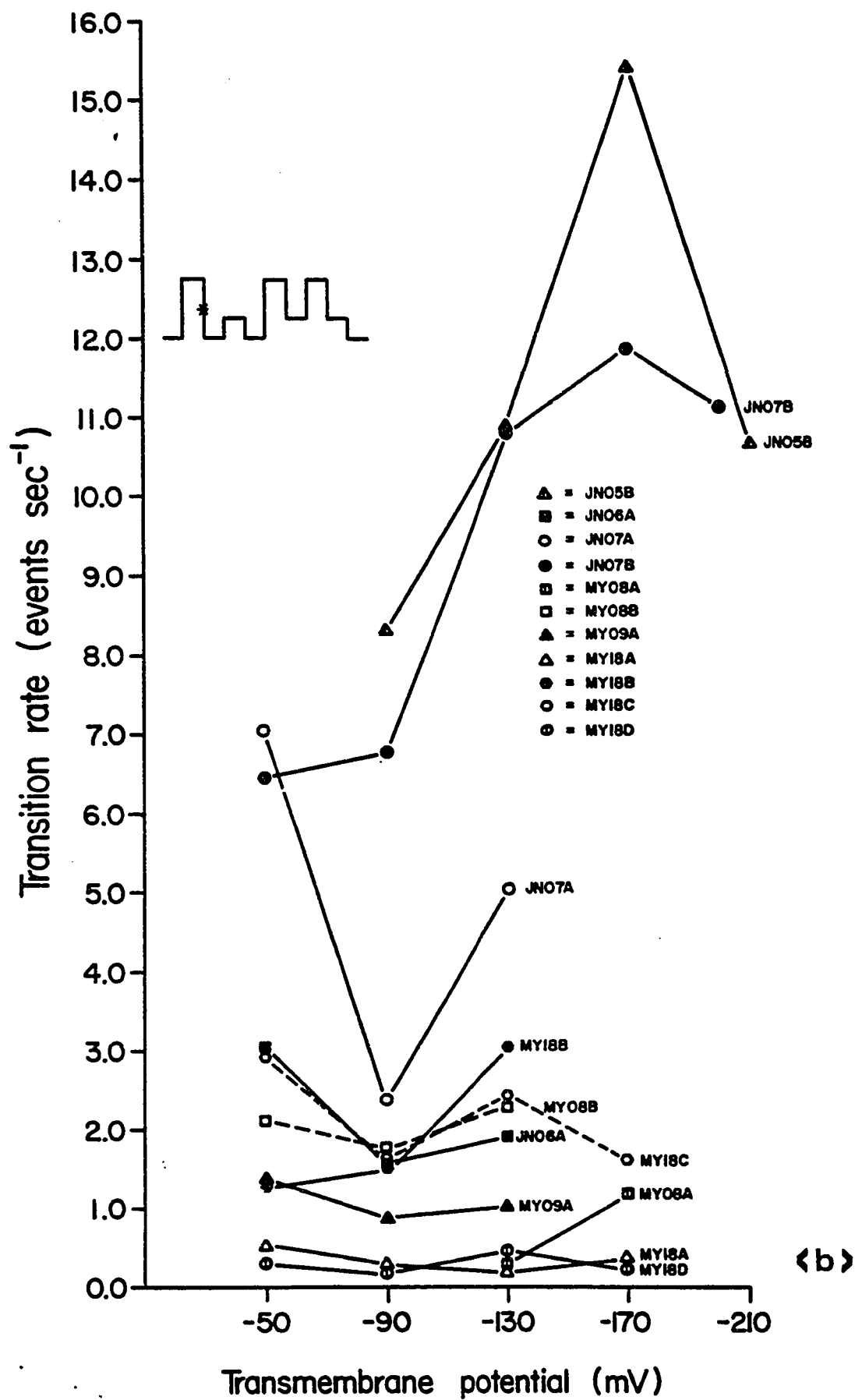
Transition rate as a function of voltage in curare-induced activation of AChR/channel complex of G8 cells.

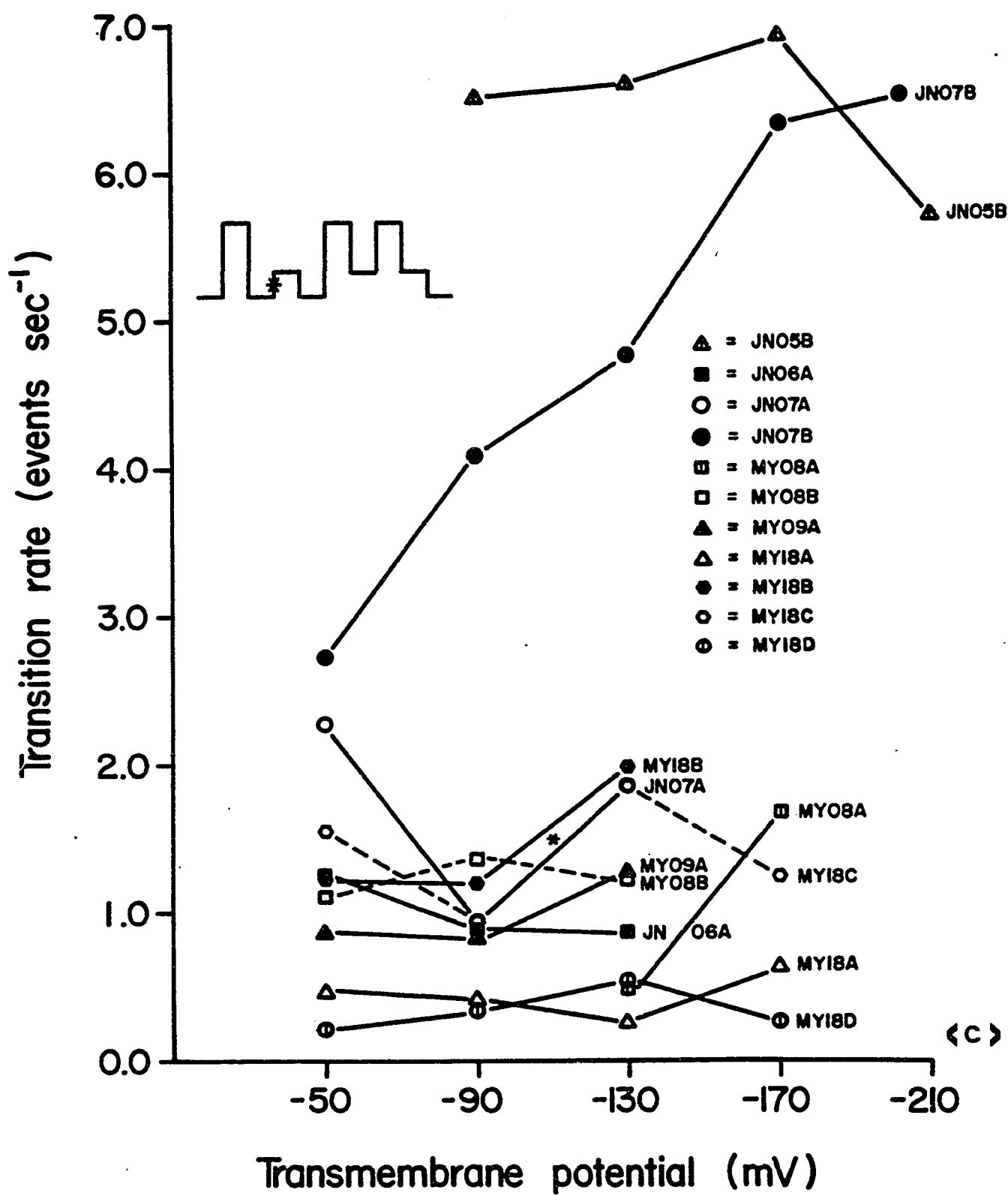
Eleven patches were analyzed for occurrence of specific types of channel openings at a series of standard voltages (-50mV, -90mV, -130mV, -170mV). All patches were cell attached and activated by 10^{-5} M curare. The type of transition implied in the ordinate is indicated as an asterisk placed on a specific transition in the schema of possible channel open configurations. Main conductance openings are the tallest and S1 are the smallest amplitude openings in the schema of upward current jumps.

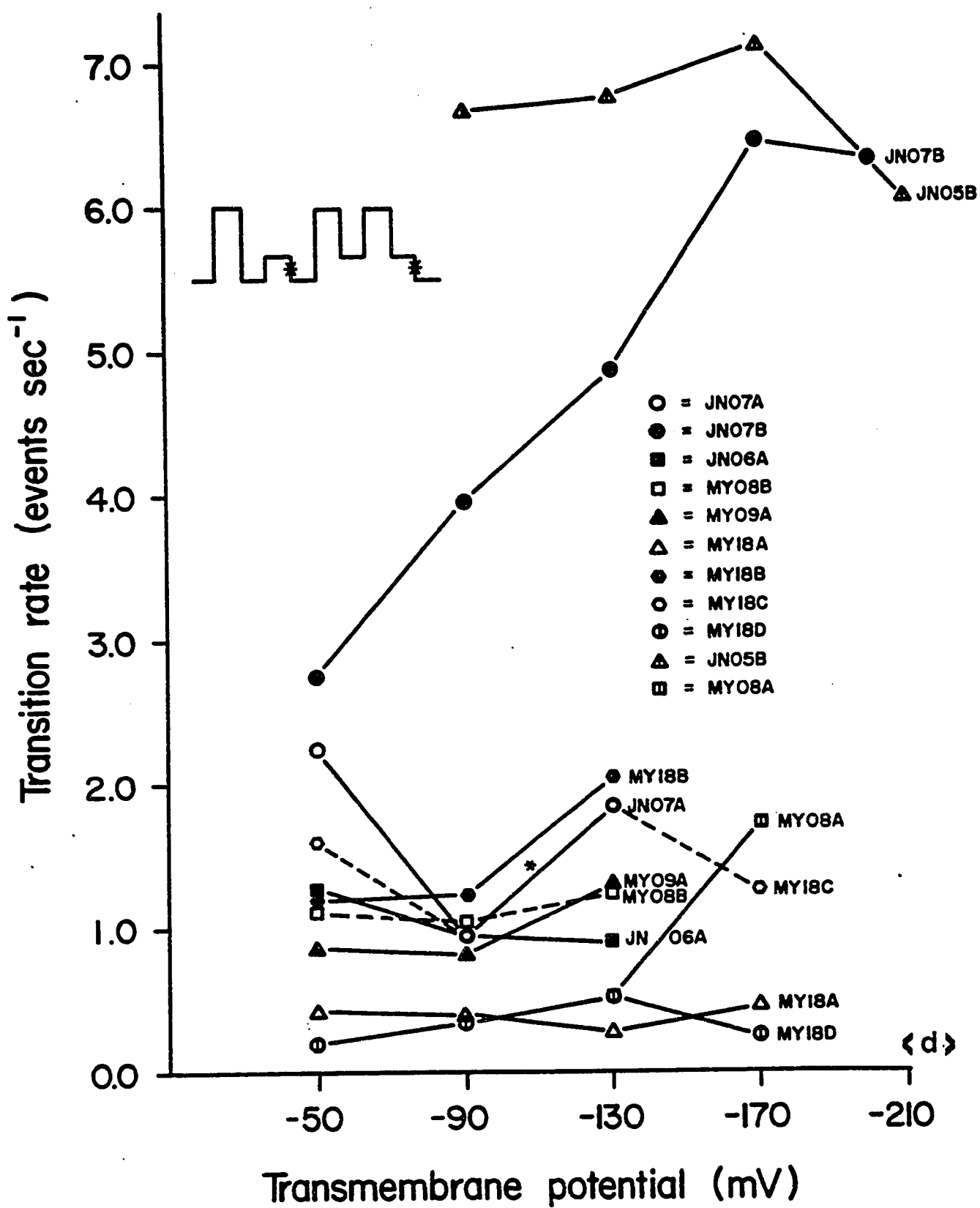
- 8a) Transition between closed state to main open state
- 8b) Transition between main open state to closed state
- 8c) Transition between closed state to substate S1 opening
- 8d) Transition between substate S1 opening to closed state
- 8e) Transition between S1 open state to main open state
- 8f) Transition between main open state to closed state

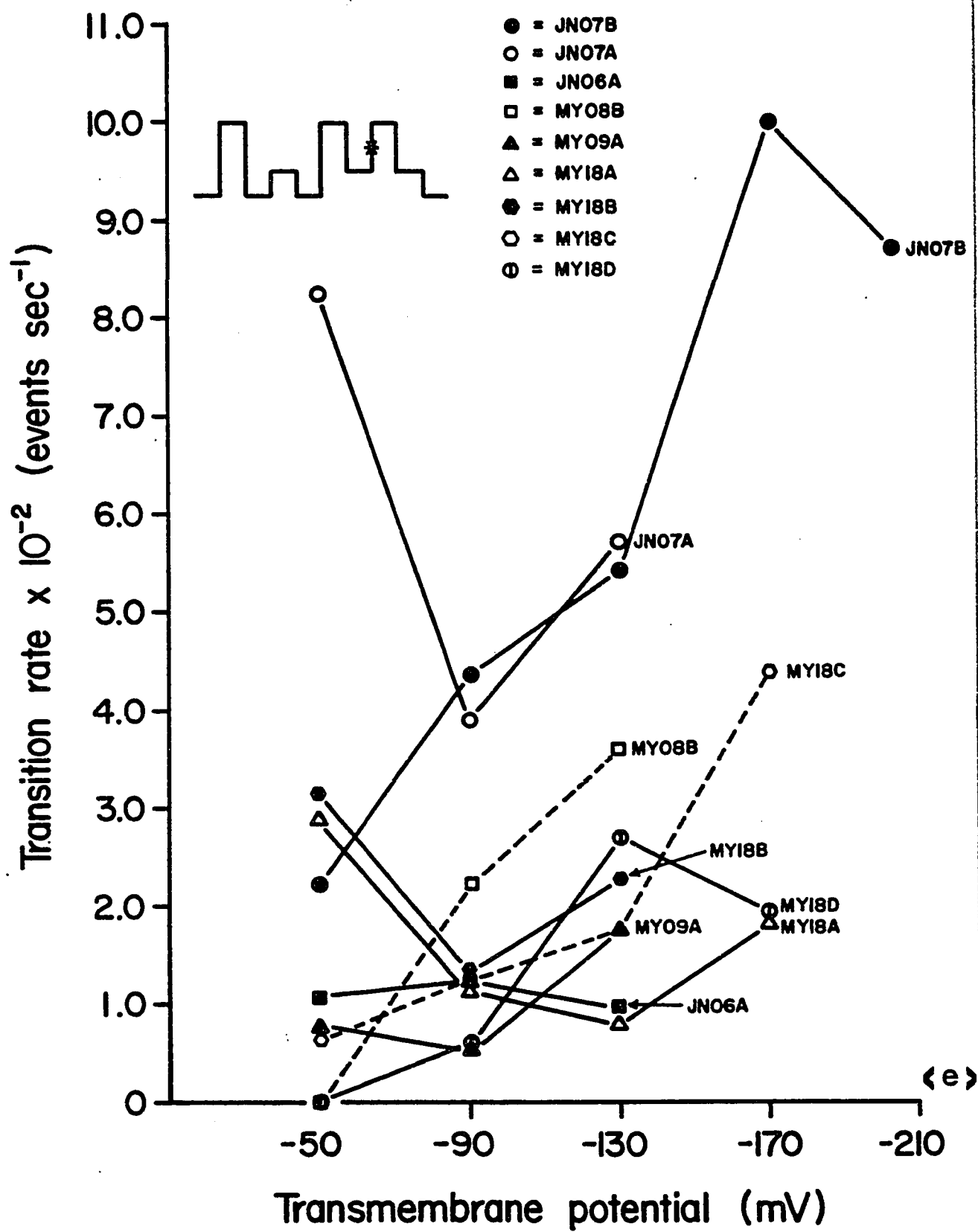


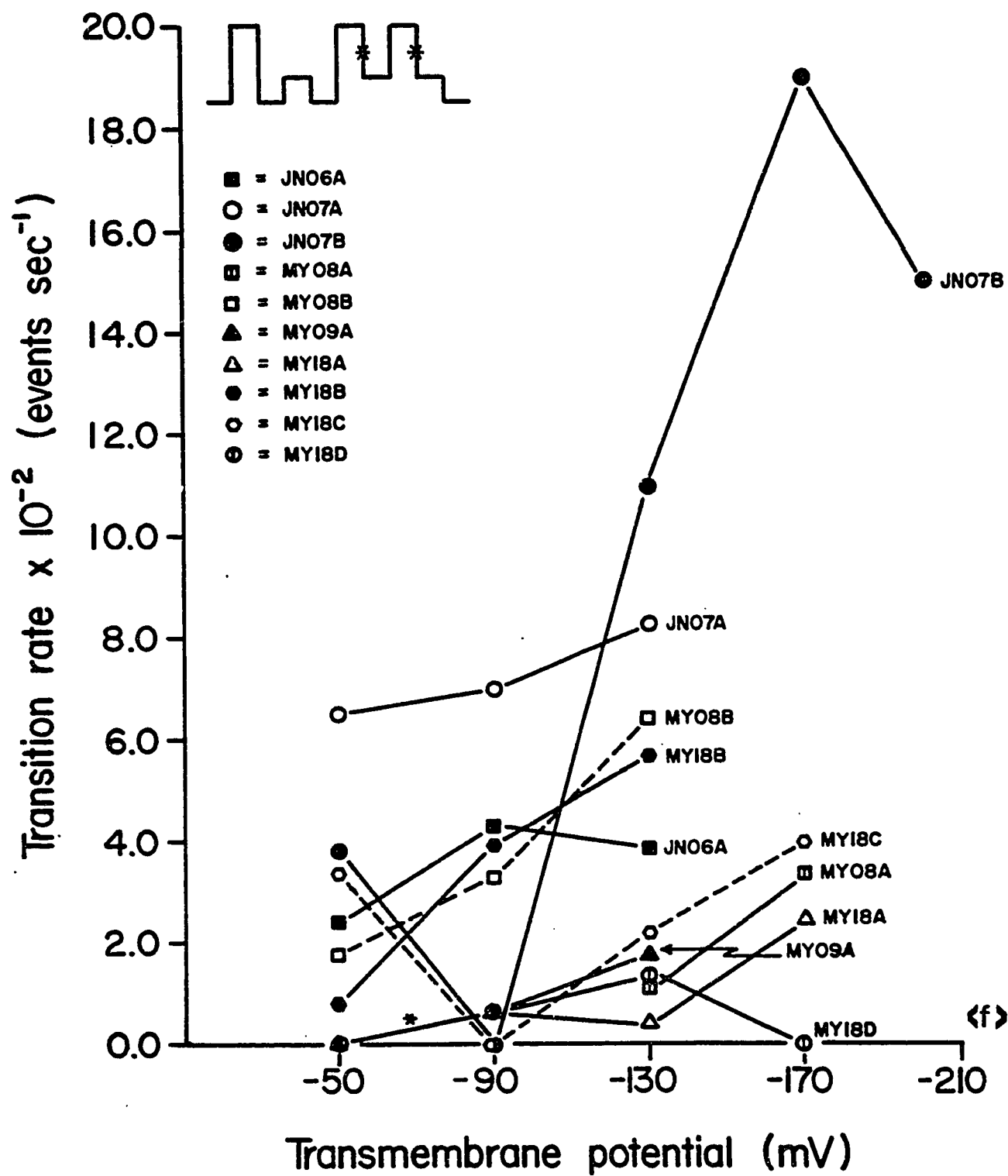
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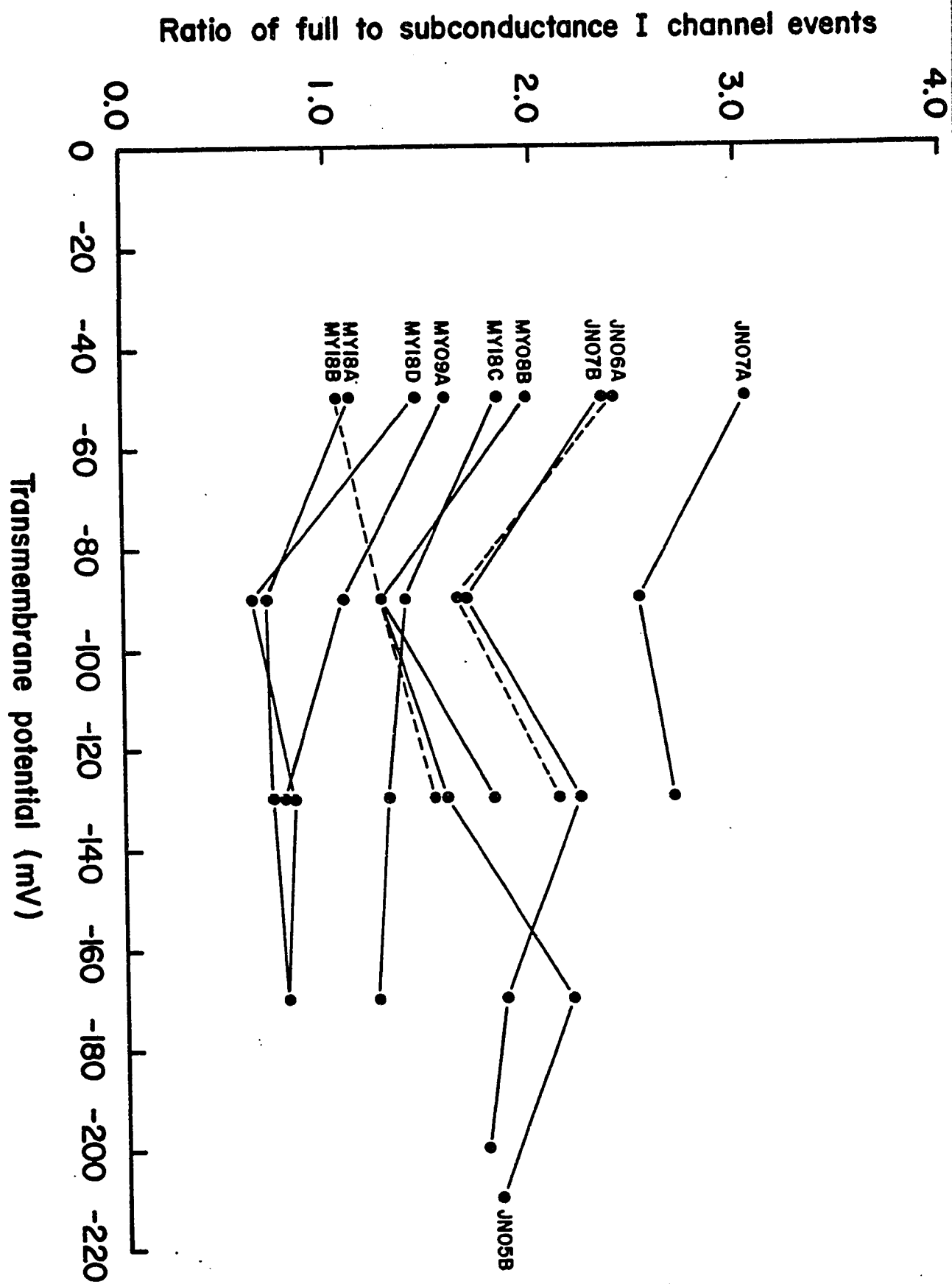


much patch-to-patch scatter and finally some displayed increased activation with hyperpolarization. Variability in transition rates between patches is not surprising since patch size (dependent on pipette tip geometry), channel density, and to some extent the state of AChR development are not expected to be constant. Ideally, a patch containing a single channel would have been more appropriate for such a study.

A potentially more interesting display of kinetic variability is seen, when the ratios of M:S1 events is considered (figure 9). Although the ratio is evidently not a function of voltage, the ratio differs significantly between patches as well as within patches. Problems with exaggeration of subconductance numbers due to difficulty in distinguishing true channel events from noise at low driving forces may be responsible for the elevated S1 values at $V_m = -50\text{mV}$. As well, non-stationarity of the channels (due to desensitization, phosphorylation etc.) may be affecting the values obtained at one voltage and not the others. Therefore, while some trends may be statistically significant between certain test voltages, on a whole no correlation between driving force and M/S1 openings has been revealed. The variability between patches may in fact be a reflection of differences in the developmental state of the AChRs found in the different patches; some AChRs may be S1 competent while others may be S1 incompetent. The differences in the proportion of S1 competent to incompetent may be reflected in the ratios in Figure 9. Inhomogeneity in the gating kinetics of

FIGURE 9

Relationship between ratio of conductance states and the transmembrane potential of the patch. Data graphed was obtained from 9 patches. Channel activation was induced by 10^{-5}M curare with patches in the cell-attached configuration. Patches were clamped at standard voltages (-50mV, -90mV, -130mV, -170mV etc.) for about 5 minutes or until sufficient data on all transition types was obtained.



individual Na^+ channels has been observed in frog skeletal muscle (Patlak, Oritz and Horn, 1986); such heterogeneity in the AChR of rapidly developing cells does not seem unexpected.

B/Characterization of single channel conductance openings of the AChR of G8 cells with various agonists.

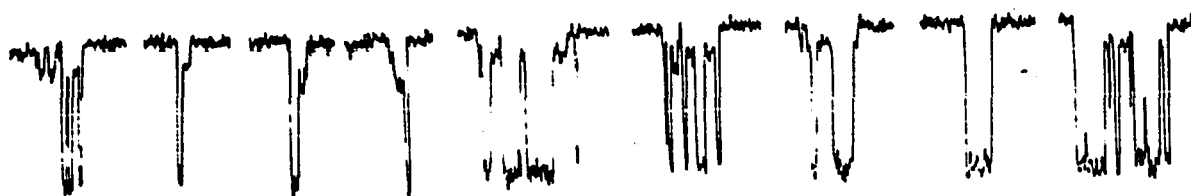
Having seen multiple conductance openings of the AChR of G8 cells for both curare and suberyldicholine in our initial studies, we undertook further characterization of these open states with a variety of agonists. All seven agonists (see Table 1) used in these experiments, displayed activation at the single channel level, of the AChRs of G8 myotubes. Figure 10 illustrates a sampling of conductance states recorded from each agonist. Due to a change in micropipette construction at this stage in the investigation the background noise was reduced drastically and it became possible to distinguish an even smaller conductance state of the receptor/channel complex. As well, the open channel noise of the substate events was greater than both the open channel noise of the M type event and the background noise observed with the closed channel. The term "open channel noise" refers to the flickering seen in the current amplitude of the open AChR/channel. It is important to point out that the events chosen to illustrate different fully resolved conductance levels induced by various agonists under-emphasize the kinetic differences among agonists (not typical kinetic behavior). The most frequent openings remained those previously

FIGURE 10

Sample single channel currents for the agonists Chol, Carb, Nic, SucCh, SubCh, and Cur. For all records, $V_m = -130\text{mV}$. Inward current is downward. For each agonist, all traces are from a single patch. Example of S1 events (double dot), S2 events (single dots) and A events (triple dots) are pointed out in the SubCh traces. For SubCh (*) the time scale is 75ms instead of 30ms. The illustrations underemphasize the briefness of the events. The longer-lasting events are over-represented here to better illustrate the channel conductances.



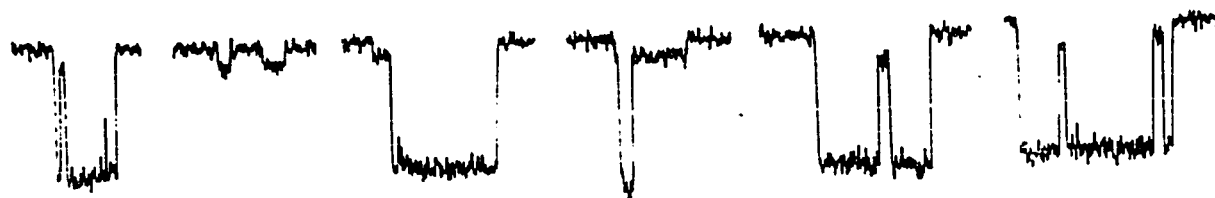
Chol



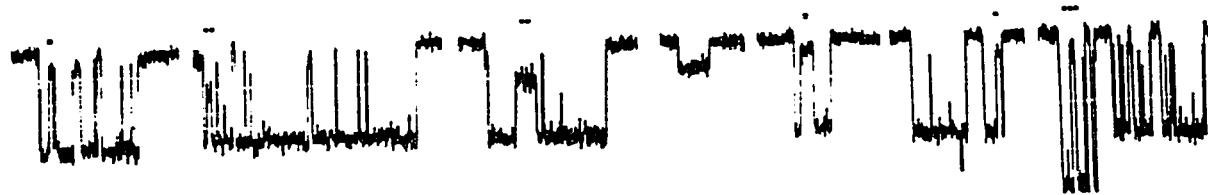
Carb



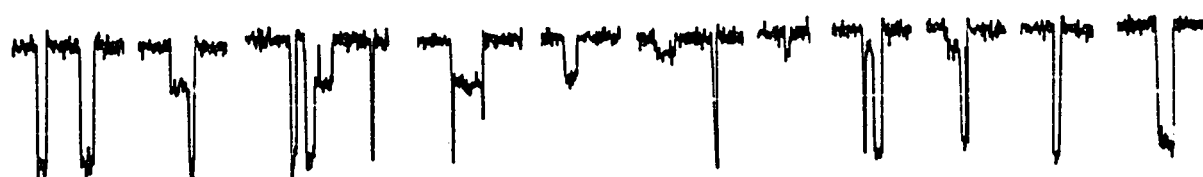
Nic



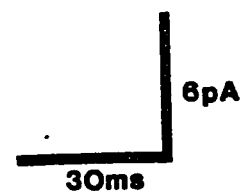
SucCh



SubCh[∞]



Cur



referred to as main (M) type which had a slope conductance of approximately 40pS. Subconductance openings of two classes became apparent in this data. The open state previously referred to as S1 appeared more or less rarely in patches depending on the agonists used; it represents current jumps of about 1/3 the magnitude of the M-type channels. A second, more frequent type of opening with a magnitude of approximately 1/5 of the main type was observed in all patches with all agonists. Both the S1 and S2 subconductance openings appeared in transition with M type events, however, they were also observed to occur in isolation. Transitions between S1 and S2 were extremely rare events. Because transitions occur between main and substates, it has been assumed that S1 and S2 do not represent discrete channel types but are subconductance states of channels of the main (M) type.

Figure 10 also illustrates some channel openings commonly referred to by some workers as adult-(A) type events (Trautmann, 1983) because of their similarity with adult neuromuscular AChR/channel openings. These were approximately 40% greater than the M type. Type A events were very short-lived, rare and not found in all patches. Their appearance did not correlate with the agonist being used. Transition between A and any of the subconductance levels occurred but were extremely rare. No transitions between main and adult type openings were observed. Consequently, these two current levels (main and adult) were assumed to be due to two distinct channel types.

Finally events whose amplitudes were approximately 50% of

the M state were observed very rarely and only on a few patches. These openings may deserve their own designation, however, because no transitions between them and the M or A states have been observed to date we cannot assume that they are associated with the AChR. As a result they were not included in the I/V analysis.

A summary of observations on subconductances from all agonists tested (see Table 1 for abbreviations) follows;

- * Type S2 was present in all patches for all agonists

- * For agonists other than Cur, ACh, Carb and Nic, records did not necessarily contain >20 well-resolved type S1 events at 3 or more of the standard voltages, so slope conductance from I/V curves are omitted in Table 4.

- * ACh, Nic; type S1 openings were only observed on some patches.

- * Carb, SubCh; type S1 openings found in all patches but very infrequent.

- * Cur; type S1 openings found in all patches and frequent.

- * SucCh; type S1 events were rare. Events that may have been a larger type channel (i.e. 50% of M type were observed) were not included in the analysis.

- * ACh; one patch had both the normal and larger type S1 conductances, however, transitions between these were not observed.
- * Chol; extremely few S1 events occurring in transition with M or A type channel events.
- * No agonist controls; very rare unequivocal subconductances (due to the briefness of these channel events - unresolved M type openings could erroneously be categorized as S1 or S2).

Conductance

The conductance values for the different open states of the AChR calculated for each agonist are summarized in Table 4. The slope conductance was measured whenever possible from I/V curves. Certain substate conductances are, however, expressed as percentages of the M-type, because these rare substates did not appear at several voltages in the record. This is especially true for S1 events which (except when curare is the agonist) were sporadic or even absent in some patches. Conductance values found in Table 4 suggest that there exist more-or-less discrete open states of the AChR with stereotypical current flow and that except in the case of SubCh there is no agonist - dependence in the determination of subconductance current levels. Values in the conductance tables come from raw data similar to that displayed in figure 11 and 12. The amplitude of ≥ 20 events of each current type were measured and averages were plotted on I/V curves specific for each batch and agonist used.

TABLE 4: Magnitude of RChR conductance states
(% of type H)

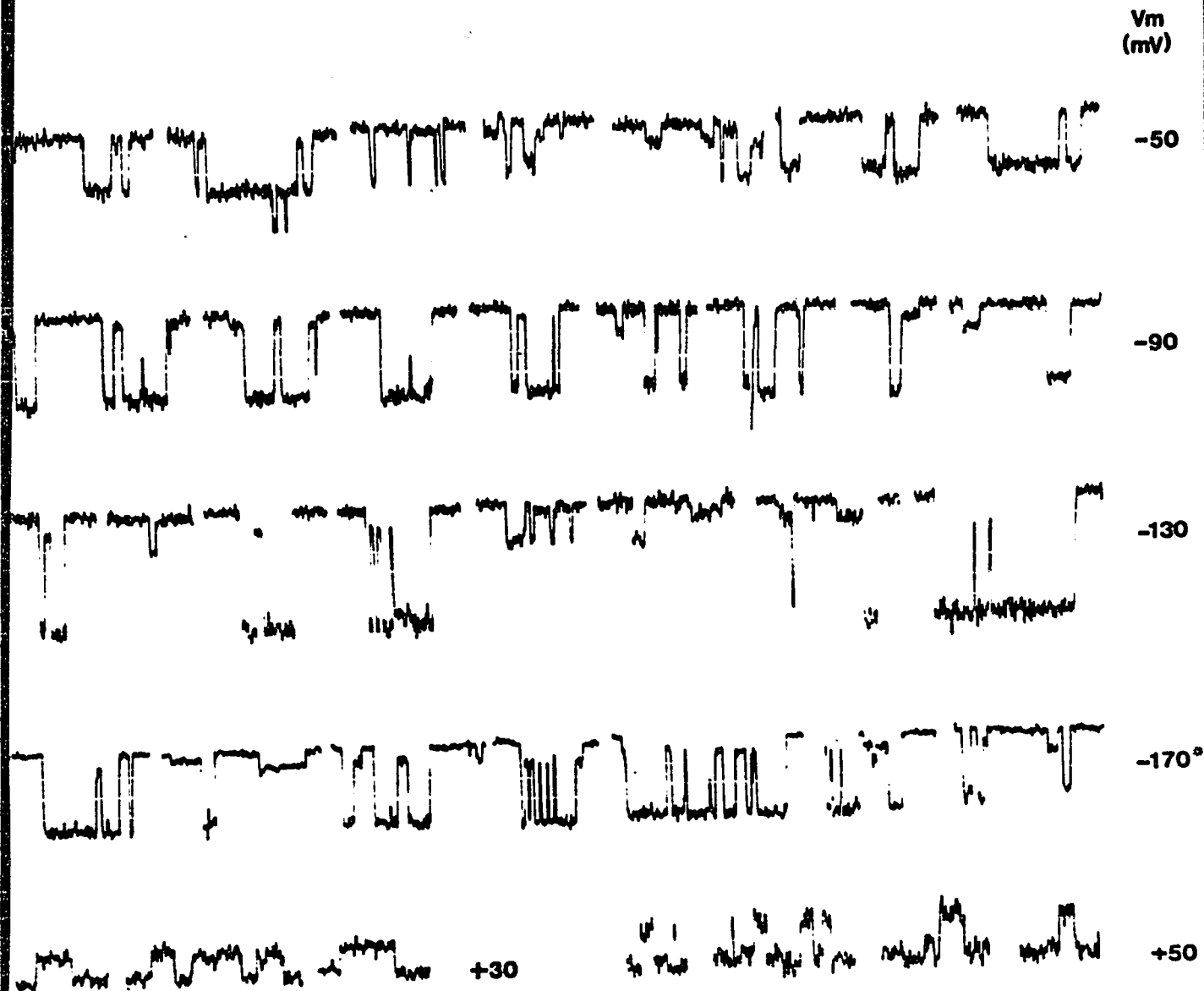
Agonist	H	Conductance Types		No. of patches
		S1	S2	
Chol	42.6 ± 2.3	----- (31.3)	----- (18.4)	3
RCh	40.8 ± 1.0	11.6 ± 0.8 (28.4)	7.3 ± 1.1 (17.9)	4
Carb	41.5 ± 1.9	11.0 ± 1.3 (28.4)	7.1 ± 0.6 (17.1)	4
Nic	40.6 ± 2.6	12.5 ± 0.6 (30.7)	8.1 ± 1.3 (20.0)	6
SucCh	43.4 ± 2.5	----- (33.9)	7.1 ± 0.4 (16.3)	3
SubCh	34.8 ± 2.6	----- (31.6)	----- (21.8)	6
Cur	36.9 ± 2.5	14.0 ± 2.9 (37.8)	6.1 ± 0.8 (16.5)	5
Control	39.8 ± 2.7	----- (34.6)	7.0 ± 0.7 (17.5)	5

Values are in picosiemens ± SE (percentage of type H openings)

Conductances for different agonists were not significantly different from conductance values found with RCh as the standard agonist.
Student's t test; (P < 0.05).

FIGURE 11

ACh-induced events recorded at the four test voltages (-50, -90, -130 and -170mV) as well as two voltages which caused outward current flow (+30, +50mV). For -170mV (*) the scale is 12pA instead of 6pA.



10⁻⁶M ACh M.W. 146

FIGURE 12

Curare-induced events recorded at the four test voltages (-50, -90, -130 and -170mV). Channel events were chosen so as to best illustrate various conductance levels and transition sequences between various open states. Because fully-resolved events were specifically chosen, the briefness of typical curare activated channel openings is underemphasized.

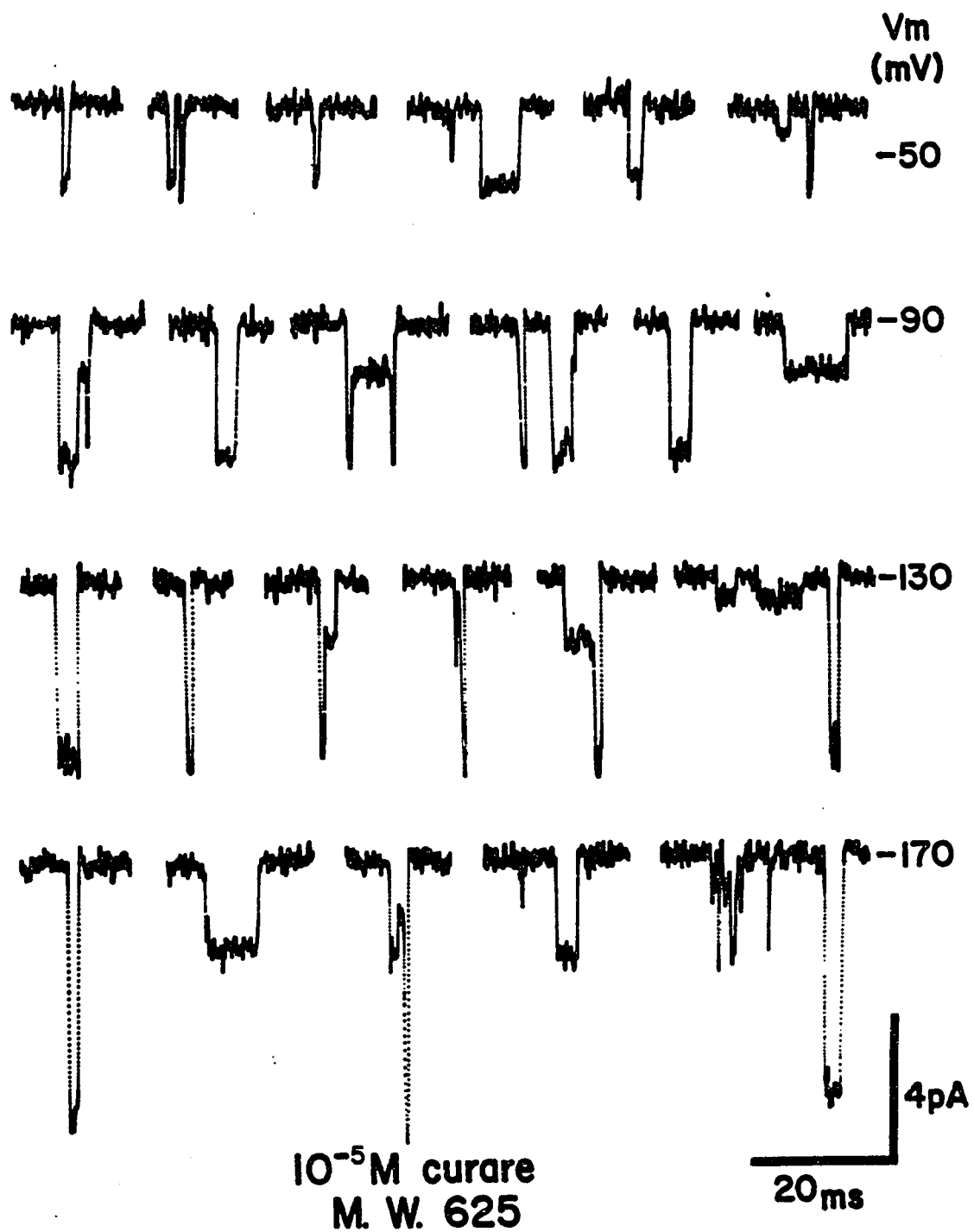
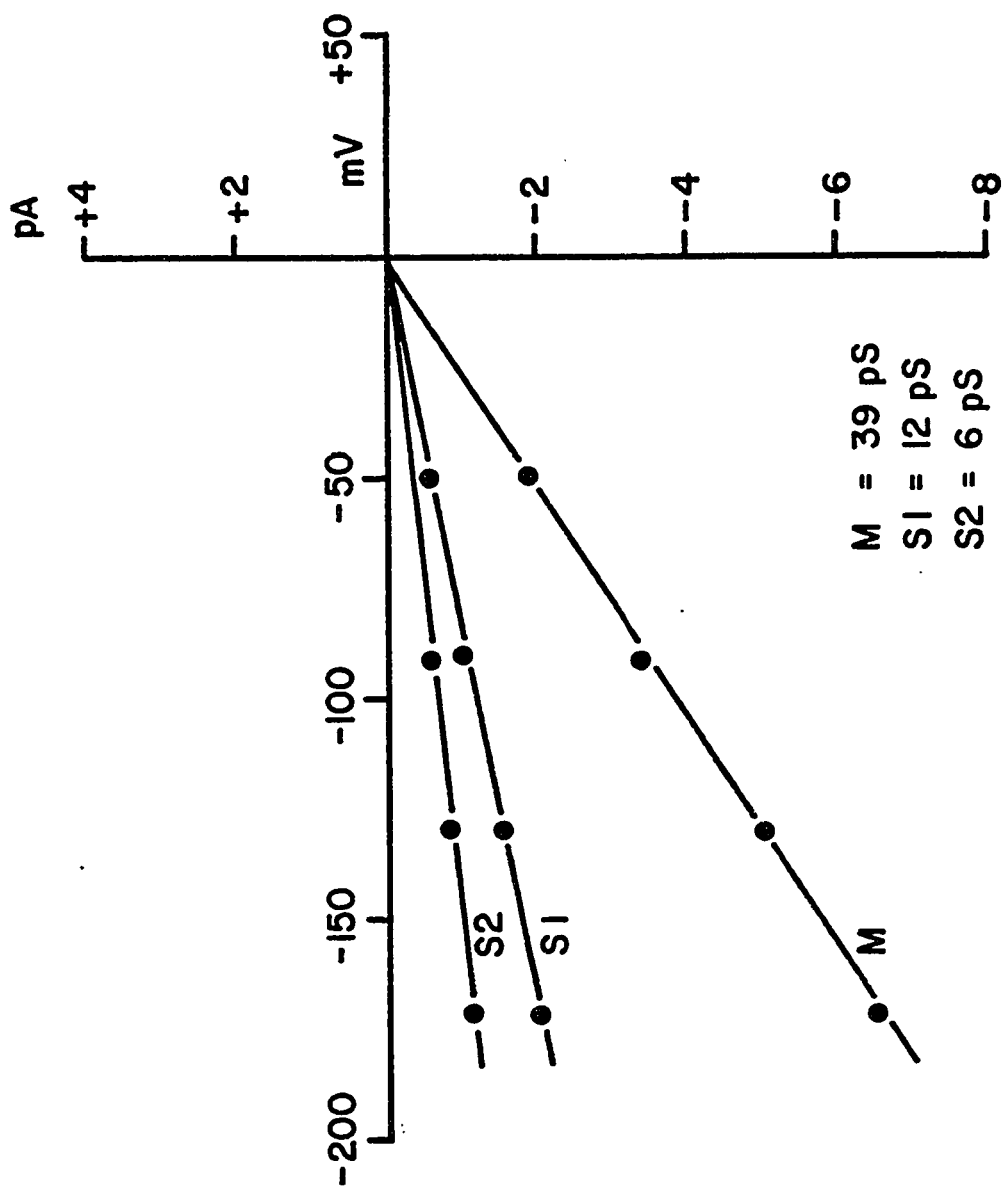


FIGURE 13

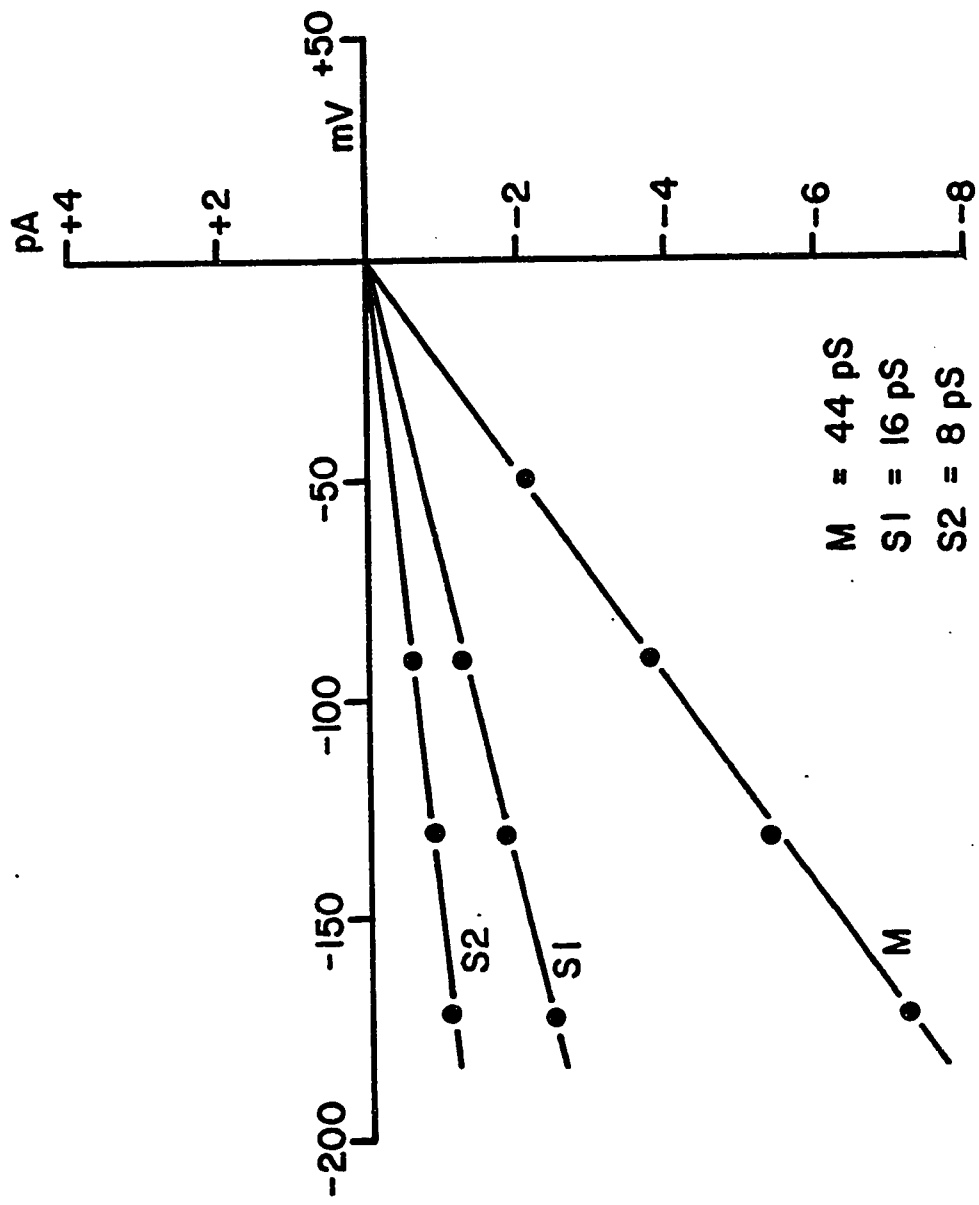
Single channel I/V relations for events of types M, S1, S2 for a patch exposed to ACh. The lines are linear least squares regression fits, whose slope (in picosiemens) is indicated. Note that the extrapolated x-axis intercept of each line indicates the reversal potential for the respective conductance class.



10^{-6} M ACETYLCHOLINE M.W. 146

FIGURE 14

Single channel I/V relation for events of types M, S1 and S2 for a patch exposed to Cur. The lines are linear least squares regression fits, whose slope (in picosiemens) is indicated. Note that the extrapolated x-axis intercept of each line indicates the reversal potential for the respective conductance class.



$10^{-5} \text{ M CURARE M.W. 625}$

Figures 13 and 14 are samples of curves on which linear regression analysis has been performed and slope conductances have been extracted. Values appearing in the conductance table are means from several patches. Table 5 summarizes the values obtained for zero-current intercepts (i.e. reversal potentials $\pm$ S.E.) of the I/V curves extrapolated from the slope conductances for each open state and agonist.

When S1 and S2 type events were too small or brief to be resolved at -50mV, the measurements of conductances were based on events at -90, -130, and -170mV. No correlation was seen in this data between the subconductance values and the molecular weight of the agonist used (increasing M.W. - descending order in Table 1). S1-type events of about the same size were observed regardless of the agonist. The same was true for S2 events for all agonists.

A-type channel states

A-type openings were observed in at least one patch per agonist. In most cases, these were easily distinguished from the M-type of channel openings. A-type versus M-type event amplitude ratios for various drugs are summarized in Table 6 ($\pm$ S.E.). A-type openings were so infrequent that they generally were only obtained from the long records made at -130mV for kinetic analysis. As a result, no I/V curves could be constructed and the conductance measurements represent ratios with the M-type current amplitudes measured in the same record. This

TABLE 5: Reversal potentials of the different conductance states using various agonists

Agonist	No. of patches	Reversal potentials *	
		S1	S2
Chol	3	-----	-----
		-0.7mV $\pm$ 0.4	
ACH	4	-2.8mV $\pm$ 0.8	-0.9mV $\pm$ 0.3
		-1.2mV $\pm$ 0.3	
Carb	4	0.2mV $\pm$ 1.7	-2.8mV $\pm$ 1.7
		1.3mV $\pm$ 1.0	
Nic	6	0.5mV $\pm$ 0.8	1.8mV $\pm$ 0.8
		1.3mV $\pm$ 1.1	
SuccCh	3	-----	-0.3mV $\pm$ 1.6
		2.4mV $\pm$ 1.8	
SubCh	6	-----	-----
		-2.8mV $\pm$ 1.7	
Cur	5	-4.2mV $\pm$ 1.1	-2.9mV $\pm$ 0.9
		-3.6mV $\pm$ 0.7	
control	5	-----	-3.4mV $\pm$ 0.8
		-3.8mV $\pm$ 0.4	

* Reversal potentials represent averages of the zero-current voltage for several patches.
 $\pm$ S.E.

TABLE 6: Conductance of R-type channel events in 68 cells.

Agonist	No. of patches	No. of events	Conductance Ratio*
Cho1	3	29	1.38 ± 0.015
Rich	3	18	1.41 ± 0.019
Carb	2	18	1.37 ± 0.025
Nic	2	13	1.37 ± 0.029
SucCh	1	13	1.42 ± 0.025
SubCh	1	6	1.28 ± 0.024
Cur	3	12	1.31 ± 0.061

Conductance ratios = R-type currents/M-type currents

R-type openings in no-agonist controls could not be differentiated from M-type spontaneous openings. In both cases, the majority of channel events were unresolved (ie. closing of the channel occurred before the recording system reached the full open amplitude for current measurements).

The conductance of Curare-activated R-type events was not shown to be significantly different from the other agonist induced R-type openings.

SubCh activated R-type openings were however significantly different; Student's t test ($P > 0.05$)

assumes that M and A events share the same reversal potential. SubCh A-type channels were of marginally lower (relative) conductance than the other agonists. It has been assumed, nevertheless, that these A-type openings were indeed associated with AChR/channel complexes because they shared reversal potentials of $0\text{mV} \pm 10\text{mV}$; they have been observed in transition with S1 and S2 type openings; their kinetic behavior was similar to M-type events with the same agonist and their conductance and kinetic properties resemble that of adult AChR opening observed in other preparations (Leibowitz and Dionne, 1984).

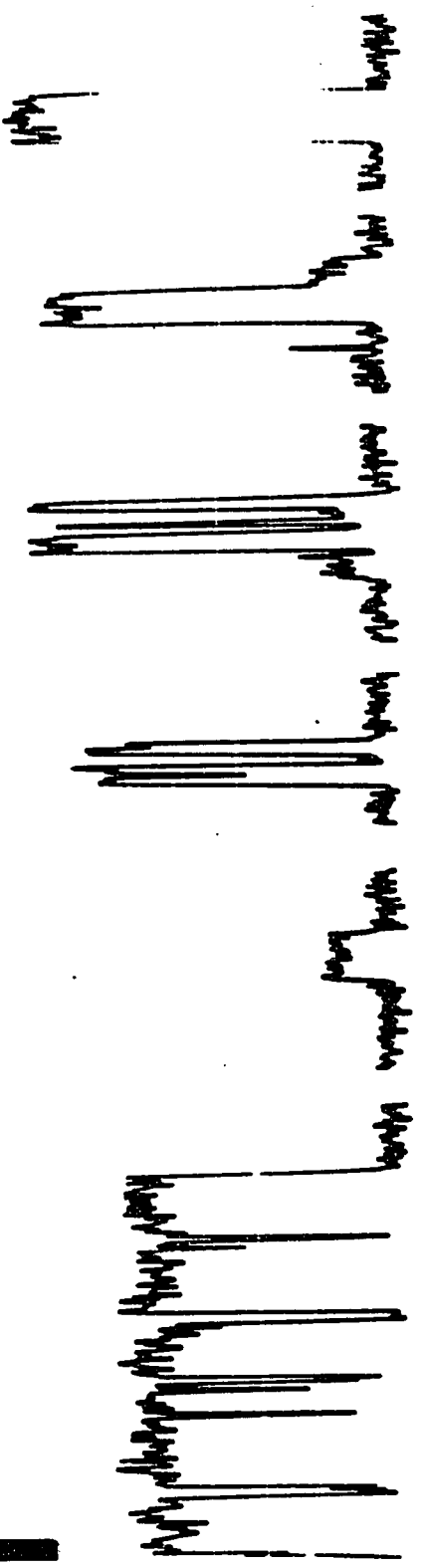
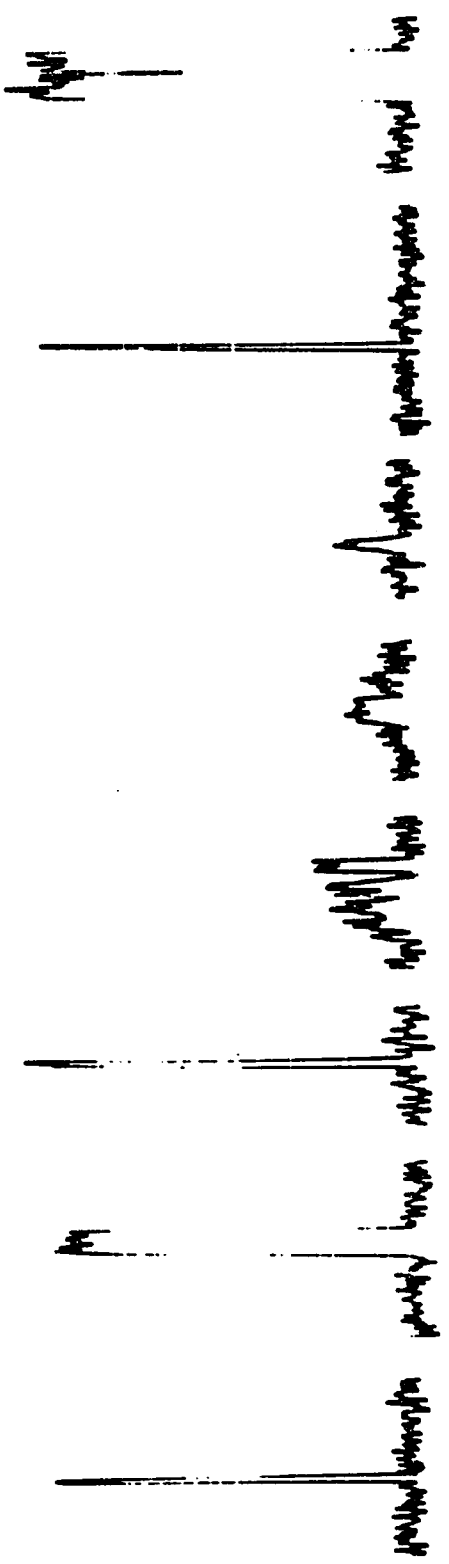
No agonist controls

In recent reports on primary cultured mouse myotubes, Jackson (1984, 1986) revealed that immature AChRs are able to open spontaneously, albeit with very low frequency and only very briefly. Spontaneous openings were observed with the clonal myotubes, G8 (figure 15).

No-agonist controls were carried out on 20 patches; of these, four showed no activity. Fourteen patches showed extremely low activity (figure 15, top line); activation frequencies were variable between (0.009-0.333 events/sec for type M openings $\bar{x}=0.117 \pm 0.031$) (0-0.074 events/sec for type S2 openings $\bar{x}=0.024 \pm 0.006$) and two other patches showed very high activity.

FIGURE 15

A selection of events recorded in the absence of agonist. V_m is -130mV . The events on the first line are from a patch which showed such occurrences less than 10 times/minute (one of the low frequency patches described in the text), whereas those on the second line are from one of the two high frequency patches. Small conductance events occurring either in isolation or in combination with large events were observed in the high frequency patches. Channels illustrated from the low frequency patch underemphasize the briefness of the average openings. These long events were chosen to best illustrate the conductance levels observed in the spontaneous openings.



No agonist

20 ms

4 pA

In the patches with low spontaneous activity, M and S2 type events were frequent enough for slope conductance determination (Table 4), however S1 openings were extremely infrequent and their conductance was determined as a percentage of the M type openings. Events categorized as S1 and S2 openings were found mostly in isolation (i.e. not in transition with M or A type events), therefore we are assuming they are in fact AChR openings on the basis of their conductance, similarities with subconductances seen with agonists (i.e. 1/3 and 1/5 of M type events respectively). As well, the reversal potential of S2 openings is $-3.0\text{mV} \pm 0.04\text{mV}$ while M-type openings $V_{\text{rev}} = -3.4\text{mV} \pm 0.8\text{mV}$; these values are in agreement with typical AChR reversal potentials. Because of their brevity, unresolved M type (or even A-type) openings may have been erroneously categorized as S1 or S2 openings.

Open times for these spontaneous channel events could not be determined by fitting histograms (see kinetics section) because the events were too few in number. The most straightforward method for estimating the mean open time was to use the maximum-likelihood formula;

$$\tau = \bar{\tau} - t_{\text{min}}$$

where τ = estimate of lifetime in specific open state, $\bar{\tau}$ = mean of observation of specific open states and t_{min} = lower limit of observations included in τ (0.150ms) (c.f. Colquhoun and Sigworth, 1983).

Because it was necessary for events to be of long enough duration for classification according to conductance level, those events measured were longer than the norm. However, subtracting t_{min} corrects for this, assuming (and the assumption may not be justified) that the remaining events are part of a single exponential distribution;

$$M = 0.39ms (0.542 - 0.150ms) \pm 0.04ms$$

$$S1 = 0.43ms (0.583 - 0.150ms) \pm 0.06ms$$

$$S2 = 0.41ms (0.560 - 0.150ms) \pm 0.02ms$$

This problem seems less critical for the S1 and S2 spontaneous openings which opened for longer periods of time with much fewer of the brief openings. However, some of these patches displayed one or two exceptionally long openings which were presumed to relate to some other phenomenon (eg. M type (16.450ms), S1(3.35 ms) and S2 (7.100ms). Measurements were taken on events occurring at -130mV.

On the two patches which were perhaps from hot spots, a much higher frequency of spontaneous activity was observed (figure 15; line two). This activity consisted of M type events which appeared to be from two different kinetic distributions. Very short-lived openings, similar to those in the low frequency patches, were observed along with others with much longer open times. The latter looked disconcertingly as if they had been agonist activated. Scrupulous care was taken, however, to avoid any contamination in these control experiments. These abnormal patches also contained S1 and S2 type events which were found in

transition with the brief and longer M type openings as well as in isolation. In light of this last observation it is impossible to dismiss these channels as non-AChR associated; at the same time, labelling them as AChR/channel complex openings solely on the presence of sublevel openings and conductance measurements would be premature. These events may be due to AChRs activated either spontaneously or by some unknown agonist. A possibility may be that they are "hot spot" AChRs undergoing some processing or maturation which renders them unstable and prone to bursting behavior. If there are "hot spots" on these cells, they would not have interfered with normal agonist recording since agonist induced activity would have been so high in a "hot spot" patch that one would have been forced to discard the data due to openings of several channels at one time.

Probabilities for various open states

Even from visual inspection of the single channel data for different agonists, it is apparent that some drugs are more effective than others at activating the AChR/channel complexes of G8 myotubes (SubCh, ACh, Nic, SucChol, Carb, Chol and Cur; in descending order of agonistic ability). These results were not unexpected given previous studies with these known agonists. What was surprising, however, was that when the conditional probabilities (i.e. the probability that given a channel was open, it was open in a specific state (M, S1, S2)) were calculated, there were no striking agonist-dependent differences

TABLE 7: Probability that, given a channel is open; it is open in state M, S1, S2.

Agonist	S2	S1	M	No. of patches
Chol	0.057 ± 0.012	0.029 ± 0.005	0.914 ± 0.017	3
ACH	0.102 ± 0.033	0.012 ± 0.003	0.063 ± 0.037	4
Carb	0.007 ± 0.029	0.007 ± 0.003	0.904 ± 0.033	4
Nic	0.147 ± 0.014	0.036 ± 0.026	0.009 ± 0.059	6
SucCh	0.110 ± 0.049	0.034 ± 0.019	0.020 ± 0.011	3
SubCh	0.105 ± 0.051	0.027 ± 0.016	0.004 ± 0.050	6
Cur	0.264 ± 0.045	0.135 ± 0.022	0.602 ± 0.065	5

S2; Both SubCh and Cur values are significantly different [($P > 0.05$) with ACh as the standard].

S1; Both Carb and Cur values are significantly different from the values found with ACh as the standard agonist.

M; Cur values are significantly different from the values found for the standard agonist ACh.

except with curare (Table 7). The greatest variability in the data were observed in the probabilities of being in S1 conductance state. The conditional probability of being in the S1 open state, for curare-induced events was 5X that for Chol, SubCh, SucCh, and Nic. Carb and ACh activated events, however, had probabilities of being in the S1 state lower than the other five agonists. For G8 cells, the conditional probability of being in the S2 state was similar between agonists other than curare and suberyldicholine. Regardless of the agonist and the relative probabilities of the subconductances, the M conductance state was the most probable open state. Curare was the only agonist for which other substates can be regarded as commonplace.

Reversibility between transitions

Previous single channel data (figure 11 and 12) have shown that the G8 AChR/channel complexes, when activated, may open to several different conductance levels which can in turn oscillate among each other. The most frequent transitions observed were between M and S1 type events and M and S2 type events. However, transitions between the two subtypes did occur though extremely rarely. These transitions were sufficiently infrequent that it is possible that they were in fact transitions from one substate to an unresolved M-type opening then back to another substate.

TABLE 8: Reversibility measurements: Ratios of one sequence of transition events to its mirror image.

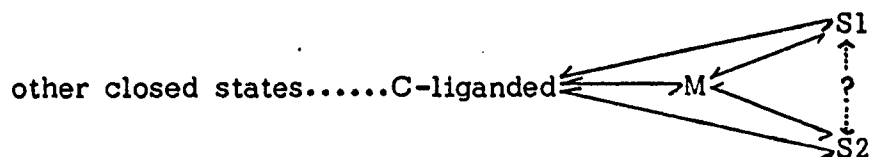
Transition sequences	Agonists					
	Chol	RCh	Carb	Nic	SuccCh	SubCh
No. of patches	3	4	4	6	3	6
Cur	5					
S1→H, H→S2	1.04±0.09	0.99±0.02	1.04±0.12	0.90±0.02	0.97±0.05	0.98±0.03
S2→H, H→S2	0.79±0.16	0.97±0.03	0.92±0.04	0.96±0.09	1.03±0.01	0.97±0.01
S2→S1, S1→S2	0.99±0.02	0.89±0.06	1.04±0.10	0.05±0.06	1.00±0.003	0.92±0.07
C→H→S2→C, C→S2→H→C	1.06±0.24	1.10±0.06	1.11±0.08	1.42±0.38	1.05±0.29	1.02±0.04
C→H→S1→C, C→S1→H→C	1.00x	0.97±0.03	0.88±0.43x	1.06±0.12	0.47±0.24x	1.16±0.23
						1.70x

xVery low n values i.e. (for C→H→S1→C/C→S1→H→C for curare; 6/2 events out of 28,307 transitions).

Note; C=closed state, H=main state, S1=30% and S2=20% of H-type conductances

; Ratios=1 indicate that the transition sequence is freely reversible.

Alternatively, $S1 \longleftrightarrow S2$ transitions may be spurious products of the automatic analysis routine. Open channel noise for a single subconductance event could have contained peaks which were attributed to the $S1$ and $S2$ categories. Data on these apparent transitions are included for completeness, but the information we have is not sufficient to convince us that they represent real transitions. Table 8 summarizes data for different transition sequences; values represent the ratio of transitions of a given sequence compared to the reverse sequence. If the channel system is in equilibrium, it is expected that the transitions from one open state to another would be equal in number to their reverse transitions. For most agonists, the data displays the reversibility characteristics of a system in equilibrium (re. Table 8, first 3 transition sequences and their reciprocals). Perhaps the only noteworthy deviation from a ratio of unity is the case of $S1 \rightarrow M$, $M \rightarrow S1$ for curare. As a test for microscopic reversibility, which is expected in a cyclic scheme at equilibrium, we compared the number of four state transitions (closed to substate to main to closed) sequences in one direction with the number of occurrences of the reciprocal sequences. From the data in table 8, we see that the various transition sequences tested for were reversible; there was no significant difference between transition types ($P < 0.05$). This implies that M , $S1$, $S2$ open states may in fact be communicating states within the AChR/channel complex and oscillations between states may involve a cyclic pattern.



Note that, for the reasons mentioned above, we have chosen not to include a direct link between S1 and S2, although this difficult-to-resolve transition may be real. For those values with large standard errors and those with asterisks, the variability can probably be attributed to the low number of event sequences of interest which occurred in the records. For example, one patch, included in the curare mean ratio for $C \rightarrow M \rightarrow S1 \rightarrow C / C \rightarrow S1 \rightarrow M \rightarrow C$, had only 2 openings in one direction and 1 in the other. In such cases, the data was not subjected to a statistical test. However, it is evident that there are no severe discrepancies from microscopic reversability present in the data.

Open times

Kinetic analysis of open time distributions was performed to determine the number of kinetically distinguishable open states which the ACh/channel can assume when activated by the seven different agonists. Ideally, one would prefer to use data from one-channel patches containing several thousand transitions for such analysis. However, in practice, data from several multi-channel patches were lumped together into a larger data set. Computerized fitting of data, using the Marquardt nonlinear least squares algorithm yielded rate-constants for

the transitions which were extracted from regressions of the sum of exponential probability density functions.

Probability density for:

one exponential: $N(t) = N_f(1/T_f) \exp(-t/T_f)$

two exponential: $N(t) = N_f(1/T_f) \exp(-t/T_f) + N_s(1/T_s) \exp(-t/T_s)$

three exponential: $N(t) = N_f(1/T_f) \exp(-t/T_f) + N_s(1/T_s) \exp(-t/T_s) + N_z(1/T_z) \exp(-t/T_z)$

Here $N(t)$ denotes the number of events with duration between time t and $t+dt$. The probability density function itself is continuous, but to fit real data, it is, of course necessary to chose bins of some finite binwidth, as described in the Methods. T_f , T_s and T_z are the mean durations for the fast, slow and extra slow components for the open time distributions. The inverse of these means are referred to as rate constants; the means themselves are often termed time constants or τ . N_f , N_s and N_z are the estimated number of each type of event obtained by integrating over the probability density function from $t=0$ to $t=\infty$. The open time distributions of the various types of open states (ie. M, S1, S2) consistently fit better to a sum of two exponentials than to a single exponential. Examples of open time histograms for M, S1 and S2 type openings activated by curare are illustrated in figures 16, 17 and 18. Analysis of the data for closed times was not performed since the focus of this work is on open states. A summary of mean open times for various conductance states activated by the seven agonists appears in Table 9. Examples of different fits to the same data are illustrated in

TABLE 9: Mean open times (τ) of various conductance states activated by a variety of agonists.

Conductance levels	Agonists						
	Cur	Chol	Carb	SucCh	Nic	RCh	SubCh
R-type openings							
$\langle \tau \rangle$ fast	0.23 ± 0.01	0.54 ± 0.06	0.20 ± 0.04	0.32 ± 0.05	0.33 ± 0.05	0.25 ± 0.00	0.55 ± 0.04
$\langle \tau \rangle$ slow	1.47 ± 0.07	3.06 ± 0.33	6.77 ± 0.43	7.15 ± 0.35	0.06 ± 1.43	9.69 ± 0.49	19.14 ± 0.99
S1-type openings							
$\langle \tau \rangle$ fast	0.05 ± 0.002	0.07 ± 0.01	0.06 ± 0.01	0.00 ± 0.003	0.07 ± 0.01	0.09 ± 0.01	0.13 ± 0.01
$\langle \tau \rangle$ slow	1.45 ± 0.33	1.25 ± 0.24	0.59 ± 0.31	1.35 ± 0.25	0.50 ± 0.06	0.52 ± 0.16	1.00
S2-type openings							
$\langle \tau \rangle$ fast	0.07 ± 0.004	0.07 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.04 ± 0.01	0.08 ± 0.01
$\langle \tau \rangle$ slow	0.56 ± 0.11	0.34 ± 0.05	0.57 ± 0.11	0.47 ± 0.10	0.40 ± 0.06	0.39 ± 0.06	0.35 ± 0.07
$\langle \tau \rangle$ very slow	-----	-----	-----	1.96 ± 0.98	-----	1.32 ± 0.13	1.53 ± 0.99

Note: $\langle \tau \rangle$ is expressed in ms.

$\langle \tau \rangle$ was calculated from the inverse of rate constants found in open time histograms (see methods)

$\langle \tau \rangle$ for each drug is extracted from compiled open time histograms containing several patches;
Cur 5 patches, Chol 3, Carb 4, SucCh 3, Nic 6, RCh 4, SubCh 4.

FIGURE 16

Open time histogram for curare-activated M-type openings of the AChR/channel of G8 myotubes. Histograms were constructed with data from 5 cell-attached patches held at -130mV . The time constants associated with the fitted function (solid line drawn over histogram bins) are indicated. Although data were rebinned for the fitting process (see methods) it is displayed here with uniform size binwidths.

OPEN TIME DISTRIBUTION OF M-TYPE EVENT

CURARE AS AGONIST, $V_m = -130\text{mV}$

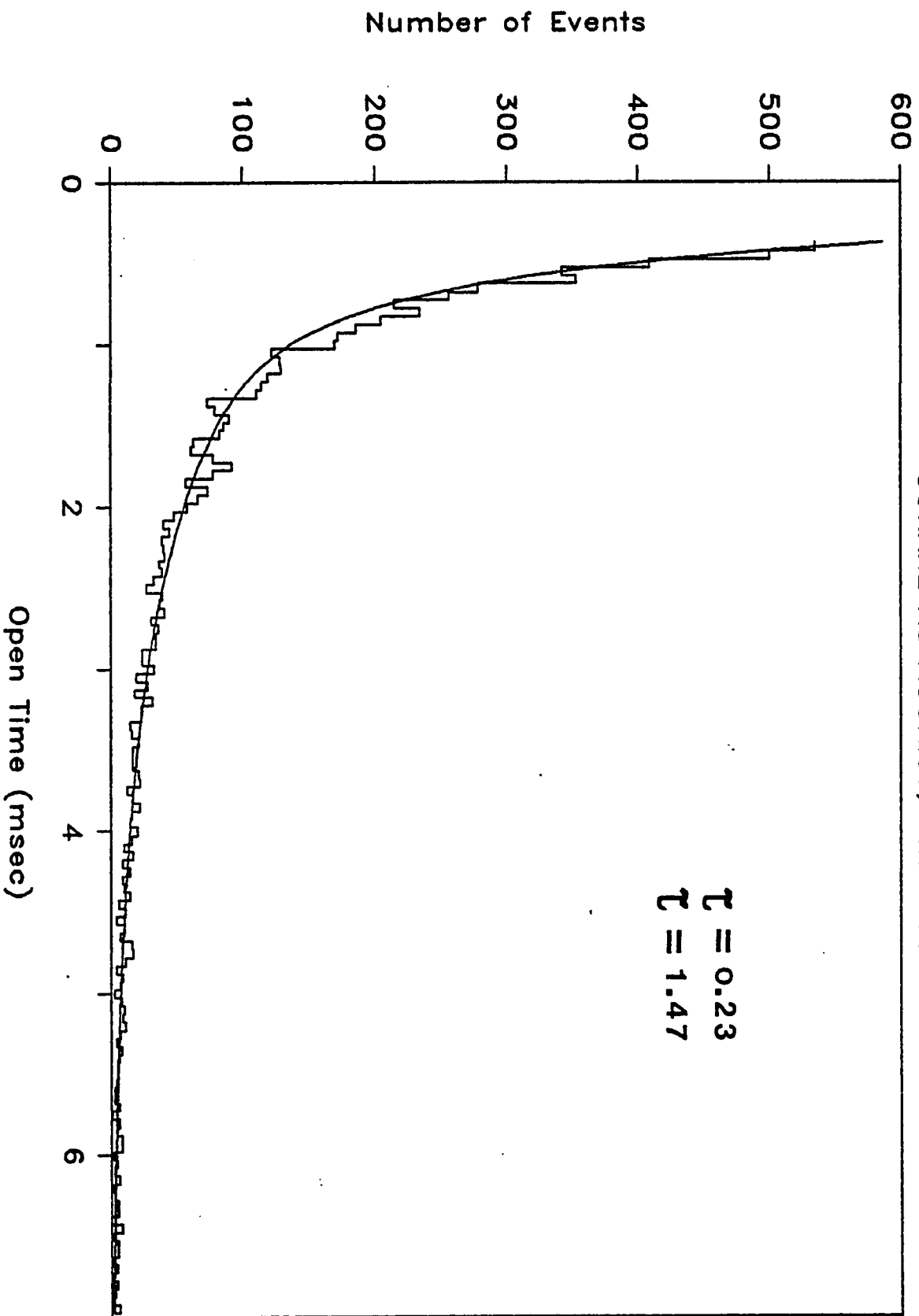


FIGURE 17

Open time histogram for curare-activated, S2 type openings of the AChR/channel in G8 myotubes. Histograms constructed with data from 5 cell-attached patches held at -130mV . The time constants associated with the fitted function (continuous line drawn over histogram bins) are indicated. Although data were rebinned for the fitting process (see methods) it is displayed here with uniform size binwidths.

OPEN TIME DISTRIBUTION OF S2 EVENTS

CURARE AS AGONIST, $V_m = -130\text{mV}$

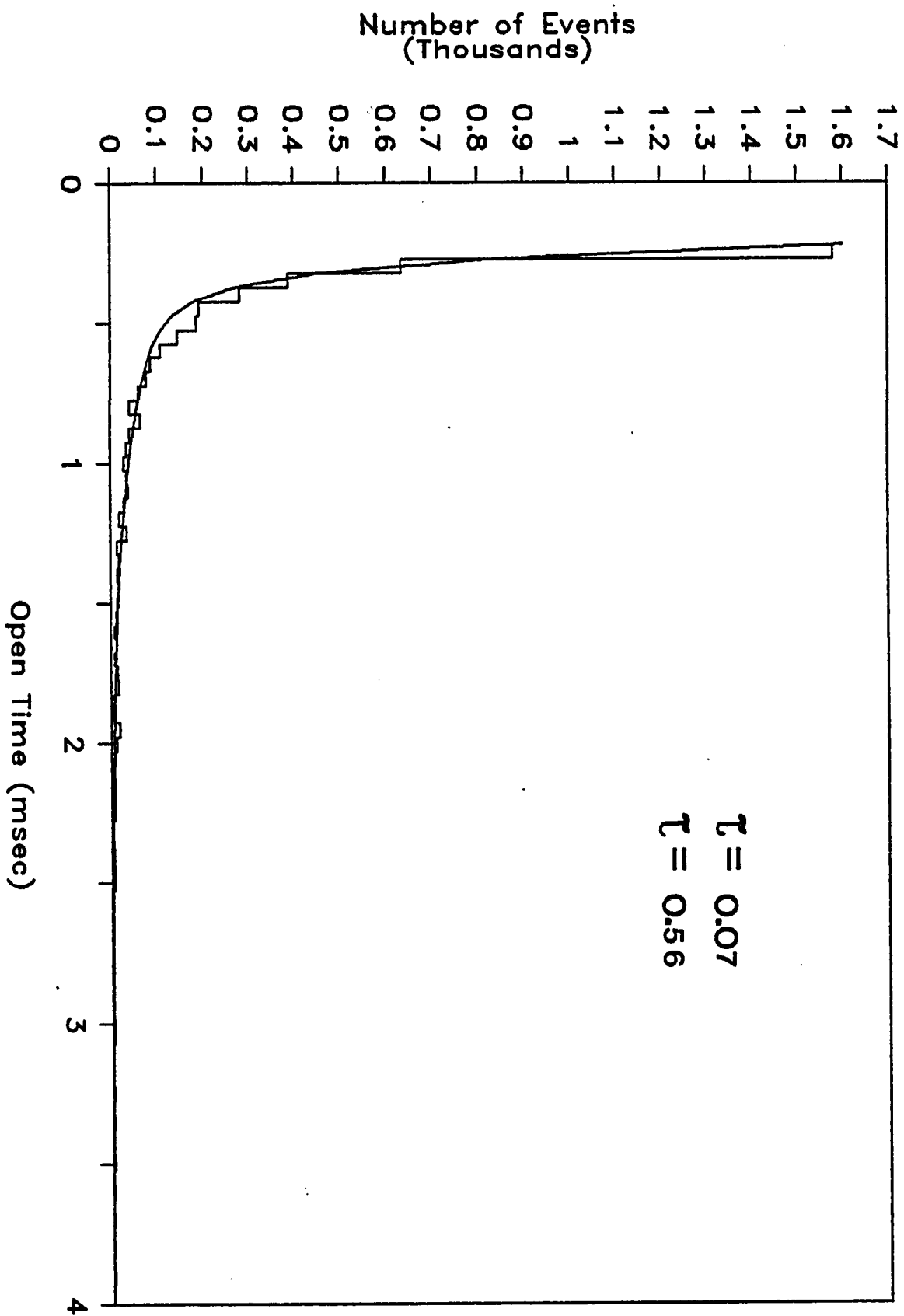


FIGURE 18

Open time histogram for curare-induced, S1 type openings of the AChR/channel in G8 myotubes. Histograms constructed with data from 5 cell-attached patches held at -130mV . The time constants associated with the fitted function (continuous line drawn over the histogram bins) are indicated. Although data were rebinned for the fitting process (see methods) it is displayed here with uniform size binwidths.

OPEN TIME DISTRIBUTION OF S1 EVENTS

CURARE AS AGONIST, $V_m = -130\text{mV}$

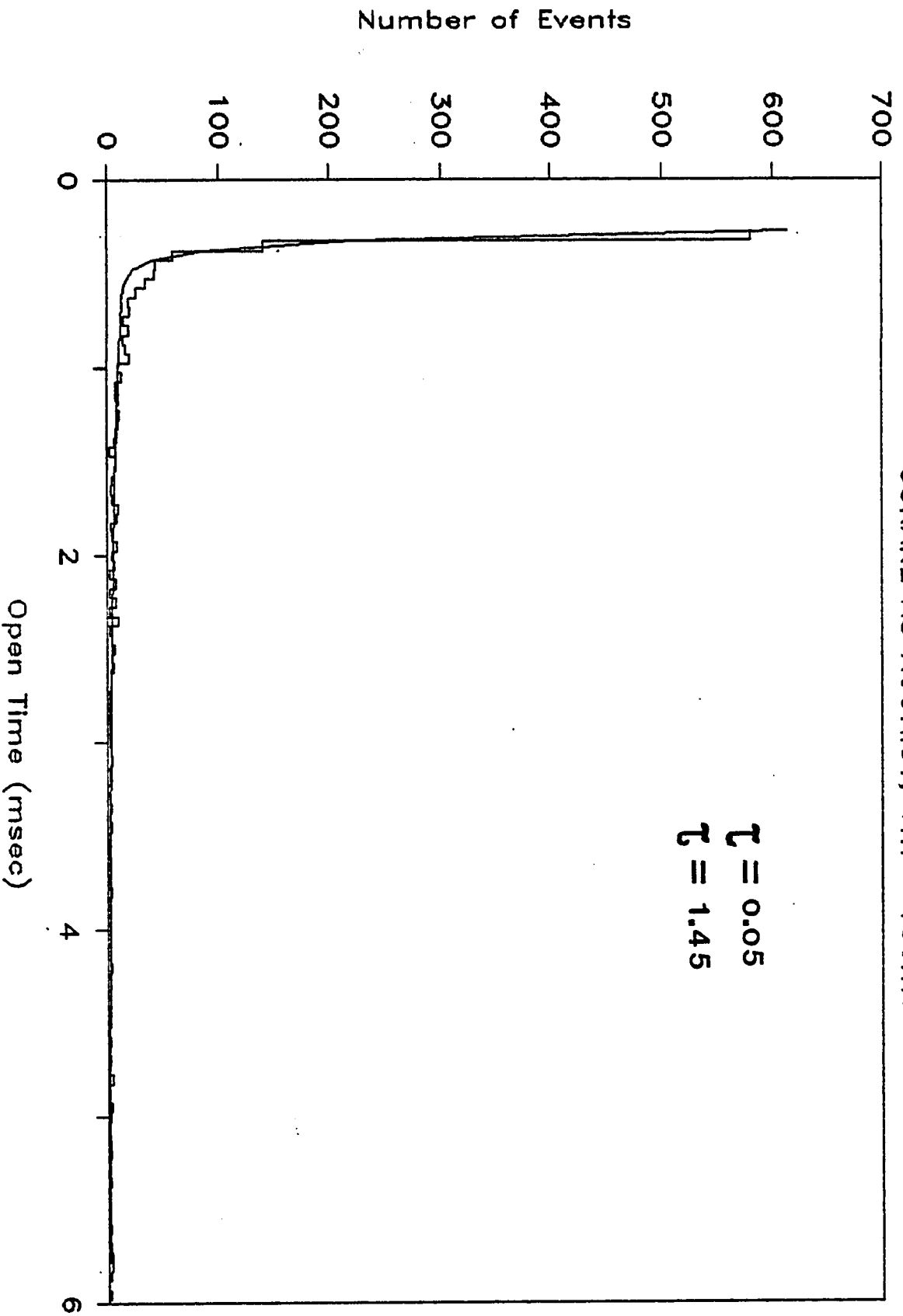


FIGURE 19

Open time histograms for ACh-activated S2 type openings of the AChR/channel in G8 myotubes. Histograms were constructed with data from 4 patches held at -130mV .

A) Data fit with a 2 exponential function (continuous line drawn over histogram bins); the two time constants extracted are indicated. Minimized chi squared=3860.9

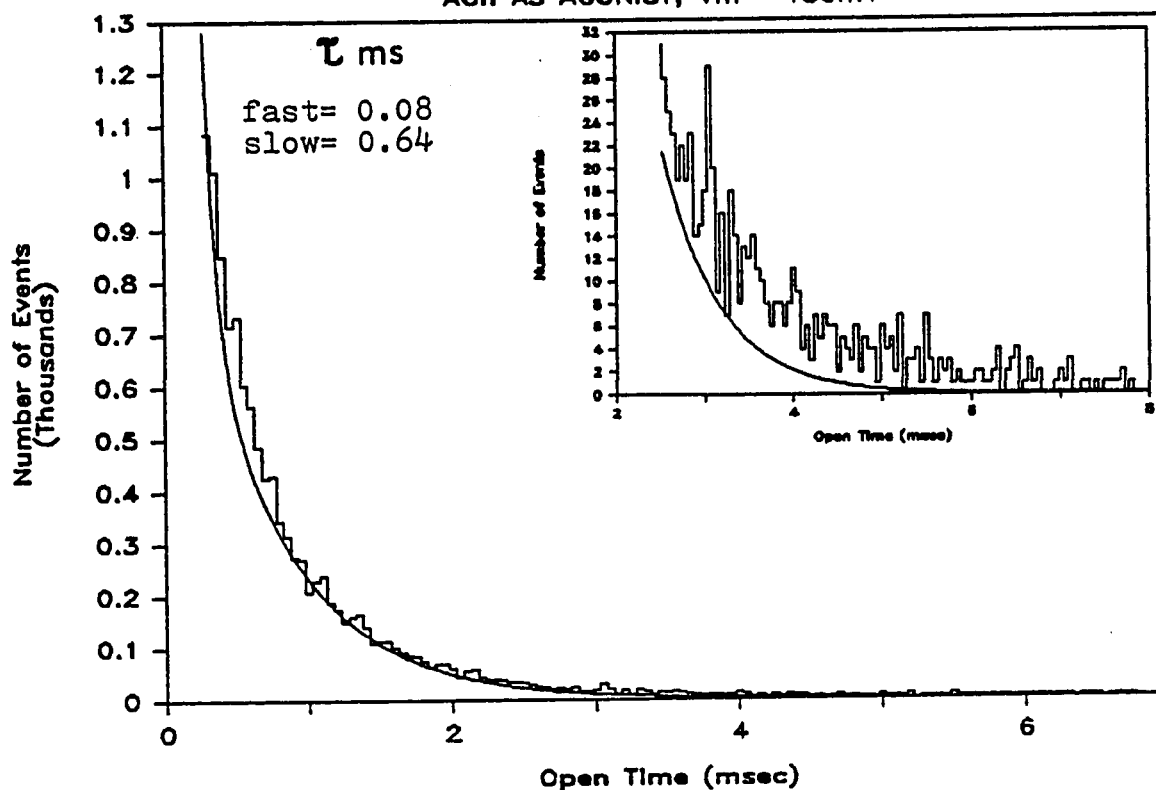
The tail of the 2-exponential fit is displayed in an inset.

B) Same data fit with 3 exponential function; the three time constants are indicated. Minimized chi square=231.6

The tail of the 3-exponential fit is displayed in an inset.

OPEN TIME DISTRIBUTION OF S2 EVENTS

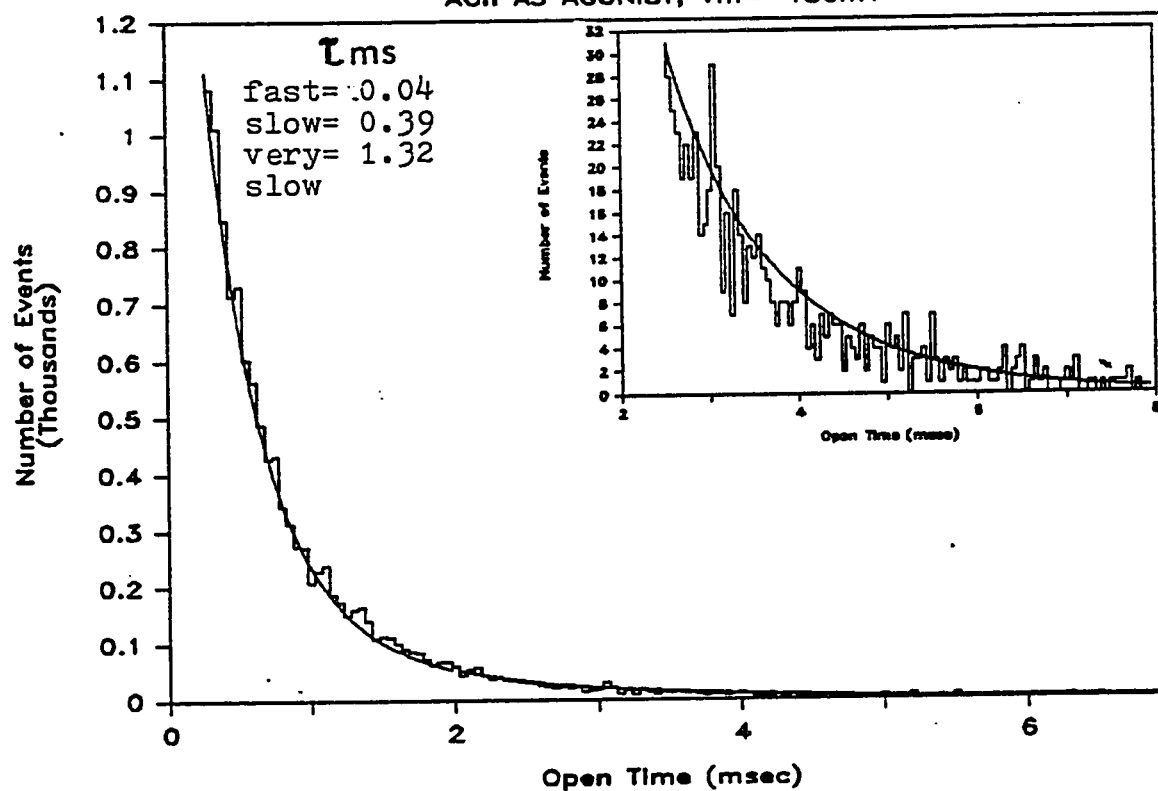
ACH AS AGONIST, $V_m = -130\text{mV}$



a

OPEN TIME DISTRIBUTION OF S2 EVENTS

ACH AS AGONIST, $V_m = -130\text{mV}$



b

figure 19 (a and b) for open time histograms of ACh-activated S2 type channel events. The number of exponential terms to best fit our data was chosen on the basis of a minimum chi square value derived from the fitting routine. For all conductance types, the fits indicate that there are at least two kinetically distinct open states, one fast and another more long-lived. This is true for all agonists studied. In the case of S1 and S2 conductance types, the distribution of fast openings is almost certainly contaminated by a) background noise and b) unresolved openings to larger conductance states. Due to the bandwidth limitations of the recording system and the cleaning of data files (i.e via program CLEAN), channel events of such brevity (0.035-0.112ms for S2, 0.047-0.125ms for S1) could not have been distinguished; the fast τ for the subconductance events will therefore not be discussed further. However, the slow τ extracted from the data is not beyond the bandwidth limitation of the system and is therefore considered valid for both types of subconductance events.

All substates of a particular conductance type have time constants which fall between 0.3 and 2.0ms regardless of the agonists inducing channel opening. Subconductances are, thus, short-lived by comparison with main conductance events where the range of open times is from 1.5 to about 20ms for the same agonists. In general, subconductance open time trends are unrelated to M open times in that longer-lived M events do not co-exist with longer-lived substate events. In the case of

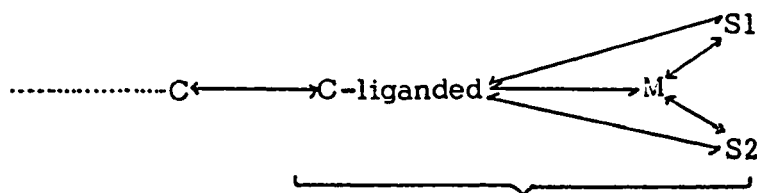
S1 type events, two significantly different tendencies in mean channel open times appear in the data when values of τ for different agonists were compared to the time constant for ACh-activated events ($P < 0.05$). Cur, Chol, SucCh and SubCh all display τ s over 1.2ms while Carb, Nic, ACh mean open times are closer to 0.5ms. The reason for the appearance of these two trends for S1 openings is not known, however, the size and the valence of the agonists does not seem to be important in determining whether the S1 event will be of the fast or slow variety. The mean open times (τ_{s1}) for S2 conductance events were even less variable between agonists than S1 mean open times. The τ_{s1} values obtained with different agonists were not significantly different from the value obtained for ACh-induced events. However, open time histograms for S2 events activated by SucCh, ACh and SubCh displayed a third time constant of even longer duration. These agonists appear to activate S2 type events with two kinetically distinct, resolvable open states. It is possible that the extra-long S2 open state results from contamination of the data by a channel other than the AChR/channel. This explanation would be unlikely if the third time constant appeared in all patches exposed to SucCh, ACh, and SubCh and not in patches exposed to other agonists. Unfortunately, in order to obtain subconductance open time histograms with sufficient density for fits, it was necessary to lump data from all patches; patch-by-patch testing for the third exponential is therefore not possible. Another possible explanation for this third exponential is

that its lifetime may be agonist dependent; strong agonists may better stabilize this kinetic state resulting in the appearance of longer-lived S2 openings in the records. Weaker agonists may produce an analogous opening that is more short-lived and therefore not distinguishable from the first two components of the distribution.

As with the subconductance open time distributions, M type openings displayed two populations of open times. For this conductance type, the fast τ was long enough that it could not be produced by misinterpretation of noise and is therefore considered valid. Values obtained for different agonists did not vary significantly ($P < 0.05$) from the value of τ obtained for ACh-activated events and were similar in value to the mean open time of spontaneous openings discussed previously. The values for the longer-lived open state of the M-type channel events, however, showed significant differences between agonists. Curare was able to invoke openings of the channel to this longer-lived (τ_{sl}) type of open state but was unable to keep the channel in this kinetic state for long. SubCh, at the other extreme, was able to maintain the channel in this state for much longer periods of time. Values in the τ slow column for the M-type openings can be taken as an indication of the relative activating abilities of the various agonists on the G8 AChR.

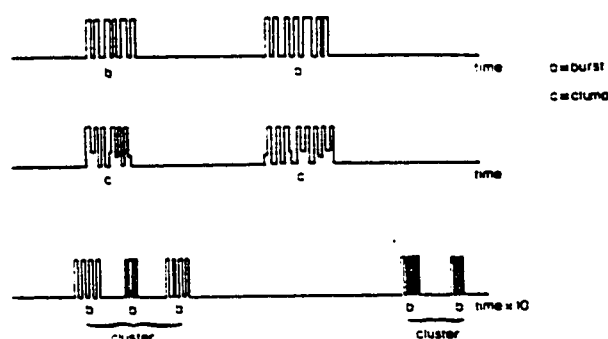
Burst analysis

Several AChR/channel preparations have been reported to display bursting tendencies when activated by strong agonists (Nelson and Sachs, 1979; Auerbach and Sachs, 1984). Bursting may be defined as periods of channel activation interrupted by one or more brief excursions to a closed, zero conductance state. Most studies of burst analysis have involved the oscillations between only two conductance states: open and closed. Because of the multiple open levels in our data, modifications to the analysis schemes were required. Our programs for burst analysis make the assumption that channel activation could behave according to the following model;



Bursts are defined as any oscillation or channel activity taking place within the scheme defined by the open bracket. Whenever the channel leaves the end of the scheme defined by the bracket for more than X ms (the interburst interval), we arbitrarily designate this as an end of burst. This may be compared to the Sine and Steinbach model (1984) reviewed in the introduction where bursting involved transitions between a closed but fully liganded state of the AChR and the open state (s). Ends of bursts according to this model represent the dissociation

of the liganded molecule (s). Estimates of burst durations of the channel with various agonists were calculated from open times regardless of the actual current level and are therefore based on a two state system (i.e. opened or closed). Since our assumption and definition of a burst differ from established criteria (Colquhoun and Hawkes, 1982), we have chosen to refer to these events as clumps of openings rather than bursts. This is not to be confused with the term cluster which, by convention, refers to a group of bursts closely spaced.



Clump analysis was performed to elucidate the effect of agonists on channel open behavior; results are summarized in Table 10. Figure 20 (a-d), represent clump analysis data for a single patch; in this case AChR/channel openings were ACh-activated. Values appearing in Table 10 are calculated from averages for several patches.

Like the mean open times for the long-lived M type openings, the clump durations of the G8 AChRs vary with the strength of the agonist used. The clump duration is smallest for the partial agonist curare, while the very strong agonist suberyl-dicholine displays longer periods of opening. An example

of clump duration estimates for ACh-activated openings is presented in Figure 20 (a) where the y-intercept of the graph in the region of interburst interval insensitivity (plateau in curve) represents the rough estimate of the clump duration.

The values for closings per clump represent the average number of S1, S2, M and C-liganded closings within the estimated clump duration. This data permits us to evaluate whether agonists that produce longer bursts necessarily induce more within-burst activity. While the differences are not dramatic, one can observe more frequent closings per clump in the longer-lived openings activated by the strong agonists SubCh and ACh; weak agonists such as Cur and Chol appear to open channels that when they close remain closed. Carb, SucCh and Nic-induced channel openings are not for the most part frequently interrupted with channel closings (i.e. quiet).

An interburst interval of 4.0ms was arbitrarily chosen from the data because it best represented the midpoint of the plateau in graphs of clump duration versus interburst interval for different agonists: plateaus represent areas where the estimated length of a clump is insensitive to the time in between defined clumps (i.e. see figures 20a). The same value was used throughout clump analysis calculations for different agonists so as to standardize data between agonists.

Openings of less than 1.0ms were omitted from the analysis because they usually represented single, isolated, fast events

TABLE 18: Clumping of G8-fCHR channel openings with various agonists

Agonist	No. of patches	Estimate of clump duration $\pm$ S.E.	No. of closings clump $\pm$ S.E.	S1 openings clump $\pm$ S.E.	S2 openings clump $\pm$ S.E.	M openings clump $\pm$ S.E.
Cur	5	3.1 ± 0.2	0.2 ± 0.04	0.6 ± 0.1	0.2 ± 0.01	0.5 ± 0.1
Chol	3	6.8 ± 1.3	0.2 ± 0.03	0.4 ± 0.1	0.1 ± 0.01	0.7 ± 0.2
Carb	3	10.4 ± 2.8	0.4 ± 0.06	0.9 ± 0.1	0.1 ± 0.02	0.6 ± 0.2
SucCh	3	11.8 ± 2.0	0.5 ± 0.10	1.0 ± 0.3	0.2 ± 0.1	0.8 ± 0.1
Nic	6	15.4 ± 3.2	0.5 ± 0.13	1.0 ± 0.2	0.2 ± 0.02	0.9 ± 0.2
ACH	4	19.5 ± 1.4	1.0 ± 0.14	1.7 ± 0.4	0.3 ± 0.1	1.4 ± 0.3
SubCh	6	22.8 ± 2.7	0.9 ± 0.11	1.5 ± 0.1	0.2 ± 0.1	0.9 ± 0.2

Estimates of clump duration are expressed in milliseconds.

Note: The term clump shall be used in reference to bursting type behavior because we include all open states in a clump, whereas a burst implies openings of a single conductance. Estimates of clump duration, closing and openings to various states per clump are calculated from averages for several patches (using 4.8ms as interburst interval and excluding openings of less than 1.8ms from the data).

FIGURE 20

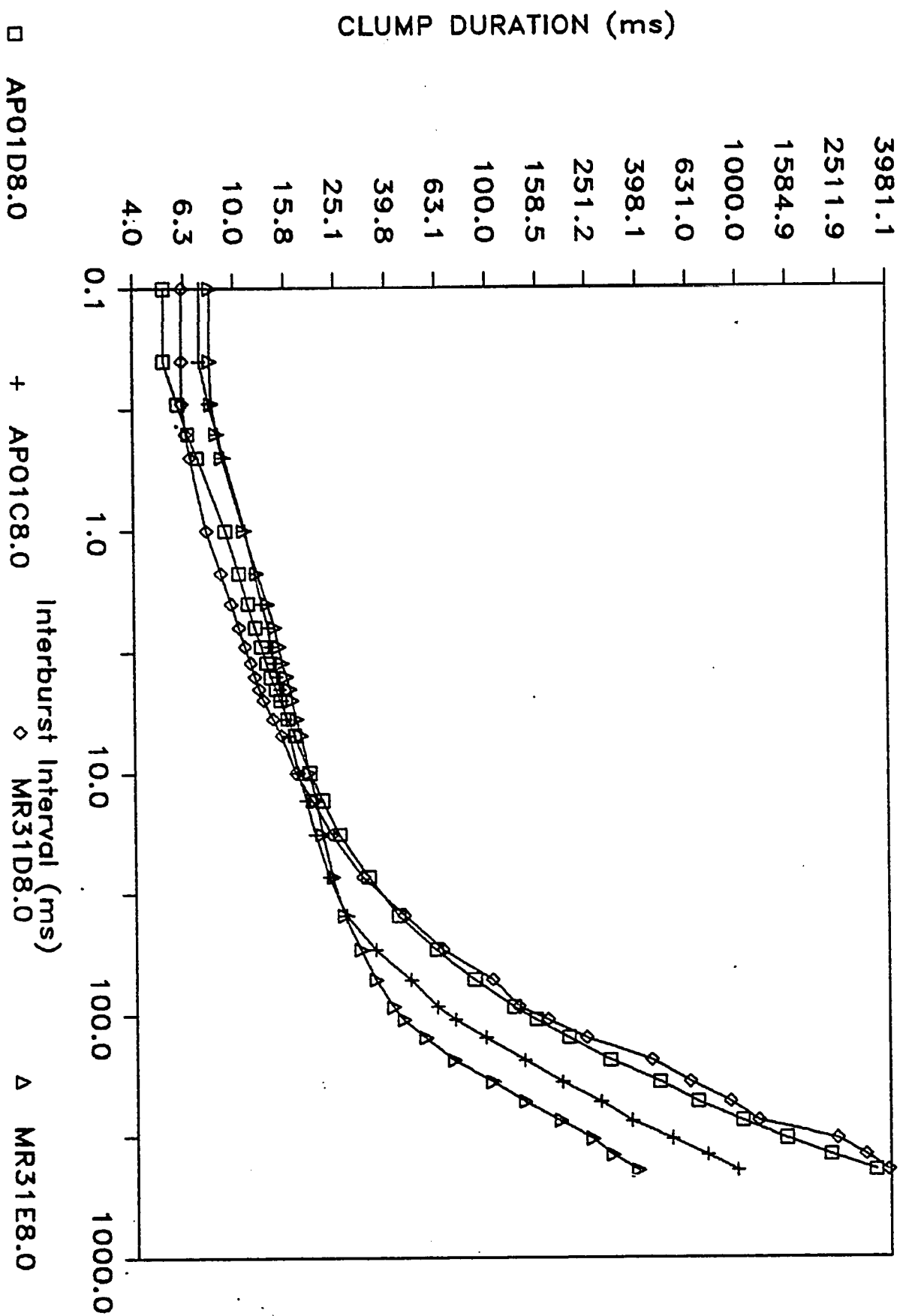
Burst analysis data from channels exposed to ACh ; graphs include data from four patches. All recordings were made on cell-attached patches clamped at -130mV . Estimates of clump durations found in table 10 were made using interburst intervals of 4.0ms and excluding all openings of less than 1ms duration. Data represented in the graphs include the brief openings.

A) average clump duration

B) closings per clump

AVERAGE CLUMP DURATION

ACETYLCHOLINE AS AGONIST, $V_m = -130\text{mV}$



CLOSINGS PER BURST

ACETYLCHOLINE AS AGONIST, $V_m = -130\text{mV}$

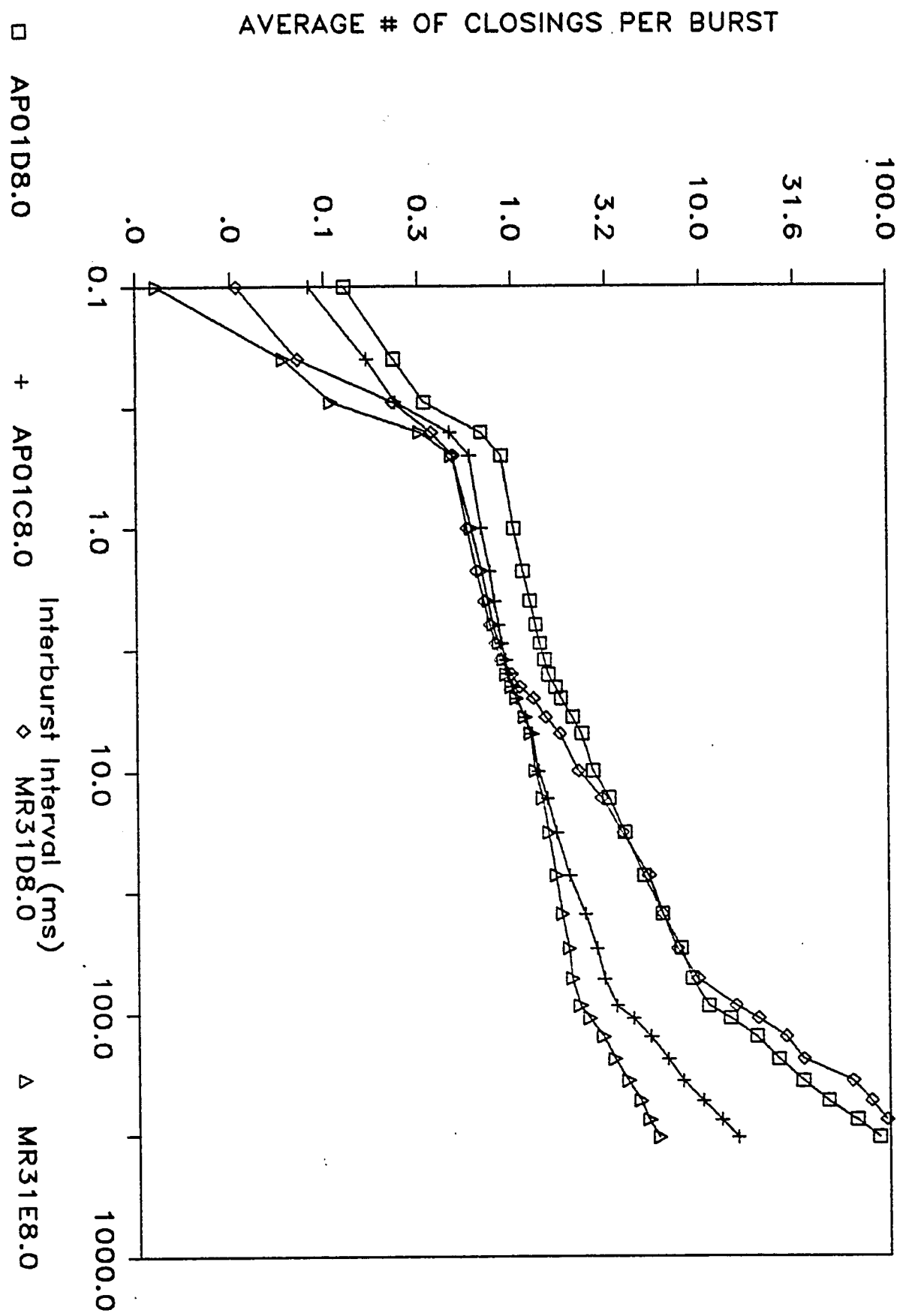


FIGURE 21

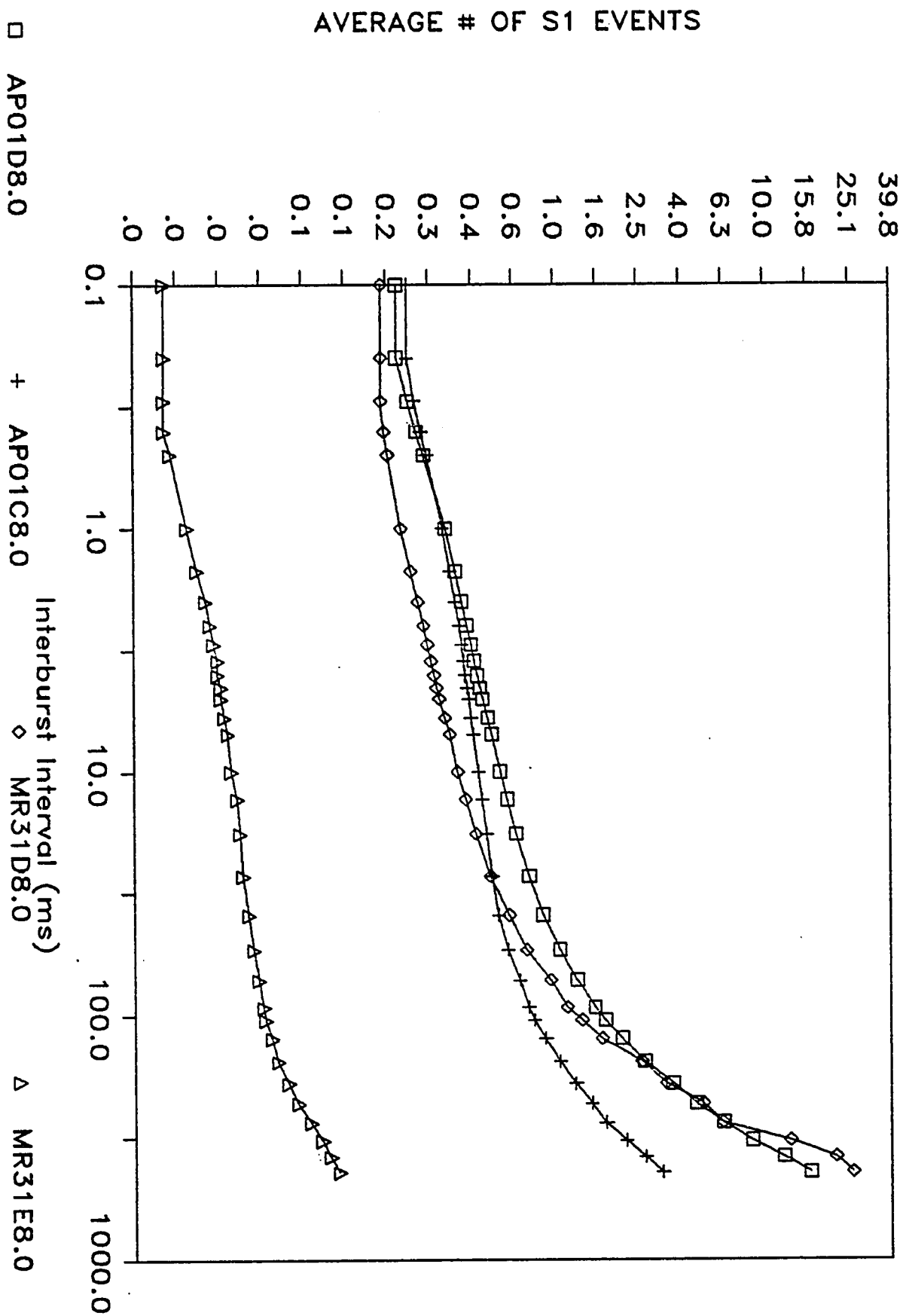
Burst analysis data; graphs include data from four patches. All recordings were made from cell-attached patches clamped at -130mV. Channels were exposed to 10^{-6} M ACh.

- A) S1 openings per clump
- B) S2 openings per clump
- C) M openings per clump

a)

S1 SUBCONDUCTANCE OPENINGS PER BURST

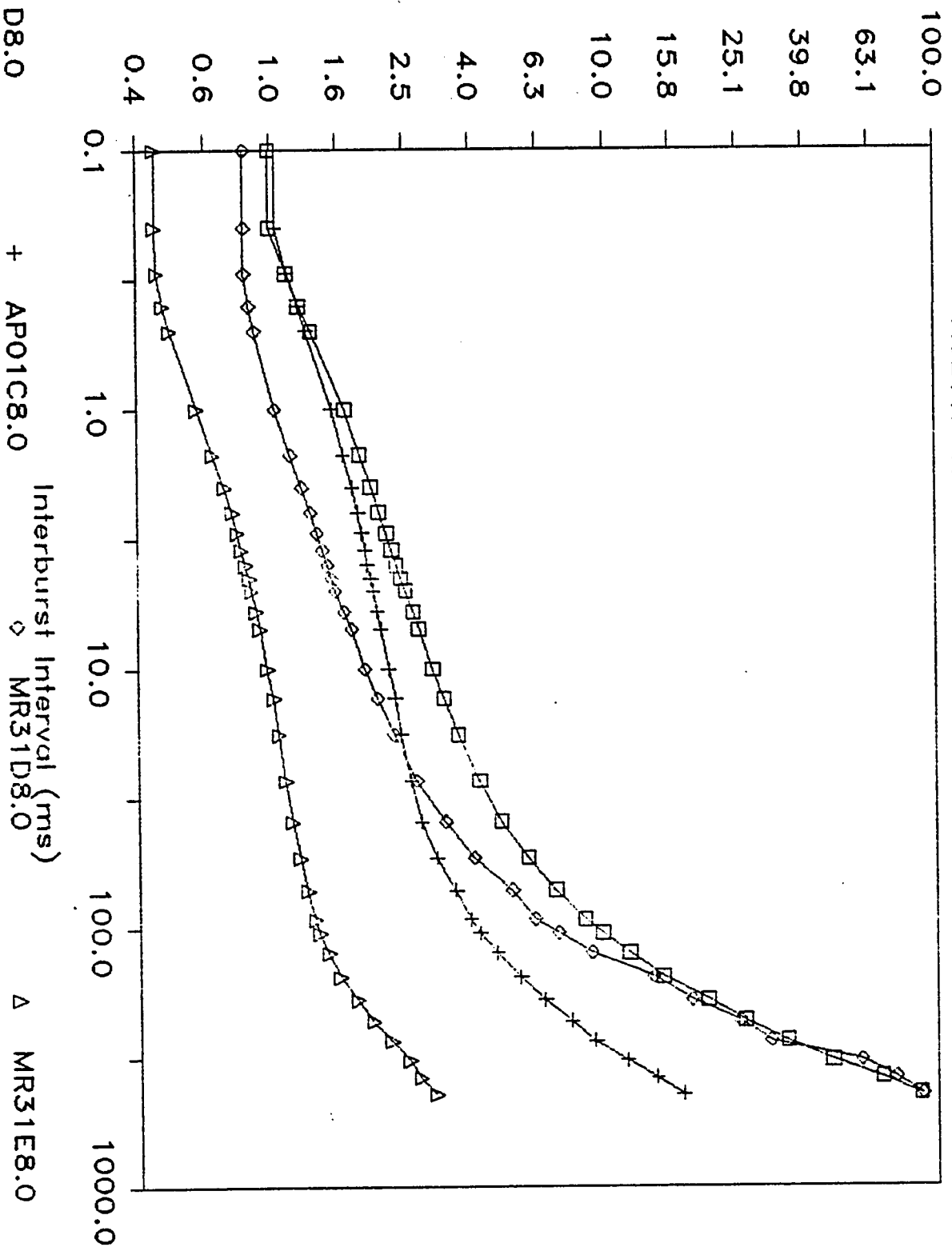
ACETYLCHOLINE AS AGONIST, $V_m = -130\text{mV}$



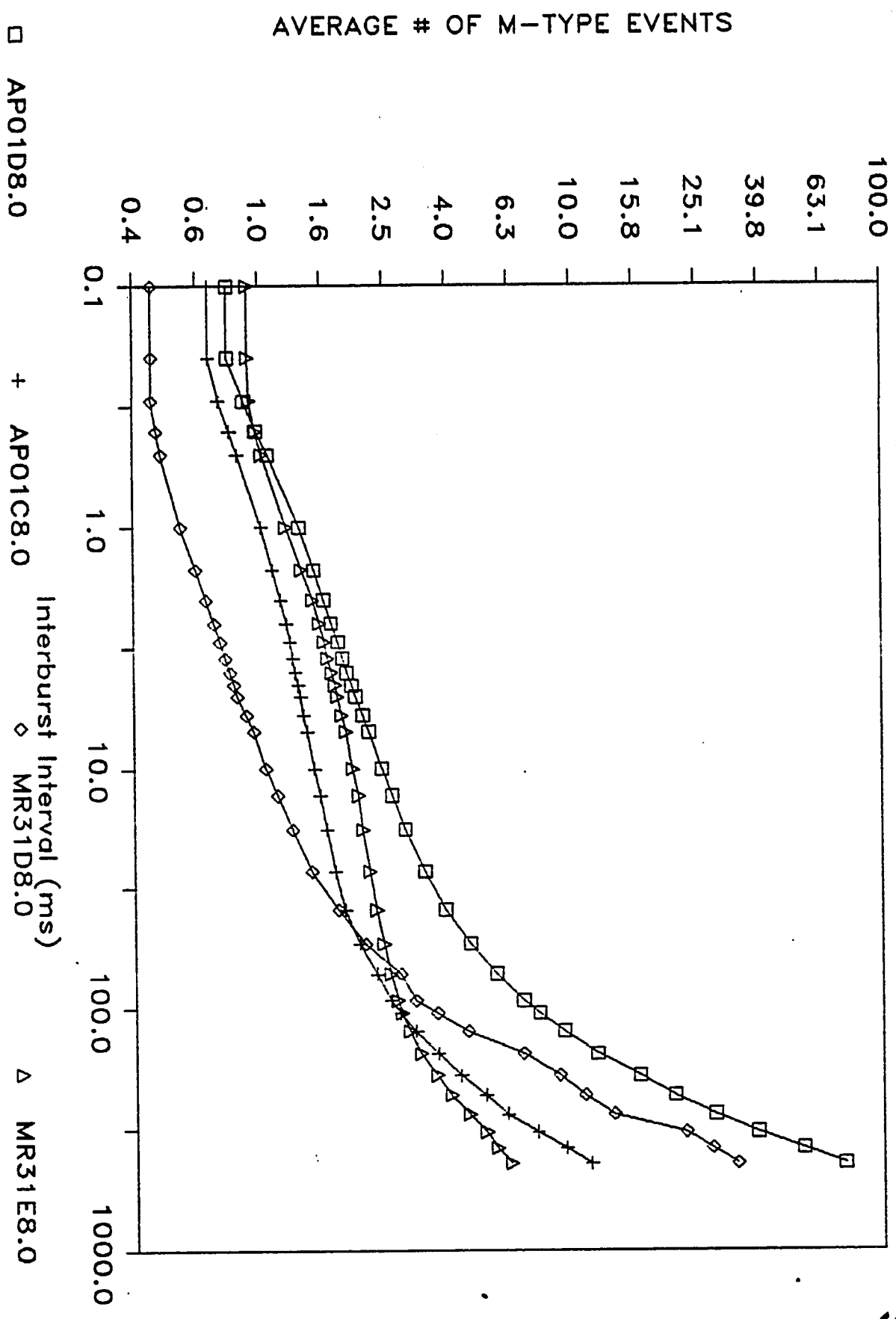
S2 SUBCONDUCTANCE OPENINGS PER BURST

ACETYLCHOLINE AS AGONIST, $V_m = -130\text{mV}$

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M-TYPE OPENINGS PER BURST ACETYLCHOLINE AS AGONIST, $V_m = -130\text{mV}$



not characteristic of a clump. Their presence in the data would have artificially reduced the estimated clump duration.

With the dimension of multiple conductance states added to the burst analysis models, the interpretation of data can prove to be very difficult. The open channel in a complex burst may not oscillate between the open and closed states but may instead show frequent transitions between different non-zero conductance states. Auerbach and Sachs (1984) observed that flickering of the channel from fully open to a small conductance open state could be misinterpreted as closings when bandwidth limitations of the experimental set-up did not permit clear resolution of short-lived single channel events. To demonstrate flickering between different conductance levels, values for the number of S1, S2, and M type openings per clump were extracted from the data. The question being asked was - do agonists that produce few closings per clump, as determined from our crude analysis, display flickering behavior between open states? For example, SubCh activates channels with average clump durations of 23ms. Table 10, one can expect that within this time period a SubCh-activated channel will close at least once. However, the data also suggest that the channel will oscillate from the S1 conductance state (1.5 S1 openings/clump) to the full conductance state (0.9 M openings/clump) at least once. There is a low probability for the SubCh-clumps to contain an S2 opening, however, they do occur

rarely during clumps. Conversely, Chol clumps are not interrupted by numerous closings and do not contain many transitions between conductance states; they represent quiet, simple single channel openings. To summarize the observations in Table 10, and Figures 20-21, SubCh and ACh induce burst-like activity in the AChR of G8 cells. These bursts are noisy in that they include flickering between different open states and the closed state. Nic, SucCh and Carb display less of a tendency to initiate bursting in G8 AChRs and activate fairly quiet uninterrupted openings. Finally, Chol and Cur activate very short-lived channel openings which are less frequently grouped together. These agonist molecules appear to invoke brief events which occur in isolation; if they remain bound to the receptor for long periods of time, they do not induce repetitive oscillations between closed and open states.

It would appear from this analysis, that the effects of agonists as allosteric effectors is very diversified. It is not the case, for instance that agonist size correlates with the lifetime of open states or with the propensity to induce subconductances.

DISCUSSION

A/Initial AChR/channel characterization

Whole cell studies

To establish whether the clonal cell G8 was an appropriate preparation in which to study single nicotinic AChR/channel behavior, it was necessary to first ascertain whether these myotubes behave similarly at the whole cell level to other well documented muscle cell preparations. To test for cholinergic channels within the cell membrane, the G8 cells were challenged with the ACh analogue suberyldicholine. The resulting depolarizations confirmed the presence of ACh-modulated channels within the myotube membrane. Depolarization of the whole cell is a direct manifestation of the inward ionic flux that occurs subsequent to the agonist-induced opening of a large population of AChR channels on the cell surface exposed to the agonist.

Further macroscopic characterization of the behavior of G8 AChR channels was undertaken using curare. Curariform drugs as mentioned previously have a diverse repertoire of actions. On some muscle preparations they may induce depolarization and thereby display weak agonistic abilities (Morris et al., 1983). The better known effects of curare on other excitable cells are antagonistic ones which reduce or even abolish responses invoked by other cholinergic agonists (Jenkinson, 1960).

The agonistic ability of curare was not demonstrable at the macroscopic level on this muscle preparation since G8 myotubes

challenged with curare failed to depolarize. However, the antagonistic properties of curare were apparent in studies in which it abolished agonist-induced depolarizations of the myotubes. These results with curare support the assumption that the depolarizations observed in the myotubes when challenged with suberyldicholine were in fact AChR/channel associated and not due to openings of some other channel type. We know that in skeletal muscle, curare binds specifically and with high affinity to the AChR. If the depolarization response of the cells when exposed to suberyldicholine was unrelated to AChR/channel openings, suberyldicholine-induced depolarizations would not have been abolished by curare binding to the AChRs. The whole-cell findings indicate, therefore, that these two drugs have a common site of action - the AChR/channel.

Having established that G8 cells are a good experimental preparation for electrophysiological studies of the AChR/channel, I initiated investigations at the single channel level.

Correlation between whole cell findings and single channel data for G8 myotubes.

Single channel openings induced by suberyldicholine were observed in G8 AChR/channels; these events were long-lived, numerous and permitted significant current flow. It is therefore not surprising that depolarizations from the resting potential

are observed when this agonist simultaneously induces opening of thousands of AChR/channels.

The situation for curare is however not as clear cut. Like suberyldicholine, curare-activated channel openings are observed in the single channel data. This appears to contradict the findings in the macroscopic studies where there were no apparent depolarizations in myotubes challenged with curare. At least a partial explanation for this paradox may be found in the kinetic and conductance properties of Cur-activated channel openings. Unlike SubCh-invoked events, Cur-activated openings are extremely brief: the channel closing rate is fast so that less charge is moved across the channel during each opening. Cur-activated openings, again unlike SubCh, display subconductance events at high frequency. These lower conductance states of the AChR/channel further diminish the effectiveness of curare as a depolarizing agent. As well, many curare-AChR interactions will be of an antagonistic rather than agonistic nature (witness the ability of 10^{-5}M curare to abolish depolarizations induced by 10^{-5}M SubCh); this is not the case for SubCh. The frequency of activation of curare is also lower, this is displayed in the fact that one hundred fold greater concentration of curare was needed to activate channels at a frequency comparable to that of SubCh. Presumably any depolarization produced by these very small responses was not greater than the background voltage noise and was not detectable with our intracellular recording set-up.

Effects of voltage on the AChR activation frequency.

The effect of transmembrane voltage on the transition rates between states has been investigated in several studies in an attempt to understand what forces influence conformation changes of the AChR/channel, however, no conclusive trends have been demonstrated. Sine and Steinbach (1986) reported an increase in the rate of transition from the closed to the open state with increases in potential differences across the patch membrane. Takeda and Trautmann (1984), however, observed the opposite relationship between the transition rates and voltage; they report a decrease in β with hyperpolarization of the patch membrane.

In our initial single channel studies, we observed very clearly patches which displayed an increase in the number of single channel events/unit of time with increased hyperpolarization of the membrane. However, upon further investigation, an increased activation frequency (a manifestation of the increase in β) with increased potential difference across the patch was shown not to occur in all patches; while some patches displayed voltage dependent transition rates, others did not. The possibility exists that, over the duration of our experimental period the activation frequency of the channels was non-stationary. If for instance, there are fluctuations in the extent of desensitization on the time scale of several minutes, this could make it difficult to detect the effects of voltage with the

protocol we used. Additional difficulties arise because of the occurrence of subconductance openings; at low driving forces (where resolution of events over background noise is difficult), it is more difficult to make correct assignments of a given opening transition than it is at higher driving forces. Consequently, our findings have not contributed to a resolution of the general question of whether channel opening is voltage dependent. More particularly, this line of investigation did not help us throw any light on the mode of action of curare as a partial agonist.

B/Multiple conductance states

It has been widely accepted that the ion channel can adopt multiple closed states. In the case of the AChR/channel these include unliganded-closed, liganded closed and desensitized closed states (Sakmann et al., 1980; Sine and Steinbach, 1980). These are all of zero conductance but can be distinguished electrophysiologically on the basis of their kinetic behavior. Multiple open states exist in two forms.

- 1) Multiple kinetic open states of the same conductance have been extracted from single channel data on several experimental preparations of AChRs. Like the closed states, their existence is revealed electrophysiologically by multiple exponentials in the open time distributions.

2) The second form of open state is more easily recognized in single channel data since it involves the opening of AChR/channels to several discrete conductance states. Evidently, models of channel activation involving only two conductance states, one of zero current (closed channel) and one of non zero current (open states), are not appropriate for the AChR channels of all experimental preparations.

In previous studies reporting subconductance events, only two open states associated with the AChR complex were reported: the classical full or main type opening and a sublevel type opening whose conductance was always less than 50% of the full (Hamill and Sakmann, 1981; Auerbach and Sachs, 1984; Takeda and Trautmann, 1984). Though the conductance value for these sublevel openings varied somewhat between studies it was constant throughout any one study (i.e. it did not vary significantly within patches or between patches in any specific study). G8 myotubes are therefore, a particularly interesting preparation since they demonstrate the existence of two distinct subconductance levels for the same channel (Morris and Montpetit, 1986).

Evidence for the association of subconductance events with the AChR/channel.

A major piece of evidence suggesting that subconductances are AChR-related and not the activity of a separate class of channels lies in the fact that they share a common reversal

potential with the main type of cholinergic agonist-induced channel openings. As will be discussed later, this suggests that the selectivity of the channels producing the full and sublevel events are identical. Secondly, transitions between subconductance and main type openings are far too frequent to be attributed to random simultaneous openings and closings of two different channel types closely spaced in time. Moreover, if the different conductance states represented different channel types, at higher agonist concentrations, we would have expected to see some full type channel events superimposed on subconductance channel types. This occurred very infrequently. We feel confident, therefore, in assuming that the small conductance events observed are produced by activation of the AChR/channel as are the main type of openings.

Models for the origin of subconductances.

Several hypotheses have been suggested to explain the origins of subconductance openings in the AChR/channel complex (Morris and Montpetit, 1986). Two models have been put forth:

MODEL I: First postulated by Hamill and Sakmann, (1981), this model suggests that subconductance openings are discrete, allosterically activated conformations of the ionic pore. The AChR/channel can in other words adopt several different open conformations. The configurations giving rise to the distinct

subconductance events or partially open states, must therefore represent a conformation where the access resistance of the channel for cations has been increased (i.e. narrowing of some region of the channel). With increased access resistance, the channel can support the movement of fewer ions per second and this is manifested as the lower conductance values characteristic of partial open states.

MODEL II: Takeda and Trautmann (1984) suggest that subconductance openings are simply main type openings in which a third molecule of agonist (assuming two molecules of agonist are needed to activate the AChR) binds to a specific site in the lumen, partially occluding the channel. This partial blockade site must be accessible in either open or closed channel conformations since sublevel events are not necessarily preceded by full amplitude openings. For our data, which contains two major classes of subconductances, the scheme requires a second partial blockade site. In this model, the steric hindrance and electrostatic repulsion of the permeant cations by the cationic agonist molecule would be responsible for the lower conductance values (Morris and Montpetit, 1986).

In characterizing the multiple conductance states of the G8 AChRs, a test was devised to determine which of these two models was more tenable. The hypotheses that our research was based upon follow;

1. If subconductance events occur when agonist molecules bind to site(s) partway along the channel lumen, thereby partially occluding the conductance pathway (model II), one would accordingly expect that the large divalent agonists (Cur, SubCh, SucCh) should be associated with markedly smaller subconductance levels than small monovalent agonists (Chol, Carb, ACh, Nic)
2. If on the other hand, subconductance events occur when the channel protein assumes a conformation which has a higher luminal resistance (model I), one could expect that the magnitudes of subconductance events should not be affected by agonist size.

EVIDENCE FOR MODEL I

Our findings on the G8 AChR support Hamill and Sakmann's original contention that subconductances are due to allosteric properties of the channel. The evidence can be summarized as follows:

A) NO EFFECT OF AGONIST ON THE CONDUCTANCE VALUES.

* All agonists regularly activated the AChR channel to the S2 level; this level shows no tendency to decrease with increasing molecular size or valence.

* Although S1 events were only abundant when curare was used to activate the channel, data on S1 openings was obtained for other agonists; the data shows no tendency for the conductance of the S1 state to vary with agonist size or valence.

* As a control, we also demonstrated that the conductance of M type openings did not vary significantly with the different agonists. This control would have been even more important for comparison's sake had a difference in sublevel conductances between agonists been observed.

This subconductance data for G8 cells provides no support for the hypothesis that subconductance type S1 and S2 are produced by the binding of an agonist molecule to a specific site within the channel lumen, however it does suggest that the AChR/channel possesses allosteric properties which give rise to subconductances.

B) SUBCONDUCTANCE EVENTS CAN OCCUR SPONTANEOUSLY.

Spontaneous AChR/channel openings were first reported by Jackson (1984). He demonstrated that the AChR channel could open without the binding of two agonist molecules; these openings displayed the same conductance values as SubCh-activated openings, were α -bungarotoxin sensitive and had a reversal potential of 0mV. When Jackson selectively impaired the binding site of the AChR so that no contaminating agent (i.e. agonist or ion etc.) could activate the protein complex, he still observed spontaneous openings. Few studies on spontaneous AChR channel openings have been reported (Jackson 1984; 1986). Ours is the first to provide information on observations of spontaneous subconductance-like events.

Spontaneous openings can be attributed to thermodynamic fluctuations in the channel's immediate vicinity. Let us assume that channels require a certain "activation energy" which will permit them to undergo a transition between closed and open configurations. Since the channel exists in a thermal environment, it will receive energy from collisions with ions and water molecules. We can postulate that, infrequently, this energy may be sufficient to overcome the energy barrier for a conformational change and the channel will open spontaneously. Using the same analogy, the binding of agonist molecules is thought to lower the energy barrier so that the available thermal energy is frequently sufficient to allow for closed open transitions. Since agonist-induced openings are generally longer than spontaneous openings, the agonist can also be attributed with increasing the energy barrier for the open closed transition.

Spontaneous M-type openings were characterized on the basis of their conductance and kinetics, and were shown to be similar to openings seen by Jackson (1986). Our single channel data also displayed what appear to be spontaneously occurring subconductance events. We cannot, however, categorically state that sublevel events seen in the low frequency no-agonist patches represent subconductance openings; the events occurred in isolation rather than in transitions with the M type spontaneous openings. It should be remembered, however, that transitions between multiple conductance levels are relatively infrequent

even for agonist-induced openings. It would not therefore be surprising if their occurrence in no-agonist control situations were rare. We have, moreover, additional reasons to suspect that these sublevel current jumps are AChR-associated.

- 1) they disappear at 0mV transmembrane potential; this would be expected for events associated with the AChR/channel whose reversal potential is 0mV.
- 2) they appear as current jumps whose magnitudes at various transmembrane potentials are similar to agonist-induced subconductance openings of the AChR/channel complex.
- 3) recordings from the control patches referred to in the results as "hot spot patches" contained obvious subconductance events in transition with the main open state. These would undoubtedly have further supported our claim of spontaneous subconductance if the kinetic behavior of the spontaneous M type openings appearing in these high activity patches had not been so abnormal. If we are correct in assigning the small conductance events in the no-agonist data to the AChR/channel, this strongly suggests that the multiple conductances seen in AChR channel openings are not the result of partial blockade of the channel lumen, but are part of the allosteric make-up of the channel and can occur without the presence of agonist molecules.

C) SUBCONDUCTANCES ARE OBSERVED AT LOW AGONIST CONCENTRATIONS

The concentrations used for each agonist were near their respective K_d values for the receptor binding site (Taylor et al., 1983). In cases where it has been tested, K_d for channel blockade tends to be higher than that for receptor binding. Thus, our experimental conditions were chosen to be relatively unfavourable for entry of agonist molecules into the channel lumen, and yet we regularly observed subconductances. It should also be pointed out that, because agonists are charged, the incidence of subconductance events should increase with hyperpolarization if the partial blockade model (Model II) is correct. The ratio of S1/M events should therefore have increased with hyperpolarization; tests with curare did not show this. Once again, the allosteric model seems more tenable.

D) OPEN CHANNEL NOISE OF SUBCONDUCTANCE EVENTS IS GREATER THAN THE OPEN CHANNEL NOISE SEEN IN M TYPE EVENTS

Sigworth (1985) studied the small fluctuations in ion transport that can be observed in single open AChR/channel data. He showed that open channel noise was greater than the background noise of a patch. Open channel noise has high and low frequency components. The high frequency component is attributed to the "shot noise" of discrete ion permeation events. The low frequency component represents small fluctuations in the electrostatic or chemical environment of the ions in the channel;

Sigworth suggests that it results from small conformational fluctuations in the protein molecule.

It has been shown that subconductance openings have noise levels greater than the M type open noise (Auerbach and Sachs, 1984; Sigworth, 1985; our own findings). This increase in noise above the M type open noise, as well as making sublevel events harder to resolve at low driving force, suggests that the conformational substate is not identical to or as stable as the full state. Perhaps, "micro"-fluctuations in conformation occur more readily in the substate. This interpretation suggests that subconductances are indeed discrete conformational states of the AChR.

E) LOW CONDUCTANCE DUE TO LIMITED BANDWIDTH

Low amplitude events are not always due to channel conformations of low conductance. Unresolved high frequency transitions between the open and closed states could for example, give the mistaken impression of a reduced conductance (Colquhoun and Sigworth, 1983). What light does our data throw on the possibility that sublevel events are in fact an illusion produced by the limited bandwidth of the recording system? The apparent amplitude of events produced by protracted high-frequency flickering would depend on the opening and closing rates of this "flicker" mode (Morris and Montpetit, 1986). Since characteristic magnitudes of the two subconductance states were essentially the same for all agonists, these rates would have had

to be invariant for all agonists. This stricture renders it extremely unlikely, though not impossible, that rapid flickering between the fully open and fully closed states is responsible for the apparent subconductance events.

F) LOW CONDUCTANCE DUE TO CHANNEL BLOCK BY IONS.

While channel blockade has been a recognized mode of action of some noncompetitive antagonists, it has only recently been reported that agonist molecules and permeant ions can also cause occlusion of the open AChR/channel by binding to some intraluminal site (Sine and Steinbach, 1984).

This study has ruled out the possibility that agonist block produces the subconductance events. Additional investigation is required, however, to adequately rule out the possibility that subconductances occur when an ion such as Ca^{++} binds to sites in the lumen. The likelihood of monovalent ion involvement in the production of subconductances through channel blockade seems remote since substitutions of Na^+ with Cs^+ or Li^+ (Hamill and Sakmann, 1981) produced no obvious change in the ratio of substate to full openings.

As with the agonist blockade hypothesis, one would expect that if ions were binding to luminal sites, producing partial occlusion, different ions should produce different subconductance levels. Recordings of single channel openings from rat myotube AChRs using salines with elevated Mg^{2+} and Ba^{2+} (external solution) show that these ions can interfere with the permeation

of monovalent ions (Dani and Eisenman, 1985). However, the effects seen are in the nature of general lowering of channel conductance values, not the production of discrete transient low conductance states. The fact that some aspects of the kinetics of subconductances are agonist-dependent makes the Ca^{++} hypothesis unlikely. Therefore, while the possibility of ion blockade has not been tested in this research, the findings of other researchers suggest that it is not responsible for the subconductance events.

G) SUBCONDUCTANCES AND THE SELECTIVITY FILTER

Our data suggests that different conformational states of the AChR/channel are responsible for subconductances. We can assume from the reversal potential data, where values were essentially the same ($0\text{mV} \pm 5\text{mV}$) for different conductance types, that the conformational changes in the protein during transition between subconductances and full openings do not affect the selectivity filter of the channel.

This conclusion needs some amplification. As mentioned previously, the reversal potential of the AChR/channel is near 0mV because the channel is almost equally permeable to Na^+ and K^+ ions. Given the usual low intracellular Na^+ and high K^+ , the reversal potential of a purely Na^+ selective channel (under the saline conditions used) would be about $+55\text{mV}$ while that of a purely K^+ selective channel would be nearer to -70mV . If conformational rearrangements associated with subconductance events were to take place at the selectivity filter of the

AChR/channel, one would expect a change in the relative permeabilities of the pore to the various permeant cations. This type of change in channel selectivity would be reflected as a dramatic shift of the channel's reversal potential towards the V_{rev} characteristic for the new predominant permeant ion. Since we did not see any such trends in the reversal potentials for full, S1 and S2 open states, one can assume that the configurational changes resulting in the increased access resistance of subconductances does not occur near the rate-limiting resistive barrier that is the selectivity filter.

If agonists do not affect the conductance values of the different open states, do they affect any other property of the AChR channel?

The choice of agonist used does not appear to influence the distinct conductances of fully open or partial open states of the G8 myotubes AChR/channels; that is, the ease with which the ionic current is carried through the channel in various open states is not agonist-dependent. However, the frequency of occurrence of the different conductance states within the single channel data is agonist-dependent. This is readily apparent when one compares the number of subconductance openings activated by curare per unit time with the number of ACh-induced sublevel openings per unit time. If one returns to the activation energy argument previously mentioned one can suggest that

agonists destabilize the closed state to varying agonist-dependent degrees and reduce the configurational energy barriers for transition thereby favoring a conformational change to an open state. The inequality between agonists in their ability to destabilize the closed state is manifested in the differences in concentrations required of different agonists to produce similar channel activation frequencies. This could be quantified more rigourously by studying the effect of various agonists on the rates of channel transition from closed to various open states, using the outside-out configuration so that a fixed population of channels could be tested with different agonists. Again, using the energy barrier analogy, we can suggest that because the S1 and S2 open states occur more infrequently than M type open states, the energy barrier for transitions to these subconductance states must be higher than the energy required to open to the full conductance state.

In the same line, the energy required to induce a conformational change of the receptor protein to the S1 state must be greater than that for the S2 state because of the fewer occurrences of the former in single channel records.

In models of channel activation, the role of the agonist is to accelerate the rates of transitions (Jackson, 1984) from closed to open by the reduction of the energy barrier between the closed and open channel. In a situation where there are three open states for the AChR channel (M, S1, S2) which do not occur in the same proportion when activated by different ago-

nists, one can assume that the agonists have differential abilities in lowering the energy barriers of the different conductance states. For example, Cur-activated S1 channel openings occur much more frequently in the data than ACh-activated S1 subconductances. One can therefore speculate that curare is better than ACh at lowering the transition energy barrier for S1 openings and thereby increasing the transition rate to this configurational state. The relative abilities (in descending order) of the various agonists at opening channels to subconductance states follow:

S1.....Cur>>> Nic $\approx$ SucCh > Chol > SubCh > ACh > Carb

S2.....Cur > SubCh > Nic > SucCh > ACh > Carb > Chol

Therefore, in terms of activation to the subconductance states, the conditional probability data we obtained suggests that curare is the best agonist for reducing the barrier to subconductance openings while other drugs such as carbachol do not accelerate the opening rates towards subconductance states as efficiently.

Effects of agonists on the open time kinetics of the multiple conductance states

In the past, kinetic studies on single channel data have served to distinguish different types of AChR/channels based on their open time properties (e.g. embryonic/adult). However, recently several distinct kinetic open states have also been observed within the same channel type. Two kinetically distinct

open states having identical non-zero conductance have been observed for the AChRs of various preparations (Colquhoun and Sakmann, 1981; Morris et al., 1983; Jackson et al., 1983; Gardner and Barnard, 1985).

As an indication that the time constants we have extracted from the data (for the easiest to resolve conductances) are in line with what is in the literature, we have summarized some values obtained for rat and mouse myotubes by other researchers and compared them to our values for M-type openings.

Morris et al (1983)	Cur $\tau_f=0.29\text{ms}$	$\tau_s=1.40\text{ms}$
Takeda and Trautmann (1984)	Cur $\tau_f=0.3\text{ ms}$	$\tau_s=1.7\text{ ms}$
G8	Cur $\tau_f=0.23\text{ms}$	$\tau_s=1.47\text{ms}$
Jackson (1986)	Carb $\tau_f=0.25\text{ms}$	$\tau_s=3.5\text{ms}$
G8	Carb $\tau_f=0.28\text{ms}$	$\tau_s=6.8\text{ms}$
Trautmann (1983)	ACh -----	$\tau_s=7.3\text{ms}$
Takeda and Trautmann (1984)	ACh -----	$\tau_s=12.6\text{ms}$
G8	ACh $\tau_f=0.25\text{ms}$	$\tau_s=9.7\text{ms}$

Differences in τ values may occur for several reasons, the most important of which are: the differences in the temperature of the experimental preparation at the time of data recording, differences in the V_m at which kinetic analysis takes place and differences in agonist concentrations (high concentrations of

agonist may lead to a decreased mean open time when channel blockade occurs). From this comparison, we see that the values we obtained were similar to those reported in the literature.

Like many other AChR preparations, full conductance events of G8 cells have two kinetically different open states.

1) The short-lived M-type openings do not vary in mean open time when activated by different agonist molecules. The τ for these fast events was similar to that estimated for spontaneous channel openings of this conductance type; thus it is reasonable to assume that the mean open time of the fast M-type events is a property inherent in the channel. By contrast, the probability of occurrence of short-lived M events is agonist-dependent. In other words, all agonists increase the probability of short-lived M events and some agonists are more effective than others at inducing the state, but once in the state, the dwell time is independent of the activating agonist.

It was originally thought that the fast component in the M-type open histogram reflected the opening of monoliganded channels; conversely the longer-lived events were attributed to a biliganded AChR complex (Takeda and Trautmann, 1984). However, studies by Sine and Steinbach (1986) have demonstrated that this is not the case for the AChRs of the clonal line BC3H-1. They varied the agonist concentrations (thereby varying the ratio of mono-to-biliganded channels) and found no variation in the ratio of fast to slow events. As well, experiments by Morris et al., (1983) demonstrated that curare at two different concentrations

does not alter this ratio for rat myotubes. While still a controversial point, most researchers believe that this fast τ can be regarded as a kinetically different open state of the biliganded channel.

2) The situation is very different for long-lived M-type openings. The G8 results demonstrate that the slow time constant for the M-type events is agonist-dependent. This means that different agonists differentially stabilized these long-lived state(s) of the G8 AChR/channel. SubCh was by far the most effective at stabilizing the channel in this fully conducting long-lived open state while curare's ability to maintain the channel in this configuration was poor. Agonist induced stabilization of an open state implies that the binding of an agonist molecule in some way decreases the leaving (or transition rate (s)) from this state.

The kinetic properties of subconductance openings have only been superficially studied to date (Hamill and Sakmann, 1981; Auerbach and Sachs, 1984). Characterization of the effects of two different agonists on the kinetic behavior of subconductances was first attempted on AChRs by Auerbach and Sachs (1984). These researchers showed that in tissue cultured chick muscle, bursts of current contained numerous low conductance gaps. These were 12% of the main conductance state which is similar to our S2 openings. Open time analysis revealed a single kinetic component in these subconductance events. They demonstrated that the mean lifetime for SubCh-induced subconductances was

longer than for ACh ($1.13 \text{ ms} \pm 0.28$ and $0.61 \text{ ms} \pm 0.25$ respectively). In reviewing our results for the mean open times of subconductance events it might first be important to remind ourselves that the fast time constants for both S1 and S2 openings with all agonists have been disregarded due to the very real possibility that this data is more related to electrical noise than actual channel events. After having said this we can now focus our attention on the longer-lived component of the S1 and S2 open time histograms. Essentially our data agree with Auerbach and Sach's (1983) in that the actual subconductance S1 and S2 open time histograms may be fit by a single exponential i.e. τ_{sl}).

With the exception of SucCh, ACh and SubCh, the lifetimes of S2 events (τ_{sl}) were shown not to be significantly affected by the agonist used to activate the sublevel openings. It was found however that the strong agonists SubCh, ACh and SucCh activated S2 channel events whose open time histograms are best fit by three exponentials. The detection of this additional component for these drugs may be related to the greater density of the open time histograms obtained for these strong agonists. In other words it is possible that a comparable component exists for the other agonists but that these long-lived openings are too rare to provide enough density for a three exponential fit of the open time histograms. Alternatively, this particular open state may be agonist-dependent. Which ever argument one

accepts, it is evident that the agonists SubCh, ACh and SucCh are better than the other agonists at stabilizing long-lived S2 subconductances.

Kinetic analysis of S1 openings demonstrates that there are two mean open time tendencies in the data. The agonists Cur, Chol, and SucCh all induce S1 subconductance openings with mean open times two fold greater than those seen in subconductances activated by Carb, Nic and ACh. The significance of these two tendencies is not understood; there seems to be no common factor distinguishing the agonists which give rise to long and the agonists inducing the short S1 openings. SubCh-activated S1 openings have τ part way between the other two tendencies. It should be noted that the chi-squared value obtained for the fit of the open time histogram with this drug was fairly large.

In summary, each subconductance type appears to have at least one state whose closing rate is agonist-independent. However, the appearance of extra τ s for some agonists and not others suggests that there could exist agonist-dependent open states not yet characterized kinetically.

Burst analysis

Bursts may be defined as groups of channel openings separated by very short closed periods less than a specified length. Any closed period lasting longer than this critical time is arbitrarily considered to be dividing different bursting episodes of the channel. Colquhoun and Sakmann (1981) first coined the

term Nachschl  g to describe this flickering type of behavior in the AChR/channel. However, since this time, flickering activities have been found in other types of channels and seem to be a common feature of ion channels (Cull-Candy et al., 1981; Barrett et al., 1982). A complex model for bursting has emerged from analysis of experimental results (Jackson et al., 1983; Sine and Steinbach, 1984; see review in introduction). It is thought that the bursting channel is a doubly-bound open channel which is repeatedly oscillating between a fully open conductance state and a closed state (Jackson et al., 1983) or a subconductance state (Auerbach and Sachs, 1984). The interburst interval is thought to be related to dissociation of agonist molecules(s).

In our kinetic analysis, bursts were recognized as any group of openings to any non-zero conductance state with closed periods of less than 4ms between them. Bursts were renamed clumps for our purposes to recognize the fact that oscillations to different open conductance states were included in each episode. From the previous analysis routines for mean open times, we knew that there existed several populations of open times per state in our channel data. Mean clump duration was calculated with the maximum likelihood equation which assumes the data has a single exponential distribution. Therefore, in an attempt to validate this estimation of clump duration, we ignored all openings of less than 1.0ms, so as to eliminate the error induced by the population of brief isolated channel openings. Our data reveals that those agonists which open individual channels for longer

periods of time are also more likely to induce burst type openings which last longer than those seen with "weaker" agonists (i.e. partial agonists). Therefore, the mean open times for clumps (Table 10) activated by partial agonists, such as curare or choline are very short while the AChRs activated by "classically stronger" agonists, such as ACh and SubCh, which are more efficient at stabilizing the open state (or destabilizing the closed state), display clumps that last over five times longer.

Since the definition of a burst implies flickers of the channel to a closed state, we determined the relative number of closings per clump for different agonists. The results obtained seem surprising in that for all agonists, the channel experienced very few closings during this flickering behavior. Those agonists which induced the most number of closings per clump were again ACh and SubCh. This is somewhat expected since they have longer openings which thereby increases the probability for a transition to occur.

If the channel is not predominantly in the closed state during a clump, what type of openings are occurring? For most of these agonists, the channel spends the majority of its clump time in the M conductance state. This is determined by a) recognizing that the channel opens to the M state at least once per clump (table 10), b) comparing the values of clump duration in table 10 with those for mean open time of the M type channel event in table 9 and c) recognizing the similarity in these values. Therefore in a clump, once the M type event has occurred,

not much time is left for other events. For example, SubCh-induced single channel openings display τ_{sl} of approximately 19ms, while its clump duration is 23ms. If the channel is opened to the full state even only once during a clump, this type of opening will account for over 80% of the clump duration. This leaves only 4ms for closing or subconductance openings.

At the other extreme, however, we find Cur-activated channels which have a clump duration of 3ms, very few closings per clump and the same number of M type openings as S1 type openings per clump. If one compares these findings with curare's τ_{sl} (1.47ms) for individual M-type channel events, it appears as if this partial agonist spends as much time in the subconductance state S1 as in the M-state in a clump.

The complexity of burst analysis results obtained for a system with multiple conductance and kinetic states can be appreciated when trying to interpret the results. While this burst analysis has been fairly crude in nature (i.e. specifying an interburst interval of 4.0ms for all agonists etc.), it still gives us some indication of the kinetic patterns that can be observed when channels are activated by different agonists. Reassuringly, the clump duration findings paralleled the agonist-dependent trends found in the open times for the slower component of the M type open time histograms using different agonists.

SUMMARY OF FINDINGS

1. The data indicate that the AChR/channels of G8 myotubes possess a minimum of four different conformational states (i.e. M, S1, S2 open states and at least one closed state) each possessing distinct conductances. (approximately 40pS, 13pS, and 7pS respectively)

2. Although conductance values for these states are not agonist-dependent, some of the kinetic parameters of the multiple conductance states are agonist-dependent. Given that a channel is open, the likelihood of it being in a specific state differs considerably among different activating molecule(s).

3. For most agonists it was possible to demonstrate that transitions between closed, subconductance and full open states of the AChR/channel are microscopically reversible, suggesting that there are cyclic transitions in the overall activation scheme. In general the system appears to be in equilibrium since the ratio of forward and backward transition rates for all possible transition pairs either did not deviate significantly from, or were close to unity.

4. The several kinetically different open states which can be observed in the data are distinguished by their mean open times. For the subconductance state S1, only one kinetic open state was resolved. The subconductance state S2 for agonists SucCh, ACh and SubCh, displays two kinetically different open states while revealing only one for Cur, Chol, Nic and Carb. In

general, open times for the subconductance are agonist independent or (in the case of τ_{slow} for S1) show no trends that can be related to parameters of the agonist such as molecular weight or valence. From M type openings of the G8 AChRs, two kinetic open states can be resolved. The duration of the short-lived openings are agonist-independent while the more long-lived openings vary with the stabilizing ability ("agonist strength") of the activating molecule.

5. Burst analysis on the single channel data confirms the trends observed in the mean open time studies; those drugs which activated long channel openings also displayed a preference for longer-lived bursts.

GENERAL IMPLICATIONS OF OUR FINDINGS

Several researchers have speculated that the conformational flexibility of the five subunit AChR structure could be the source of subconductance events (Hamill and Sakmann, 1981; Auerbach and Sachs, 1983, Takeda and Trautmann, 1984; Morris and Montpetit, 1986). The implications of this statement are :

Channels are essentially enzymes, for which agonists are allosteric activators (Changeux et al., 1983). Subconductance states in channels demonstrate that a given allosteric effector can enhance the stability of more than one active state of the enzyme.

The AChR is a pentameric structure. In an immature condition it exhibits conformational degrees of freedom not detected in AChRs at the neuromuscular junction. Should it be shown, using in vivo embryonic preparations that subconductances are a normal feature of developing AChRs rather than a form of tissue culture pathology, a maturation process that constrains the channel to avoid allosterically-activated subconductance states should be sought. Since G8 cells can be co-cultured with neurons to form functional synapses (Christian et al., 1977) they might be a useful preparation for continued study of such maturation processes.

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APPENDIX

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PROGRAM Ksaml: {Continuous A/D sampling to disk}
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```
{Uses SYSLIB non-wait I/O using double buffering algorithm given in
Section 2.4.62 of RT-11 Advanced Programmers Guide. Maximum
sampling rate is approximately 20 Khz using the AR11 A/D
and an RK05 disk on a 11/40.}
```

```
{A version(19 Aug 83, K. IWASA) of THRU to acquire series of data from
the same cells, with generating LOG-file}
```

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CONST
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```
bufsize=8192; {32 BLOCKS}
NonWaitMode=10100B;
maxlen=14; {32*2 *14 = 896 blocks}
bell=7;
```

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TYPE
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```
  databuf=array[1..bufsize] of integer;
```

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VAR
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```
  OutputFile, LogFile: text;
  buffer1,buffer2:databuf;
  i,wordcount,block,channel,error,adcount, adch:integer;
  ring, ch, more:char;
  blocking, exnum, dlen :integer;
  rat: real;
  ExpName:array[1..6] of char;
  FName:array[1..10] of char;
  procedure LBADC(var buffer:databuf; adchannel:integer; var count:integer);
    external;
  procedure LBCLK(rate:REAL);external;
  function iwrite(var wordcount:integer; var buffer:databuf;
    var block,channel:integer):integer;fortran;
  function iwait(var channel:integer):integer;fortran;
  function getc:char;external;
  function GetChannel(VAR f:text):integer;external;
```

```
PROCEDURE FILLBUF1;
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```
  {Waits for interrupt driven A/D sampling routine to fill buffer1}
  begin
    if adcount<=bufsize then writeln('FB1-Buffer overflow',adcount);
    while adcount>bufsize do;
  end;
```

```
PROCEDURE FILLBUF2;
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```
  {Waits for interrupt driven A/D sampling routine to fill buffer2}
  begin
    if adcount>bufsize then writeln('FB2-Buffer overflow',adcount);
    while adcount<=bufsize do;
  end;
```

```
PROCEDURE WRIT(var buffer:databuf);
var error:integer;
```



```

begin
  error:=iwrite(wordcount,buffer,block,channel);
  if error<0 then
    case error of
      -1:writeln('Attempt to write past end of file');
      -2:writeln('Hardware error');
      -3:writeln('Channel not open');
    end;
    block:=block+blockinc
  end;
end;

PROCEDURE WAIT;
var error:integer;
begin
  error:=iwait(channel);
  if error=1 then writeln('Channel not open');
  if error=2 then writeln('Hardware error');
end;

PROCEDURE EXPERIMENT(var adchannel:integer; var rate:real);
VAR
  cell: array[1..80] of char;
  cond: array[1..80] of char;
  routine:char;
begin
  writeln('sampling program version 2.0KI
    autostop is on
    record length is set to 896 blocks
    if longer one is needed use KTHRU instead');
  writeln('
    this program will create a log file, expname.LOG');
  write('Experiment name:'); readln(ExpName);
  rewrite(LogFile,expname,'.LOG');
  writeln('what kind of cells?'); readln(cell);
  writeln(logfile,'[cell type]');
  writeln(logfile,cell);
  writeln('what sln, Temp, and drug?'); readln(cond);
  writeln(logfile,'[condition]');
  writeln(logfile,cond);
  write('routine sampling [channel: 0, sampling: 0.1 ms]? [y]');
  readln(routine);
  IF(routine='n') or (routine='N') then begin
    write('A/D channel(0-15):'); readln(adchannel);
    write('Sampling interval(msec):'); readln(rate) end
  else begin adchannel:=0; rate:=0.1 end;
  writeln(logfile,'[sampling interval] ',rate:1:3,' m sec');
end;

PROCEDURE INITIALIZE(var adchannel:integer; var rate:real);
VAR
  FileSize,error, j:integer;
  name: array[1..31] of char;
  volt: array[1..80] of char;
  jsw origin 44B:integer;
  f:text;
BEGIN

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    FileSize:=-1; {Request largest free space on disk}
    {following thing should be removed to manually input extension
    names}

```

```

    ( writeln('extension in 3 chrs');readln(name);
    for j:=1 to 6 do
        fname[j]:=expname[j];
    fname[7]:='.';
    for j:=8 to 10 do
        fname[j]:=name[j-7];
    rewrite(f,'nl:');
    rewrite(f);
    writeln(f,expname,'.',exnum:1);
    reset(f);
    readln(f,fname);

```

```

    {above 5 lines should be removed to manually input extension
    names}

```

```

    rewrite(outputfile,fname,'DAT',FileSize);
    writeln('xxxxxx now after next input, data will be taken xxxxx');
    writeln('voltage, on or off cell or something?');

    readln(volt);
    RING:=CHR(BELL); WRITELN(RING);
    writeln(logfile, fname,': ',volt);
    writeln(' xxxxx SAMPLING ',EXNUM:1,' STARTED xxxxx');
    writeln; writeln('Type Q(upper case) to manually terminate sampling');
    writeln('autostop on');
    blockinc:=bufsize div 256;
    jsr:=jsr or NonWaitMode;
    wordcount:=bufsize;
    block:=0;
    channel:=GetChannel(OutputFile);
    adcount:=-bufsize*2;
    LBADC(buffer1,adchannel,adcount);          {Sample into both buffers in}
    LBCLK(rate);                               {continuous wrap-around mode}

```

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END;

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```

PROCEDURE MAIN(var adchannel:integer;var rate:real);
BEGIN {MAIN}
    initialize(adchannel,rate);
    fillbuf1;
    for dlen:=1 to maxlen do begin
        writ(buffer1);
        ch:=getc; if ch='Q' then exit;
        fillbuf2;
        wait;
        writ(buffer2);
        ch:=getc; if ch='Q' then exit;
        fillbuf1;
        wait;
        if false then exit;
    end;
    LBCLK(0.);          {Stop sampling}
    close(OutputFile)
END;

```

```
BEGIN
  experiment(adch,rat);
  exnum:=1;
  repeat
    main(adch,rat);
    writeln('*** sampling STOPed ***');
    ring:=chr(bell); writeln(ring);
    write('more data?[Y]');
    READLN(MORE);
    exnum:=exnum+1;
  until (more='n') or (more='N');
  close(logfile)
END.
```

```

program analysis;
{open-close time analysis program in Pascal
 version 1: 22 Sep 82 K.I.}
{display used ddraw instead of ddot}
label 1; label 2; label 3;

Const
done=true;
reclen=4096; blocknumber=16;{seek number is related!}
specialmode=10100B;
lowerCaseDisable=137777B;
draw=1; erase=0;{var for procedure pointing}
baseline=200;{baseline for y coordinate}
delay=100;{baseline for time coordinate}
type
  block=array[1..256] of integer;
  forinput=record
    case integer of
      1:(total:array[1..blocknumber] of block);
      2:(each:array[1..reclen] of integer);
    end;
  str14= array[1..14] of char;
Var
fetch,search,cook,check, opened, closed, quit, thrown, pthrown
  :boolean;{control status }
groundlevel, openlevel, increment, threshold, openpoint,
closepoint, eventnumber, number, length : integer;
opentime, closetime, scale:real;
outfile, g: text;
outname, fname: str14;
databuff:forinput;
f:file of block;
x, y, pointer,movestep:integer; {used for moving pointer}

upsidedown:boolean;
c:char;
sum, denominator, start:integer;
samplingtime, cduration :real;
jsw ORIGIN 44B: integer;{Job status switch}
{----- external procedures -----}
procedure dini; external;
procedure derase; external;
procedure ddot( x,y:integer); external;
procedure dtext(var g:text); external;
procedure dmove( x,y:integer); external;
procedure ddraw( x,y:integer); external;
procedure dcolor(color:integer);external;
function getc:char; external;

{----- internal procedures -----}
procedure getn(var infile:block; i:integer);
Begin seek(f,i); infile:=f^; End;
{the above routine is the core of datatransfer}
{----- }
Procedure ask;
Var
  j, len:integer;
  answer:char;
  procedure changename(givenname:str14; var changedname:str14);
    var

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```

i, dotloc, emptloc :integer;
begin
  dotloc:=1;
  for i:=1 to 14 do begin
    changedname[i]:=givenname[i];
    if givenname[i]='.' then dotloc:=i; end;
  for i:=dotloc+1 to dotloc+3 do
    if givenname[i]=' ' then changedname[i]:='k' else emptloc:=i;
    if emptloc=(dotloc+3) then changedname[emptloc]:='k';
  end;
Begin
  writeln('Welcome to open - close analysis!
  version 1a in Pascal[Ki-28Sep83]');
  writeln('data must be without header. ');
  repeat
    write('data file name? [devise:name.ext] '); readln(fname);
    reset(f, fname, 'temp.dat/seek/buffersize:4096.', length);
    if (length<0) then writeln('no such file');
  Until length<=-1;
  write('opening = upward ?[y]'); readln(answer);
  if (answer='n') or (answer='N') then upsidedown:=true
  else upsidedown:=false;
  write('sampling time: 0.1 ms? [y]'); readln(answer);
  if (answer='n') or (answer='N') then readln(samplingtime)
  else samplingtime:=0.1 ;
  write('scaling factor:1/21 ? [y]'); readln(answer);
  if (answer='n') or (answer='N') then readln(scale)
  else scale:=1/21;
  writeln('increment to judge rise and fall');
  read(increment);
  writeln('threshold for opening and closing jumps');
  read(threshold);
  changename(fname,outname);
  writeln('outputfile=',outname);
  repeat
    rewrite(outfile, outname, '26au1.2k ',len);
    writeln('outfile length:',len);
  until len<=-1;
  writeln(outfile, 'inputfile=',fname);
  writeln(outfile, 'outputfile=',outname);
  dini; derase;
  rewrite(q,'nl:');
  {initial flag setting:}
  fetch:=false;cook:=false; check:=false; opened:=false;
  quit:=false; closed:=false; thrown:=true; pthrown:=true;
  {end of init setting}
  pointer:=0; movestep:=1; sum:=0;
End;
----- }

procedure fetchdata;
var
  dat, addr, mean, j , sign: integer;
  sumf:real;
Begin
  addr:=pointer*blocknumber;
  for j:=1 to blocknumber do getn(databuff.total[j],addr+j);
  sumf:=0;
  with databuff do begin
    for j:=1 to reclen do sumf:=sumf+each[j];
    mean:= round(sumf/reclen);

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writeln('sumf=',sumf,'mean=',mean);
if upsidetdown=true then sign:=-1 else sign:=1;
for j:=1 to reclen do
  each[j]:=(each[j]-mean)*sign;
end;
start:=1;
writeln('mean value of data:',mean);
sum:=0;start:=1;number:=0;denominator:=1;
writeln('data is fetched');
pointer:=pointer+1;
End;
C ----- }

Procedure pointing(i:integer);
const
  wing=50;
var
  xend,yend, upordown:integer;
  { x, y: global variable }
begin
  dcolor(i);
  dmove(x,y);
  if (opened=true) then upordown:=1
    else upordown:=-1;
  xend:=x+wing;
  yend:=y+wing*upordown;
  ddraw(xend, yend); dmove(x,y); ddraw(xend, y);
  dcolor(draw);
end;
C ----- }

Procedure display(changepoint:integer);
Const
  displen=511;
Var
  j, dispvalu, disppoint:integer;
Begin
  if changepoint<reclen then begin
    writeln('display starts at: ',changepoint);
    pointing(erase);
    derase;
    j:=1; disppoint:=1; dmove(1,baseline);
    while (disppoint<reclen) and (j<=displen) do
      begin
        disppoint:=changepoint+j-delay;
        if disppoint>0 then begin
          dispvalu:=databuff.each[disppoint]+baseline;
          ddraw(j,dispvalu); end;
        j:=j+1;
      end {while};
    x:=delay;
    if opened=true then y:=openlevel+baseline
      else y:=groundlevel+baseline;
    pointing(draw);
    dmove(50,30); dtext(q);
    end{if};
    else writeln('display refused');
  End;
C ----- }

```

Procedure help;

```
begin
  writeln('list of commands

  G -good transition point
  B -bad transition point
  J -jump ahead 300 points
  M -minus jump(backward) 100 points
  N -skip to the next record
  Q -quit, with an option to analyse new data
  T -throw out because of uncertain change');
  writeln('
    move pointer with arrows
    pf1-key: multiply step size
    pf2-key: reset step size')
end;
```

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< ----->
Procedure findopen;

```
Var
  rise, jump :boolean;
  average, point, i :integer;
  diff: array[1..3] of integer;

begin
  write(q, 'looking for opening');
  point:=start;(<starts search from point:=start>)
  writeln('search for up starts from ',point,' in the',
    pointer,'th rec');
  repeat with databuff do begin
    if (point>=reclen-5) then search:=done    else search:=false;
    number:=number+1;
    if number<4 then groundlevel:=each[point]
    else if number<20 then begin
      denominator:=denominator+1;
      sum:=sum+each[point]; end
    else
      sum:=sum+each[point]-each[point-19];
    average:=round(sum/denominator);
    groundlevel:=average;

    for i:=1 to 3 do
      diff[i]:=each[point+i+1]-each[point+i];
    rise:=(diff[1]>increment) and (diff[2]>increment)
      and (diff[3]>increment);
    if number>5 then
      jump:=(each[point+1]-groundlevel>threshold)
        and (each[point+2]-groundlevel>threshold)
        and (each[point+3]-groundlevel>threshold)
    else
      jump:=false;
    openpoint:=point;
    point:=point+1;
  end;
  until (jump=true) or (rise=true) or (search=done);
  if search=done then writeln('search is over at: ',point);
  if rise=true then writeln('rise at: ',point);
  if jump=true then writeln('jumped at: ',point);
end;
```

< ----->
Procedure findclose;

```

Var
  point, average, i:integer;
  fall, jump:boolean;
  diff: array[1..3] of integer;

Begin
  write(g,'looking for closing');
  point:=start;
  writeln('search for down starts at:',point);

  repeat with databuff do begin
    if (point>=reclen-5) then search:=done
    else search:=false;
    number:=number+1;
    if number<4 then openlevel:=each[point]
    else if number<20 then begin
      sum:=sum+each[point];
      denominator:=denominator+1;

      average:=round(sum/denominator);openlevel:=average end
    else begin
      sum:=sum+each[point]-each[point-19];
      average:=round(sum/denominator); openlevel:=average end;
    for i:=1 to 3 do
      diff[i]:=each[point+i]-each[point+i];
    fall:=(diff[1]<-increment) and (diff[2]<-increment)
      and (diff[3]<-increment);
    jump:= (each[point+1]<openlevel-threshold) and
      (each[point+2]<openlevel-threshold) and
      (each[point+3]<openlevel-threshold);
    closepoint:=point;
    point:=point+1;
    end;
    until (jump=true) or (fall=true) or (search=done);
    if search=done then writeln('search is over at: ',point);
    if fall=true then writeln('fall at: ',point);
    if jump=true then writeln('jumped at: ',point);
  end;
  -----

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Procedure movepointer;

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Const

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  uparrow=101B; downarrow=102B;
  rightarrow=103B; leftarrow=104B;
  pf1=120B; pf2=121B;

```

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Var

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  command: integer;

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Begin

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  repeat c:=getc until ord(c)<>0;
  command:=ord(c);writeln('moved');
  pointing(erase);
  case command of

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    uparrow:    y:=y+movestep;
    downarrow:  y:=y-movestep;
    rightarrow: x:=x+movestep;
    leftarrow:  x:=x-movestep;

```



```

pf1:      movestep:=movestep*3;
pf2:      movestep:=1;
else      writeln('out of commands');
end;
pointing(draw);
End;
( ----- )
Procedure checkchange(var changed:boolean; var changepoint:integer);
Var
  change, savejsw:integer;
Begin
  savejsw:=jsw;
  jsj:=jsj and lowercasedisable;
  jsj:=jsj or specialmode;
  quit:=false;
  write('command<');
  repeat c:=getc until ord(c)<>0;
  change:=ord(c);
  if change=33B then begin
    movepointer; check:=false end
  else begin
    check:=done;
    case c of
      'G': begin changed:=true; writeln(c); pthrown:=thrown;
            thrown:=false end;
      'B': begin changed:=false; start:=changepoint+4;
            sum:=0; denominator:=1; number:=0; writeln(c) end;
      'J': begin changed:=false;
            changepoint:=changepoint+300;
            if changepoint>reclen then search:=done;
            writeln(c); display(changepoint); check:=false end;
      'M': begin opened:=false; changepoint:=changepoint-100;
            writeln(c); display(changepoint); check:=false end;
      'Q': begin quit:=true; writeln(c) end;
      'N': begin writeln(c); changed:=false; search:=done end;
      'H': begin writeln(c); check:=false; changed:=false; help; end;
      'T': begin writeln(c); changed:=true; start:=changepoint+4;
            pthrown:=thrown; thrown:=true; end;
            {pthrown:previously thrown out}
    else begin changed:=false; check:=false end;
    end;{case}
  end;{else}
  jsj:=savejsw;
End;
( ----- )
Procedure prepare;{prepare to find closing}
Var
  a,b:real;
Begin
  openpoint:=openpoint+x-delay;
  a:=pointer; a:=a*reclen; b:=openpoint;
  opentime:=a+b;
  if (pthrown=true) or (thrown=true) then cduration:=0
  else cduration:=(opentime-closetime)*samplingtime;
  groundlevel:=y-baseline;
  start:=openpoint+4;
  number:=0; denominator:=1; sum:=0;
  closed:=false; opened:=true;
  writeln('prepared to find down');
  derase;

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End;
C ----- }
Procedure finish;
var
  more: char;
Begin
  writeln('events so far analyzed: ',eventnumber);
  readln; write('more data to analyze?[n] ');
  readln(more);
  if (more='y') or (more='Y') then begin fetch:=true;
    write('which record to examine?'); readln(pointer);
    pointer:=pointer-1;
    thrown:=true; pthrown:=true;
    start:=1;
  end
  else begin fetch:=false;close(outfile); end;
end;
C ----- }
Procedure preparenext;
  (input:  groundlevel, x, y, eventnumber)
  (output: openlevel,eventnumber, start; SCREEN,OUTPUTFILE)
var
  oduration, magnitude,a,b: real;
Begin
  eventnumber:=eventnumber+1;(global)
  closepoint:=closepoint+x-delay;
  a:=pointer; a:=a*reclen; b:=closepoint;
  closetime:=a+b;
  if (thrown=true) or (pthrown=true) then oduration:=0 else
    oduration:=(closetime-opentime)*samplingtime;
  openlevel:=y-baseline;
  magnitude:=(openlevel-groundlevel)*scale;
  start:=closepoint+4;
  writeln(eventnumber:3, oduration:12:3, magnitude:12:3, cduration:12:3);
  writeln(outfile, eventnumber:3, oduration:12:3,
    magnitude:12:3, cduration:12:3);
  number:=0; sum:=0;denominator:=1;
  closed:=true; opened:=false;
  writeln('prepared to find up');
  erase;
End;
C ----- main program ----- }
Begin
  ask;
  repeat (fetch)
    2: fetchdata;
    repeat (cook)
      repeat (opening)
        if (length<=blocknumber*pointer) then goto 1;
        findopen;
        if search=done then goto 2;
        display(openpoint);
        repeat
          checkchange(opened,openpoint);
          if quit=true then goto 1;
          if search=done then goto 2;
        until check=done;
        until opened=true;
        prepare;
        repeat (closing)

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3:   if (length<=blocknumber*pointer) then goto 1;
      findclose;
      if search=done then begin fetchdata; goto 3; end;
      display(closepoint);
      repeat
        checkchange(closed,closepoint);
        if quit=true then goto 1;
        if search=done then begin fetchdata; goto 3;end;
      until check=done;
      until closed=true;
      preparenext;
      until cook=done;
      1: finish;
      until fetch=false;
End.

```

```

(program to analise height distribution
 to aid effective channel event analysis)
(modified 26 sep 83 to didplay levels)
program hite3;
const
  reclen=4096; blocknumber=16; (reclen=blocknumber*256)
  maxhi=500; step=1;
type
  block=array[1..256] of integer;
  forinput=record
    case integer of
      1:(total:array[1..blocknumber] of block);
      2:(each:array[1..reclen] of integer);
    end;
  str10=array[1..10] of char;
  str14=packed array[1..14] of char;
var
  dataB: forinput;
  distr: array[1..maxhi] of integer;
  pointer, subzero, toohi, n, basehite: integer;
  f: file of block;
  g: text;
  fname: str14;
  reply:char;
  ( ----- external procedures ----- )
  procedure dini; external;
  procedure derase; external;
  procedure ddot( x,y:integer); external;
  procedure dtext( var g:text);external;
  procedure dmove( x,y:integer); external;
  procedure ddraw( x,y:integer); external;
  procedure dcolor(color:integer);external;
  function getc:char; external;
  procedure mtxhp;external;
  function lower(ch: char):char; external;
  procedure show(name:str14); external;
  ( ----- internal procedures ----- )
  procedure getn(var infile:block; i:integer);
  Begin seek(f,i); infile:=f^; End;
  ( ----- )
  procedure initialize;
  var
    ch: char;
    length : integer;
  begin
    write('need help?'); readln(ch); ch:=lower(ch);
    if ch='y' then show('plt:hite3.doc ');
    write('base height:'); readln(basehite);
    repeat
      write('input file:'); readln(fname);
      reset(f, fname,'temp.dat/seek/buffersize:4096.',length);
      if (length<0) then writeln('no file') ;
    Until length>1;
    pointer:=0;
    dini; derase;
    subzero:=0; toohi:=0;
    rewrite(g,'nl:');
  end;
  ( ----- )
  procedure erase;

```

```

var
  i:integer;
begin
  for i:=1 to maxhi do distr[i]:=0;
end;
( ----- )
Procedure fetchdata;
var
  dat, addr, mean, j ,j1: integer;
  sumf:real;
Begin
  addr:=pointer*blocknumber;
  for j:=1 to blocknumber do getn(dataB.total[j],addr+j);
  sumf:=0;
  for j1:=1 to reclen do
    begin
      dat:=dataB.each[j1];
      sumf:=sumf+dat;
    end;
  mean:= round(sumf/reclen);
  writeln('sumf=',sumf,'mean=',mean);
  writeln('mean value of data:',mean);
  writeln('data fetched');
  pointer:=pointer+1;
End;
( ----- )
procedure filter3;
var
  data: array[1..reclen] of integer;
  j: integer;
begin
  with dataB do begin
    data[1]:=round((each[1]+each[2])/2);
    data[reclen]:=round((each[reclen-1]+each[reclen])/2);
    for j:=2 to reclen-1 do
      data[j]:=round((each[j-1]+each[j]+each[j+1])/3);
    end;
  with dataB do begin
    for j:=1 to reclen do
      each[j]:=data[j];
    end;
  end;
( ----- )
procedure disprec;
const
  displen=512; maxint=9999;
var
  start, offset, dispval,i,j, maxdat, mindat: integer;
  dispbuff: array[1..displen] of integer;
  scale: real;
begin
  start:=0; offset:=350;derase;
  maxdat:=0; mindat:=maxint;
  for i:=1 to reclen do begin
    if dataB.each[i]>maxdat then maxdat:=dataB.each[i];
    if dataB.each[i]<mindat then mindat:=dataB.each[i];
  end;
  scale:=100/(maxdat-mindat);
  for j:=1 to 4 do begin
    for i:=1 to displen do

```

```

    dispbuff[i]:=round((dataB.each[2*i+start]-mindat)*scale);
    dmove(1,dispbuff[i]+offset);
    for i:=2 to displen do begin
        dispvalu:=dispbuff[i]+offset;
        ddraw(i,dispvalu); end;
    start:=start+displen*2; offset:=offset-100;
end;
dmove(50,20); writeln(g,'data file=',fname,'rec.#',pointer:1);
dtext(g);
end;

```

```

C -----
procedure discriminate;
var
    i, height: integer;
begin
    for i:=1 to reclen do
        begin
            height:=round(dataB.each[i]/ step)+basehite;
            if (height>=1) and (height<=maxhi) then
                distr[height]:=distr[height]+1
            else if height>maxhi then
                toohi:=toohi+1
            else
                subzero:=subzero+1;
        end;
    end;
end;

```

```

C -----
Procedure checkloc;
const
    specialmode=10100B;
Var
    change, savejsw, x, movestep, j:integer;
    c:char;
    xst: array[1..10] of integer;
    jsw origin 44B: integer;
    channelhite, number:integer;
    Procedure movepointer;
    Const
        rightarrow=103B; leftarrow=104B;
        pf1=120B; pf2=121B;
        draw=1; erase=0;
    Var
        command: integer;
        c:char;
    Procedure pointing(i:integer);
    begin
        dcolor(i);
        dmove(x,10);
        ddraw(x,500);
        dcolor(draw);
    end;

    Begin
        repeat c:=getc until ord(c)<>0;
        command:=ord(c);writeln('moved');
        pointing(erase);
        case command of

            rightarrow: x:=x+movestep;

```

```

leftarrow: x:=x-movestep;

pf1:      movestep:=movestep*3;
pf2:      movestep:=1;
else      writeln('out of commands');
end;
pointing(draw);
End;
(-----)

Begin
movestep:=2; xst[1]:=400; xst[2]:=100;
for j:=3 to 10 do xst[j]:=200;
write('How many cursors(<=10)?'); readln(number);
savejsw:=jsw;
jsw:=jsw or specialmode;
for j:=1 to number do begin
x:=xst[j];
repeat
write('command<');
repeat c:=getc until ord(c)<>0;
change:=ord(c);
if change=33B then movepointer
else if (c='g') or (c='G') then exit
else writeln('no such command');
until false;
writeln('cursor loc=', x-basehite);
dmove(x,400); write(g,x-basehite:1); dtext(g);
xst[j]:=x;
end;
End;
(-----)

procedure findpeaks;
var
highest, j, area, level: integer;
begin
highest:=0;
for j:=1 to maxhi do if distr[j]>highest then highest:=distr[j];
level:=highest;
repeat
area:=0;
for j:=1 to maxhi do
if distr[j]>level then area:=area+distr[j]-level;
until area>=0.05*reclen;
nd;
(-----)

procedure display;
const
baseline=100; labelline=60;
var
i, highest, dispvalu:integer;
norm:real;
begin
derase;
highest:=0;
for i:=1 to maxhi do
if distr[i]>highest then highest:=distr[i];
norm:=400/highest;
dmove(1, distr[i]+baseline);
for i:=2 to maxhi do begin
dispvalu:=round(distr[i]*norm);

```

```
ddraw(i,dispval+baseline); end;
writeln('subzero=', subzero);
writeln('too high=',toohi);
writeln(q,'data file = ',fname);
writeln(q,'record no ',pointer);
dmove(100,labelline);
dtext(q);
end;
(-----)

begin(main program)
  initialize;
  repeat
    erase;Cerase array distr>
    fetchdata;
    disprec;
    discriminate;
    display;
    checkloc;
    write('hard copy[h], quit[q], or continue[ret]?'); readln(reply);
    if reply='h' then mtxhp;
    if reply='q' then exit;
  until false;
end.
```



```

Program fivestates; {with variance analysis}
{channel analysis program in Pascal
classifies events into open1,open2,open3,open4 and closed states
version 6 of burst family: 28 Feb 1984: K.I.
no vm version, no variance analysis: 17 Apr 1984: ki }
{calculates variance to see what kind of fit this is producing}
{see note on Mar 86 change in 3CAT.PAS source code. Same applies here.}
Const
  reclen=4096; blocknumber=16; {seek number is related!}
  baseline=200; {baseline for y coordinate}
  displen=512; {display length on matrox}
  yes=1; no=-1; dunno=0;
type
  block=array[1..256] of integer;
  fordata=record
    case integer of
      1:(total:array[1..blocknumber] of block);
      2:(each:array[1..reclen] of integer);
    end;
  str14= array[1..14] of char;
  unsigned=0..65535;
  reply=-1..1;
  state=0..4;
Var
  upward, adjust: boolean;
  currentlevel: array[0..4] of integer;
  previousstate: state;
  threshold1, threshold2, threshold3, threshold4: integer;
  length, eventnumber: unsigned;
  printlines, size1, size2, size3, size4, number, filelength,
  closecutoff, opencutoff:
  integer;
  sum, scale, maxvar: real;
  g, forhist, help, sf: text;
Co: for dtext, for hist: for HIST )
  name, hname: str14;
  data:fordata;
  f:file of block;
  reduced: array[1..reclen] of integer;
  x, y, pointer, lastrec:integer; {used for moving pointer}
  c:char;
  samplingtime :real;
  {----- external procedures -----}
  procedure dini; external;
  procedure derase; external;
  procedure ddot( x,y:integer); external;
  procedure dtext(var g:text); external;
  procedure dmove( x,y:integer); external;
  procedure ddraw( x,y:integer); external;
  procedure dcolor(color:integer);external;
  function getc:char; external;
  procedure show(name:str14);external;
  {----- internal procedures -----}

function answer:reply;
var
  ch: char;
function lower(ch:char):char; external;
begin
  readln(ch); ch:=lower(ch);

```

```

if ch='y' then answer:=yes
else if ch='n' then answer:=no
else answer:=dunno;
end;

procedure getn(var infile:block; i:integer);
Begin seek(f,i); infile:=f^; End;

Procedure ask;
var
  j, len:integer;
  looseness: real;
procedure chname(givenname:str14; ch: char; var changedname:str14);
external;
procedure changeroutine;
begin
  write('sampling time: 0.05 ms? [y]');
  if answer=no then readln(samplingtime)
  else samplingtime:=0.05 ;
  write('scaling factor:0.0488 ? [y]');
  if answer=no then readln(scale)
  else scale:=0.0488;
end;
Begin
write('Welcome to five level analysis [version 6c, 17 Apr 84, KI]');
  writeln('data must be without header. ');
repeat
  write('data file name? [device:name.ext] '); readln(fname);
  reset(f, fname, 'temp.dat/seek/buffersize:4096.', filelength);
  if (filelength<0) then writeln('no such file');
until filelength<>-1;
  write('routine condition[sampling=0.05ms; scale=0.0488]?');
  if answer=no then changeroutine
  else begin samplingtime:=0.05; scale:=0.0488; end;

  write('channel opening is upward?');
  {this is to reverse the data so that upward in the display is open}
  if answer=no then upward:=false else upward:=true;
  write('openlevel 4='); readln(currentlevel[4]);
  write('openlevel 3='); readln(currentlevel[3]);
  write('openlevel 2='); readln(currentlevel[2]);
  write('openlevel 1='); readln(currentlevel[1]);
  write('closedlevel='); readln(currentlevel[0]);
  threshold1:=round((currentlevel[1]+currentlevel[0])/2);
  threshold2:=round((currentlevel[2]+currentlevel[1])/2);
  threshold3:=round((currentlevel[3]+currentlevel[2])/2);
  threshold4:=round((currentlevel[4]+currentlevel[3])/2);
  size1:=abs(currentlevel[1]-currentlevel[0]);
  size2:=abs(currentlevel[2]-currentlevel[0]);
  size3:=abs(currentlevel[3]-currentlevel[0]);
  size4:=abs(currentlevel[4]-currentlevel[0]);
  if upward=false then
    for j:=0 to 4 do currentlevel[j]:=-currentlevel[j];
  { write('default cutoff [2 pts]?');
  if answer=no then begin
    write('open time cutoff='); readln(opencutoff);
    write('close time cutoff='); readln(closecutoff);
    end
  else begin opencutoff:=2; closecutoff:=2; end;
}

```

```

write('what extension character for outputfile?');readln(c);
chname(fname,c, hname);
writeln('outputfile=',hname);
repeat rewrite(forhist, hname, '.dat', len) until len <>-1;
writeln(forhist, 'inputfile=', hname);
dini; derase;
rewrite(g,'nl:');
sum:=0;
write('Entire data file to be analysed?');
if answer=yes then begin pointer:=0;
                        lastrec:=round(filelength/blocknumber); end
else begin
writeln('file length=',round(filelength/blocknumber):2,' records');
write('starting record number='); readln(pointer); pointer:=pointer-1;
write('last record number='); readln(lastrec);
end;
length:=0;
{
maxvar:=size2;
maxvar:=maxvar*size2;
maxvar:=maxvar*displen;
write('routine variance check?');
if answer=no then begin
write('routine value: 0.2 which corresponds to half channel size
deviation.
A smaller value imposes stricter criterion.
value=');
readln(looseness); end
else looseness:=0.2;
maxvar:=maxvar*looseness;
}

{maxvar:=size^2*displen/5 avoid integer overflow!!}
printlines:=0;
End;

Procedure fetchdata;
var
    addr, j : integer;
Begin
    addr:=pointer*blocknumber;
    with data do begin
        for j:=1 to blocknumber do getn(total[j],addr+j);
        if upward=false then for j:=1 to reclen do each[j]:=-each[j];
    end;
    pointer:=pointer+1;
    writeln('working on record ', pointer:1);
End;
----- }

Procedure display(n:integer);
{displays and calculates variance}
type
    str50= array[1..50] of char;
Var
    j, dispvalu, st:integer;
    variance:real;
    line: str50;
{
Procedure estvariance;
var

```

```

    j: integer;
    diff: real;
begin
    variance:=0;
    with data do
        for j:=st+1 to st+displen do begin
            diff:=each[j]-reduced[j];
            variance:=variance+diff*diff;
        end;
    end;
end;
)
Begin
    derase; st:=n*displen;
    with data do begin
        dmove(1,each[1+st]+baseline);
        for j:=2 to displen do ddraw(j,each[j+st]+baseline);
    end;
    dmove(1, reduced[1+st]+baseline);
    for j:=2 to displen do ddraw(j, reduced[j+st]+baseline);
    dmove(50,100); writeln(q,'rec ',pointer:2); dtext(q);
    (
        estvariance;
        reset(sf);
        if (variance<maxvar) and (printlines>=1) then
            for j:=1 to printlines do begin
                readln(sf, line); writeln(forthist, line);
                writeln(line);
            end
        else if variance>=maxvar then
            writeln('data rejected based on variance analysis');
    )
    printlines:=0; (rewrite(sf));
End;

```

Procedure find;

```

var
    i, n: integer;
    currentstate: state;
    displflg :boolean;

```

procedure print;

```

var
    duration, magnitude: real;
begin
    eventnumber:=eventnumber+1;
    duration:=length*samplingtime;
    if length=0 then magnitude:=0 else magnitude:=sum*scale/length;
    if eventnumber=1 then duration:=0;
    writeln(forthist, eventnumber:3, duration:12:3, magnitude:12:3,
        previousstate:2, currentstate:2);
    writeln(eventnumber:3, duration:12:3, magnitude:12:3, previousstate:2,
        currentstate:2);
    printlines:=printlines+1;
end;

```

procedure level(n: state);

```

begin
    currentstate:=n;
    reduced[i]:=currentlevel[n];
    if previousstate<>n then

```

```

    begin
      print; length:=0; sum:=0;
    end;
    length:=length+1;
    previousstate:=n;
  end;

Begin
  n:=0;
  with data do
    begin
      displflg:=false;
      for i:=1 to reclen do begin
        if (each[i]<threshold1) then level(0)
        else if (each[i]=threshold1) and (each[i]<threshold2) then
          begin level(1); displflg:=true; end
        else if (each[i]=threshold2) and (each[i]<threshold3) then
          begin level(2); displflg:=true; end
        else if (each[i]=threshold3) and (each[i]<threshold4) then
          begin level(3); displflg:=true; end
        else
          begin level(4); displflg:=true; end;
        sum:=sum+each[i]-currentlevel[0];
        if i mod(512)=0 then
          begin
            if displflg=true then
              display(n); n:=n+1; displflg:=false; end;
          end;
      end;
    end;
  end;
End;

----- main program -----
Begin
  ask;
  repeat
    fetchdata;
  until pointer>=lastrec;
  close(forhist);
End.

```

Program TALLY; {scans through output files from CLEAN to give a reading
on the number of each possible transition type as well
as the relative probabilities of observing each state.
Cathy Morris, Mar 1985}

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```
const
  openstates=4; {number of distinguishable open states}

type
  state=0..openstates;
  opnstat=1..openstates;
  str14=array[1..14] of char;
  unsigned=0..65535;

var
  inf:text;
  fname:str14;
  tally:array[state,state] of integer;
  condopnprob:array[opnstat] of real;
  dwell,prob:array[state] of real;
  i,j:state;
  count:unsigned;
  totaldurn,opndurn,cutoff:real;

procedure initialize;
type
  str50 = array[1..50] of char;
var
  len:integer;
  title:str50;
  ans:char;

begin
  repeat
    writeln('What CLEANed file is being TALLYed?');
    write('input filename = '); readln(fname);
    reset(inf,fname,'temp.dat',len);
    if len=-1 then
      writeln('You must be kidding! Try again.');
```

```

procedure transitions;
var
  previousstate,currentstate:state;
  eventnumber:unsigned;
  duration,magnitude:real;

begin
  totaldurn:=0; count:=0; opndurn:=0;
  repeat
    readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);
    tally[previousstate,currentstate]:=tally[previousstate,currentstate]+1;
    dwell[previousstate]:=dwell[previousstate]+duration;
    totaldurn:=totaldurn+duration;
    if previousstate>0 then opndurn:=opndurn + duration;
    count:=count+1;
  until eof (inf);
  for i:=0 to openstates do
    begin prob[i]:=dwell[i]/totaldurn;end;
  for i:=1 to openstates do
    begin condopnprob[i]:=dwell[i]/opndurn; end;
  end;

procedure createfiles;
var
  outfile:text;
  status:integer;

begin
  status:=20;
  rewrite(outfile,'DK:Trans.tal','temp.dat',status);
  writeln('output file allocation = ',status);writeln;
  writeln(outfile,'Tally of transitions from ',fname);
  writeln(outfile);
  writeln(outfile,'A CUTOFF OF ',cutoff:10:3,' WAS USED IN MAKING THIS ');
  writeln(outfile,'ANALYSIS. ALL EVENTS <= THIS DURATION IGNORED. IF');
  writeln(outfile,'MEAN TIMES ARE NOT TOO DIFFERENT FROM CUTOFF, DONT ');
  writeln(outfile,'FORGET THIS FACTOR WHEN INTERPRETTING DATA!');
  writeln(outfile);writeln(outfile);
  writeln(outfile,'total number of transitions = ',count:6);
  writeln(outfile,'total duration of CLEANed record = ',totaldurn:12:3);
  writeln(outfile);
  writeln(outfile,'Number of i-state to j-state transitions found in recor
  writeln(outfile);
  for i:=0 to openstates do
    for j:=0 to openstates do
      begin if tally[i,j]>0 then
        writeln(outfile,i:2,' to ',j:2,' - ',tally[i,j]:6);
      end;
  writeln(outfile);
  writeln(outfile,'All other types of transitions were not seen in this');
  writeln(outfile,'record.');
```

```

writeln(outfile);writeln(outfile);writeln(outfile);
writeln(outfile,'Probabilities for the various states.');
```

```

writeln(outfile);
for i:= 0 to openstates do
  writeln(outfile,i:2,' ',prob[i]:6:5);
writeln(outfile);
writeln(outfile,'Probability that, given that a channel is open, it is')
writeln(outfile,'in open state 1,2,3...etc');
```

```

for i:=1 to openstates do
  writeln(outfile,i:2,' ',condopnprob[i]:6:5);

```

```
writeln(outfile);  
close(outfile);  
writeln('The output file created by this run is called DK:TRANS.TAL and');  
writeln('the next running of the program will create a new file with the');  
writeln('same name which will therefore erase the present version. SO...');  
writeln('IF YOU WANT TO KEEP THE CONTENTS OF TRANS.TAL YOU MUST RENAME IT');  
writeln('APPROPRIATELY IMMEDIATELY ON EXITING THIS RUN!');  
end;
```

```
BEGIN(main)  
  initialize;  
  startfresh;  
  transitions;  
  createfiles;  
END.
```


Program Mirror; {Uses CLEANed files. Checking for the number of occurrences
of a particular sequence of channel states and the number
of occurrences of the mirror image of this sequence. 185
Sequences of from 2 to 10 can be examined.
Cathy Morris June 1986 }

Const

openstates=5;

type

state=0..openstates;

str14=array [1..14] of char;

str50=array [1..50] of char;

var

inf:text;

fname:str14;

series,posn:array [1..10] of state;

seqsize,j,model1,model2,count,eventnumber:integer;

duration,magnitude:real;

first,previousstate,currentstate:state;

title:str50;

procedure initialize;

var

len:integer;

begin {initialize}

repeat

writeln('Which CLEANed file is to be examined?');

write('input filename = ');readln(fname);

reset(inf,fname,'temp.dat',len);

if len=-1 then

writeln('Wake up little Susie. Try again.');

until len>0; writeln;readln(inf,title);writeln(title);readln(inf,title);

writeln(title);writeln;

write('How many positions are there in the sequence?[2-10] ');

readln(seqsize);

for j:=1 to seqsize do posn[j]:=0;

for j:=1 to seqsize do

begin write('State for position ',j:2,' is? [0-5] ');readln(posn[j]);end;

writeln;

writeln('Before continuing, doublecheck: you are going to compare the ');

writeln('number of sequences ...');

for j:=1 to seqsize do writeln(posn[j]);

writeln('...to the number of mirror-image sequences...');

for j:=seqsize downto 1 do writeln(posn[j]);

writeln;writeln('Be patient. This program was not written for speed!');

writeln('Long data files can take a few minutes and then some.');

count:=0; model1:=0; model2:=0;

end; {initialize}

procedure compare;

var

check,mircheck,comp,mircomp:integer;

begin

comp:=0;mircomp:=0;check:=0;mircheck:=0;

for j:=1 to seqsize do

begin

comp:=abs(series[j]-posn[j]);

mircomp:=abs(series[j]-posn[seqsize+1-j]);

```

    check:=check+comp;
    mircheck:=mircheck+mircomp;
end;
    if check = 0 then model1:=model1 + 1;
    if mircheck = 0 then model2:=model2 + 1;
end;

procedure slide;
begin
    for j:=2 to seqsize do series[j-1]:=series[j];
    readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);
    series[seqsize]:=currentstate;
    count:=count+1;
end;

procedure showandtell;
var
    outf:text;
begin
    rewrite(outf,'DK:MIRSEQ.LST','temp.dat');
    writeln; writeln('Output file has been opened. Almost finished...');
    writeln(outf,'CLEANed file used: ',fname);
    reset(inf);readln(inf,title);
    writeln(outf); writeln(outf,title); readln(inf,title); writeln(outf,title);
    writeln(outf);
    writeln(outf,'Sequence of states examined was ');
    for j:= 1 to seqsize do write(outf,' ',posn[j]:2);
    writeln(outf);
    writeln(outf,'The number of times this sequence occurred was ',model1:6);
    writeln(outf);
    writeln(outf,'The reverse sequence occurred ',model2:6,' times.');
```

```

    writeln(outf); count:=count+seqsize;
    writeln(outf,'The total number of transitions was ',count:9);
    writeln;
    writeln('The output file MIRSEQ.LST should be printed NOW if you want to ');
    writeln('keep the information.');
```

```

end;

begin (main)
    initialize;
    first:=posn[1];
    for j:=1 to seqsize do series[j]:=0;
    for j:=1 to seqsize do
        begin
            readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);
            series[j]:=currentstate;
        end;
    repeat
        if (series[j]=first) or (series[seqsize]=first)
            then compare;
        slide;
    until eof(inf);
    showandtell;
end.
```

Program zfbHST; Ca simple program to arrange data that has been through the CLEAN program into a histogram format. It is assumed that the data contains substates, fullstates and closed intervals. User must make appropriate hard copy of the outputfiles immediately after running. This program only deals with one state at a time. N.B. This program does NOT plot the histogram or lump the bins in any way. It does, however, provide a mean for all events longer than a stated duration, and this can be used in maximum likelihood estimates with the appropriate precautions and additional calculations. Nov 1984, Cathy Morris. Updated Feb 1986 to include ZERO FREQUENCY BINS in the output. These are needed in some of the curve-fitting procedures. Files from this program will take a long time to print out compared to files with the zeroes suppressed. March 1986. To facilitate transfer to the Compaq without need for editing, the histogram data and the associated information have been divided into separate files. The bincentre is calculated within the program - a feature not present in earlier versions.

```
CONST
  capacity=2048; (number of bins used in histograms)

TYPE
  state=0..3;
  unsigned=0..65535;
  str14=ARRAY [1..14] OF char;
  mult=1..200;
VAR
  inf:text;
  pickstate:state;
  fname:str14;
  unitduration,cut1,cut2,smcut1durn,smcut2durn:real;
  count,j,cut1cnt,cut2cnt:unsigned;
  maxbin:unsigned;
  cntlong:unsigned;
  bin:ARRAY[1..capacity] OF unsigned;
  binwidth,bwsf:mult; (binwidth scaling factor value)
  (-----)

PROCEDURE initialize;
TYPE
  str50=ARRAY [1..50] OF char;
VAR
  len:integer;
  title,secndline:str50;
  ans1,ans2:char;
BEGIN
```

```
  writeln('If you didn't delete DK:FHIST.* before starting, crash out ');
  writeln('and do so now to avoid filesize problems.');
```

```
  writeln;
  REPEAT
    write('input filename='); readln(fname);
    reset(inf, fname, 'temp.dat', len);
    IF len=-1
      THEN writeln('No such file. Check device, name, extension');
```

```

UNTIL len>0;
readln(inf, title); writeln (title);
readln(inf, secndline); writeln (secndline); (assumes 2 lines before data)
( proper starts)
writeln;
write('Digitized sample time (msec):'); readln(unitduration);
writeln;
writeln('If binwidths wider than sample time are needed,');
); writeln('use what scaling factor?. Choose 1, for binwidths to = sample time
write('      Choose from: [2..200] in integers '); readln(binwidth);
writeln;
writeln('Histogram for which state?for closed type 0');
writeln('      for smallest open type 1');
writeln('      for next open type 2');
writeln('      for next open type 3 '); readln(pickstate)
writeln('Mean times that can be used for single-expo max lik estimates');
writeln(' are being made but you must choose truncation point. ');
cut1:=0.300; cut2:=0.450;
write('Cutoff for first estimate is <=0.3 msec. O.K? [y/n]'); readln(ans1);
IF (ans1 = 'n') OR (ans1 = 'N')
  THEN
    BEGIN
      write('First cutoff <= ?? [msec] '); readln(cut1);
    END;
write('Cutoff for second estimate is <=.45 msec. O.K? [y/n]'); readln(ans2);
IF (ans2 = 'n') OR (ans2 = 'N')
  THEN
    BEGIN
      write('Second cutoff <= ?? [msec] '); readln(cut2);
    END;
; writeln('A reminder: if you want to keep the outputfiles at the end of this
; writeln('run they should be renamed when program terminates. They have the
writeln('"generic" names FHIST.CHS and FORFIT.HST .');
writeln;writeln;
writeln('This takes a while....you will be notified when you are needed. ');
writeln;
END;
(-----)

```

PROCEDURE fillup;

VAR

```

maxsize,rem:real;
duration,magnitude,length,largest:real;
previousstate,currentstate:state;
eventnumber:integer;
leng,intlen:unsigned;

```

BEGIN

```

count:=0;
maxsize:=0;
cntlong:=0;
cut1cnt:=0;cut2cnt:=0;smcut1durn:=0;smcut2durn:=0;

```

REPEAT

```

  readln (inf,eventnumber,duration,magnitude,previousstate,currentstate);

```

```

  IF previousstate=pickstate

```

THEN

BEGIN

```

  count:=count+1;

```

```

  IF duration>maxsize

```

```

    THEN maxsize:=duration;

```

END;

```

UNTIL eof(inf);
largest:= maxsize/unitduration;
IF largest<= 32767.000
THEN
  BEGIN
    maxbin:=round(maxsize/unitduration);
    IF maxbin>capacity
    THEN bwsf:=binwidth
    ELSE bwsf:=1;
  END
ELSE
  BEGIN
    maxbin:=capacity; bwsf:=binwidth;
  END;
writeln('Binwidths have been set. Histograms being constructed.');
```

<Initializes bins for each histogram.>

```

FOR j:=1 TO capacity DO
  bin[j]:=0;
  reset(inf);readln(inf);readln(inf);
  REPEAT
    readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);
    IF (previousstate=pickstate)
    THEN
      BEGIN <pickstate>
        IF duration > cut1
        THEN
          BEGIN
            cut1cnt:=cut1cnt+1;
            smcut1durn:=smcut1durn+duration;
          END;
        IF duration > cut2
        THEN
          BEGIN
            cut2cnt:=cut2cnt+1;
            smcut2durn:=smcut2durn+duration;
          END;
        <last 2 ifs were for max lik mean estimates>
        <next stuff is to organize bins for hist>
        length:=duration/unitduration; <must be real for this test>
        IF length<32767.000
        THEN
          BEGIN <length>
            intlen:=round(length);
            rem:=intlen MOD bwsf; <Erects appropriate bin size >
            IF rem=0
            THEN leng:=round(length/bwsf)
            ELSE leng:=(trunc(length/bwsf))+1;
            IF (leng<=capacity)
            THEN bin[leng]:=bin[leng]+1
            ELSE cntlong:=cntlong+1;
          END <length>
        ELSE
          IF (length>32767.000)
          THEN cntlong:=cntlong+1;
        END;
        <pickstate>
      UNTIL eof(inf);
    END;
  <----->
PROCEDURE createfiles;
```

```

VAR
  chos,hst:text;
  status,len:integer;
  binsize,binmid,bincent:real;
BEGIN
  len:=20; status:=200;
  rewrite(chos,'DK:FHIST.chs','temp.dat',len);
  rewrite(hst,'DK:FORFIT.HST','temp.dat',status);
  writeln('output file allocation for data = ',status); writeln;
  writeln(chos,'Distribution of ',pickstate:1,' state events from ',fname);
  writeln(chos); writeln(chos);
  writeln(chos,'      total number of ',pickstate:1,' state events: ',count:6)
  writeln(chos);
  writeln(chos,'# of ',pickstate:1,' state events too long for histogram');
  writeln(chos,'      ie >512*unitduration*scalingfactor : ',cntlong:6);
  writeln(chos); writeln(chos);
  writeln(chos,'Bin size is ',unitduration*bwsf:10:3,' msec');
  writeln(chos);writeln(chos);
  writeln(chos,'top of BIN(MSEC.)      FREQUENCY');

  writeln(hst,fname,' Capacity given on next line...');
  writeln(hst,capacity);
  binsize:=unitduration*bwsf; binmid:=binsize/2;
  FOR j:=2 TO capacity DO
    BEGIN
      bincent:=(j*binsize)-binmid;
      writeln(hst,bincent:12:3,'      ',bin[j]);
    END;
  writeln(chos);writeln(chos);
  writeln(chos,'A mean that can be used for max lik est on single expo data');
  writeln(chos,'has been made. N.B. Only for SINGLE EXPONENTIAL!');
  writeln(chos);
  IF (cut1cnt > 0)
    THEN
      BEGIN
        writeln(chos,'...estimate with ',cut1:10:3,' msec as cutoff is ');
        '); writeln(chos,'      ',smcut1durn/cut1cnt:10:3,' - ',cut1:10:3,' mse
      END;
  IF (cut2cnt > 0)
    THEN
      BEGIN
        writeln(chos,'...estimate with ',cut2:10:3,' msec as cutoff is ');
        '); writeln(chos,'      ',smcut2durn/cut2cnt:10:3,' - ',cut2:10:3,' mse
      END;
  close(chos); close(hst);
  writeln('Logfile DK:FHIST.CHS created. Rename or print it out. ');
  writeln;
  writeln('Datafile DK:FORFIT.HST created. Rename this now,according');
  writeln('to some appropriate convention. ');
  writeln;
  writeln(cntlong:6,' of the ',count:6,' ',pickstate:1,' state events');
  writeln('      were too long. ');

  writeln;
  writeln('Do not forget - print out the files you want now!');
  writeln('If you get eof errors increase value of var status');
  writeln('in the source version of the program and recompile. ');
END;

```

(main)

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```
BEGIN
  initialize;
  fillup;
  createfiles;
END.
```

ogram ERSTAN; Uses CLEANed data files and outputs two files related to 192 burst parameters. File BURST.BST can be used for doing kinetic analysis of burst and closed durations, by feeding it through one of the XXXHST.sav programs. File BURST.LST lists the averages which can be obtained immediately. When the program terminates, the .LST file should be printed immediately and the .BST file should be renamed and saved. Because the input has already been CLEANed, no further provision is made in this program for discarding brief transitions. To change the number of openstates, change the appropriate constant and recompile. One additional output file is created; BURST.OUT which is an ASCII file suitable for transmitting to the Compaq for the Lotus program. Cathy Morris March 1986. This version has a provision for batch processing of up to 50 clean files. Wade, Aug/86 }

```

const
  openstates=4; {number of distinguishable open states}

type
  state=0..openstates;
  str14=array[1..14] of char;
  unsigned=0..65535;
  trls=1..50;

var
  inf:text;
  outname:str14;
  fname:array[1..50] of str14;
  interburstmin:real;
  trials:trls;
  stop,wades,both:boolean;
  i:trls;
  tempoutf,outputf,dataoutf:text;
  valu:real;
  val:array[trls] of real;
  z,len,batchnumber,trialsdone :integer;

procedure changename1st(givename:str14; var changedname:str14);
var
  i, dotloc, emptloc :integer;
begin
  dotloc:=1;
  for i:=1 to 14 do
    begin
      changedname[i]:=givename[i];
      if givename[i]='.' then dotloc:=i;
    end;
    changedname[dotloc+1]:='1';
    changedname[dotloc+2]:='s';
    changedname[dotloc+3]:='t';
  end;

procedure changenameout(givename:str14; var changedname:str14);
var
  i, dotloc, emptloc :integer;
begin
  dotloc:=1;
  for i:=1 to 14 do

```



```

begin
  changedname[i]:=givenname[i];
  if givenname[i]='.' then dotloc:=i;
end;
changedname[dotloc+1]:='o';
changedname[dotloc+2]:='u';
changedname[dotloc+3]:='t';
end;

procedure changenamebst(givenname:str14; var changedname:str14);
var
  i, dotloc, emptloc :integer;
begin
  dotloc:=1;
  for i:=1 to 14 do
    begin
      changedname[i]:=givenname[i];
      if givenname[i]='.' then dotloc:=i;
    end;
    changedname[dotloc+1]:='b';
    changedname[dotloc+2]:='s';
    changedname[dotloc+3]:='t';
  end;
end;

procedure batch;
var
  reply:str14;
  x,k: integer;

procedure checkfile;
var
  foundfile: boolean;

begin
  reset(inf,fname[k],'temp.dat',len);
  if len=-1 then
    begin
      writeln('You must have goofed! Try again. ');
      k:=k-1;
    end;
end;

begin
  begin
    k:=0;
    write('How many files to be processed? '); readln(batchnumber);
    writeln('Enter files names to be processed. ');
    for k:=1 to batchnumber do
      begin
        write('File #',k:3,' = '); readln(reply);
        fname[k]:=reply; checkfile;
      end;
    end;
    writeln('The files about to be processed are ... ');
    for x:=1 to batchnumber do
      begin
        writeln(fname[x]);
      end;
    end;
  end;
end;

```

```

procedure setintervals;
var
  reply: char;
  k,int: integer;
begin
  trials:=34;
  val[1]:=0.1; val[2]:=0.2; val[3]:=0.3; val[4]:=0.4;
  val[5]:=0.5; val[6]:=1; val[7]:=1.5; val[8]:=2;
  val[9]:=2.5; val[10]:=3; val[11]:=3.5; val[12]:=4;
  val[13]:=4.5; val[14]:=5; val[15]:=6; val[16]:=7;
  val[17]:=10; val[18]:=13; val[19]:=18; val[20]:=27; val[21]:=39;
  val[22]:=54; val[23]:=72; val[24]:=93; val[25]:=105;
  val[26]:=125; val[27]:=155; val[28]:=190; val[29]:=230;
  val[30]:=275; val[31]:=325; val[32]:=380; val[33]:=440; val[34]:=505;

  writeln('The current preset interburst intervals are: ');
  for i:=1 to trials do
    writeln('Interval #',i:3,' = ', val[i]:5:1);

  write('Do you want to extend or reduce the number of intervals used? n/y ');
  readln(reply);
  if reply='y' then
    begin
      write('Answer with an "E/e" to extend or a "R/r" to reduce. ');
      readln(reply);
      if (reply='E') or (reply='e') then
        begin
          write('How many extra intervals? '); readln(int);
          for k:= trials+1 to int+trials do
            begin
              write('Interval # ',k:3,' = ? [msec]'); readln(valu);
              val[trials]:=valu;
            end;
          writeln('The number of intervals is now ',trials+int);
        end;

      if (reply='R') or (reply='r') then
        begin
          write('How many of the long intervals do you want deleted? ');
          readln(int);
          trials:= trials - int;
          writeln('The number of intervals is now ',trials);
        end;
    end; {modifications}
end; {procedure}

procedure initialize;
type
  str50 = array[1..50] of char;
var
  title:str50;
  ans,resp: char;
begin
  writeln('Do you want BOTH the time-sequence file and the listing of ');
  write('averages for the burst parameters? [y/n] '); readln(resp);
  if (resp='y') or (resp='Y') then

```

```

begin
  both:=true; trials:=1
end
else
begin
  both:=false;
  writeln('Only the output file for average parameters will be made. ');
  write('Do you want to use the preset interburst times? y/n ');
  readln(resp);
  if (resp='y') or (resp='Y') then
    setintervals
  else
begin
  write('How many minimum interburst times do you want to try? [1-50] ');
  readln(trials);
  writeln('WARNING. Enter interburst times in ascending order. ');
  for i:=1 to trials do val[i]:=0;
  for i:=1 to trials do
begin
  write('Value # ', i:3, ' = ? [msec]'); readln(valu);
  val[i]:=valu;
end;
end; {user set intervals}
writeln('Do you want the # of closings for' );
write('all states output in filename.OUT? y/n '); read(resp);
if (resp='y') or (resp='Y') then
  wades:=false
else
  wades:=true;
end; {interval definitions}
? both=true then
begin
  interburstmin:=1.0; {default value}
  writeln('Minimum interburst interval is currently set at 1.0 msec. ');
  write('Do you want to leave this as is? [y/n] '); readln(ans);
  if (ans='n') or (ans='N') then
begin
  write('New minimum interburst interval = ? [msec] ');
  readln(interburstmin);
end
else writeln('Default value of 1.0 msec is being used. ');
end;
writeln; writeln('Program is running. Dont type anything for the moment. ');
writeln;
writeln('WARNING, using 34 intervals on a large clean file will cause ');
writeln('this program to run as long as 1 hour, so take a nap! ');
writeln;
end;

procedure findmaxduration;

var
  eventnumber:unsigned;
  k,x:integer;
  duration,maxduration,magnitude:real;
  currentstate,previousstate: state;

begin
  maxduration:= 0; k:=0;
  reset(inf,fname[z]);

```

```

readln(inf); readln(inf); {gets to the data}
repeat
begin
  readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);
  if previousstate=0 then
    if duration > maxduration then maxduration:=duration;
  end;
until eof(inf);
for x:=trials downto 1 do
begin
  if val[x] > maxduration then
    k:=k+1;
  end;
trials:=trials - k;
end; {procedure}

-----bursts -----}

procedure bursts;
const
  dummyevnum=9999;
  dummymag=99.999;
  dummyps=1;
  dummyps=0; {these are to provide fillers for the time sequence files}
var
  j,previousstate,currentstate:state;
  eventnumber:unsigned;
  duration, magnitude,thisburstdurn,totlbrstdurn:real;
  outforhist:text;
  k,ctnbursts,status,blks:integer;
  local,global:array[state] of integer;
  localdurn,smfrctn:array[state] of real;
begin {bursts}
  reset(inf,fname[z],'temp.dat',len);
  blks:=5*trials;
  changename1st(fname[z],outname);
  rewrite(dataoutf,outname,'temp.dat',blks);
  rewrite(tempoutf,'DK:TEMP.DAT','temp.dat',blks);
  writeln('Output listing file (.LIST) for ',fname[z],' has been opened. ');
  writeln;
  writeln(batchnumber-z,' file(s) to be processed. ');
  findmaxduration;
  if both=true then
  begin
    status:=300;
    writeln; changenamebst(fname[z],outname);
    writeln('Output histogram (.BurST) for ',fname[z],' has been opened. ');
    rewrite(outforhist,outname,'temp.dat'); {,status};
    writeln(outforhist,'Data from ',fname[z],' analyzed for bursts ');
    writeln(outforhist,'with a minimum interburst time of ',interburstmin:12:3);
  end;

  for i:=1 to trials do
  begin {analysis}
    reset(inf,fname[z]);readln(inf); readln(inf); {gets past 2 info lines in file}
    if both=false then interburstmin:=val[i];
    repeat
      readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);

```

```

until ((previousstate=0) and (duration>=interburstmin) or eof(inf));
  {this step will cover the possibility that the record begins in the middle
  of a burst. It gets you safely to the first stretch of baseline}

totlbrstdurn:=0; cntbursts:=0;
for j:= 0 to openstates do
  begin global[j]:=0;
    smfrctn[j]:=0; end;

repeat {go through data}
  thisburstdurn:=0;
  for j:= 0 to openstates do
    begin local[j]:=0;
      localdurn[j]:=0; end;
  repeat {newburst}
    readln(inf,eventnumber,duration,magnitude,previousstate,currentstate);
    if ((previousstate <>0) or ((previousstate=0)and(duration<interburstmin)))
      then
        begin thisburstdurn:=thisburstdurn + duration;
          local[previousstate]:=local[previousstate] + 1;
          localdurn[previousstate]:=localdurn[previousstate] + duration;
        end;
    until((previousstate=0)and(duration>=interburstmin))or eof(inf));
  {end of newburst repeat}
  totlbrstdurn:=totlbrstdurn + thisburstdurn;
  cntbursts:=cntbursts + 1;
  for j:=0 to openstates do
    begin smfrctn[j]:=smfrctn[j] + localdurn[j];
      global[j]:=global[j] + local[j]; end;
  if both=true then
    begin
      writeln(outforhist,cntbursts:7,thisburstdurn:12:3,dummymag:12:3,dummysps:4,
        dummyscs:2);
      writeln(outforhist,dummyevnum:7,duration:12:3,magnitude:12:3,previousstate:4,
        currentstate:2);
      {this should create an outputfile with open - that is bursting - events
      and closed events all sorted out for processing through an XXXHST.sav
      program. The dummy values are there as spacers except for ps and cs
      which are for real and can be used in subsequent programs}
    end;
  until eof(inf); {end of go through data repeat}

if both=true then close(outforhist);

writeln(dataoutf,'Listing of burst analysis from ',fname[z]);
writeln(tempoutf,fname[z]);
writeln(dataoutf,'The interburst minimum was ',interburstmin:12:3,' msec');
writeln(tempoutf,interburstmin:12:3);
writeln(dataoutf,'See also the appropriate time sequence file for this run. ');
writeln(dataoutf);
writeln(dataoutf,'Rough average of burst duration = ');
writeln(dataoutf,' ',totlbrstdurn/cntbursts:12:3,' msec');
writeln(tempoutf,totlbrstdurn/cntbursts:12:3);
writeln(dataoutf);
writeln(dataoutf,'This figure is only of a ballpark nature; to get an ');
writeln(dataoutf,'accurate version, histograms must be fit to data from ');
writeln(dataoutf,'the burst.BST file. ');
writeln(dataoutf); writeln(dataoutf);writeln(dataoutf);

for j:= 0 to openstates do

```

```

begin
  writeln(dataoutf,'Average # of ',j:2,'-state events per burst= ');
  writeln(dataoutf,'          ',global[j]/cntbursts:12:3);
  writeln(tempoutf,global[j]/cntbursts:12:3);
  writeln(dataoutf);
end;

for j:= 0 to openstates do
begin
  writeln(dataoutf,'Average proportion of bursting time spent in state-',j:2);
  writeln(dataoutf,'          = ',smfrctn[j]/totlbrstdurn:12:3);
  writeln(tempoutf,smfrctn[j]/totlbrstdurn:12:3);
  writeln(dataoutf);
end;
writeln(dataoutf,'Total number of bursts counted was ',cntbursts:9);
writeln(tempoutf,cntbursts:9);
for k:=1 to 16 do (adds enough lines to cause a new page to be made on )
begin (the printer for the next interburst interval)
  writeln(dataoutf);
end;
end; (analysis)
end; (bursts)

Procedure rearrangeOutputfile;
var
  blks,j,x:integer;
  burstavg,onestateprop,zerostate,twostate,threestate:real;
  onestate,fourstate:real;

begin
  blks:=1*trials;
  changenameout(fname[z],outname);
  rewrite(outputf,outname,'temp.dat',blks); (,'temp.dat',blks);
  reset(tempoutf,'dk:temp.dat','temp.dat',blks);
  for j:=1 to trials do
    begin
      if wades=true then
      begin
        readln(tempoutf);readln(tempoutf);
        readln(tempoutf,burstavg);
        readln(tempoutf,zerostate);
        for x:=1 to 5 do
          readln(tempoutf);
        readln(tempoutf,onestateprop);
        for x:=1 to 4 do
          readln(tempoutf);
        writeln(outputf,burstavg:12:3,onestateprop:12:3,zerostate:12:3);
      end
      else
      begin
        readln(tempoutf);readln(tempoutf);
        readln(tempoutf,burstavg);
        readln(tempoutf,zerostate); readln(tempoutf,onestate);
        readln(tempoutf,twostate); readln(tempoutf,threestate);
        readln(tempoutf,fourstate);
        for x:=1 to 6 do
          readln(tempoutf);
        reestate:12:3,zerostate:12:3,onestate:12:3,twostate:12:3,th
      end;
    end;
  end; (finish writing filename.OUT)

```

```
close(outputf);  
end; (procedure)
```

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```
( -----main ----- )
```

```
BEGIN (main)  
  batch;  
  initialize;  
  for z:=1 to batchnumber do  
    begin  
      bursts;  
      close(dataoutf);  
      close(tempoutf);  
      rearrangeOUTputfile;  
    end;  
  for i:= 1 to 10 do  
    write (chr(7));  
  if both=true then  
    begin  
      writeln('The output files created by this run are:');  
      for i:=1 to z do  
        begin  
          writeln(fname[z],'.BST and', fname[z],'.LST.');        end;  
      writeln('You should immediately process these files. ');  
    end  
  else  
    writeln('Print out:');  
    for i:=1 to z do  
      begin  
        writeln(fname[z],'.LST and ', fname[z],'.OUT (input for lotus)... NOW!');      end;  
  ND.
```

```

'Program Marqfit.bas -- a non-linear least squares fitting
'routine using the marquardt algorithm
'(c) 1985 W. Schreiner, M. Kramer, S. Krischer, & Y. Langsam
30 '-----initialize program settings-----
40 REV = 4.3: PI = 3.14159265#
40 ON ERROR GOTO 19000: RECORD = 0: KEY OFF
60 DEF SEG=64: POKE 23,(PEEK(23) OR 64): DEF SEG 'set caps lock
65 ' The values MXVAR%, MXOBS% AND MDI% can be changed
70 ' be sure to change the DIM sizes correspondingly
80 MXVAR%=30: MXOBS%=300: MDI%=MXVAR%*(MXVAR%+1)/2
90 DIM X(300), DTA(300), UNBINY(300),WGT(300),BF(300),FXP(300),FYP(300),XP(300)
100 DIM YP(300), C(265), A(265), B(30), DPARM(30), PARM(30), KFIX(30),
    DERIV(30), G(30), GRAD(30)
110 DEF FNLGT(X)=LOG(X)/LOG(10)
120 ON KEY(1) GOSUB 10200: ON KEY(2) GOSUB 10300
130 ON KEY(3) GOSUB 10310: ON KEY(4) GOSUB 10400
140 KEY(1) ON : KEY(2) ON : KEY(3) ON: KEY(4) ON
160 FG=2: BG=0: FG1=14: BG1=0: FG2=4: BG2=0: ' color attributes
165 FOR I= 1 TO MXOBS%: WGT(I)=1 : NEXT
190 ' -----plot routine initialization
200 PLOTSET%=0 :PLOTFLG%=0 ' plot definition flag; user input of BF for function
210 XTOTAL.PIX = 639: YTOTAL.PIX=199
220 ' (for lo_res use xtotal.pix = 319)
230 ' xmin.pix is lower left hand xmin in pixel coords;
240 ' ymin.pix is lower left hand ymin in pixel coords;
250 'xmin and ymin are the same but user coordinates
260 XMIN.PIX = CINT(.11*XTOTAL.PIX): XMAX.PIX=(.94*XTOTAL.PIX)
270 YMAX.PIX= CINT(.07*YTOTAL.PIX)-2: YMIN.PIX= YTOTAL.PIX-12
280 DELTAX.PIX = XMAX.PIX - XMIN.PIX+1
290 DELTAY.PIX = YMIN.PIX-YMAX.PIX+1
300 ' functions to convert x & y in user coords --> pixel coords
310 DEF FNSX(N)= XMIN.PIX + CINT((N-XMIN)*DELTAX.PIX/(XMAX-XMIN))
320 DEF FNSY(N) = YMIN.PIX - CINT((N-YMIN)*DELTAY.PIX/(YMAX-YMIN))
335 ' -----start up -----
340 IPFLG = 0 : 'pause in fitting flag
350 ISVFL =-1 : ' data saved flag
360 CLS: LOCATE 10 : COLOR FG2,BG2: PRINT TAB(15) "MARQUARDT LEAST SQUARES
    - REV" ;REV:
362 PRINT: PRINT"Minimizes Chi-squared."
363 PRINT "This program uses data from (prog) REBINned histograms (at least 5 cb
    s/bin)."
```

364 PRINT "For graphic comparison of exp with obs. appropriate adjustments"

365 PRINT "are made on rebinned observations."

366 COLOR FG,BG: FOR I =1 TO 1000: NEXT : LOCATE 20

370 INPUT "USE DATA ON DRIVE (ENTER LETTER):"; DRV\$: DRV\$=LEFT\$(DRV\$,1): IF DRV\$

<> "" THEN DRV\$ = DRV\$+":"

380 GOTO 420

390 ' ----- MENU -----

400 PRINT "PRESS ANY KEY TO CONTINUE": WHILE INKEY\$ = "" :WEND

410 RETCOD=0: 'reset error flag

420 F1%=0: SCREEN 0,0: CLS: GOSUB 10000: COLOR FG2,BG: LOCATE 1,20:PLOTFLG%=0

425 PRINT "MENU OPTIONS:":COLOR FG,BG: PRINT: COLOR FG1,BG1

430 PRINT TAB(2) " GENERAL:":;COLOR FG,BG

435 PRINT TAB(21) "1 - ENTER TITLE " 'line 800

440 PRINT TAB(21) "2 - PRINTER "; 'line 850

450 IF PFLG%=0 THEN PRINT "ON/";:COLOR FG1,BG1:PRINT "OFF": COLOR FG,BG ELSE

COLOR FG1,BG1: PRINT "ON";: COLOR FG,BG: PRINT "/off"

460 PRINT TAB(21) "3 -. SOLVE " 'line 900

470 PRINT TAB(21) "4 - PLOT " 'LINE 15000

480 PRINT TAB(21) "5 - QUIT" 'line 1300

490 PRINT : COLOR FG1,BG1: PRINT " ENTER DATA:":; COLOR FG,BG: PRINT TAB(21)

"6 - MANUAL " 'line 1400

500 PRINT TAB(21) "7 - UREAD" 'line 1800


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10 PRINT
20 COLOR FG1,BG1: PRINT "  MODIFY DATA: ";: COLOR FG,BG: PRINT TAB(21)
   "8 - EDIT" 'LINE 2000
30 PRINT TAB(21)"9 - SCALE" 'LINE 2200
40 PRINT TAB(21)"10 - ZERO" 'LINE 2400
50 PRINT:COLOR FG1,BG1: PRINT "  PARAMETERS: ";: COLOR FG,BG: PRINT TAB(20)
   "11 - ENTER/REVIEW/CHANGE" 'line 2500
60 PRINT " 12 - FIX" 'line 2800
70 PRINT " 13 - FREE" 'line 3000
80 PRINT
90 PRINT
100 COLOR FG1,BG1: PRINT "  DATA & PARAM: ";: COLOR FG,BG: PRINT " 14 - LIST"
    'LINE 3200
110 PRINT " 15 - SAVE ON DISK" 'LINE 3400
120 PRINT " 16 - READ FROM DISK" 'line 3600
130 COLOR 15,BG: INPUT;"ENTER CHOICE"; KMND: COLOR FG,BG: PRINT
140 ON KMND+1 GOTO 420,800,850,900,15000,1300,1400,1800,2000,2200,2400,2500,2800
    ,3000,3200,3400,3600
150 PRINT "***** ERROR*****"; KMND;" INVALID COMMAND": GOTO 400
160 'enter a title for documentation -----
170 INPUT "ENTER TITLE: "; TITL$: LOCATE 25,1: PRINT TITL$; SPC(11)
180 GOTO 420
190 ' toggle printer on/off -----
200 PFLG% = 1-PFLG%: GOTO 420
210 'solve the mrqdt non-linear lst sq fit problem
220 ITER% = 0: IF IPFLG<> 0 THEN ITER%= NITER%
230 IPFLG= 0: ISVFL=0: CLS: GOSUB 10000
240 INPUT "HOW MANY ITERATIONS? "; MXITER%
250 IF MXITER% <0 THEN MXITER% = 0
260 GOSUB 6000 ' go fit
270 IPFLG = -1 : NITER% = NITER% + ITER%
280 PRINT: PRINT: PRINT TITL$: PRINT NITER%; "ITERATIONS": PRINT
290 IF PFLG%=1 THEN LPRINT : LPRINT TITL$ : LPRINT NITER%; "iterations"
300 GOSUB 9000
310 IF FLG%=1 THEN PRINT: PRINT "CHOLESKY NEGATIVE DIAGONAL --";
    "UNABLE TO SOLVE WITH SUPPLIED INITIAL PARAMETERS"
320 PRINT : PRINT "PRESS ANY KEY FOR FITTING STATISTICS"
330 WHILE INKEY$="":WEND
340 GOSUB 1100: PRINT : GOTO 400 'print statistics and return
350 'print fit statistics -----
360 PRINT : PRINT "SOME FITTING STATISTICS:"
370 PRINT "SIGMA="; SIG;"R= ";R;: PRINT TAB(41); "MINIMIZED CHI SQUARED = ";WSS
380 CHI2 = INT(10*CHI2)/10
390 PRINT "CHI SQUARED= ";CHI2;"/";NF;"DEG OF FREEDOM"
400 PRINT "# of calls to chi squared ="; NSSC;: PRINT TAB(41); "# of deriv call
    s="NDC: PRINT "# INC IN LAMBDA"; INCR;"LAMBDA=";LAMBDA
410 IF PFLG%<>1 THEN RETURN
420 LPRINT:LPRINT "SOME FITTING STATISTICS:"
430 LPRINT "SIGMA=";SIG;"R= ";R;:LPRINT TAB(41); "MINIMIZED CHI SQUARED = ";WSS
440 LPRINT "CHI SQUARED= ";CHI2;"/";NF;"DEG OF FREEDOM"
450 LPRINT "# OF CALLS TO CHI SQUARED =";NSSC;:LPRINT TAB(41); "# OF DERIV CALLS
    ="NDC: LPRINT "# INC IN LAMBDA="; INCR;"LAMBDA=";LAMBDA
460 RETURN
470 'quit -----
480 IF ISVFL <>0 THEN 1360
490 INPUT "PRESENT DATA NOT SAVED. SAVE IT?? (Y/N)"; T1$
500 IF LEFT$(T1$,1)= "Y" THEN 3400 'go save
510 IF LEFT$(T1$,1)<> "N" THEN 420
520 DEF SEG=64: POKE 23,(PEEK(23) AND 191): DEF SEG 'clear caps
530 KEY ON: CLS:END
540 ' manual data entry-----
550 PRINT "MANUAL DATA ENTRY - ": PRINT "  (, TO EXIT)"
560 'set flags to indicate data points that currently exist
570 FOR K=1 TO NOBS%: XP(K)=1: NEXT: FOR K= NOBS%+1 TO MXOBS%: XP(K)=0: NEXT
580 PRINT "POINT      X,MEASUREMENT"
590 PRINT "===== "
600 FOR I=1 TO MXOBS%

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440 PRINT I;: INPUT " ";T1$,T2$
450 IF T1$="" THEN NOBS%= I-1: GOTO 1480
460 X(I)=VAL (T1$): DTA(I)=VAL(T2$): XP(I)=1
470 NEXT
480 INPUT "WEIGHTS - ENTER 'NO', 'STAT' OR 'EXPLICIT'?"; T1$
490 IF T1$="NO" THEN FOR I=1 TO NOBS%: WGT(I)=1: NEXT: GOTO 1580
500 IF T1$="STAT" THEN FOR I=1 TO NOBS%: WGT(I)=SQR(DTA(I)): NEXT: GOTO 1580
510 IF T1$ <> "EXPLICIT" GOTO 1480
520 PRINT : PRINT "POINT          X          Y          WEIGHT":
      PRINT "=====          =====          ====="
530 FOR I = 1 TO NOBS%
540 PRINT I; "          ";X(I); "          ";DTA(I); "          ";WGT(I);
550 INPUT "New Weight="; WGT$: IF WGT$="" THEN 1570
560 WGT(I)= VAL(WGT$)
570 NEXT
580 NOBS%=0: PRINT "NOW ORDERING & REVIEWING DATA"
600 FOR I=1 TO MXOBS%
610 IF XP(I)=0 GOTO 1650 'ZERO IT IF PT IS TO BE DELETED
620 NOBS%=NOBS%+1
630 X(NOBS%)= X(I): DTA(NOBS%)= DTA(I): WGT(NOBS%)= WGT(I)
640 IF I<> NOBS% THEN XP(I)=0
650 NEXT
680 IPFLG=0: ISVFL=0: GOTO 420
800 'user defined read routine -----
810 PRINT " NO USER DEFINED ROUTINE IMPLEMENTED"
820 GOTO 400
2000 'edit data
2005 FOR K=1 TO NOBS%: XP(K)=1: NEXT
2010 FOR K=NOBS%+1 TO MXOBS%: XP(K)=0: NEXT
2015 PRINT "ENTER 'W' TO CHANGE ONLY WEIGHTS,";: INPUT "'D' TO CHANGE DATA & WEI
      GHTS";T1$
2020 IF T1$="W" GOTO 1480
2030 PRINT "ENTER DATA PT RANGE FOR EDITING (N1,N2)"
2040 INPUT "(, TO EXIT)"; T1$.T2$
2050 IF T1$="" GOTO 1580
2060 I=VAL(T1$):J=VAL(T2$)
2070 IF I<1 OR J>MXOBS% THEN PRINT "INVALID RANGE": GOTO 2030
2080 FOR K=I TO J
2090 PRINT "CURRENT VALUE OF X, DTA, WGT FOR PT #";K;" =": PRINT X(K); " ";DTA(K)
      : " "; WGT(K)
2100 PRINT "ENTER NEW VALUE FOR X(";K;")"
2105 PRINT "(ENTER 'D' TO DELETE PT, OR 'enter' ";: INPUT " TO LEAVE UNCHANGED)"
      : T1$
2110 IF T1$="D" THEN XP(K)=0 : GOTO 2150
2120 IF T1$="" THEN 2150
2130 X(K)=VAL(T1$): XP(K)=1
2140 INPUT "ENTER NEW VALUES FOR DTA,WGT";DTA(K),WGT(K)
2150 NEXT
2160 GOTO 2030
2200 'SCALE X,DTA,WGT -----
2210 INPUT "ENTER X-COORDINATE SCALE FACTOR";XSCALE
2220 INPUT "ENTER DATA POINT SCALE FACTOR";DSCALE
2230 INPUT "ENTER WEIGHT SCALE FACTOR";WSCALE
2240 FOR I=1 TO NOBS%
2250 X(I) =X(I)*XSCALE: DTA(I)=DTA(I)*DSCALE: WGT(I)=WGT(I)*WSCALE
2280 NEXT
2290 IPFLG= 0: ISVFL=0: GOTO 420
2400 'zero data & parameters -----
2410 FOR I=1 TO MXOBS%: X(I)=0: DTA(I)=0: WGT(I)=0: NEXT : NOBS%=0
2420 FOR I=1 TO MXVAR%: PARM(I)=0: DPARM(I)=0: NEXT: NPRM%=0
2430 IPFLG=0: ISVFL=-1: GOTO 420
2500 'enter/review/change param -----
2510 CLS: GOSUB 9000: IF NPRM%<1 THEN 2580
2530 PRINT "CHANGE PARAMETERS? ENTER: 'A' TO CHANGE ALL": PRINT TAB(29) "'S' TO
      SELECTIVELY CHANGE (or add parameters)"
2540 PRINT TAB(29) "press RETURN to leave unchanged": INPUT T1$

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550 IF T1$="" GOTO 420
560 IF T1$="S" THEN 2700
570 IF T1$<>"A" THEN 2530
580 PRINT "ENTER PARAMETERS ONE AT A TIME.":PRINT " (NULL TO EXIT)"
590 FOR I=1 TO MXVAR%
600 PRINT I;" "; INPUT T$: PRINT
610 IF T$="" THEN NPRM%=I-1: GOTO 2660
620 IF LEFT$(T$,1)=". " OR LEFT$(T$,1)="- " THEN 2640
630 IF LEFT$(T$,1) <"0" OR LEFT$(T$,1)>"9" THEN PRINT T$" INVALID, RETYPE":
    GOTO 2600
640 PARM(I) = VAL(T$)
650 NEXT
660 IF NPRM% <=0 GOTO 420
670 FOR I=1 TO NPRM% : DPARM(I)=0: NEXT
680 IPFLG=0: ISVFL=0: GOTO 420
690 'change some of the parameters -----
710 PRINT "ENTER PARM#, AND VALUE": PRINT SPC(10)"(, TO EXIT)"
720 INPUT T1$,T2$: IF T1$="" THEN GOSUB 9000: GOTO 400
730 IF VAL(T1$)= NPRM%+1 THEN NPRM%=NPRM%+1
740 IF VAL(T1$) < 1 OR VAL(T1$) > NPRM% THEN PRINT " INVALID PARM#, RANGE IS 1-
    NPRM%+1: GOTO 2720
750 IF LEFT$(T2$,1)=". " OR LEFT$(T2$,1)="- " THEN 2770
760 IF LEFT$(T2$,1) < "0" OR LEFT$(T2$,1) > "9" THEN PRINT T2$"INVALID, RETYPE"
    : GOTO 2720
770 PARM(VAL(T1$)) = VAL (T2$)
780 GOTO 2720
790 'fix some parameters -----
810 GOSUB 9000
820 IF NPRM% <1 THEN 400
830 INPUT "ENTER PARM# TO BE FIXED (NULL TO EXIT)";I
840 IF I=0 GOTO 2870
850 IF I<1 OR I>NPRM% THEN PRINT "INVALID PRAM#. RANGE IS 1-"; NPRM%: GOTO 2800
860 KFIX(I)= 1: F1%=1: GOTO 2830
870 IF F1%=0 GOTO 400 'nothing changed
880 GOSUB 9000: IPFLG=0: ISVFL=0: GOTO 400
890 'free some previously fixed param. -----
910 GOSUB 9000: IF NPRM%<1 GOTO 400
930 INPUT "ENTER PARM# TO BE FREED (NULL TO EXIT)"; I
940 IF I=0 GOTO 3070
950 IF I<1 OR I>NPRM% THEN PRINT "INVALID PARM#, RANGE IS 1-";NPRM%: GOTO 3000
960 KFIX(I)=0 : F1%=1: GOTO 3030
970 IF F1%=0 GOTO 400
980 GOSUB 9000: IPFLG=0: ISVFL=0: GOTO 400
990 'print data and param. values -----
1010 CLS: GOSUB 10000: PRINT "MARQUARDT LEAST SQUARES - REV";REV
1020 PRINT TITL$: PRINT NITER%:"ITERATIONS": PRINT
1030 IF PFLG%=1 THEN LPRINT TITL$:LPRINT NITER%:"ITERATIONS"
1040 GOSUB 9000: GOSUB 1100' print parameters and statistics
1060 PRINT: PRINT NOBS%:" DATA POINTS BEING FITTED": PRINT " X DTA
    WGT BF FMM"
1070 IF PFLG%=1 THEN LPRINT : LPRINT NOBS%:"DATA POINTS BEING FITTED":LPRINT "
    X DTA WGT BF FMM"
1080 IF NOBS%<=0 GOTO 3360
1090 PFORM$="##.##^"
1100 FOR I=1 TO NOBS%
1110 IF BRKFLG% THEN BRKFLG%=0: PRINT "BREAK IN LISTING": GOTO 400
1120 GOSUB 20000: FMM=FUNCTN-DTA(I)
1130 PRINT USING PFORM$;X(I),DTA(I),WGT(I),BF(I), FMM
1140 IF PFLG%=1 THEN LPRINT USING PFORM$;X(I),DTA(I),WGT(I),BF(I),FMM
1150 NEXT
1160 GOTO 400
1170 'save data & parameters -----
1180 PRINT "THESE ARE YOUR CURRENT FILES": FILES DRV$+"*.*"
1190 PRINT :INPUT "SAVE DATA AS DISK FILE NAMED";T1$
1200 IF MID$(T1$,2,1)<> ":" THEN T1$ = DRV$+T1$
1210 OPEN "O",2,T1$: IF RETCOD<>0 THEN CLOSE 2: GOTO 400

```

```

460 WRITE #2, TITL$: WRITE #2, NOBS%,BW
470 FOR I=1 TO NOBS%: PRINT #2,X(I),DTA(I),WGT(I),BF(I): NEXT
480 WRITE #2, NPRM%
485 FOR I=1 TO NPRM%: WRITE #2,PARM(I),DPARM(I),KFIX(I): NEXT
490 CLOSE 2: IPFLG=0: ISVFL=-1: GOTO 420
600 'read data & param. from disk -----
610 PRINT "THESE ARE YOUR CURRENT FILES": FILES DRV$+"*. *"
630 PRINT : INPUT " READ DATA & PARAM. FROM DISK FILE NAMED";T1$
635 IF MID$(T1$,2,1)<> ":" THEN T1$= DRV$+T1$
640 OPEN "I",3,T1$: IF RETCOD<> 0 THEN CLOSE 3: GOTO 400
660 INPUT #3, TITL$: INPUT #3, NOBS%,BW
670 FOR I=1 TO NOBS%: INPUT #3,X(I),DTA(I),WGT(I),BF(I): NEXT
680 INPUT #3,NPRM%
685 FOR I=1 TO NPRM%: INPUT #3, PARM(I),DPARM(I),KFIX(I): NEXT
690 CLOSE 3
700 IPFLG=0: ISVFL=-1: GOSUB 9000: PRINT
705 LOCATE 25,1: PRINT TITL$;: LOCATE 21 : PRINT : GOTO 400
7000 'subroutine to compute chi-squared -----
7010 CHI2=0
7020 FOR I=1 TO NOBS%
7030     IF WGT(I) =0 THEN 4060
7040     GOSUB 20000
7050     CHI2=CHI2+((FUNCTN-DTA(I))^2/dta(I))
7060 NEXT
7070 RETURN
7500 'subroutine obtains CHOLESKY backward solution of matrix A -----
7510 G(1)=G(1)/A(1)
7520 IF NFIT%<=1 THEN 4630
7530 L=1
7540 FOR I=2 TO NFIT%
7550 K=I-1
7560 FOR J=1 TO K
7570     L=L+1: G(I)=G(I)-A(L)*G(J)
7580 NEXT
7590     L=L+1: G(I)= G(I)/A(L)
7600 NEXT
7610 MDI%=(NFIT%*(NFIT%+1)/2
7620 G(NFIT%)=G(NFIT%)/A(MDI%)
7630 IF NFIT% <= 1 THEN RETURN
7640 FOR K1=2 TO NFIT%
7650     I=NFIT%+2-K1
7660     K=I-1: L=I*K/2
7670     FOR J=1 TO K
7680         G(J)=G(J)-G(I)*A(L+J)
7690     NEXT
7700     G(K)=G(K)/A(L)
7710 NEXT
7720 RETURN
5000 'subroutine to perform CHOLESKI decomposition of matrix a
5010 FLG%=0
5020 FOR J=1 TO NFIT%
5030     L=J*(J+1)/2
5040     IF J<= 1 THEN 5120
5050     FOR I=J TO NFIT%
5060         K1=I*(I-1)/2+J: F=A(K1): K2=J-1
5070         FOR K=1 TO K2: F=F-A(K1-K)*A(L-K): NEXT
5080         A(K1)=F
5090     NEXT
5100     IF A(L) >0 THEN 5160
5110     FLG%=1: PRINT "CHOLESKI - NEG. DIAG J,L,A(L)=";J,L,A(L)
5120 RETURN
5130 F=SQR(A(L))
5140 FOR I =J TO NFIT%
5150     K2=I*(I-1)/2+J: A(K2)=A(K2)/F
5160 NEXT
5170 NEXT

```

```

220 RETURN
500 'subroutine to calculate the chi square (wss=chi^2) -----
510 WSS=0: NSSC=NSSC+1
520 FOR I=1 TO NOBS%
530     GOSUB 20000
540     WSS = WSS + ((FUNCTN - DTA(I))^2/FUNCTN)
550 NEXT
570 RETURN
700 'subroutine to calculate the sum of squares -----
710 SS=0: NSSC=NSSC + 1
720 FOR I=1 TO NOBS%
730     GOSUB 20000: SS=SS+ (FUNCTN - DTA(I))^2
750 NEXT
770 RETURN
900 'subroutine to calculate the sum of DTA(I)^2 -----
910 R=0
920 FOR I=1 TO NOBS%: R=R + DTA(I)^2: NEXT
950 RETURN
990 'subroutine to do the main mathematics of the fitting -----
000 NFIT%=0: NX%=0
010 IF NPRM%<1 THEN RETURN 2500
020 FOR I=1 TO NPRM%
030     IF KFIX(I)=0 GOTO 6070
040     NX%=NX%+1
050     DPARM(NX%)=0!
060     GOTO 6090
070     NFIT%=NFIT%+1
080     B(NFIT%)=PARM(I)
090 NEXT
100 IF NFIT%<=0 THEN NITER%=ITER%: RETURN 420
110 'preform MARQUARDT fitting
120 IF NOBS%>=NFIT% THEN 6180
130 PRINT "# OF DATA POINTS (NOBS%=";NOBS%;")"; " IS LESS THAN"; TAB(41);
140     "# OF PARAM. TO BE FIT (NFIT%=";NFIT%;")"
150 PRINT "MODEL IS UNDETERMINED"; CHR$(7)
160 NITER%=ITER%: RETURN 400
170 PRCSN=2.5E-07: LAMBDA=.01: LMIN=1E+20: PHI=1
180 MDI%=NFIT%*(NFIT%+1)/2
190 NSSC=0: NDC=0: NITER%=0: INCR=0
200 GOSUB 5500 'Cholesky decomposition
210 PRINT "CHI SQUARED =";WSS
225 IF PFLG%=1 THEN LPRINT "CHI SQUARED =";WSS
230 GOTO 6280
240 ' next iteration -----
250 NITER%=NITER%+1
260 IF (NITER%>4 AND LAMBDA<LMIN) THEN LMIN=LAMBDA
270 LOCATE ,13 : PRINT WSS: IF PFLG% =1 THEN LPRINT WSS: " ";
280 IF NITER%>=MXITER% THEN PRINT MXITER%:"ITERATIONS - PAUSE": GOTO 6920
290 IF BRKFLG% THEN BRKFLG%=0 : PRINT "OPERATOR INTERRUPT": GOTO 6920
300 COLOR FG2,BG2: PRINT "COMPUTING ";: COLOR FG1,BG1
305 PRINT " ITERATION #";NITER%+1;: COLOR FG,BG : PRINT
310 '----- decrease lambda -----
320 LAMBDA=.31622777# * LAMBDA: INCR=0
330 '----- CALCULATE A=JTJ AND JTF
340 FOR I=1 TO MDI%: A(I)=0: NEXT
350 FOR I=1 TO NFIT%: GRAD(I)=0: NEXT
360 FOR I=1 TO NOBS%
370     GOSUB 21000
380     FMM=(F - DTA(I))*WGT(I)
390     J=0
400     FOR K=1 TO NPRM%
410         IF KFIX(K)=0 THEN J=J+1: DERIV(J)=DERIV(K)*WGT(I)
420     NEXT
430     FOR J=1 TO NFIT%
440         GRAD(J) =GRAD(J) +DERIV(J) * FMM
450         L=J*(J-1)/2
460

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6470     FOR K=1 TO J: A(L+K)=A(L+K)+DERIV(J)*DERIV(K): NEXT
6480     NEXT
6490 NEXT
6500 NDC=NDC+1
6510 '----- save "A" matrix and current parameter values "B"
6520 FOR I=1 TO MDI%: C(I)=A(I) : NEXT
6530 FOR I=1 TO NFIT%: DERIV(I) =B(I): NEXT
6540 '----- doctor "A" matrix diagonal elements to be:
6550 '       a=a(1+lambda) + phi*lambda
6560 DA=PHI *LAMBDA
6570 FOR J=1 TO NFIT%
6580     G(J)=-GRAD(J)
6590     L=J*(J+1)/2
6600     A(L)=C(L)*(1+LAMBDA)+DA
6610     K=J-1
6620     IF K>0 THEN FOR I=1 TO K: A(L-I)=C(L-I): NEXT
6630 NEXT
6650 GOSUB 5000 'Cholesky decomposition
6660 IF FLG%<>0 GOTO 6840
6680 GOSUB 4500 'calculate g ( the change in parameters)
6690 ' ----- find new parameters b=d+g
6700 '       (no counts the # of zero elemetns in g)
6710 NO=0: I=0
6720 FOR J=1 TO NPRM%
6730     IF KFIX(J)<> 0 GOTO 6780
6740     I=I+1
6750     B(I)=DERIV(I) +G(I)
6760     IF ABS(G(I)) <=ABS(PRCSN*DERIV(I)) THEN NO =NO+1
6770     PARM(J)=B(I)
6780 NEXT
6790 IF NO=NFIT% GOTO 6890
6800 OLDWSS=WSS: GOSUB 5500 'update the wss
6820 IF WSS < OLDWSS THEN GOTO 6250 ' next iteration
6830 '----- last step too big; increase lambda
6840 IF LAMBDA <PRCSN THEN LAMBDA = PRCSN
6850 INCR=INCR+1
6860 LAMBDA= 10! * LAMBDA
6870 IF LAMBDA <= 100000!* LMIN GOTO 6560
6880 '----- convergence reached
6890 RI=LAMBDA/LMIN
6900 CLS: GOSUB 10000 :PRINT CHR$(7): PRINT "          CONVERGENCE REACHED"
6905 PRINT "# OF PARAMETERS FIT=";NFIT%
6907 PRINT "# OF PARAMETERS CONVERGED="; NO
6910 PRINT "LAMBDA / L(MIN)="; RI
6920 NF= NOBS% - NFIT%
6930 IF NF<= 0 GOTO 6990
6940 GOSUB 5700 'calc sum of squares
6950 SIG= SQR(SS/NF)
6960 GOSUB 5900 'calc sum of dta^2
6970 R = SQR(SS/R)*100!
6980 GOSUB 4000 'compute chi-squared
6990 IF NITER%<= 0 GOTO 7130
7000 FOR J=1 TO MDI%: A(J) = C(J): NEXT: GOSUB 5000
7010 IF FLG%=0 GOTO 7060
7020 FOR J=1 TO NPRM%
7030     IF KFIX(J)=0 THEN PARM(J)=999.0001
7040 NEXT
7050 GOTO 7130
7060 K4=0
7070 FOR I4=1 TO NPRM%
7080     IF KFIX(I4) <> 0 GOTO 7120
7090     K4=K4+1
7100     FOR J=1 TO NFIT%: G(J) =0!: NEXT: G(K4)=1!
7110     GOSUB 4500: DPARM(I4)=SQR(ABS(G(K4)))*SIG
7120 NEXT
7130 RETURN

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0000 'list the current values of the parameters -----
0010 IF NPRM% <1 THEN PRINT "NO PARAM. ENTERED": RETURN
0020 PRINT
0025 PRINT NPRM%; " FITTING PARAMETERS & THEIR ERRORS": PRINT
0030 IF PFLG% = 1 THEN LPRINT : LPRINT NPRM%; "FITTING PARAMETERS & THEIR ERRORS"
LPRINT
0040 FOR I=1 TO NPRM%
0050 IF PFLG%=0 GOTO 9080
0060 LPRINT I;") ";PARM(I);
0070 IF KFIX(I)=0 THEN LPRINT "+-";DPARM(I) ELSE LPRINT " (FIXED)"
0080 PRINT I;") ";PARM(I);
0090 IF KFIX(I) =0 THEN PRINT "+-";DPARM(I) ELSE PRINT " (FIXED)"
0100 NEXT
0110 PRINT : RETURN
9999 '-----Function Key and Help subroutines -----
10000 'display "help" / title line -----
10005 XCURLIN =CSRLIN: YCURSOR = POS(0): LOCATE 25,1
10010 IF SHOWTITLE THEN COLOR FG2,BG:PRINT TITL$; ELSE COLOR FG2,BG:
PRINT " 1 - SHOW FUNCTION KEYS"; " 2 - SHOW TITLE 3 - BREAK 4 - QUICK
PLOT";:COLOR FG,BG
10020 LOCATE XCURLIN, YCURSOR : RETURN
10200 SHOWTITLE =0: GOSUB 10000: RETURN 'display "HELP" line --- F1
10300 SHOWTITLE =-1: GOSUB 10000: RETURN 'display title ----- F2
10310 BRKFLG%=1: RETURN 'user interrupt (break function) ----- F3
10400 CLS: PRINT TAB(15); "QUICK PLOT " ' ----- F4
10410 PRINT TAB(10); "Y ";YSTYL$;" VS."; "X ";XSTYL$;" PLOT"
10420 IF PLOTSET%=0 THEN PRINT "PLOT NOT DEFINED - FIRST USE MENU OPTION #4":
PRINT "PRESS ANY KEY TO CONTINUE": WHILE INKEY$="": WEND: RETURN
10430 GOSUB 16000 'scale data log or linear
10440 GOSUB 16300 'calculate fitting function
10450 IF XNEG THEN PRINT "WARNING: NEGATIVE X VALUE. X LOG PLOT INVALID":
XNEG=0: GOTO 10470
10460 GOSUB 16500 'plot
10470 SCREEN 0,0: RETURN
15000 'plot data points and fitting function -----
15010 'plot data points and fitting function -----
15020 CLS: PLOTFLG%=1
15030 IF NPRM%<1 AND NOBS%<1 THEN PRINT TAB(20); "NO DATA POINTS OR PARAMETERS E
NTERED": GOTO 400
15040 FOR I=1 TO NPRM%
15050 IF PARM(I) <> 0 THEN GOTO 15080
15060 NEXT
15070 PRINT." WARNING : all parameters are currently = 0"
15080 RESPLOT =0 'flag to indicate plot for residuals
15090 IF NOBS% <1 THEN GOTO 15170
15100 PRINT TAB(15); "CHOOSE PLOT": PRINT
15110 PRINT "1 - PLOT DATA AND FITTING FUNCTION"
15120 PRINT "2 - PLOT RESIDUALS [ABS(FIT MINUS MEASUREMENT, FMM)]"
15130 PRINT "3 - PLOT RESIDUALS [% FIT MINUS MEASUREMENT]"
15140 INPUT "ENTER CHOICE:";KMND
15150 IF KMND=2 THEN RESPLOT=1: PCNT%=0
15160 IF KMND=3 THEN RESPLOT=1: PCNT%=1
15170 PRINT TAB(15); "CHOOSE GRAPH STYLE:"
15180 PRINT "1 - Y LINEAR VS. X LINEAR"
15185 PRINT "2 - Y LINEAR VS. X LOG"
15190 IF RESPLOT=1 GOTO 15210
15200 PRINT "3 - Y LOG VS. X LINEAR": PRINT "4 - Y LOG VS. X LOG"
15210 OLDSTYL%=GSTYL%: PRINT : INPUT "ENTER CHOICE:" ; GSTYL%
15220 IF GSTYL%=0 THEN 400
15230 IF GSTYL%<> OLDSTYL% THEN PLOTSET% =0
15240 XSTYL$="LINEAR"
15245 IF (GSTYL%=2 OR GSTYL%=4) THEN XSTYL$="LOG"
15250 YSTYL$="LINEAR"
15255 IF (GSTYL%=3 OR GSTYL%=4) THEN YSTYL$="LOG"
15260 CLS: PRINT TAB(15); "Y ";YSTYL$;" VS. "; "X ";XSTYL$;" PLOT"
15270 IF NOBS% <1 THEN 15460

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15280 'go scale the data into plotting array -----
15290 GOSUB 16000 'scale determine minimum and maximum of DTA -----208-----
15300 IF XNEG THEN PRINT "WARNING: NEGATIVE X VALUE. X LOG PLOT INVALID": XNEG=0
      : GOTO 15170
15320 PRINT : PRINT "DATA POINTS:"
15330 PRINT "X VALUES range from: ";XP(1);" to ";XP(NOBS%)
15340 PRINT "Y VALUES range from: ";MINDTA;" to ";MAXDTA
15350 IF PLOTSET%=0 THEN 15420
15360 PRINT "Current graph boundaries are:"
15370 PRINT "      xmin =";XMIN;"      XMAX = ";XMAX
15380 PRINT "      ymin =";YMIN;"      YMAX = ";YMAX
15390 PRINT : PRINT "Do you wish to change any of the graph boundaries (Y/N)"
15400 INPUT "(null to leave unchanged)";T1$
15410 IF T1$="" OR T1$="n" THEN 15460
15420 PRINT: INPUT "ENTER GRAPH XMIN,XMAX,YMIN,YMAX";XMIN,XMAX,YMIN,YMAX
15425 IF XMIN=0 AND XMAX=0 GOTO 420
15430 PRINT: PRINT "ENTER xinterval, yinterval";
      "(THE # OF INTERVALS ON THE x,y AXIS)";
15435 INPUT XINTERVAL,YINTERVAL
15440 PLOTSET%=1
15450 IF RESPLOT=1 OR NPRM%=0 THEN XINC=2*(XMAX-XMIN)/MXOBS%: GOTO 15720
15460 PRINT: PRINT "PLOT OF FITTED FUNCTION";
      "      (FROM CURRENT VALUE OF PARAMETERS):"
15470 IF NOBS%<1 AND PLOTSET%=0 GOTO 15530
15480 PRINT "The function will be plotted in the range";XMIN;" to ";XMAX
15490 INPUT "  ENTER X INCREMENT for plotting the function";XINC
15495 INPUT "Enter bin width FACTOR for plotting function";FBF
15500 IF XINC<=0 OR FBF<=0 THEN PRINT "INVALID ENTRY": GOTO 15480
15510 IF (XMAX-XMIN)/XINC > MXOBS% THEN PRINT
      "too many points, must be fewer than ";MXOBS%
      : GOTO 15480
15520 GOTO 15570
15530 PRINT "      NO DATA CURRENTLY ENTERED"
15540 PRINT:PRINT "ENTER XMIN,XMAX AND X-INCREMENT"
15550 INPUT "for evaluating fitting functions";XMIN,XMAX,XINC
15560 IF (XMAX-XMIN)/XINC > MXOBS% THEN PRINT
      "too many points. must be fewer than";MXOBS%: GOTO 15530
15570 GOSUB 16300 'evaluate function
15580 PRINT :PRINT "MINIMUM of fitted function= ";MINYP
15590 PRINT "MAXIMUM of fitted function = ";MAXYP
15600 PRINT : PRINT "current graph boundaries are:"
15610 PRINT "      xmin= ";XMIN;"      xmax= ";XMAX
15620 PRINT "      ymin= ";YMIN;"      ymax= ";YMAX
15630 IF NOBS%<1 THEN 15670
15640 PRINT
15645 PRINT "do you wish to change THESE GRAPH BOUNDARIES? (Y/N)"
15650 INPUT "(null to leave unchanged)";T1$
15660 IF T1$="" OR T1$="N" THEN 15720
15670 PRINT : PRINT "ENTER NEW YAXIS BOUNDARY VALUES -- YMIN, YMAX"
15680 INPUT "(ENTER , TO CHANGE ALL GRAPH BOUNDARIES)";T1$,T2$
15690 IF T1$ = "" THEN 15420
15700 YMIN = VAL(T1$): YMAX = VAL(T2$)
15710 PRINT : PRINT "ENTER xinterval, yinterval";
      "(THE # OF INTERVALS ON THE x,y AXIS)"
15715 INPUT XINTERVAL, YINTERVAL
15720 GOSUB 16500 'go and plot
15730 SCREEN 0,0: GOTO 420 'finished plotting
16000 'scale data & find the minimum and maxima of date in yp(i)
16010 XNEG =0: PLOTFLG%=1
16020 MINDTA= 9.999999E+37: MAXDTA = -9.999999E+37
16030 FOR I=1 TO NOBS%
16035 UNBINY(I) = CINT((BF(I) * BW) * DTA(I))
16040 XP(I) =X(I): YP(I)=UNBINY(I)
16050 IF RESPLOT <> 1 THEN 16090
16060 NORM = UNBINY(I): IF NORM = 0 THEN NORM=1E-10
16070 GOSUB 20000: YP(I)=FUNCTN-DTA(I) 'ves - calc residuals

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6080 IF PCNT% <> 0 THEN YP(I) = YP(I)/NORM 'plot % residuals ?
6090 IF (GSTYL%=1 OR GSTYL%=3) GOTO 16110 'xlog plot?
6100 IF X(I)>0 THEN XP(I)=FNLGT(XP(I)) ELSE XNEG=1: RETURN
6110 IF (GSTYL%=1 OR GSTYL%=2) GOTO 16130 'ylog plot?
6120 IF UNBINY(I)>0 THEN YP(I)=FNLGT(YP(I)) ELSE YP(I)= YMIN
6130 IF YP(I)>= MAXDTA THEN MAXDTA=YP(I)
6140 IF YP(I)<= MINDTA THEN MINDTA= YP(I)
6150 NEXT
6160 RETURN
6290 'calculate function using current parm values -----
6300 PRINT : PRINT "CALCULATING FUNCTION...."
6305 IF NPRM% <1 THEN RETURN
6310 I=0: KK=0: MINYP=9.9E+37: MAXYP=-9.9E+37
6320 FOR XPT=XMIN TO XMAX STEP XINC
6330   KK=KK+1: FXP(KK)=XPT
6340   X(I)=XPT: IF (GSTYL%=1 OR GSTYL%=3) GOTO 16360
6350   X(I)=10^XPT 'X-LOG PLOT
6360   GOSUB 20000
6370   FYP(KK) =FUNCTN 'FOR NON-LOG Y PLOT
6380   IF (GSTYL%=1 OR GSTYL%=2) GOTO 16400
6390   IF FUNCTN > 0 THEN FYP(KK)=FNLGT(FUNCTN) ELSE FYP(KK) = YMIN
6400   IF FYP(KK) >= MAXYP THEN MAXYP =FYP(KK)
6410   IF FYP(KK) <= MINYP THEN MINYP = FYP(KK)
6420 NEXT XPT
6430 KMAX =KK
6440 RETURN
6500 'plotting subroutine -- first axes and tick marks -----
6510 DELTAX = XMAX-XMIN: DELTAY = YMAX-YMIN: LETWID = 8: LETHGT=8
6515 'hi resolution screen & choose color 2 - for pc & 100% compatibles
6520 SCREEN 2,0: DEF SEG = &H0: OUT &H3D9,2
6530 LINE (XMIN.PIX,YMIN.PIX) - (XMAX.PIX,YMAX.PIX),,B
6540 IF XINTERVAL <1 THEN XINTERVAL = 5
6550 ' X TICKS
6560 MINCOL =0
6570 FOR TICK=0 TO XINTERVAL
6580   TICKPLACE = XMIN.PIX + CINT(DELTAX.PIX*TICK/XINTERVAL)
6590   LINE(TICKPLACE,YMIN.PIX-2) - (TICKPLACE,YMIN.PIX+2)
6600   LINE(TICKPLACE,YMAX.PIX-2) - (TICKPLACE,YMAX.PIX+2)
6610   TICCOL = INT(TICKPLACE/LETWID)-1
6620   VAL.LAB = XMIN + (TICK/XINTERVAL)*DELTAX
6630   IF (GSTYL%=2 OR GSTYL%=4) THEN VAL.LAB = 10^VAL.LAB
6640   TICLAB= VAL.LAB: GOSUB 18000
6650   TICCOL = TICCOL - LEN(FORMAT$)/2 +2
6660   IF TICCOL + LEN(FORMAT$) > 80 THEN 16700
6670   IF TICCOL < MINCOL GOTO 16700
6680   LOCATE 25,TICCOL: MINCOL=TICCOL+LEN(FORMAT$)
6690   PRINT USING FORMAT$; TICLAB;
6700 NEXT TICK
6710 IF YINTERVAL < 1 THEN YINTERVAL =5
6720 ' y ticks
6730 OLDROW=26
6740 FOR TICK=0 TO YINTERVAL
6750   TICKPLACE = YMIN.PIX - CINT(DELTAY.PIX*TICK/YINTERVAL)
6760   LINE(XMIN.PIX-2,TICKPLACE) - (XMIN.PIX+2,TICKPLACE)
6770   LINE(XMAX.PIX-2,TICKPLACE) - (XMAX.PIX+2,TICKPLACE)
6780   TICROW = INT(TICKPLACE/LETHGT) + 1
6790   IF TICROW >= OLDROW-1 THEN GOTO 16860
6800   VAL.LAB = YMIN + (TICK/YINTERVAL)* DELTAY
6810   IF (GSTYL%=3 OR GSTYL%=4) THEN VAL.LAB = 10^VAL.LAB
6820   TICLAB= VAL.LAB : GOSUB 18000
6830   TICCOL = 8-LEN(FORMAT$): IF TICCOL < 1 THEN TICCOL=1
6840   LOCATE TICROW,TICCOL: OLDROW=TICROW
6850   PRINT USING FORMAT$; TICLAB;
6860 NEXT TICK
17000 'PLOT POINTS AND FUNCTION -----
17010 FOR I=1 TO NOBS%

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7020   CIRCLE (FNSX(XP(I)),FNSY(YP(I))),4
7030 NEXT
7040 IF RESPLOT = 1 OR NPRM% < 1 GOTO 17120 'NO FUNCTION PLOT
7050 IWENT%=0
7060 FOR K=1 TO KMAX
7070   XPT = FNSX(FXP(K)): YPT =FNSY(FYP(K))
7080   IF XPT < 0 OR XPT > XTOTAL.PIX OR YPT < 0 OR YPT > YTOTAL.PIX THEN
       17110 'pt out of range
7090   IF IWENT%=0 THEN PSET (XPT,YPT): IWENT%=1
7100   LINE -(XPT,YPT)
7110 NEXT K
7120 GTITL$=TITL$: PCNT$="": IF PCNT%=1 THEN PCNT$="(%)"
7130 IF RESPLOT =1 THEN GTITL$=GTITL$+" - residuals"+PCNT$
7140 LOCATE 1,50-LEN(GTITL$): PRINT GTITL$;
7150 WHILE INKEY$ = "" : WEND: RETURN
18000 ' subroutine to format a label for axis -----
18020 FORMAT$="#"
18030 IF TICLAB=0 THEN RETURN
18040 ORDER=INT(LOG(ABS(TICLAB))/2.30258)
18050 IF ABS(ORDER)>=4 THEN FORMAT$ = "##.#####": RETURN
18060 IF ORDER <0 THEN FORMAT$ = FORMAT$+"." : GOTO 18080
18070 FORMAT$=FORMAT$+STRING$(ORDER+1,"#")+ "."
18080 T=TICLAB
18090 TOL=ABS(TICLAB/100000!)
18100 FP=ABS(T-FIX(T)): IF 1-FP<FP THEN FP=1-FP
18110 WHILE FP>TOL
18120   T=T*10: TOL=TOL*10
18130   FP=ABS(T-FIX(T)): IF 1-FP<FP THEN FP=1-FP
18140   FORMAT$=FORMAT$+"#"
18150 WEND
18160 RETURN
19000 'error trap -----
19010 IF RETCOD = 0 THEN RETCOD = -1: ERRCNT= 1: GOTO 19030
19020 ERRCNT=ERRCNT+1 'error flagged again in same routine
19030 IF ERR=5 THEN PRINT "ILLEGAL FUNCTION CALL"
19040 IF ERR=6 THEN PRINT "OVERFLOW ERROR"
19050 IF ERR=53 OR ERR = 54 THEN PRINT "FILE NOT FOUND"
19060 IF ERR=61 THEN PRINT "DISK FULL ERROR"
19070 IF ERR=70 OR ERR=71
       THEN PRINT "DISK NOT READY OR WRITE PROTECTED"
19080 PRINT "ERROR #";ERR;" ON LINE NUMBER "; ERL
19090 IF ERRCNT > 5 THEN PRINT : PRINT
       "ROUTINE TERMINATED DUE TO ERROR COUNT": GOTO 400
19100 PRINT "PRESS ANY KEY TO CONTINUE .....": WHILE INKEY$="" : WEND
19110 RESUME NEXT
20000 'subroutine to evaluate the function chosen to be fit
20001 'The function code here is three exponentials.
20002 IF PLOTFLG%=1 THEN BF=FBF ELSE BF=BF(I)
20003 ELAMT= EXP(-PARM(4)*X(I))
20004 EBETAT= EXP(-PARM(5)*X(I))
20005 EGAMMAT = EXP(-PARM(6)*X(I))
20006 IF PLOTFLG%=1 THEN BF=FBF ELSE BF=BF(I)
20010 FUNCTN=(PARM(1)/BF)*PARM(4)*ELAMT + (PARM(2)/BF)*PARM(5)*EBETAT + (PARM(3)
/BF)*PARM(6)*EGAMMAT
20020 RETURN
20050 '
21000 'subroutine to evaluate the derivative of the function
21005 'with respect to each parameter. Only 4 parameters.
21010 GOSUB 20000
21020 F=FUNCTN
21030 DERIV(1)= (PARM(4)*ELAMT)/BF
21040 DERIV(2)= (PARM(5)*EBETAT)/BF
21041 DERIV(3)= (PARM(6)*EGAMMAT)/BF
21044 DERIV(4)= (PARM(1)/BF)*ELAMT*(-PARM(4)*X(I)+1)
21045 DERIV(5)= (PARM(2)/BF)*EBETAT*(-PARM(5)*X(I)+1)
21046 DERIV(6)= (PARM(3)/BF)*EGAMMAT*(-PARM(6)*X(I)+1)

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21050 RETURN

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