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POSSIBLE ROLE OF PKC IN REGULATION OF NEURONAL SEROTONIN UPTAKE AND 5-HT_{2A} RECEPTOR

by

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DEDICATION

To my husband "Saeed Gharib", my dear aunt "Nezhat" and my son "Arta".

ABSTRACT

A component of the 5-HT system that has recently attracted considerable interest is the serotonin transporter. Many drugs used to treat depression are serotonin reuptake inhibitors and 5-HT uptake was shown to be decreased in platelets and brain samples of depressed patients. Protein kinase C (PKC) and the cAMP-dependent second messenger system were recently shown to be involved in the regulation of 5-HT uptake in some non-neuronal cells (e.g. platelets). Since the characteristics of neuronal and platelet 5-HT uptake are similar, and the primary structure of the cloned 5-HT transporter from human brain and platelet is identical, we have decided to investigate whether neuronal serotonin uptake is also regulated by PKC.

[^{3}H]5-HT uptake and its kinetics parameters (Km and Vmax) were measured in the synaptosomal (P_2) fraction of brain tissue *in vitro* in the presence of different concentration of 4ß-phorbol-12 myristate-13-acetate (PMA), a compound known to stimulate PKC activity, and of a selective 5-HT uptake inhibitor, citalopram. Exposure of cortical synaptosomes to citalopram (1 μM) produced a marked decrease of 5-HT uptake (to 13%, 6.8% and 9.6%, respectively of control) at all (2, 5, 10 minutes) time intervals of pre-incubation. However, the PKC activator, PMA failed to produce unequivocal changes in the rate of 5-HT uptake by cortical synaptosomes of the rat brain. Under certain experimental conditions, PMA (0.1 μM) produced a moderate inhibition of 5-HT uptake measured at a single concentration of 5-HT. Low concentration (0.1 μM) of PMA did not significantly

alter the kinetics parameters (Km and Vmax) of 5-HT uptake in synaptosomes. However, higher PMA concentrations (1 and 10 μM) decreased significantly the affinity of the transporter for 5-HT without altering the rate of uptake. This may represent an important aspect of regulation of transporter protein by PKC, and it could involve allosteried changes in transporter configuration that would result in altered affinity. There are several possible reasons that could explain different results in platelets and neuronal tissue, including a more complex regulation of the transporter, involvement of different PKC subspecies or other modulators in regulation of 5-HT uptake.

It has been reported that chronic treatment with 5-HT_{2A} receptor antagonists down-regulates antagonist labelled 5-HT_{2A} receptors. This is in contrast to the situation with other receptors, including 5-HT_{1A}, where treatment with antagonists results in up-regulation of the receptors. Pervious studies on agonist-induced changes in 5-HT_{2A} receptor density have been limited by lack of a highly specific 5-HT_{2A} agonists. The new 5-HT_{2A} ligands including 1-(2,5-dimethoxy-4-iodo-phenyl)2-amino-propane (DOI) may be valuable tools in studying the regulation of 5-HT_{2A} receptors, since they act as agonists on 5-$HT_{2A/2C}$ receptors. Recently, it has been shown that DOI also down-regulates the 5-HT_{2A} receptors in rat cortex, not only after repeated but also after a single exposure. It has also been reported that *in vitro* exposure of cortical slices to DOI causes a translocation of PKC activity from cytosol to membrane. 5-HT_{2A} receptors utilize the PI-PKC signalling system.

It is assumed that activation of this system by repeated (or even single) adminstration of DOI may result (via phosphorylation ?) in down-regulation of 5-HT_{2A} receptor sites. Therefore, this study was undertaken to explore further 5-HT_{2A} receptor regulation by agonist treatment *in vivo* and the role of PKC in this regulation.

Single injection of DOI (10 mg/kg; i.p.) down-regulated 5-HT_{2A} receptor density in cortical synaptosomal preparation assayed 24 hours later by [^{3}H]ketanserin binding as well as in homogenate of rat cerebral cortex (by 50%). Single high doses (5 or 10 mg/kg; i.p.) but not a low dose (1 mg/kg) of DOI reduced the Bmax of 5-HT_{2A} receptors in cortical synaptosomes labelled with [^{3}H]ketanserin (by 23% and 50%, respectively), without significant changes in Kd. Repeated doses of DOI further down-regulated (by 65%) 5-HT_{2A} sites in cortical synaptosomes, without altering the Kd value. DOI acts *in vivo* as an agonist not only at the 5-HT_{2A} but also at the 5-HT_{2C} receptors that may be located both pre- and postsynaptically. Both are linked to PI-PKC signalling system. Our finding of a similar degree (50%) of down-regulation of 5-HT_{2A} receptors in cerebral cortex of rats with lesion to serotonergic terminals by PCA suggests that the site action of DOI in down-regulation of 5-HT_{2A} receptor in rat brain is post-synaptic.

PKC activity in both particulate and soluble fraction of rat cortical synaptosomes was measured by incorporation of [^{32}P]ATP into a specific target of PKC. The increase (by 38%) of PKC activity in the particulate fraction of the

cortical synaptosomal tissue following single injection of DOI parallelled the decrease in 5-HT_{2A} receptor density assessed with [^{3}H]ketanserin, suggesting that 5-HT_{2A} sites may be down-regulated as a result of phosphorylation of the receptor by activation of PKC after receptor stimulation with agonist. This possibility is supported by the observation that three consecutive daily injection of DOI resulted in an even larger decrease in 5-HT_{2A} receptor density as well as in a significant decrease (by 19%) in cytosolic PKC activity and an increase (by 24%) of PKC activity in particulate fraction. Single injection of DOI also induced translocation of PKC from cytosolic to membrane fraction in PCA-lesioned rat. These data are in good agreement with findings from receptor binding study (down-regulation of 5-HT_{2A} receptors seen in PCA-lesioned rats) and support the notion that changes in PKC activity took place in postsynaptic elements.

The present investigation has shown that down-regulation of 5-HT_{2A} receptors in rat cerebral cortex by *in vivo* DOI treatment is accompanied by translocation of PKC activity from the cytosolic to membrane fraction. This may be a consequence of intracellular elevation in diacylglycerol (DAG) resulting from receptor mediated PI hydrolysis. Increased phosphorylation of 5-HT_{2A} receptor may be, at least in part, responsible for the observed down-regulation of 5-HT_{2A} receptor density.

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ABBREVIATION USED IN THESIS

AC	Adenylyl cyclase
ATP	Adenosine-5'-triphosphate
ßARK	ß-adrenergic receptor kinase
cAMP	3',5'-cyclic adenosine monophosphate
CNS	Central nervous system
5-CT	5-carboxamidotryptamine
DAG	Diacylglycerol
DMSO	Dimethyl-sulfoxide
DOI	1-(2,5-dimethoxy-4-iodo-phenyl) 2-amino-propane
DOM	1-(2,5-dimethoxy-4-methyl-phenyl) 2-amino-propane
EGTA	Ethylene glycol-bis(ß-aminoethyl ether) tetraacetic acid
5-HT	5-hydroxytryptamine
i.p	Intraperitoneal
IP_3	Inositol (1,4,5)-triphosphate
MAO	Monoamine oxidase
MAOIs	Monoamine oxidase inhibitors
MDA	3,4-methylendioxyamphetamine
MDMA	3,4-methylendioxymethamphetamine
NE	Norepinephrine
8-OH-DPAT	8-hydroxy-2-(di-n-propyl-amino)tetralin
P_2	Crude synaptosomal fraction
PC	Phosphatidylcholine
PCA	*p*-chloroamphetamine
PDBu	Phorbol dibutyrate
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol (4,5)-diphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMA	4ß-phorbol 12-myristate 13-acetate
PLC	Phospholipase C
PLD	Phospholipase D
SEM	Standard error of mean
SSRIs	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
ß-TPA	4-ß-12-tetradecanoyl-phorbol 13-acetate
Tris	Tris (hydroxy methyl) methylamine

I. INTRODUCTION

A. THE SEROTONERGIC SYSTEM

1. Serotonin functions, pathway and Innervation

It has been known since the mid-nineteenth century that, after blood clots, the serum possesses a substance that constricts vascular smooth muscle so as to increase vascular tone. Around the turn of the twentieth century, platelets were identified as the source of this substance, and then, in the late 1940's, Page and his collaborators isolated and characterized this "tonic" substance in serum as serotonin (Rapport et al, 1948). Serotonin (5-hydroxytryptamine; 5-HT; Fig. 1) is widely distributed in nature, being found in plant and animal tissues, venoms, and stings. In mammals (including human), over 90% of the serotonin in the body is found in enterochromaffin cells in the gastrointestinal tract. In the blood, serotonin is found in platelets, which are able to concentrate the amine by means of an active carrier mediated mechanism. Serotonin is also contained in brain and has been identified as a central nervous transmitter in 1950's. Because of its hydrophillic structure, serotonin does not pass the lipophilic blood-brain barrier readily.

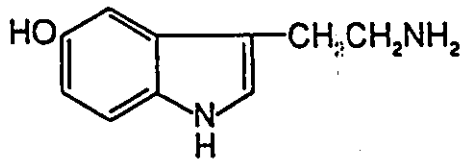

Fig. 1. 5-Hydroxytryptamine

The brain serotonergic system has been shown to be involved in regulation of various physiological functions such as sleep, body temperature, blood pressure and perception of pain. Many psychotherapeutic drugs that are effective in masked treatment of depression, anxiety disorders, and schizophrenia, and in some cases selective, have effects on the serotonergic system. In the last two decades, various theories have arisen linking abnormalities of 5-HT function to the development of a number of psychiatric disorders, particularly depression, anxiety and schizophrenia.

In brain, serotonin-containing neuronal cell bodies are largely restricted to discrete clusters or groups of cells located along the midline of the brainstem (raphe nuclei, cell body groups B1-B9 described by Dahlstrom and Fuxe (Consolazione and Cuello, 1982). Their axons, however, innervate nearly every area of the central nervous system (Fig. 2). Some serotonergic neuronal cell bodies, however, are found outside the raphe nuclei, and not all raphe neurons are serotonergic. Two main ascending serotonergic pathways to the forebrain emerge from the midbrain raphe nuclei -the dorsal periventricular path and the ventral tegmental radiations. Both pathways converge in the caudal hypothalamus where they join the medial forebrain bundle (MFB). The dorsal and median raphe nuclei give rise to multiple, distinct sets of axons that form separate pathways to different brain regions (Consolazione and Cuello, 1982). Functionally related structures in the brain are innervated by the same group of serotonergic neurons. Ascending projections from the median raphe, for example, are found preferentially in the

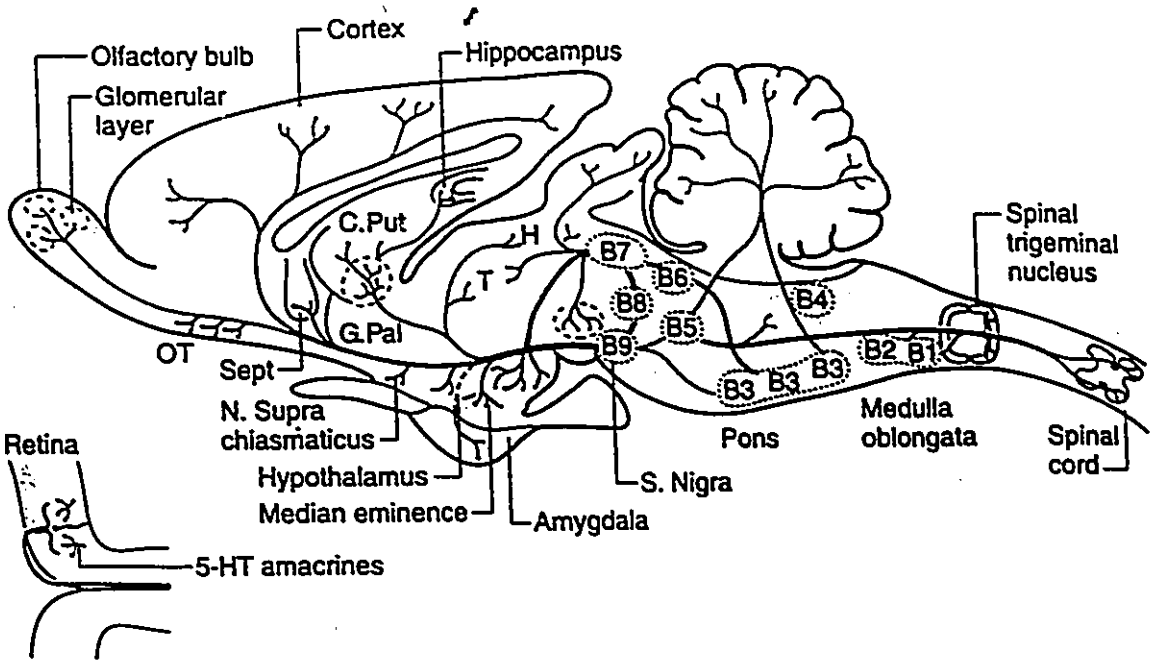

Fig. 2. Schematic drawing depicting the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections. (OF) olfactory tuberculum; (Sept) septum; (C.Put) nucleus caudate-putamen; (G.Pal) globus pallidus; (T) thalamus; (H) habenulae; (S.Nigra) substantia nigra. (Adapted from Consolazione and Cuello, 1982)

hippocampus and septum (limbic structures), and are characterised morphologically by coarse, beaded axons with terminal varicosities. In contrast, projections from the dorsal raphe project most heavily into the cortex and striatum, and possess fine axons that are particulary vulnerable to neurotoxic amphetamines such as *p*-chloroamphetamine (Ricaurte et al, 1985). The fine terminals of the dorsal raphe projection to the cortex are associated with 5-HT_2 receptors, suggesting that distinct anatomical projections may interact specifically with certain

5-HT receptor subtypes (Molliver, 1987). In addition, there is evidence that 5-HT cell bodies in the median and dorsal raphe differ in their responsiveness to pharmacological agents. For example, the 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) is three times more potent in suppressing the firing of neurones in the dorsal raphe than in the median raphe (Blier et al, 1990). Taken together, these findings suggest that serotonergic innervation in the brain may have a high degree of specialization subserving different function and these can be differentially affected by pharmacological means.

2. 5-HT receptor subtypes and signal transduction mechanisms

5-HT receptors consist of at least three distinct types of molecular structures (Table 1): guanine nucleotide binding protein (G protein) coupled receptors, ligand-gated ion channels, and transporters (Peroutka, 1993). Prior to introduction of molecular biological techniques, the classification of 5-HT receptors was based predominantly on the pharmacological properties of the receptors.

In the 1970's, it was realised that radioligands such as [^{3}H]5-HT and [^{3}H]spiperone labelled at least two different types of recognition sites, termed 5-HT_1 and 5-HT_2 by Peroutka et al (1979). Subsequently, Bradley et al (1986) defined "5-$HT_{1\text{-like}}$" receptor by their susceptibility to antagonism by methiothepin and/ or methysergide, resistance to antagonism by 5-HT_2 antagonist, and potent agonism by 5-carboxamidotryptamine (5-CT). These classification systems were dependent upon the availability of selective pharmacological agents.

TABLE 1. Overview of 5-HT receptors

G protein-coupled receptors
5-HT_1 "family" and 5-HT_4, 5-ht_6, 5-ht_7 (linked to AC)
5-HT_2 "family" (linked to PI)
Ligand-gated ion channels
5-HT_3, 5-ht_5
Transporters
5-HT uptake site

Recent molecular biological data, have also confirmed the existence of multiple 5-HT receptors. According to their data, 5-HT receptors can now be classified into at least seven classes (or groups) of receptors (Table 1) that comprise the 5-HT_1, 5-HT_2, and 5-HT_3 classes, as well as the "uncloned" 5-HT_4 receptor. The 5-ht_5, 5-ht_6, and 5-ht_7 receptor genes have been cloned recently, but the receptors have yet to be fully characterised operationally and transductionally in intact tissues.

All 5-HT_1 receptor subtypes have been cloned and shown to share a high degree of homology (Van Wijngaarden et al, 1990). They also have high affinity for 5-HT and share a common transduction system in being negatively coupled to

adenylyl cyclase, presumably via a common or similar G-protein link. It appears that 5-HT_{1A} receptor, like the other members of the 5-HT_1 receptor family, negatively couples to adenylyl cyclase (AC) via G-inhibitory protein (subunit *αi*) (Bertin et al, 1992) (Fig 3). Transduction systems other than adenylyl cyclase have been described for this receptor. Andrade et al (1986) reported the presence of a pertussis toxin sensitive G-protein that couples 5-HT_{1A} receptors in hippocampal pyramidal cells to a K^+ channel. Activation of the receptor leads to channel opening and hyperpolarization (Bobker et al, 1990).

The 5-HT_{1C} receptor originally included in 5-HT_1 family was shown to signal via phosphatidylinositol (PI) system and has high degree of homology with 5-HT_2 receptor. For this reason, it has recently been renamed 5-HT_{2C} receptor (Humphrey et al, 1993).

Post-synaptic 5-HT_{1A} receptors mediate the effects of 5-HT released from nerve terminals; the characteristic response of the post-synaptic cell is hyper-polarization due to entry of potassium ions (Bobker et al, 1990). The hippocampus, amygdala and some hypothalamic nuclei contain a high density of 5-HT_1 sites, most of which belong to the 5-HT_{1A} subtype (Pazos et al, 1988). High concentration of 5-HT_{1A} receptors is also found on the cell bodies of 5-HT neurones in the raphe nuclei. These are considered autoreceptors, since they have an inhibitory effect on 5-HT neuron firing and their stimulation by a selective 5-HT_{1A} agonists such as 8-OH-DPAT was shown to decrease the release of 5-HT in terminal fields (Sharp et al, 1989).

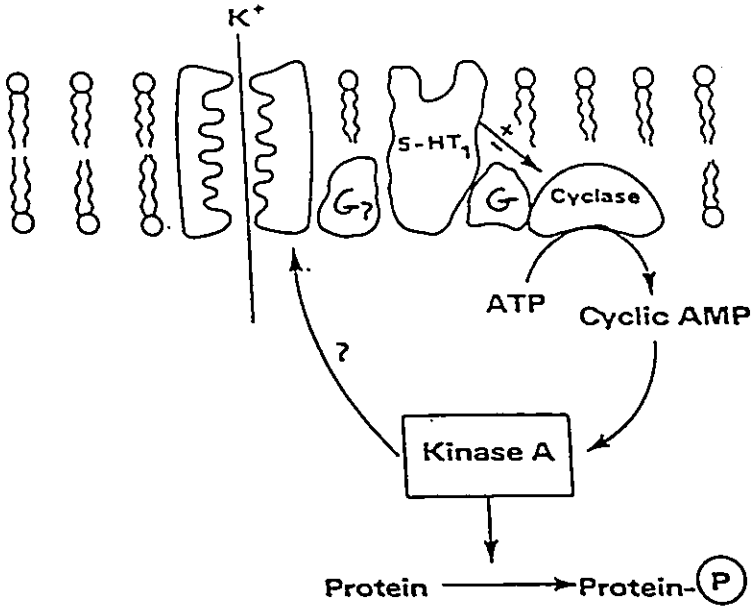

Fig. 3. 5-HT_1 receptor and signal transduction. (ATP) Adenosine-5'-triphosphate; (Cyclic AMP) 3',5'-cyclic adenosine monophosphate; (G) Guanine nucleotide regulatory protein. (Adapted from Roth and Chuang, 1987).

The activation of central 5-HT_{1A} receptors induces a behavioural syndrome, which is characterised by flat body posture, reciprocal forepaw treading, head weaving (Tricklebank, 1985), and lower lip retraction (Berendson et al, 1989). A variety of 5-HT_{1A} receptor agonists, especially those considered to be partial agonists, such as buspirone, ipsapirone and gepirone have anxiolytic effects in animal models of anxiety (Traber and Glaser, 1987). Characteristics and functional correlates of other members of 5-HT_1 family are summarized in Table 2.

Until recently, there was no compelling evidence to subdivide 5-HT_2 receptors which are widespread and mediate many of the actions of 5-HT throughout the body (Leysen et al, 1984; Mylecharane, 1990). However, the close structural homology of the 5-HT_{1C} receptor with 5-HT_2 receptor (Julius et al, 1990), together with a shared second messenger transduction system (Conn and

TABLE 2

5-HT RECEPTORS IN THE CNS

5-HT_1 family (linked to AC)

Subtype	Location	Response/ Transduct.	Agonists	Antagonists	Function/ Clin.relev.
5-HT_{1A}	DR,Hc, Cx Pre- & postsyn.	Hyperpolar. Inh. AC Open K^+ channel	8-OH-DPAT Flesinoxan Buspirone	Pindolol Spiperone	Behav.sy (rod.) PRL release Anxiety Depression
5-HT_{1B} (rod.)	SN Bas.gglia Presyn.	Inhibit release Inh.AC	Metergoline	Methiotepine Pindolol	Locom.(↑
5-HT_{1D} α,β (hum.)	SN Bas.gglia Sup.coll. Presyn.	Inhibit release Inh. AC	Metergoline Sumatriptan	Methiotepine Mianserin	Migraine Huntington
5-ht_{1E}	Only CNS	Inh. AC	5-HT	None	?
5-ht_{1F}	Mainly CNS	Inh. AC	5-HT	None	?

(DR) Dorsal raphe; (HC) Hippocampus; (Cx) Cortex; (Presyn.) Presynaptic; (Postsyn) Postsynaptic; (SN) Substantia nigra; (Bas.gglia) Basal ganglia;.(Sup.coll) Superior colliculus; (Hyperpolar) Hyperpolarization; (Inh.AC) Inhibitor of adenylate cyclase; (Behav.sy) Behavioural syndrome; (8-OH-DPAT) 8-hydroxy-2-(di-n-propyl-amino)tetralin.

Sanders-Bush, 1985, 1986) and very similar operational characteristics (Sahin-Erdemli et al, 1991), have lead to the inclusion of 5-HT_{1C} receptor into the family of 5-HT_2 receptors as 5-HT_2 subtype (Hoyer, 1988; Hartig, 1989).

This now makes the "classical" 5-HT_2 receptor a 5-HT_{2A} subtype. A newly sequenced rat stomach fundus 5-HT receptor has been classified as 5-HT_{2B} subtype (Foguet et al, 1992a,b). In the rest of this thesis, therefore the term "5-HT_{2A}" will be used instead of "5-HT_2". All three subtypes have been cloned and shown to be a G-protein-linked single protein molecule of similar size and close homology, linked to the activation of phosphoinositide metabolism (Table 3).

5-HT_{2A} receptors are widely distributed in peripheral tissues (e.g. platelet, bronchial) (Bradley et al, 1986). The effects mediated by these receptors include platelet aggregation and bronchospasm. The only central actions classified by Bradley and colleagues as 5-HT_{2A} receptor mediated are some behavioural effects in rodents (head twitch, wet-dog shake) and neuronal depolarization. Nevertheless, 5-HT_{2A} receptors are found in high concentration in the cerebral cortex and in certain regions of hypothalamus. It is well established that 5-HT_{2A} receptors are linked to PI turnover. This has been demonstrated in rat cortex, aortic smooth muscle, and human platelets (Conn and Sanders-Bush, 1984, 1985; Roth et al, 1984; De Chaffoy et al, 1985). The receptors are coupled to phospholipase C (PLC), and their stimulation increases phosphoinositide hydrolysis, producing inositol triphosphate, which mobilizes intracellular Ca^{2+} from internal storage sites (Affolter et al, 1984) (Fig 4).

TABLE 3

5-HT RECEPTORS IN THE CNS
5-HT_2 family (linked to PI)

Subtype	Location	Response/ Transduct.	Agonists	Antagonists	Function/ Clin.relev.
5-HT_{2A}	Cortex (L.IV) Olf.tub (Plat.)	Depolariz. ↑ PI	DOI α-Me5-HT	Ketanserine Spiperone Mianserin	Behav.sy (rod.) Depression
5-HT_{2B}	Mainly periph.?	↑ PI	DOI α-Me5-HT		?
5-HT_{2C}	Choroid pl., GP, SN,Hc	↑ PI	DOI α-Me5-HT mCPP	Metergoline Ritanserin	CSF prod. Anxiety?

(L.IV) Laminae IV (rat); (Olf.tub)Olfactory tubercule; (Plat.)Platelet; (Periph) Peripheral; (PI) Plexus; (GP) Globus pallidus; (SN) Substantia nigra; (Hc) Hippocampus; (Depolariz) Depolarization; (PI) Phosphatidylinositol; (DOI) 1-(2,5-dimethoxy-4-iodo-phenyl) 2-amino-propane; (α-Me5-HT) α-Methyl-5-Hydroxytryptamine; (mCCP) Methyl-chlorophenylpiperazine; (Behav.sy) Behavioural syndrome; (rod) Rodent; (CSF prod) Cerebrospinal fluid production.

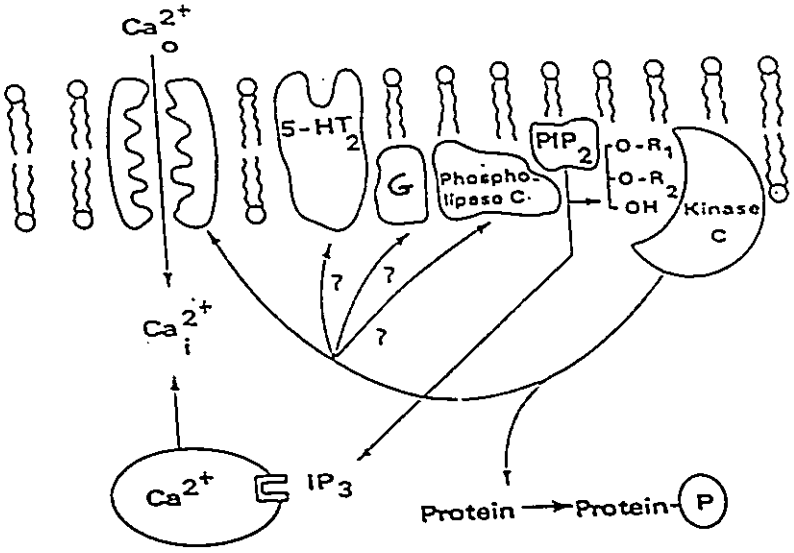

Fig. 4. 5-HT_2 receptor and signal transduction. (PIP_2) Phosphatidylinositol (4,5)-diphosphate; (IP_3) Inositol (1,4,5)-triphosphate; (G) Guanine nucleotide regulatory protein. (Adapted from Roth and Chuang, 1987).

The greatest density of 5-HT_{2C} receptors is seen in the choroid plexus, but significant levels of 5-HT_{2C} binding are also apparent in the frontal cortex and some areas of the hippocampus, in the basal ganglia and hypothalamus (Pazos et al, 1988). This distribution suggests that 5-HT_{2C} receptors may participate in the modulation of cognitive and emotional functions. 5-HT_{2C} receptors have been suggested to play a role in a variety of processes such as locomotion, feeding and anorexia nervosa, penile erection (Berendsen et al, 1990), migraine, obsessive compulsive disorders and anxiety (Lucki, 1992). Based on *in situ* hybridization histochemistry, localization of 5-HT_{2C} receptors might be not only at post-synaptic site but also be at pre-synaptic site, since 5-HT_{2C} receptor mRNA expression has been found in all areas containing serotonin-producing cells (e.g. raphe reticular formation) (Hoffman and Mezey, 1989).

The 5-HT_3 receptors were first identified in the peripheral nervous system,

where they mediated the pain and flare reaction associated with intradermal lesions (Richardson et al, 1985). More recently, 5-HT_3 receptor binding sites and functional responses have been detected in the central nervous system (CNS) (Table 4). 5-HT_3 receptor is unique and differs from the 5-HT_1 and 5-HT_2 family of receptors in that it is directly coupled to an ion channel without an intervening G protein or second messenger system (Peroutka, 1988). The 5-HT_4 receptor has been identified in a variety of tissues, including the brain (Bockaert et al, 1992). Despite being operationally distinct from 5-HT_1 receptors, the 5-HT_4 receptor appears to be positively linked to adenylyl cyclase and may, therefore, be more closely related structurally to 5-HT_1, and particularly to the recently cloned 5-ht_6 and 5-ht_7 receptors (also linked positively to adenylyl cyclase), than to the 5-HT_2 or 5-HT_3 receptors.

Involvement of the components of brain serotonergic system in the action of some psychotropic drugs is schematically depicted in Fig 5.

3. 5-HT Uptake

The high-affinity neuronal uptake of 5-hydroxytryptamine is an important carrier-mediated mechanism for the removal of this amine from the synaptic cleft. The uptake process involves several steps; after binding of the 5-HT molecule to the substrate recognition site, the molecule is translocated by a carrier to the inside of the cell where it then dissociates from the carrier (Fig 6).

TABLE 4

5-HT RECEPTORS IN THE CNS
other

Type	Location	Response/ Transduct.	Agonists	Antagonists	Function/ Clin.impli
5-HT_3	Area postr. Limbic sy	Depolariz. (ion chann.)	2-Me5-HT	Granisetron Zacopride	Emesis Anxiety
5-HT_4	Hc Bas.gglia	↑ cAMP	5-MeO-T Cisapride	Tropisetron	?
$5\text{-ht}_{5A\ 5B}$	Cx,HC (mRNA)	?	5-HT	Methiotepin	?
5-ht_6	Str., Cx,Hc (mRNA)	↑ AC	5-HT	Methiotepin	
$5\text{-ht}_{7\ A,B,C}$	Hy,Th, supra-chiasm. nn (mRNA)	↑ AC	5-HT	Methiotepin	Regul. circ.rh.?

(Area postr) Area postrema; (Limbic sy) Limbic system; (Hc) Hippocampus; (Bas.gglia) Basal ganglia; (Cx) Cortex; (Str) Striatal mRNA; (Hy) Hypothalamus; (Th) Thalamus; (chiasm.nn) Chiasmatic nucleus; (Depolariz) Depolarization; (cAMP) 3',5'-cyclic adenosine monophosphate; (AC) Adenylate cyclase; (2-Me5-HT) 2-Methyl 5-Hydroxytryptamine; (5-MeO-T) 5-Methoxy-tryptamine; (5-HT) 5-Hydroxytryptamine; (SSRI) Selective serotonin reuptake inhibitor; (Regul. circ.rh) Regulation of circadian rhythms.

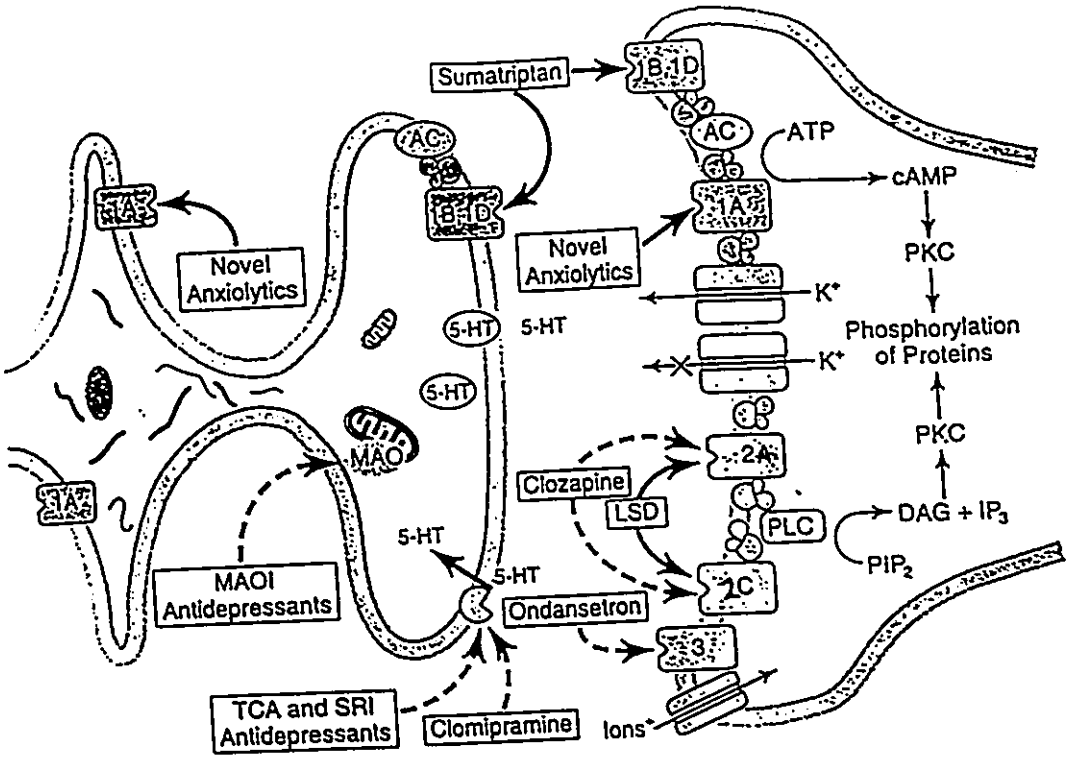

Fig. 5. Serotonergic sites as targets for action of psychotropic d. ·gs. (MAOI) Monoamine oxidase inhibitor; (TCA) Tricyclic antidepressant; (SSRI) Selective serotonin reuptake inhibitor; (5-HT) 5-Hydroxytryptamine; (AC) Adenylyl cyclase; (ATP) Adenosine-5'-triphosphate; (cAMP) 3',5'-cyclic adenosine monophosphate; (PKC) Protein kinase C; (DAG) Diacylglycerol; (IP_3) Inositol (1,4,5)-triphosphate; (PIP_2) Phosphatidylinositol (4,5)-diphosphate; (PLC) Phospholipase C. (Adapted from Siegel et al, 1994).

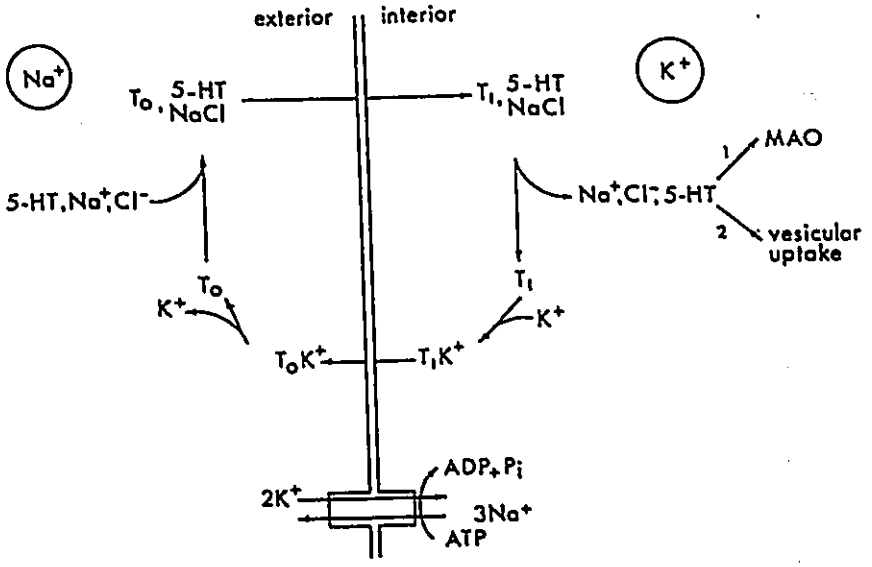

Fig. 6. 5-HT uptake process. (T) Transporter; (5-HT) 5-Hydroxytryptamine; (MAO) Monoamine oxidase; (ATP) Adenosine-5'-triphosphate; (ADP) Adenosine-5'-diphosphate.

The two transporter systems for 5-HT translocation from the synaptic cleft and its storage in vesicle are localized in the presynaptic cytoplasmic and the storage vesicle membranes, respectively. The uptake of 5-HT is an active process that is temperature dependent and has an absolute requirement for external Na^+ and Cl^-. The energy for this process is provided by Na^+, K^+-ATPase pump.

Certain drugs used in treatment of depression, including the tricyclic antidepressants and particularly the selective serotonin reuptake inhibitors (SSRIs), are known to inhibit the neuronal uptake of 5-HT, thus increasing the amount of transmitter in the synapse and hence its action on both pre- and postsynaptic receptors. Specific recognition sites for the tricyclic antidepressant drugs such as [^{3}H]imipramine have been demonstrated in brain (Raisman et al, 1979) and platelets (Briley et al, 1979) of various species, including humans (Langer et al, 1981). Because [^{3}H]imipramine binding was found to be associated with 5-HT uptake sites on serotonergic neurons (Raisman et al, 1979), this radioligand was suggested for labelling the 5-HT transporter and the density of serotonergic innervation in various experimental and clinical conditions. However, it has been demonstrated that [^{3}H]imipramine binds in brain tissue to two classes of sites: high- and low affinity (Hrdina, 1984). Only the high-affinity sites were found to be functionally related to the 5-HT uptake process (Hrdina, 1987, 1988). Recently, more selective 5-HT uptake inhibitors such as [^{3}H]citalopram (D'Amato et al, 1987) or [^{3}H]paroxetine (Hrdina et al, 1990) were utilized to label 5-HT uptake sites in

brain. [^{3}H]paroxetine was shown to bind selectively with very high affinity to a single class of sites on the 5-HT transporter that are likely to be the same as the recognition sites for 5-HT. In late 1991, the 5-HT transporter was cloned from basophilic leukemia cells (Hoffman et al., 1991) and rodent brain (Blakely et al, 1991). Expression and characterization of the 5-HT transporter revealed that the molecule possesses the pharmacological and functional properties expected of the 5-HT uptake site. Recently, Lesch et al (1993b) have shown that the 5-HT transporter in brain and platelet is identical (Fig 7). The neuronal 5-HT transporter is an important target of action of antidepressant drugs, particularly the selective serotonin uptake inhibitors such as fluoxetine.

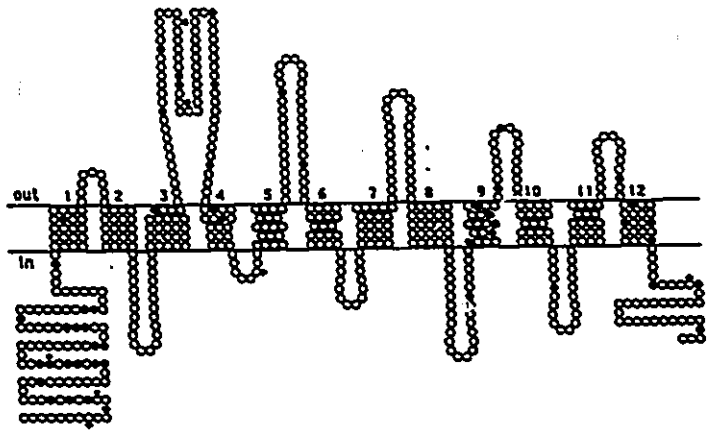

Fig. 7. Structural model of the human platelet 5-HT uptake site/transporter. Circles represent individual amino acids. Solid circles indicate amino acids differing from those of the rat brain 5-HT transporter. Squares reflect putative glycosylation sites. Potential phosphorylation sites for cyclic AMP-dependent protein kinase and protein kinase C are indicated by crosses and triangles, respectively. (Adapted from Lesch et al, 1993b).

4. Adaptive changes in serotonin receptors, transporter and signalling mechanism during treatment with antidepressants

A number of experiments have looked at the effect of chronic antidepressant treatment on the density and number of amine receptors in the brain. Banerjee et al (1977), Wolfe et al (1978), Kopanski et al (1983), and Baron et al (1988) reported that repeated adminstration of most tricyclic antidepressants, MAO inhibitors and electroconvulsive shock, induced a reduction of the number and function of ß-adrenoceptors and an associated reduction in noradrenaline-stimulated production of cAMP. Peroutka and Snyder (1980) also reported that chronic treatment with some antidepressants induced homologous desensitization of 5-HT-stimulated phosphoinositide hydrolysis as well as simultaneous downregulation of 5-HT_{2A} receptors. Previous studies of adaptive changes in the 5-HT_{2A} receptor after chronic adminstration of the tricyclic antidepressants, imipramine and desipramine (Kendal et al 1985), and of the atypical antidepressant, mianserin (Conn and Sanders-Bush, 1984; Roth and Ciaranello, 1991) have revealed a desensitization of 5-HT-stimulated hydrolysis of phosphatidyl inositols in conjunction with a downregulation of 5-HT_{2A} binding sites in rat brain. It was hypothesized that this downregulation might in part account for the alleviation of depressive symptoms, as the downregulation was observed to correlate with the onset therapeutic effect.

There are however some problems with the antidepressant-induced 5-HT_{2A}

receptor downregulation hypothesis. The new selective 5-HT uptake inhibitors such as fluoxetine, fluvoxamine and sertaline have not been shown to consistently alter the number of 5-HT_{2A} receptors (Sanders-Bush et al, 1989). In contrast, autoradiography studies have been shown that chronic treatment with a selective serotonin uptake inhibitor, fluoxetine, produced significant upregulation in the density of 5-HT uptake sites labelled with [^{3}H]paroxetine as well as 5-HT_{2A} receptor sites in the frontoparietal and striate cortex and in the hippocampus and of vesicular amine uptake sites in caudate-putamen (Hrdina and Vu, 1993). These alterations are likely a part of adaptive neuronal changes that occur after repeated adminstration of the drug. Conversely, desipramine, which does not posses significant 5-HT uptake blocking ability, also induces a downregulation of 5-HT_{2A} receptors after chronic treatment (Peroutka and Snyder, 1980). Another point to consider is that changes in 5-HT_{2A} receptor density has not always been correlated with changes in phosphoinositide turnover after chronic antidepressant treatment. For example, sertaline, a potent 5-HT uptake inhibitor, causes no downregulation of 5-HT_{2A} receptors, and yet produces a decreased in [^{3}H]inositol phosphate formation upon stimulation with 5-HT (Sanders-Bush et al, 1989).

Since receptor changes only represent the first step in the action of antidepressants, recent studies have also been focused on neuronal signal transduction processes beyond the receptor level as potential targets for the action of these drugs. Racagni et al (1992) has shown the chronic treatment with

desipramine and fluoxetine alters the cAMP-dependent phosphorylation system in rat brain associated with the microtubule fraction and suggested that this system could represent an intracellular target involved in the biochemical mechanism of action of antidepressant drugs. PKC is another pivotal enzyme in phosphorylation of cellular proteins and its activity has been associated with regulation of serotonin receptor-triggered signals (e.g. 5-$HT_{2A/2C}$), neurotransmitter release and neuronal plasticity (Nishizuka, 1988). Recently, imipramine was reported to prevent *in vitro* the inhibitory effect of PKC activator phorbol ester on norepinephrine (NE)-induce accumulation of inositide phosphates in brain slices (Nalepa and Vetulani, 1991).

B. ROLE OF PROTEIN KINASE C IN NEURONAL FUNCTION

Protein kinase C (PKC) is present in high concentrations in neuronal tissues and has been implicated in a broad spectrum of neuronal functions. Nerve cells can transmit signals over long distances by means of electrical impulses. Opening of voltage-gated Ca^{2+} channels following depolarization of the presynaptic membrane by an action potential normally translates the electrical signal into several chemical messages. The influx of Ca^{2+} triggers an exocytotic release of a variety of neurotransmitters from synaptic vesicles. The chemical messages are then reverted back to electrical through channel-linked receptors such as glutamate receptors located on postsynaptic membranes. Many proteins related to these processes of synaptic transmission may be the prime targets of PKC

action. Activation of this enzyme in nerve cells is frequently associated with the modulation of ion channels (Shearman et al 1989), desensitization of receptors (Huganir and Greengard, 1990), and enhancement of neurotransmitter release (Robinson, 1992). The PKC pathway may modulate the efficacy of synaptic transmission, thus providing a basis for some forms of memory.

On the other hand, the non-channel-linked receptors (e.g. 5-HT_{2A} receptor) respond to agonists, by initiating a cascade of enzymatic reactions. The first step in this cascade is activation of G protein, which may either interact directly with ion channels or control the production of intracellular second messengers. When phospholipases are activated via G protein-linked receptors, they catalyze the breakdown of membrane-bound phosphatidylinositol (4,5)-diphosphate (PIP_2) into the second messengers inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG) (Fig. 8). IP_3 stimulates the release of intracellular calcium stores from storage vesicles through direct action on receptors linked to calcium channels (Berridge and Irvine, 1989). On the other hand, PKC is activated by increased amounts of DAG formed in the membrane as a result of agonist-induced hydrolysis of inositol phospholipid by phospholipase C (Nishizuka, 1984).

Upon cell stimulation, a DAG increase is detected in various intracellular compartments at different times during the cellular responses. The early peak of DAG is transient and reverts back to basal line within seconds, at most minutes, temporally corresponding to the formation of IP_3 and to the rise in intracellular Ca^{2+} concentration. At a relatively later phase of cellular responses, the formation

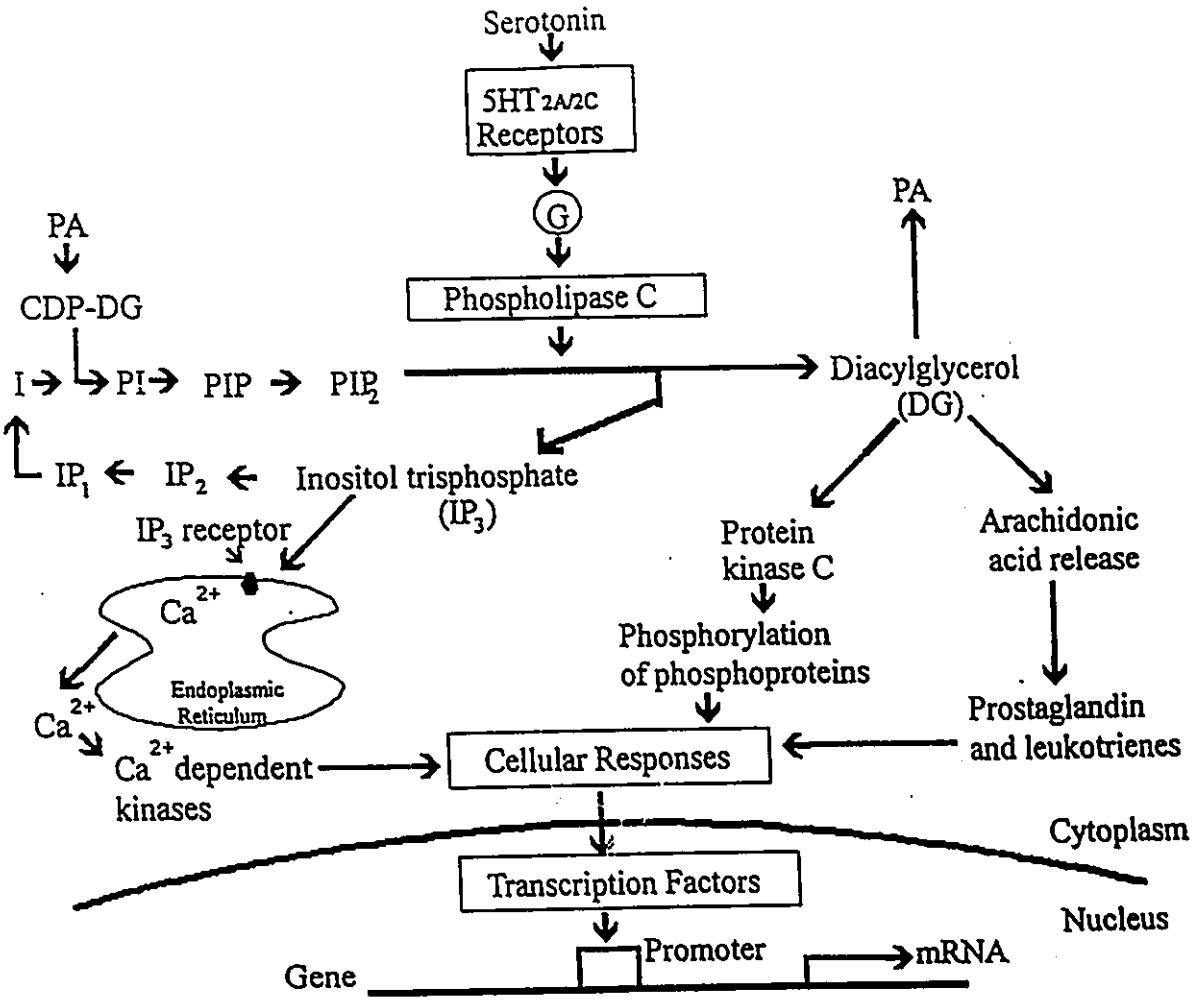

Fig. 8. PI-PKC signalling system. (PA) Phosphatidic acid; (CDP) Cytidine-diphosphate; (PI) phosphatidylinositol; (PIP) phosphatidylinositol-monophosphate; (PIP_2) phosphatidylinositol-diphosphate; (IP_2) Inositol diphosphate; (IP_1) Inositol monophosphate; (I) Inositol. (Adapted from Pandey et al, 1995).

of DAG has a slow onset but is more sustained. DAG binds to and stimulates PKC in the presence of phospholipids (principally phosphatidylserine) and calcium ions, perhaps by lowering its requirement for calcium. PKC is distributed in both cytoplasmic and membranous cellular compartments, and upon activation (either by DAG or by the DAG analogues, phorbol esters), translocation of the enzyme from cytosol to membrane occurs (Kraft et al, 1983). This translocation of PKC to the membrane appears to be essential in its activation. PKC proceeds to phosphorylate serine and threonine residues on a range of intracellular proteins,

resulting in their activation. It is believed that the agonist-induced cascade of degradation of various membrane phospholipids is necessary for transducing full information from extracellular signals across the membrane.

So far, ten isoforms of PKC have been identified in mammalian tissues (Table 5). These isoforms show subtly different enzymological properties, different tissue expression, and specific intracellular localization (Tanaka and Saito, 1992). The four PKC isoforms, α, βI, βII, and (cPKC), originally emerged from the initial screening (Bell and Burns, 1991). They have four regions of conserved sequence (C1-C4) with five regions of variable sequence (V1-V5). These isoforms are activated by DAG and Ca^{2+}. Tumour-promoting phorbol esters act as an analog of DAG, a physiological activator of PKC, and they activate PKC *in vitro*. The repeat of the cysteine-rich sequence present in the C1 region is essential for phorbol ester binding. The C2 region appear to be related to Ca^{2+} sensitivity of the enzyme. The C3 region includes the catalytic site, and the C4 region seems to be necessary for recognition of the substrate to be phosphorylated. The α, βI, βII, and isoforms were identified in isolated fractions of type III, II and I, from the soluble fraction of rat brain on a hydroxyapatite column (Kikkawa et al, 1987). Type I PKC is brain-specific and high amounts have been reported in rat cerebellum. In contrast, type II PKC is predominant in the cortex, and type III PKC in the olfactory bulb (Huang et al, 1987). cPKC isoform-containing neurons have been demonstrated in the neocortex, hippocampus, striatum, substantia nigra, and cerebellum of rat.

TABLE 5

PKC ISOFORMS IN MAMMALIAN TISSUES

Isoforms	Activators	Tissue expression	Intracellular localization (in neurons)
cPKC			
α	Ca^{2+}, DAG, PS, FFA, LysoPC	Universal	Golgi complex, dendrites, periphery of perikarya
βI	Ca^{2+}, DAG, PS, FFA, LysoPC	Some tissues	Periphery of perikarya
βII	Ca^{2+}, DAG, PS, FFA, LysoPC	Many tissues	Golgi complex, dendrites
γ	Ca^{2+}, DAG, PS, FFA, LysoPC	Brain, spinal cord	Throughout cytoplasm; nucleus, plasma membrane
nPKC			
δ	DAG, PS	Universal	Golgi complex, axons, nerve terminals
ϵ	DAG, PS, FFA	Brain and others	
η	?	Lung, skin, heart	
θ	?	Skeletal muscle	
aPKC			
ζ	PS, FFA	Universal	
λ	?	Ovary, testis, and others	

(DAG) Diacylglycerol; (PS) Phosphatidylserine; (FFA) *cis*-unsaturated fatty acid; (Lyso PC) Lysophosphatidylcholine.

1. Possible role of PKC in regulation of serotonin uptake

A component of the 5-HT system that has recently attracted considerable interest is the serotonin transporter. Many drugs used to treat depression are serotonin reuptake inhibitors and 5-HT uptake was shown to be decreased in platelets and brain samples of depressed patients (Paul et al, 1981; Perry et al, 1983).

The active transport of serotonin across the plasma membrane has been well characterized both in neurons and platelets (Rudnick and Nelson, 1978). Some recent evidence indicates that serotonin transporter may be linked to the second messenger system. It has been reported that protein kinase C (PKC) and the cAMP-dependent second messenger system are involved in the regulation of 5-HT uptake in some non-neuronal cell. Myers et al (1989) found that inhibition of 5-HT uptake in cultured endothelial cells was associated with the phorbol ester-induced translocation of PKC from cytosol to membrane. Furthermore, Hoffman (1991) reported that endogenous cAMP analogues increased the 5-HT uptake rate in rat basophilic leukemia cells, whereas phorbol esters had a pronounced inhibitory effect. These data suggested that 5-HT uptake may be under dual control of the two main intracellular regulatory systems involving PKC and protein kinase A (PKA). Anderson and Horn (1992) have demonstrated that exposure of human platelets to activators of PKC, 4-ß-12-tetradecanoyl-phorbol 13-acetate (ß-TPA) and mezerein produced substantial reductions in the rate of platelet serotonin uptake. The mean Vmax observed after 5 minutes exposure to 1 μM ß-TPA was

decreased to 66% of the control value, and with 1 μM mezerein to 78% of control. The maximum inhibitory effects of both were reached after 20 minute exposure and could be blocked by previous exposure to staurosporine (PKC inhibitor). Recently, Chudzik et al (1994) have shown that exposure of human platelets to PKC activators such as phorbol esters and diacylglycerol significantly inhibits the rate of serotonin uptake but does not alter the binding of [^{3}H]paroxetine to the 5-HT recognition site on the transporter. Characteristics of neuronal and platelet 5-HT uptake are similar, and it was shown recently that the primary structure of the cloned 5-HT transporter from human brain and platelet is identical (Lesch et al, 1993b). Hence, both the neuronal and platelet serotonin transporter may be regulated by similar intracellular mechanisms.

2. Possible involvement of PKC in down-regulation of 5-HT$_{2A}$ receptor by agonist treatment

It has been repeatedly reported that chronic treatment with 5-HT$_{2A}$ receptor antagonists down-regulates antagonists-labelled 5-HT$_{2A}$ receptors (Gandolfi et al, 1985). This is in contrast to the situation with other receptors, including 5-HT$_1$, where treatment with antagonists results in up-regulation of the receptors. The reason for this phenomenon remains unexplained. Previous studies have been, however, limited by lack of highly specific 5-HT$_{2A}$ agonists. Glennon and co-workers (1987) have proposed that the apparent low affinity of classical serotonin

agonists for the 5-HT_{2A} site is due, in part, to the use of 5-HT antagonists as labelling radioligands. They recently identified several phenylisopropylamine derivatives as potential 5-$HT_{2A/2C}$ agonists (Glennon et al, 1986). These include 1-(2,5-dimethoxy-4-substituted-phenyl) 2-amino-propanes, such as DOM (methyl substitution), DOB (bromo-substitution) and DOI (iodo-substitution). These new 5-$HT_{2A/2C}$ ligands became a valuable tool in studying the regulation of 5-$HT_{2A/2C}$ receptors which have been implicated in mediating the psychoactive properties of phenylalkylamine hallucinogens (Glennon et al, 1984). Leysen et al (1989) found that the 5-HT_{2A} receptors in the rat cortex became rapidly desensitized by repeated treatment with DOM. Recently Pranzatelli (1991) has shown that DOI also down-regulates the 5-HT_{2A} receptors in rat cortex, not only after chronic treatment but also after a single exposure. Furthermore, *in vitro* exposure of cortical slices to DOI (but not to 5-HT_{1A} agonists) causes a translocation of PKC activity from cytosol to membrane (Wang and Friedman, 1990a). This effect was inhibited by selective 5-HT_{2A} receptor antagonists (e.g. ketanserin), and was dependent on extracellular calcium. Wang and Friedman (1990b) also reported that serotonin and 5-HT_{2A} receptor agonist (DOI) but not the 5-HT_{1A} or 5-HT_{1B} agonists induced a dose-dependent PKC translocation in human blood platelets. The brain 5-HT_{2A} receptor has been shown to be linked to the phosphoinositide pathway (Conn and Sanders-Bush, 1986). Clauster at al (1988) found an increase in cortical and hippocampal [^{3}H]inositol phosphate ([^{3}H]IP) formation following stimulation with 5-HT_{2A} agonists. This effect could be blocked by 5-HT_{2A} antagonist, ketanserin.

Platelets also contain serotonin receptors and thus have been used as a tool for studying serotonergic transmission and the signal transduction systems which are linked to serotonin receptors. De Chaffoy et al (1988) reported that 5-HT_{2A} receptor stimulation by serotonin in platelets is linked to phosphoinositide metabolism.

Several experiments have provided support for the notion that translocation of PKC to the membrane serves, at least in part, to homologously desensitize the 5-HT_{2A} receptor. In platelets, intracellular calcium is mobilized by stimulation with serotonin. This mobilization can be inhibited by ketanserin and thus is likely to be mediated by the 5-HT_{2A} receptor (Kagaya et al, 1990). A role for the PKC in this apparent acute desensitization of the 5-HT_{2A} receptor was proposed based on the finding that mezerein (activator of PKC) also inhibited the calcium release response of platelets to 5-HT. Similarly, Dillon-Carter and Chuang (1989) reported a decrease in inositol phosphate accumulation upon stimulation of cerebellar granular cells with 5-HT_{2A} agonists, as well as a decrease in basal PLC activity upon adminstration of a PKC activator, 4ß-phorbol 12-myristate 13-acetate (PMA). They proposed a model in which PKC would phosphorylate some component of the 5-HT_{2A} receptor system (receptor, G-protein or PLC) and hence inhibit further agonist-induced propagation in the cascade. In rat aorta, the PKC activator, phorbol dibutyrate (PDBu) was demonstrated to desensitize 5-HT_{2A} receptor-mediated phosphoinositide turnover and to attenuate the aortic contraction which is induced in this tissue by 5-HT (Roth et al, 1986), suggesting again an acute

negative feedback effect of PKC on the transducing system of the 5-HT_{2A} receptor. This PKC-mediated negative feedback regulation is not exclusive to the 5-HT_{2A} receptor's second message. It has been shown that other receptor transducing system are also acutely downregulated by PKC activation (Nishizuka, 1986).

C. CHEMICAL LESION AS A TOOL TO STUDY SEROTONERGIC FUNCTION

Amphetamine and its derivatives are indirectly acting sympathomimetic amines which are believed to exert their effects by releasing endogenous biogenic amines from nerve terminals. Certain amphetamine derivatives, including *p*-chloroamphetamine (PCA), fenfluramine, 3,4-methylendioxyamphetamine (MDA), and 3,4-methylendioxymethamphetamine (MDMA), preferentially release serotonin both *in vivo* (Fuller et al, 1965) and *in vitro* (Schmidt et al, 1987). It has been shown that the serotonin release induced by these compounds is mediated by serotonin transport systems (Rudnick and Wall, 1992). PCA interacts with serotonin transporter by competing with serotonin for transport and stimulating the efflux of serotonin. In addition to releasing serotonin, PCA also leads to a long-term depletion of serotonin (Ricaurte et al, 1985; Hrdina et al, 1990) which correlates with morphological damage to serotonergic nerve endings (Ricaurte et al, 1985; Molliver, 1990). Hrdina et al (1990) reported that treatment with PCA dramatically decreased 5-HT uptake (by 80%), as well as levels of 5-HT (by 76%) and 5-hydroxyindolacetic acid (5-HIAA; by 55%) but not NE when compared with control rats. Unlike the serotonergic neurotoxins, 5,6- and 5,7-dihydroxytryptamine

which do not cross the blood-brain barrier and have to be given intraventricularly or directly into brain tissue, PCA can be given systemically to deplete serotonin in the CNS (Fuller, 1978). PCA is thus a useful pharmacologic tool for selectively lesioning serotonergic terminals in brain in studies on the possible sites of drug action.

The serotonin transporter has been implicated in the neurotoxicity of PCA since inhibitors of serotonin uptake block the destruction of serotonergic terminals produced by PCA (Schmidt et al, 1987; Fuller, 1980). These results suggest that the serotonin transporter either mediates the entry of neurotoxic amphetamines into serotonergic terminals or participates in sequelae leading to serotonin release and depletion or both (Fuller, 1980). It is of interest that transient depletion of 5-HT was shown to provided substantial protection against subsequent PCA-induced degeneration of 5-HT axon terminals (Berger et al, 1992). The neurotoxicity induced by PCA thus appear to be dependent on the presence of endogenous stores of 5-HT.

AIMS, RATIONALE AND HYPOTHESES

A. POSSIBLE ROLE OF PKC IN THE REGULATION OF NEURONAL SEROTONIN UPTAKE

It has been shown that PKC and PKA are involved in intracellular regulation of serotonin uptake in some non-neuronal cells including platelets. The

characteristics of platelet and neuronal 5-HT transport are similar and the primary structure of the cloned 5-HT transporter from brain and platelets is identical. It was reasonable to assume that similar intracellular mechanisms involving PKC and PKA may regulate the function of serotonin transporter in brain and platelets.

The aim of these experiments was to test hypothesis that activation of PKC, inhibits the neuronal serotonin transport in brain.

B. POSSIBLE ROLE OF PKC IN THE DOWN-REGULATION OF 5-HT_{2A} RECEPTORS BY AGONIST TREATMENT

Recently, it has been shown that the new selective 5-$HT_{2A/2C}$ receptor agonists of phenylisopropylamine derivatives series (e.g. DOI) downregulate the density of 5-HT_{2A} receptors in cerebral cortex. Since DOI activates PI signalling system and causes translocation of PKC activity, it can be assumed that activation of PKC by receptor agonist, DOI may contribute (via phosphorylation ?) in the down-regulation of 5-HT_{2A} receptors.

The aim of this project was to test the hypothesis that down-regulation of 5-HT_{2A} receptors in cerebral cortex by agonist treatment is associated with the activation of PKC and that the site of this action is at the post-synaptic level.

II. MATERIALS AND METHODS

A. STUDY OF NEURONAL 5-HT UPTAKE REGULATION BY PKC ACTIVATORS

1. Experimental design

Male Sprague-Dawley rats weighing 200 to 250 g were obtained from Charles-River Inc., and were used for all experiments. Animals were housed under standard laboratory conditions (12 hours light/dark cycle) with access to food and water. To assess the role of PKC, [^{3}H]5-HT uptake by crude synaptosomal (P_2) fraction of brain tissue "*in vitro*" was measured in the presence of different concentrations of 4ß-phorbol-12 myristate-13-acetate (PMA), a compound known to stimulate PKC activity, and of a selective 5-HT uptake inhibitor, citalopram. The concentrations of PMA used were 0.1, 1 and 10 μM, and that of citalopram was 1 μM. PMA (12 mg) was dissolved in 2 mL ethanol or dimethylsulfoxide (DMSO) to make a 10 mM of PMA stock solution. Aliquots of this stock solution were used to make serial dilution to final concentrations used. Citalopram (1 mg) was dissolved in 5 mL of incubation buffer. Two μL aliquot of this stock solution was added to 1 mL of sample to make a final concentration of citalopram (1 μM). Different *in vitro* exposure times to these drugs were used. Synaptosomes were pre-incubated for 2, 5 or 10 minutes in the presence of either citalopram or PMA, and uptake of [^{3}H]5-HT by synaptosomes was measured at a single concentration 100 nM.

2. Experimental procedures

2.1 [^{3}H]5-HT uptake and its kinetic parameters

[^{3}H]5-HT uptake was measured as previously described (Mann and Hrdina, 1992).

2.1.1 Preparation of tissue

Rats were killed by decapitation and their brains quickly removed and dissected out. Cortex was homogenized in 10 volume of sucrose buffer (0.32 M sucrose, 0.002 M Tris, pH 7.4) for 15 seconds with a teflon pestle homogenizer (setting 6). The homogenate was centrifuged in a Beckman centrifuge at 1,000 xg and 4°C for 10 minutes. The crude nuclear pellet was discarded and the resulting supernatant centrifuged at 17,000 xg and 4°C for 17 minutes. The final pellet, representing the crude synaptosomal (P_2) fraction, was resuspended in the original volume of buffer and kept on ice until assayed (approximately 1 to 2 hours).

2.1.2 [^{3}H]5-HT uptake assay

Fifty microliters of homogenate corresponding to 200 to 300 μg protein per tube was added to assay tubes (with final incubation volumes of 0.5 mL) containing 400 μL of incubation buffer (20 mM Tris, 5 mM KCl, 1.2 mM $MgCl_2.6H_2O$, 2.5 mM $CaCl_2.2H_2O$, 10 mM glucose, 0.1 mM pargyline, 1 mM

ascorbic acid, pH 7.4); aerated with 95% O_2 & 5% CO_2. [^{3}H]5-HT in concentration of either 100 nM or 50, 65, 100, and 200 nM (for kinetics parameters) was added to each tube in triplicate and a 5 minute incubation at 37°C followed. Blank tubes representing non-specific uptake were incubated at 0°C. Incubation was terminated by the addition of 5 mL of ice-cold incubation buffer and subsequent filtration through Whatman GF/B filters. The filters were washed twice with 5 mL of ice-cold incubation buffer, dried, and the retained radioactivity determined by liquid scintillation spectrometry (Beckman LS 7800) at a counting efficiency of 46%. Net uptake was defined as the difference between the total (at 37°C incubation) and non-specific (at 0°C incubation) uptake. Net uptake amounted to between 75 and 85% of total binding. The values were expressed as pmol/mg protein/5 minutes.

2.1.3. Determination of protein content

The following modified version of Lowry's assay (Lowry et al, 1951) was used to determine the protein content of tubes for [^{3}H]5-HT uptake assay in order to avoid the interference of reagents in the homogenate buffer with the chemicals in the protein assay: Aliquots of samples were made up to a volume of 950 μL with distilled water, and 50 μL of trichloroacetic acid (100%) was added to each tube, mixed immediately and left for 45 minutes to allow precipitation of proteins. The tubes were then centrifuged (Eppendorf 5412 bench-top centrifuge) for 5

minutes. The resulting supernatants were aspirated and discarded, and the protein pellets were resuspnded in 200 μL of 0.1 M NaOH. The original method of Lowry et al (1951) was then used to determine the protein content of each tube. A Beckman DU-7 spectrophotometer was used to measure the absorbency of light by proteins at a wavelength of 640 nm.

2.1.4. Determination of uptake parameters

The maximum uptake rate (Vmax) and affinity constant (Km) were calculated from Lineweaver-Burke plots using four concentrations of [^{3}H]5-HT. The total, non-specific and the net uptake of [^{3}H]5-HT by cortical synaptosomes, under these experimental conditions, are shown in Fig 9. The double reciprocal plot of net [^{3}H]5-HT uptake as shown in Fig 9 (insert) was linear and yielded in control tissue the following kinetic constants: Vmax = 6.292 (pmol/mg protein/5 min), Km = 0.118 (μM), r = -0.966.

2.2. Drugs and chemicals

[^{3}H]5-HT creatinine sulphate (specific activity, 27.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Phorbol-12 myristate-13-acetate (PMA) and dimethyl-sulfoxide (DMSO) were purchased from Sigma Chemicals Co., and citalopram hydrobromide was obtained from Lundbeck A/S, Copenhagen. Other chemicals used were of purest grade available.

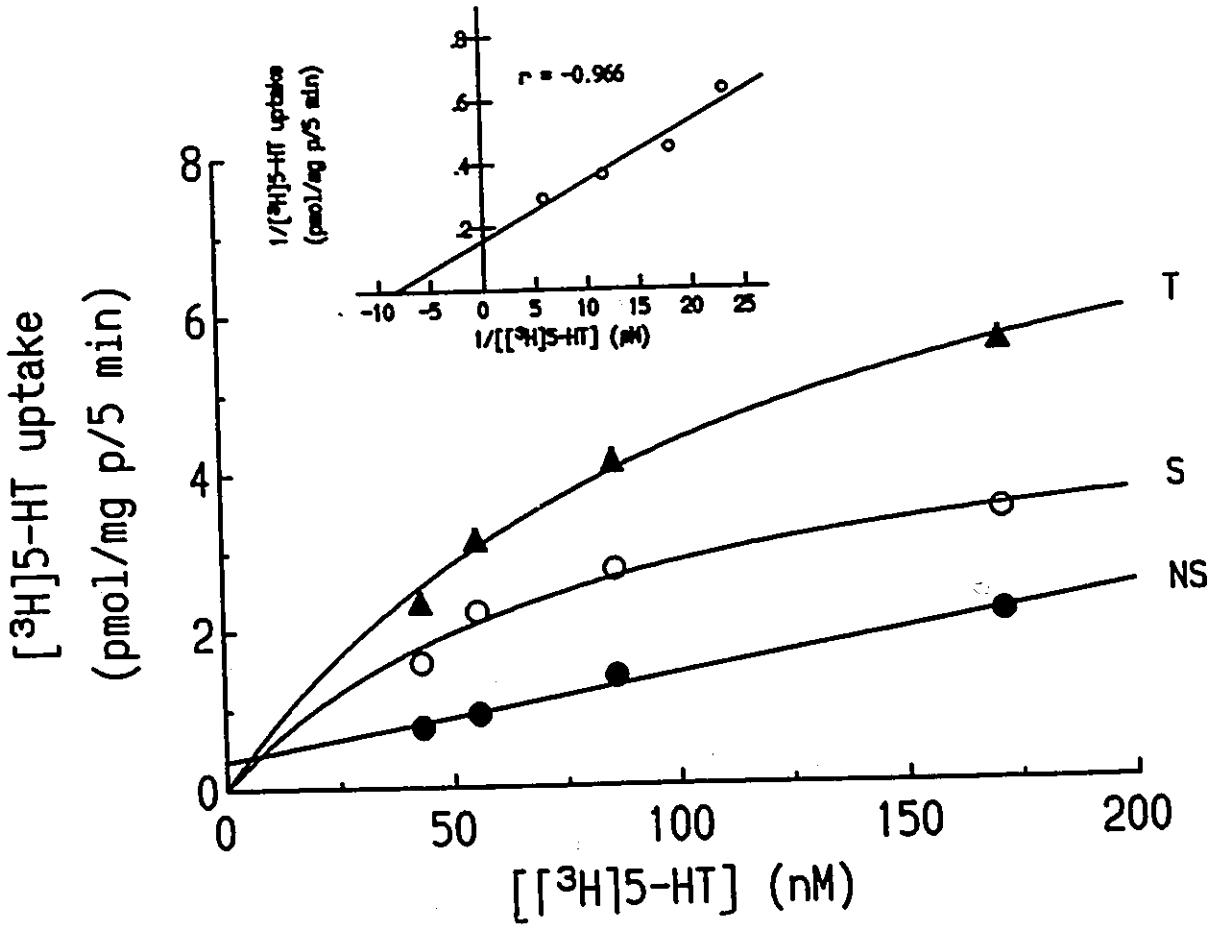

Fig. 9. [^{3}H]5-HT uptake in cortical synaptosomes (P_2 fraction) of a control rat brain. Uptake was determined over a concentration range of 50 to 200 nM [^{3}H]5-HT. Net uptake (S) was defined as the difference between uptake at 37°C (T) and 0°C (NS). **Insert:** Same data expressed in Linweaver-Burke plot. The Vmax value was 6.292 pmol/mg p/5 min and Km 0.118 μM.

B. STUDY OF THE POSSIBLE ROLE OF PKC IN 5-HT_{2A} RECEPTOR DOWN REGULATION AFTER AGONIST TREATMENT

1. Experimental design

Experiments were performed to evaluate the effect of acute treatment with 1-(2,5-dimethoxy-4-iodo-phenyl)-2-aminopropan (DOI), as a selective 5-$HT_{2A/2C}$ receptor agonist, on the kinetic parameters of [^{3}H]ketanserin binding in both homogenate and synaptosomal preparation and on the PKC activity in soluble and particulate fraction of rat brain tissue. Groups of naive male Sprague-Dawley rats weighing 200 to 250 g were injected intraperitonealy (i.p.) with either a single dose of DOI (10 mg/kg), or with the same dose (10 mg/kg, i.p.) for three consecutive days. The control animals were given the equivalent volume of 0.9% saline (vehicle). In studies examining dose dependency of DOI effect, DOI was administered to groups of animals in single doses of 1, 5 or 10 mg/kg; i.p. The doses of DOI were selected on the basis of previous reports (Pranzatelli et al, 1987; Pranzatelli, 1990). Animals were sacrificed 24 hours after the single or the last of three consecutive injections.

In studies examining the effect of a lesion to the serotonergic neuronal system, animals were injected with *p*-choloroamphetamine, PCA, (10 mg/kg; i.p./day) for three consecutive days and sacrificed 7 days after the last injection. Two additional groups of PCA-lesioned animals were injected with a single dose of DOI (10 mg/kg; i.p.) or with DOI (10 mg/kg;i.p.) for three consecutive days and

sacrificed 24 hours after the last injection of DOI.

The time period (24 hours) between the last daily dose of DOI and sacrifice was chosen to minimize interference of residual drug with the assays, and was based on the plasma half life of the drug ($t_{1/2}$ of DOI = 12 hours).

2. Experimental procedures

2.1 [^{3}H]Ketanserin binding in brain homogenate

[^{3}H]ketanserin binding in brain homogenate was determined as described below.

2.1.1 Preparation of tissue

Rats were killed by decapitation, the brain quickly removed and dissected. Frontal cortex was homogenized in 200 volumes of ice-cold incubation buffer (50 mM Tris-HCl pH 7.7) for 15 seconds with a Polytron (setting 6). The homogenate was pre-incubated for 15 minutes at 37°C before centrifugation in a Beckman centrifuge at 4°C and 30,000 xg for 30 minutes. The resulting pellet was resuspended in the original volume of buffer, homogenized and centrifuged again under the identical conditions. The final pellet obtained was homogenized in the original volume of buffer and kept on ice until assayed (approximately 1 to 2 hours).

2.1.2 Binding assay

Assay tubes (with final incubation volumes of 2.5 mL) contained 1 mL of incubation buffer (50 mM Tris-HCl pH = 7.7, 1 μM prazocin and 1 μM tetrabenazine to prevent [^{3}H]ketanserin binding to α_1-adrenergic receptors and vesicular amine transporter, respectively), in the absence or presence of 1 μM mianserin (for determination of total and non-specific binding, respectively) and 0.5 mL [^{3}H]ketanserin at concentrations of 0.05, 0.1, 0.25, 1.0 and 2.0 nM. Incubation was started by the addition to each tube of 1 mL homogenate, corresponding to final protein concentrations of 200 to 300 μg. Incubation was performed in duplicate, proceeded for 15 minutes at 37°C and was terminated by the addition of 5 mL of ice-cold incubation buffer. The content of tubes was filtered through Whatman GF/F filter discs (presoaked in ice-cold incubation buffer containing 0.1% polyethyleneimine for at least 2 hours), with two additional washes. The filters were dried and the retained radioactivity was determined by liquid scintillation spectrometry (Beckman LS 7800) at a counting efficiency of 46%.

2.1.3 Determination of binding parameters

The maximum number of binding sites (Bmax) and equilibrium dissociation constant (Kd) were calculated by Scatchard analysis of saturation binding data. Under these experimental conditions, the specific binding of [^{3}H]ketanserin to

cerebrocortical homogenates reaches saturation at above 1 nM concentration (Fig. 10). The Scatchard transformation indicated that [^{3}H]ketanserin binds to a single class of sites of high affinity (Fig. 10, insert) with a Kd of 0.358 nM and Bmax of 291 fmol/mg protein.

2.2 [^{3}H]ketanserin binding in brain synaptosomes (P_2 fraction)

[^{3}H]ketanserin binding in brain synaptosomes was determined as described below.

2.2.1 Preparation of tissue

Rats were killed by decapitation and their brains quickly removed and dissected out. Fontal cortex was homogenized in 200 volume of sucrose buffer (0.32 M sucrose, 0.002 M Tris-HCl, pH 7.4) for 15 seconds with a teflon pestle homogenizer (setting 6). The homogenate was pre-incubated for 15 minutes at 37°C and then centrifuged at 1,000 xg and 4°C for 10 minutes. The resulting supernatant was centrifuged at 17,000 and 4°C for 20 minutes. The final pellet, representing the crude synaptosomal (P_2) fraction, was resuspended in the original volume of buffer and kept on ice until assayed (about 1 to 2 hours).

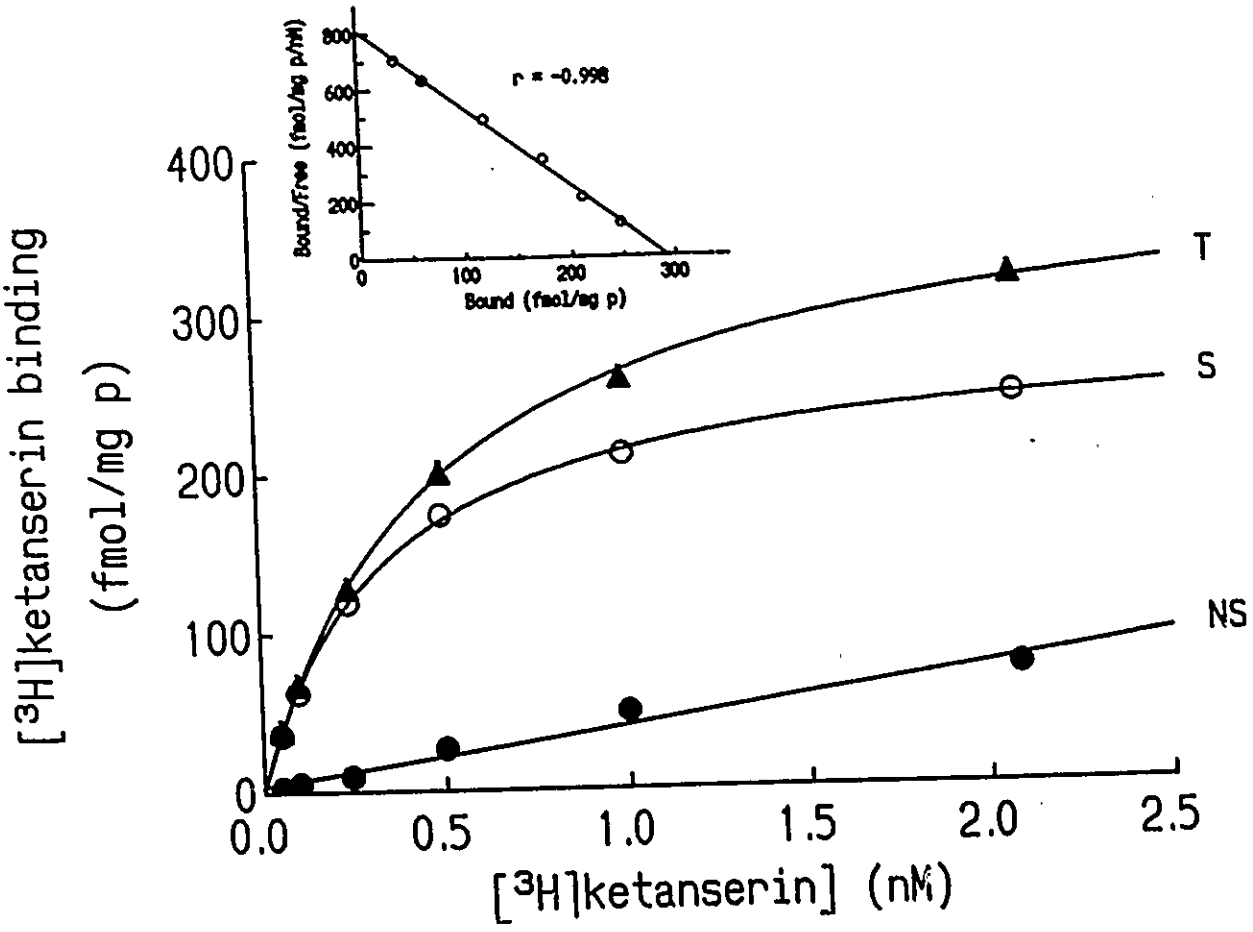

Fig. 10. Saturation curves for binding of [^{3}H]ketanserin in cerebrocortical homogenate of rat. Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding (S) was defined as the difference between binding in the absence (T) or presence (NS) of 1 μM of mianserin. **Insert:** Scatchard analysis of [^{3}H]ketanserin binding. The Bmax value was 291 fmol/mg of protein and Kd 0.358 nM.

2.2.2 Binding assay

As described above (in section B:2.1) for [^{3}H]ketanserin binding in brain homogenate.

2.2.3 Determination of binding parameters

As described above (in section B:2.1) for [^{3}H]Ketanserin binding in brain homogenate. The kinetics of [^{3}H]ketanserin binding to cortical synaptosomes was similar to that seen in the homogenate although the Bmax was lower.

2.3 Measurement of protein kinase C activity

Protein kinase C (PKC) activity in subcellular fractions was measured using a commercially available enzyme assay system (Amersham, Des Plaines, IL). A PKC-specific target peptide and all necessary co-factors are provided in the kit. PKC activity in soluble and particulate fractions was determined after modification of the method described by Wang and Frideman (1990a).

2.3.1 Preparation of tissue

Rats were killed by decapitation, the brains quickly removed and dissected.

Cortical tissue was homogenized in 10 volumes of sucrose buffer (0.32 M sucrose, 0.002 M Tris-HCl, pH 7.4) for 15 seconds using polytron homogenizer (setting 6). The homogenate was first centrifuged at 1,000 xg and 4°C for 10 minutes, and the resulting supernatant centrifuged again at 17,000 xg and 4°C for 17 minutes. The final pellet, representing the crude synaptosomal (P_2) fraction, was resuspended in 1 mL of assay buffer (0.32 M sucrose, 20 mM Tris-HCl/pH 7.5, 5 mM Hepes/pH 8.00, 5 mM benzamidine, 2 mM dithiothreitol, 3 mM EGTA/pH 7.5, 0.5 mM $MgSO_4$, 0.5 mM $ZnSO_4$, 0.1 mM phenylmethylsulfonylfluoride, 0.1 mg/mL leupeptin, 0.05 mg/mL pepstatin and 0.1 mg/mL aprotinin), sonicated (Mettler Electronic Sonicator) for 1 minute, and homogenized in the same buffer for 15 seconds (setting 6). The homogenates were then centrifuged (Beckman L7-35) at 100,000 xg and 4°C for one hour. The supernatants (after dilution in assay buffer) were used for determination of PKC activity in the cytosolic fraction. The resulting pellets were solubilized on ice for 1 to 2 hours in 1 mL of above buffer containing 1% Nonidet P-40, and recentrifuged at 2,500 xg and 4°C for 15 minutes. The supernatants obtained (after dilution in assay buffer) were used immediately for determination of PKC activity in the membrane fraction.

2.3.2 Activity assay

Eppendorf assay tubes (with final incubation volume of 75 μL) contained 25 μL of component mixture (3 mM $Ca(C_2H_3O_2)$, 2 mol % Lα phosphatidyl-L-serin, 6 μg/mL phorbol 12-myristate 13-acetate, 225 μM peptide and 7.5 mM dithiothreitol

in 50 mM Tris-HCl containing 0.05% W/V sodium azide, pH 7.5) and 25 μL sample, corresponding to 1 to 3 μg and 2 to 4 μg protein in the soluble and particulate fractions, respectively. Blank tubes representing non-specific phosphorylation contained 25 μL sample buffer instead.

The reaction was initiated by addition of 25 μL of magnesium-[^{32}P]ATP buffer (10 μCi/mL [^{32}P]ATP, 150 μM ATP and 45 mM $(CH_3COO)2Mg.4H_2O$ in 50 mM Tris-HCl containing 0.05% W/V sodium azide, pH 7.5) to each tube. Incubation proceeded for 15 minutes at room temperature (25°C) and was terminated by the addition of 100 μL of "stop" reagent (dilute acidic reaction-quenching reagent) to each tube. An aliquot of solution from each tube (125 μL) was spotted onto individual peptide-binding papers which were then placed in a 5% V/V acetic acid bath (at least 10 mL of the wash reagent per paper) for 10 minutes at room temperature with intermittent gentle mixing to ensure that each paper remains separated from neighbouring papers. The washing solution was then decanted and replaced with a similar volume of fresh 5% V/V acetic acid for a second 10 minutes wash. Papers were then dried and the retained radioactivity of ^{32}P was determined in a Beckman LS 7800 counter.

2.3.3 Calculation of PKC activity

The PKC activity in subcellular tissue fractions was determined using the Amersham enzyme assay system. A PKC-specific target peptide and all necessary

co-factors were provided in the kit. The enzyme present in the samples will catalyze the transfer of the -phosphate of adenosine-5'-triphosphate (ATP) to the threonine group of the target peptide which is specific for protein kinase C. The assay is a modification of a mixed micelle assay (Hannun et al, 1985), activating the enzyme with phorbol 12-myristate 13-acetate. The reaction is performed at pH 7.5 in a tris aminomethane buffer and is optimized for co-factor concentrations to exhibit maximum enzyme activity. The assay shows a linear incorporation of ^{32}P into substrate peptide corresponding to at least 10% ATP conversion, providing samples are suitably diluted. The phosphorylated peptide is separated on binding paper. After washing the paper, the extent of phosphorylation may be detected by scintillation counting. The principle of the assay is shown in the following scheme:

$$\text{Peptide} + [^{32}P]\text{ATP} \xrightarrow[Mg^{++}\ Ca^{++}\ \text{PS / Phorbol}]{\text{PKC}} [^{32}P]\text{peptide} + \text{ADP}$$

The ^{32}P incorporated into the synthetic target peptide is quantitatively measured by the binding papers. The result obtained should be corrected for any non-specific effects using an appropriate blank. The extent of phosphorylation representing protein kinase C activity is determined as follows:

Calculation of specific radioactivity (R) of 150 μM Mg[^{32}P]ATP

$$R = \frac{\text{cpm per 10 } \mu\text{L Mg[}^{32}\text{P]ATP}}{1.5 \text{ nM}}$$

(10 μL of 150 μM ATP contains 1.5 nmoles).

Calculation of total phosphate (T) transferred to peptide

$$T = (\text{sample cpm} \times 1.4^{*}) - \text{blanks cpm}$$

* This factor is derived from the total assay volume divided by the volume applied to the binding paper.

In our experiments the blank value did not change significantly with the volume applied.

Calculation of pmoles phosphate (P) transferred per minute by PKC to the PKC-specific peptide substrate:

$$P = \frac{T \times 1{,}000 \text{ pmoles/minute}}{I \times R}$$

I = incubation time (15 minutes)

2.4 Protein determination

The protein content of subcellular tissue fractions was determined as described above (in section A:2.1.3) for P_2 fraction of rat brain.

2.5 Methods of data analysis

Analysis of equilibrium binding data to determine the equilibrium dissociation constant (Kd) and the maximum number of binding sites (Bmax) of [^{3}H]ketanserin were performed using the BDATA computer program (EMF Software, Inc., Knoxville, TN) for non-linear regression fitting to a one-site binding model.

2.6 Statistics

All results are expressed as means ± standard error of means (SEM). Student's t-test and one-way ANOVA with a Newman-Keuls test were used to calculate the significance of differences between the means whenever appropriate.

2.7 Drugs and chemicals

[^{32}P]ATP tetra (triethylammonium) salt (specific activity 3000 Ci/mmol) and the protein kinase C enzyme assay system were purchased from Amersham (Des Plaines, IL), 1-(2,5-dimethoxy-4-iodo-phenyl)2-amino-propane (DOI) hydrochloride was obtained from Research Biochemical In. (Natick, MA). Nonidet-P 40 were purchased by Sigma Chemicals Co. Other chemicals were of purest grade available.

III. RESULTS

A. POSSIBLE ROLE OF PKC IN REGULATION OF NEURONAL SEROTONIN UPTAKE

1. Effect of PKC activation on 5-HT uptake

In initial experiments, the time course of the effect of a single concentration of a PKC activator, PMA in comparison with a known serotonin uptake inhibitor, citalopram on [^{3}H]5-HT uptake by cortical synaptosomes was examined. In these experiments, the synaptosomes were pre-incubated for various length of time (2 , 5 or 10 min) in the presence of either citalopram (1 μM) or PMA (1 μM) and the uptake by synaptosomes was measured at a single concentration (100 nM) of [^{3}H]5-HT. The results are shown in Table 6 and Fig 11.

As expected, exposure of cortical synaptosomes to a selective serotonin uptake inhibitor, citalopram (1 μM) produced a marked decrease of 5-HT uptake (to 13%, 6.8% and 9.6%, respectively of control) at all three time intervals of pre-incubation. Exposure of cortical synaptosomes to the PKC activator, PMA (1 μM) resulted in a moderate decrease of serotonin uptake that was significant in comparison with controls only at 5 minutes pre-incubation period (2.89 $\pm$ 0.23 vs 4.35 $\pm$ 0.32 pmol/mg protein/5 minutes; $p < 0.01$). This exposure time was therefore chosen for further experiments.

TABLE 6

Time course of the effect of PMA (1 μM) and citalopram (1μM) on the [^{3}H]5-HT uptake (pmol/mg protein/5 minutes) by cortical synaptosomal fraction of the rat brain. Each value represents the mean ± SEM of separate determination in brain tissue for five animals in each group. The uptake assay were performed in triplicate.

Treatment	PREINCUBATION-TIME (MIN)		
	2	5	10
None (Control)	3.8 ± 0.57 (100%)	4.35 ± 0.32 (100%)	3.04 ± 0.48 (100%)
PMA	3.21 ± 0.38 (84%)	2.89 ± 0.23** (66%)	2.43 ± 0.37 (80%)
Citalopram	0.50 ± 0.08** (13%)	0.296 ± 0.12** ++ (6.8%)	0.293 ± 0.11** (9.6%)

Effect of treatment at various pre-incubation time:

2 minute: Significant effect (ANOVA: f = 19.69; p < 0.01). ** Significantly different vs both control and PMA group (p < 0.01, Newman-Keuls test).

5 minute: Significant effect (ANOVA: f = 72.36; p < 0.01). ** Significantly different vs control (p < 0.01, Newman-Keuls test); ++ Significantly different vs PMA group (p < 0.01, Newman-Keuls test).

10 minute: Significant effect (ANOVA: f = 16.5, p < 0.01). ** Significantly different vs both control and PMA group (p < 0.01, Newman-Keuls test).

ANOVA has shown no significant effect of preincubation time in any of the groups.

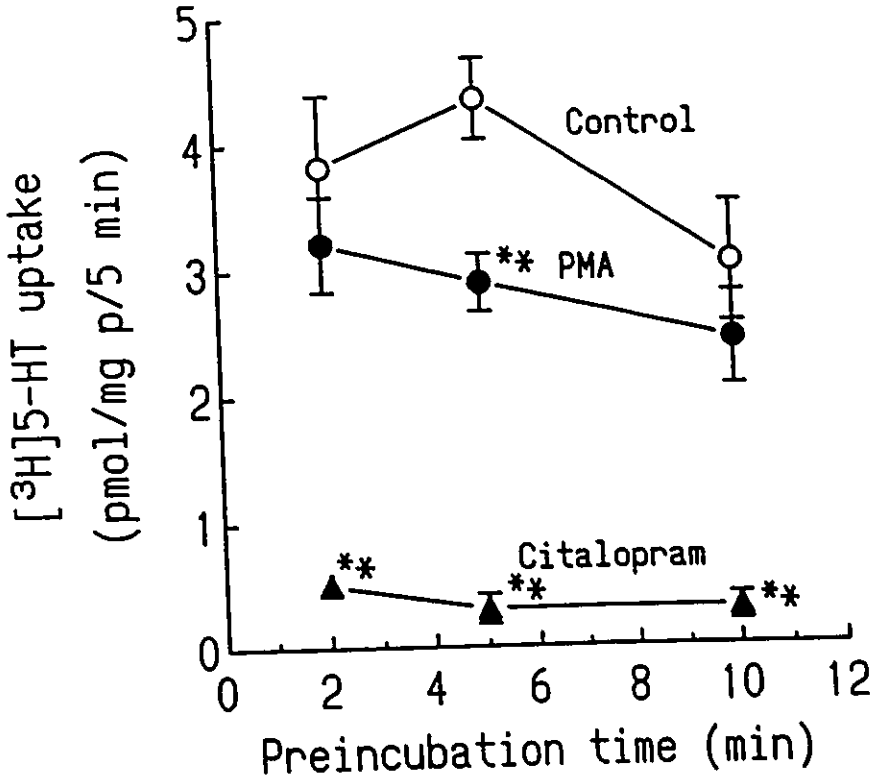

Fig. 11. The time course effect of PMA (1μM) and citalopram (1μM) on [^{3}H]5-HT uptake (pmol/mg protein/5 minutes) by cortical synaptosomal fraction of rat brain. Data are means ± SEM of five separate experiments as presented in Table 6. Control (O), PMA (●), citalopram (▲).

** Significantly different vs control (Newman-Keuls test, $p < 0.01$).

2. Evaluation of the effect of PMA solvents

Since PMA used in our initial experiments was dissolved in ethanol, we proceeded to examine the possible effect of this solvent alone present in various concentrations (0.01%, 0.1%, and 1%) on the 5-HT uptake and compare it to the effect of varying concentrations of PMA (0.1 μM, 1 μM and 10 μM) dissolved in ethanol at the above concentration of solvent. The exposure time was 5 minutes. The results are shown in Table 7 and Fig 12. Under this conditions, PMA at a concentration of 1 μM produced only a modest (16%) decrease in 5-HT uptake, and the difference versus ethanol alone was not significant (2.89 $\pm$ 0.23 vs 3.42 $\pm$ 0.04 pmol/mg protein/5 minute; $p > 0.05$). The 5-HT uptake in synaptosomes exposed to 0.1 μM or 10 μM PMA was not different from that seen in preparations exposed to ethanol alone. From this experiment, we have concluded that ethanol used as a solvent may have contributed to the result seen with PMA in our initial experiments.

Another solvent routinely used in experiments with PMA is dimethylsulfoxide (DMSO). The effect of PMA dissolved in DMSO on 5-HT uptake in synaptosomes, in comparison with exposure of synaptosomes to corresponding concentrations (0.01%, 0.1% and 1%) of DMSO alone is shown in Table 8 and Fig 13. ANOVA has shown no significant differences between the effect of various concentration of DMSO or PMA. However, at the lowest concentration used (0.1 μM) PMA

TABLE 7

Effect of different concentration of PMA (dissolved in ethanol) and ethanol alone on the [^{3}H]5-HT uptake by cortical synaptosomal fraction of rat brain. Each value represents the mean ± SEM of three separate experiments performed in triplicate. ANOVA has shown no significant differences between the effect of various concentrations of the solvent alone or PMA dissolved in ethanol.

TREATMENT	[^{3}H]5-HT UPTAKE (pmol/mg p/5 min)
Ethanol(0.01%)	2.41 ± 0.58
Ethanol (0.1%)	3.42 ± 0.04
Ethanol (1%)	2.60 ± 0.23
PMA (0.1 μM)	2.32 ± 0.24
PMA (1 μM)	2.89 ± 0.23
PMA (10 μM)	2.57 ± 0.26

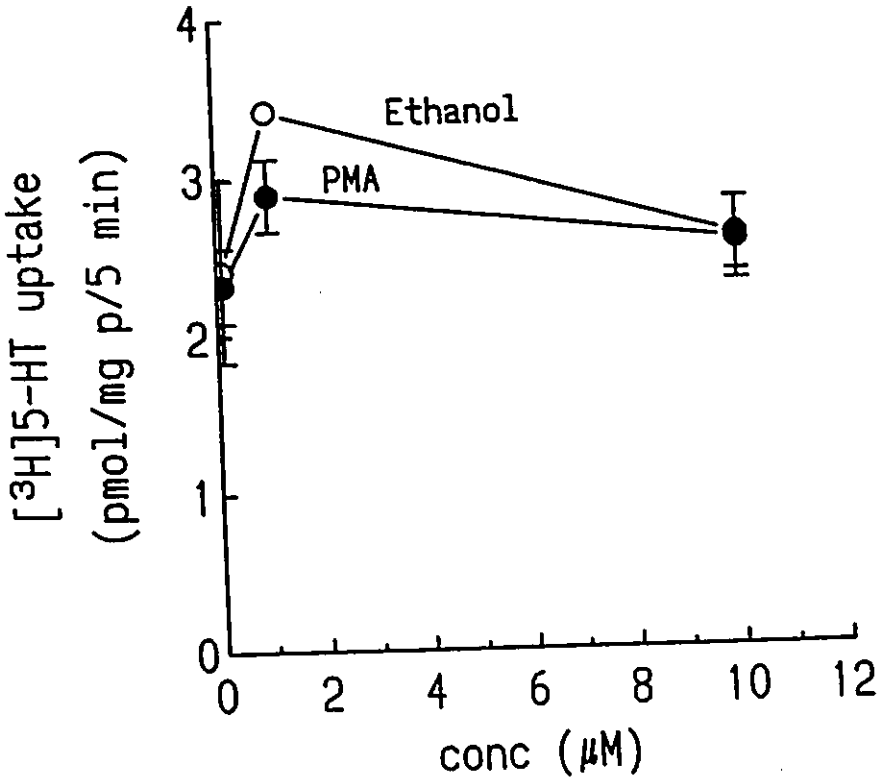

Fig. 12. Effect of different concentrations of PMA (dissolved in ethanol) and ethanol alone on the [^{3}H]5-HT uptake by cortical synaptosomal fraction of rat brain. Each point represents the mean ± SEM of three separate experiments as presented in Table 7. Ethanol (O), PMA (●).

TABLE 8

Effect of different concentrations of PMA (dissolved in DMSO) and DMSO alone on the [^{3}H]5-HT uptake by cortical synaptosomal fraction of rat brain. Each value represents the mean ± SEM of six separate experiments performed in triplicate. ANOVA has shown no significant differences between the effect of various concentrations of DMSO or PMA.

TREATMENT	[^{3}H]5-HT UPTAKE (pmol/mg p/5 min)
DMSO (0.01%)	3.10 ± 0.32
DMSO (0.1%)	2.00 ± 0.48
DMSO (1%)	2.76 ± 0.32
PMA (0.1 μM)	2.12 ± 0.21 *
PMA (1 μM)	2.66 ± 0.33
PMA (10 μM)	2.23 ± 0.26

* Significantly different from corresponding concentration of the solvent (Student's t-test, $p < 0.05$).

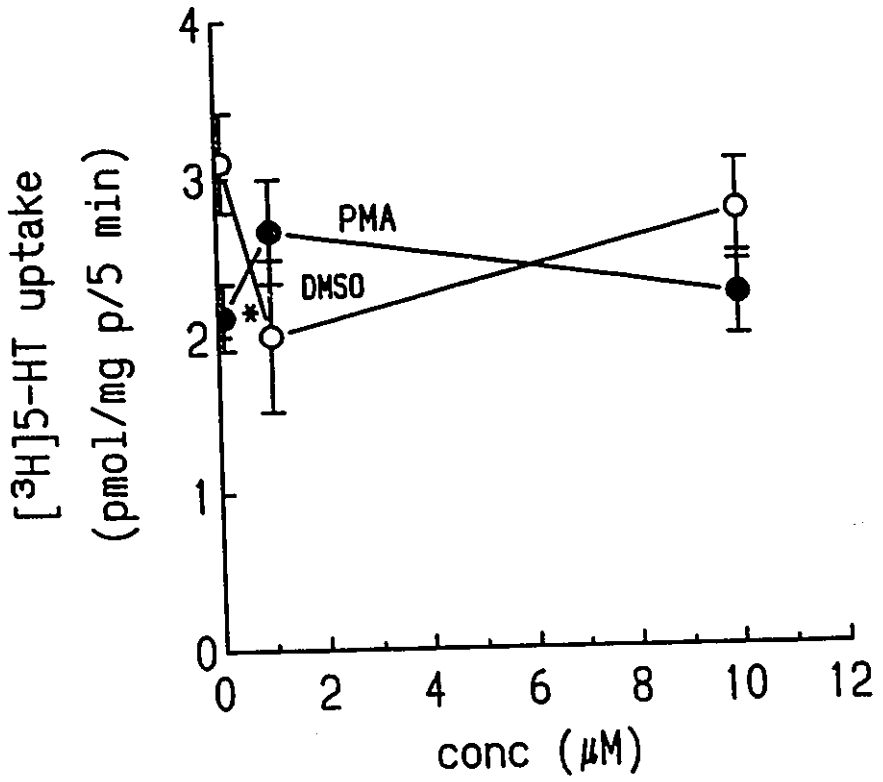

Fig. 13. Effect of different concentration of PMA (dissolved in DMSO) and DMSO alone on the [^{3}H]5-HT uptake by cortical synaptosomal fraction of rat brain. Data are means ± SEM of six separate experiments as presented in Table 8. DMSO (O), PMA (●).

* Significantly different from DMSO alone (Student's t-test, $p < 0.05$).

produced a significant inhibition of 5-HT uptake in comparison to the solvent alone (2.12 ± 0.21 vs 3.10 ± 0.32 pmol/mg protein/5 minute; $p < 0.05$). We have therefore decided use this concentration of PMA dissolved in DMSO for examining the effect of the PKC activator on the kinetic parameters of 5-HT uptake.

3. Effects of PMA on kinetics of 5-HT uptake

Data presented in Table 9 and Fig 14, show the kinetics of [^{3}H]5-HT uptake by cortical synaptosomes of rat brain in the presence of 0.1 μM PMA (dissolved in DMSO) or DMSO alone (0.01%). It is evident that this concentration of PMA did not produce a significant changes in kinetic parameters (Vmax, Km) of [^{3}H]5-HT uptake when compared with solvent control. We have then examined whether higher concentrations of PMA (1 μM and 10 μM) will produce some alteration in [^{3}H]5-HT uptake by cortical synaptosomes. The analysis of 5-HT uptake kinetics (Table 10 and Fig 15) has revealed that the exposure of cortical synaptosomes to these higher concentrations of the phorbol ester did not alter significantly Vmax of [^{3}H]5-HT uptake by cortical synaptosomes. However, the affinity constant (Km) was significantly increased by both concentrations of PMA in comparison to control value (ANOVA: $f = 6.25$, $p < 0.05$; Newman-Keuls test and Student's t-test: $p < 0.05$ for differences of 1 μM and 10 μM of PMA-treated groups versus control).

TABLE 9

Effect of PMA (0.1 μM) and DMSO (0.01%) on the kinetics of [^{3}H]5-HT uptake by rat cortical synaptosomes. Each value represents the mean ± SEM of four separate experiments performed in triplicate. ANOVA has revealed no significant effect of treatment on either Km or Vmax.

TREATMENT	Km (μM)	Vmax (pmol/mg p/5 min)
Control	0.119 ± 0.01	5.76 ± 0.55
DMSO (0.01%) (Solvent ctrl)	0.097 ± 0.02	5.75 ± 1.00
PMA (0.1 μM)	0.129 ± 0.02	6.72 ± 1.00

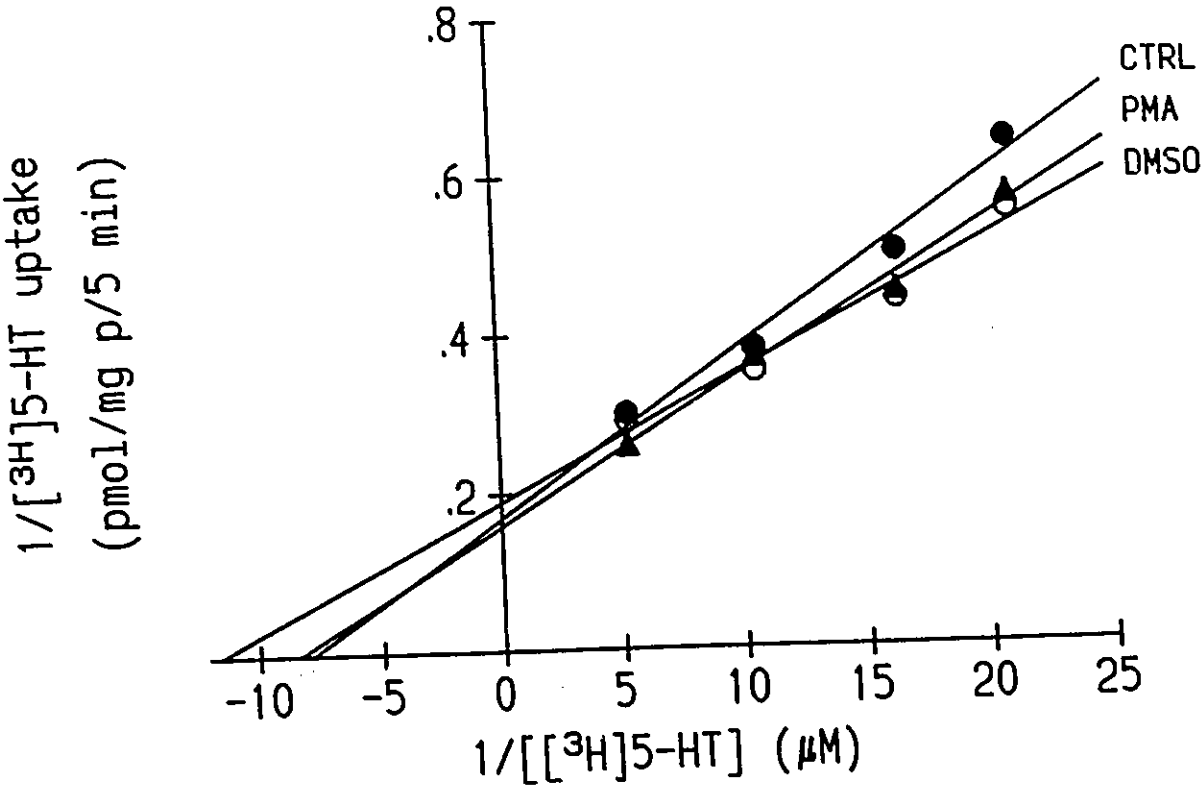

Fig. 14. Lineweaver-Burke plots of [^{3}H]5-HT uptake in cortical synaptosomes of rat brain. Effect of PMA (0.1 μM) and DMSO (0.01%) on the kinetics of [^{3}H]5-HT uptake: Control (●), DMSO (O) and PMA (▲). Uptake was determined over a concentration range of [^{3}H]5-HT of 50 to 200 nM. Net uptake was defined as the difference between uptake at 37°C (total) and 0°C (non-specific). Each point represents the mean ± SEM of four separate experiments performed in triplicate. The corresponding Vmax values and Km values has been presented in Table 9.

TABLE 10

Effect of PMA (1 or 10 μM) on the kinetics of [^{3}H]5-HT uptake by rat cortical synaptosomes. Each value represents the mean ± SEM of five separate experiments performed in triplicate.

TREATMENT	Km (μM)	Vmax (pmol/mg p/5 min)
Control	0.114 ± 0.02	6.00 ± 0.27
PMA (1 μM)	0.198 ± 0.01 *	6.71 ± 0.37
PMA (10 μM)	0.163 ± 0.02 +	6.08 ± 0.53

ANOVA revealed that means of Km but not of Vmax were significantly different ($f = 6.25$, $p < 0.05$). * Significantly different from control ($p < 0.05$, by both Newman-Keuls test and Student's t-test). + Significantly different vs control ($p < 0.05$; Student's t-test).

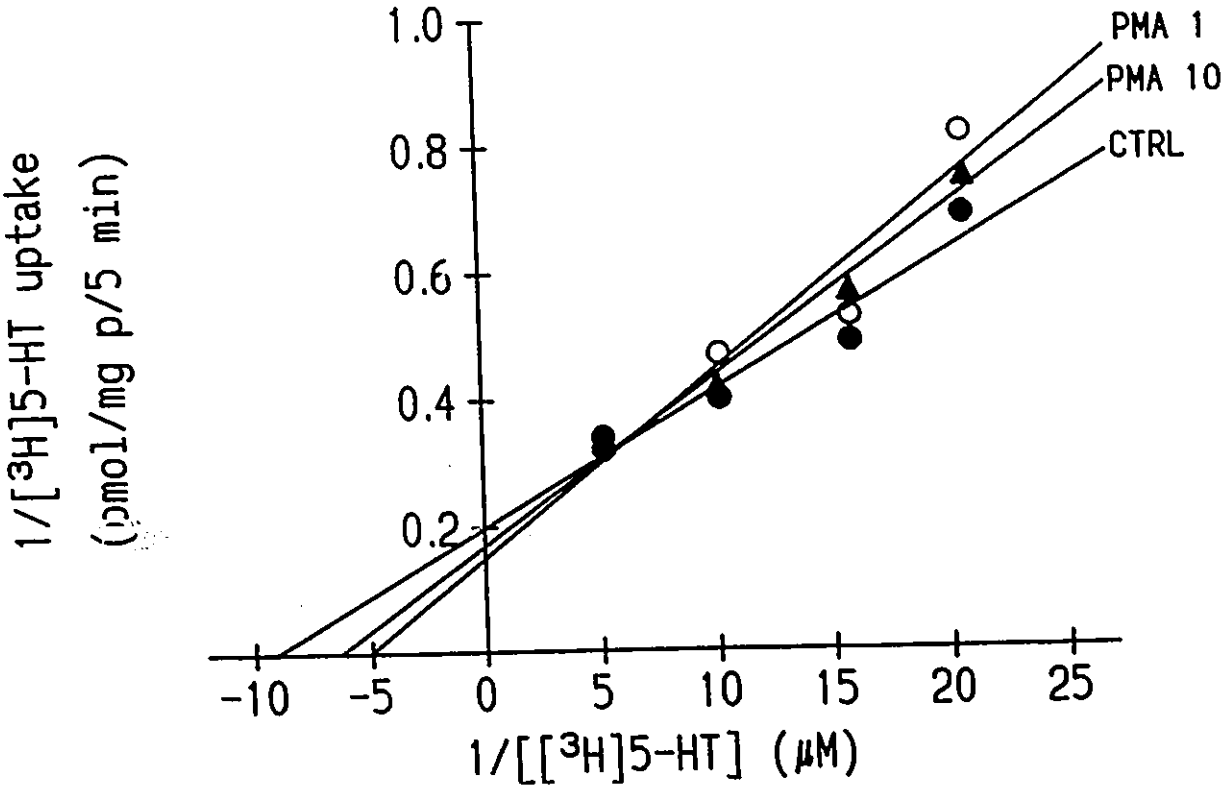

Fig. 15. Lineweaver-Burk plots of [^{3}H]5-HT uptake in cortical synaptosomes of rat brain. Effect of PMA (1, 10 μM) on the kinetics of [^{3}H]5-HT uptake: Control (●), PMA 1 μM (O) and PMA 10 μM (▲). Uptake was determined over a concentration range of [^{3}H]5-HT of 50 to 200 nM. Net uptake was defined as the difference between uptake at 37°C (total) and 0°C (non-specific). Each point represents the mean ± SEM of five separate experiments performed in triplicate. The corresponding Vmax values and Km values has been presented in Table 10.

4. Conclusion

Activation of PKC by a phorbol ester, PMA failed to produce unequivocal changes in the rate of 5-HT uptake by cortical synaptosomes of rat brain. Under certain experimental conditions, PMA (0.1 μM and 1 μM) produced a moderate inhibition of 5-HT uptake measured at a single concentration of 5-HT. Low concentration (0.1 μM) of PMA did not significantly alter the kinetics parameters (Vmax, Km) of 5-HT uptake in synaptosomes. However, higher PMA concentrations (1 and 10 μM) decreased significantly the affinity of the transporter for 5-HT without altering the rate of uptake. Caution should be exercised in selecting PMA solvent concentrations in uptake experiments.

B. STUDY OF THE POSSIBLE ROLE OF PKC IN DOWN-REGULATION OF 5-HT_{2A} RECEPTORS AFTER AGONIST TREATMENT

5-Hydroxytryptamine$_{2A}$ (5-HT_{2A}) receptors in rat brain have been reported to be down-regulated not only by chronic treatment with various antidepressants (Peroutka and Snyder, 1980) but also with 5-HT_{2A} antagonists (Gandolfi et al, 1985) that would be expected to produce a supersensitivity and up-regulation of the receptor. The effect of agonist treatment has been less investigated because of their lack of selectivity for the 5-HT_{2A} receptor, low affinity and mixed agonist-antagonist properties of the drugs used. Recently, Pranzatelli (1991) has shown that the 5-HT_{2A} receptor mediated phosphatidyl inositol response in rat cortex became downregulated by acute or chronic treatment with 1-(2,5-dimethoxy-4-iodo)-2-aminopropane (DOI). This drug has been described as a relatively selective 5-$HT_{2A/2C}$ agonist and is one of the most potent agonists known for these receptor (Glennon et al, 1986). Wang and Friedman (1990a) also reported that *in vitro* exposure of cortical slices to DOI causes PKC activity translocation from cytosol to membrane that is inhibited by ketanserin as a selective 5-HT_{2A} receptor antagonist. In order to explore further 5-HT_{2A} receptor regulation *in vivo* and the role of PKC in this regulation, we investigated the 5-HT_{2A} receptor mediated phosphatidyl inositol response and the binding properties of the 5-HT_{2A} receptor in rats treated acutely or repeatedly with DOI.

[^{3}H]ketanserin widely used as a selective ligand for 5-HT_{2A} receptors (Leysen et al, 1984). In the frontal cortex, which has a high 5-HT_{2A} receptor density, 80% of [^{3}H]ketanserin binding is specifically inhibited by various 5-HT_{2A} antagonists (Leysen et al, 1988). We have therefore used [^{3}H]ketanserin to label the binding sites on the 5-HT_{2A} receptor.

1. Effects of acute DOI treatment on [^{3}H]ketanserin binding in rat cerebral cortex

Data presented in Table 11 and Fig 16 demonstrate the effect of acute treatment with DOI on the kinetics of [^{3}H]ketanserin binding in homogenate of rat cerebral cortex. Control animals were injected with a corresponding volume of saline. A single injection of DOI (10 mg/kg; i.p.) significantly ($p < 0.01$) down-regulated 5-HT_{2A} receptor binding sites labelled 24 hours later by [^{3}H]ketanserin compared to control rats. The density of [^{3}H]ketanserin binding sites (120 ± 13 fmol/mg protein) was decreased by 50% compared to that in controls (255 ± 14 fmol/mg protein). The effect of acute treatment with DOI (10 mg/kg i.p. 24 hours before death) on the kinetics of [^{3}H]ketanserin binding in synaptosomal fraction of rat cerebral cortex is shown in Table 12, Fig 17.

TABLE 11

Effect of acute treatment with DOI (10 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in homogenate of rat cerebral cortex determined 24 hours later. Values are mean ± SEM obtained by Scatchard analysis of saturation binding data in brains from four animals in each group. The binding assays were performed in duplicate.

TREATMENT	Kd (nM)	Bmax (fmol/mg protein)
Control (saline)	0.507 ± 0.10	244 ± 12.0 (100)
DOI	0.567 ± 0.11	120 ± 13.0 * (50)

* Significantly different from control (Student's t-test, $p < 0.01$). Percentage values are given in parentheses.

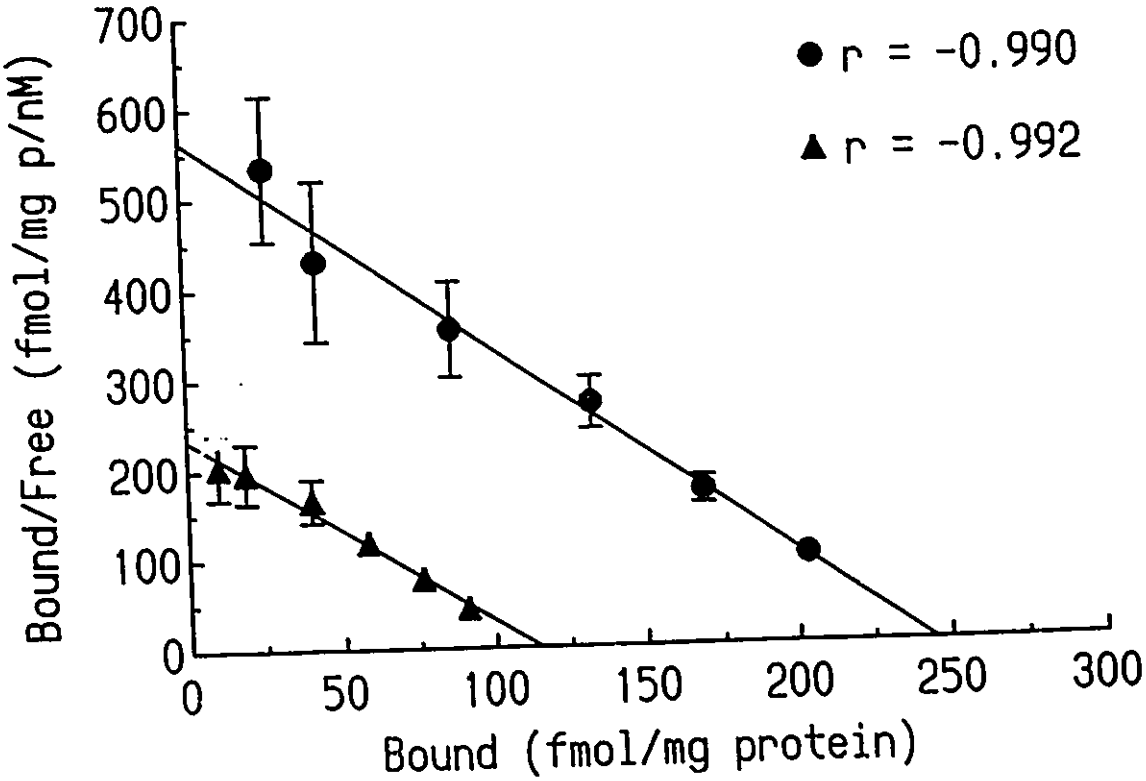

Fig. 16. Scatchard plot of [^{3}H]ketanserin binding in homogenate of cerebral cortex of rat treated with a single i.p. injection of DOI (10 mg/kg) 24 hours before death. Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 1 μM mianserin. Each point represents the mean ± SEM of four separate experiments as presented in Table 11. Control (●), DOI (▲).

TABLE 12

Effect of acute treatment with DOI (10 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomes of rat brain determined 24 hours later. Values are mean ± SEM obtained by Scatchard analysis of saturation binding data in brains from four animals in each group. The binding assays were performed in duplicate.

TREATMENT	Kd (nM)	Bmax (fmol/mg protein)
Control (saline)	0.465 ± 0.05	157 ± 12.0 (100)
DOI	0.410 ± 0.02	79 ± 4.0 * (50)

* Significantly different from control (Student's t-test, $p < 0.01$). Percentage values are given in parentheses.

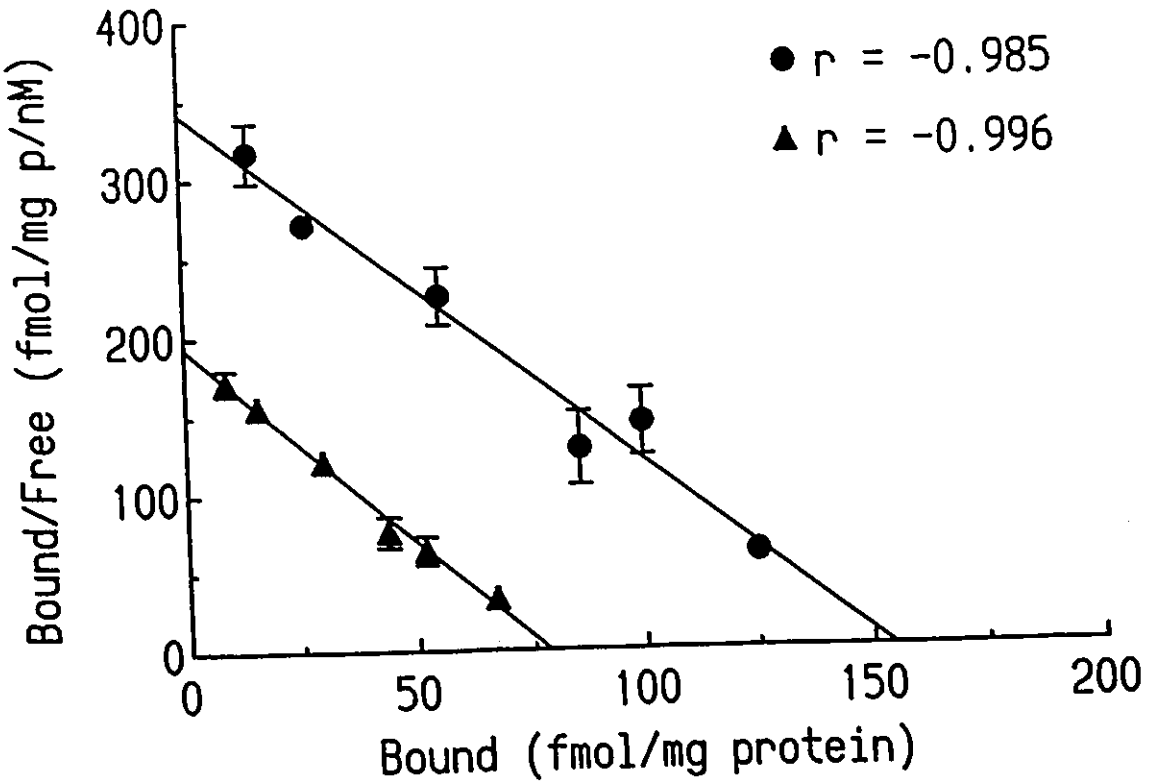

Fig. 17. Scatchard plot of [^{3}H]ketanserin binding in cortical synaptosomes of rat treated with a single i.p. injection of DOI (10 mg/kg) 24 hours before death. Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 1 μM mianserin. Each point represents the mean ± SEM of four separate experiments as presented in Table 12. Control (●), DOI (▲).

A single injection of DOI also markedly reduced ($p < 0.01$) the density of 5-HT_{2A} receptor binding sites in cortical synaptosomes of rat brain. The specific binding amounted to 79 ± 4 fmol/mg protein compared to 157 ± 12 fmol/mg protein in controls. There were no significant changes in affinity constant (Kd) of [^{3}H]ketanserin for its binding sites, compared to corresponding controls in either homogenate or cortical synaptosomal fraction. The Bmax of [^{3}H]ketanserin binding in the cortical synaptosomes of rat brain was lower than that in the homogenate.

2. Dose-dependency of down-regulation of [^{3}H]ketanserin binding in cortical synaptosomes of rat by DOI treatment

To determine whether the ability of DOI to down-regulate 5-HT_{2A} receptor sites is dose-dependent, groups of four naive rats were injected intraperitonally (i.p.) with a single dose of DOI (1, 5 and 10 mg/kg) or the equivalent volume of saline as a vehicle.

The effect of acute treatment with varying doses of DOI (1 and 5 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomes of rat brain is shown in Tables 13 and 14 and Figs 18 and 19. A single low dose of DOI (1 mg/kg; i.p.) did not markedly down-regulate [^{3}H]ketanserin labelled 5-HT_{2A} sites compared to controls, but the 5 mg/kg dose of DOI significantly reduced ($p <$

TABLE 13

Effect of acute treatment with DOI (1 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomal fraction of rat brain determined 24 hours later. Values are means ± SEM obtained by Scatchard analysis of saturation binding data in brains from four animals in each group. The binding assays were performed in duplicate. Percentage values are given in parentheses.

TREATMENT	Kd (nM)	Bmax (fmol/mg protein)
Control (saline)	0.384 ± 0.05	122 ± 2.0 (100)
DOI	0.349 ± 0.02	99 ± 11.9 (82)

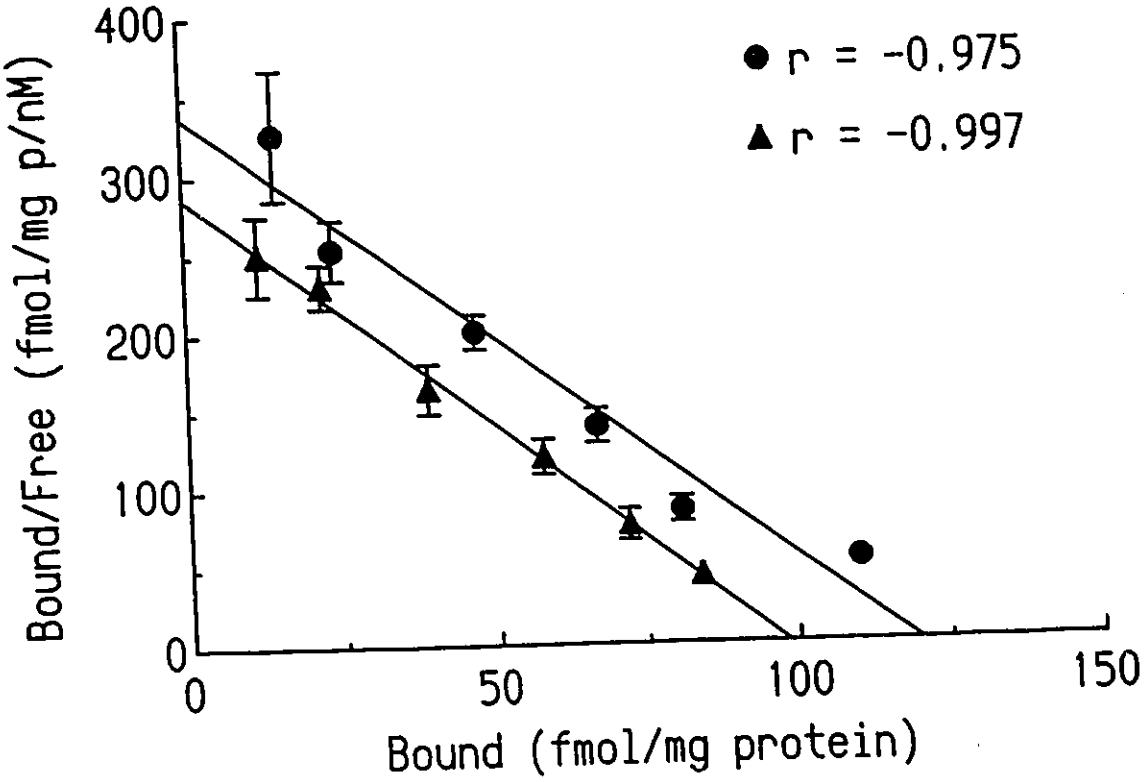

Fig. 18. Scatchard plot of [^{3}H]ketanserin binding in cortical synaptosomal fraction of rat treated with a single i.p. injection of DOI (1 mg/kg) 24 hours before death. Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence(non-specific) of 1 μM mianserin. Each point represents the mean ± SEM of four separate experiments as presented in Table 13. Control (●), DOI (▲).

TABLE 14

Effect of acute treatment with DOI (5 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomal fraction of rat brain determined 24 hours later. Values are means ± SEM obtained by Scatchard analysis of saturation binding data from four animals in each group. The binding assays were performed in duplicate.

TREATMENT	Kd (nM)	Bmax (fmol/mg protein)
Control (saline)	0.418 ± 0.02	104 ± 4.6 (100)
DOI	0.414 ± 0.02	80 ± 4.3 * (77)

* Significantly different from control (Student's t-test, $p < 0.01$). Percentage values are given in parentheses.

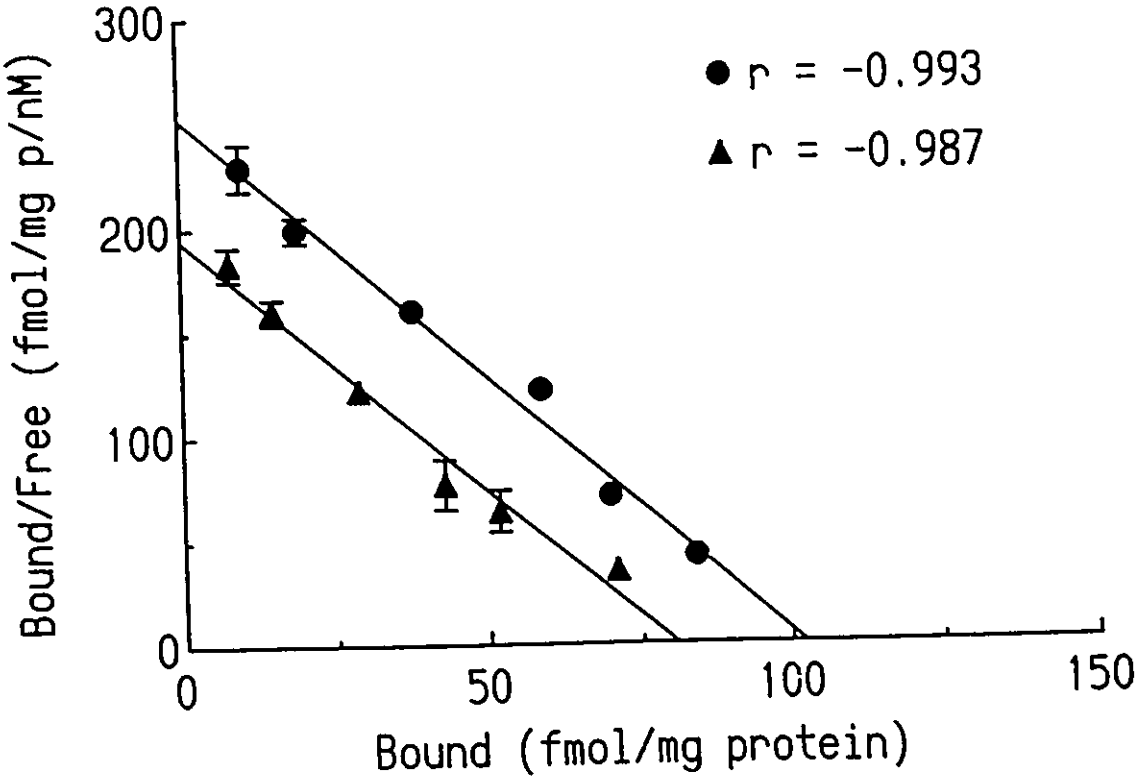

Fig. 19. Scatchard plot of [^{3}H]ketanserin binding in cortical synaptosomal fraction of rat treated with a single i.p. injection of DOI (5 mg/kg) 24 hours before death. Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding was defined as the difference between in the absence (total) or presence (non-specific) of 1 μM mianserin. Each point represents the mean ± SEM of four separate experiments as presented in Table 14. Control (●), DOI (▲).

0.01) the density of 5-HT_{2A} receptors in cortical synaptosomes of rat brain, without changing in the binding affinity (Kd). The present data show that the ability of DOI in down-regulation of 5-HT_{2A} receptors is a dose-dependent phenomenon (Fig 20).

3. Effect of repeated doses of DOI on the [^{3}H]ketanserin binding in cortical synaptosomes of rat

We have also examined the effect of repeated doses of DOI on the [^{3}H]ketanserin binding in cortical synaptosomes of rat brain to establish whether repeated injections of DOI would alter the extent of down-regulation of 5-HT_{2A} receptor sites seen with a single injection of the drug. In this study, groups of three naive rats were injected i.p. with 10 mg/kg of DOI for three consecutive days and killed 24 hours after the last injection. Table 15 and Fig 21 demonstrate the effect of three consecutive daily injections of DOI (10 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomes of rat brain. Three consecutive daily injections of DOI further down-regulated (by 65%) 5-HT_{2A} receptor sites in cortical synaptosomes as compared to control ($p < 0.01$). Specific [^{3}H]ketanserin binding amounted to 35 $\pm$ 2.1 (fmol/mg protein) compared to 101 $\pm$ 3.8 (fmol/mg protein) in controls. Affinity constant of [^{3}H]ketanserin for its binding sites increased (by %23), but this difference compared to corresponding controls was not significant.

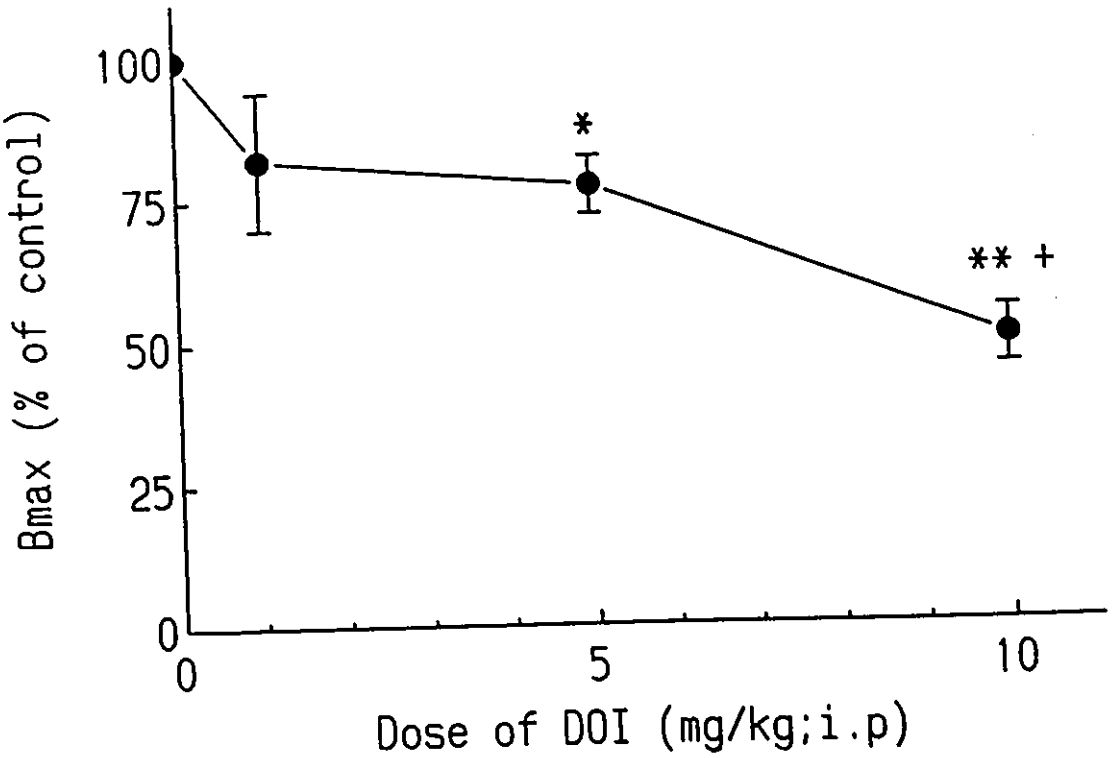

Fig. 20. Dose-dependency of acute DOI (1, 5, 10 mg/kg; i.p.) effect on the density of 5-HT_{2A} receptors in cortical synaptosomal fraction of rat brain. The results are expressed as percentage of 5-HT_{2A} receptor density (Bmax) with value in control animals taken as 100%. Each point represents the mean ± SEM of four separate experiments.
ANOVA revealed significant differences between the means ($f = 10.54$; $p < 0.01$).
** Significantly different from controls and the group treated with 5 mg DOI ($p < 0.01$, Newman-Keuls test). $^{+}$ Significantly different from the group treated with 1 mg/kg DOI ($p < 0.05$, Newman-Keuls test).
* Significantly different from control (Student's t-test, $p < 0.01$).

TABLE 15

Effect of three consecutive daily injections of DOI (10 mg/kg; i.p., the last injection 24 hours before death) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomal fraction of rat brain. Values are means ± SEM obtained by Scatchard analysis of saturation binding data from three animals in each group. The binding assays were performed in duplicate.

TREATMENT	Kd (nM)	Bmax (fmol/mg protein)
Control (saline)	0.388 ± 0.005	100 ± 3.8 (100)
DOI	0.506 ± 0.04	35 ± 2.1 * (35)

* Significantly different from control (Student's t-test, $p < 0.01$). Percentage values are given in parentheses.

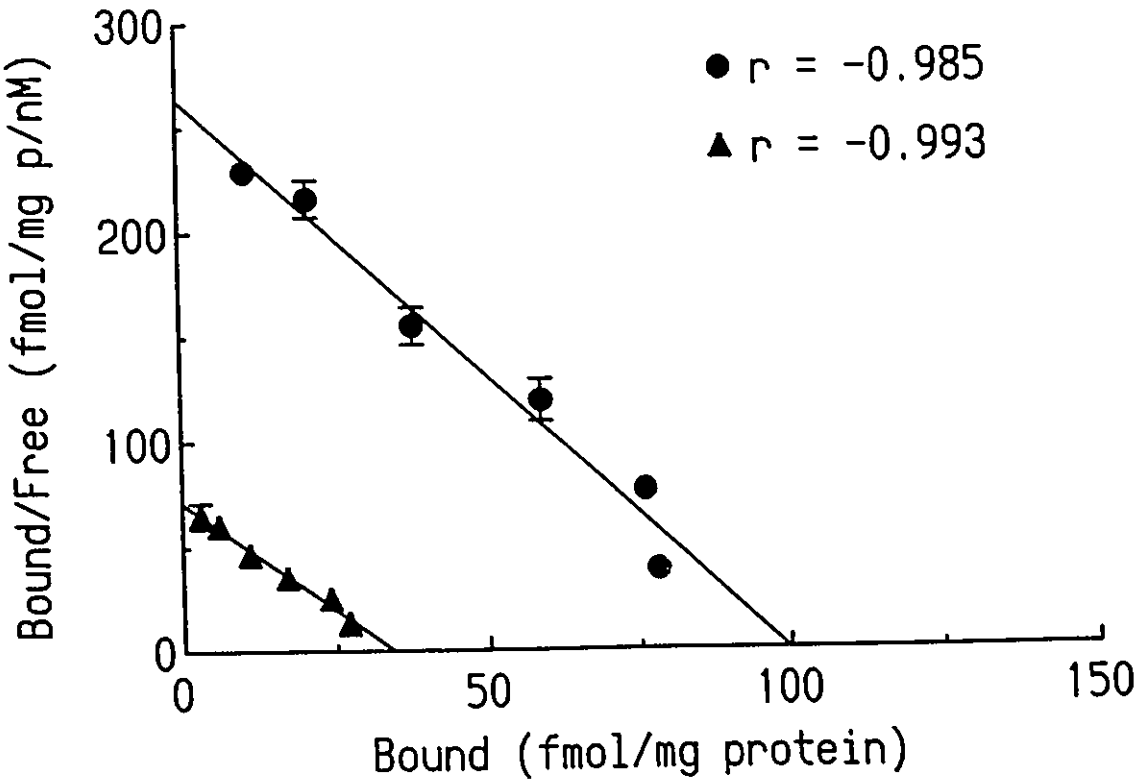

Fig. 21. Scatchard plot of [^{3}H]ketanserin binding in cortical synaptosomal fraction of rat treated with three consecutive daily injections of DOI (10 mg/kg; i.p., the last injection 24 hours before death). Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 1 μM mianserin. Each point represents the mean ± SEM of three separate experiments as presented in Table 15. Control (●), DOI (▲).

4. [^{3}H]ketanserin binding and response to DOI treatment in PCA-lesioned rats

PCA is a useful pharmacologic tool for selectively lesioning serotonergic nerve terminals in neocortex and other forebrain structures (Ricaurte et al, 1985). DOI acts *in vivo* as an agonist not only at 5-HT$_{2A}$ but also at the 5-HT$_{2C}$ receptors that may be located both pre- and postsynaptically (Hoffman and Mezey, 1989). Therefore, a lesion to 5-HT terminals by PCA could help to identify the site of DOI action. In experiments to assess the effect of lesion to serotonergic terminals on DOI effect, rats were injected i.p. with 10 mg/kg of PCA for three consecutive days and killed seven days after the last injection.

There was no significant change in Bmax of [^{3}H]ketanserin binding sites in cortical synaptosomes from PCA-lesioned rats in comparison to control animals (153 ± 10 vs 154 ± 9 fmol/mg protein). Acute treatment of rats with DOI (10 mg/kg; i.p. 24 hours before death) after lesion of serotonergic nerve terminals by PCA produced a significant ($p < 0.01$) decrease (by 47%) in the density of 5-HT$_{2A}$ receptor sites labelled by [^{3}H]ketanserin in cortical synaptosomes of rat brain (Table 16 and Fig 22). Binding amounted to 81 ± 15 fmol/mg protein compared to 153 ± 10 fmol/mg protein in controls. There was no significant change in affinity constant in DOI-treated animals compared to corresponding controls. These data provide evidence for the notion that the site of DOI action in down-regulating 5-HT$_{2A}$ receptors is likely to be a post-synaptic one.

TABLE 16

Effect of acute treatment with DOI (10 mg/kg; i.p.) on the kinetics of [^{3}H]ketanserin binding in cortical synaptosomal fraction of PCA-lesioned rats determined 24 hours later. Values are means ± SEM obtained by Scatchard analysis of saturation binding data from four animals in each group. The binding assays performed in duplicate.

TREATMENT	Kd (nM)	Bmax (fmol/mg protein)
PCA + saline	0.530 ± 0.06	153 ± 10 (100)
PCA + DOI	0.526 ± 0.09	81 ± 15 * (53)

* Significantly different from PCA-lesioned rats treated with saline (Student's t-test, $p < 0.01$).
Percentage values are given in parentheses.

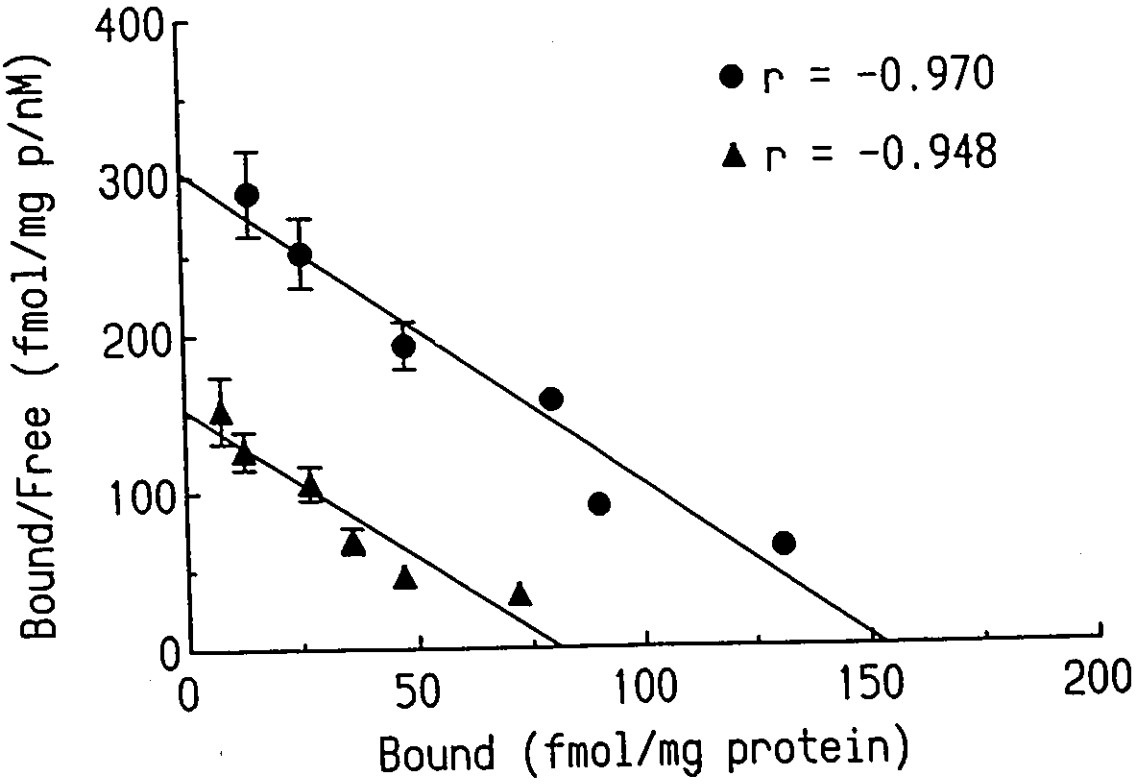

Fig. 22. Scatchard plot of [^{3}H]ketanserin binding in cortical synaptosomal fraction of PCA-lesioned rats treated with a single i.p. injection of DOI (10 mg/kg) 24 hours before death. Binding was determined over a concentration range of [^{3}H]ketanserin of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 1 μM mianserin. Each point represents the mean ± SEM of four separate experiments as presented in Table 16. Control (●), DOI (▲).

5. Effect of DOI treatment on PKC activity in subcellular fractions of rat brain

We have examined the effect of DOI treatment on the PKC activity in subcellular fraction of rat brain to explore whether changes in PKC signalling system induced by 5-$HT_{2A/2C}$ receptor agonist treatment play a role in the down-regulation of 5-HT_{2A} receptors. As shown in Table 17 and Fig 23, treatment of rats with a single dose of DOI (10 mg/kg; i.p. 24 hours before death) resulted in a significant increase (by 38%) in PKC activity in particulate fraction of the cortical synaptosomes (from 1.46 ± 0.11 to 2.37 ± 0.11 pmol/min/μg protein; $p < 0.01$). However, treatment with the 5-$HT_{2A/2C}$ receptor agonist produced no significant change in PKC activity in soluble fractions when compared to control animals.

5.1. PKC activity in PCA-lesioned rats and response to DOI treatment

In experiments to assess the effect of a lesion to serotonergic terminals on PKC activity, rats were injected i.p. with 10 mg/kg of PCA for three consecutive days and killed seven days after the last injection.

PKC activity in both soluble and particulate fractions of cortical synaptosomes from PCA-lesioned rats was significantly higher than that seen in control rats. Table 18 and Fig 24 demonstrate the effect of a single dose of DOI (10 mg/kg; i.p. 24 hours before death) on PKC activity in PCA lesioned-rats. DOI treatment resulted in a significant increase of PKC activity in the particulate fraction

TABLE 17

Effect of acute treatment with DOI (10 mg/kg; i.p.) on PKC activity in soluble and particulate fractions of cortical synaptosomes from brains of naive rats. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of sample buffer alone. Value are mean ± SEM from four animals. The PKC assay was performed in duplicate.

TREATMENT	SOLUBLE FRACTION (pmol/min/μg p)	PARTICULATE FRACTION (pmol/min/μg p)
Control	7.63 ± 0.32 (100)	1.46 ± 0.11 (100)
DOI	7.68 ± 0.28 (100)	2.37 ± 0.11 * (138)

* Significantly different from control rats ($p < 0.01$).
Percentage changes are given in parentheses taking values in control animals as 100%.

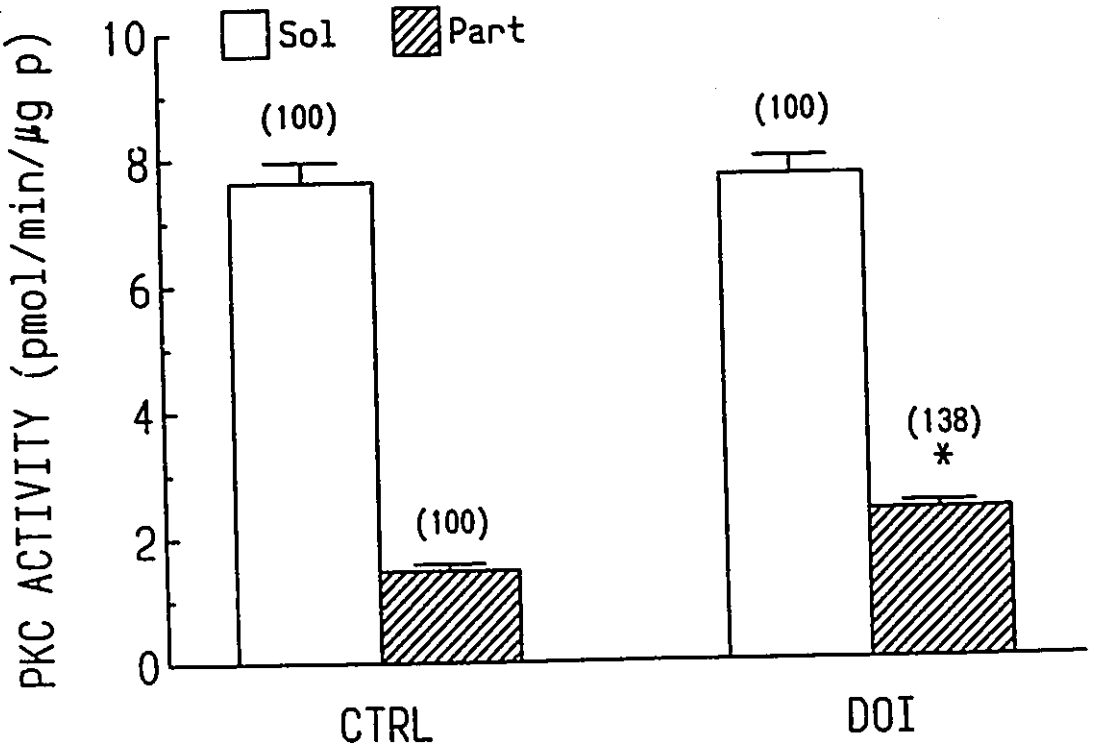

Fig. 23. Effect of acute treatment with DOI (10 mg/kg; i.p.) on PKC activity in soluble and particulate fractions of cortical synaptosomes from brains of rats. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of sample buffer alone. Each bar represents the mean ± SEM of values from four animals.

* Significantly different from control rats (Student's t-test, $p < 0.01$).

TABLE 18

Effect of acute treatment with DOI (10 mg/kg; i.p.) on PKC activity in soluble and particulate fractions of cortical synaptosomes from brains of PCA-lesioned rats. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of sample buffer alone. Value are mean ± SEM from four animals. The PKC assay was performed in duplicate.

TREATMENT	SOLUBLE FRACTION (pmol/min/μg p)	PARTICULATE FRACTION (pmol/min/μg p)
PCA + Saline	9.79 ± 0.38 (100)	1.96 ± 0.08 (100)
PCA + DOI	8.50 ± 0.59 (87)	2.36 ± 0.03 * (117)

* Significantly different from PCA-lesioned rats ($p < 0.01$) receiving saline. Percentage changes are given in parentheses taking values in PCA + Saline treated animals as 100%.

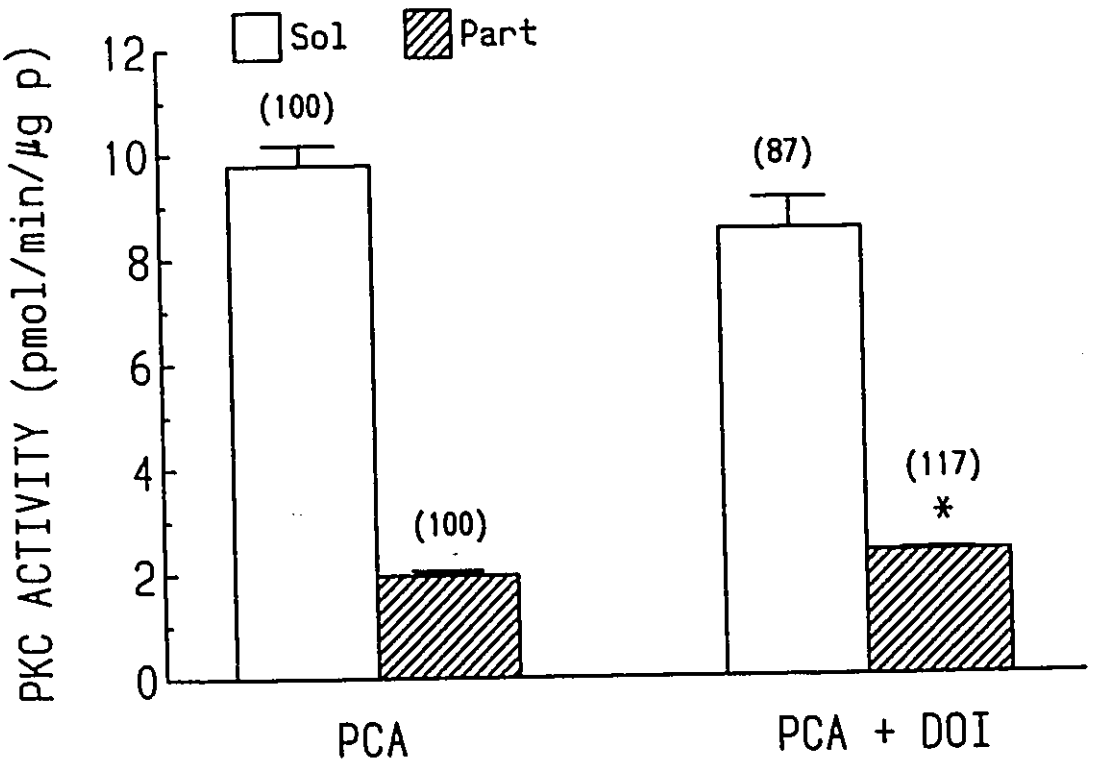

Fig. 24. Effect of acute treatment with DOI (10 mg/kg; i.p.) on PKC activity in soluble and particulate fractions of cortical synaptosomes from brains of PCA-lesioned rats. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of sample buffer alone. Each bar represents the mean ± SEM of values from four animals.

* Significantly different from PCA-lesioned rats (Student's t-test, $p < 0.01$) receiving saline.

(from 1.96 ± 0.08 to 2.36 ± 0.03 pmol/min/μg protein ; $p < 0.01$) but only a modest non-significant decrease in the cytosolic fraction in comparison with PCA-lesioned rats receiving only saline.

We have also examined the effect of three consecutive daily injections of DOI (10 mg/kg; i.p.) on PKC activity in subcellular fractions from brains of PCA-lesioned rats. The result are shown in Table 19 and Fig 25. Under these experimental condition, the PKC activity in the soluble fraction from DOI-treated animals decreased significantly from 10.09 ± 0.33 to 8.14 ± 0.20 pmol/rnin/μg protein ($p < 0.01$). In contrast, the PKC activity in the particulate fraction increased from 2.04 ± 0.22 to 2.70 ± 0.26 pmol/min/μg protein, although the difference was not statistically significant. The relative decrease of PKC activity in the soluble fraction was 19% and the relative increase in particulate fraction amounted to 24%. Treatment with the 5-$HT_{2A/2C}$ receptor agonist, DOI, thus produced in PCA-lesioned rats a translocation of PKC activity from cytosolic to membrane fraction similar to that seen with DOI in naive control animals.

TABLE 19

Effect of three consecutive daily injections of DOI (10 mg/kg; i.p.) on PKC activity in soluble and particulate fractions of cortical synaptosomes from brains of PCA-lesioned rats. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of sample buffer alone. Values are mean ± SEM from four animals. The PKC assay was performed in duplicate.

TREATMENT	SOLUBLE FRACTION (pmol/min/μg p)	PARTICULATE FRACTION (pmol/min/μg p)
PCA + Saline	10.09 ± 0.33 (100)	2.04 ± 0.22 (100)
PCA + DOI	8.14 ± 0.20 * (81)	2.70 ± 0.26 (124)

* Significantly different from PCA-lesioned rats (Student's t-test, $p < 0.01$) receiving saline.
Percentage changes are given in parentheses taking values in PCA + Saline treated animals as 100%.

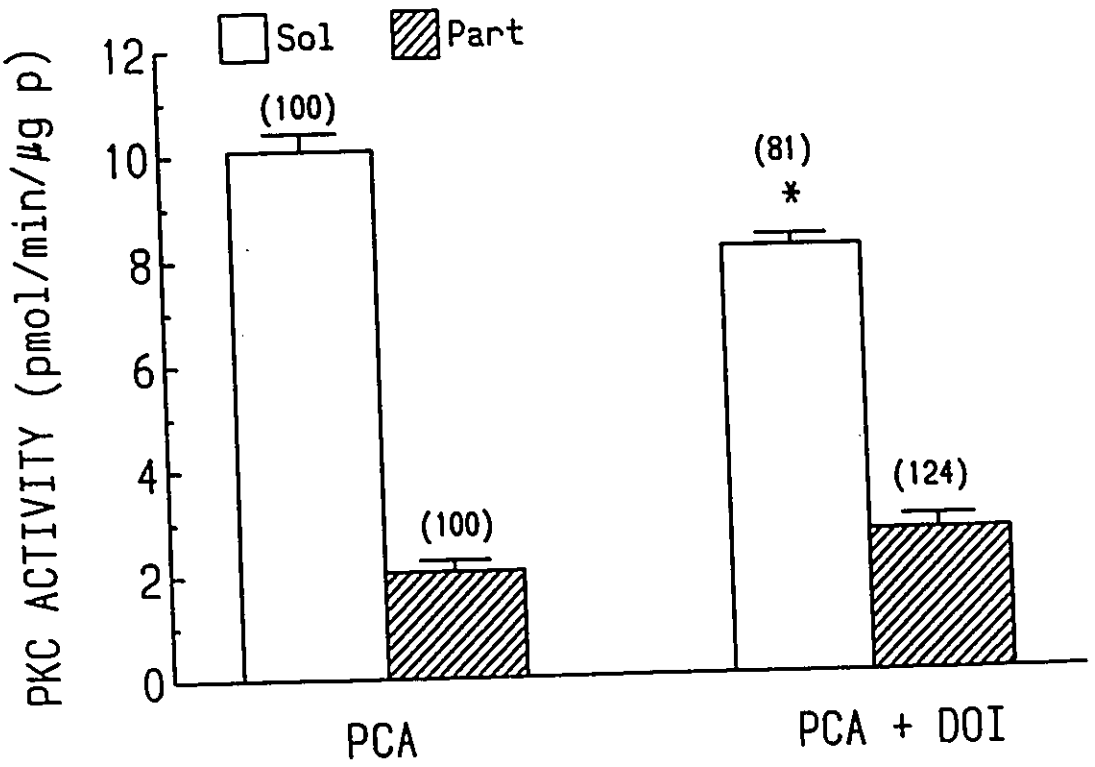

Fig. 25. Effect of three consecutive daily injections of DOI (10 mg/kg; i.p.) on PKC activity in soluble and particulate fractions of cortical synaptosomes from brains of PCA-lesioned rats. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of sample buffer alone. Each bar represents the mean ± SEM of values from four animals.

* Significantly different from PCA-lesioned rats (Student's t-test, $p < 0.01$) receiving saline.

6. Conclusion

We have found that acute treatment with a 5-$HT_{2A/2C}$ receptor agonist (DOI) increases significantly (by 38%) the PKC activity in the particulate fraction of cortical synaptosomes of rat brain. Treatment of PCA-lesioned rats with a single dose of DOI resulted in significant increase of PKC activity in particulate fraction compared to PCA-lesioned rats receiving vehicle only. These data suggest that treatment with the 5-$HT_{2A/2C}$ agonist (DOI) in PCA-lesioned rats produces a translocation of PKC activity from cytosol to membrane fraction similar to that seen in naive control animals.

The present data thus suggest that the down-regulation of 5-HT_{2A} receptor in cerebral cortex by DOI treatment is accompanied by translocation of PKC activity from cytosolic to membrane fraction.

IV. DISCUSSION

A. POSSIBLE ROLE OF PKC IN REGULATION OF NEURONAL SEROTONIN UPTAKE

The high-affinity neuronal uptake of 5-hydroxytryptamine (5-HT) is an important carrier-mediated mechanism for the removal of this amine from the synaptic cleft. It is an active process that is temperature dependent and has an absolute requirement for external Na^+ and Cl^-. The energy for this process is produced by Na^+-K^+ ATPase pump. Many drugs used in treatment of depression, including the tricyclic antidepressants and particularly the selective serotonin reuptake inhibitors (SSRIs), are known to inhibit the neuronal uptake of 5-HT, thus increasing the amount of transmitter in the synapse and hence its action on both pre- and postsynaptic receptors. Specific recognition sites for the tricyclic antidepressant drugs such as [^{3}H]imipramine have been demonstrated in brain (Raisman et al, 1979) and platelets (Briley et al, 1979) of various species, including humans (Langer et al, 1981). [^{3}H]Imipramine sites in both the brain and platelets have been shown to be associated with the 5-HT uptake mechanism (Paul et al, 1981). Because [^{3}H]imipramine binding was found to be associated with 5-HT uptake sites on serotonergic neurons (Raisman et al, 1979), It was thought that 5-HT uptake was regulated by an allosteric site to which [^{3}H]imipramine binds or an endogenous compound. However, it has been demonstrated that [^{3}H]imipramine binds in brain tissue to two classes of sites: high- and low affinity (Hrdina, 1984), only the high-affinity sites were found to be functionally related to the 5-HT uptake

process (Hrdina, 1987, 1988), and the role of low-affinity [^{3}H]imipramine sites is uncertain and the endogenous molecules that may regulate the transporter function have not been definitely demonstrated.

There are other possibilities for regulation of neuronal 5-HT transporter including presynaptic receptor activation as shown for glucose (Hoffman et al, 1991), norepinephrine (Boyd et al, 1985), amino acid (Hansson and Ronnback, 1989), and ion transport (Brown et al, 1988). However, it has not yet been shown that activation (or other alteration) of presynaptic 5-HT receptors could alter 5-HT transporter function. Intracellular mechanisms (e.g. protein kinases) could also regulate 5-HT transporter protein by phosphorylation (dephosphorylation) similar to regulation (desensitization) of some receptors (e.g. ß-adrenergic receptors by ßARK). Indeed, it has recently been shown that PKC and PKA are involved in intracellular regulation of serotonin uptake in some non-neuronal cells including platelets. Myers et al (1989) have demonstrated that inhibition of 5-HT uptake in cultured endothelial cells was associated with the phorbol ester-induced translocation of PKC from cytosol to membrane. Furthermore, Hoffman (1991) studied the involvement of second messenger systems in the regulation of 5-HT uptake in rat basophilic cells and reported that endogenous cAMP analogues substantially increased the 5-HT uptake rate, whereas phorbol esters showed a pronounced inhibitory effect. These data suggested that 5-HT uptake may be under dual control of the two main intracellular regulatory systems involving PKC and protein kinase A (PKA). Cool et al (1991) have reported on the cAMP-

dependent regulation of the high affinity serotonin transport in human placental choriocarcinoma cells and their findings have been recently confirmed by King et al (1992). Recently, Anderson and Horn (1992) have demonstrated that activation of PKC by phorbol esters inhibited serotonin transport in human platelets. Chudzik et al (1994) have also shown that exposure of human platelets to PKC activators such as phorbol esters and diacylglycerol significantly inhibits the rate of serotonin uptake but does not alter the binding of [^{3}H]paroxetine to the 5-HT recognition site on the transporter. Very little is known, however, about the mechanism of serotonin uptake regulation in the central nervous system. Cloning of the gene for serotonin transporter and subsequently derived amino acid sequence predict the presence of one consensus site for the cAMP-dependent PKA and two potential PKC phosphorylation sites, which have been identified near the end of the cytoplasmic N-terminus (Hoffman et al, 1991; Lesch et al, 1993a, 1993b). It has been documented that platelet 5-HT uptake characteristics are similar, if not identical to the neuronal one (Da Prada et al, 1988). Only recently the gene encoding the platelet 5-HT transporter has been cloned (Lesch et al, 1993b). The analysis of its structure confirms that the human brain and platelet 5-HT transporter are identical in terms of their amino acid composition (Lesch et al, 1993a, 1993b) and structure. Thus, it is reasonable to assume that similar intracellular mechanisms involving PKC and PKA may be involved in regulation of neuronal serotonin uptake.

In the present study, activation of PKC by a phorbol ester, PMA failed to produce unequivocal changes in the rate of 5-HT uptake by cortical synaptosomes of rat brain. Under certain experimental conditions, PMA (0.1 μM and 1 μM) produced a moderate inhibition of 5-HT uptake measured at a single concentration of 5-HT. Low concentration (0.1 μM) of PMA did not significantly alter the kinetics parameters (Vmax, Km) of 5-HT uptake in synaptosomes. However, higher PMA concentrations (1 and 10 μM) have produced a significant effect on 5-HT Km (decreased the affinity of the transporter to 5-HT). This is a new finding and may represent an important aspect of regulation of transporter protein by PKC. The mechanism of this effect is not clear but could involve allosteried changes in transporter configuration that would result in altered affinity.

There are several possible reasons that could explain the different results obtained with PMA in platelets and neuronal tissue. Although there are many similarities in 5-HT uptake by neurons and platelets, there are also some important differences between them. Firstly, there is no 5-HT synthesis in platelets, but there is in neurons. Secondly, there is no synapse in platelets but serotonergic neurones receive rich synaptic input. Thus, regulation of transporter in neurons is likely to be much more complex. Thirdly, PKC subtypes in platelets and neuronal tissue and their distribution may be different.

On the other hand, the critical enzyme involved in regulation of neuronal 5-HT transporter may be PKA. Although Chudzik et al (1994) found that cAMP-dependent second messenger system was probably not involved in the regulation

of 5-HT transporter in platelets, it was shown to have a role in this respect in some other non neuronal cells (Myers et al, 1989; Hoffman, 1991; Cool et al, 1991; King et al, 1992). Other transporters have been shown to be regulated by presynaptic receptor activation (e.g. glucose). It appears from recent evidence that the activity of the presynaptic 5-HT transporter is regulated by at least one presynaptic receptor (Cool et al, 1991). Since most presynaptic 5-HT receptors are of 5-HT_{1A} type linked to AC-PKA signalling system, it may be important in future experiments to examine the effect of PKA stimulation on the activity of the serotonin transporter. Another possibility to consider is that brain synaptosomal preparation may be still too complex for dissecting the effect of PKC stimulation on a single process such as 5-HT uptake. Future experiments should examine the role of PKC and PKA on transporter function in less complex preparation (e.g. cultured neuronal cells).

Clearly, more work is needed to investigate the possible role of PKC in the regulation of neuronal serotonin transporter and to define the mechanism of phorbol ester-induced inhibition of the 5-HT transporter in platelets. It is not yet known whether the transporter molecule is a target protein for phosphorylation by PKC. The results from human platelet study do not warrant any speculation on the involvement of signal transduction pathways which may be linked to the inhibition of phorbol esters, induced inhibition of 5-HT uptake. These may include phosphatidylinositol hydrolysis, elevation of intracellular Ca^{++}, arachidonate cascade and/or G protein activation. Future experiments using neuronal tissue,

platelets or transfected cell lines may provide elucidation of above mechanisms. Establishing the mechanisms by which platelet and neuronal serotonin transporter are regulated may provide a new insight into the mechanisms of antidepressants action. It is known, for example that many drugs including antidepressant imipramine which is also a 5-HT uptake inhibitor have been found to inhibit PKC by interacting with the phospholipid binding domain of this enzyme (Mori et al, 1980).

B. POSSIBLE INVOLVEMENT OF PKC IN DOWN-REGULATION OF 5-HT_{2A} RECEPTOR BY AGONIST TREATMENT

1. Effects of acute DOI treatment on [^{3}H]ketanserin binding in rat cerebral cortex

Experimental studies have documented changes in drug responsiveness caused by increase or decrease in the number of receptor sites or by alterations in the efficiency of coupling of receptors to distal effector mechanisms. It is likely that these changes account for much of the individual variability in response to some drugs, particularly those that act at receptors for hormones, biogenic amines and neurotransmitters. In some cases, the change in receptor number is caused by other hormones (e.g., thyroid hormones). In other cases, the agonist ligand itself induces a decrease in the number ("down-regulation") or coupling

efficiency of its receptors (e.g. β_2 adrenergic agonist; Clark, 1986). Receptor-specific desensitization mechanisms presumably act physiologically to allow cells to adapt to changes in rates of stimulation by hormones and neurotransmitters in their environment. An antagonist may increase the number of receptors in a critical cell or tissue by preventing down-regulation caused by an endogenous agonist. Various therapeutics strategies can be used to deal with receptor-specific changes in drug responsiveness, depending on the clinical situation.

It has been reported that chronic treatment with 5-HT_{2A} receptor antagonists (e.g. ketanserin) down-regulates antagonists-labelled 5-HT_{2A} receptors (Gandolfi et al, 1985). This is in contrast to the situation with other receptors, including 5-HT_1 subtypes, where treatment with antagonists results in up-regulation of the receptors (Conn and Sanders-Bush, 1987). The reason for this phenomenon remains unexplained. The effect of agonist treatment has been less investigated because of their lack of selectivity for the 5-HT_{2A} receptor, low affinity and mixed agonist-antagonist properties of the drugs used. Recently, Glennon and co workers (1986) identified several phenylisopropylamine derivatives as selective 5-$HT_{2A/2C}$ agonists. These include 1-(2,5-dimethoxy-4-substituted-phenyl) 2-amino-propanes, such as DOM (methyl substitution), DOB (bromo-substitution) and DOI (iodo-substitution). [^{3}H]DOB has been used to label a binding site on the 5-HT_{2A} receptor for which 5-HT agonists show greater affinity than antagonists (Titeler et al, 1985), in contrast to the 5-HT_{2A} site labelled with [^{3}H]ketanserin (Leysen et al, 1984). It is currently unclear whether the agonist- and antagonist-labelled 5-HT_{2A}

receptors represent a high (5-HT_{2H}) and low (5-HT_{2L}) affinity state of the same site (Glennon et al, 1986) or two separate receptors (Pierce and Peroutka, 1989). Under these circumstances, studies of 5-HT_{2A} receptor regulation are complex. The new 5-$HT_{2A/2C}$ ligands may be a valuable tool in studying the regulation of 5-$HT_{2A/2C}$ receptors.

Recently, Pranzatelli (1991) has shown that DOI down-regulates the density of 5-HT_{2A} receptors in rat cerebral cortex not only after chronic treatment but also after a single exposure. The mechanism of down-regulation of 5-HT_{2A} receptors by agonist treatment is not clear, but since DOI activates PI signalling system and causes translocation of PKC activity, it can be assumed that activation of PKC by receptor agonist, DOI may contribute (via phosphorylation ?) in the down-regulation of 5-HT_{2A} receptors.

In the present study, we have confirmed that acute treatment with 5-$HT_{2A/2C}$ agonist, DOI down-regulated (by 50%) of the density of 5-HT_{2A} receptors in cortical synaptosomal preparation (P_2 fraction) as well as in homogenate of rat cerebral cortex assayed 24 hours later by [^{3}H]ketanserin binding . Although, the Bmax in the P_2 fraction of rat brain was lower than that in the tissue homogenate, the extent of down-regulation was the same in both preparations. In agreement with Buckholtz's study (1988), we have demonstrated that effect of DOI on down-regulation of 5-HT_{2A} is dose-dependent; single high doses (5 and 10 mg/kg; i.p) but not a low dose (1 mg/kg) of DOI markedly reduced the density of 5-HT_{2A}

receptors in P_2 fraction of rat brain, (by 23% and 50%, respectively). Our data also showed that repeated administration of DOI further down-regulated (by 65%) 5-HT_{2A} sites in cortical synaptosomes labelled 24 hours later by [^{3}H]ketanserin, without a significant change in the affinity constant.

DOI acts *in vivo* as an agonist not only at 5-HT_{2A} but also at the 5-HT_{2C} receptors that may be located both pre- and postsynaptically (Hoffman and Mezey, 1989). 5-HT_{2C} receptors are most abundant in the choroid plexus but present also in cerebral cortex and hippocampus. However, the density of receptors in these area is far less than that in choroid plexus (Hoffman and Mezey, 1989). In brain membrane preparations, it is difficult to differentiate between presynaptic and postsynaptic receptors as these two areas of the neuron are not easily separated. Electrophysiological studies suggest that 5-HT_{2A} receptors are postsynaptic, but the possibility that 5-HT_{2C} receptor also stimulated by DOI may be situated presynaptically as well can not be ruled out (Hoffman and Mezey, 1989). Although [^{3}H]ketanserin is a selective ligand of choice for 5-HT_{2A} receptors, it has some, although much lower (pA_2 = 6.5), affinity for 5-HT_{2C} sites as well. We have thus asked the question of whether the site of DOI action in down-regulation of 5-$HT_{2A/2C}$ receptors labelled with [^{3}H]ketanserin is predominantly postsynaptic. PCA is a useful pharmacologic tool for selectively lesioning serotonergic nerve terminals in neocortex and other forebrain structures (Ricaurte et al, 1985). It interacts with serotonin transporter by competing with serotonin for transport and stimulating the

efflux of serotonin. In addition to releasing serotonin, PCA also leads to a long-term depletion of serotonin (Ricaurte et al, 1985; Hrdina et al, 1990) which correlates with morphological damage to serotonergic nerve endings (Ricaurte et al, 1985; Molliver, 1990). Hrdina et al (1990) reported that treatment with PCA dramatically decreased 5-HT uptake (by 80%), as well as levels of 5-HT (by 76%) and 5-hydroxyindolacetic acid (5-HIAA; by 55%). Therefore, a lesion to 5-HT terminals by PCA could help to identify the site of DOI action. In agreement with previous findings (Stockmeier and Kellar, 1986) density of [^{3}H]ketanserin labelled sites was not altered in PCA-lesioned rats. DOI effect on 5-HT_{2A} receptors in PCA-lesioned rats has not yet been examined. Our finding that DOI produces similar (about 50%) down-regulation of 5-HT_{2A} receptors in PCA-lesioned rats as in control rats, suggests that the site of DOI action in down-regulation of 5-HT_{2A} receptor in rat brain is post-synaptic.

2. Effect of DOI treatment on PKC activity in subcellular fractions of rat brain

A large body of evidence indicates that PKC is present in high concentrations in brain (Nishizuka, 1988), where it plays a pivotal role in neurotransmitter- or hormone-mediated signal transduction. PKC-mediated neuronal protein phosphorylation mediates a wide variety of neurophysiological events such as membrane conductance, ion channel conductivity, and axonal transport (Nishizuka, 1988). Serotonergic neurons through activation of 5-HT_{2A}

receptors may regulate their specific target cells by receptor mediated PI hydrolysis and/or PKC activation. Our study as well as previous studies by other investigators (Buckholtz et al, 1988; McKenna et al, 1989; Pranzatelli, 1991) have demonstrated a 5-HT_{2A} receptor down-regulation by acute and chronic DOI treatment. The focus of the present study was to examine the influence of DOI treatment on the PKC activity in the signal transduction pathway. In previous studies, DOI has been used as a 5-$HT_{2A/2C}$ agonist *in vitro* (cortical slices) to demonstrate PKC translocation from cytosolic to the membrane following 5-HT_{2A} receptor activation (Wang and Friedman, 1990a). This effect appears to be due to a selective 5-HT_{2A} receptor stimulation since it is prevented by 5-HT_{2A} receptor antagonists. The brain 5-HT_{2A} receptor has been shown to be linked to the phosphoinositide pathway (Conn and Sanders-Bush, 1984, 1986). Clauster et al (1988) found an increase in cortical and hippocampal [^{3}H]inositol phosphate ([^{3}H]IP) formation following stimulation with 5-HT_{2A} agonists. In order to explore 5-HT_{2A} receptor regulation by agonist treatment *in vivo* and the role of PKC in this regulation, we have examined PKC activity changes in the same design used for [^{3}H]ketanserin binding to the 5-HT_{2A} receptor.

We found an increase of PKC activity in the particulate fraction of the cortical synaptosomal tissue (by 38%) following single injection of DOI that paralleled the decrease of 5-HT_{2A} receptor density labelled with [^{3}H]ketanserin. This is, to our best knowledge, the first report of PKC translocation following the *in vivo* adminstration of DOI. *In vitro*, the early peak of DAG after receptor stimulation is

transient and reverts back to basel line within seconds to minutes, temporally corresponding to the formation of IP_3 and to the increase in intracellular Ca^{2+} concentration. The decrease in cytosolic PKC could persist for a longer time period. A sustained activation of PKC is necessary for neuronal plasticity and nerve growth. It was also shown that in the later stage of cellular responses, the formation of DAG has a slow onset and is more sustained (Nishizuka, 1992). The sustained elevation of DAG, which may come from phosphatidylcholine (PC) hydrolysis, is often seen in response to various long-lasting signals that can stimulate PKC. This could expiain the sustained translocation of PKC found in the present study. Several mechanisms have been postulated for signal-induced formation of DAG from PC. PC is hydrolysed by phospholipase D (PLD) in a signal-dependent fashion to produce phosphatidic acid, which is then dephosphorylated to DAG (Exton, 1990). PLD was detected in the rat brain (Saito and Kafner, 1975), and PLD from rat synaptosomes is activated in the presence of low concentration of Ca^{2+} (Chalifa et al, 1990). Because PLD is also stimulated by phorbol ester or membrane-permeant DAG, sometimes synergistically with the Ca^{2+} ionophore, PKC, once activated initially by PI hydrolysis, may play a role in formation of DAG from PC and prolonged PKC activation. It is possible that PC is first converted to PI by exchange of the choline moiety with free inositol, and the resulting PI is subsequently hydrolysed by PLC to produce DAG and inositol phosphate (Nishizuka 1992). This apparent transphosphatydylation reaction is activated by phorbol esters.

The 5-HT_{2A} receptor might be down-regulated as the result of phosphorylation of the receptor by activation of PKC after stimulation of 5-HT_{2A} receptor by agonist treatment. This possibility is supported by observation that three consecutive daily DOI injections to rats resulted in an augmented decrease in 5-HT_{2A} receptors and a significant decrease in cytosolic PKC activity and an increase of PKC activity in particulate fraction. The relative decrease in the cytosolic fraction was 19% and the increase in membrane fraction amounted to 24%.

We found that the effect of DOI treatment on PKC activity (increase in the particulate fraction) was the same in PCA-lesioned rats and controls. Single injection of DOI induced translocation of PKC from cytosolic (-13%) to membrane fraction (+17%) in PCA-lesioned rat. These data also are in good agreement with our results from receptor binding assay (down-regulation of 5-HT_{2A} receptors seen in PCA-lesioned rats). These results support the notion that changes in PKC activity took place in postsynaptic elements. PKC activity in both soluble and particulate fraction was higher in rats with lesioned serotonergic nerve terminals compared to naive rats. The reason for this may be in the different group of rats examined for these two series experiment.

In conclusion, our results extend previous *in vitro* observations indicating that activation of 5-HT_{2A} receptor-linked phospholipase C results in PI hydrolysis and PKC activation. The present investigation has shown that down-regulation of

5-HT_{2A} receptor in rat cerebral cortex by *in vivo* DOI treatment is accompanied by translocation of PKC activity from cytosolic to membrane fraction. This may be a consequence of intracellular elevation in diacylglycerol resulting from receptor mediated PI hydrolysis. Increased phosphorylation of 5-HT_{2A} receptor may be, at least in part, responsible for the observed changes in 5-HT_{2A} density.

3. Future work

This report is the first demonstration that the 5-$HT_{2A/2C}$ receptor agonist, DOI administered *in vivo* induces activation of PKC in rat cerebral cortex and suggests that the down-regulation of 5-HT_{2A} receptors in cortical synaptosomes may occur through a mechanism involving protein kinase C. More work remains to be done to define the exact mechanism of PKC involvement in regulation of 5-HT_{2A} receptors after *in vivo* treatment with receptor agonists.

1) To provide further evidence that the observed changes in PKC activity after DOI treatment were due to 5-HT_{2A} receptor stimulation, it would be important to examine whether 5-HT_{2A} receptor antagonists could abolish or decrease DOI-induced activation of PKC.

2) Conversely, if PKC is, at least in part, responsible for down-regulation of 5-HT_{2A} receptors, it would be important to see whether inhibitors of PKC activation, which bind to either the regulatory or the catalytic domain of PKC, would alter the down-regulation of 5-HT_{2A} receptors by DOI.

3) In order to confirm that changes in PKC activity following DOI adminstration were indeed due to stimulation of 5-HT_{2A} receptor, it would be also important to assess the time course of 5-HT_{2A} receptor down-regulation and PKC activity changes.

4) In the present study, we have only measured the total PKC activity. It would be important to examine which isoform of the enzyme is involved in the effect of DOI.

5) To study further the effect of DOI on the subcellular distribution of PKC, crude homogenates of frontal cortex from control and DOI-treated rats could be sub-fractionated into crude nuclear (P_1), crude synaptosomal (P_2), crude microsomal (P_3), and cytosolic (C) fractions for analysis of enzyme activity.

6) Recent studies suggest that PKC may also be activated in cells by processes independent of translocation. Chakravarthy et al (1994) reported that the membranes of some cells (e.g. 3T3-L1, WEHI-2) maintain a pool of activable PKC that responds to lower levels of extracellular stimuli than cytosolic PKC, and that can be activated by signals which result in formation of DAG via the breakdown of phospholipids other than in PIP_2 pathway. Further studies should be done to investigate the possibility of existence of membrane PKC pools in brain and their role in regulation of receptor function.

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