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The Potentiating Effects of Neuropeptide Y On Vascular Smooth Muscle

by

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A thesis submitted in conformity with the requirements of the degree of Masters of Science in the University of Ottawa

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Abstract

Neuropeptide Y (NPY) has potent pre- and postsynaptic effects in vascular tissue. Presynaptically, it inhibits transmitter release by activating Y_2-receptors on sympathetic nerve endings (Wahlestedt et al., 1990). Postsynaptically, it has only a poor direct contractile effect on most isolated peripheral blood vessels. However, it potentiates the response to nerve stimulation and other vasopressor agents (Lundberg et al., 1989; Cheung, 1991). There are studies which suggest that voltage-sensitive calcium channels are involved in the potentiating effects of NPY (Pernow et al., 1986; Cheung, 1991; Andriantsitohaina & Stoclet, 1990). The endothelium has also been implicated by some but not others to play a significant role in NPY potentiating effects (Hieble et al., 1989; Daly & Heible., 1987; Andriantsochaina & Stoclet, 1988; Budai et al., 1989; Gustafsson & Nilsson, 1990).

In the present study, the roles of nifedipine-sensitive calcium channels and the endothelium in the potentiating effect of NPY in the rat tail artery were investigated. Contractile responses to KCl, α,ß-methylene ATP (mATP), and NA were compared. KCl- and mATP-induced vasoconstriction is closely linked to nifedipine-sensitive calcium channels while NA-induced vasoconstriction is not.

The role of the endothelium in potentiating effects of NPY

was also tested by comparing intact arterial ring segments with denuded arterial ring segments. Freshly isolated single smooth muscle cells in which the influences of the endothelium and the nerves were unequivocally eliminated were examined in order to verify the results of the ring segments. Contractile responses to KCl and mATP were potentiated by NPY (50 nM) while NA responses were not potentiated at 50 nM but were at 500 nM NPY. The potentiating effect of NPY was antagonized by nifedipine. The intact and denuded arteries responded similarly throughout the experiment. As in the ring segments, the shortening of single smooth muscle cells in response to KCl and mATP was potentiated by NPY (50 nM) while the noradrenaline response was potentiated by NPY at 500 nM but not at 50 nM.

These results suggest that the potentiating effect of NPY is more specific to contraction mediated by nifedipine-sensitive calcium channels and is not dependent on the presence of an intact endothelium.

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Introduction

Historical Background

Isolation of Neuropeptide Y

Neuropeptide Y (NPY) was first isolated from porcine brain extracts (Tatemoto, 1982; Tatemoto *et al.*, 1982). This peptide was 36 amino acids long with tyrosine on the C-terminal. As the single letter code for the amino acid tyrosine is Y, and the fact that it was isolated from neuronal tissue, this peptide was named neuropeptide Y. NPY has now been isolated from many species. The structural similarities of NPY among the various species from which NPY was isolated strongly suggests that the structure of NPY has been well conserved during evolution. Further evidence of this evolutionary conservation lies in the high degree of sequence homology among mammals, chickens, goldfish (*Carassius auratus*) and horned sharks (*Heterodontus francisci*) (Blomqvist *et al.* 1990) (Table 1).

Chemical Structure

In addition to the similarity of the chemical structure of NPY among a number of different species, NPY also shows structural similarities to peptide YY (PYY) (approximately 70

Table I Species

human		(Keast et al., 1985)
squirrel monkey (Saimiri sciureas)		(Smith et al., 1985)
baboon		(Bruun et al., 1986)
lemur (Primate lemurian)		(Bons et al., 1990)
marmoset		(Keast et al., 1985)
cow		(Fried et al., 1986)
pig		(Bruun et al., 1986)
dog	MAMMALS	(Keast et al., 1985)
cat		(Bruun et al., 1986)
pigeon		(Bruun et al., 1986)
chicken		(Bruun et al., 1986)
rat		(Gall et al., 1986)
guinea pig		(Keast et al., 1985)
toad (Bufo marinus)	AMPHIBIANS	(Morris et al., 1986)
frog		(Bruun et al., 1986)
red-earred turtle	REPTILES	(Anderson & Reiner, 1990)
anglerfish		(Noe et al., 1986)
skates (Elasmobranchii)		(Bruun et al., 1985)
lamprey		(Van Dongen et al., 1985)
goldfish	FISH	(Bruun et al., 1986)
tench (Tinca)		(Bonn, 1990)
carp		(Bruun et al., 1986)
insects	INVERTEBRATES	(Yui et al., 1985)
molluscs		(Yui et al., 1985)
monogenean gill-parasite (Diclidophora merlangi)		(Maule et al., 1990)

% homology) and pancreatic polypeptide (PP; approximately 50 % homology) (Tatemoto, 1982). These three peptides are members of the pancreatic polypeptide (PP)-fold family of peptides. This family is different from other similarly sized peptides in having a rigid conformation and a high degree of secondary structure in aqueous solution (Schwartz et al.,

1990). One member of the family, avian PP (APP), has been characterized in sufficient detail using X-ray crystallography to establish its three-dimensional structure (Glover et al., 1983). Although NPY and APP only share about 50 % homology, the residues which are common to both peptides are the C-terminal residues and the core residues which are important for the stabilization of the PP-fold (Glover et al., 1985; Schwartz et al., 1990). Based on the known three-dimensional structure of APP, assumptions can be made about the three-dimensional structure of NPY. This structure is shown in figure 2 (Schwartz et al., 1990). The residues of the N-terminus form a left-handed polyproline II-like helix which lies in close proximity of an alpha helix formed by residues 14 to 32. A beta turn is responsible for drawing together the two helical configurations. Residues 33 to 36 project from the alpha helix at a 90 ° angle.

Distribution of NPY

Central Nervous System

NPY, as its name would suggest, is found in neuronal tissue. Radioimmunoassay studies and immunocytochemical studies have demonstrated a widespread distribution throughout the brain and spinal cord. In the rhinencephalon, NPY-like immunoreactivity (NPY-ir) is found in numerous olfactory

nuclei (Bonn, 1990) and in all layers in the telencephalon of the cerebral cortex.

Especially dense NPY-ir is found in the hippocampus (Martel, et al., 1990). Many cell bodies and a few fibres containing NPY-ir are found within the caudate-putamen and the nucleus accumbens (Salin et al., 1990). The amygdala demonstrates dense NPY-ir as does the septal area, the preoptic areas, the hypothalamus and the stria terminalis which connects the amygdala to the latter three structures (Reuss et al., 1990).

Within the hypothalamus NPY-ir containing cell bodies are most dense in the arcuate nucleus (Gray & Morley, 1986; Mormede et al., 1990). Within the thalamus, NPY-ir is found in a number of discrete nuclei (Danger et al., 1990). Dense NPY-ir was found also within the locus coeruleus (Pammer et al., 1990) ventral tegmentum area, the substantia nigra, the inferior colliculus and the reticular formation (Bons et al., 1990; Martel et al.,1990). Significant amounts of NPY-ir were found in the nucleus tractus solitarius and area postrema (Martel et al., 1990) implicating NPY as an important regulator of cardiovascular parameters (Vallejo & Lightman, 1986).

In the spinal cord the highest density of NPY-ir in terminals is in superficial layers (I-II) of the dorsal horn (Gray & Morley, 1986). Cell bodies with NPY-ir have not been

observed (Gibson et al., 1984; Gray & Morley, 1986; Martel et al., 1990).

Peripheral Nervous System

Shortly after NPY was isolated from porcine brain (Tatemoto, 1982), NPY-like-immunoreactivity (ir) was localized in peripheral noradrenergic neurons (Lundberg et al., 1982) and in other tissues (Furness et al., 1984; Sundler et al., 1983; Stjernquist et al., 1983; Edvinsson et al., 1983). In December, 1983, Lundberg et al. (1983) used a highly specific radioimmunoassay for NPY and immunohistochemistry to probe the peripheral nervous system of guinea-pig, cat, pig, and man. High levels of NPY-ir were found in sympathetic ganglia, gastrointestinal, respiratory and urogenitary tracts, and in tissues which receive a dense sympathetic innervation, such as vas deferens, heart, spleen, kidney and blood vessels. NPY-like-ir was also found in the adrenal medulla. The immunohistochemical staining results demonstrating colocalization of NPY with NA in noradrenergic neurons were obtained using antibodies raised against NPY as well as an enzyme, dopamine-beta-hydroxylase, which is responsible for noradrenaline synthesis in these neurons. The evidence necessary to demonstrate that these noradrenergic neurons were in fact sympathetic neurons, came from two types of studies. The first type were those in which neurons, which stained for

DBH and NPY in a control animal, were compared to those which stained for DBH and NPY in animals in which guanethidine was used to destroy the sympathetic neurons. The second type of investigations used were those which showed differential effects of reserpine, which selectively destroys neuronal storage vesicles containing NA, and 6-hydroxydopamine (6-OHDA), which selectively destroys noradrenergic neurons. One would expect that after adding reserpine, no addition loss of NPY would occur with the addition of 6-OHDA, if, in fact, all the NPY were costored with NA in the same vesicles. The amount of NPY that is stored with NA as opposed to that which is stored by itself varies as much as 25 to 75 % from one tissue to another (Allen et al., 1986). Using the approaches discussed, a number of studies revealed NPY colocalization with noradreneric-sympathetic neurons in tissues (Table II).

Although NPY is ubiquitous throughout the sympathetic nervous system, Corr et al. (1990) found NPY-containing neurons innervating coronary arteries in rats after the rat had been sympathectomized with guanethidine. Further evidence which supports the idea of the localization of NPY in the parasympathetic nervous system come from studies by Suzuki et al. (1990). NPY was found to co-exist with vasoactive intestinal polypeptide (VIP) and acetylcholine (ACh) in parasympathetic cerebrovascular nerves of sympathectomized rats. These reports of NPY localization in parasympathetic

Table II NPY colocalization with NA in sympathetic noradrenergic tissue.

rat and guinea-pig irides	(Zhang et al., 1984)
dental pulp	(Uddman et al., 1984a)
respiratory tract	(Sheppard et al., 1984) (Uddman et al., 1984b)
adrenal gland	(Varndell et al., 1984)
juxtaglomerular apparatus	(Ballesta et al., 1984)
thyroid gland	(Grunditz et al., 1984) (Hedge et al., 1984)
spleen	(Lundberg al., 1984)
genital tract	(Adrian et al., 1984)
heart	(Gu et al., 1984)
perivascular fibres	(Ekblad et al., 1984)

nerves are, however, anecdotal. In fact, Yokoyama et al. (1990) found NPY-like-ir nerves innervating the prostate gland of the monkey which he believed were not sympathetic or parasympathetic due to the lack of Ach and NA. He referred to these fibres as non-adrenergic.

NPY can exist by itself or , more commonly, with other neuroactive substances. NPY is co-localized with a number of other peptides and neurotransmitters. NPY is co-localized with GABA in the cortex and striatum (Aoki & Pickel, 1990) with somatostatin in the same fibres in various regions of

the brain such as the basal ganglia, hypothalamus and striatum (Chronwall et al., 1984; Anderson and Reiner, 1990). In parasympathetic cerebrovascular nerves NPY is co-localized with VIP and Ach (Suzuki et al., 1990), and NPY has been co-localized with VIP in submucosal plexus neurons of the small intestine (Pataky et al., 1990). In some noradrenergic neurons, enkephalins are costored with NPY (Kong et al., 1990). However, the substance most commonly co-localized with NPY in the sympathetic nervous system is noradrenaline.

Synthesis of NPY

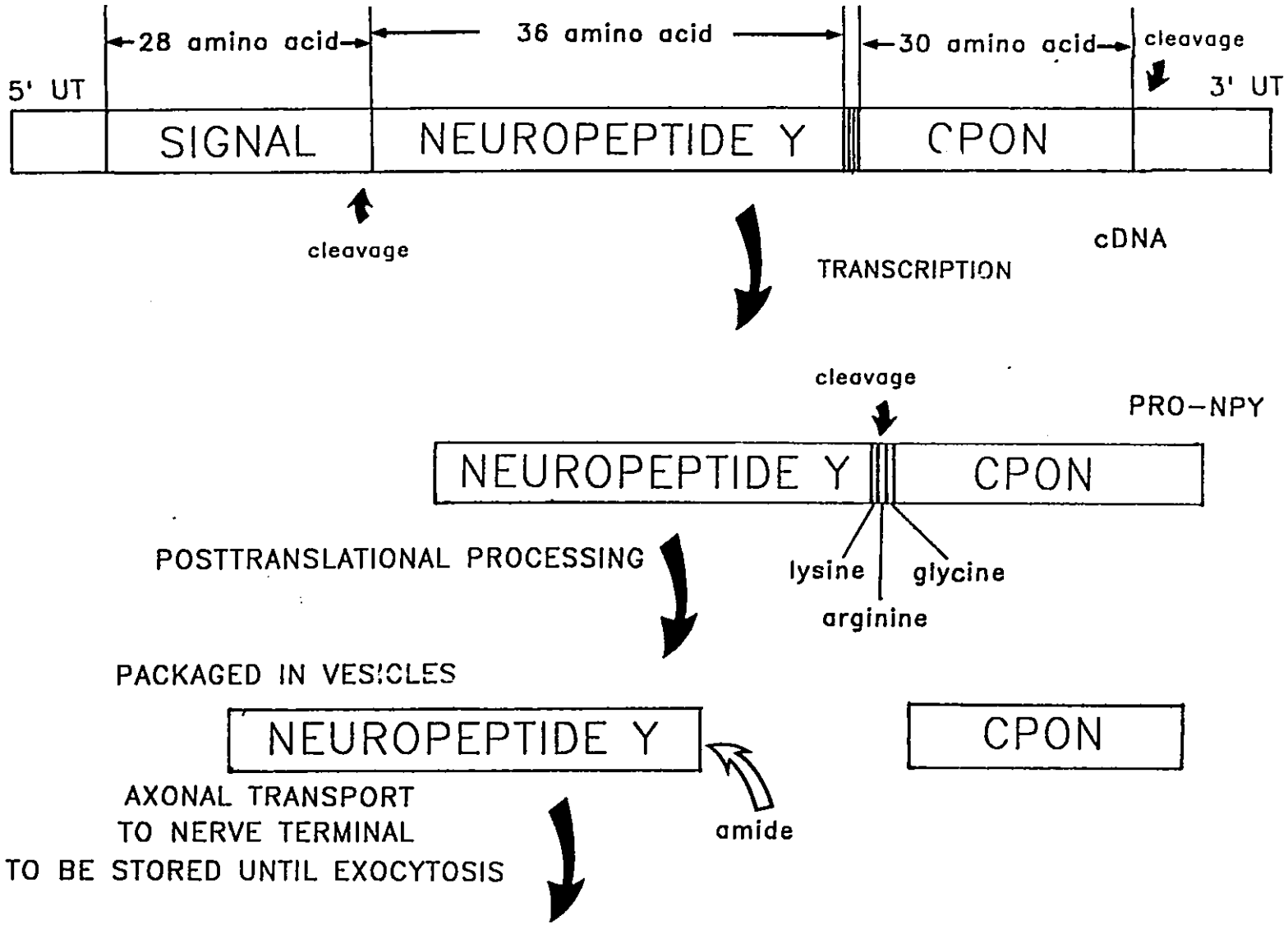
The synthesis of NPY begins with the transcription of the genome in the nucleus of the cell. From this, a 98 amino acid precursor is produced (Allen, 1990). This precursor is translated in the endoplasmic reticulum resulting in a cleavage of the 28 amino acid signal peptide leaving a 69 amino acid peptide consisting of NPY (36 amino acids), CPON (30 amino acid C-flanking peptide) and 3 amino acids necessary for post-translational processing in the golgi apparatus (Allen, 1990). The enzymes responsible for cleaving the peptide precursor are not yet known (Dickerson et al., 1990). Pro-NPY undergoes a single cleavage at the -LYS-ARG- during posttranslational processing to yield the final 36 amino acid product (Allen, 1990). It is packaged in the golgi apparatus and transported along the axon to the nerve terminal to be

stored until it is released by exocytosis (Cooper et al., 1986; Fried et al., 1985) (Figure 1). In the nerve terminals it is stored in large dense core vesicles and not in the small dense or small opaque vesicles in the sympathetic nerve terminals (Fried et al., 1985).

Release of NPY

It was established in the vas deferens that ATP and NA were co-released from sympathetic nerves and each was responsible for only one component of the biphasic neurogenic contraction (Meldrum & Burnstock, 1983; Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984). Direct evidence necessary proving that NPY, ATP and NA are all co-released upon stimulation of the sympathetic nerve did not appear until 1988 (Kasakov et al., 1988). The mechanical activity, the fractional rate of [^{3}H]NA release and ATP and NPY levels in the superfusate were measured at several points before, during and after electrical stimulation of the sympathetic nerve supplying the guinea pig vas deferens. The increase over the basal amount of [^{3}H]NA in the superfusate and the contractile response were synchronized with the increase over the basal ATP and NPY levels in the superfusate. Tetrodotoxin (TTX) blocked all of the responses to stimulation indicating that the release in response to stimulation was from the nerves. The post-synaptic effects of each transmitter were selectively blocked by prazosin or

Figure 1. Synthesis of NPY. The cDNA is transcribed in the nucleus to produce NPY mRNA. The 98 amino acid preprohromone yields a 69 amino acid prohormone containing NPY, C-flanking peptide (CPON) and 3 amino acids necessary for post-translational processing in the golgi apparatus. After translation by ribosomes on the endoplasmic reticulum the prohormone is packaged in a vesicle and sent to the golgi apparatus for sorting and post-translational modifications. A single cleavage between lysine and arginine occurs and NPY is amidated, packaged in vesicles. NPY is then sent to the nerve terminal via axonal transport to be stored until exocytosis.


28 amino acid
36 amino acid
30 amino acid
cleavage
5' UT
3' UT
SIGNAL
NEUROPEPTIDE Y
CPON
cleavage
TRANSCRIPTION
cDNA
cleavage
PRO-NPY
NEUROPEPTIDE Y
CPON
POSTTRANSLATIONAL PROCESSING
lysine
glycine
arginine
PACKAGED IN VESICLES
NEUROPEPTIDE Y
CPON
amide
AXONAL TRANSPORT
TO NERVE TERMINAL
TO BE STORED UNTIL EXOCYTOSIS

α,β,methylene-ATP. This study provides the first clear evidence for a concomitant release of NA, ATP, and NPY from sympathetic nerves. In general, NPY is released preferentially upon high frequency stimulation (Lundberg et al., 1990) while ATP is released at lower frequencies of stimulation (Thuresson-Klein, 1983), and NA is released over a broad range of frequencies of stimulation overlapping with ATP and NPY (Lundberg et al., 1986). The importance of the frequency dependent release of these neuroactive substances will become evident when their physiology is discussed.

Physiological Function

NPY has many effects throughout the body. It has effects in the brain and on the endocrine system which affect behavior. It also has effects in the renal system, gastrointestinal system, and, most importantly, the cardiovascular system. These effects are at both pre- and post-synaptic sites.

Central Nervous System

Within the central nervous system, NPY is found in very high concentrations in the hypothalamus and has been shown, by many, to have a variety of effects. By far the most pronounced effect observed following an intracerebro-

ventricular injection of NPY, is an dramatic increase in feeding behavior in rats (Stanley & Leibowitz, 1984; Levine & Morley, 1984). This increase in feeding behavior was found to be specific for carbohydrates when 3 pure macronutrient (protein, fat, and carbohydrate) diets were made available to the rats (Stanley et al., 1985). In one study where feeding behavior was induced upon the intraventricular (ivt) administration of NPY, male rats, not female rats, exhibited decreased sexual motivation (Clark et al., 1985).

NPY has been shown to have suppressive effects on several components of sexual behavior by acting on the median preoptic area in rats (Beck et al., 1990). Sutton et al. (1988a) has implicated NPY in the initiation of puberty in monkeys. The effects of NPY on reproductive hormones vary greatly depending on the animal's sex steroid background (O'Halloran et al., 1990). NPY can decrease the release of growth hormone (GH) (McDonald et al., 1985), thyrotropin (TSH) (Härfstrand et al., 1987), and prolactin (PRL) (Härfstrand et al., 1987) as well as luteinizing hormone (LH) (McDonald et al., 1985) in ovarectomized rats. In ovarectomized rats receiving estrogen supplements, NPY had a stimulatory effect on the release of LH, GH and follicle stimulating hormone (FSH) (McDonald et al., 1985). NPY stimulated LHRH secretion in the presence of physiological levels of plasma estrogen and decreased LHRH secretion in the absence of estrogen due to ovariectomy

(MacdDonald, 1990). NPY also potentiates the gonadotrophs response to LHRH (Crowley et al., 1987) and GnRH (Sutton et al., 1988b).

NPY, whether superfused over the suprachiasmatic nucleus (SCN) (Albers & Ferris, 1985) which is believed to be the circadian rhythm pacemaker, or simply released from neurons in the intergeniculate leaflet of the dorsal thalamus which project to the SCN (Card & Moore, 1982), produce dramatic changes in the phase of the circadian activity rhythm (Albers, 1986). The responses to NPY are variable depending on the animal's exposure to light and original rhythm upon receiving NPY. This latter observation suggests that NPY and light cooperate to regulate circadian phase (Moore & Card, 1990).

The actual physiological function of NPY in the hippocampus is not clearly understood yet although NPY is thought to enhance memory by inhibiting the release of gamma-butyric acid (GABA) from basket cells (Morley & Flood, 1990). NPY is a potent inhibitor of excitatory input into the hippocampus by decreasing the feedforward excitation and inhibition without decreasing the release of the excitatory transmitter, glutamate or changing the properties of the post-synaptic circuitry, such as recurrent inhibition (Colmers, 1990).

Systemic Effects of NPY

NPY produces a wide range of effects on peripheral targets

Table III Peripheral Target Tissues for NPY.

1. Blood vessels (vasoconstriction)
2. Heart (chronotropy; inotropy)
3. Intestine (secretion & motility)
4. Pancreas (insulin & glucagon)
5. Kidney (collecting tubule; renin)
6. Atrial cardiocytes (ANF)
7. Autonomic nerves (inhibition)

(Table 3). Some may be mediated by NPY activity as a classical neurotransmitter while others may be as a result of NPY acting as a neuromodulator. The most pronounced effect of NPY is vasoconstriction in vivo (see below). There are however, a few other important peripheral targets upon which NPY acts.

NPY has natriuretic properties as well as potent vasoconstrictor effects on renal arteries, especially in the juxtaglumerular apparatus, resulting in a decrease in urine production (Allen et al., 1985; Raine et al., 1984). The reports (Smyth et al., 1988; Smyth et al., 1989; Hackenthal et al., 1987) of NPY increasing sodium and water excretion and decreasing renin release in rat could not be confirmed by Aubert et al. (1988) or Echtenkamp & Dandridge (1989) in primates. The way in which NPY causes natriuresis is not clear and remains to be determined. It could even have something to do with atrial natriuretic factor (ANF) or

antidiuretic hormone (ADH), as NPY injections significantly increased plasma levels of IR-ANF (Baranowska et al., 1987) and NPY perfusion of renal cortical collecting tubules resulted in an inhibition of ADH-induced increases in hydraulic conductivity (Dillingham & Anderson, 1989).

As NPY is found localized around both arterial and ductal structures of the pancreas (Carlei et al., 1985), it is not surprising that NPY has effects on insulin and glucagon. The effects of NPY on insulin secretion are stimulatory in the CNS and directly inhibitory in the pancreas (Pettersson et al., 1987; Moltz & McDonald, 1985; Kuenzel & McMurtry, 1988) while the effect on glucagon secretion is inhibitory and only mediated in the pancreas (Pettersson et al., 1987).

In the gastrointestinal tract, NPY has effects on ion absorption, gastric acid secretion, and muscle contractility that are independent of the central nervous system effects that NPY has on feeding behavior. Sympathetic innervation of the esophagus yields NA and NPY which both increase blood flow and motility (Cunningham & Sawchenko, 1990) whereas NPY causes a dose dependent inhibition of electrically stimulated, neurally mediated contraction of longitudinal muscle from guinea-pig terminal ileum by decreasing ACh release presynaptically (Allen et al., 1987). Also by inhibiting the release of ACh from postganglionic cholinergic neurons, NPY has been shown to decrease gastric acid secretion in isolated

rat stomach (Tsai & Cheng, 1990). In the rat, NPY decreases gastric acid secretion following administration into the lateral cerebral ventricle of conscious rats (Penner et al., 1990). In the small intestine, both the ileum and the jejunum, NPY increases net absorption, from mucosa to serosa, of Cl^- (MacFadyen et al., 1986; Brown et al., 1990) while contradictory reports of increased (Friel et al., 1986) and decreased (Hubel & Renquist, 1986) absorption of Na^+ exist.

Cardiovascular Effects of NPY

The most important role NPY plays involves the control of the cardiovascular system. NPY exercises its control via the central nervous system by acting on the hypothalamus, medulla oblongata and parts of the midbrain including the nucleus tractus solitarii (NTS). Microinjections of NPY into the posterior hypothalamic nucleus resulted in an increase in blood pressure (Westfall et al., 1988) while intramedullary microinjections into the nucleus tractus solitarii (Tseng et al., 1989) and general i.c.v. administration (Scott et al., 1989) of NPY resulted in a decrease in heart rate, systolic blood pressure and diastolic blood pressure due to decreases in the release of NA from catecholamine neurons by NPY.

The main effects of intravenous administration of NPY in vivo in anaesthetized rats are an increase in the mean arterial pressure (MAP), and total peripheral resistance

(TPR), and a decrease in cardiac index (CI) and stroke volume (SV) (Zukowska-Grojec et al., 1987). The basal heart rate is decreased in studies of conscious animals (Minson et al., 1987; Minson et al., 1989; Minson et al., 1990; Zukowska-Grojec et al., 1987; Itoi et al., 1986). In studies of isolated hearts the same observations are made (Balasubramaniam et al., 1988; Allen et al., 1983; Mabe et al., 1987; Franco-Cereceda et al., 1985). NPY decreases the contractility in the atria and ventricles of the heart by exhibiting negative inotropic and chronotropic effects (Balasubramaniam et al., 1988; Piper et al., 1989; Rigel et al., 1989). The mechanism for this is thought to be a coupling of NPY to adenylate cyclase (AC) via an inhibitory G-protein and a resultant decrease in cAMP (Kassis et al., 1987; Piper et al., 1989; Millar et al., 1988). Decreases in the contractility of the heart may also be due to presynaptic inhibition of parasympathetic and sympathetic nerves innervating the heart. NPY decreases release of NA and ACh from sympathetic and parasympathetic systems innervating the heart (Revington & McCloskey, 1990; Michel et al., 1989). In this way, acting presynaptically, NPY can decrease the cardiac responses evoked by field stimulation without affecting the chronotropic and inotropic responses to exogenous NA and ACh (Lundberg et al., 1984).

The main function of NPY, increasing vascular tone, can be

readily observed upon the i.v. administration of a low dose of NPY (Lundberg & Tatemoto, 1982). NPY causes vasoconstriction in many isolated organs: spleen (Saxena et al., 1989), kidney (Minson et al., 1989), lung (Martling et al., 1990), heart (Komaru et al., 1990). NPY also has potent direct contractile effects on some isolated blood vessels but in most isolated vessels, has poor direct contractile effects (Lundberg et al., 1989). In cerebral, coronary and skeletal muscle arteries, the most potent effects are observed. Pernow et al. (1987a) and Pernow et al. (1987b) showed that, in skeletal muscle arteries, NPY was a more potent vasoconstrictor than NA as 50 nM NPY caused a contraction similar in magnitude to that of 10 μM NA. In cerebral arteries of the rabbit, NPY caused a contraction with a mean EC_{50} of 2.7 nM (Abel & Han, 1989). An EC_{50} of 10 nM NPY was calculated by Prieto et al. (1991) for the vasoconstrictor response in isolated coronary arteries and 30 nM NPY evoked a contraction whose magnitude was 55% of that produced by 125 mM KCl whereas 30 nM NPY in the rabbit ear artery did not evoke any contraction (Edvinsson et al., 1984). The vasoconstrictor response by NPY in most peripheral resistant blood vessels such as the femoral, saphenous, rabbit ear, rat tail, and mesenteric arteries is weak and variable (Andriansitohaina & Stoclet, 1988; Hieble et al., 1989; MacLean & McGrath, 1990; Pernow et al., 1987; Pernow et al.,

1986).

There are three types of action by NPY at the sympathetic neuroeffector junction in vascular smooth muscle: 1) direct contractile effects on the post-synaptic target, 2) indirect potentiating effects on the post-synaptic target and, 3) pre-synaptic inhibitory effects (Dahlof et al., 1985b; Edvinsson et al., 1984). Although there are earlier reports in which NPY was not found to decrease electrically stimulated release of [^{3}H]-NA (Ekblad et al., 1984) many reports since have been made in which NPY inhibits the release of electrically stimulated [^{3}H]-NA presynaptically (Lundberg et al., 1985; Lundberg et al., 1987; Pernow et al., 1987; Pernow et al., 1986; Pernow, 1988; Kahan et al., 1988; Pernow, 1989b; Westfall et al., 1987; Wong-Dusting & Rand, 1988).

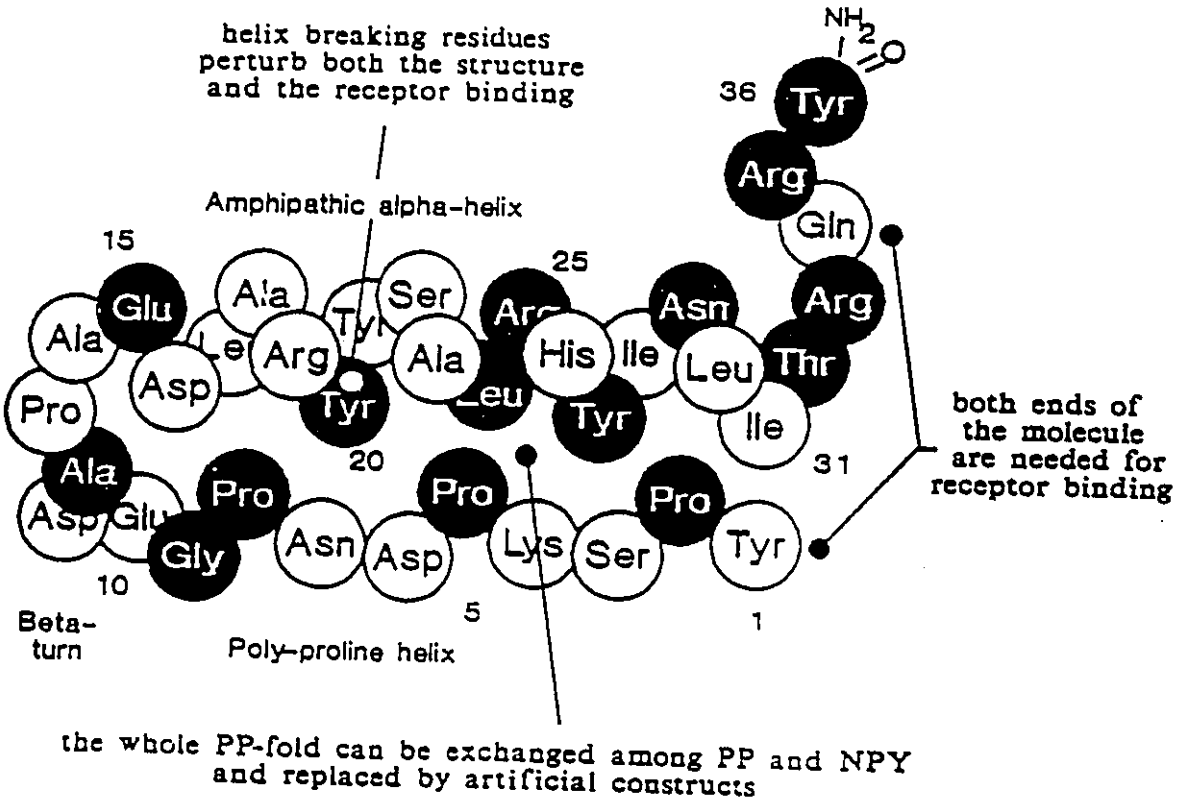
NPY potentiates the pressor response post-synaptically and is usually more pronounced in vessels in which NPY exerts minor or no direct contractile effects (Ekblad et al., 1984; Lundberg et al., 1987; Pernow, 1988). NPY was first found to potentiate the adrenergic component of vasocontriction (Dahlof et al., 1985a). As more investigations were carried out, it was demonstrated by many that NPY could also potentiate vasoconstriction induced by histamine (HIS) (Abel & Han, 1989), prostaglandin $F_{2\alpha}$ (Andriantsitohaina & Stoclet, 1988), mATP (Saville et al., 1990), 5-HT (Prieto et al., 1991), potassium depolarization (Oshita et al., 1989; Neild, 1987).

Just in the last year, there have been reports that NPY potentiates purinergic responses preferentially over adrenergic responses (Cheung, 1991; Ellis & Burnstock, 1990). The post-synaptic stimulatory effects of NPY are the opposite with respect to its presynaptic inhibitory effects. Potentiation of vasoconstriction was the predominant effect of exogenous NPY in response to electical stimulation of sympathetic nerves.

Structure-function relationship

The relative importance of the structure of NPY (Figure 2) to its function can be established with analogs in which a specific residue has been changed. There are two receptor subtypes which bind NPY and PYY. These receptor subtypes, Y_1 and Y_2, were established based on bioassays (Wahlestedt et al., 1986) and binding studies (Sheik et al., 1989). The bioassays were based on the fact that NPY has both pre- and post-synaptic actions. The post-synaptic receptors Y_1 caused an increase in intracellular calcium by inhibiting adenylate cyclase in the HEL cells (Aakerlund et al., 1990) whereas the presynaptic inhibitory actions of NPY are via Y_2 receptors which are linked to G proteins (Foucart et al., 1990). Y_1 receptors are also predominantly the postsynaptic NPY receptors in the vasculature. Y_2 receptors can be located post-synaptically in the vasculature as well but the majority

Figure 2. Structure of NPY based on X-ray structure of related peptide avian PP. Residues common to NPY, PYY and PP are indicated in white on black. Some of the structural elements which have been determined using synthetic analogs in structure-function studies are indicated (Schwartz et al., 1990).


helix breaking residues perturb both the structure and the receptor binding
Amphipathic alpha-helix
NH2
36
Tyr
Arg
Gln
Arg
Thr
Leu
Ile
Asn
His
Ile
Tyr
Leu
Ala
Arg
Tyr
Ala
Ser
Ala
Glu
Ala
Pro
Asp
15
25
20
31
both ends of the molecule are needed for receptor binding
Pro
Pro
Pro
Gly
Asn
Asp
Lys
Ser
Tyr
Asp
10
5
1
Beta-turn
Poly-proline helix
the whole PP-fold can be exchanged among PP and NPY and replaced by artificial constructs

of post-synaptic vascular NPY receptors are Y_1. The distribution of NPY receptors in the vasculature is similar to the distribution of α-adrenoceptors. The Y_1 receptor in the vasculature is responsible for the vasoconstrictive properties of NPY whereas the Y_2 receptor is responsible for the presynaptic inhibitory effects of NPY. The Y_2 receptor binds the C-terminal of NPY_{18-36} as well as the parent NPY molecule while the Y_1 receptor will only bind NPY if both the N-terminal and the C-terminal are present. The binding of NPY_{18-36} to the Y_2 receptor increased the intracellular calcium of the HEL cells by only 10 percent of the increase in intracellular calcium by the parent NPY molecule (Fuhlendorff et al., 1990). This weak activity by the C-terminal fragment suggests that both ends of the molecule are needed for full activity. Further evidence supporting the necessity of the presence of both ends of the molecule for full activity come from studies in which the proline residue was introduced at position number 20, in an attempt to disrupt the alpha helix of the C-terminal (Fuhlendorff et al., 1990). As in the studies using NPY_{18-36}, the binding to Y_2 receptors was weak and no binding to Y_1 receptors was observed. Using an analog of NPY in which residues 5-24 had been replaced with a simple, non-peptide bridge, Krstenansky et al. (1989) found that full binding activity on the Y_2 receptor could be obtained

indicating that the epitope which binds to the receptor is composed of residues in the N- and C-terminal of the molecule. Furthermore, results from binding studies with NPY molecules which underwent substitutions of residues 34 and 4, suggest that residue 34 is the key to differential recognition of NPY/PYY and pancreatic polypeptide (PP) by Y_2 and PP receptors but not by Y_1 receptors (Fuhlendorff et al., 1990) and residue 4 is responsible for the differential recognition of NPY/PYY and PP by Y_1 and PP receptors (Schwartz et al., 1990). In addition to bringing the two crucial segments, the N- and C-terminal, together so that they are available for recognition by the receptors, the PP-fold motif is amphiphilic across the axis of the fold and maintains a strong electrostatic dipole moment along the axis. The dipole moment contributes to the stability of the PP-fold motif. The degree with which the amphiphilic moment and the dipole moment contribute to receptor binding are currently being investigated by substituting residues which would alter the amphiphilic or electrostatic nature of the molecule. As the importance of the individual residues and the role they play in maintaining the PP-fold motif is understood, the structure-function relationship will also be understood.

Agonists and Antagonists

The studies using fragments of NPY will also lead to the

realization of new antagonists, agonists and partial agonists based on the binding affinity of the analog or fragment and its activity. If a fragment binds to a Y_1 or Y_2 receptor with high affinity yet does not activate that receptor, it is, by definition, an antagonist. There are, as yet however, no widely accepted antagonists for NPY. Balasubramanium & Sheriff, 1990, found NPY_{18-36} to be a competitive antagonist of NPY in rat cardiac ventricular membranes. Kazuhiko Tatemoto's lab has actually come up with two NPY receptor antagonists designated PYX-1 and PYX-2 using an analog mixture-screening strategy on NPY analogs which had little or no activity, (i.e. were unable to increase the intracellular calcium in human erythroleukemia (HEL) cells). PYX-1 corresponded to Ac-[3-(2-6-dichlorobenzyl)-Tyr^{27}, D-Thr^{32}] NPY_{27-36}, and PXY-2, to Ac-[3-(2-6-dichlorobenzly)-$Tyr^{27,36}$, D-Thr^{32}] NPY_{27-36} (in preparation) (Tatemoto, 1990). Michel & Motulsky (1990), also using HEL cells, demonstrate He 90481's ability as a competitive antagonist of NPY receptors, to inhibit porcine NPY-induced increases of intracellular calcium. Benextramine (Doughty et al., 1990) and D-myo-inositol-1.2.6-triphosphate (Edvinsson et al., 1990) are two more substances shown to have antagonistic effects . Perhaps some of the above mentioned will prove to be effective antagonists of NPY receptors in a number of systems with time and more testing.

Mechanism of Action of NPY

Neuropeptide Y has both pre- and post-synaptic effects. Presynaptically it has an inhibitory effect mediated usually by a decrease in transmitter release. Postsynaptically, it has stimulatory effects mediated in two ways. It enhances coreleased neurotransmitter action and it has a direct stimulatory effect.

In neurons, NPY suppresses excitability. This effect of NPY is due to the inhibition of calcium influx through voltage-sensitive calcium channels (Ewald et al., 1989; Perney & Miller, 1989; Hirning et al., 1990; Wiley et al., 1990). The inhibitory effect that NPY has on calcium influx is through the activation of a pertussis toxin-sensitive G-protein (Go). In myenteric neurons, NPY inhibits calcium influx through N-type calcium channels via a direct receptor-G-protein-channel coupling (Hirning & Miller, 1990). In C-cells of the Rana pipiens sympathetic ganglion, NPY suppresses calcium currents (Schofield & Ikeda, 1988). More recently, also in support of the idea that NPY decreases neuron excitability, inwardly rectifying potassium channels have been shown to be activated by NPY (Zidichouski et al., 1990). Perney & Miller, (1989) have demonstrated in dorsal root ganglion neurons, that NPY, through a pertussis toxin-sensitive G-protein, activates the synthesis of IP_3 and diacylglycerol (DAG). The IP_3 production results in an

increase in intracellular calcium for which a physiological role has not been determined. The production of DAG is thought to be responsible for the inhibitory effects of NPY in sympathetic neurons. This is based on the findings that activators of protein kinase C and synthetic analogs of DAG partially mimicked the inhibitory effects of neurotransmitters on calcium currents (Rane & Dunlap, 1986).

Presynaptic inhibition of release of neurotransmitter from nerve terminals by NPY is via activation of Y_2-receptors on sympathetic nerve endings (Wahlestedt et al., 1990). The transduction pathway for the Y_2-receptor is not yet clearly understood. In guinea-pig vas deferens, the mechanism of presynaptic action by NPY was shown to be different than that of α_2-adrenoceptor activation (Cheung & Dukkipati, 1991). The involvement of N-type calcium channels was also ruled out in this preparation. It was found that NPY prevented nerve action potential invasion of the nerve terminals. This was by a mechanism which did not involve sodium channels (Cheung & Dukkipati, 1991). NPY (50-1000 nM) also failed, in cortical and hippocampal synaptosomes, to modify calcium influx via N or L type calcium channels (Lundy & Frew, 1991). There has been one report recently (Chernaeva, 1990), which has demonstrated in the guinea-pig vas deferens, that NPY reduces [^{3}H]NA release via adenylate cyclase (AC) and the processes responsible for calcium mobilization.

Mechanism of Action of Vasoconstriction

Depolarization-induced Vasoconstriction

Contraction of vascular smooth muscle dependent on membrane depolarization can be induced by increasing the concentration of potassium in the physiological bathing solution to 30 mM or more (Bolzon & Cheung, 1989). The membrane potential is determined primarily by the concentration gradient for potassium, as shown in the Nernst's equation.

$$Em = \frac{RT}{Fz} \log \frac{[K]_o}{[K]_i}$$

where...

Em is membrane potential R is Boltzman's constant

T is absolute temperature F is Faraday's constant

z is the valence of the ion

[K] is potassium concentration inside (i) or out (o)

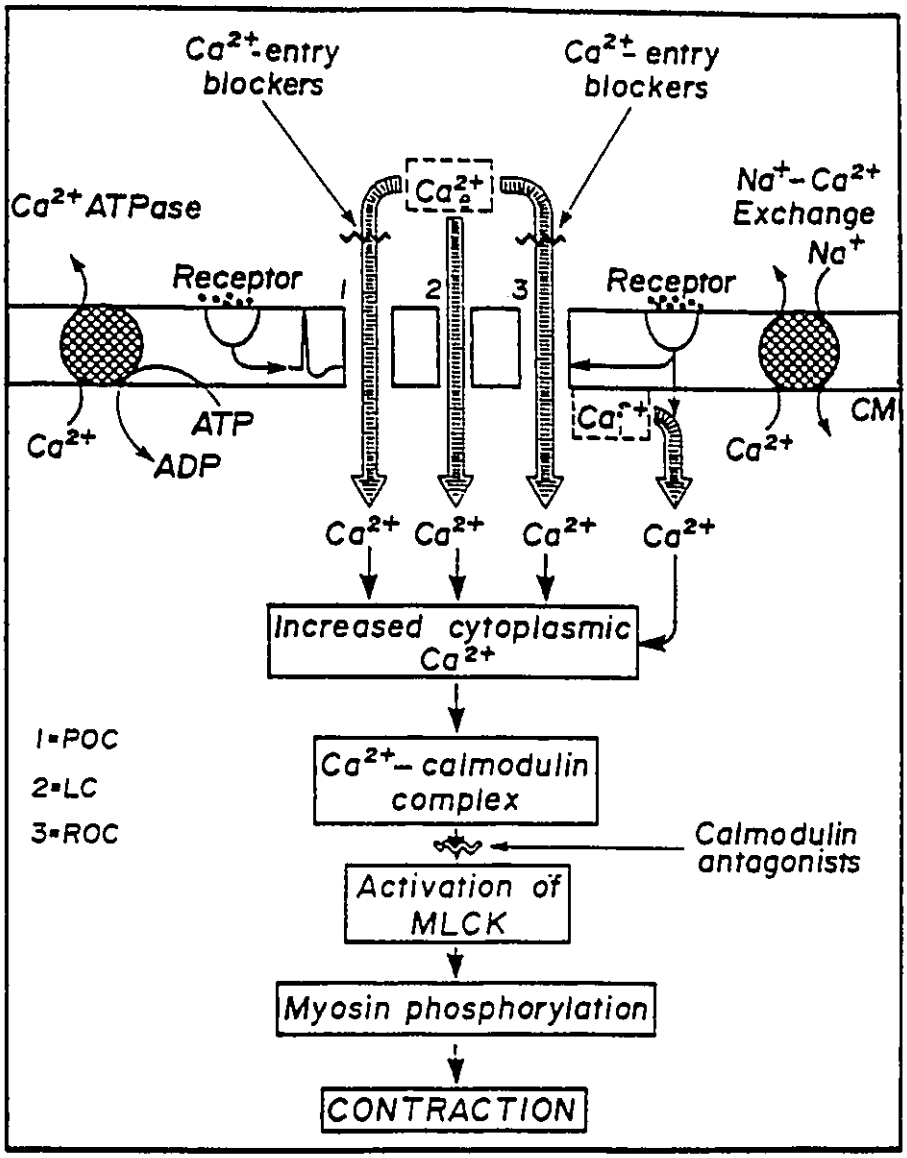
As the membrane depolarizes from a resting potential of about 68 mV in the rat tail artery to a potential less negative than --49 mV, contraction could be observed (Cheung, 1984). This is thought to be due to the opening of calcium channels with depolarization. From voltage clamp studies, voltage-dependent calcium channels, both transient (T)- and sustained (L)-type, could also be demonstrated (Benham et al., 1987a). The

dihydropyridine, nifedipine, blocks the influx of calcium through these channels (Figure 3) (Rimele & Vanhoutte, 1984). Its ability to inhibit vasoconstriction depends directly on the relative contribution of external calcium influx through voltage-sensitive calcium channels. The inhibitory effect that nifedipine has on KCl-induced contraction is substantial. There is virtually a complete block of KCl-induced contractions by nifedipine.

Agonist-induced vasoconstriction

ATP and NA are both released from the sympathetic nerve terminals upon neurogenic stimulation and are responsible for a slow tonic contraction due to NA and a phasic contraction due to ATP (Cheung, 1984). The transduction mechanism for ATP-mediated contraction is not completely understood as yet. Using an ATP analog, L-APPCP which is selective for the P_{2x} receptor (Hourani et al., 1986), it was established that, by activating this receptor, the membrane is depolarized (Benham et al., 1987b; Benham & Tsien, 1987). Both voltage sensitive and ATP-activated cation channels have been shown to be responsible for the calcium influx which results in contraction (Benham et al., 1987b; Benham & Tsien,, 1987). In addition to these results, there is evidence that suggests that ATP-induced contraction is also mediated by a release of calcium from internal stores via activation of IP_3 (Phaneuf et

Figure 3. Vasoconstriction mediated by influx of calcium. Intracellular calcium levels are increased by calcium influx through potential operated calcium channels (POC), a passive calcium leak pathway (LC) and receptor activated calcium channels (ROC). Receptor activation by specific agonists may also activate POCs by depolarizing the cell membrane and may induce calcium release from intracellular stores. Once calcium levels are elevated in the cell, calcium binds to calmodulin and activates myosin light chain kinase (MLCK) which phosphorylates myosin resulting in contraction. The calcium is removed from within the cell by membrane pumps and transporters such as the Ca_{2+}-ATPase pump and the Na_{+}/Ca_{2+} exchanger shown.


Ca^{2+} entry blockers
Ca^{2+} entry blockers
Ca^{2+} ATPase
Ca^{2+}_o
$Na^+ - Ca^{2+}$ Exchange
Na^+
Receptor
1
2
3
Receptor
Ca^{2+}
ATP
ADP
Ca^{2+}
CM
Ca^{2+}
Ca^{2+} Ca^{2+} Ca^{2+} Ca^{2+}
Increased cytoplasmic Ca^{2+}
1=POC
2=LC
3=ROC
Ca^{2+} – calmodulin complex
Calmodulin antagonists
Activation of MLCK
Myosin phosphorylation
CONTRACTION

al., 1987; Tawada et al., 1987). Nifedipine blocks a large component of the mATP-induced vasoconstriction. This is due to the fact that a large component of the contraction is mediated by an influx of calcium through voltage sensitive calcium channels (Benham, 1987).

NA causes vasoconstriction by acting on post-synaptic α_1 (Cheung, 1984) and α_2 (Medgett, 1985) receptors. The activated α_2 receptor causes an influx of external calcium through receptor-operated calcium channels and voltage sensitive calcium channels (Cheung, 1985) while the activated α_1 receptor causes mainly a release of calcium from internal stores via IP_3 in most arterial preparations (Somlyo et al., 1988; Chiu et al., 1987). The relative contributions of the two alpha receptors to contraction and hence, the source of calcium for contraction, vary considerably from one arterial preparation to another (Van Nueten et al., 1988). As the amount of calcium influx due to activation of alpha-adrenoceptor varies from one arterial preparation to another, the nifedipine sensitivity of the contraction mediated by NA also varies from one preparation to another. The external calcium influx that is responsible for vasoconstriction in each of the above cases is different (Omote et al., 1989; VanHoutte et al., 1983). The transduction mechanisms of KCl- and ATP-induced contractions are considered to be electro-mechanically coupled whereas the NA-induced contractions are

via a pharmo-mechanical coupling (Cheung, 1991). For this reason nifedipine has a much greater ability to block KCl and mATP mediated vasoconstriction than that which is mediated by NA.

Neuropeptide Y

There are indications that voltage-sensitive calcium channels may be involved in NPY-induced vasoconstriction. Voltage-sensitive calcium channel blockers inhibit vasoconstriction to NPY in cerebrovascular smooth muscle (Edvinsson, 1985), coronary arteries (Aizawa et al., 1989; Hayashi et al., 1986), peripheral rat blood vessels (Pernow et al., 1986; Zukowska-Grojec et al., 1986; Mabe et al., 1985; Dahlöf et al., 1985a), and peripheral human blood vessels (Pernow et al., 1987). There are also numerous reports of NPY's potentiating effects on vascular smooth muscle which show the involvement of voltage-sensitive calcium channels. Nifedipine inhibited the potentiating effects of NPY on vasocontriction mediated by a number of vasoconstrictors in human (Lundberg et al., 1985), rabbit (Duesler et al., 1990), guinea-pig (Cheung, 1991) and rat blood vessels (Pernow et al., 1986). In calcium-free solution, NPY's potentiation of NA-induced vasoconstriction in rabbit mesenteric arteries was absent and could only be observed upon the readdition of

calcium (Oshita et al., 1989). Andriansitohaina & Stoclet (1988) suggested that NPY partially depolarizes the arterioles and induces an increase in calcium entry through voltage-sensitive channels, thus enhancing contractions elicited by agonists or by KCl-depolarization. Hence the potentiation of NA was thought to be mediated through a different mechanism (Andriantsitohaina & Stoclet, 1988). From studies of guinea-pig basilar artery (Fallgren et al., 1990) and rabbit cerebral artery (Abel & Han, 1989) NPY's ability to depolarize the membrane of vascular smooth muscle at very low concentrations (3 nM), has been demonstrated. Neild (1987) found also that NPY caused a depolarization that was associated with contraction in the rat tail artery. However, later (1990) Neild and Kotecha demonstrated a lack of membrane depolarization by NPY when potentiation of vasoconstriction occurred in arterioles of the submucosa of the guinea-pig small intestine. Neild's findings in the guinea-pig small intestine are similar to those by Wahlestedt et al. (1985) in the rabbit femoral artery. NPY-induced membrane depolarizations also have not been observed in rat cerebral arteries (Brayden & Conway, 1988), renal vascular smooth muscle (Bührle et al., 1986), or arterioles of the guinea-pig small intestine (Neild & Kotecha, 1990) using concentrations of NPY as high as 1 μM.

For the potentiating effect of NPY on NA-induced

contraction, a G-protein has been implicated based on the pertussis toxin-sensitive nature of NPY-potentiated vasoconstriction (Andriantsitohaina et al., 1990) and increased intracellular calcium (Mihara et al., 1989). Further studies must be carried out on the effects of NPY on intracellular calcium as Erne & Hermsmeyer (1988) failed to observe any changes in intracellular calcium in vascular smooth muscle cells from primary cultures obtained from azygous veins of neonatal WKY rats with NPY before or after NA-induced increases in intracellular calcium. Reynolds & Yokota (1988) also failed to see any changes in intracellular calcium in vascular smooth muscle cells in response to NPY but did observe a decrease in forskolin-stimulated cAMP levels.

Role of Endothelium

Since Furchgott & Zawadzki (1980) demonstrated ACh's ability to relax rabbit aorta in an endothelium-dependent fashion, a number of substances, including ATP, substance P (SP), and 5-hydroxytryptamine (5-HT), have been shown to act on endothelial cells causing release of endothelium derived relaxing factor(s) (EDRF) (Vanhoutte & Rimele, 1983; Furchgott, 1984; Gordon, 1986). EDRF has been identified as a nitric oxide (NO)-like substance which is cleaved from L-

arginine by specific enzymes (Ignarro et al., 1987; Palmer et al., 1987). It is released from the endothelial cell in response to physical stimuli or after stimulation by any of the above mentioned substances (Cocks & Angus, 1983; Rubanyi et al., 1986). It acts on the smooth muscle cell to increase c-GMP levels which attenuate agonist-induced increases of intracellular free calcium thereby relaxing preconstricted vessels (Pohl & Busse, 1990). In addition to EDRF, the endothelium releases endothelium-derived hyperpolarizing factor (EDHF) which makes the membrane potential of vascular smooth muscle more negative and less responsive to vasoconstrictor stimuli (Chen et al. 1988). The endothelium also produces substances which causes contraction instead of vasodilation. One of these is a 21-amino acid protein called endothelin (ET) which causes potent vasoconstriction (Yanagisawa et al., 1988). The stimuli responsible for the release of ET is unknown other than the fact that an influx of external calcium is necessary. ET acts on the vascular smooth muscle by activating receptors linked to phospholipase C and arachidonic acid (Lüscher, 1989). Activation of these second messengers result in the accumulation of intracellular calcium likely through the formation of inositol triphosphate and possibly through activation of protein kinase C through the formation of diacylglycerol. The endothelium is thought to be the source of many other substances which regulate vascular

activity: prostacyclin, thromboxane A2, angiotensin II, superoxide and as yet unidentified endothelium-derived contracting factors (Lüscher, 1990). This has created a foundation for the idea that NPY might mediate its effects through the endothelium. Controversy exists as to whether the endothelium is necessary for the effects of NPY. There are reports of NPY effects which are endothelium-independent (Pernow & Lundberg, 1988; Pernow, 1989; Andiantsitohaina & Stoclet, 1990; Fallgren et al., 1990; Westfall et al., 1990; Kwan et al., 1990) as well as reports of NPY effects that are endothelium-dependent (Daly and Hieble, 1987; Hieble et al., 1988; Hieble et al., 1989; Maclean & McGrath, 1990; Prieto et al., 1991). MacLean & McGrath (1990) found that NPY (0.01-1.0 nM) caused vasoconstriction in the isolated vascular bed of the rat tail which had been precontracted with 2.5-10 nM ET or 5-20 nM 5-HT only in those preparations which had not been denuded of the endothelium by perfusing with the detergent CHAPS (0.3 % (w/v)) at 2 ml/min for 90 s. These findings were contradictory to those by Pernow & Lundberg (1988) who found, in human skeletal arteries and pig splenic arteries, that NPY (5-500 nM) caused potent contractions (101 ± 16 % (skeletal arteries; n=8) and 103 ± 6 % (splenic arteries; n=8) of the contractions evoked by NA and phenylephrine (PE), respectively). After removal of the endothelium by gently rubbing the intimal layer of the vessel against holders in the

organ bath, similar contractions to NPY (5-500 nM) were observed (90 ± 13 % (skeletal arteries; n=7) and 111 ± 6 % (splenic arteries; n=7) of the contractions evoked by NA and phenylephrine (PE), respectively).

In superfused isolated rabbit ear artery segments, potentiation of the contractions elicited by field stimulation of perivascular nerves (10 Hz, 500 ms train duration, 80 V, 0.7 ms pulse duration) was found to be dependent on the endothelium as attempts with 1, 10 and 100 nM NPY failed to potentiate these contractions when the endothelium had been removed (Daly and Hieble, 1987). There was, however, some intrinsic contractile activity observed, at a concentration of 100 nM NPY, which was independent of the endothelium. The contractions were measured using a force transducer and drugs were superfused. The endothelium was removed by rubbing the intimal surface of the vessels with a polyethylene cannula and its presence tested with the cholinergic muscarinic agonist, carbachol (1 μM) in segments which had been precontracted with NE (0.1 μM). The same group, working with isolated perfused rabbit ear artery , isolated superfused rabbit ear artery and isolated superfused canine saphenous artery, measured perfusion pressure as a measure of contractility as well as tension developed using superfused segments as before. NPY was administered both intraluminally and extraluminally in the perfused segments while in the superfused segments NPY was

administered by superfusion. In this study responses to stimulations and exogenously applied NA were monitored. It was observed that NPY at 1, 10 and 100 nM potentiated neurogenic responses in the perfused vessels preferentially when the NPY was administered intraluminally. NPY (100 nM) potentiated the contractile response of the superfused rabbit ear artery to exogenous NA and neurogenic stimulation only when the endothelium was able to produce a relaxation in response to ACh (1 μM). Similar results were obtained with the dog saphenous artery (Hieble et al., 1989). In contrast, Budai et al., 1989, also investigated the effect of NPY (30 nM) on the neurogenic response of perfused isolated rabbit ear artery to transmural stimulations (8 Hz every 2.5 min, 40 V, 1 ms pulse duration) and reported that the endothelium was not necessary for the potentiating effects of NPY. The endothelium was removed by running a 2 inch length of doubled 00 silk back and forth through the lumen. The presence of the endothelium was tested using ACh (1 μM) to relax vessels precontracted with methoxamine (0.3 μM). The addition of NPY (30 nM) caused no direct contractile effect of its own yet it did potentiate the contractile response to stimulation by 96 % in the control and 101 % in the tissues in which the endothelium had been removed, clearly indicating that the endothelium was not neccessary for the potentiation observed. Another study by Gustafsson and Nilsson (1990) also

demonstrated that NPY (10 nM) potentiated the contractile response to electrical field stimulation of isolated superfused rabbit ear arteries and small mesenteric arteries of the rat independent of the endothelium. The presence of the endothelium was confirmed by *en face* silver impregnation and successful relaxation by ACh (1 μM) of vessels precontracted with NA.

Andriantsitohaina & Stoclet (1988) found, in 3rd generation mesenteric arteries of the rat, that NPY (100 nM) did not induce contraction by itself but was still able to potentiate the contractile response to exogenously applied NA, 5-HT and $PGF_{2\alpha}$ in preparations in which ACh (1 μM) relaxation of NA (10 μM)-induced contractions was less than 50 % (10-50 %). Prieto *et al*. (1991) found, measuring perfusion pressure of isolated coronary arterial ring segments, that NPY caused both a concentration-dependent contraction and that NPY (20 nM) increased the sensitivity of the segments to NA and 5-HT. Furthermore, not only were the effects of NPY still present when the endothelium had been mechanically removed, but denudation actually increased the sensitivity and maximal response to NPY.

Hypothesis to be tested:

The endothelium is necessary for the NPY-induced potentiation of vasoconstriction (Daly & Hieble, 1987).

Objectives:

1. Test the potentiation by NPY of vasoconstriction in intact and denuded tail artery segments and in isolated vascular smooth muscle cells where the influence from nerves and endothelium were completely eliminated.

2. Compare the potentiation by NPY of contractions mediated by membrane depolarization (KCl) and by adrenoceptors and purinoceptors.

Experimental Design

1. Tissue Preparations

The effects of NPY were tested in two preparations from the rat tail artery. The rat tail artery was chosen because a technique for isolating single cells already exists and the cells obtained from this preparation are well characterized (Bolzon & Cheung, 1989). The physiology and pharmacology of the rat tail artery preparation is also well characterized making it a good model in which to study the effects of NPY.

Ring Segments:

The first preparation consisted of intact and denuded ring segments of the tail artery. The denuded arterial ring segments had their endothelial lining removed by gently rubbing the intimal layer of the vessel with a coarse wire while the endothelial layer of the intact ring segments was left undisturbed. Both intact and denuded ring segments were studied simultaneously in a paired study. The treatment of both groups was identical.

Isolated Smooth Muscle Cells:

The second preparation consisted of single vascular smooth muscle cells isolated from the tail artery. The isolated cells were free of any influence from the nerves or the

endothelium. The cells, once obtained, were separated into two groups (control and NPY treated), which were studied simultaneously and received the same manipulations with one exception. The NPY treated group received NPY and the control group did not.

2. Drugs-agonists

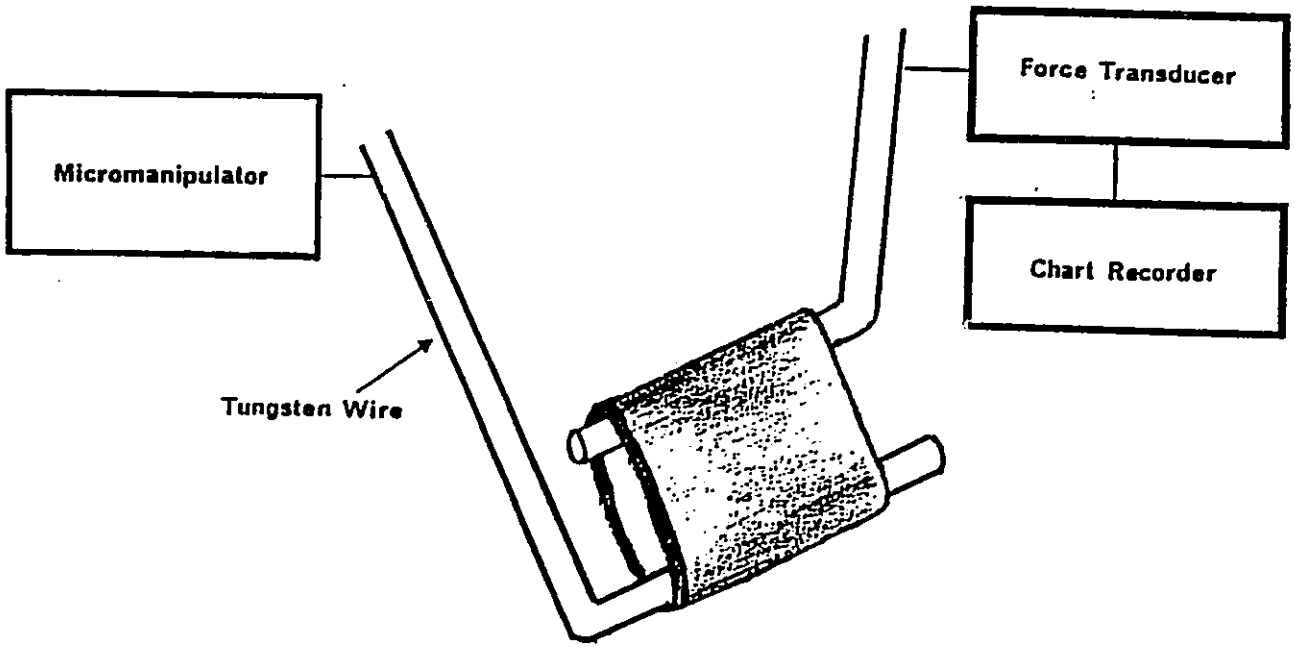
The role of voltage-sensitive calcium channels in NPY potentiation of vasoconstriction was studied using three different modes of vasoconstriction. First, increasing concentrations of KCl were used to depolarize the membrane and induce contraction via the influx of calcium through voltage-sensitive calcium channels. This is a direct test for the action of NPY on voltage-sensitive calcium channels. ATP and NA are endogenous vasoconstrictor neurotransmitters. Previous studies from our lab have demonstrated NPY to preferentially potentiate the response to activation of the purinoceptors (Cheung, 1991). This was suggested to be due to a larger dependence of the purinergic response on the voltage operated calcium channel. In the present study, the effects of NPY on purinoceptors and adrenoceptors were compared and these effects were tested to see if they were dependent on the endothelium. Nifedipine, a voltage-sensitive calcium channel blocker, was used to probe the relationship between the calcium channels and the potentiating effects of NPY.

Methods

The arterial ring segment preparation

Male Wistar rats (10-12 weeks old), (Charles River) were first stunned and then sacrificed by cervical dislocation. The tail was immediately cut away and pinned down to a rectangular piece of styrofoam. Using #5 fine forceps (Fine Science Tools) and microscissors, the ventral tail artery was lifted and dissected from the tail under a dissection microscope. The artery was then immediately submersed in physiological solution (PSS1) in a petrie dish previously coated with a 1 cm layer of Sylgard. After cleaning off the connective tissue, the portion of the artery 5 to 6 cm from the proximal end was cut into segments 3 to 5 mm. Half of the segments were carefully pulled onto a coarse piece of wire (0.7 μm) which was fixed in the bottom of the dish. These segments were each pulled on and off the wire twice. This was sufficient to denude their endothelial lining. From this point on, all of the ring segments were treated identically in order that a paired comparative study would be carried out. With the aid of a dissecting microscope, the segments were put on a tungsten wire fixed to a micromanipulator (Figure 4). A second tungsten wire (254 μm) was gently slipped into the lumen of the segments taking care not to damage the

Figure 4. Illustration of an arterial ring segment mounted on the apparatus for measuring the force of contraction produced in response to various stimuli. One of the parallel tungsten wires is fixed to a micromanipulator while the other is attached to the force transducer.


Micromanipulator
Force Transducer
Chart Recorder
Tungsten Wire

endothelium. This second wire (254 μm) was attached to a force transducer (Grass Instruments FT03). The force transducer fed to a Buxco computer which would display a continuous record of developed force over time. The contraction apparatus shown in Figure 4 was fixed to a plexiglass frame which held a 2.0 ml bath of PSS1 to ensure that the vessel would remain submersed at all times. Once the ring segment was mounted on the two parallel tungsten wires the plexiglass frame was placed in a Tek bath filled with distilled water to maintain the 2.0 ml bath of PSS1 at a temperature of 37° C. A resting tension of 0.5 g was applied to the ring segment using the micromanipulator. The PSS1 bathing the tissue was replaced with fresh, 37° C PSS1 every 15 minutes and the resting tension was maintained at 0.5 g. After a 1 hour equilibration period, 30 μM noradrenaline (NA) was added to the bath and the resulting tension was recorded until the maximum tension had been reached (usually within 3 to 5 minutes). At this point the tissue was washed with PSS1 several times over 60 to 90 minutes. Any decrease in the resting tension to less than 0.5 g was accommodated for in order to maintain a stable resting tension during that period. NA (30 μM) was added again in the same way and washed again, until the maximal tension developed in response to the NA was stable (usually 3 to 4 times). After establishing a stable response to NA, the functional integrity of the endothelium

was tested (described below). Following this, the tissue was challenged with increasing cumulative doses of one of three contractile agents; NA, KCl, or α,β,methylene-ATP (mATP). The volume of solution used to carry the agent and that was added to the 2.0 ml bath was small (10 to 50 µl) so as not to increase the volume of the bath significantly. In the case of mATP, no desensitization was detected when the cumulative doses of mATP were added within 8 minutes. In the experiments using KCl as a contractile agent, 3.5 µM phentolamine was present in the bath in order to block the effect of NA released from perivascular nerves due to the depolarizing effects of KCl. Following the addition of the last dose of the agent, the tissues were washed with PSS1 for 60 to 90 minutes, except in the case of mATP which was washed for 90 to 120 minutes in order to allow the tissue to fully recover from the desensitization.

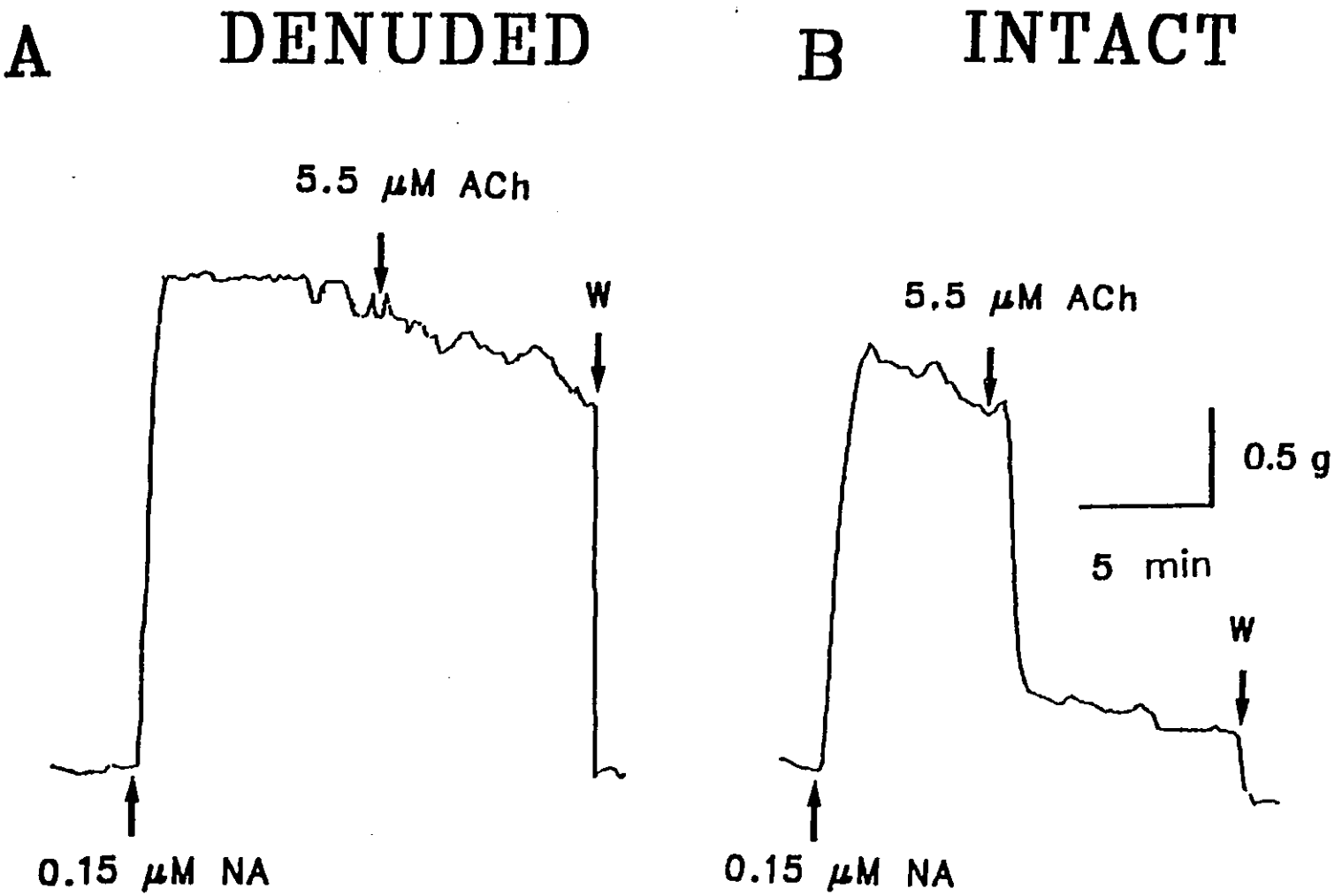
In experiments designed to look at the potentiating effect of NPY, 50 or 500 nM NPY was added to the bath and the resulting developed tension, if any, was recorded. Once the tension had either returned to the 0.5 g resting value or plateaued at a value near that, usually 5 to 15 minutes later, the addition of the cumulative doses of the contractile agent was carried out as before. In the experiments designed to study the effect of nifedipine (NIF) on NPY potentiation, after a control dose-response had been obtained from a ring

segment, 2.8 μM NIF was added to the bath and left for 15 minutes, at which point the contractile agent was added in the same fashion as in the control above. The tissue was washed for 60 to 90 minutes with PSS1 and 2.8 μM NIF was added as above, then 50 or 500 nM NPY was added in the manner above and finally the contractile agent was then added as it had been in the control period.

Acetylcholine test

Once the ring segments had equilibrated and the response to 30 μM NA was consistent, all of the ring segments were challenged with 0.15 μM NA and the resulting force that developed was recorded. After a sustained force had developed (~ 5 minutes), 5.5 μM acetylcholine was added to the bath. In the ring segments that were denuded there was only a relaxation of 6 ± 1 % (n=36) of the sustained force developed in response to 0.15 μM NA, whereas the intact ring segments relaxed 55 ± 2 % (n=41). The substantial relaxation observed in the intact ring segments upon the addition of acetylcholine clearly indicated that a functional endothelium was present (Figure 5 B). The inability of ACh to cause a significant relaxation (Figure 5 A) was evident of a successfully denuded vessel.

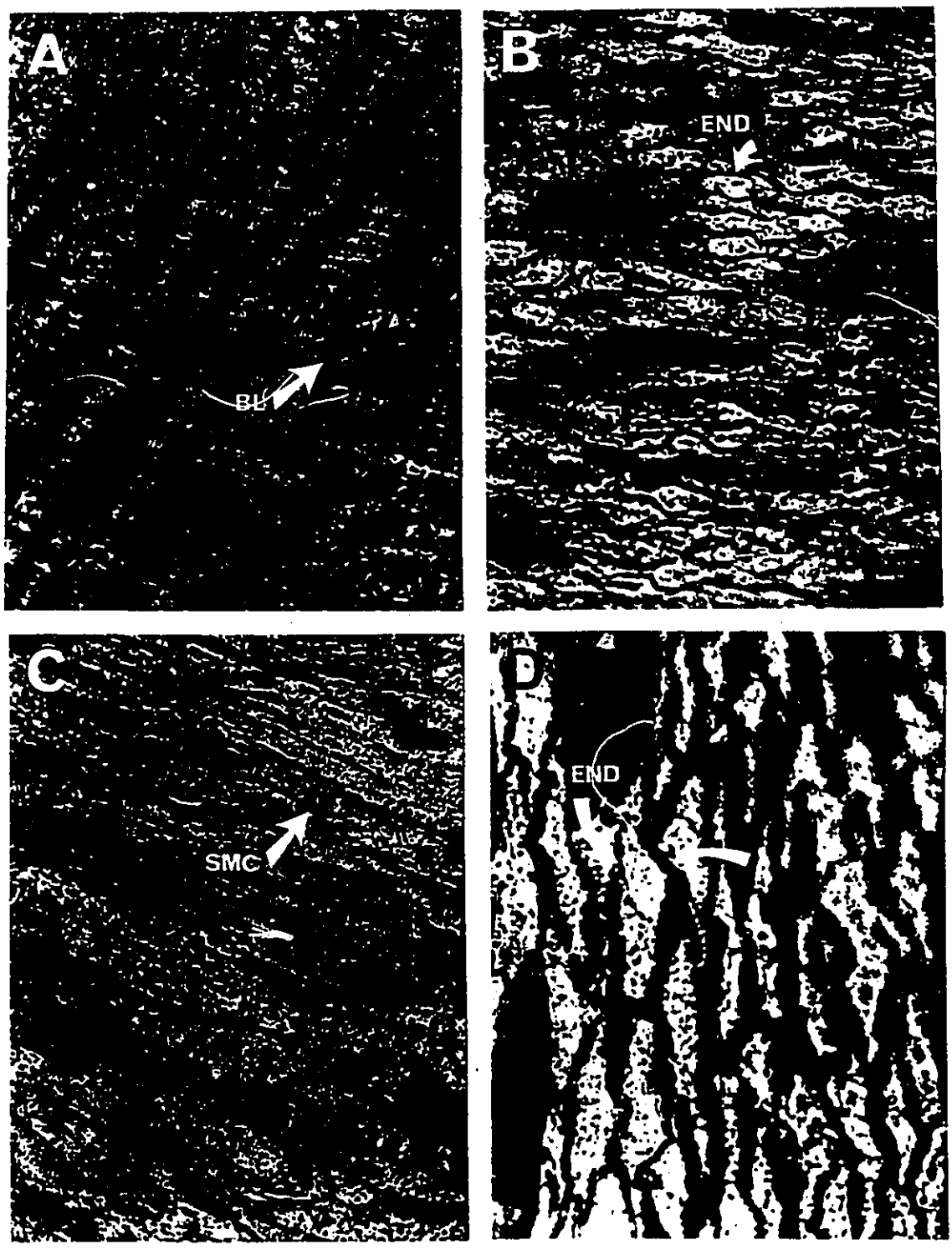
Figure 5. Example traces of the ability of acetylcholine (ACh) to relax two precontracted arteries with and without their endothelial lining. ACh is unable to relax the denuded vessel A which has been contracted by the application of NA (0.15 μM). The same concentration of ACh is able to produce a substantial relaxation of the precontracted intact vessel B. The (W) represents the points at which the vessel was washed with normal PSS1 to remove any drugs and their effects.


A
DENUDED
5.5 μM ACh
W
0.15 μM NA
B
INTACT
5.5 μM ACh
0.5 g
5 min
W
0.15 μM NA

Silver staining

In order to test the morphological integrity of the endothelium the endothelial cell borders were stained using a technique modified from that described by Nakatsu et al., 1988. The ring segments were cut open longitudinally with microscissors and pinned with the adventitia face down in a Sylgard coated petrie dish so that the endothelial lining was exposed. The tissue was pinned with tungsten pins (127 μm) ensuring that the tissue was pulled taut to eliminate folds in which the stain would collect. The tissue was washed twice for ~1 minute in 280 mM dextrose solution, and then immersed in 15 mM $AgNO_3$ solution for exactly 1 minute. It was washed twice more in the dextrose solution to remove any silver chloride precipitate before being immersed in $(NH_4)_2S$ for 20 seconds. Finally it was washed thoroughly once more with PSS to remove any excess silver sulphide which may have formed. It was then mounted on a slide with the adventia face down on the glass. In the segments that had been pulled over the coarse wire the silver sulphide stain was deposited uniformly over the entire face of the intimal surface of the vessel sometimes allowing visualization of the basal lamina (Figure 6 A) or the smooth muscle cells (Figure 6 C) underlying the basal lamina. The vessels which had not been denuded, clearly showed the presence of the intact endothelial lining producing somewhat of a honey comb-like pattern (Figure 6 B & D).

Figure 6. Light micrographs of segments of rat tail artery en face stained with a silver nitrate solution. After removal of the endothelium by gently rubbing the intimal surface of the vessel, the basal lamina (BL; shown with arrow) is visible as very long parallel bands roughly 15 μm in width and unestimatable length A). When the rubbing of the segment is continued, the vascular smooth muscle cells (SMC; shown with arrow) become visible with the staining C). They appear as elongated spindle-like cylinders with pointed ends that are interlocked and overlapping. When the vessel is not pulled over the coarse wire, the endothelium is left undisturbed and the staining produces a pattern which looks a honeycomb or tree bark in B and D. The endothelial cells (END; shown with arrows), about 30 μm in length, are much smaller than the smooth muscle cells which are about 100 μm long. The scale bar represents 45 μm in A-C and 20 μm in D.

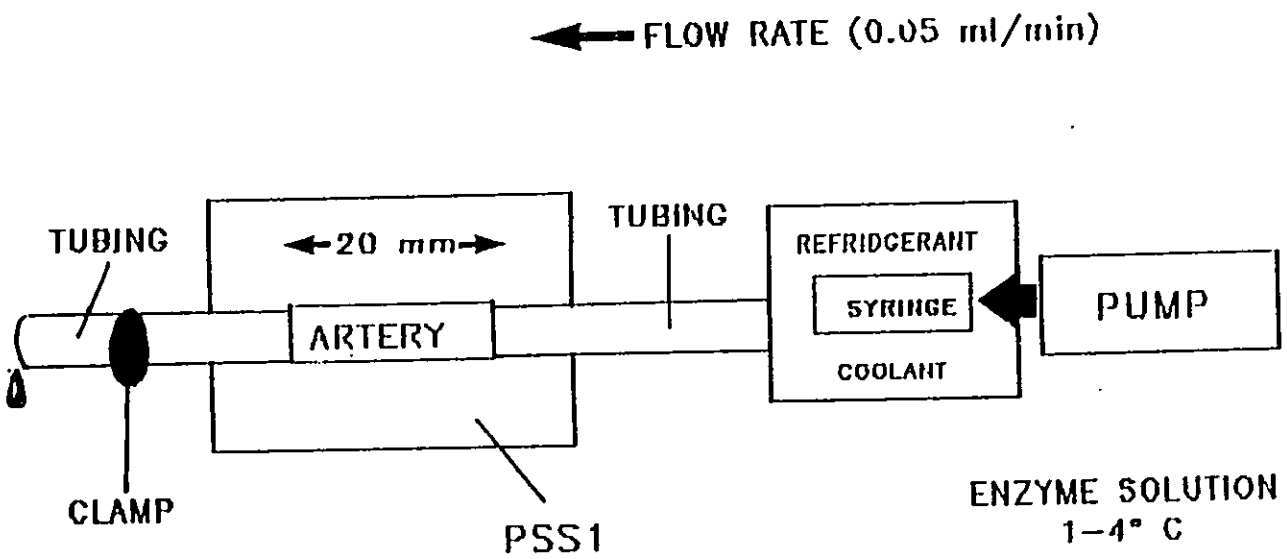

A
BL
B
END
C
SMC
D
END

Cell isolation study

Cells were isolated according to the procedure of Bolzon and Cheung (1989). Following the excision of the ventral rat tail artery as described above, the most proximal end of the artery was pulled, using fine forceps, onto the tapered end of a 15 cm long piece of PE 60 tubing that was fastened to the bottom of the petrie dish. Using silk suture thread, the artery was tied securely so that a 10 cc syringe filled with PSS2 and fitted with a 20 Ga needle could be used to inject the PSS2 through the tubing and into the vessel with enough pressure to distend the vessel slightly. The other end of the artery was then tied onto another 15 cm long piece of tubing and the artery was transferred to an isolation chamber shown in figure 7 where it was fixed to the bottom and superfused with PSS1 at a temperature of 36.5° C. The syringe, filled with PSS1, was placed in a Harvard syringe infusion pump 22 so that the artery could be perfused at a rate of 0.05 ml/min. After 90 minutes an enzyme solution of PSS2 containing 0.02% collagenase, 0.1% papain, and 4 mM dithiothreitol (DTT) was passed through a 0.45 µm hydrophilic nylon membrane filter unit. The activity of papain depends closely on the oxidation

Figure 7. A schematic of the cell isolation apparatus. The pump ensures that the artery is perfused with the chilled enzyme solution at a constant rate and the clamp permits an elevation of the perfusion pressure while maintaining the perfusion rate.

CELL ISOLATION



state of its sulfydyl groups, thus necessitating the presence of the reducing agent, DTT. The syringe perfusing the artery was filled with this enzyme solution and placed in a jacket of frozen gel refrigerant in order to maintain the syringe and it's contents at 1-4° C. The low temperature served to preserve the activity of the digestive enzymes. The artery was perfused at the same rate as before yet under increased pressure accomplished by increasing the flow resistance in the tubing attached to the distal portion of the artery. During the exposure to the enzyme, the artery gradually distended and increased in diameter and length by roughly 50%. After 75 minutes, when the artery became almost transparent, the artery was removed from the isolation chamber and placed in a 35 mm petrie dish where the tubing was then discarded and the artery was cut longitudinally using microscissors. The artery was then gently teased with fine forceps allowing the single cells to dislodge from the tissue and settle on the bottom of two 35 mm petrie dishes. The dishes were fixed in place next to one another on the stage of a microscope (Leitz Laborlux 12). Using 20 Ga needles, 15 cm pieces of fine latex tubing, and a sage peristaltic tubing pump model 375A, the cells were superfused with PSS3 at 22° C and a rate of 0.5 ml/min. The cells were followed using a video camera (JVC TK-10) and a system monitor (JVC TM-9010) and after 15 minutes, were photographed in 4 fields of 15 to 25 cells at 250 X

magnification with a mitsubishi video printer using K61S thermal paper. The dish of cells which were arbitrarily chosen as the treated group received 50 or 500 nM NPY. After 15 minutes of exposure to the NPY, more photographs of the same cells were taken. While the treated group continued to received NPY, both dishes were administered a low dose of one of three contractile agents: NA, mATP or KCl. After the cells had been exposed to the agents for 15 minutes and all shortening in response to the agent had stopped, more photographs of the same cells were taken. This was repeated with increasing doses of the contractile agent until a complete dose-response had been achieved. Once the photographs of the cells had been enlarged by 150 %, 30 cells from each group were arbitrarily chosen with the exclusion of cells not responding and cells which were already short (<40 μm). These cells were presumably damaged and or leaky to calcium. The lengths of these 60 cells were measured using a digitizing tablet (Jandel Scientific 2210) and Sigma Scan computer program (version 2.5).

Solutions

The composition of the physiological salt solutions (PSS1-PSS3) are shown in Table 4. The composition of the osmotically balanced KCl solutions are shown in Table 5. The solutions required for staining were: dextrose 280 mM, $AgNO_3$

15 mM, and $(NH_4)_2S$ 2% w/v. The nifedipine was prepared from a 2.8 mM stock solution of 50% ethanol which was prepared daily and diluted such that the final concentration of ethanol was 0.05%. The NPY was prepared from stock solutions of 5 and 50 μM that were stored at -20° C. All other chemicals were prepared fresh daily and were dissolved in PSS1 for ring segment studies and PSS2 or PSS3 for single cell studies.

Source of chemicals

The NPY (human, rat) was purchased from Peninsula Laboratories. The nifedipine was purchased from Miles Laboratories. The aqueous $(NH_4)_2S$ was purchased from Mallinckrodt. The α,β,methylene-ATP (mATP), NA ((-)-arterenol), dithiotheitol (DTT), and collagenase (type II, 400 Units/ml) were all purchased from Sigma. The papain, Hepes, silver nitrate, and all other chemicals were purchased from BDH.

Statistical analysis

The EC_{50}s were obtained from the curve which was estimated using an iterative Marquardt Levenberg curve fitting algorithm and the following four parameter logistic equation (Rodbard & Hutt, 1974):

$$Y = \frac{(a-d)}{(1+(\frac{X}{C})^{b})}$$

where a = asymptotic maximum response

b = slope of curve

c = inflexion point of curve or EC_{50}

d = asymptotic minimum response

x = concentration of agonist

and y = response by preparation

The results are all expressed as means ± standard error. All EC_{50}s were compared using a Z-score test. Paired students t-tests were used to compare the denuded and intact arterial ring segments. Paired students t-tests were used to compare the control with the NPY treated groups and analysis of variance between and within groups was used to ensure that the variability between animals used was not greater than the variance between the cells obtained from one animal. A value of $p<0.05$ was considered statistically significant. All students t-tests were performed using the computer program Sigmaplot (version 4.0) and the analysis of variance was performed using the computer program PC ANOVA.

Table 4. Ionic composition of physiological solutions (mM).

	PSS1	PSS2	PSS3
NaCl	120.0	137.0	120.0
KCl	5.0	5.4	5.0
KH_2PO_4	-----	0.4	-----
$NaHCO_3$	25.0	4.2	25.0
$CaCl_2$	2.5	-----	2.5
$MgSO_4$	1.0	-----	1.0
glucose	11.0	5.6	11.0
NaH_2PO_4	1.0	0.4	1.0
Hepes	-----	10.0	10.0
gas phase			
% oxygen	95	100	100
% CO_2	5	-----	-----
pH	7.4	7.4	7.4

Table 5. Sodium and potassium concentrations of depolarizing solutions (mM).

depolarizing solution #	1	2	3	4	5	6
KCl	10	20	30	40	60	90
NaCl	115	105	95	85	65	35

Results

Arterial Ring Segments

Effects of NPY on potassium-induced contractions

Increasing concentrations of KCl produced a concentration-dependent increase in the force of contraction by the arterial ring segments (Figure 8A). The average maximum contraction of the intact arteries, produced by the depolarizing solution was 62.8 ± 4.4 % (n=15) of the maximum contraction produced by NA (30 μM). The effect of NPY (50 nM) alone was a small contraction in 2 of 14 intact arteries. The contractions were 3.3 % of the maximal response to NA. Contractions to 30, 40, and 60 mM KCl were potentiated by NPY (50 nM) as seen in the example traces (Figure 8B). The maximum responses to KCl were unchanged before and after the addition of NPY. NPY produced the largest increases in the contractile response to KCl at 30 and 40 mM KCl. Figure 10A represents the mean log KCl-concentration response curve for 5 endothelium-intact arteries before and after the addition of NPY (50 nM). The curves illustrate the potentiation by NPY (50 nM) of the contractile response to 30, 40, and 60 mM KCl. There is no significant potentiation by NPY of the response to 10 or 20 mM KCl and the maximum response to KCl is not changed by NPY. The EC_{50} of the control curve was significantly shifted to the left from 42.6

Figure 8. Examples of original recordings illustrating the potentiating effects of NPY (50 nM) on potassium chloride (KCl)-induced contractions of a denuded arterial ring segment. The numbers under the arrows represent the KCl concentration (mM) of the solution bathing a single arterial ring segment. The ring segment was washed with physiological solution containing normal KCl concentration (5 mM) at W following the first cumulative concentration response and the tissue was allowed to recover for 60 minutes before adding NPY.

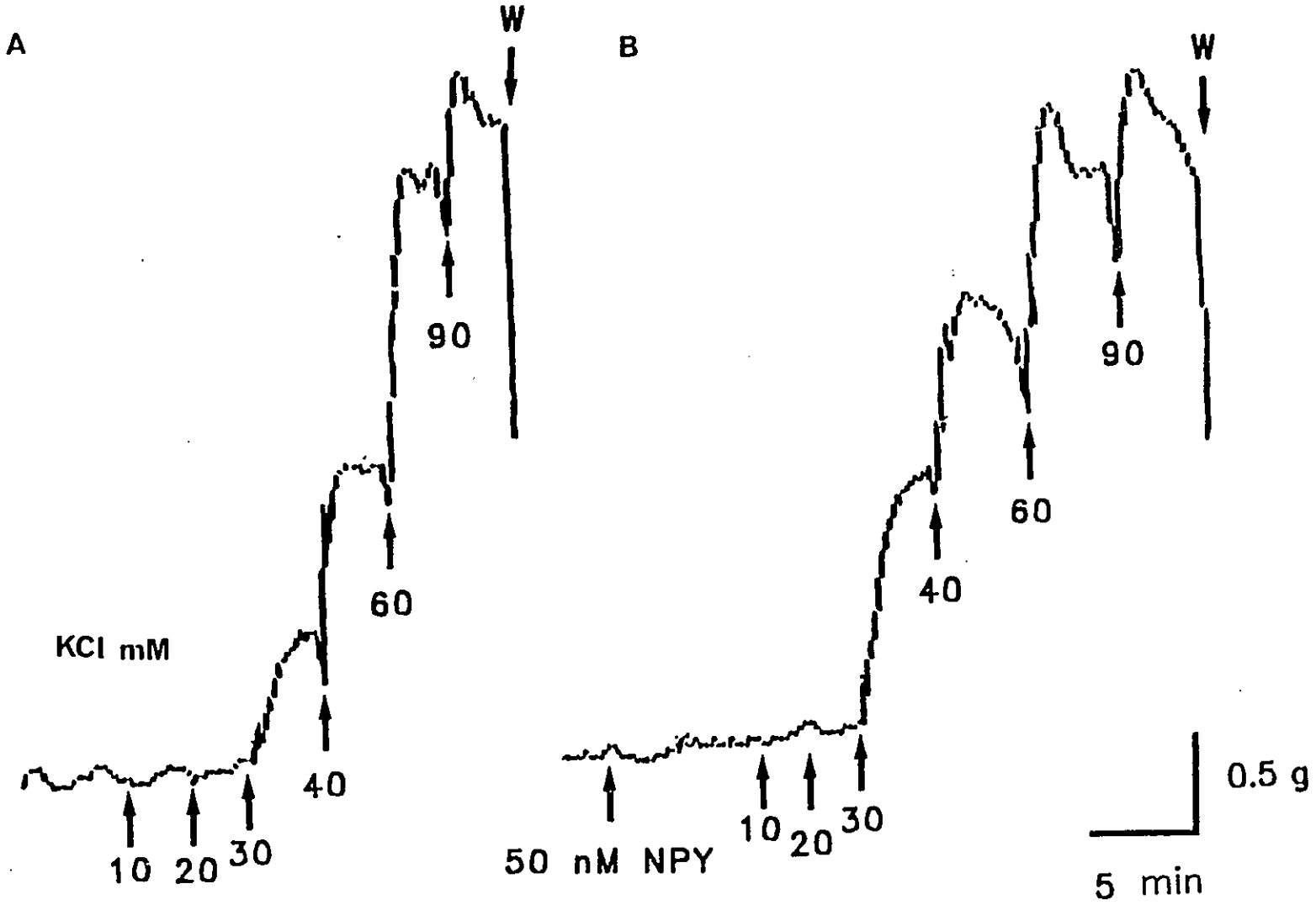
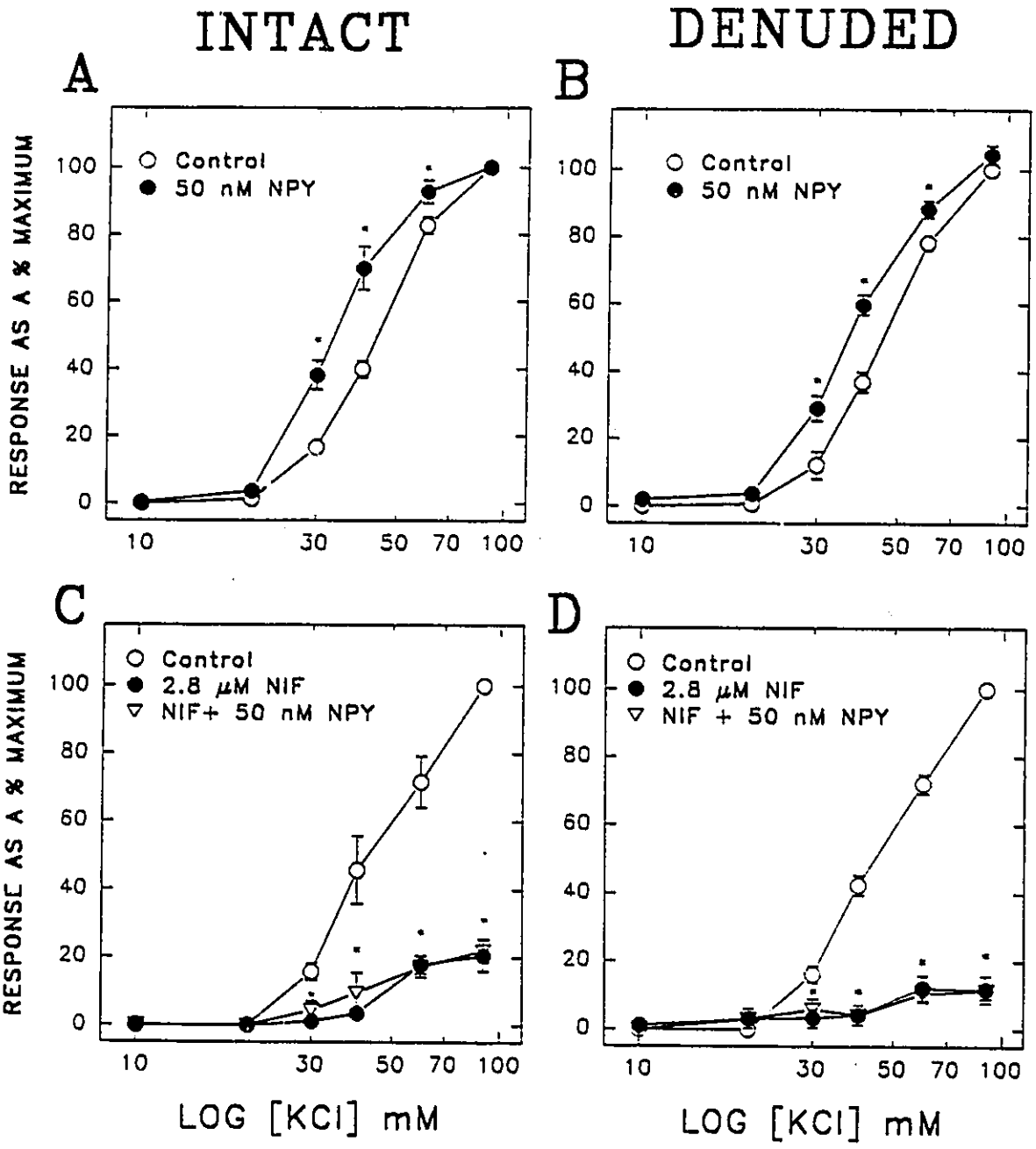

A
W
90
60
40
KCl mM
10
20
30
B
W
90
60
40
50 nM NPY
10
20
30
0.5 g
5 min

Figure 9. KCl-induced concentration response curves for intact and denuded arterial ring segments. Control responses for **A**, **B**, **C**, and **D** are open circles. The closed circles in **A** intact ring segments (n=14) and in **B** denuded ring segments (n=14) represent responses after the addition of NPY (50 nM). The closed circles and the open triangles in **C** intact ring segments (n=6) and in **D** denuded ring segments (n=6) represent responses after the addition of 2.8 μM nifedipine alone and 2.8 μM nifedipine with 50 nM NPY, respectively. All data points with error bars shown are means with S.E.M. and statistical significance using paired students t-test is indicated with asterisks ($p<0.05$).


INTACT
DENUDED
A
B
C
D
RESPONSE AS A % MAXIMUM
Control
50 nM NPY
2.8 μM NIF
NIF+ 50 nM NPY
NIF + 50 nM NPY
LOG [KCl] mM
0
20
40
60
80
100
10
30
50
70
100

± 1.0 mM to 35.6 ± 2.5 mM (n = 12) after the addition of NPY.

The KCl-induced contractile response was similar in the denuded arteries as in the intact arteries (Figure 9A and 9B). The maximum contraction produced by KCl in the denuded arteries was 55.2 ± 7.5 % (n=12) of the maximum contraction produced by NA. The addition of NPY (50 nM) caused a contraction that was about 3.4 % of the maximal response to NA in 3 of 14 denuded arteries. This response to NPY was similar in intact and denuded arteries. The potentiating effect of NPY (50 nM) on the KCl-induced contractions was also similar in the intact and denuded arteries. For 5 denuded arteries in Figure 9B, the control KCl concentration response curve, with an EC_{50} of 45.0 ± 1.2 mM, was shifted to the left in the presence of NPY yielding an EC_{50} of 38.5 ± 1.4 mM. Thus NPY potentiates the KCl-induced contractile response in both groups of arteries.

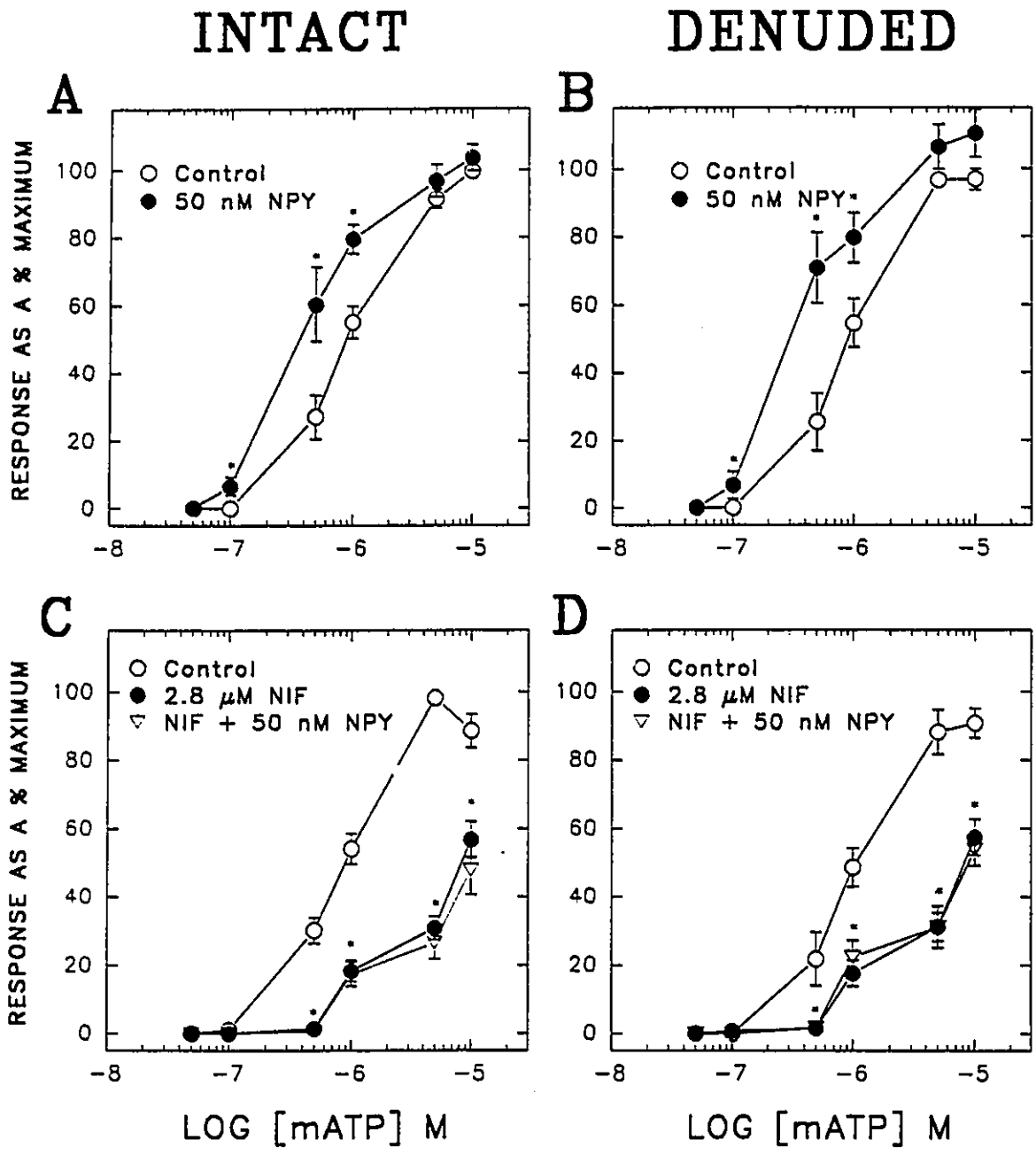
In both the intact and denuded arteries the addition of nifedipine (2.8 μM) to the bath greatly reduced the KCl-induced contractile response over the entire concentration range of KCl (Figure 9C, D). The maximum contractile response to KCl was reduced by 80 % and 87 % in intact and denuded arteries, respectively. This dramatic decrease in the contractile response to KCl with the addition of nifedipine suggests that the contraction is mediated by an influx of extracellular calcium through voltage operated calcium

channels (VOCC). The third curve in Figure 9C and D represents the KCl-induced response of arteries in the presence of nifedipine (2.8 μM) and NPY (50 nM). The contractions produced by KCl in the presence of the dihydropyridine was not potentiated by NPY. The KCl concentration response curves of the arteries in the presence of nifedipine alone and nifedipine with NPY are virtually superimposable with a maximum contraction of 21 % and 20 % of the maximum control response in the intact arteries and 13 % and 13 % in the denuded arteries. This clearly illustrates the nifedipine sensitivity of the potentiation of the KCl-induced response by NPY.

Effects of NPY on mATP-induced contractions

The vessels responded to mATP in a concentration dependent manner producing a sigmoid concentration response curve which plateaued at about 10 μM mATP. The contractile response to mATP was similar in intact and denuded arteries over the entire concentration range used (Figure 10A, B). The EC_{50}s were 953 ± 134 (n=6) and 955 ± 138 (n=6) (nM mATP) for the intact and denuded arteries respectively. The maximum contractions produced by mATP in intact and denuded arteries were 50.9 ± 2.4 % (n=12) and 47.4 ± 2.8 % (n=12) of the maximum response to NA. In both the intact and denuded arteries, NPY (50 nM) significantly potentiated the responses

Figure 10. Dose-dependent mATP-induced contractile responses of ring segments with and without an intact endothelium. Control responses for A, B, C, and D are open circles. In A) intact ring segments (n=9) and in B) denuded ring segments (n=9), the closed circles represent the responses after the addition of NPY (50 nM). Intact segments are on the left in C (n=8) while the denuded segments are in D (n=8). In both C and D the responses after the addition of 2.8 μM nifedipine alone are represented by the closed circles and the responses after the addition of both 2.8 μM nifedipine and 50 nM NPY are represented by triangles. Data points are means with S.E.M. with statistical significance shown with asterisks ($p<0.05$).


INTACT
DENUDED
A
B
C
D
RESPONSE AS A % MAXIMUM
Control
50 nM NPY
2.8 μM NIF
NIF + 50 nM NPY
LOG [mATP] M
100
80
60
40
20
0
-8
-7
-6
-5

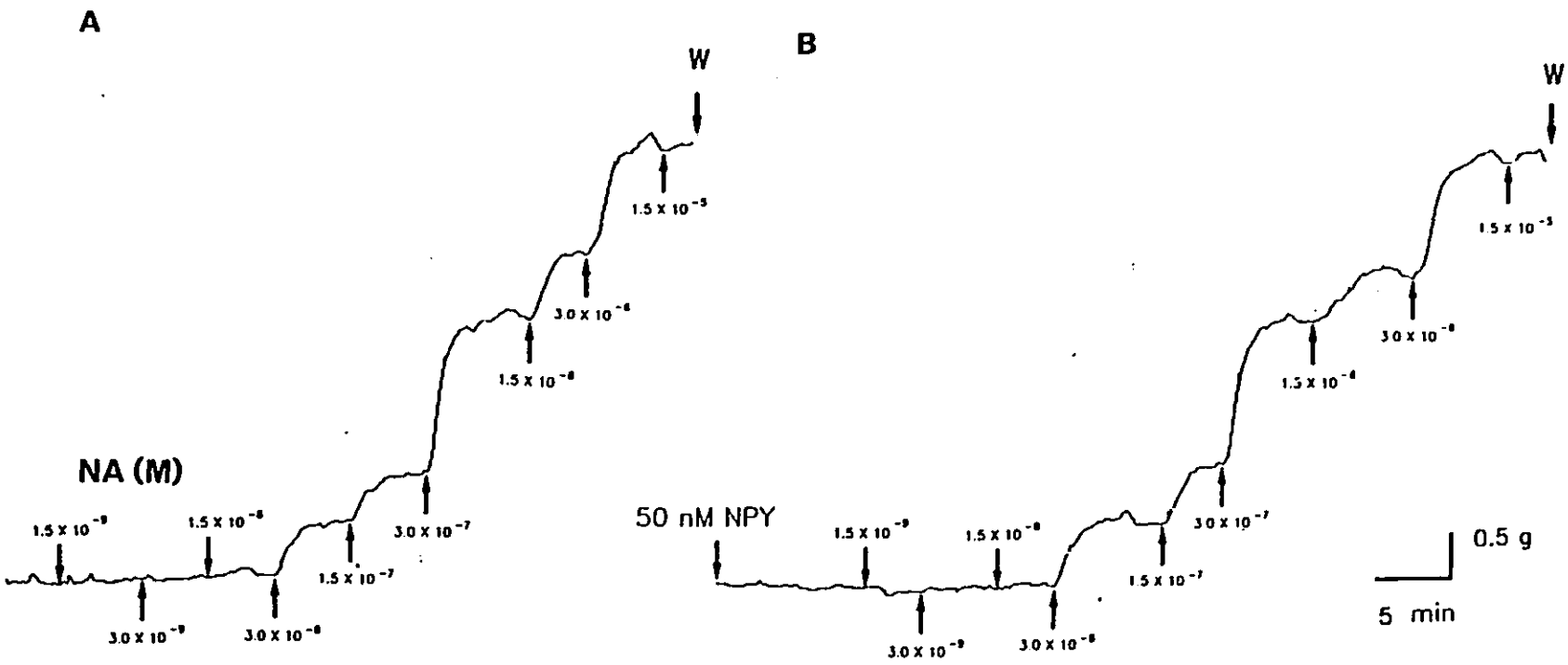
to 0.1, 0.5 and 1.0 μM mATP causing a leftward shift in the concentration response curves and decreased the EC_{50}s to values of 511 ± 103 and 511 ± 064 (nM mATP) respectively.

The addition of nifedipine (2.8 μM) to intact and denuded arteries in Figure 10C and D decreased the contractile responses to mATP by over fifty percent. Hence, the nifedipine sensitive component accounts for more than half of the contractile response to mATP. The subsequent addition of 50 nM NPY with 2.8 μM nifedipine resulted in responses which were virtually identical in magnitude as indicated by the second and third curves of Figure 10C and D. The mATP concentration response curves with nifedipine in Figure 10C and D, are superimposable, whether or not NPY is present. This suggests that all of the potentiation of the mATP response by NPY is blocked by nifedipine.

Effects of NPY (50 nM) on NA-induced contractions

The same concentration of NPY (50 nM) that produced a significant potentiation of KCl and mATP responses did not affect contraction induced by noradrenaline (NA). Figure 11A illustrates an example of an original recording of dose-dependent increases in contractile force developed by a denuded arterial segment in response to NA. After the removal of NA and 1 hour of recovery, the artery was exposed to 50 nM NPY (Figure 11B). The same cumulative doses of NA were then

Figure 11. Examples of original recordings of NA-induced contractile responses illustrating the lack of potentiation by NPY (50 nM). The arrows indicate the addition of cumulative concentrations of NA to a single denuded arterial ring segment **A.** The W represents the point at which the artery was washed with ringers and left to recover for 60 minutes. **B** After adding 50 nM NPY the concentrations of NA are added cumulatively.


A
W
NA (M)
1.5 X 10⁻⁹
1.5 X 10⁻⁸
1.5 X 10⁻⁷
3.0 X 10⁻⁷
1.5 X 10⁻⁶
3.0 X 10⁻⁶
1.5 X 10⁻⁵
3.0 X 10⁻⁹
3.0 X 10⁻⁸
B
W
50 nM NPY
1.5 X 10⁻⁹
1.5 X 10⁻⁸
1.5 X 10⁻⁷
3.0 X 10⁻⁷
1.5 X 10⁻⁶
3.0 X 10⁻⁶
1.5 X 10⁻⁵
3.0 X 10⁻⁹
3.0 X 10⁻⁸
0.5 g
5 min

added on top of the NPY. There was no change in the contractile response to NA after the addition of NPY (50 nM).

This lack of effect of NPY (50 nM) on the NA-induced contraction can also be seen in the concentration response curves of Figure 12A and B. The sigmoid curves of control and NPY treated tissue are superimposable, clearly illustrating the inability of NPY, at this concentration (50 nM), to have any significant effects on NA-induced contractions in intact and denuded arteries.

Effects of NPY (500 nM) on NA-induced contractions

When the concentration of NPY is increased from 50 nM to 500 nM there is a small but significant potentiation of the NA-induced contraction in the ring segments. This effect of NPY is similar in both the intact and denuded arteries (Figure 13A and B). The EC_{50}s for intact (138 ± 034 nM NA (control) and 95 ± 29 nM NA (NPY); n = 9) and denuded (199 ± 23 nM NA (control) and 158 ± 24 nM NA (NPY); n = 10) are significantly decreased and the concentration response curve is shifted to the left upon the addition of NPY (500 nM). This potentiation by NPY, however, is less effective on NA-induced responses at 500 nM than it is on KCl- and mATP-induced responses at just 50 nM.

The inhibitory effect of nifedipine (2.8 μM) was also smaller on NA-induced contractions than on those induced by

Figure 12. Noradrenaline concentration response curves before and after the addition of NPY (50nM). Control responses are open circles. Closed circles are responses to NA while exposed to NPY (50 nM). The responses by intact segments are shown in A (n=6) and the responses by denuded segments are shown in B (n=6). All symbols represent the mean and S.E.M.. Paired students t-tests were carried out for statistical significance and none were found to be different ($p<0.05$).

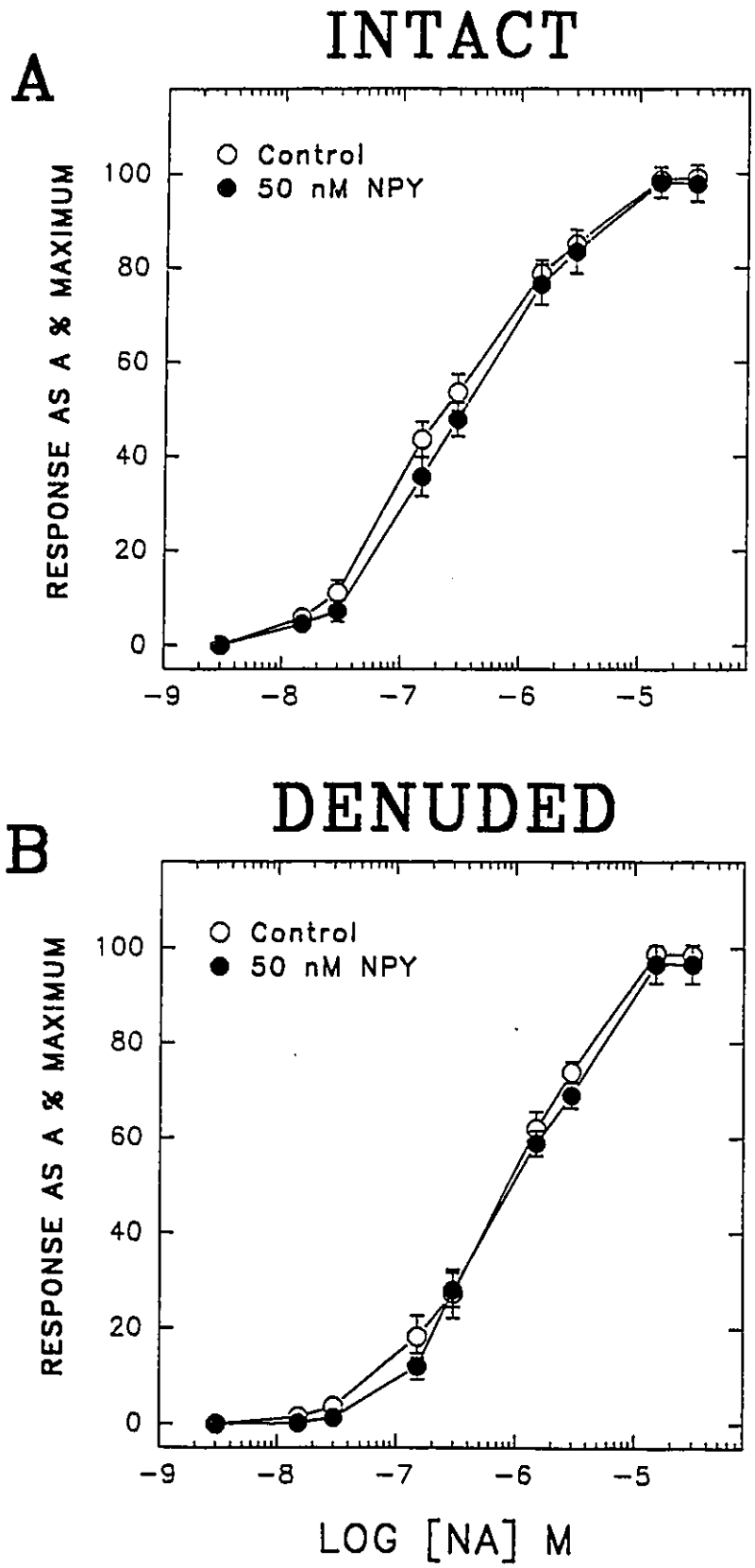
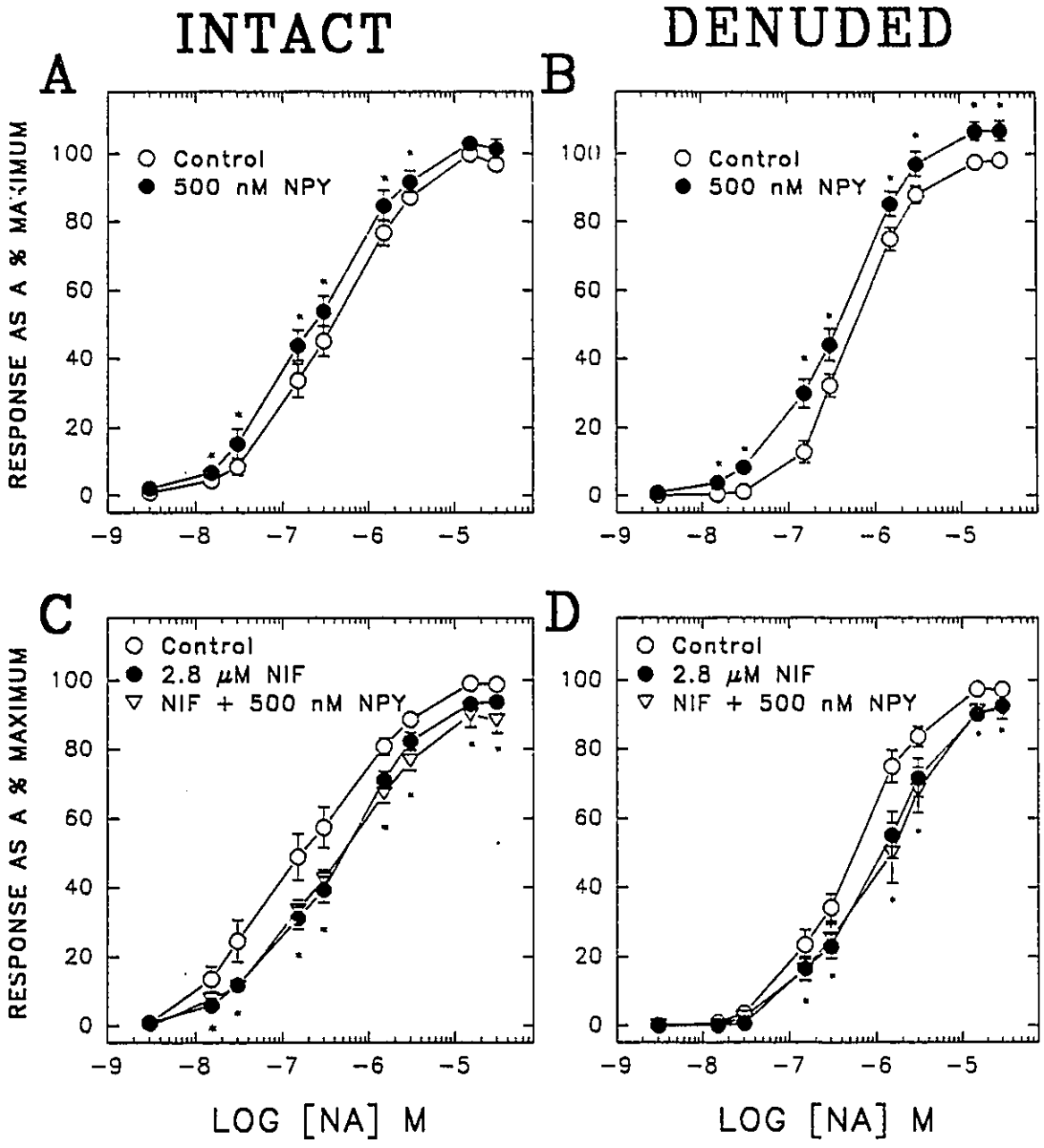

A
INTACT
Control
50 nM NPY
RESPONSE AS A % MAXIMUM
100
80
60
40
20
0
-9
-8
-7
-6
-5
B
DENUDED
Control
50 nM NPY
RESPONSE AS A % MAXIMUM
100
80
60
40
20
0
-9
-8
-7
-6
-5
LOG [NA] M

Figure 13. The effects of nifedipine (2.8 μM) and NPY (500 nM) on noradrenaline concentration response curves for intact and denuded arteries. Open circles represent control responses in A, B, C, and D. In A) the intact arteries (n=10) and B) the denuded arteries (n=9), the responses to NA after the addition of NPY (500 nM) are plotted with closed circles. The responses to NA with nifedipine (2.8 μM) present in C) the intact arteries (n=9) and D) the denuded arteries (n=7) are represented by the closed circles. The responses with nifedipine and NPY (500 nM) in C) and D) are shown with open triangles. Data shown are the means with S.E.M.. Statistical significance is indicated by an asterisk.


INTACT
DENUDED
A
B
C
D
RESPONSE AS A % MAXIMUM
RESPONSE AS A % MAXIMUM
Control
500 nM NPY
Control
500 nM NPY
Control
2.8 μM NIF
NIF + 500 nM NPY
Control
2.8 μM NIF
NIF + 500 nM NPY
0
20
40
60
80
100
−9
−8
−7
−6
−5
LOG [NA] M
LOG [NA] M

KCl and mATP. The NA-induced response was significantly attenuated 10-20 % by nifedipine. This effect of nifedipine was similar in intact and denuded arteries (Figure 13C and D). The EC_{50}s were significantly increased from 80.5 ± 20.6 nM to 17.3 ± 3.9 nM NA (n = 9) for intact vessels and from 20.0 ± 3.2 to 45.9 ± 11.2 nM NA (n = 7) for denuded vessels as the concentration response curve shifted to the right. When NPY (500 nM) was added in addition to nifedipine, there was no change from the responses with nifedipine and NA alone. The concentration response curves were superimposable and the EC_{50}s remained essentially unchanged (intact 197 ± 28 nM; denuded 677 ± 170 nM). The NPY potentiation of the responses to NA in both arteries in Figure 13A and B was completely blocked by nifedipine as it was in Figures 9 and 10 with KCl and mATP.

Single vascular smooth muscle cells

Morphology and viability of single cells

The isolation technique described earlier yielded 5.6 x 10^4 cells/ml of which 98% exclude trypan blue (Figure 14). More sensitive tests of viability were 1) the calcium tolerance of the cells and their ability to remain relaxed (>40 μm) in physiological calcium (2.5 mM) and 2) the functional integrity of the contractile proteins and machinery necessary for contraction. The percentage of cells that were relaxed (>40

Figure 14. A light micrograph illustrating a typical field of freshly isolated vascular smooth muscle cells in PSS1.

100 µm

μm) was 93, and of those 93 %, less than 4 % of these cells failed to contract in response to NA. The mean cell length for the cells of the control group was 113.14 ± 0.96 μm (n = 570 cells from 19 arteries, 30 cells per artery) and 112.90 ± 0.98 μm (n = 570 cells from 19 arteries, 30 cells per artery) for the NPY group. These lengths are in agreement with the lengths of the smooth muscle cells that can be seen in the silver nitrate staining (Figure 6C). The cells contract to receptor activation by NA (Figure 15) and mATP and by depolarizing with high potassium solutions (Figure 16 A-E). These contractions are concentration dependent. As the cells contract and their length decreases, their width increases. Eventually, a 120 μm long cell at rest, shaped like a cylinder with tapered ends, becomes a 20 or 30 μm spherical ball after contracting maximally (Figure 15 A-D).

Effects of NPY on KCl-induced cell shortening

The addition of increasing concentrations of KCl produced a dose-dependent shortening of the cells (Figure 16A-E). The resting length of the cells in the control group (111.89 ± 1.88 μm) was not statistically different from the length of the cells in the paired NPY treated group (115.14 ± 2.27 μm; n = 150 cells from 5 arteries). When NPY (50 nM) was added, the cells contracted slightly, shortening in length to 104.02

Figure 15. A series of light micrographs depicting the contraction by two cells in response to increasing concentrations of NA. Two cells at rest in PSS1 **A** are exposed to 3 x 10^{-9} M NA **B** and undergo some shortening. The cells shorten considerably in **C** when the concentration of NA is increased to 3 x 10^{-7} M. The maximizing of the volume/surface area ratio can be seen as the cells reach a point in the contraction **D** (3 x 10^{-5} M NA) where they are spherical balls and cannot shorten any further.

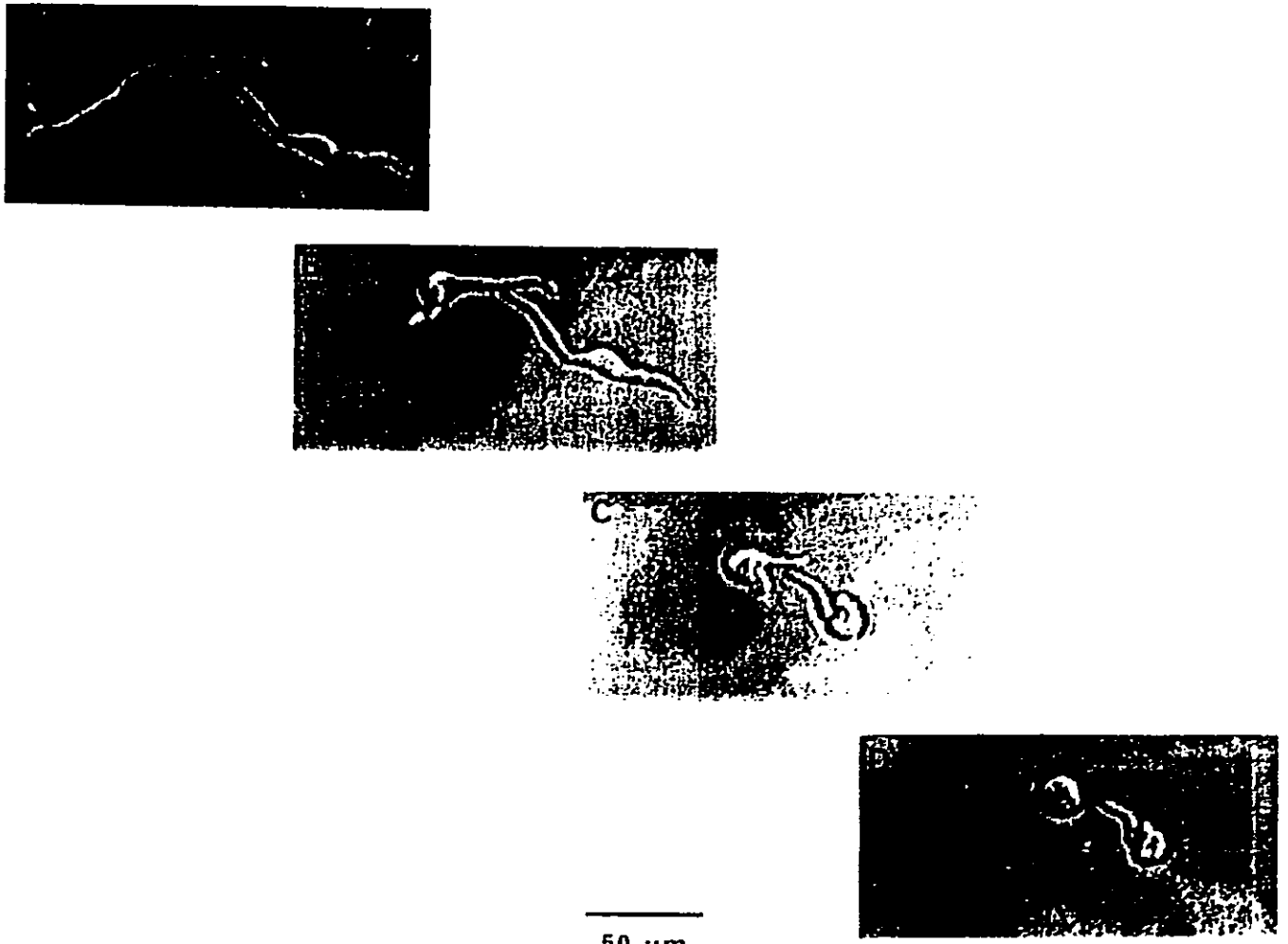
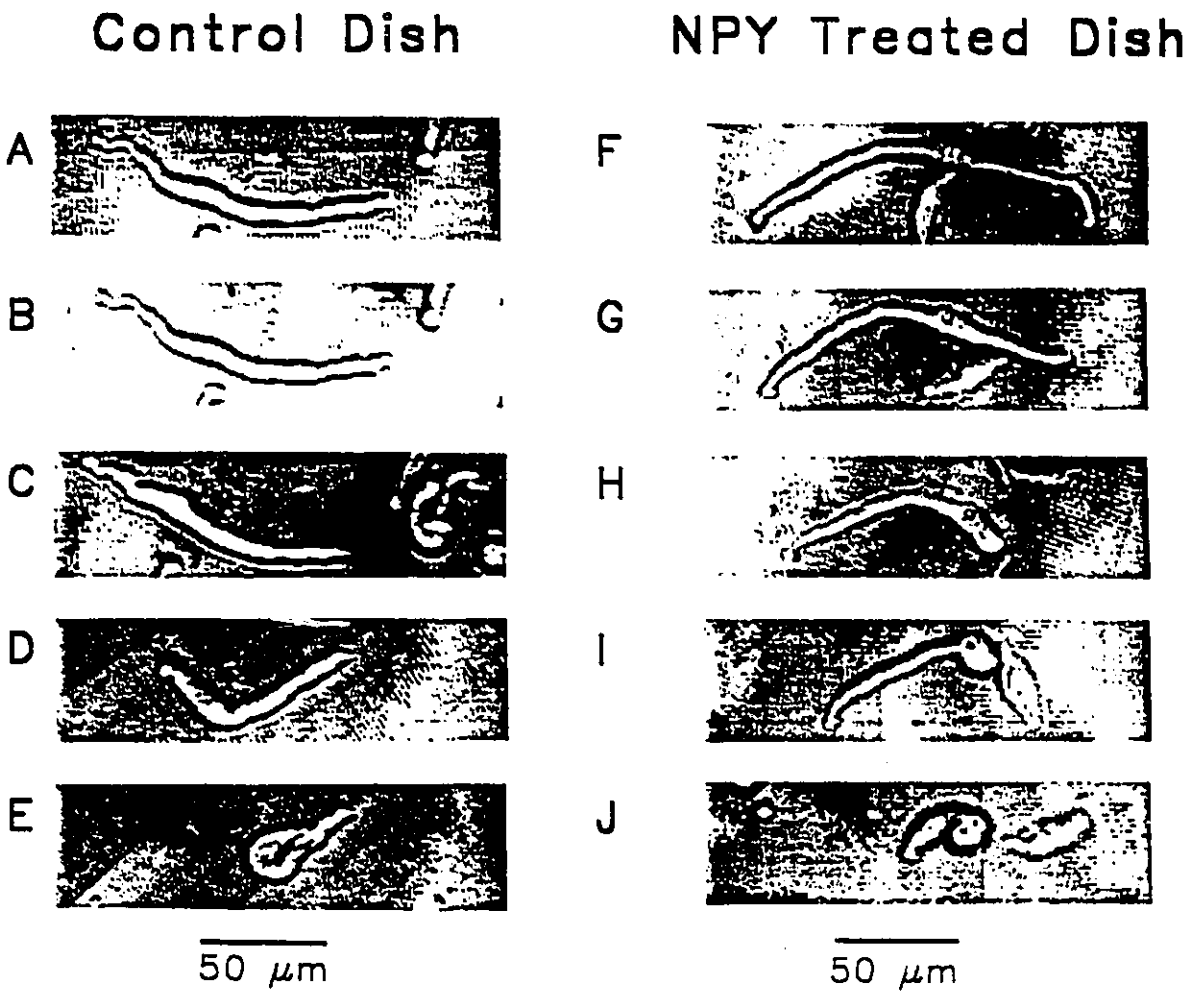

C
50 µm

Figure 16. Example of NPY (50 nM) potentiation of KCl-induced cell shortening. The panels A-E are light micrographs of a single cell in the control dish while the images to the right in F-J are of a cell in the NPY treated dish. Panels A and F show both cells at rest in PSS3. The cell in panel G shortens slightly when NPY (50 nM) is applied, while the cell in panel B remains at rest in PSS3 without NPY. The remaining panels depict the cells' response to increasing concentrations of KCl; (20 mM) C and H, (40 mM) D and I, and (90 mM) E and J. The cell in the presence of NPY contracts more, at each concentration of KCl, and reaches a shorter final length J than the cell without NPY E.


Control Dish
NPY Treated Dish
A
B
C
D
E
F
G
H
I
J
50 μm
50 μm

± 2.18 μm. Further addition of KCl to the cells in the presence of NPY (50 nM) (Figure 16H-J) resulted in contraction, shortening the cells to a much greater extent than control (Figure 16C-E). The shortening in response to 90 mM KCl resulted in a length of 45.08 ± 1.87 μm in the NPY group of cells and 57.70 ± 2.07 μm in the control group of cells which did not receive NPY. This significant potentiation was similar to that seen in the ring segments.

This potentiation by NPY can be seen also in the cell length frequency histogram of Figure 17. The distribution of the cells of a given length is normal around the mean. There is no shift in the distribution of cells in the control dish in Figure 17 A and B. When 40 mM KCl is added the histogram shifts to the left indicating a shortening of the cells. In the NPY treated dish there is a slight left-ward shift in the histogram upon application of NPY (50 nM). There is also a left-ward shift when 40 mM KCl is subsequently added. This shift is greater in magnitude than the shift in the control cells. The mean lengths of the control cells are statistically different from the NPY cells in panels B and E and panels E and F but not panels A and D. The skewed appearance of a cell length histogram, such as in panel F, has been observed before by others (Singer & Fay, 1977) and is due to the arrangement and design of the contractile machinery in the smooth muscle cells (Fay & Delise, 1973) which prevents

Figure 17. Histograms illustrating the direct effects of NPY (50 nM) on the cell length and the effects on cell shortening in response to 40 mM KCl. In the top series of histograms the distributions represent the percentage of cells at a given length at rest in PSS3 **A** again in PSS3 **B** and after 40 mM KCl had been added **C**. The bottom histograms represent the percentage of cells a given length at rest **D** as in **A** and after the addition of NPY (50 nM) **E** as opposed to the corresponding cells at rest in **B**. The histogram in **F** represents cells that have been exposed to NPY and 40 mM KCl and are paired with the cells in **C**.

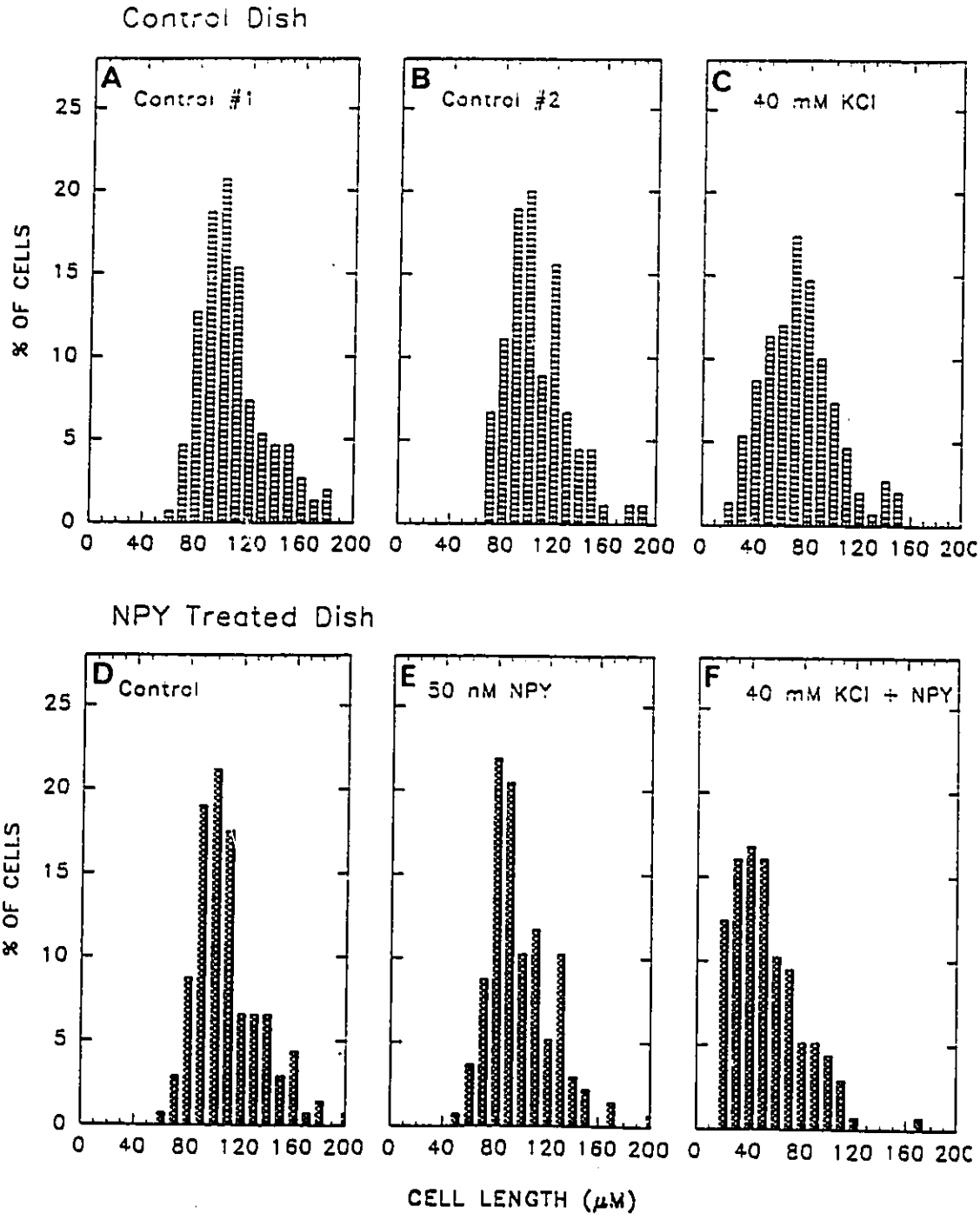
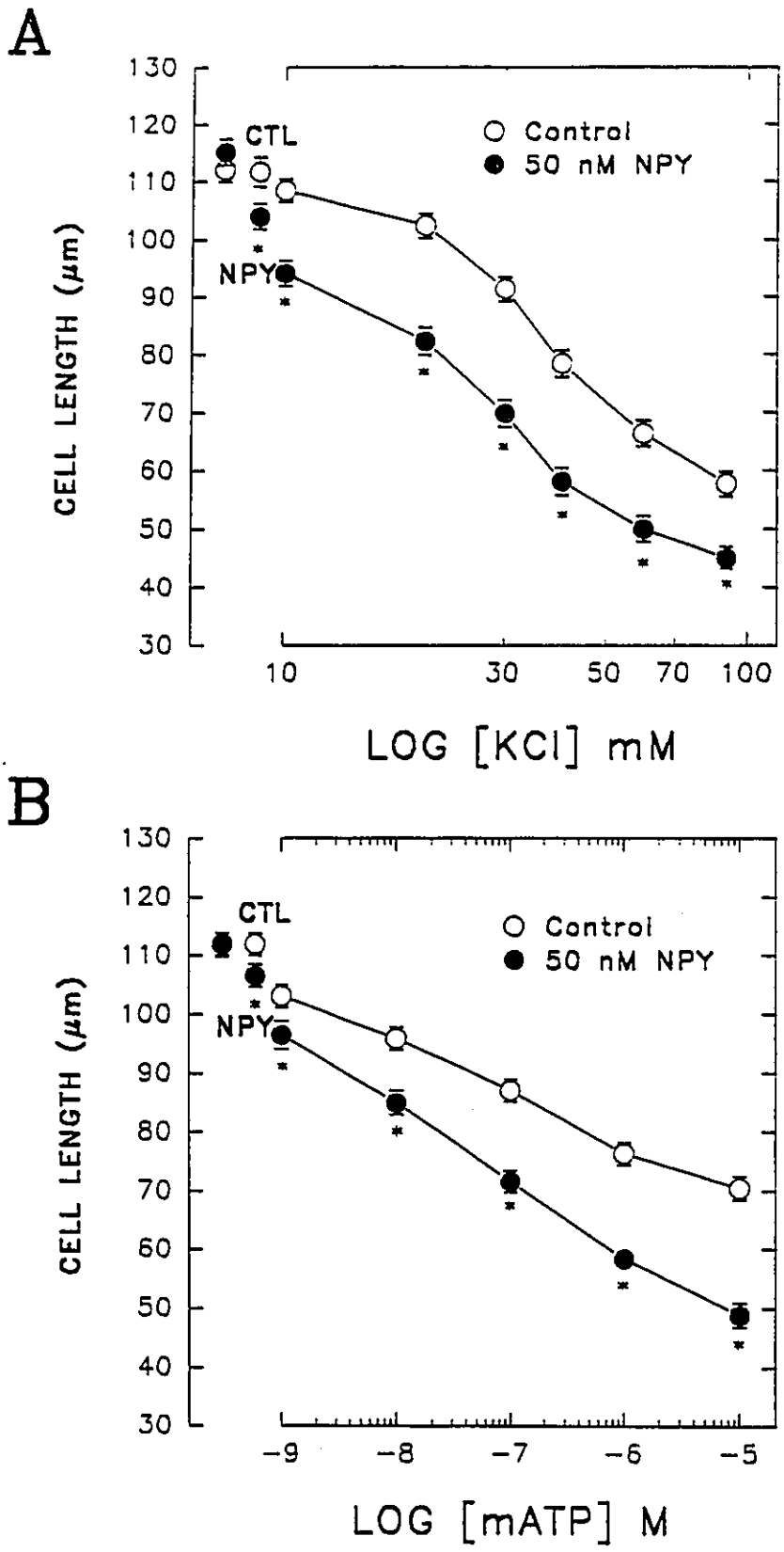

Control Dish
A
Control #1
B
Control #2
C
40 mM KCl
NPY Treated Dish
D
Control
E
50 nM NPY
F
40 mM KCl + NPY
% OF CELLS
0
5
10
15
20
25
0 40 80 120 160 200
CELL LENGTH (μM)

Figure 18. NPY (50 nM) induced potentiation of KCl- and mATP-concentration response curves. In **A**, the control KCl concentration response curve (open circles) is shifted to the left by the addition of NPY (50 nM) (closed circles). A similar shift of the mATP concentration response curve (open circles) in **B** occurs when NPY (50 nM) (closed circles) is added. All data points are the means and S.E.M. of 150 cells of 5 arteries, 30 cells per artery. The asterisks represent statistical significance using paired student's t-test ($p<0.05$).


A
CELL LENGTH (μm)
130
120
110
100
90
80
70
60
50
40
30
CTL
NPY
Control
50 nM NPY
10
30
50
70
100
LOG [KCl] mM
B
CELL LENGTH (μm)
130
120
110
100
90
80
70
60
50
40
30
CTL
NPY
Control
50 nM NPY
−9
−8
−7
−6
−5
LOG [mATP] M

further shortening of the cells beyond 20-30 μm.

The potentiating effects of NPY (50 nM) on KCl-induced cell shortening can also be seen in the concentration response curves of Figure 18A. The control KCl-concentration response curve is significantly shifted to the left upon the addition of NPY such that the EC_{50} of the control cells (40.74 ± 3.57 mM) is statistically larger than that of the NPY treated cells (29.71 ± 3.09 mM; n = 150 cells of 5 arteries, 30 cells per artery).

Effects of NPY on mATP-induced cell shortening

The resting length of cells in the control group (111.89 ± 1.88 μm) was not statistically different from the length of the cells in the paired NPY treated group (111.78 ± 2.07 μm; n = 150 cells from 5 arteries). The addition of mATP induced a concentration dependent decrease in the cell length (Figure 18B). The ability of mATP to induce cell shortening was about 50 % as effective as NA. This difference in the effectiveness of the two drugs was similar in the contraction studies of the ring segments. The application of NPY (50 nM) caused a significant potentiation of the mATP-induced cell shortening. At 10 μM of mATP, the cell length for the NPY group (49.94 ± 1.50 μm) was significantly shorter than the cell length for the control group (69.01 ± 1.83 μm) (Figure 18B). The EC_{50}s were also significantly different for the control groups (8.43 ± 2.80 μM) with respect to the NPY treated cells (0.25 ± 0.11

μM) resulting in a left-ward shift in the control mATP-concentration response curve.

Effects of NPY (50 nM) on NA-induced cell shortening

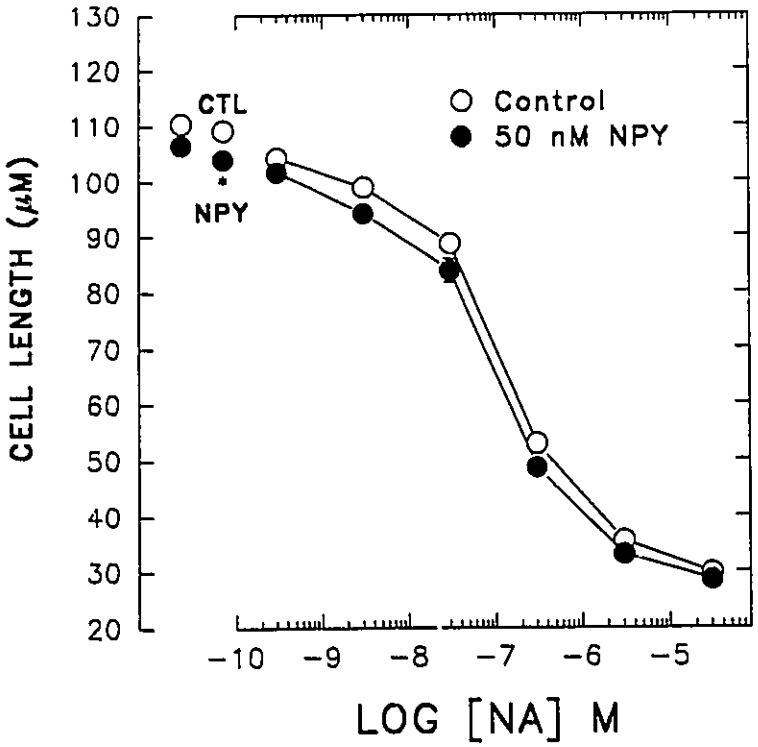
The resting cell length of the control group (110.45 ± 1.81 μm) was not statistically different from the NPY treated group (108.78 ± 1.97 μm; n = 120 cells from 4 arteries). Dose-dependent shortening of the cells occurred following the addition of increasing concentrations of NA (Figure 19A). Although the cell length of the control group (109.16 ± 1.90 μm) was statistically different from the NPY treated group (104.03 ± 1.72 μm) after the application of NPY (50 nM), the presence of NPY did not affect responses to NA. At no concentration of NA was the cell length of the control group statistically different from the NPY treated group. The cell length of the control group at 30 μM NA was 29.73 ± 0.76 μm as opposed to the cell length of the NPY treated group (28.48 ± 1.12 μm).

Effects of NPY (500 nM) on NA-induced cell shortening

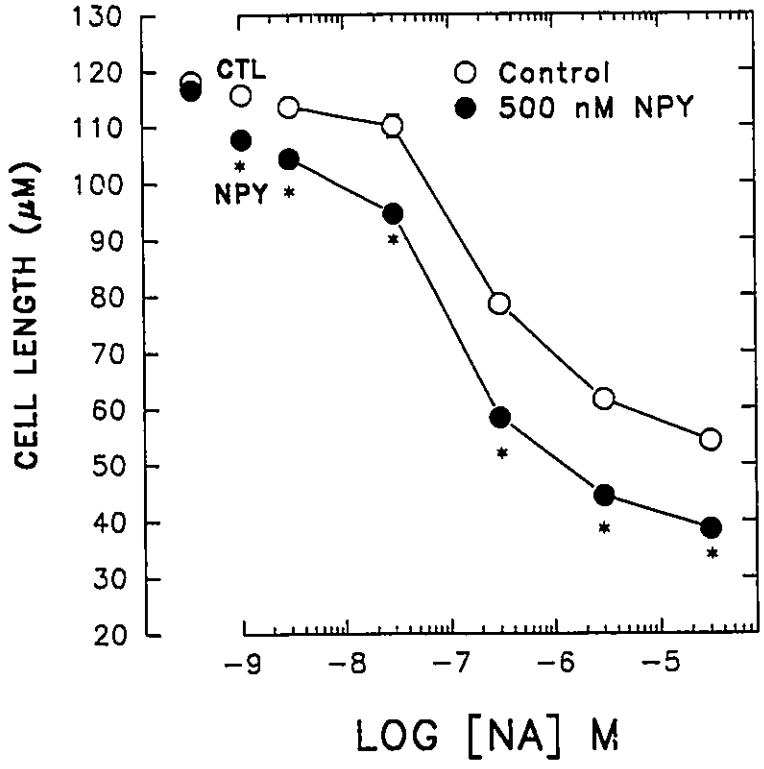
The cells of the control group contracted in response to NA as they did previously (Figure 19B). The resting cell length for the control group (118.18 ± 1.90 μm) and for the NPY treated group (116.77 ± 1.69 μm; 150 cells of 5 arteries, 30 cells per artery) were not statistically different. The

Figure 19. The effects of NPY at concentrations 50 and 500 nM on NA-induced cell shortening. The lack of effect of 50 nM NPY (closed circles) on the NA concentration response curve (open circles) is shown in **A**, whereas the significant potentiation by the higher concentration of NPY (500 nM) (closed circles) of the NA concentration response curve (open circles) is illustrated in **B**. All data points are the means and S.E.M. of 150 cells of 5 arteries, 30 cells per artery. Statistical significance using paired student's t-test ($p<0.05$) is represented by the asterisks.

A


CELL LENGTH (μM)
130
120
110
100
90
80
70
60
50
40
30
20
CTL
NPY
Control
50 nM NPY
−10
−9
−8
−7
−6
−5
LOG [NA] M

B


CELL LENGTH (μM)
130
120
110
100
90
80
70
60
50
40
30
20
CTL
NPY
Control
500 nM NPY
−9
−8
−7
−6
−5
LOG [NA] M

addition of NPY (500 nM) produced a significant decrease in cell length to 107.76 ± 1.66 μm. This was greater than the decrease in length produced by NPY (50 nM) (Figure 19A). In addition to causing a contraction by itself, NPY (500 nM) potentiated the contractile response to NA whereas NPY (50 nM) did not. At 30 μM NA the cells shortened to 38.68 ± 1.11 μm in the NPY treated group as compared to 54.18 ± 1.68 μm in the control group.

Discussion

Using two separate preparations, the involvement of voltage-sensitive calcium channels and the role of the endothelium in the mechanisms involved in the potentiating effects of NPY were examined. The first preparation consisted of arterial ring segments in half of which, the endothelium was removed while the other half were left undisturbed. These two groups were then compared. The second preparation consisted of freshly isolated vascular smooth muscle cells which were free from any influence from the nerves or the endothelium. The results from this preparation were compared to the results using the arterial segments.

The evidence implicating voltage-sensitive calcium channels in the actions of NPY is substantial. Contractions of 3rd generation mesenteric arterioles of the rat, elicited by potassium-chloride depolarization or the addition of calcium chloride to vessels already depolarized with potassium-chloride were potentiated by NPY (Andriantsitohaina & Stoclet, 1988). These contractions were inhibited by nitrendipine, a voltage-sensitive calcium channel blocker. NPY produced no effect on the contractions in the presence of nitrendipine other than to enhance the ability of the inhibition by nitrendipine. Nitrendipine blocks the voltage-sensitive calcium channel better when the channel is in the open state.

Hence, the NPY is likely enhancing the opening of the voltage-sensitive calcium channels. When a contraction occurs in response to an agonist, NPY potentiates only the component of the contraction which is sensitive to calcium channel blockers. The results of Andriantsitohaina & Stoclet, (1990) show, in rat mesenteric arterioles, that NPY potentiated only the calcium entry blocker sensitive component of contraction induced by stimulation of α_1-adrenoceptors. Nifedipine was also found to block the NPY potentiating effects of NA-induced contractions in isolated rat femoral arteries and mesenteric arteries (Pernow et al., 1986). Similarly, NPY potentiates only the nifedipine sensitive component of neurally-induced contractions of isolated rat femoral arteries and mesenteric arteries (Pernow et al., 1986) and guinea-pig saphenous arteries (Cheung, 1991). In the guinea-pig saphenous artery NPY specifically potentiated the purinergic response to stimulation. This was most likely due to the fact that ATP activates calcium channels in vascular smooth muscle cells (Benham, 1989).

This study investigated the role of voltage-sensitive calcium channels by comparing the potentiating effect of NPY on contractions to KCl, mATP and NA. A low concentration of NPY (50 nM) significantly potentiated KCl- and mATP-induced contractions. Although the responses to the maximum and the minimum concentrations of KCl were unchanged by 50 nM NPY, the

responses to the concentration of KCl that produced a contraction that was roughly 50 % of the maximum response was greatly enhanced by NPY. A similar potentiation by NPY (50 nM) of the mATP-induced contractions was observed. The responses to the maximum and minimum concentrations of mATP were similar in magnitude but the EC_{50} was significantly decreased. There already exist reports of NPY potentiation of KCl-induced contractions of rat resistance vessels (Andriantsitohaina & Stoclet, 1988) and of the purinergic component of nerve stimulation of guinea-pig saphenous artery (Cheung, 1991) and rabbit ear artery (Saville et al., 1990).

In the NA-induced contractions, NPY at the same concentration (50 nM) did not produce any significant potentiation. The contractile response to NA remained unchanged when NPY (50 nM) was used. The NA-concentration response curves are virtually superimposable. Only at the high concentration of NPY (500 nM) was any potentiation of the NA-induced contractions observed. Using this concentration, NPY caused an increase in the NA response smaller but similar to the increase of responses to KCl and mATP that just 50 nM NPY did. Neither the response to the maximum nor the minimum concentration of NA was altered by NPY (500 nM) but the responses to NA at concentrations between maximum and minimum were significantly potentiated. There was an obvious difference in the efficacy of NPY to potentiate contractions

to NA as opposed to KCl and mATP. A similar difference between NPY potentiation of contractions to NA and mATP released by nerve stimulation in guinea-pig saphenous artery was observed (Cheung, 1991). Also, in the guinea-pig vas deferens, NPY greatly enhanced responses to mATP and to a lesser extent to exogenous NA (Ellis & Burnstock, 1990). In order to establish whether this difference was due to the difference in the relative amounts of calcium influx through voltage-sensitive calcium channels, nifedipine was used.

First, the component of the contraction that was nifedipine-sensitive was identified. The ability of nifedipine to block the contractions was much greater when KCl and mATP were used than when NA was used. The contractions to KCl were almost completely abolished by nifedipine. The maximum response to KCl in the presence of nifedipine was less than 25 % of the maximum control response. The remaining component of the response to KCl that was nifedipine resistant could have been due to the slowing down of the Na^{+}/Ca^{2+}-exchanger. This protein allows an influx of 3 sodium in exchange for 1 calcium. It is driven by the concentration gradient for sodium and when sodium was replaced by potassium in the high potassium solutions, the driving force would be decreased and there would be a build up of calcium in the cell. Nifedipine also had a substantial inhibitory effect on the contractions induced by mATP with the maximum response

decreased by more than 50 %. This nifedipine sensitivity of mATP related contractions has been previously reported (Pernow et al., 1987b). The nifedipine-insensitive component of the contraction was due to mechanisms other than those involving voltage-sensitive calcium channels. The primary action of mATP is to activate P_{2x} receptors which would allow an influx of calcium and sodium which results in depolarization (Benham, 1989). There are also reports that activation of ATP receptors may produce an increase in intracellular calcium via a second messenger but the primary mechanism of contraction by ATP is via depolarization (Fujino, et al., 1991). The results of this study show that in the rat tail artery, the contractions in response to NA are not very nifedipine-sensitive relative to the KCl and mATP responses. The maximum response to NA in the presence of nifedipine was still greater than 90 % of the control response. This could be explained by the fact that mATP and KCl induce contraction primarily via electromechanical coupling and NA induces contraction primarily via pharmo-mechanical coupling. KCl depolarizes the membrane by altering the electrochemical gradient for potassium. This depolarization is sufficient to activate the voltage sensitive calcium channels which results in an influx of calcium and contraction. The process by which mATP induces contraction is similar to KCl in that the membrane is depolarized. NA is much different. NA interacts with α-

adrenoceptors initiating two distinct processes of calcium mobilization for contraction. One process involves the metabolism of phosphatidylinositol resulting in release of intracellular calcium stores. The other involves the influx of calcium and is independent of phosphatidylinositol turnover (Chiu et al., 1987). The potentiating effect of NPY does not involve phosphatidylinositol turnover (Reynolds & Yokota, 1988), nor does NPY have any effect on the intracellular calcium stores (Erne & Hermsmeyer, 1988).

The greater nifedipine-sensitivity of the KCl- and mATP-induced contractions corresponded to the greater ability of NPY to potentiate these contractions. Once the component of the contraction which was due to the influx of calcium through voltage sensitive calcium channels had been blocked by nifedipine, the nifedipine was tested on the NPY potentiation. After the addition of nifedipine, NPY (50 nM) had no effect on the contractile responses to KCl, mATP, nor did NPY (500 nM) have any effect on NA-induced contractions. The potentiation by NPY was completely blocked by nifedipine (2.8 μM). These findings are similar to those in which NPY potentiates only the component of the contraction which is sensitive to calcium channel blockers (Pernow, et al., 1986; Andriantsitohaina & Stoclet, 1990).

There are some suggestions that NPY potentiation is mediated via membrane depolarization. KCl-induced

depolarization of the mesenteric arterioles of the rat produced a similar potentiation as NPY of NA- and 5-hydroxytryptamine-induced contractions (Andriantsitohaina & Stoclet, 1988). Intracellular recordings show that NPY depolarizes the membrane of rabbit cerebral arteries (Abel & Han, 1989), guinea pig basilar artery (Fallgren et al., 1990), and rat tail artery (Neild, 1987). However, NPY has no effect on the membrane potential in rat cerebral arteries (Brayden & Conway, 1988), submucosal arterioles of the guinea-pig small intestine (Neild & Kotecha, 1990), and the guinea-pig saphenous artery (Cheung, unpublished observations). Potentiation of vasoconstriction in response to nerve stimulation of the guinea-pig saphenous artery has been reported (Cheung, 1991) and potentiation of vasoconstriction of submucosal arterioles of the guinea-pig has been reported (Neild & Kotecha, 1990). Since the potentiating effect of NPY could be seen in preparations in which NPY had no depolarizing effect, it is not likely that membrane depolarization plays a key role in the potentiating effects of NPY. Rather, NPY potentiates, specifically, the contractile responses mediated by membrane depolarization such as KCl and mATP. This potentiation is occurring in close association with the voltage sensitive calcium channels. Evidence for this comes from the results of the experiments in which nifedipine blocked the NPY potentiation.

There is controversy as to whether the endothelium is necessary for the potentiating effects of NPY. It was reported in isolated rabbit ear artery that NPY potentiation of transmural nerve stimulation occurred only if there was an intact and functional endothelium (Daly & Hieble, 1987). In the same preparation and in canine saphenous artery, similar observations were made by the same group (Hieble et al., 1989). NPY potentiation of contractions to agonists as well as to nerve stimulation was dependent on the endothelium. These results could not be repeated, however, by a different group investigating the same preparation that Hiebles group used; the rabbit ear artery (Budai et al., 1989). They found that NPY potentiated the contractile response to nerve stimulation with and without the endothelium equally. This endothelium-independent NPY potentiation reported (Budai et al., 1989) in the rabbit ear artery was verified by others also using the rabbit ear artery (Gustafsson & Nilsson (1990). Results from the present study show that NPY caused a similar potentiation of KCl-induced responses in those arteries which had a functionally intact endothelial lining and those arteries which had the endothelial lining removed. The KCl-induced responses were inhibited by nifedipine in a similar manner in intact and denuded vessels. The addition of nifedipine also blocked the potentiation by NPY of KCl actions. The control curves for mATP were virtually

superimposable for the intact and denuded vessels. This was the case for the curves after the addition of NPY, nifedipine and nifedipine with NPY. The doses of KCl at which significant potentiation was observed in the intact vessels were the same as those doses which were significantly potentiated by NPY in the denuded vessels. This was the case for the responses in the presence of nifedipine with or without NPY. NPY (50 nM) had no effect on NA at any concentration in both intact and denuded vessels. All concentration response curves, control for intact and denuded, and NPY for intact and denuded, were superimposable. The curves for the NPY (500 nM) potentiation and the effects of nifedipine on this potentiation were also similar for intact and denuded vessels. These results demonstrating an endothelium-independence of the potentiation by NPY in the rat tail artery are in agreement with the observations of NPY potentiation in small mesenteric arteries of the rat and the central ear artery of the rabbit (Gustafsson & Nilsson, 1990; Budai et al., 1989) and opposes the observations in the rabbit ear artery in which the potentiation by NPY of vasoconstrictor responses is dependent on the endothelium (Daly & Heible, 1987; Heible et al., 1989).

The single cell studies reported here present a model in which there is absolutely no influence from nerves or the endothelium. Any potentiation by NPY in this pure vascular

cell preparation would be indisputable evidence for the fact that the endothelium is not necessary for the effects of NPY and that the target of NPY is the vascular smooth muscle. The shift in the frequency distribution histograms and the shifts in the KCl-concentration response curve for the cells show the significant potentiation by NPY (50 nM) of the cell shortening. The magnitude of this potentiation by NPY is greater than the potentiation of cell shortening in response to the other agonists used. There was also significant potentiation of mATP-induced cell shortening by NPY (50 nM). This potentiation occurred, as for the KCl responses, at every concentration of the agonist. The greater sensitivity of the cells with respect to the ring segments has been observed by others. Isolated bovine carotid arterial cells demonstrated an increased threshold sensitivity to histamine (Warshaw et al., 1986) and in isolated rat tail artery cells an increased threshold sensitivity to NA- and KCl-induced cell shortening (Bolzon, 1989). This increased sensitivity of the cells might be explained in a number of ways: The contractions in the cells are occurring in the absence of the endothelium and the relaxing factors from the endothelium. The concentration of exogenously applied stimulating agent should be greater at the membrane of the vascular muscle cell in the single cell preparation than in the ring segments because of the removal of extracellular barriers in the cell preparation (Bolton,

1979). In ring segment preparations, developed force is recorded and in cell preparations, cell shortening is measured. The ability to measure the cell shortening of unattached single cells which are not subjected to a resting tension, may be more sensitive than the ability to record tension changes in the ring segments, since these are subjected to a set resting tension due to their inherent compliance. NPY (50 nM) failed to increase the cell shortening in response to NA. At no concentration of NA was there a significant difference in the cell length regardless of whether NPY was present or not. When the concentration of NPY was increased to 500 nM, however, there was a significant potentiation of the NA-induced response at every concentration of NA used. The cells significantly shorten in response to the addition of NPY. The contractile response to NPY was greater at 500 nM than at 50 nM. The fact that there were responses to NPY at 50 nM in the cells and not in the ring segments may be explained by the same phenomenon that explained the increased sensitivity of the cells to the other agonists used. The demonstration of the potentiation by NPY of the contractile responses to KCl, mATP and NA in the freshly isolated vascular smooth muscle cells provides unequivocal evidence that neither the nerves or the endothelium are necessary for the potentiating effects of NPY. These findings are in agreement with reports which show Y_1

receptors, believed to be responsible for the postsynaptic effects of NPY, located primarily on the vascular smooth muscle cells as opposed to the endothelium or nerves (Sheikh et al., 1991).

Conclusions

The present study supports the proposal that NPY mediates its potentiating effects primarily through voltage-sensitive calcium channels. This was demonstrated by the fact that the potentiation by NPY was greatly attentuated with nifedipine. The fact that NPY could only potentiate NA-induced responses at high concentrations as compared to the responses to KCl and mATP also suggests that NPY acts through voltage-sensitive calcium chanrels.

In all cases, NPY potentiated the contractile responses to KCl, mATP and NA in intact and denuded arteries equally. The single cells studies provide further evidence of this. In this pure cell preparation, in which the influence of both the nerves and the endothelium had been removed, there was significant potentiation by NPY of all contractions induced by KCL, mATP and NA. Therefore, the potentiation by NPY is independent of the endothelium.

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