May 1994

Denervation Supersensitivity of the Rat Vas Deferens: A Role for Protein Kinase C

Sonny T. Abraham
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Denervation supersensitivity of the rat vas deferens: A role for protein kinase C

Abraham, Sonny Thomas, Ph.D.
East Tennessee State University, 1994
DENERVATION SUPERSENSITIVITY OF THE RAT VAS DEFERENS:
A ROLE FOR PROTEIN KINASE C

Dissertation
Presented to the Faculty of
the Department of Pharmacology
James H. Quillen College Of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Science

by
Sonny Thomas Abraham B.S.
May 1994
APPROVAL

This is to certify that the Graduate Committee of

SONNY THOMAS ABRAHAM

met on the

17th day of November, 1993.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council, Associate Vice-President for Research and Dean, School of Graduate Studies, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science.

Chairman, Graduate Committee

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean, School of Graduate Studies
ABSTRACT

DENERVATION SUPERSENSITIVITY OF THE RAT VAS DEFERENS: A ROLE FOR PROTEIN KINASE C

by

Sonny Thomas Abraham

A role for protein kinase C (PKC) in the denervation-induced supersensitivity of the rat vas deferens was investigated. Chronic, surgical denervation of the rat vas deferens (up to 8 days) resulted in tissues that produced enhanced contractile responses to norepinephrine (NE) in isolated organ baths. Single challenges of NE (10 μM) produced 0.6 ± 0.1 g of maximal tension in the control vas whereas in the paired, denervated tissue 2.2 ± 0.3 g of tension was recorded (n=6). Cumulative concentration-effect curves to NE produced in the denervated vas deferens were shifted 18-fold to the left of the control response. Neurokinin A (NKA) responses after denervation of the tissue were not significantly different from the control. Denervation did not alter the contractile response to phorbol diacetate (PDA), a PKC activator. Pretreatment of denervated and control vas deferens with 100 μM nifedipine (a calcium channel blocker), significantly attenuated the contractile response to NE. The responses in the control tissues were depressed by 88%, those in the denervated vasa were only antagonized by 65% after nifedipine treatment.

Exposure of denervated and control vas deferens to 100 μM NE resulted in no significant accumulation of diacylglycerol (DAG) from basal values. The molecular species of DAG produced after receptor stimulation, in either tissue group, were not different from those found in resting tissues. Denervation also had no effect on the binding characteristics of membrane-associated PKC when assayed using the specific ligand, [3H]phorbol dibutyrate. Binding maxima of 4.8 ± 0.9 and 4.1 ± 0.4 pmol/mg protein (n=4) were determined in the membrane fraction isolated from paired control and denervated vas deferens, respectively. The dissociation constant for the ligand was found to be 4.3 ± 0.6 and 3.5 ± 0.1 nM in the control and denervated groups, respectively.

The PKC activity of resting vas deferens was not altered by chronic surgical denervation. Denervated and control vas deferens that were stimulated with 100 μM NE showed a time-dependent translocation of PKC from the cytosolic to the membrane fraction of the tissue. In both tissue groups exposure to NE resulted in a 3-4 fold increase in the membrane-bound PKC activity, which remained elevated above basal values for up to 20 min. The rate of translocation of PKC was faster in denervated vasa (maximal at 5 min after NE) when compared to the control.
at 20 min), but the maximal amount of the enzyme activated was the same for the two tissue groups. The ability of NKA, 60 mM K+-depolarization and PDB (PKC activator) to produce translocation of the PKC was not altered by denervation of the vas deferens.

In conclusion, surgical denervation of the rat vas deferens produced tissues that exhibited enhanced contractile activity when exposed to NE but not NKA. The apparent resistance of the denervated tissue to calcium channel blockade (with nifedipine) may indicate either an enhanced sensitivity of the contractile apparatus to Ca2+ or a decreased dependence of the tissue on extracellular Ca2+ after denervation. The unaltered basal DAG or PKC levels after denervation would indicate that the supersensitive response is not due to their elevation under resting conditions. However, the increased rate of activation of PKC in denervated tissue, in response to NE, is consistent with a positive role for the enzyme in the enhanced contractile activity of the rat vas deferens.
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1994
DEDICATION

To my wife, Theresa, and children, Joshua, Alyssa and Jacob, who made the long hours in the laboratory, bearable.

The fear of the Lord is the beginning of wisdom.

Psalms 111:10
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I thank the faculty and staff of the Department of Pharmacology for their assistance over the past six years. I appreciate your kindness and efforts on my behalf. The constructive criticism of my committee members: Drs. Donald Hoover, Michael Miyamoto, Peter Rice, Mitchell Robinson and Barbara Turner was most helpful. This work would not have been possible without the aid of many persons but I am particularly grateful to Dr. Mitchell Robinson for taking time to train me and allow the liberal use of his laboratory, in which all the lipid studies were conducted.

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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>binding maximum</td>
</tr>
<tr>
<td>C-E</td>
<td>concentration-effect</td>
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<tr>
<td>DAG</td>
<td>1,2-sn-diacylglycerol</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective concentration giving 50% of maximal response</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>MAG</td>
<td>monoacylglycerol</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane sulfonic acid</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NKA</td>
<td>neurokinin A</td>
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<tr>
<td>PDA</td>
<td>4β-phorbol-12,13-diacetate</td>
</tr>
<tr>
<td>PDB</td>
<td>4β-phorbol-12,13-dibutyrate</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PMA</td>
<td>4β-phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PS</td>
<td>L-α-phosphatidyL-serine</td>
</tr>
<tr>
<td>PSS</td>
<td>physiological salt solution</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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CHAPTER 1

Introduction

Survey of Supersensitivity in Various Organs

The first report of denervation-induced supersensitivity appeared in 1855 when Budge observed a greater pupillary dilation of irides after sectioning of the postganglionic fibers from the superior cervical ganglia as compared with sectioning the preganglionic fibers cited by (Fleming et al., 1973). A mechanism for this phenomenon was not described until about fifty years later when Langendorff discovered that the "paradoxical" dilation of the denervated iris (postganglionic nerve sectioned) versus the decentralized iris (preganglionic nerve sectioned) was due to circulating substances from the adrenal glands. This phenomenon is now known to be due to enhanced sensitivity of denervated irides to circulating catecholamines released from the adrenal medulla (Fleming et al., 1973).

Thesleffe and others (Thesleffe, 1960) reported the development of supersensitivity of skeletal muscle when denervation resulted in greatly enhanced contractile response to acetylcholine (ACh). The area of the muscle sensitive to ACh, which is normally confined to the motor end-plate, spread outward to cover the entire surface of the muscle fiber. Electrophysiological and radiolabelled studies indicated a spread in nicotinic receptors from the end-plate region to cover the entire surface of the muscle (Fleming et al., 1973; Askmark and Gillberg, 1987). The increase in the number of nicotinic receptors, as determined by binding studies, after denervation
(Askmark and Gillberg, 1987) was corroborated by reports of increased mRNA for the α-subunit of the receptor protein after denervation (Shieh et al., 1987). These findings, however, did not entirely explain the resulting contractile response of denervated skeletal muscle to non-cholinergic agents (e.g. histamine, serotonin, bradykinin and caffeine) which are not seen in normally innervated muscle.

Westfall and Fleming (1968a) showed that chronic catecholamine depletion of adrenergic nerves by reserpine resulted in enhanced sensitivity of cardiac muscle to the chronotropic effects of norepinephrine and calcium in the dog as well as the guinea pig (Westfall and Fleming, 1968b). However, changes in the inotropic effect of various agonists after reserpine treatment was not consistently seen. Chronic reserpine treatment of the rat has also been shown to produce enhanced chronotropic sensitivity of spontaneously beating right atria to isoproterenol (Rice et al., 1987).

Denervation studies have also been conducted in a number of smooth muscle organs from various species. Trendelenberg (1963) and Langer (1966) describe extensive work done in producing sensitivity changes in the cat nictitating membrane by reserpine treatment or sectioning of the preganglionic fibers innervating the organ. Akhtar and Abdel-Latif (1986) were able to denervate the iris by surgically removing the superior cervical ganglion in the rabbit. In isolated organ baths denervated irides were 17-fold more sensitive to the contractile effect of NE as control tissues.

Chronic treatment of guinea pigs with the ganglionic blocking agent chlorisondamine resulted in increased sensitivity of the isolated ileum to acetylcholine (Fleming, 1968). The production of supersensitivity in vascular smooth muscle after
adrenergic neurotransmitter depletion with reserpine has been observed less consistently. This is probably due to the fact that changes in sensitivity were usually measured in the intact animal where difficulty in delineating purely postjunctional responses was encountered. Changes in resting blood pressure, basal adrenergic tone, and uptake and metabolism of pressor agents that often results from reserpine treatment may account for much of the inconsistency (Fleming et al., 1973).

Supersensitivity of the Rat Vas Deferens.

Birmingham (1968) showed that stripping the serous coat around the vas deferens caused a loss of NE content as well as catecholamine fluorescence in the tissue eight days after the procedure. Vas deferens denervated in this manner exhibited about a 20-fold increase in sensitivity to the contractile stimulus of NE in vitro.

Kasuya et al. (1969) were able to produce supersensitivity in the rat vas deferens by destroying the hypogastric plexus from which short postganglionic fibers innervate the vas deferens (Sjöstrand, 1965). This was achieved by crushing the hypogastric nerve plexus and stripping the serous coat from the most prostatic end of the vas (before the deferential artery meets the body of the vas deferens). This method had the advantage of causing less bleeding of the tissue when compared to the method of Birmingham (1968). Vasa denervated in this manner also showed marked loss in NE content and catecholamine fluorescence four days after the procedure, implying a nearly complete destruction of adrenergic nerves in the tissue. In vitro,
these tissues showed a greatly enhanced contractile response to NE that was characterized by significant leftward shifts in the concentration-effect (C-E) curve and increases in the maximal contractile force generated by the tissue. This denervation-induced supersensitivity was also exhibited by acetylcholine, angiotensin II, high potassium and barium. The authors concluded that surgical denervation produced a nonspecific supersensitivity of the vas deferens since a number of dissimilar agents revealed the enhanced sensitivity of the tissue.

Pharmacological destruction of adrenergic nerves with 6-hydroxydopamine was also shown to produce equivalent supersensitivity in the rat vas deferens as the surgical procedure (Westfall, 1977). In the same study chronic reserpine treatment produced more modest changes in sensitivity with smaller shifts in the NE concentration-effect curves without a change in the maximal force produced by the tissue.

Mechanisms of Supersensitivity in the Rat Vas Deferens

Westfall et al. (1977) made the initial observation of about 100% increase in nexal contacts between smooth muscle cells from denervated vas deferens but not reserpine-treated tissues, as compared to control vasa. The authors postulated increased intercellular coupling, via nexal contacts, would mediate the increased maximal contractile force in response to various agents. However such nexal contacts may be largely artifactual due to the preparative procedure used (permanganate-fixation) since Paton et al. (1976) did not observe any nexal junctions between cells
when the normal vas deferens was fixed with glutaraldehyde prior to electron microscopy.

The electrophysiological characteristics of the smooth muscle cells after denervation of the vas deferens were studied by Goto et al. (1978). This group reported no change in the resting membrane potential after denervation, however, a 6.5 mV reduction in the threshold potential to depolarization (by an external electrode) was observed. The authors also measured the propagation of electrotonic potential through the tissue and found that the spread of excitation through denervated vasa was markedly improved when compared to control tissues. It was concluded that the denervation-induced change in threshold potential and electrical propagation would result in increased sensitivity of the tissue to agents that depolarize the cell membrane. The change in the electrical conduction of denervated tissues was also consistent with the authors' earlier observation of increased nexal contacts between cells in denervated vasa.

Since catecholamines initiate contractile response of the vas deferens by activating the α₁-adrenoceptor, changes in receptor characteristics after chronic denervation were studied by Hata et al. (1981). Using [³H]WB4101 as a specific ligand for α₁-adrenoceptors these authors described a significant decrease in receptor number without a change in the affinity of the receptor, after chronic denervation of the vas deferens. However, the ability of epinephrine and phenylephrine to displace [³H]WB4101 binding was significantly enhanced in denervated vasa whereas that of phentolamine and ergotamine did not change. The authors concluded that an increased
affinity of $\alpha_1$-adrenoceptors for agonists (epinephrine, phenylephrine) but not antagonists (WB4101, phentolamine) resulted after denervation of the vas. Such an increase in agonist affinity for the receptor was proposed to mediate the observed supersensitivity.

Rice (1983) found no change in the $\alpha_1$-adrenoceptor population of rat vas deferens after denervation when assayed using [3H]WB4101 as the ligand. Further, the ability of Epi to displace bound [3H]WB4101 in denervated or control vasa was not found to be different. The reason for the discrepancy between these two studies is not readily apparent.

More evidence for a lack of denervation-induced change in receptor characteristics was presented by Abel et al. (1985) who found no difference in the $\alpha_1$-adrenoceptors from denervated and control tissues. This group used [125I]BE2254 as a specific ligand for the receptor binding assay. The ability of NE to displace this ligand from its binding site was not different in the two tissue groups either. Additionally, these authors reported that neither the $K_d$ of NE nor the number of receptors was altered by denervation when determined in intact vasa by the receptor-inactivation method of Furchgott (1966).

Evidence for post-receptor events mediating enhanced sensitivity after denervation was reported by Takenawa et al. (1983) who showed an increased accumulation of phosphatidic acid (PA) after NE stimulation in these tissues. However, NE-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) in denervated tissues was not significantly different from that in control vas. The authors
thought their results were consistent with a role for phosphatidic acid in increasing calcium-influx into supersensitive cells thereby mediating enhanced contractile response to agonists. A role for PA in mediating influx of cations has been proposed by various groups in the past (Putney et al., 1980; Salmon and Honeyman, 1980), but is not well accepted currently. Thus the significance of increased accumulation of PA in denervated tissues after receptor stimulation remains to be determined.

A possible link between enhanced contractile response and increased calcium mobilization in denervated rat vas deferens was investigated by Minneman et al. (1988). In 18-day denervated vasa, NE was found to be 10-fold more potent in causing total \(^{3}H\) inositol phosphate accumulation when compared to control tissues. No difference in the maximal amounts of \(^{3}H\)IPs formed was noted between the two tissues. In the presence of uptake and metabolism inhibitors, control tissues showed an equivalent increase in potency to NE-induced production of \(^{3}H\)IPs as in the denervated group. The authors reasoned that the increased potency of NE in causing IP formation after denervation was due to loss of uptake and metabolism of exogenously applied NE (prejunctional phenomenon). This was supported by findings that the potency of phenylephrine and carbachol (not subject to uptake and metabolism as NE) in elevating IP levels was unchanged after denervation. The conclusion was reached that chronic denervation of the rat vas deferens does not result in an enhanced coupling of \(\alpha_{1}\)-adrenoceptors to PIP\(_{2}\) hydrolysis.
General Considerations in the Production of Supersensitivity

Slow Time-course of Development.

Structures that are denervated or depleted of neurotransmitter take a number of days before maximal supersensitivity results. Daily administration of reserpine for 7-14 days was required to see optimal sensitivity development in the cat nictitating membrane (Fleming and Trendelenburg, 1961). Supersensitivity was maximally expressed about 4 days after surgical denervation of the rat vas deferens (Kasuya et al., 1969).

Nonspecific Nature of the Enhanced Sensitivity.

Chronic reserpine treatment resulted in increased sensitivity of rabbit aortic strips to NE, acetylcholine and potassium (Hudgins and Fleming, 1966). Kasuya et al. (1969) reported that denervation of the rat vas deferens resulted in supersensitivity of the organ to various dissimilar contractile agents as well. This nonspecific nature of the change in sensitivity is good evidence for some common post-receptor alteration in the cells which results in enhanced sensitivity to a number of different contractile agents.

Prejunctural and Postjunctural Supersensitivity.

Tissues which receive predominantly adrenergic innervation appear to produce different types of supersensitivity when denervated or depleted of transmitter with
reserpine. Prejunctional supersensitivity is seen with catecholamines which are subject to neuronal uptake (Trendelenburg, 1966). Thus tissues lacking adrenergic nerve endings (denervated) or with impaired uptake into the nerve terminal (cocaine treated) would exhibit a decreased threshold for activation by NE simply due to increased concentration of agonist at the receptor. This would result in a leftward shift in the agonist concentration-effect curve. Postjunctional supersensitivity is independent of neuronal uptake and would be exhibited by adrenergic as well as non-adrenergic agents. The enhanced sensitivity to NE in the denervated vas deferens was observed as an 18-fold shift in the agonist C-E relationship as well as an increase in the maximal contractile response of the tissue (Abraham and Rice, 1992a). Using inhibitors of neuronal and extra-neuronal NE uptake (cocaine, hydrocortisone) and metabolism (U-0521) in normal tissue it was determined that prejunctional supersensitivity probably accounted for about a six-fold leftward shift in the NE C-E curve. The remaining three-fold shift in the NE C-E curve as well as the increase in the maximal contractile response appear to be due to postjunctional alteration in tissue sensitivity.

Excitation-Contraction Coupling in Smooth Muscle

The Central Role of Calcium

Early studies by Ringer (1883) showed the importance of calcium in muscle contraction when the isolated frog heart ceased to beat and became insensitive to
stimulation in isotonic sodium chloride solution. Addition of calcium chloride to the solution restored the spontaneous activity of the heart which was maintained for a prolonged period. Production of contractile activity in skeletal muscle fibers by intracellular application of calcium via micropipette was more direct evidence for a role for calcium ions in muscle contraction (Ebashi, 1980). A central role for calcium has also been shown in smooth muscle contraction (Bozler, 1969). Since then work by numerous laboratories has shown that the elevation in intracellular free calcium is the primary initiator of contractile activity in smooth muscle (Ramos et al., 1986).

As in skeletal muscle, smooth muscle has been shown to possess actin and myosin filaments but in less than parallel orientation with the cell. Unlike in skeletal muscle the Z-line which anchors actin and myosin filaments is missing and the fibers appear to be attached to cytoskeletal elements via dense bodies (Somlyo et al., 1977). The myosin isolated from various smooth muscle cells appears to be much like that in skeletal muscle, having one pair of heavy polypeptide chains (200 kDa M.W.) and two pairs of light chains (15-20 kDa).

Adelstein et al. (1977) described a cellular kinase which was able to phosphorylate the light chains of smooth muscle myosin in the presence of calcium. They also showed that this light chain phosphorylation increased the ability of actin to stimulate the ATPase activity of the myosin head. From these results they proposed a model by which elevation in intracellular calcium above 0.1 - 1 μM would activate myosin light chain kinase. This enzyme would in turn phosphorylate the 20 kDa light chains of myosin resulting in actin-mediated ATPase activity of the myosin head with
subsequent actin-myosin crossbridge cycling and muscle contraction. With the
discovery of a calcium binding protein (calmodulin) which activates MLCK, the
cascade for production of contractile response can now be linked to elevations in
cytoplasmic calcium (Kamm and Stull, 1985).

Contractile force in smooth muscle is thought to develop when the myosin
head interacts with the actin filament and undergoes a conformational change at the
"hinge" region of the heavy chain. This causes the myosin head to pull on the actin
filament to produce a power stroke resulting in the myosin and actin filaments sliding
past each other (Murphy, 1976). The energy for this stroke is derived from bound
ATP which is hydrolyzed to ADP and P_i by the slow ATPase activity of the myosin
head. During the power stroke the bound ADP and P_i are released and this exposes
the site for ATP binding which results in detachment of the myosin head from actin.
Subsequent ATP hydrolysis returns the myosin head to the "cocked" state in position
to interact with a new site on the actin filament further down its length and the cycle
is repeated. This results in the myosin head "walking" along the actin filament and
many such events in parallel eventually shorten the cell.

**Coupling of Receptors to Calcium Mobilization**

It is quite clear that intracellular Ca^{2+} is the fundamental second messenger for
the production of muscle contraction and it appears that receptor activation results in
its mobilization (Michell, 1975). What remains to be determined is how cell-surface
receptor stimulation results in this event. Since early observations of hormone-induced
phosphoinositide metabolism (Hokin and Hokin, 1953) it has become increasingly accepted that Ca$^{2+}$ mobilization by hormones is linked to this early membrane event. These receptors appear to be linked via guanine nucleotide binding proteins (G proteins) to a plasma-membrane bound phospholipase C which is able to hydrolyze membrane phospholipids, primarily phosphatidylinositol-4,5-bisphosphate (PIP$_2$), to produce inositol-1,4,5-trisphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) (Berridge, 1984).

Evidence for a link between IP$_3$ and Ca$^{2+}$-mobilization arose from work in permeabilized hepatic and pancreatic cells where IP$_3$ was able to release cellular stores of Ca$^{2+}$ in a low calcium environment (Streb et al., 1983). This release of intracellular Ca$^{2+}$ was shown to be from non-mitochondrial, non-secretory granule microsomal fraction of cells and quite specific for IP$_3$ since inositol-1,4-bisphosphate, inositol-1-phosphate or inositol-1,2-cyclic phosphate were not able to elicit this action. Photolysis of caged IP$_3$ by laser pulse has also been shown to produce contractile activity in smooth muscle (Walker et al., 1987). A role for other inositol polyphosphates (IP$_4$, IP$_5$, etc.) in Ca$^{2+}$-mobilization from extracellular sources has also been proposed but remains without firm supportive evidence.

In the rat vas deferens, Fox et al. (1985) showed that concentration-dependent activation of $\alpha_1$-adrenoceptors by NE resulted in significant inositol phosphate accumulation. It was also reported that partial agonists which were only able to produce 60% or less of the contractile response of NE were able to elicit 70-98% of the NE-induced IP accumulation. The same group (Han et al., 1987) also published a
report showing that incubation of the vas with $\alpha_1$-adrenoceptor antagonists WB4101 and benoxathian prior to NE exposure inhibited the contractile activity of the vas to a greater extent than it did the elevation in IPs.

This dissociation of inositol phosphate accumulation from contractile response revealed by partial agonists and antagonists was used by the authors to postulate two distinct subtypes of $\alpha_1$-adrenoceptor, one which appears to mediate contractile response ($\alpha_{1b}$-type) and the other coupled to PIP$_2$ hydrolysis ($\alpha_{1\alpha}$-type). Additionally the same paper showed that the normal vas deferens was very dependent on extracellular Ca$^{2+}$ for the production of contractile force in response to NE. These results taken together make the physiological role of IP$_3$ in mediating contractile response in the vas deferens rather ambiguous. To explain such discrepancies Putney (1986) and Irvine and Moore (1986) have proposed models in which release of intracellular Ca$^{2+}$ promotes influx of Ca$^{2+}$ to maintain high enough cytosolic Ca$^{2+}$ to mediate cellular response as well as to refill depleted intracellular stores.

The Role of Protein Kinase C

A role for 1,2-diacylglycerol (DAG), produced as a result of PIP$_2$ hydrolysis (Fig. 1), was first proposed by Nishizuka's group who showed that protein kinase C (PKC) was dependent on unsaturated diacylglycerol for activation (Takai et al., 1979). They were able to show that PKC was activated during thrombin stimulation of platelets resulting in phosphorylation of specific intracellular proteins (Kawahara et al., 1980). Since then PKC has been identified in almost all cell types and found to
perform multiple functions (Nishizuka, 1988). The protein is found mostly in the cytosol in soluble form with smaller amounts associated with the plasma membrane, nuclear membrane and cytoskeletal filaments. This 82kDa protein possesses a regulatory domain which binds Ca\(^{2+}\), phosphatidylserine (PS) and DAG or phorbol esters as well as a catalytic domain which binds ATP.

Figure 1 Scheme for Ca\(^{2+}\)-mobilization and contractile activity in smooth muscle. G\(_p\), G-protein; PLC, phospholipase C; PIP\(_2\), phosphatidylinositol-4,5-bisphosphate; IP\(_3\), inositol-1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; PKC, protein kinase C; MLCK, myosin light chain kinase; MLC, myosin light chain.

The enzyme functions optimally in the presence of DAG, PS and Ca\(^{2+}\) which
results in the exposure of the catalytic domain to serine and threonine phosphorylation sites on substrate proteins (Bell and Burns, 1991). Phorbol esters (PDB, PMA etc.) appear to bind to the same site on PKC as DAG and thus are able to activate the enzyme in a manner similar to the endogenous activator (König et al., 1985). During physiological stimulation cytosolic PKC is thought to become associated with the plasma membrane, where it is in proximity with PS and DAG in the presence of Ca\(^{2+}\) (Huang; 1989). The primary stimulus for this translocation of PKC appears to be the rise in intracellular free Ca\(^{2+}\) (Ho et al., 1988), however, others maintain that sustained elevation in membrane DAG may be responsible for PKC translocation (Rasmussen et al., 1987).

Molecular cloning techniques have identified several isozymes of PKC, most notably \(\alpha, \beta I, \beta II, \gamma, \delta, \epsilon, \zeta, \eta\) and L, of which \(\alpha - \gamma\) are dependent on Ca\(^{2+}\) for activity while \(\delta - L\) appear to be Ca\(^{2+}\)-independent (Bell and Burns, 1991). Smooth muscle cells contain the Ca\(^{2+}\)-dependent \(\alpha, \beta\) and \(\gamma\) subtypes (Singer et al., 1992) as well as the Ca\(^{2+}\)-independent \(\epsilon\) and \(\zeta\) isoforms (Khalil et al., 1992). The role of the various subtypes of PKC in smooth muscle is largely unknown although there does appear to be some differential activation of the different isoforms during receptor stimulation (Singer et al., 1992; Khalil et al., 1992).

Protein kinase C has been proposed to maintain sustained contraction of smooth muscle since: (i) PKC-mediated contractile response by phorbol esters (PDB) takes over an hour to develop maximally (Merkel et al., 1991); (ii) PKC-mediated phosphorylation of cellular proteins desmin, synemin and caldesmon appear after about
phosphorylation of cellular proteins desmin, synemin and caldesmon appear after about 1 hour of agonist stimulation. The role of PKC in smooth muscle has been proposed to be to phosphorylate these cellular proteins which may then play a role in modulating sustained muscle contraction (Rasmussen et al., 1987). However, a role for PKC in initiating and maintaining smooth muscle contraction has been presented in non-vascular smooth muscle (Menkes et al., 1986; Abraham and Rice, 1992b).

In intact iris smooth muscle Howe and Abdel-Latif (1988) have shown that PKC activation is able to phosphorylate myosin light chain (MLC) at a site distinct from that of myosin light chain kinase. The relevance of this action on muscle contraction is unknown but the phosphorylation of MLC preceded the production of contractile response in the tissue by phorbol dibutyrate (PDB). Lacerada et al. (1988) as well as Souhrada and Souhrada (1989) have shown that PKC activation can enhance Ca^{2+}-influx into muscle cells and have offered this as the mechanism by which PKC mediated contractile response occurs.

**Statement of the Problem**

The work of numerous groups has shown that supersensitivity in smooth muscle results from the withdrawal of the endogenous neurotransmitter from the nerve endings in the tissue (Fleming et al., 1973). In the rat vas deferens the resulting supersensitivity is thought to be due to postjunctional alteration in the smooth muscle cells (Kasuya et al., 1969). However, work by various investigators has failed to provide clear evidence of cellular changes which would explain the nonspecific
supersensitivity to dissimilar contractile agents.

The increase in nexal contacts between smooth muscle cells after denervation of the vas deferens has been proposed to increase electromechanical coupling between cells and result in enhanced contractile response to depolarizing agents. However, it is not clear whether the nexal contacts reported to exist in control vasa alone, as reported by Westfall et al. (1977), are an artifact since one other group has reported no nexal connections in the normal rat vas deferens (Paton et al., 1976) while a second group (Daniel et al., 1976) has shown that the technique used by Westfall's group may actually contribute to increased nexal structures.

The elevation in threshold potential after denervation of the vas deferens has also been proposed to result in enhanced response to depolarizing agents (Goto et al., 1978). This change would result in increased ease by which agents could depolarize the muscle cell membrane and thus conceivably decrease the threshold concentration of agonists in initiating contractile activity (resultant leftward shift in the C-E curve). However, apart from a decrease in the threshold concentration and lowered EC₅₀, a significant elevation in the maximal contractile force results after denervation. This phenomenon cannot be explained by the alteration in the threshold potential of the cell membrane.

Takenawa et al., (1983) noted an enhanced accumulation of phosphatidic acid in response to NE in denervated vasa as compared to control responses. The PA was thought to increase Ca²⁺ mobilization and thus promote the enhanced contractile response to various agonists. Such a role for PA is not borne out by current
investigations and in any case, would not explain the increased sensitivity to high K$^+$ and Ba$^{2+}$ which presumably do not occur by elevation in cellular second messengers.

A role for altered $\alpha_1$-adrenoceptors in mediating the supersensitivity phenomenon was ruled out when no change in the number or binding affinities of the receptor were observed after denervation (Rice, 1983; Abel et al., 1985). Coupling of $\alpha_1$-adrenoceptors to PIP$_2$ hydrolysis was not found to be significantly changed after surgical denervation of the vas deferens (Takenawa et al., 1983; Minneman et al., 1988) and so increased inositol phosphate production could not explain the supersensitivity response.

Increased sensitivity to agonists in other smooth muscle systems have implicated changes in protein kinase C activity as a causative factor. Unilateral superior cervical ganglionectomy in rabbits has produced supersensitivity to NE in isolated iris muscle (Akhtar and Abdel-Latif, 1986). The authors reported an increase of 30% in cytosolic PKC activity of denervated irides when compared to control tissues.

The caudal artery (Aqel et al., 1987) and thoracic aorta (Lograno et al., 1989) from spontaneously hypertensive rats (SHRs) has been shown to exhibit enhanced contractile response to NE and elevated K$^+$ relative to the response of tissues from Wistar-Kyoto (WKY) rats. Phorbol esters (PMA) as well as non-phorbol activators of PKC (mezerein) have been shown to produce greater contractile response in the mesenteric arteries of stroke-prone SHRs when compared with WKY vessels (Turla and Webb, 1987). An increase in PKC activity was proposed to mediate the enhanced
contractile response of aortic muscle strips from SHRs since the NE response in these tissues was more susceptible to antagonism by H-7 (PKC inhibitor) when compared to WKY tissues (Shibata et al., 1990). Renal arteries from SHRs showed increases in cytosolic PKC activity relative to WKY controls, but such differences were not seen in the aortas of the same animals (Silver et al., 1992). Bazan et al. (1992) also reported no difference in PKC activities from aortas of SHRs and WKY rats, although the ability of phorbol esters to activate PKC (translocation from cytosolic to membrane compartment) was greater in the SHR tissue.

There is significant evidence that various pathological and adaptive conditions involve increased receptor or tissue sensitivity. Altered responsiveness of blood vessels from hypertensive animals to contractile agents has been outlined above. Rebound hypertension after rapid withdrawal of clonidine during antihypertensive therapy may be due to increased NE released from sympathetic nerves as well as increased receptor sensitivity of the heart and vascular muscle to circulating catecholamines (Hansson et al., 1973). Normal subjects receiving guanethidine (which interferes with sympathetic neurotransmission) for 3 days developed supersensitivity to the arrhythmic effects of administered catecholamines (Fleming et al., 1973). Supersensitivity of cardiac muscle to circulating catecholamines has been proposed to maintain myocardial function of the transplanted heart in the absence of sympathetic innervation (Donald, 1973).

The rat vas deferens affords a smooth muscle system whereby sensitivity changes may be consistently induced and then studied. Application of this knowledge
may further a better understanding of such pathophysiological alterations and eventually lead to better therapeutic regimes for their management.

At a more fundamental level, such investigations would lead to a better understanding of smooth muscle function and plasticity. Although the primary initiation of contractile activity in smooth muscle appears to be via myosin light chain phosphorylation by a specific kinase (MLCK), many other cellular proteins (PKC, Caldesmon, desmin, etc.) may play a role in regulating this activity (Rasmussen et al., 1987). What is unclear is the extent to which these various cellular proteins may function in smooth muscle response. The present work attempts to define the contribution of PKC in mediating contractile function in the vas deferens. Additionally, if alterations in cellular proteins are found with chronic denervation, a mechanism by which smooth muscle cells may enhance cell surface signaling would be implicated.

**Aims of the Study**

The present study was designed to evaluate the role of protein kinase C in the tissue supersensitivity that results from chronic denervation of the rat vas deferens. Experiments were done to determine a number of parameters which would be indicative of altered PKC function in denervated tissues when compared to the control vas deferens. The experimental goals of the study are as follows:
1. to determine the functional relevance of PKC in the contractile response of the vas deferens by use of PKC activators as well as inhibitors. These experiments would provide information as to the extent to which PKC mediates normal contractile function in response to receptor and non-receptor agents.

2. to compare the ability of the phorbol PKC activators to produce contractile responses in the denervated and control vasa. If enhanced response after denervation is due to nonspecific increases in PKC activation, these experiments could provide evidence for such alterations.

3. to determine the amounts of 1,2-diacylglycerol produced in denervated and control vasa in response to receptor stimulation as well as to analyze the various molecular species of DAG produced. These experiments would indicate an increased activation of PKC in denervated tissues due to enhanced levels of the endogenous activator (DAG) formed subsequent to receptor stimulation. Changes in the molecular species of DAGs produced could indicate changes in $\alpha_1$-adrenoceptor coupling to different membrane phospholipases (PLC, PLD, etc.).
4. to evaluate the binding characteristics of membrane-bound PKC from denervated and control vasa using the ligand [³H]phorbol-12,13-dibutyrate. An increase in basal PKC in the membranes or enhanced affinity of the protein for activators following denervation would provide a mechanism by which PKC activation may be altered.

5. to determine the activation of PKC in denervated and control vas deferens as indicated by translocation of PKC from the cytosolic to the membrane fraction of the cell. These experiments would reveal differences in the ability of receptor stimulation to activate PKC in the two tissue groups. Non-receptor-mediated (K⁺ and phorbol esters) translocation of PKC in the two groups would also be studied.
CHAPTER 2

Materials and Methods

Animals and Tissues

Male CD rats (VAF) bred from Charles River CD rats in the Division of Laboratory Animal Resources were used in the study. On occasion additional male CD (VAF) rats were purchased from Charles River Labs (Wilmington, Ma.). Animals were housed and cared for in the animal care facility according to the guidelines of the American Association for Accreditation of Laboratory Animal Care. They had free access to food and water and were maintained on a 12 hour light/dark cycle. Rats weighing 300-600 g were used to obtain tissue for the experiments herein. The animals were euthanized by exposure to carbon dioxide gas until loss of consciousness, followed by cervical dislocation.

Whole vas deferens from the base of the bladder to the epididymis were dissected and placed in ice-cold, oxygenated, physiological salt solution (PSS). Vasa were carefully cleaned of the connective tissue sheath, adherent blood vessels and fat, while under ice-cold PSS, using fine forceps. When appropriate, tissue wet weights were obtained soon after tissues were cleaned and while still cold.
Surgical Denervation Procedure

The denervation procedure was performed essentially according to the method introduced by Kasuya et al. (1969). Rats were anesthetized with 60 mg/kg pentobarbital sodium, administered via intraperitoneal (i.p.) injection. Later the anesthetic was changed to a combination of ketamine (60 mg/kg) and xylazine (12 mg/kg) also administered by the i.p. route. The ketamine/xylazine combination was found to produce better surgical analgesia and anesthesia as evidenced by decreased incision and pedal reflex. The dose of pentobarbital used was also less consistent in inducing rapid anesthesia or recovery of the animal after surgery. Butorphanol (2 mg/kg, subcutaneous injection) was also given to the animals prior to surgical incision. This long-acting analgesic helped to speed the recovery of the animal after surgery.

The surgical procedure was carried out according to aseptic guidelines using sterile instruments and materials (Cunliffe-Beamer, 1993). The abdomen was shaved using animal clippers and the area cleaned with 95% ethanol followed by povidone-iodine solution. A 3-4 cm long, 1 cm off-midline, incision was made in the skin and abdominal muscle layer of the rat using a scalpel. The bladder as well as the seminal vesicle and prostate on the surgical side were exposed and immobilized with hemostats. The testicle on the surgical side was pulled out of the scrotal sack until the area where the vas deferens meets the base of the bladder was exposed (Fig. 2). The adherent fat in the area was removed with forceps and the vas deferens was freed from its mesenteric attachments.
Figure 2. View of visceral organs during denervation surgery. Dashed lines indicate nerves entering the vas deferens and the arrow points to the area of phenol application.

Under a magnifying lens up to 3 individual nerve fibers could be seen entering the vas deferens via the connective tissue sheath, while, a fourth usually entered along with the deferential artery. Using fine forceps the connective tissue sheath around the tissue, from the base of the bladder to the junction of the deferential artery and vas deferens was carefully removed with minimal bleeding.

To destroy the nerves which entered with the artery, as well any that had been missed with the forceps, 3 to 4 drops of 5% phenol (wt./vol in water) were applied to the area. Subsequently, the abdominal musculature was closed using silk surgical suture and the skin incision was secured with metal wound clips. The animal was then wrapped in paper and placed in the cage for recovery. The right or left vas deferens was randomly denervated in each animal and the contralateral tissue was left
intact as the control. The rats were allowed to recover usually for 6 to 8 days before 
the denervated and control tissues were dissected out.

Contractile Studies of the Vas deferens

Tissue Preparation and Equilibration

Adult, male CD rats (300-600 g) were used as the source of fresh vas deferens 
in these studies. The animals were anesthetized with carbon dioxide and killed by 
cervical dislocation. Both vasa deferentia were rapidly removed and placed in ice-
cold, oxygenated, physiological salt solution (PSS). The tissues were cleaned of 
blood vessels and adhering connective tissue (under cold buffer) using fine forceps. 
The vasa were then placed in individual jacketed organ baths containing PSS (see 
Appendix A for preparation scheme) of the following composition (mM): NaCl (113); 
KCl (4.7); CaCl₂ (2.5); MgSO₄ (0.57); NaHCO₃ (25); NaHPO₄ (1.01) and dextrose 
(5.55). The buffer was continuously aerated with a mixture of 95% O₂ and 5% CO₂ 
and maintained at 37°C throughout the experiment.

Measurement of Contractile Response

Vasa deferentia were suspended vertically in 25 ml organ baths with one end 
attached to Grass force-displacement transducers (FT03C) and the other end to the 
bottom of the organ bath via cotton thread. The tissues were maintained at 250-300 
mg resting tension, and isometric contractile responses were recorded by a Grass
Model 7D polygraph (Quincy, MA.). Following a 60-90 min equilibration period, all tissues were exposed to 10 μM norepinephrine (NE) challenge as this allowed subsequent responses of the tissue to be more consistent. An additional washout and re-equilibration period (45 min) preceded further exposure to contractile agents. The buffer in the organ baths was replaced with pre-warmed PSS every 15-20 min during the course of the experiments. Contractile agents and antagonists were applied in 10-25 μl volumes of solvent using Eppendorf digital pipettes. Cumulative concentration-effect curves (C-E curves, van Rossum, 1963) to contractile agents were constructed by their addition to each organ bath in approximately 0.5 log unit increments to the final concentrations indicated.

Assessment of the Denervation Procedure

The success of the denervation procedure was determined in vitro by the use of tyramine which is able to enter intact adrenergic nerve terminals and elicit a contractile response by causing the release of endogenous NE (Birmingham, 1970). In the organ baths denervated and control vasa were exposed to 100 μM tyramine and the contractile responses recorded. Only tissues producing 10% or less of the control tyramine response were considered sufficiently denervated to be used in the study.

PKC and Normal Contractile Function

The role of PKC in the contractile activity of normal rat vas deferens was studied using PKC activators and inhibitors to assess the extent to which these agents
would influence the contractile activity of the tissue. In most experiments exposure to phorbol-12,13-diacetate (PDA), phorbol-12,13-dibutyrate (PDB), mezerein or 4α-phorbol followed a 1 min pre-exposure of rat vas deferens to PSS containing 20 mM K⁺. In each case that buffer concentrations of K⁺ were increased, equivalent Na⁺ was removed to maintain osmolarity (see Appendix B for preparation).

Tissues were incubated with 30 nM nifedipine for 10 min or 30 μM isoH-7 for 20 min prior to agonist exposure. Paired vas deferens exposed to vehicle (ethanol or distilled water, respectively) served as control for these experiments. Modulation of the NE response by PDA was studied by adding 2 μM of the agent to the tissue baths 10 min prior to the construction of concentration-effect curves for NE. Control NE responses were elicited in the presence of the vehicle (0.1% DMSO, v/v).

**PKC and Denervation Supersensitivity**

Contractile response to NE (single concentration or cumulative C-E curves, 10 nM-30 μM) were produced in denervated and control vasa to characterize the supersensitivity that develops after chronic denervation of the tissue. Cumulative C-E curves to neurokinin A (NKA, 1 nM-3 μM) were also produced in pairs of control and denervated tissues. The role of voltage-dependent calcium channels on the NE response in denervated and control tissues was determined after treatment of both groups of vasa with 100 nM nifedipine for 10 min. This concentration of nifedipine was used since it was found to significantly antagonize the NE response without abolishing it. The effect of direct PKC activation in denervated and control tissues
was determined by producing contractile responses (cumulative C-E curves) to PDA in both groups in PSS with 20 mM K\(^+\) and 10 \(\mu\)M phentolamine. Phentolamine was used to antagonize the contractile response that could result from the release of endogenous NE in adrenergic nerve terminals, especially in the control tissues. The concentration of phentolamine used was sufficient to completely antagonize the response to 10 \(\mu\)M NE applied exogenously.

**Diacylglycerol Production in the Rat Vas Deferens**

**Tissue Preparation**

Whole vas deferens (denervated and control) were dissected from freshly killed rats and placed in cold, oxygenated PSS and cleaned of adhering connective tissue, blood vessels and fat. Tissues were allowed to equilibrate under physiological conditions in organ baths for about 1 hour before exposure to a 10 \(\mu\)M NE challenge. This was followed by a 45 min washout and equilibration period before subsequent exposure to 100 \(\mu\)M NE for 0, 30, 60, 120, 300 and 600 s. At this time the stimulation was terminated by quickly placing the tissue in liquid nitrogen.

**Sample Preparation**

Immediately following the freezing of the tissue with liquid nitrogen, it was placed in a ground glass homogenizer containing 3 ml chloroform/methanol (2:1 vol/vol) which had been cooled to -10\(^\circ\)C. The tissue was homogenized completely
with rapid strokes and then the homogenate was pipetted out and placed in 20 ml scintillation vials. The homogenizer was rinsed with 1 ml of the chloroform/methanol mixture which was also pipetted out and added to the scintillation vial. Vials were tightly capped and stored at -20°C until extraction and analysis (within two weeks).

**Neutral Lipid Extraction**

The lipids in the sample were extracted according to the Bligh and Dyer (1959) technique. Into each scintillation vial 2 ml of chloroform/methanol (2:1, v/v) followed by 4 ml methanol and 2.5 ml water were added. The vials were vortexed and methanol was added drop-wise until the cloudy suspension became clear. Three ml chloroform and 3 ml H₂O were added, the vials vortexed and centrifuged at 800 X g for about 1 min. Separation of the mixture into an aqueous (above) and organic (below) phase was achieved by centrifugation. The lower phase was carefully pipetted into 10 ml tubes and the upper phase was re-extracted with 3 ml of chloroform which was also pipetted out after further centrifugation. The lipids of interest were extracted into the chloroform layer which was then dried under a stream of nitrogen with repeated rinsing of the tubes with chloroform to concentrate the lipids at the bottom of the tube. Once all the solvent was removed 200 µl of chloroform was added to each sample. Tubes were tightly capped and stored at -20°C or samples were immediately spotted onto thin layer chromatography (TLC) plates.
Thin Layer Chromatography

Silica gel G plates (20 X 20 cm, Analtech Inc., Newark, DE.) were activated at 70°C for at least an hour before use. The plates were channelled with lanes of 1 cm width and only the middle 18 lanes were used. Chromatography tanks were prepared by equilibrating them with 100 ml of hexane/diethyl ether/acetic acid (20:80:1, v/v/v) for about 1 hour before use. Known amounts of cholesterol and 1,2-dipalmitoyl glycerol standards (0.4 - 10 µg), dissolved in chloroform, were applied to each plate.

Five and 50 µl of each sample in chloroform were applied to the TLC plate using disposable glass microcapillary pipets (Fisherbrand®, Fisher Scientific, Pittsburgh, PA.). The plates were placed in the developing tank until the solvent front traveled about 14 cm (in about 30-40 min). Plates were air dried and then sprayed with 10% copper sulfate in 10% phosphoric acid and allowed to dry further. Lipids were visualized by charring the plates over a hot plate (Thermolyne, Dubuque, Iowa) which had been warmed to setting 6 for at least 10 min prior to use.

Analysis and Quantification of Lipids.

Charred TLC plates were scanned by a Howtek 3+ Digital scanner and the image was recorded and analyzed by using Bio-Image (Millipore, Milford, MA.) image analysis system on a SUN workstation. Density profiles for each spot on the plate were produced, from which the integrated optical density was obtained. The amount of tissue lipids (cholesterol and DAG) were determined from the standard curve obtained by plotting the mass of cholesterol or dipalmitin (0.4-10µg) against the
integrated optical density obtained for these standards. GraphPad's InPlot (San Diego, CA.) curve fitting program was used to produce the standard curves from which the mass of the sample DAG and cholesterol could be determined. The amount of diacylglycerol in each tissue sample was then corrected for recovery rate of extraction and variation in tissue weight simply by dividing the mass of DAG by the cholesterol mass in the same sample. This could be done since cholesterol mass remains quite stable and is proportional to the number of cells in the tissue, and thus the tissue mass (Datta, 1987). The final data were expressed as ng DAG/μg cholesterol for each tissue sample.

**Molecular species of DAG by HPLC**

**Sample Preparation.** The molecular species of DAG that resulted from receptor activation in denervated and control tissues were resolved by reverse-phase HPLC. Three previously extracted neutral lipids samples from denervated and control vasa, stimulated with 100 μM NE for 0, 60 and 300 s, were pooled and then dried under a stream of nitrogen. The lipids were dissolved in 300 μl of chloroform, spotted on TLC plates and resolved in the hexane/diethyl ether/acetic acid system along with the appropriate standards.

The DAG spots on the plates were visualized by spraying the plates with 0.5% dichloroflourescein (in ethanol) with the stain being enhanced by holding the plate in a tank of ammonia. The silica gel containing the DAG spots was scrapped off the plate
and placed in 10 ml tubes containing 2 ml ethanol (95%). Two ml of hexane and 2 ml of water were added to the tubes which were vortexed and then centrifuged at 800 X g for 30 s. The upper hexane layer was carefully pipetted out and placed in a second 10 ml tube. The lipid mixture contained in the silica gel was re-extracted by repeating the same process twice with 2 ml of hexane. The hexane containing the sample lipids was dried off under nitrogen with repeated rinsing of the walls of each tube to isolate the lipids at the bottom.

**Benzoylation Procedure.** Sample DAGs were converted to the benzoyl derivatives before separation by HPLC. To each tube 100 µl of benzene, 20 µl of benzoic anhydride (50 mg/ml, in benzene) and 10 µl of dimethyl aminopyridine (40 mg/ml, in benzene) were added. The tubes were shaken, flushed with nitrogen, tightly capped and allowed to sit at room temperature for about 24 hours. At that time 200 µl of ammonium hydroxide was added to each tube to stop the reaction. Ten minutes later 2.5 ml acetonitrile, 0.5 ml water and 3 ml hexane were added to the tubes followed by vortexing and centrifugation to separate the organic and aqueous layers. The upper hexane layer was pipetted into a second tube and the contents of the first tube were re-extracted twice with 3 ml of hexane. The tubes were dried under nitrogen stream with frequent rinsing of the walls, and the lipid at the bottom was dissolved in 100 µl of acetonitrile/isopropanol (70:30, v/v).
Reverse-phase HPLC. The various species of diacylglycerol were resolved by reverse-phase HPLC using an Ultrasphere-ODS column (Altex, Berkeley, CA., 4.5 X 250 mm) and mobile phase of acetonitrile/isopropanol (70:30, v/v) at 35°C. The mobile phase flow rate of 1.4 ml/min was generated by a Perkin-Elmer Series 410 LC pump and DAG derivatives detected by a Perkin-Elmer LC-95 UV/visible spectrophotometer with absorbance at 230 nm. For data collection the detector was interfaced to Maxima 820 chromatography workstation (Dynamic Solutions, Millipore, Milford, MA.) by which the resulting chromatograms were stored for later analysis. Molecular species of DAG in the sample were compared to known species that had been previously derivatized to the benzoyl form. The standard mix of DAG derivatives that were resolved by HPLC contained 1,2-dimyristoyl glycerol (14:0-14:0), 1,2-dipalmitoyl glycerol (15:0-15:0), 1,2-dioleoyl glycerol (18:1-18:1), 1-palmitoyl-2-oleoyl glycerol (16:0-18:1), 1,2-dipalmitoyl glycerol (16:0-16:0), 1-stearoyl-2-oleoyl glycerol (18:0-18:1), 1,2-distearoyl glycerol (18:0-18:0) and 1-stearoyl-2-arachidonyl glycerol (18:0-20:4).

**DAF Mass by High-Pressure Liquid Chromatography**

**Sample Preparation.** Tissues and samples were prepared essentially as indicated for determining DAG mass by thin layer chromatography with minor changes. Control vasa were exposed to 10 μM NE for 0-10 min under physiological conditions and then rapidly frozen in liquid nitrogen. Whole tissues were then placed
in scintillation vials containing 3 ml of chloroform/methanol (2:1 v/v) while still
frozen and stored at -20°C until the extraction procedure (usually 1 week later). [³H]
cholesterol (200,000 dpm) and [³H] dipalmitin (10,133 dpm) were added to each
scintillation vial before the neutral lipid extraction procedure outlined above (Bligh
and Dyer, 1959), was undertaken. The radiolabelled lipids were eventually used to
estimate the recovery of sample lipids from the vials.

**Column Fractionation.** The extracted lipids were dried under nitrogen and
dissolved in 500 μl of hexane/methyl-tert-butyl ether (96:4, v/v) in preparation for
column fractionation. A Fisher Prep-Sep Si (300 mg) column was conditioned by
rinsing with 10 ml isopropanol, followed by 2 X 5 ml methyl-tert-butyl ether (MTBE)
and then 2 X 10 ml of hexane. All of the extracted lipid samples were applied to the
column and the tubes containing the lipid samples were rinsed with two further
aliquots (500 μl) of hexane/MTBE which were also applied to the column. Each 500
μl aliquot of sample was allowed to enter the column and then rinsed with 2 X 2 ml
of hexane/MTBE (96:4) to elute triglycerides and cholesterol esters in the sample.
The columns were then rinsed with 2 X 1.5 ml of hexane/MTBE/acetic acid
(100:2:0.2, v/v/v) to remove fatty acids. Cholesterol, diacyl- and monoacylglycerols
(MAG) were eluted from the column by 2 X 3 ml of MTBE/acetic acid (100:0.2) and
collected in 10 ml tubes. The solvent was evaporated under nitrogen with repeated
washings using chloroform to isolate the lipid sample at the bottom of the tubes.
Lipid Separation and Quantification by HPLC. The isolated cholesterol, DAG and MAG were benzoylated and collected just as described above for DAG from the TLC extract. The benzoylated derivatives were then dissolved in 100 μl of 3.2% MTBE in cyclopentane (v/v). The lipid components were then resolved by normal-phase HPLC using an Ultrasphere SI column (Altex, Berkeley, CA.) in a mobile phase of 3.2% MTBE in cyclopentane (v/v) at a flow rate of 1.0 ml/min and 35°C.

The lipids that eluted from the column were detected by a Perkin-Elmer LC-95 UV/visible spectrophotometer with absorbance set at 230 nm. The resulting chromatograms were stored onto the hard disk drive of a NEC AP-CIV personal computer by the use a Maxima 820 chromatography workstation. The cholesterol fraction that eluted from the column was collected in 10 ml tubes while the DAG that eluted was collected in 20 ml scintillation vials.

The cholesterol fraction was dried under nitrogen and dissolved in 400 μl acetonitrile/isopropanol (63:37, v/v). About 20 μl of this sample was then resolved by reverse-phase HPLC using an Ultrasphere-ODS column, acetonitrile/isopropanol (63:37) mobile phase and 1.4 ml/min flow rate. The chromatograms were stored on disk for further analysis and the cholesterol fraction eluting from the column was collected in a second set of scintillation vials.

Scintillation vials containing the earlier collected DAG and the later cholesterol were dried under nitrogen and the lipids were dissolved in 200 μl methanol. Ten ml of scintillation cocktail were added to the vials before radioactivity determination in a liquid scintillation counter (LS 6800, Beckman Instruments, Irvine CA.). The counts
in each sample were a measure of the recovery rate of cholesterol and DAG from each sample. The area under the peaks corresponding to cholesterol, DAG and MAG were calculated using Maxima's peak integrating function. Using the peak area and correcting for the recovery of lipid in each sample, the amount of cholesterol, DAG and MAG in each sample was determined in volt•second. Using the molar absorptivity of 13,200/M•cm for the benzoyl ester (Warne and Robinson, 1991) and conversion factor 802140 μV•s/nmol for normal-phase (DAG and MAG) and 572957 μV•s/nmol for reverse-phase chromatograms (cholesterol), the lipid mass was determined in nmoles. The conversion factors were determined by chromatography of known amounts of [3H]1,2-dipalmitoylglycerobenzoate and [3H]cholesteryl benzoate and lipid mass verified by scintillation counting. The amount of DAG and MAG was then normalized to the amount of cholesterol in each sample to account for differing mass of tissue, with the final data being represented as nmol DAG or MAG/μmol of cholesterol.

**Binding Studies in the Rat Vas Deferens**

**Tissue and Sample Preparation**

Freshly dissected vasa from animals seven days after surgery were placed in ice-cold, oxygenated PSS and cleaned of connective tissue and attached blood vessels. Denervated and control vas deferens were mounted in isolated organ baths, bathed in oxygenated PSS and under 250-300 mg of tension. After about an hour of
equilibration, each tissue was exposed to 100 μM tyramine to verify successful
denervation of the experimental tissues. Denervated vasa that did not respond to
tyramine were exposed to 10 μM NE and then all tissues were washed with fresh PSS
and incubated for a further 45 min.

Each vasa was placed in a ground glass homogenizer and manually
homogenized in 2.5 ml of ice-cold 25 mM MOPS buffer containing 250 mM sucrose,
2.5 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 5 mM dithiothreitol and 10
μg/ml leupeptin. The homogenate was transferred to 15 ml polycarbonate tubes and
centrifuged in a Beckman J2-21M centrifuge (Beckman Instruments, Irvine, CA) at
50,000 X g for 30 min. The resulting supernatant was discarded and the pellet
resuspended in 5 ml of MOPS buffer (containing 5 mM CaCl₂ and 8 mM MgCl₂)
using a Kontes microtip sonicator/cell disrupter (Electro-Mech Instruments, Perkasie,
PA.). The suspension was centrifuged a second time at 50,000 X g for 30 min and the
supernatant was discarded. The pellet was resuspended in 3 ml of 25 mM MOPS
buffer (5 mM CaCl₂, 8 mM MgCl₂, pH 7.4 at 37°C) using a sonicator and then either
kept on ice until the binding assay was performed or stored at -20°C for up to 2 weeks
before assay.

Binding Assay Procedure

Protein Assay. Aliquots (100 μl) of the membrane samples in MOPS buffer
were assayed for protein content using a modified procedure of the method introduced
by Lowry et al. (1951, see Appendix C) and using bovine serum albumin as the protein standard.

**Saturation Binding Assay.** The binding characteristics of membrane-bound protein kinase C from denervated and control samples were compared using the \(^{3}H\)phorbol dibutyrate binding assay. The assay was done in 1.5 ml microcentrifuge tubes at 37°C in a shaking incubator/water bath (Precision Instruments, Chicago, IL.). Each microfuge tube contained 0.1 - 80 nM of \(^{3}H\) phorbol dibutyrate (\(^{3}H\)PDB, diluted from the stock in MOPS buffer) and 50 - 150 μg of protein sample (in MOPS) in 250 μl total volume. On occasion 10 μM of mezerein (a non-phorbol PKC activator) was present in each tube to account for non-specific binding of the ligand. The microfuge tubes were incubated in the water bath for 20 min at 37°C and pH 7.4, with constant agitation.

At the end of the incubation period, the tubes were quickly removed from the incubator and placed in a microcentrifuge. The tubes were centrifuged at 16,800 X g for 15 min to separate free and bound ligand. An aliquot of the supernatant (100 μl) was pipetted into 20 ml scintillation vials and counted to determine the free concentration of the \(^{3}H\)PDB. The rest of the supernatant was pipetted out and discarded into appropriate storage containers. The tip of each microfuge tube containing the membrane pellet was cut into a 20 ml scintillation vial using a sharp blade. The pellet was treated with 200 μl of 0.5 N sodium hydroxide in 0.5% sodium lauryl sulfate for about 30 min to digest the pellet protein. Bio-Safe II scintillation
cocktail (10 ml) was added to the vials which were then allowed to sit overnight before the radioactivity was determined. Vials were counted for 10 min each in a Beckman LS 9800 scintillation counter (Beckman Instruments, Irvine, CA.) at 35-41% efficiency.

**Competitive Binding Assay.** The binding characteristics of unlabelled PDA, PDB, mezerein and 4α-phorbol in control vasa were determined by competitive binding of these agents with [3H]PDB. As with the procedure outlined above, the assay was performed in 1.5 ml microfuge tubes in 250 μl MOPS buffer with 50-150 μg protein and 5-10 nM of [3H]PDB. Various concentrations of PDA (1-10000 nM), PDB (0.1-3000 nM), mezerein (0.1-3000 nM) and 4α-phorbol (0.1-300 μM) were added to each tube. Tubes were incubated for 20 min at 37°C and pH 7.4 in a shaking incubator. Bound and free ligand were separated by centrifugation as indicated above and the radioactivity was determined by liquid scintillation counting.

**Protein Kinase C Activity in Rat Vas Deferens**

**Tissue and Sample Preparation**

Denervated and control vasa were prepared and incubated in PSS as outlined previously. To determine differences in the ability of α-adrenoceptor stimulation to activate PKC, each tissue was exposed to 100 μM NE. The tissues were stimulated with NE for 0, 1, 2, 5, and 20 min at which time they were quickly blotted on tissue
paper and manually homogenized in a ground glass apparatus containing ice-cold buffer. The volume of homogenizing buffer used for each tissue was 50 volumes of the tissue wet weight, and consisted of 25 mM MOPS (pH 7.4 at 25°C) 250 mM sucrose, 2.5 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 5 µg/ml leupeptin.

The homogenate was centrifuged at 50,000 X g for 30 min at 4°C and the supernatant (cytosolic fraction) was pipetted out and stored in capped 5 ml polypropylene tubes at -20°C. The pellet was resuspended in 4 ml of homogenizing buffer using a Kontes micro-ultrasonic cell disrupter (Electro-Mech Instrument Co., Perkasie, PA). The suspension was also stored frozen until the assay procedure (2 weeks later). On the day of the assay, the samples were thawed at room temperature and kept on ice.

The pellet suspension was treated with Triton X-100 (0.3% final concentration) for about an hour, while on ice, with intermittent mixing using a sonicator. The mixture was then centrifuged at 50,000 X g and 4°C for 30 min. The supernatant containing the solubilized membrane proteins was saved as the particulate fraction and kept on ice. The particulate and cytosolic fractions (100 µl aliquots) were then assayed for protein content using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA.) with bovine-γ-globulin as the protein standard (Appendix D). The samples were all diluted with the homogenizing MOPS buffer to a final protein concentration of 40 µg/ml before assaying for PKC activity.
Protein Kinase C Activity Assay

The particulate and cytosolic cell fractions were assayed for PKC activity using a commercially available kit from Amersham. The assay detects the ability of sample PKC to transfer the labelled phosphate group from [γ-32P]ATP onto a threonine residue on the peptide substrate in the presence of optimal concentrations of calcium, phosphatidylserine and phorbol myristate acetate (PMA). The peptide substrate is derived from the epidermal growth factor (EGF) receptor which has been shown to be directly phosphorylated by PKC during cell activation with EGF (Heasley et al., 1989). Twenty five μl of each diluted sample (1 μg protein content) was incubated with [γ-32P]ATP (22,000 dpm), 75 μM substrate peptide, 1mM calcium acetate, 3.75 mM magnesium acetate, 2 μg/ml phorbol 12-myristate 13-acetate (PKC activator) and L-α-phosphatidyl-L-serine in 75 μl total volume. The reaction tubes were incubated at 25°C for 15 min and the reaction stopped with the addition of 100 μl dilute acid. An aliquot from each tube (125 μl) was spotted onto a 4 cm² piece of peptide-binding paper which was then rinsed twice in 5% acetic acid (10 ml/paper) for 10 min. The papers were then placed in 20 ml scintillation vials along with 10 ml Bio-Safe II cocktail. The vials were capped and counted without delay for 32P for 10 min each on a Beckman LS 9800 counter (Irvine, CA.). Blank runs with no protein sample were performed simultaneously to account for nonspecific binding of radioactivity to the binding papers. Eight particulate and cytosolic samples from the two groups were randomly chosen and assayed for PKC in the absence of Ca2+, PMA and PS. This was to determine nonspecific kinase activity in the samples.
Calculations and Statistics

Contractile Studies

Data are usually represented by the mean (± S.E.M.) response as a percent of the paired, control response, for 3-7 animals. This method of data representation decreased the variation between animals and allowed for more rigorous comparisons. The one- and two-way analysis of variance with least significant difference (LSD) were used to compare mean responses. Bonferroni's multiple comparison test was applied to analyses that resulted in nonsignificant F-ratios. The Students t-test was only used when the variation of the means were equivalent for the two groups being compared. Levels of P < 0.05 were accepted as indicative of significant differences between the groups being compared. Statistical analyses were done on an IBM-compatible personal computer using the SPSS/PC* Studentware (SPSS Inc., Chicago, IL.) or InStat (San Diego, CA.) software packages.

Diacylglycerol Studies

The mass of DAG and MAG were expressed in terms of the cholesterol content of each sample to account for differences in lipid recovery after the extraction procedure as well as to normalize for the differing mass of the tissues. The mean ± S.E.M. or S.D. of these values were used to represent the data. Data were not reduced to percent values in order to allow for comparisons with further experiments. One-way analysis of variance (InStat software, San Diego, CA.) was used to compare
means and probability values less than 0.05 were accepted as indicating statistical
difference.

**[^3]H]PDB Binding Studies**

Data obtained from the radioligand binding assays were converted from counts
per minute to picomoles of binding per milligram of protein by using a spreadsheet
program (SwiftCalc, Northbrook, IL.) on a personal computer. In the saturation
binding assay these values were then used to determine the binding maximum ($B_{\text{max}}$)
and dissociation constant ($K_d$) of $[^3]H]PDB$ in the protein samples obtained from
denervated and control vasa. Using the non-linear regression analysis available on
GraphPad InPlot software (San Diego, CA.), the data were fit to the equation:

$$\text{Total Binding} = \frac{B_{\text{max}} [\text{Ligand}]}{K_d + [\text{Ligand}]} + N [\text{Ligand}],$$

where $N[\text{Ligand}]$ represents the nonspecific binding of the ligand. The $B_{\text{max}}$
represents the maximum number of binding sites in pmoles per mg protein whereas
the $K_d$ is the dissociation constant of $[^3]H]PDB$ for the binding site in nanomolar.

In the competitive binding studies the data were reduced to percent of maximal
binding for each of the unlabelled ligands. These values were fit to the competitive
binding equation for one or two sites using GraphPad's curve fitting program. The $K_d$
for the competing ligands were calculated by converting the $IC_{50}$ using the Cheng and
Prusoff (1973) factor,
\[ K_1 = \frac{IC_{50}}{1 + \frac{[\text{Ligand}]}{K_d}} , \]

where \( K_1 \) is the dissociation constant of the competing unlabelled compound, and \( K_d \) is that of \([\text{H}]\text{PDB}\) and \([\text{Ligand}]\) is the concentration of the \([\text{H}]\text{PDB}\). This analysis was also performed using GraphPad InPlot. The mean values of \( B_{\text{max}} \) and \( K_d \) (± S.E.M.) obtained for the denervated and control samples were compared by paired Student's t-test, with \( P < 0.05 \) as indication of statistical difference.

**PKC Activity in Vas Deferens**

The efficiency of \( ^{32}\text{P} \) counting was assumed to be 100% and counts per minute were converted to nmoles of phosphate transferred to the peptide substrate per min per mg of sample protein (nmol/min/mg), according to instructions available with the kit. These values from each of the 4 tissues (animals) in each treatment group were averaged (mean ± S.E.M.). Using one-way analysis of variance changes in PKC activity in particulate and cytosolic fractions after agonist stimulation were compared to basal activities in resting tissues. Changes in corresponding denervated and control tissue PKC activities after agonist stimulation were also compared by analysis of variance. Probability values less than 0.05 were accepted as indicating significant differences between the means under comparison.
Materials and chemicals

Contractile Studies

(-)-Norepinephrine bitartrate, 1-oleoyl-2-acetyl-sn-glycerol (OAG), PDA, PDB, mezerein, 4α-phorbol, nifedipine, and 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine (isoH-7) were obtained from Sigma Chemical Co. (St. Louis, MO.). Neurokinin A (NKA) was purchased from Bachem California, Inc. (Torrance, CA.) and phentolamine was a kind gift from Ciba-Geigy (Summit, NJ.). PDA, PDB, mezerein and 4α-phorbol were dissolved in DMSO and chloroform was the solvent for OAG. Nifedipine was initially dissolved in ethanol and dilutions were made in distilled water. Final concentration of solvents in the organ baths did not exceed 0.1% (v/v). All other agents were dissolved in distilled water or PSS before addition to organ baths. Reagents and chemicals were obtained from Fisher Scientific (Pittsburgh, PA.).

DAG Studies

Methanol, chloroform, hexane, methyl-tert-butyl ether and cyclopentane were of HPLC grade and purchased from Burdick and Jackson (Muskegon, MI.). Benzene, benzoic anhydride, benzoyl chloride and pyridine were from Aldrich Chemical Co. (Milwaukee, WI.). (1,2-3H(N)) Cholesterol (62.0 Ci/mmol) was from New England Nuclear (Boston MA.) while the [3H] dihexadecanoyl-sn-glycerol ([3H] dipalmitin) was prepared from L-α-dipalmitoyl [2-9,10-3H(N)]phosphatidylcholine (50.0 Ci/mmol,
NEN) by phospholipase C (Bacillus cereus, Type XIII, Sigma Chem. Co., St. Louis, MO.) hydrolysis (Warne and Robinson, 1991). 1,2-diaclylglycerol standards were purchased from Sigma or prepared by phospholipase C hydrolysis of the appropriate phosphatidylcholine species (Sigma Chem. Co.) as described above.

**Binding Studies**

3-(N-morpholino) propane sulfonic acid (MOPS), EDTA, EGTA, leupeptin, β-mercaptoethanol and dithiothreitol were obtained from Sigma Chem. Co. (St. Louis, MO.). [20-3H(N)] Phorbol-12,13-dibutyrate (18-20 Ci/mmol) was purchased from New England Nuclear (Boston, MA.). Bio-Safe II scintillation cocktail was from Research Products International (Mount Prospect, IL.) and all other chemicals and reagents were from Fisher Scientific.

**PKC Activity Studies**

The protein kinase C kit and [γ-32P]ATP (3000 Ci/mmol) were purchased from Amersham Life Sciences (Arlington Heights, IL.) whereas the Bio-Rad protein assay was from Bio-Rad Labs (Hercules, CA.). The bovine-γ-globulin, EDTA, EGTA, phenylmethylsulfonyl fluoride, DTT and leupeptin were from Sigma Chem. Co. (St. Louis, MO.). All other chemicals and reagents were from Fisher Scientific.
CHAPTER 3

Results

Contractile Studies in the Vas Deferens

Normal Contractile function and PKC.

Exposure of the rat vas deferens to 10 µM NE caused a rapid increase in contractile force that reached a maximum within 25-30 seconds and then decayed to a steady tonic phase within 2-3 min (Fig. 3A). Neurokinin A (NKA, 2 µM, not shown) and K* (60 mM, Fig. 7) challenges also produced similar profiles.

![Figure 3](image-url)

Figure 3 (A) Typical isometric response to 10 µM NE in the rat vas deferens. (B) Response to 20 mM K*-PSS followed by 2 µM PDA in the same tissue. (C) Response to 2 µM 4α-phorbol in 20 mM K*-PSS. Resting tension on all tissues at the beginning of the trace is 250 mg. 'W' indicates tissue rinsed with fresh PSS.
Exposure of vasa deferentia to PDA or PDB alone produced, at best, a small transient contractile response (less than 10% of the maximal NE response). However in the presence of PSS containing 20 mM K⁺, 2 μM PDA produced a prompt phasic response that was 87 ± 0.1% (n=4) of the 10 μM NE response. Contractile force was maximal within 2 min (Fig. 3B) and then decayed to a sustained tonic phase (later tonic response not shown). By varying the K⁺ concentration of the PSS from 10-40 mM maximal PDA response was found to occur in PSS containing 20 mM K⁺. Phorbol-12,13-dibutyrate (2 μM) also elicited a contractile response that was 50 ± 2.8 % of the maximal NE response (n=4; not shown) whereas the inactive, 4α-phorbol (2 μM), was unable to elicit any contractile response (Fig. 3C). The responses to PDA (in the presence of 20 mM K⁺) were concentration-dependent with an EC₅₀ of 190 ± 26 nM (n=4, Fig. 4).

![Figure 4](image_url)
Contractile responses to PDA and PDB were immediately reversed by rinsing tissues with normal PSS and were attenuated by 20 min pretreatment with 30 μM isoH-7, a PKC inhibitor. Prior treatment of vas deferens with 30 nM nifedipine, a dihydropyridine calcium channel blocker, resulted in greater than 80% decrease in the PDA contractile response (P < 0.05, Table 1). Neither chronic surgical denervation of the vas deferens (Table 1) nor 10 μM phentolamine pretreatment (not shown) had an effect on the maximal PDA response obtained in the 20 mM K*-containing PSS. The DAG analog, OAG (10 µg/ml), did not produce a significant contraction (in normal or 20 mM K*-PSS) when compared to paired tissue exposed to the vehicle (chloroform, not shown).

The contractile responses of the vas deferens to NE and NKA were non-competitively inhibited by pretreating vas deferens with 30 μM isoH-7 for 20 min. Maximal responses to NE and NKA were reduced to 42 and 30% of control (P < 0.005), respectively (Figs. 5 and 6). IsoH-7 also depressed maximal phasic responses to 60 mM K* and 2 μM PDA (65 and 49% of control, respectively, P < 0.05). IsoH-7 treatment of rat vas deferens resulted in a marked loss in the sustained (tonic) response to K* (Fig. 7) and PDA (not shown).
Table 1

The effect of various treatments on the 2 μM PDA-induced (in 20 mM K⁺-PSS) contractile response in the rat vas deferens.

One vas deferens was used as the control tissue while the paired tissue was either chronically denervated, treated with 30 μM isoH-7 (20 min) or treated with 30 nM nifedipine (10 min) prior to eliciting the PDA response. Data are represented by the mean (± S.E.M.) response of 4-6 animals, expressed as a percent of the control response.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Contractile response to PDA in 20 mM K⁺-containing PSS (% of the control response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired control</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>7-day denervated</td>
<td>6</td>
<td>128.9 ± 17.8</td>
</tr>
<tr>
<td>After isoH-7</td>
<td>4</td>
<td>49.2 ± 6.0 a</td>
</tr>
<tr>
<td>After nifedipine</td>
<td>4</td>
<td>15.9 ± 3.3 b</td>
</tr>
</tbody>
</table>

abc Significantly different from control, P < 0.05 and P < 0.005, respectively.
Figure 5. Cumulative C-E curve to NE in control and 30 μM isoH-7 treated rat vas deferens. Data are the mean (± S.E.M.) response of three animals.

Figure 6. Cumulative C-E curve for NKA in control and 30 μM isoH-7 treated vas deferens. Data are the mean (± S.E.M.) response from four animals.
Figure 7. Representative tracings of the contractile response to 60 mM K⁺ in control (A) and 30 μM ISO-H-7 treated (B) vas deferens. Resting tension on the tissues prior to stimulation was 250 mg.
Vas deferens pretreated for 10 min with 2 μM PDA in normal PSS showed no significant change in the onset of response or the EC$_{50}$ of the subsequent NE concentration-curve effect (Fig. 8). The maximal response to NE in the presence of PDA was greater than with NE alone in every case but narrowly failed to achieve statistical significance by two-way analysis of variance (P < 0.07) and Bonferroni's test.

Figure 8. NE response after 10 min PDA (2 μM; ●) or vehicle (DMSO; O) pretreatment of paired vas deferens. Responses are represented by the mean (± S.E.M.) of six animals, obtained in normal PSS.
Assessment of Denervation. Vasa deferentia that were chronically denervated and their paired controls were exposed to 100 μM tyramine in isolated organ baths and the contractile response recorded. Denervated tissues showed a time-dependent loss in responsiveness to tyramine (Fig. 9), with 2-day denervated tissues producing $21.8 \pm 5.2\%$ of the control response ($n=4$, $P < 0.001$). Vasa denervated for seven days produced a contractile response that was $1.7 \pm 0.7\%$ of control ($n=4$, $P < 0.001$). The tyramine response was essentially the same for tissues that were denervated for 4-14 days and only those producing 10% or less of the control were considered sufficiently denervated to be used in the study.

![Figure 9. Tyramine response in control and paired, 2 or 7-day denervated rat vas deferens. ** indicate significant difference from control ($P < 0.001$).](image)
NE response in denervated vas deferens. In tissues that were chronically denervated for 6-8 days, 10 μM NE was able to elicit 2.02 ± 0.3 g of contractile tension within 25 seconds (Fig. 10). The paired control tissues produced 0.63 ± 0.11 g of maximal tension at the same time point (n=6, P < 0.005). All tissues that were tested showed an initial large phasic response followed by a later tonic response of lesser magnitude. The tonic portion in the denervated tissues was significantly greater than that of control vas for up to 3 min of NE exposure (P < 0.05).

Figure 10. Time-course of contractile force generated by denervated (●) and control (O) vas in response to 10 μM NE. Response in the denervated vasa were significantly greater than in controls at all points (P<0.05).
Cumulative C-E curves to NE were produced in denervated tissues and compared to control responses to characterize the increased sensitivity of these tissues. The EC$_{50}$ for NE in denervated tissues was $5.5 \times 10^{-8}$ M while that in the control was $9 \times 10^{-7}$ M, an 18-fold shift in the C-E curve after denervation (Fig. 11). Cumulative additions of NE also resulted in $73.3 \pm 8.7\%$ greater contractile maximum in denervated than in the control vas (P < 0.01). The contractile responses to NE in denervated vasa were significantly greater than in the control at all concentrations of the agonist tested (P < 0.01).

![Figure 11](image.png)

**Figure 11.** Concentration-effect curve to NE in 7-day denervated and paired control rat vas deferens. All denervated values were significantly different from control (P < 0.05).
Response to NKA in denervated vas deferens. Construction of cumulative C-E curves to NKA in denervated and control vasa showed no significant alteration in the threshold concentration or EC$_{50}$ of the two groups (Fig. 12). Moreover, there was no overall effect of denervation on the NKA response of the vas deferens as indicated by two-way analysis of variance (P > 0.1). None of the concentrations of NKA used produced a significantly greater response in the denervated tissue, as indicated by Bonferroni's multiple-comparison test.

Figure 12. Neurokinin A contractile response in control (Δ) and denervated (▲) rat vas deferens.
Response to PDA in Denervated Vas Deferens. Cumulative additions of PDA to isolated organ baths produced significant contractile responses in both the control and denervated vas deferens (Fig.13). In the presence of 10 μM phentolamine (an α-adrenergic receptor antagonist) and 20 mM K⁺-PSS, PDA produced concentration-dependent contractile activity in both tissue groups. However the responses obtained from the two groups were not significantly different. Contractile responses to single challenges of 2 μM PDA (in 20 mM K⁺) were not significantly altered after denervation either (Table 1).

Figure 13. Concentration-effect curve to PDA (20 mM K⁺-PSS) in denervated and control vasa.
NE response in denervated vasa after nifedipine. Concentration-effect curves to NE were constructed in denervated and control tissues, before and after 10 min incubation with 100 nM nifedipine (a calcium channel blocker). The response to NE obtained after nifedipine was expressed as a percent of that before pretreatment with the channel blocker (Fig. 14). This concentration of nifedipine was selected as it significantly inhibited the contractile activity of both the denervated and control tissues in response to NE (P < 0.05) without completely abolishing it. The maximal contractile response to NE in the presence of nifedipine was 11.7 ± 1.0% (P < 0.001) and 34.8 ± 2.3% (P < 0.001) of the response before, in control and denervated tissues, respectively. Thus, nifedipine was able to inhibit 88% of the NE response in control vasa whereas only 65% of the denervated tissue response was antagonized.

Figure 14. NE contractile response in denervated and control vas deferens before and after 100 nM nifedipine treatment. Responses to NE after nifedipine treatment, in each tissue, is expressed as a percent of the NE response before nifedipine exposure.
Determination of Diacylglycerol Mass in Rat Vas Deferens.

**DAG Mass by Thin Layer Chromatography.**

Denervated and control vasa were exposed to 100 μM NE in isolated organ baths under physiological conditions, for up to 10 min. The neutral lipids in the tissue were extracted in chloroform, and DAG and cholesterol resolved along with appropriate standards by thin layer chromatography. The lipids were visualized by charring on a hot plate (Plate 1) and quantified, relative to lipid standards, using Bio-Image analysis software.

The mass of DAG in each sample was divided by the mass of cholesterol in the same sample and expressed as ng of DAG per μg of cholesterol. Exposure of denervated and control vasa to 100 μM NE resulted in no significant change in free DAG from basal values at any of the time points determined (Fig. 15). Additionally, diacylglycerol mass was not significantly different between the two groups at any of the time-points compared.
Figure 15. Diacylglycerol mass determined in denervated and control vas deferens after stimulation with 100 μM NE. Data are represented by the mean (± S.E.M.) for 3-4 animals.
Plate 1 Thin layer chromatographic plate containing the cholesterol (column 1-5) and DAG (column 14-18) standards as well as lipid samples (column 6-13) from denervated and control vas deferens treated with NE for 10 min. For each sample a low and high amount were applied to the silica plate with the low amount being used to quantify the cholesterol content and the high sample used to determine DAG mass.
DAG Mass in Control Vas Deferens by HPLC.

Control vas deferens alone were exposed to 10 µM NE for 0-10 min under physiological conditions and the neutral lipids extracted from the tissue. DAG and MAG were separated and quantified by normal-phase HPLC. The mass of DAG and MAG were divided by the amount of cholesterol in the same sample and expressed as nmol/µmol cholesterol. Basal levels of DAG determined by HPLC (35 nmol/µmol cholesterol) were similar to those obtained by TLC (29 ng/µg cholesterol). As with the TLC experiments stimulation with NE for up to 10 min did not significantly alter the mass of DAG in the tissues (Fig. 16). The mass of MAG in the same samples did not accumulate significantly until after 5 min of exposure to NE.

Figure 16. DAG (●) and MAG (○) levels in control vas after NE exposure. Lipid mass was determined by HPLC from 2 animals at each point.
Molecular Species of DAG in Rat Vas Deferens.

The molecular species of DAG from denervated and paired control vasa were compared by reverse-phase HPLC to reveal any alteration in the source of DAG following denervation. Species of DAG from denervated and control vas deferens stimulated with 100 μM NE for 0, 1 and 10 min were compared.

All samples contained DAG lipids with similar retention times as the 18:0-18:1, 16:0-16:0, 16:0-18:1, 18:1-18:1 and 18:0-20:4 DAG standards (Fig. 17). Exposure to NE for 10 min resulted in relatively higher amounts of the 18:0-20:4 DAG species in both control and denervated tissues. Chronic denervation of the vas deferens did not alter the relative amounts of the various species of DAG in resting or NE stimulated tissues (not shown).

Figure 17. Molecular species of DAG from resting and NE stimulated (10 min), denervated rat vas deferens; separated by reverse-phase HPLC. The arrow indicates the position of 18:0-20:4 DAG.
**[^{3}H]Phorbol Dibutyrate Binding Studies**

[^3H]PDB Binding in Control Vas Deferens.

Control vasa were homogenized in 0.3 mM CaCl₂/1 mM MgCl₂-containing MOPS and the particulate fraction (containing the plasma membrane) was assayed for PKC binding characteristics. The specific binding of the ligand was calculated from total binding by non-linear regression analysis (GraphPad InPlot, Fig. 18). A binding maximum ($B_{\text{max}}$) of 22.8 pmol/mg protein and dissociation constant ($K_d$) of 7.6 nM were obtained. Binding assays performed in the presence of 10 μM mezerein (to account for nonspecific binding) provided a $B_{\text{max}}$ of 27.9 pmol/mg and $K_d$ of 9.8 nM.

![Figure 18. Specific binding of [^3H]PDB to control rat vas deferens particulate calculated from total binding. Each data point is a mean of 2 replicates.](image)

$B_{\text{max}} = 22.8 \text{ pmol/mg}$  
$K_d = 7.6 \text{ nM}$
Specific binding of [³H]PDB was competitively displaced by unlabelled mezerein ($K_d = 7 \text{ nM}$), PDB ($K_d = 15 \text{ nM}$) and PDA ($K_d = 500 \text{ nM}$) but not by the non-PKC activator, 4α-phorbol (Fig. 19).

![Graph showing competitive binding of mezerein (mez), PDB and PDA but not 4α-phorbol with [³H]PDB, in control vas deferens.]

**Figure 19.** Competitive binding of mezerein (mez), PDB and PDA but not 4α-phorbol with [³H]PDB, in control vas deferens.

**³H]PDB Binding in Denervated Vas Deferens.**

Control and paired, denervated vas deferens were prepared as indicated earlier and homogenized in Ca²⁺-free MOPS containing EDTA and EGTA. The particulate fraction was isolated and assayed. As indicated above, specific binding was calculated from total binding by non-linear curve fitting. The $B_{max}$ obtained from control and denervated samples were $4.8 \pm 0.9 \text{ pmol/mg}$ and $4.1 \pm 0.4 \text{ pmol/mg}$ of protein, respectively. The $K_d$ values obtained from the two groups were also similar (Table 2).
Table 2

Specific binding of $[^3H]PDB$ in control and denervated rat vas deferens.

Whole rat vas deferens (denervated and paired control) were cleaned of extraneous tissue, incubated in PSS for 45 min and then challenged with 10 μM NE. After thorough rinsing and a further equilibration period, all tissues were homogenized in 2.5 ml MOPS containing 2.5 mM EDTA and 2 mM EGTA. The particulate fractions of the homogenates were isolated and $[^3H]PDB$ binding assays performed on them.

<table>
<thead>
<tr>
<th>Tissue Sample (n)</th>
<th>$B_{\text{max}}$ (± S.E.M.) (pmol/mg protein)</th>
<th>$K_d$ (± S.E.M.) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL 4</td>
<td>4.8 ± 0.9</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>DENERVATED 4</td>
<td>4.1 ± 0.4</td>
<td>3.5 ± 0.1</td>
</tr>
</tbody>
</table>
Protein Kinase C Activity in Rat Vas Deferens

PKC activity in control tissues.

Brain PKC activity has been characterized extensively (Nishizuka, 1988) and therefore initial studies were conducted to compare the relative amounts of PKC in the vas deferens. Samples of rat brain striatum and vas deferens were cleaned in cold, oxygenated PSS and incubated in Ca^{2+}-free (EDTA/EGTA) MOPS buffer for 15 min. The tissues were homogenized in Ca^{2+}-free MOPS and particulate and cytosolic fractions were prepared. All samples were assayed for protein content and diluted to a concentration of 40 μg/ml before being assayed for PKC activity.

Protein kinase C activity in the brain cytosolic fraction was 16.3 ± 1.1 nmol/min/mg protein (n=3) whereas that in the particulate was 5.6 ± 0.2 nmol/min/mg (n=3), giving a cytosolic to particulate ratio of 2.9. The PKC activity was 10.9 ± 0.3 and 5.3 ± 0.4 nmol/min/mg protein (n=4) in the vas cytosolic and particulate samples, respectively. This provided a cytosolic to particulate activity ratio of 2.1 in the vas deferens. The cytosolic activity of PKC in the brain sample was significantly higher than in vas deferens (P < 0.05) but particulate activities were equivalent.

Effect of denervation on NE-induced PKC activation.

Whole vasa deferentia (paired control and denervated) were prepared as indicated previously, incubated under physiological conditions and stimulated with 100 μM NE for 0-20 min. At various time-points of stimulation the tissues were quickly
blotted on tissue paper and then homogenized in Ca\(^{2+}\)-free MOPS.

The homogenates were separated into cytosolic and particulate fractions by centrifugation and following protein assay were diluted to 40 μg/ml of protein. The samples were assayed for total PKC activity as indicated earlier. Eight samples randomly selected from the particulate and cytosolic fractions were also assayed for activity in the absence of Ca\(^{2+}\), PMA and PS to determine the extent of 'nonspecific' phosphorylation activity in the assay.

Basal PKC activity of particulate and cytosolic fractions from denervated and control tissues were not significantly different. Stimulation of both denervated and control vasa with NE resulted in a time-dependent increase in particulate PKC activity with a concomitant decrease in cytosolic activity (Fig. 20 and 21).
Figure 20. Effect of NE exposure on PKC distribution in control vas deferens. Each point is the mean (± S.E.M.) of 4 rats and * indicates significantly different from 0 min activity (P < 0.05).

Figure 21. Effect of NE on PKC distribution in denervated rat vas deferens cytosolic and membrane fractions. Each point is the mean (± S.E.M.) of 4 animals. * indicates significantly different from 0 min values (P < 0.05).
In membrane preparations from control vasa, PKC activity was significantly elevated by NE at 2, 5 and 20 min. The cytosolic activity of PKC from these tissues were not significantly different from basal levels for up to 20 min of NE stimulation.

For denervated tissues the particulate activity was significantly greater than basal values after 1, 2, 5 and 20 min of NE stimulation (P < 0.05), and in the cytosolic samples PKC activity was only significantly altered at the 1 min point (P < 0.05). The maximal NE-induced PKC translocation to the membrane fraction was not different between the two groups. However, the maximal change was evident in denervated tissues within 5 min (Fig. 21) but was achieved only after 20 min in control samples (Fig. 20). Membrane PKC activity in the denervated vasa was different from that in the paired control tissue at 2, 5 and 20 min after NE stimulation (Fig. 22, P < 0.05).

Figure 22. Time-course of PKC translocation to the membrane fraction of denervated and control samples after NE treatment. * indicates values significantly different from the paired control activity (P < 0.05).
Cytosolic PKC activity was not different between the two groups at any of the time-points except 5 min, when difference in particulate activity between the two groups was maximal as well (compare Figs. 18 and 19). Activity in the absence of Ca\(^{2+}\), PMA and PS was 9.8 ± 0.5% and 21.9 ± 1.1% of total activity in particulate and cytosolic fractions, respectively (n=8, significantly different, P < 0.005).

PKC activation by other agents.

Particulate and cytosolic fractions from denervated and control vas deferens were prepared after stimulation with 60 mM K\(^+\)-containing PSS, 2 μM NKA or 2 μM PDB (in 20 mM K\(^+\)-PSS). Treatment with each of these agents significantly increased PKC activity in the particulate fractions of all the samples (P < 0.01, Table 3) without significant alteration in cytosolic activity. Within each pair of denervated and control sample there was no difference in the PKC activities in response to the various agonists except that between cytosolic activity of PDB treated denervated and control vasa (P < 0.05).
Table 3

Effect of K⁺-depolarization, NKA and PDB on PKC distribution in denervated and control rat vas deferens.

Denervated and control vasa were stimulated with 60 mM K⁺-PSS (for 2 min), 2 μM NKA (for 2 min) and 2 μM PDB (in 20 mM K⁺-PSS, for 3 min) and cytosolic and particulate fractions prepared. Each of the fractions was assayed for PKC activity and compared to values obtained from unstimulated tissue samples. The data are represented by the mean (± S.E.M.) PKC activity of 3-4 animals (n).

<table>
<thead>
<tr>
<th>Condition/Agonist</th>
<th>Cytosolic (nmol/min/mg)</th>
<th>Particulate (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denervated/None</td>
<td>6.15 ±0.24 (4)</td>
<td>4.29 ±0.17 (4)</td>
</tr>
<tr>
<td>Control/None</td>
<td>7.44 ±0.24 (4)</td>
<td>3.34 ±0.44 (4)</td>
</tr>
<tr>
<td>Denervated/K⁺</td>
<td>5.96 ±0.38 (4)</td>
<td>12.77 ±1.06 (4)*</td>
</tr>
<tr>
<td>Control/K⁺</td>
<td>6.40 ±0.31 (4)</td>
<td>14.18 ±0.88 (4)*</td>
</tr>
<tr>
<td>Denervated/NKA</td>
<td>6.20 ±0.24 (4)</td>
<td>11.22 ±0.72 (3)*</td>
</tr>
<tr>
<td>Control/NKA</td>
<td>6.14 ±0.34 (4)</td>
<td>12.11 ±0.37 (4)*</td>
</tr>
<tr>
<td>Denervated/PDB</td>
<td>5.52 ±0.54 (4)c</td>
<td>12.85 ±1.86 (3)*</td>
</tr>
<tr>
<td>Control/PDB</td>
<td>6.64 ±0.38 (4)</td>
<td>13.91 ±1.09 (4)b</td>
</tr>
</tbody>
</table>

* Significantly different from denervated/unstimulated or control/unstimulated values (P < 0.01), respectively.

C Significantly different from paired control tissues exposed to PDB (P < 0.05).
In the present study the role of protein kinase C (PKC) in mediating enhanced contractile response to receptor stimulation was examined. This enzyme has been shown to perform numerous cellular functions, including a role in the contractile activity of smooth muscle. In the smooth muscle of blood vessels, PKC activation results in contractile activity (Merkel et al., 1991), whereas PKC inhibition antagonizes the contractile response to receptor agonists (NE and angiotensin II; Shibata et al., 1990). The enzyme has also been implicated in the enhanced contractile response of blood vessels from spontaneously hypertensive rats (Turla and Webb, 1987; Bazan et al., 1992). Chronic denervation of the rabbit iris smooth muscle has also been associated with about 30% increase in cytosolic PKC activity (Akhtar and Abdel-Latif, 1986).

An enhanced role for PKC in denervation supersensitivity was tested by comparing the contractile activities of a PKC activator (PDA) in denervated and control vas deferens. The amounts of diacylglycerol (DAG, the endogenous activator of PKC) produced in response to NE stimulation were also determined in control and denervated vas deferens. The binding characteristics of membrane-bound PKC to labelled PDB in denervated and control tissues were determined and an enzymatic
method was employed to determine the ability of agonists to activate PKC in the two tissue groups.

Contractile Studies in the Vas Deferens

Normal Contractile Function and PKC

Exposure of the rat vas deferens to PDA or PDB in normal PSS produced contractile response that varied from none to small and transient. The reason for this response may be that the resting concentration of intracellular calcium is below the threshold for PKC activation by phorbol esters. The activation of PKC requires free calcium and the phorbol esters or DAG appear to decrease the calcium requirement of the enzyme (Howe and Abdel-Latif, 1988). However, if the calcium concentration is below the threshold for this activation then the phorbol esters would be unable to stimulate kinase activity. This may explain why phorbol esters (PDA, PDB) were able to elicit significant contractile activity in the presence of depolarizing PSS (20 mM K⁺) which would presumably elevate intracellular calcium concentrations. In vascular smooth muscle phorbol esters alone are able to elicit a sustained response that may take up to an hour to develop maximally (Shibata et al., 1990; Merkel et al., 1991). In Ca²⁺-free, depolarizing Tyrode buffer (80 mM K⁺), Baraban et al. (1985) obtained a contractile response of the vas deferens by addition of 1 mM Ca²⁺ and 0.2 μM PDA. Their results were obtained after prolonged exposure of rat vas deferens to non-physiological conditions, i.e. 80 mM K⁺, 0 mM Ca²⁺ and 32°C bath temperature.
In this study, responses to PDA and PDB were elicited after brief pre-exposure (1 min prior to phorbol agonists) to iso-osmolar PSS containing 20 mM K⁺. The EC₅₀ estimated from the PDA concentration-effect curve (190 nM) agrees with that obtained by Baraban et al. (1985) and with the Kₐ for PDA binding to brain PKC (König et al., 1985) i.e., 140 and 45 nM, respectively. As with receptor stimulated events, PDA and PDB (in the presence of 20 mM K⁺) produced a rapid phasic and a sustained tonic contractile response. This response was immediately reversed by rinsing tissues with fresh buffer and may indicate a membrane site for phorbol ester action. PKC appears to play the central role in the response to PDA and PDB because 4α-phorbol, which is unable to activate PKC in vitro (Howe and Abdel-Latif, 1988), was without effect in normal or 20 mM K⁺-containing buffer.

Inhibition of the K⁺ plus PDA response by nifedipine would support the idea that K⁺-depolarization only augments the action of PDA by raising intracellular Ca²⁺ concentration. It is also consistent with observations that phorbol esters only increase the affinity of PKC for Ca²⁺ (Castagna et al., 1982; Howe and Abdel-Latif, 1988). The failure of phentolamine and chronic denervation to inhibit the K⁺ plus PDA response, indicates that neither α-adrenoceptor activation nor NE release from adrenergic nerves is involved in this response. It is unclear why OAG, a synthetic DAG, was unable to elicit a response in the rat vas deferens. Rapid metabolism and poor distribution through intact tissue may be the reason. PKC activation by OAG has mostly been demonstrated in cell-free systems (Howe and Abdel-Latif, 1988) or in dispersed cells (Kaibuchi et al., 1983).
Pretreatment of the rat vas deferens with isoH-7 significantly inhibited contractile responses to NE and NKA. This agent has been shown to non-competitively inhibit PKC (Kischel et al., 1989; Ratz, 1990) presumably by interacting with the ATP binding site on the regulatory subunit of the enzyme (Hidaka et al., 1984). However, the inhibitory action of this compound on other cellular kinases (e.g. MLCK) are not known and cannot be ruled out.

The application of isoH-7 markedly attenuated the maximal phasic and subsequent tonic response to 60 mM K⁺ in the rat vas deferens. This result is consistent with reports that high extracellular K⁺ causes PKC translocation and activation (Ho et al., 1988; Haller et al., 1990). These responses are most likely mediated by the influx of Ca²⁺ via voltage-dependent calcium channels and subsequent rise in intracellular Ca²⁺. The ability of Ca²⁺ to activate PKC in the presence of phosphatidylserine alone (Howe and Abdel-Latif, 1988) and its ability to convert membrane-associated PKC to the membrane-inserted form (Huang, 1989) would also support the idea of PKC activation by high K⁺. It is unlikely that isoH-7 antagonizes the K⁺ response by inhibiting Ca²⁺ influx since Shibata et al. (1990) and Merkel et al. (1991) reported no influence of isoH-7 on the K⁺-induced ⁴⁰Ca influx in vascular smooth muscle.

In normal buffer, 10 min pretreatment of the vas deferens with PDA resulted in no change in the onset or EC₅₀ of subsequent NE-induced contractile responses. The maximal contractile force in response to NE was enhanced by PDA pretreatment in each tissue tested, but the overall result was found to be statistically non-significant.
This may indicate that the statistical analysis procedure used (Bonferroni's post-test for multiple comparisons) was too stringent. In the rat vas deferens Baraban et al. (1985) reported significant enhancement of the contractile response to a single NE (20 μM) challenge by 2-3 min pretreatment with PDA. However, we were not able to reproduce this result in our experiments using single NE challenges and similar incubation times.

**Denervation Supersensitivity in the Vas Deferens**

Vas deferens that were chronically denervated for 0, 2 and 7 days showed a time-dependent loss in tyramine-stimulated contractile response. This loss in tyramine response is very similar to the time-dependent loss in NE content of the denervated tissues (Kasuya et al., 1969; Rice et al., 1987) and consistent with the degeneration of adrenergic nerve endings after chronic denervation (Gordon-Weeks and Gabella, 1977). Birmingham (1970) also reported similar loss in tyramine response after denervation of the rat vas deferens.

Chronic denervation of the vas deferens resulted in tissues that produced an initial phasic and subsequent tonic contractile response to NE, much like the response in control tissues. However, exposure of the tissue to a single concentration of NE resulted in a maximal phasic response in denervated vasa that was 320% of the control response. Since the time to maximal response was essentially the same in both groups, the data would suggest a greatly increased rate of contractile force production after denervation. The later tonic response to NE was also significantly greater in the
denervated vas for up to 3 min of NE stimulation. The equivalent time to maximal force after denervation also argues against the hypothesis that better diffusion of NE through the tissue to the receptor sites is a factor mediating the supersensitivity response (i.e. a prejunctional phenomenon).

Cumulative additions of NE revealed that denervated vasa were about 18-fold more sensitive to the stimulatory action of the agonist when compared to control tissues. The maximum force generated by cumulative NE addition was also significantly greater in the denervated as compared to the control tissues. Much of the decrease in threshold concentration for contraction in the denervated vasa appears to be due to the loss of intact adrenergic nerve terminals. This would result in greatly reduced uptake of exogenously applied NE, resulting in higher effective concentrations of NE at the receptor (prejunctional effect). This action may be demonstrated by pretreating control vasa (having intact nerve endings) with NE uptake (cocaine and hydrocortisone) and metabolism (U-0521) inhibitors before production of C-E curves (Abraham and Rice, 1992a). The result is a 6-fold decrease in the EC$_{50}$ of the NE response without a change in the maximal response.

The largest component of the shift in the NE curve after denervation would thus be due to the loss in neuronal uptake of applied NE. The remaining 3-fold shift in the denervated tissue response would be due to postjunctional alteration in tissue response to the agonist. The enhanced maximal response to single concentrations, as well as cumulative additions of NE, are consistent with the development of significant postjunctional supersensitivity in the rat vas deferens.
NKA Response in Denervated Vas Deferens

Neurokinin A, which produces a maximum contraction equivalent to NE in normal vasa, did not reveal a significant increase in sensitivity of the tissue after chronic denervation. The EC₅₀ and maximal force developed were not significantly different between denervated and control tissues, when compared by analysis of variance. This lack of effect may reflect some basic difference in the coupling of the neurokinin receptor (NK-2, Osakada et al., 1986) to the contractile apparatus as compared to the α-adrenoceptor. Thus denervation would result in enhanced coupling of certain receptors (α₁, angiotensin and acetylcholine) but not others (NK-2), to the contractile apparatus.

An alternate explanation for the lack of supersensitivity to NKA may be that the cumulative additions of the agonists resulted in short-term desensitization of the receptors. In this case non-cumulative exposures to NKA, with rinsing of tissues in between additions, may more faithfully depict changes in response after denervation. Support for short-term desensitization may be obtained from the NE response, where the single challenge of NE produced a 320% greater response in denervated relative to control tissues. The cumulative curve to NE only showed a 178% increase in maximal response. However, any desensitization that may result from cumulative addition of NKA would be short-term because the vas deferens is able to produce two very similar NKA cumulative curves when separated by a 45 minute wash and equilibration period.
PDA Response in Denervated Vas Deferens

Phorbol diacetate (PDA) was able to elicit significant, concentration-dependent contractile activity of the denervated rat vas deferens in 20 mM K⁺-PSS. However this response to PDA was virtually identical to that obtained in the paired, control vasa. Responses to single challenges of PDA (2 μM) were not significantly different between the two groups.

The lack of an enhanced response in denervated tissues would indicate that PKC activation by K⁺ plus PDA is not significantly different in the two tissues. In addition, this result would also argue against increased nexal contacts as mediating the supersensitivity response, because this hypothesis predicts that all agonists will elicit an enhanced response.

NE Response in Denervated Vasa after Nifedipine

Cumulative C-E curves to NE were produced in control and denervated vas deferens before and after treatment of the tissues with nifedipine. This treatment eliminated about 88% of the subsequent NE response in control tissues, whereas only about 65% of the response in denervated vasa was abolished. These data are consistent with control tissues being more dependent on extracellular Ca²⁺ in producing contractile tension, as compared with denervated tissues. The denervated tissues may be more dependent on Ca²⁺ mobilized from intracellular sources via IP₃-sensitive or other mechanisms.
These results do not exclude the possibility that the net cytoplasmic concentrations of Ca\(^{2+}\) are the same in the two tissue groups during receptor stimulation, but that the contractile proteins become more sensitive to the cation after denervation. Ramos et al. (1986) demonstrated that the denervated guinea pig vas deferens was more sensitive to the rise in cytosolic Ca\(^{2+}\) than control vasa. In Tritonx-100-permeabilized guinea pig vasa, cumulative additions of Ca\(^{2+}\) revealed that denervated tissues were 1.5-fold more sensitive to the contractile effect of Ca\(^{2+}\) as compared to control vasa. These authors also observed an increased rate of tension development to Ca\(^{2+}\) in the denervated vas.

**Diacylglycerol Mass in Rat Vas Deferens**

- **DAG Mass by Thin Layer Chromatography**

Denervated and control vasa treated with NE for up to 10 min showed no significant change in DAG mass from basal levels as determined by TLC. Using such a chromatographic method Takuwa et al. (1986) were able to show significant elevation of DAG mass after stimulating bovine tracheal muscle strips with carbachol. However, Rembold and Weaver (1990), studying the effect of histamine and endothelin in swine carotid artery muscle strips, reported no change in DAG mass as assayed by the DAG kinase method.
Exposure of both denervated and control vasa to NE appeared to cause a small, transient decrease in DAG mass but this was not found to be statistically significant. DAG mass at all time points of NE stimulation were essentially the same in the two tissue groups.

These results are consistent with those reported by Takenawa et al. (1983) and Minneman et al. (1988), who observed no significant difference in PIP$_2$ hydrolysis between denervated and control vas deferens after NE stimulation. However, Takenawa et al. (1983) did report a significant accumulation of phosphatidic acid (PA) in denervated vasa after NE stimulation for 3 min. Since about a third of cellular DAG is metabolized to PA by DAG kinase (Majerus et al., 1986), this study was undertaken to determine if DAG from PIP$_2$ hydrolysis was the source of the elevated PA levels. The results suggest that the elevation in cellular PA has a source other than DAG. Membrane-bound phospholipase D has been shown to hydrolyze phosphatidylcholine to PA (Lassègue et al., 1993) and thus elevated PA levels after denervation may indicate an enhanced coupling of α-adrenoceptors to phospholipase D. However the implications for such an alteration in receptor coupling to contractile function is not clear.

**DAG Mass in Control Vas Deferens by HPLC**

Using the HPLC technique the mass of DAG in control vas deferens was determined after stimulation with NE. The mass of monoacylglycerol (MAG) in the same tissues were simultaneously measured. The basal DAG levels were very similar
to those obtained by thin layer chromatography. Exposure of the vas deferens to NE for up to 10 min resulted in no significant accumulation of DAG but rather a small, transient decrease in mass was observed again. The lack of measurable DAG accumulation in vasa after NE stimulation as determined by both TLC and HPLC methods would suggest the observation is not due to limitations in the methods employed. The initial decrease in DAG mass may be due to increased metabolism or decreased PIP$_2$ hydrolysis. This initial decrease in DAG mass was accompanied by similar decreases in MAG; thus, transient loss in DAG is not due to increased metabolism to MAG. However, after longer periods of NE stimulation (greater than 5 min), MAG began to accumulate rapidly; this is consistent with DAG levels being held in check by rapid metabolism to MAG (Majerus et al., 1986).

**Molecular Species of DAG in Rat Vas Deferens**

The molecular species of DAG were determined in denervated and control vas deferens that were at rest or stimulated with NE for 1 or 10 min. The various species of DAG in denervated and control samples appeared identical by HPLC. Resting tissues contained DAG lipids with similar retention times as 1-stearoyl-2-oleoyl glycerol, 1,2-dipalmitoyl glycerol, 1-palmitoyl-2-oleoyl glycerol, 1,2-dioleoyl glycerol and 1-stearoyl-2-arachidonyl glycerol standards. With receptor stimulation the relative proportion of the 18:0-20:4 DAG (mainly from PIP$_2$ hydrolysis) was increased. This result is consistent with PIP$_2$ hydrolysis after $\alpha_1$-receptor activation in the vas, which would result in IP$_3$ (Minneman et al., 1988) and 18:0-20:4 DAG (Howe and Abdel-
Latif, 1988) formation. However, this is in apparent contradiction with the earlier data that showed no change in total DAG mass either by TLC or HPLC methods. This discrepancy may be due to increased metabolism of total DAG as well as accumulation of 18:0-20:4 DAG upon receptor stimulation, the net result being little or no change in total DAG mass.

This portion of the study was carried out to determine if the source of DAG production had changed after chronic denervation of the vas deferens. The hypothesis was that PIP$_2$ hydrolysis would remain unchanged but DAG mass would be significantly increased due to coupling of the receptor to hydrolysis of other membrane phospholipids (e.g. phosphatidylcholine). Evidence for this type of dual coupling of the same receptor to different phospholipases has been presented by Plevin et al. (1991) and Lassègue et al. (1993). Such an alteration would have resulted in formation of additional DAG species other than 18:0-20:4, however, the evidence obtained does not support this hypothesis. The results from the present study, along with prior data from other labs, would indicate that denervation does not cause an enhanced coupling of $\alpha_1$-receptors to PIP$_2$ hydrolysis.

**$[^{3}H]$Phorbol Dibutyrate Binding Studies**

**$[^{3}H]$PDB Binding in Control Vas Deferens**

In this study the binding characteristics of membrane-bound PKC were determined using the specific ligand $[^{3}H]$PDB. Control vasa homogenized in Ca$^{2+}$-
containing MOPS buffer produced specific and saturable binding of \( ^3 \)H]PDB. The \( K_d \) values obtained in the present study (3-9 nM) agree well with those obtained for \( ^3 \)H]PDB binding in mouse brain particulate fraction (7 nM; Dunphy et al., 1980), mouse brain cytosolic fraction (4 nM; Sharkey et al., 1984), rat pituitary cells (8 nM; Jaken, 1985) and EL4 mouse thymoma cells (11 nM; Sando et al., 1982).

The specific binding of the ligand was also reversible and could be displaced by increasing concentrations of mezerein, unlabelled PDB and PDA. The non-PKC activator 4\( \alpha \)-phorbol was not able to displace \( ^3 \)H]PDB binding which suggests that this compound does not bind to PKC. This agrees with the findings of Howe and Abdel-Latif (1988) who reported no PKC activation by 4\( \alpha \)-phorbol, and Dunphy et al. (1980) who were unable to displace \( ^3 \)H]PDB binding with a 4\( \alpha \)-phorbol derivative in mouse brain. This also explains the lack of contractile response to 4\( \alpha \)-phorbol in the rat vas deferens when compared to PDA and PDB.

\( ^3 \)H]PDB Binding in Denervated Rat Vas Deferens

There was no significant difference in the specific binding characteristics of \( ^3 \)H]PDB binding between chronically denervated and the paired control vasa. The \( K_d \)s obtained from this study were similar to those obtained by other groups. The \( B_{max} \) for binding was not altered by denervation either. The similarity between the PKC binding characteristics of the two samples argues against an increased affinity of PKC for its endogenous activator (DAG) or elevated resting levels of the enzyme at the membrane mediating the supersensitive response.
Protein Kinase C Activity in Rat Vas Deferens

PKC Activity in Control Vas Deferens and Brain

The PKC assay kit utilized the transfer of $^{32}$P$_i$ from [32P]ATP to the substrate peptide (EGF receptor peptide) by the PKC in the sample. The PKC activity in rat striatal cytosolic samples was about 1.5 times greater than in vas deferens, whereas the activities in the particulate fractions from the two tissues were much the same. The ratio of cytosolic to particulate activity was almost 3 for brain samples and 2 for vas deferens. This activity ratio in the vas deferens was much smaller if the tissue was not homogenized in Ca$^{2+}$-free conditions, due to a calcium artifact of PKC adhering to the membrane preparations. Since the reported values of PKC activity vary greatly due to the assay conditions used by different laboratories, the brain samples were used as a standard with which to compare the relative amounts of PKC in the vas deferens. Considering that the brain has the highest reported amounts of PKC (Nishizuka, 1988), the vas deferens appears to have significant amounts of the enzyme (67% of brain activity).

Effect of Denervation on NE-induced PKC Activation

The basal activities of PKC in the cytosolic and particulate fractions from denervated and control vasa were not significantly different. This result is comparable to that obtained in the binding assays, with about 4 pmol/mg of PKC resulting in about 4 nmol P$_i$ transferred per min per mg protein; an effective PKC activity of about
1000 molecules of $P_i$ transferred per min per mol of enzyme. Vas deferens that were exposed to NE displayed a time-dependent increase in membrane-associated PKC activity with accompanying decreases in the cytosolic activity. This result is consistent with PKC translocating to the membrane from the cytoplasm during receptor stimulation. Much the same results were obtained by agonist activation of smooth muscle strips from bovine trachea (Langlands and Diamond, 1992); swine carotid artery (Singer et al., 1992); bovine carotid artery (Haller et al., 1990) and rat aorta (Bazan et al., 1992). However, the absolute values reported in these studies vary widely owing to differing assay conditions and tissues.

In denervated vasa the PKC translocation during receptor stimulation was maximal within 5 min of NE stimulation and remained elevated for up to 20 min of stimulation. In contrast, PKC activation in control vas deferens developed more slowly and reached maximum only after 20 min of NE stimulation. Longer stimulation times with NE were not carried out in either tissue group. This result is consistent with an increased rate of PKC activation in the denervated tissues relative to the control response. Since the amount of DAG is not significantly altered during receptor activation it would appear that intracellular $Ca^{2+}$, and not DAG, mediates the translocation of PKC to the membrane in the vas deferens. A more rapid change in cytosolic $Ca^{2+}$ would result in faster activation of PKC which could be in place to augment the receptor-mediated contractile activity.

The PKC activity in the particulate and cytosolic samples assayed in the absence of $Ca^{2+}$, PMA and PS were about 10 and 22% of total activity, respectively.
This small but significant phosphorylation activity in the absence of the cofactors for PKC would imply the presence of a second protein with similar kinase properties for the substrate peptide. According to the information in the PKC assay kit protein kinase A, phosphorylase kinase and the proteolytic fragment of PKC (Ca²⁺, PMA and PS independent) are able to generate 8, 13 and 38% of the PKC activity, respectively. Additionally, myosin light chain kinase and hexokinase were not found to have any activity when assayed with the kit. The Ca²⁺, PMA and PS-independent activity could be due to any or a combination of the active proteins that are found in this tissue. Brain cortex samples assayed with the kit also provided similar amounts of nonspecific phosphorylating activity.

**PKC Activation by Other Agents**

Denervated and control vas deferens exposed to high K⁺, NKA and PDB also exhibited significant PKC translocation to the membrane fraction, when compared to their respective unstimulated tissues. This data is similar to that obtained for K⁺-induced (Ho et al., 1988; Haller et al., 1990) and PDB-induced (Haller et al., 1990; Singer et al., 1992) PKC translocation in various tissues.

The activation of PKC between denervated and control tissues by these contractile agents was not significantly different. This is consistent with the lack of supersensitive contractile response in denervated vasa after high K⁺, NKA or phorbol ester stimulation. The ability of K⁺ to produce PKC translocation also suggests a mechanism by which PKC inhibitors are able to antagonize the contractile response to
\textbf{K}^{+}\text{-depolarization. It may also indicate that the rise in intracellular Ca^{2+} is the primary signal for PKC translocation (Ho et al., 1988).}

Cytosolic activities of PKC after treatment with these contractile agents were not significantly different from the respective untreated tissues. The activity of the enzyme in the denervated sample after PDB, however, was significantly lower than that in the paired, control sample. The relevance of this result is not readily apparent.

There is considerable evidence that PKC is able to perform multiple functions in cells, which makes it difficult to correlate its activity with a complex event such as muscle contraction. Direct biochemical studies have described an inhibitory role for the enzyme on PIP_{2} hydrolysis and MLCK-mediated phosphorylation of MLC (Nishikawa et al., 1984; Itoh et al., 1988; Leeb-Lundberg et al., 1985). Calcium fluorescence studies have also shown PKC to decrease the amplitude of the intracellular Ca^{2+} spike after agonist stimulation (Itoh et al., 1988). The enzyme has been implicated in activating the Ca^{2+}-ATPase pump on the plasma and sarcoplasmic membrane (Fukuda et al., 1990). Such an action will have the effect of extruding cytoplasmic Ca^{2+} to the extracellular space as well as increasing its sequestration into the sarcoplasmic reticulum.

There is also evidence to support a positive role of PKC on smooth muscle contraction. Itoh et al. (1988) showed that even though the intracellular Ca^{2+} spike after receptor stimulation is depressed by PKC activation, significant attenuation in contractile activity does not result. The authors ascribe a role for PKC in sensitizing the contractile elements to Ca^{2+} which leads to equivalent response in the presence of
lowered Ca\textsuperscript{2+}. This is consistent with the report by Baraban et al. (1985) where PKC activation (by phorbol esters) augments the receptor-mediated contractile event. Additionally, Souhrada and Souhrada (1989) and Mironneau et al., (1991) have presented evidence to support PKC-mediated Ca\textsuperscript{2+} influx into smooth muscle cells.

With PKC playing both inhibitory and stimulatory roles in smooth muscle, it is conceivable that these processes are chronologically separated. Thus short term PKC activation could result in positive PKC effects whereas long term activation leads to negative feedback effects. If such a scenario exists, then a more rapid translocation/activation of PKC in denervated vas deferens would put the enzyme in a position to effect positive actions with the normal contractile apparatus.

The existence of PKC isozymes adds further complexity to the role of the enzyme in situ; particularly with regard to the Ca\textsuperscript{2+}/phospholipid-independent and dependent forms. It is possible that the total amount of the enzyme remains unchanged after denervation of the vas deferens, but the relative amounts of the various isozymes becomes altered. Singer et al. (1992) and Khalil et al. (1992) have characterized 5 isozymes of PKC in smooth muscle; three of which are Ca\textsuperscript{2+}-sensitive (\(\alpha\), \(\beta\) and \(\gamma\)) and the rest Ca\textsuperscript{2+}-insensitive (\(\varepsilon\) and \(\zeta\)). Denervation of the vas deferens may trigger a shift in the PKC population from the Ca\textsuperscript{2+}-sensitive to the insensitive forms. Thus the PKC-mediated effects may become more apparent at lower cellular Ca\textsuperscript{2+} concentrations. This would result in PKC-mediated events being less dependent on Ca\textsuperscript{2+} in the supersensitive vas deferens. The greater than normal response of
denervated vasa to NE after nifedipine is consistent with such a decrease in the Ca$^{2+}$ requirement for contractile response.

Ultimately the enhanced contractile response in the denervated rat vas deferens probably reflects an increased number of actin-myosin crossbridges formed during receptor stimulation. Such an increase would be mediated by: (i) increased mobilization of Ca$^{2+}$ by receptor-mediated events; (ii) increased sensitivity of the contractile proteins to Ca$^{2+}$; (iii) decreased MLC phosphatase activity which is responsible for dephosphorylation of MLC and relaxation of muscle; or (iv) some combination of the three processes working in concert. Of the three major mechanisms, PKC has been implicated in sensitizing the contractile proteins to Ca$^{2+}$ as well as decreasing MLC phosphatase activity (Somlyo and Himpens, 1989). The results obtained herein support a positive role for PKC in the supersensitive response, but not necessarily as the sole mediator. The net enhanced response of the denervated vas deferens is most likely a culmination of several cellular processes in adaptation to the loss in neuronal input. Eventually, elucidation of the mechanism of the supersensitivity phenomenon will provide information as to how smooth muscle cells adapt to receptor signalling, as well as provide evidence that neurotransmitters such as NE are not just contractile agonists but also regulators of primary cellular function with regard to gene expression, protein synthesis and metabolic activity.
CHAPTER 5
Summary and Conclusions

1. The ability of PKC activators, such as PDA and PDB, to elicit contractile response in the rat vas deferens indicates a role for protein kinase C in the production as well as the maintenance of muscle contraction in this tissue. The effect of PKC inhibition leading to depressed receptor and non-receptor-induced contraction would attest to a fundamental role of PKC in the contractile activity of the normal vas deferens.

2. The markedly enhanced response to norepinephrine in the denervated rat vas deferens is indicative of the development of supersensitivity in this tissue. The inability of NKA and PDA to elicit an increased response after denervation may point to a difference in the way these agents produce a contractile response, relative to NE. The effect of nifedipine antagonizing the NE response in control tissues to a greater extent than in denervated vasa, is further support for a postjunctional alteration leading to the supersensitive response. It is also consistent with a change in the source for Ca^{2+} utilized for muscle contraction after denervation.
3. Stimulation of denervated and control vas deferens by NE resulted in no significant accumulation of the second messenger diacylglycerol. This may be due to rapid metabolic processes that lead to near constant amounts of tissue DAG. The molecular species of DAG found in denervated tissues were the same as those in control vasa and provide support for a lack of change in the coupling of α-receptors to multiple phospholipases. Rather the receptor appears to be linked to PIP₂ hydrolysis in both denervated and control tissues.

4. Chronic denervation of the rat vas deferens led to no change in the amount or binding affinity of membrane-bound PKC. Thus the supersensitive response is not mediated by an increased basal level of membrane-bound PKC or caused by its enhanced affinity for activators, phorbol esters or DAG.

5. NE was able to activate PKC at a faster rate in the denervated vas when compared to the control response. This alteration may result in PKC being in a position to more readily augment the Ca²⁺-mediated contractile function of the tissue. Neurokinin A, PDB and K⁺-depolarization produced equivalent PKC translocation in denervated and control vasa and this is consistent with the similar tissue responsiveness to these agents after denervation.
6. The ability of NE to cause PKC translocation in the rat vas deferens without increasing DAG levels would indicate that DAG is not the primary mediator of this process. In addition, basal levels of DAG in the tissues were found to be sufficient to activate at least 50 times the PKC that became associated with the membrane after receptor stimulation (assuming a mol per mol interaction of DAG and PKC).

7. The change in the rate of PKC activation and Ca\(^{2+}\)-dependence may eventually point to a changing role of Ca\(^{2+}\) in mediating contractile response in the supersensitive vas deferens. Assuming the tension developed by smooth muscle is proportional to the number of actin-myosin cross-bridges formed, the supersensitive response would be indicative of an increase in such an interaction. This increase in actin-myosin interaction could be mediated by: (i) increased mobilization of Ca\(^{2+}\); (ii) increased sensitivity of the contractile proteins to Ca\(^{2+}\); (iii) decreased rate of dephosphorylation of MLC; or (iv) a combination of these three events. Of these possible events this study provides evidence for increased intracellular Ca\(^{2+}\) mobilization and for PKC in increasing the sensitivity of contractile processes to Ca\(^{2+}\). Thus a direct role for PKC in mediating the supersensitive response merits further investigation.
8. Thus far, the study of denervation supersensitivity has progressed from the level of the receptor toward the intracellular events mediating contractile function. It may be more efficient to begin further studies from the more fundamental interactions of actin and myosin and design the process outward. The increased rate of contractile force production after denervation warrants the study of MLC phosphorylation following receptor activation in these tissues. The maximal phasic force produced should be proportional to the amount of MLC phosphorylated. A significant deviation from this relationship would point to secondary processes (increased Ca\(^{2+}\) sensitivity or involvement of other cytosolic proteins such as desmin, caldesmon, etc.) playing a more prominent role in the supersensitive tissue. The altered sensitivity to Ca\(^{2+}\) channel blockers in denervated tissues may reflect a change in the sensitivity of the contractile apparatus to Ca\(^{2+}\). Contractile studies with the denervated, skinned muscle preparation could provide evidence for such an alteration. Such a pursuit may eventually reveal differences in the regulation of smooth muscle after denervation and provide new insights into cell signal regulation.
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APPENDIX
Appendix A

Physiological Salt Solution

Reagents

Stock A (10X concentrate)

<table>
<thead>
<tr>
<th>Molecular Wt.</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>58.44</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>74.56</td>
</tr>
<tr>
<td>Calcium Chloride*2H2O</td>
<td>146.99</td>
</tr>
<tr>
<td>Magnesium Sulfate*7H2O</td>
<td>246.48</td>
</tr>
<tr>
<td>Dextrose</td>
<td>180.16</td>
</tr>
</tbody>
</table>

dissolve in distilled water 2000 ml

Stock B (10X concentrate)

<table>
<thead>
<tr>
<th>Molecular Wt.</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Hyd. phosphate</td>
<td>136.09</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>84.01</td>
</tr>
</tbody>
</table>

dissolved in distilled water 2000 ml

Procedure

1. To make 2 l of PSS, 200 ml of stock A and B are added in a volumetric flask and brought up to volume with distilled water.

2. Addition of distilled water (about 400 ml) between the mixing of stock solutions prevents precipitation of calcium salts.
3. The PSS is aerated with 5% carbon dioxide in oxygen for about 30 min before use.

<table>
<thead>
<tr>
<th>PSS Composition</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>112.94</td>
</tr>
<tr>
<td>KCl</td>
<td>4.75</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.52</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.19</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.55</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.18</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.00</td>
</tr>
</tbody>
</table>

**Ion Concentrations**

- Na⁺  137.9 mM
- K⁺   5.9 mM
- Ca²⁺ 2.52 mM
- Mg²⁺ 1.19 mM
- Cl⁻  122.7 mM
- PO₄³⁻ 1.18 mM
- HCO₃⁻ 25.0 mM
Appendix B

High K\(^*\)-containing PSS

Reagents

Stock A (10X concentrate)

<table>
<thead>
<tr>
<th>Molecular Wt.</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
</tr>
<tr>
<td>KCl</td>
<td>74.56</td>
</tr>
<tr>
<td>CaCl(_2)(\cdot)2H(_2)O</td>
<td>149.99</td>
</tr>
<tr>
<td>MgSO(_4)(\cdot)7H(_2)O</td>
<td>246.48</td>
</tr>
<tr>
<td>Dextrose</td>
<td>180.16</td>
</tr>
</tbody>
</table>

dissolve in distilled water 1000 ml

Stock B (10X concentrate)

<table>
<thead>
<tr>
<th>Molecular Wt.</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH(_2)PO(_4)</td>
<td>136.09</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>84.01</td>
</tr>
</tbody>
</table>

dissolve in distilled water 1000 ml

Procedure

1. To make 100 mM K\(^*\)-PSS (1 l) add 100 ml of stock A, dilute with about 200 ml off distilled water and then 100 ml of stock B in 1000 ml volumetric flask. Bring up to the mark with distilled water.

2. To make 100 ml of 20 mM K\(^*\)-PSS, the above solution is diluted with regular PSS according to the formula:
\[ [K^+] \cdot 100 \text{ ml} = \{100 \text{ mM } K^+ \cdot X \text{ ml}\} + 5.9 \text{ mM } (100 - X \text{ ml}), \]

which reduces to:

\[ X = \frac{([K^+] - 5.9)}{0.94}, \]

where \([K^+]\) is the desired \(K^+\) concentration and \(X\) is the volume of 100 mM \(K^+\) to be diluted.

Thus 15 ml of 100 mM \(K^+\)-PSS is mixed with 85 ml of regular PSS to obtain 100 ml of 20 mM \(K^+\)-PSS.
Appendix C

Lowry Protein Assay

Reagents

Reagent A:

- Sodium carbonate (2.00%) 10.00g
- Sodium dodecyl sulfate (0.50%) 2.50g
- Potassium sodium tartrate (0.02%) 0.1g
- Sodium hydroxide (0.10 N) 2.0g

Dissolve in distilled water 500 ml

Reagent B: Copper sulfate.5H₂O (0.5%, w/v)

Reagent C: Mix 50 ml of Reagent A and 1 ml of Reagent B

Reagent D: Folin and Ciocalteau reagent diluted with equal volume of distilled water

Procedure

1. Sample containing 10-150 µg of protein in 400 µl volumes are used.

2. Add 2 ml of Reagent C and mix well. Allow the mixture to stand at room temperature for at least 10 min.

3. Add 200 µl of Reagent D and vortex immediately. Allow to stand for at least 30 min at room temperature.

4. Read the absorbance of the mixture in a spectrophotometer at 750 nm, against appropriate blank mixture.
With each Lowry assay protein standards (bovine serum albumin) are also assayed. Typically 10-150 μg of BSA are assayed by the same procedure to produce a standard curve. The curve of Absorbance vs. protein content (BSA) is plotted and used to calculate the protein content in the unknown samples. This is done by the use of GraphPad's linear regression procedure.

**Figure 23.** Data represents the mean (± S.E.M.) of 4 replicates with linear regression line. The dotted line indicates the 95% confidence interval.
Appendix D

Bio-Rad Protein Assay

Reagent

Bio-Rad Protein assay dye reagent concentrate
diluted 1:5 with distilled water.

Procedure

1. Allquots of the samples (100 μl) containing 10-150 μg of protein are used.
2. Add 5 ml of the diluted dye reagent and mix by shaking.
3. Allow to sit at room temperature for at least 5 min but not longer than 1 hour.
4. Read the absorbance of the mixture in a spectrophotometer at 595 nm against appropriate blanks.
5. Standard protein amounts of bovine-γ-globulin (10-200 μg/100 μl) are also assayed at the same time.

A standard curve for bovine gamma globulin is produced by plotting the Absorbance_{595} versus the protein content of the standard. BSA is generally not used as a standard due to the high amounts of color developed, thus underestimating the protein in the samples.

This assay was used in the current study to assay for protein in buffers containing EDTA and EGTA, which give very high blanks when assayed with the Lowry method.
Figure 24. Data are represented by the mean (± S.E.M.) of 3 replicates. The dotted lines are the 95% confidence interval of the regression line.
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