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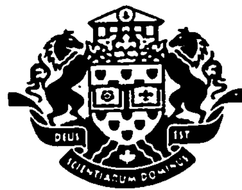

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THE ROLE OF TYROSINE KINASE IN THE CONTRACTION OF RAT MESENTERIC ARTERY

by

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

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ABSTRACT

The phosphorylation of proteins on tyrosine residues by tyrosine kinases has been suggested to contribute to signalling processes that lead to smooth muscle contraction. The aim of the current study was to further determine the role of tyrosine kinase in the contraction of rat vascular smooth muscle. The specific objectives included (i) studying the effect of tyrosine kinase inhibitors on vascular contraction induced by noradrenaline (NA), potassium chloride (KCl) and neuropeptide Y (NPY); (ii) studying the effect of sodium orthovanadate on contraction; (iii) studying the role of endothelium dependence in contraction; as well as (iv) studying the role of L-type Ca^{2+} channels in the tyrosine kinase-related response. The effects of NPY, nifedipine, sodium orthovanadate and various tyrosine kinase inhibitors were tested in intact ring segments of rat mesenteric and tail arteries. The present studies showed that the tyrosine kinase inhibitor genistein inhibits vascular smooth muscle contraction independently of the endothelium. The inactive analog of genistein, daidzein, failed to show an inhibition of NA- and KCl-induced contraction. It is suggested that genistein acts on the nifedipine-sensitive calcium channel since it produced the same level of inhibition when used alone or in combination with NPY. The tyrosine kinase inhibitors lavendustin A, tyrphostin 25, and erbstatin analog did not produce an

inhibition of contraction. This was most likely due to their specificity for membrane-associated tyrosine kinases. The phosphatase inhibitor sodium orthovanadate potentiated contractions due to its effect of prolonged tyrosine kinase phosphorylation as well as other mechanisms. This potentiation was blocked in the presence of the calcium channel blocker nifedipine, suggesting the involvement of calcium influx through the L-type Ca^{2+} channel. When genistein was used as pretreatment in the absence of calcium, there was no inhibition of NA-induced contraction, suggesting the involvement of the L-type calcium channel in its mechanism of action. NPY and sodium orthovanadate were still able to produce a potentiating effect on NA-induced contraction in the absence of calcium, which would suggest the presence of residual calcium, since a calcium chelator such as EGTA was not used.

The present studies illustrate that tyrosine kinase plays an important role in signal transduction stimulated by agonists in vascular smooth muscle.

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LIST OF ABBREVIATIONS

ATP	adenosine trisphosphate
Ca^{2+}	free calcium (II) ion
Ca_o^{2+}	extracellular calcium
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAG	1,2-diacylglycerol
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(ß-aminoethyl ether)
ERK	extracellular-signal-regulated kinase
GTP	guanosine trisphosphate
IP_3	inositol-1,4,5-trisphosphate
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase or ERK kinase
MLCK	myosin light chain kinase
NA	noradrenaline
NPY	neuropeptide Y
PDGF	platelet-derived growth factor
PIP_2	phosphatidylinositol-4,5-diphosphate
PKA	protein kinase A

PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PP	protein phosphatase
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
SR	sarcoplasmic reticulum
VSMC	vascular smooth muscle cell

INTRODUCTION

(I) General Properties of Vascular Smooth Muscle

(i) Structural and Morphological Properties

The vascular wall of arteries consists of three layers: an outer tunica adventitia, a central tunica media, and an inner tunica intima. The adventitial layer contains connective tissue, fibroblasts, mast cells, macrophages, and Schwann cells with the associated nerve axons. The connective tissue (collagen and elastin) contributes to the passive resistance to blood flow and its composition determines the elasticity or compliance of the vascular bed. Transmitter substances stored in the sympathetic nerves include noradrenaline (NA), ATP and neuropeptide Y (NPY) (Mulvany & Aalkjaer, 1990). The nerves in most arteries are confined to the adventitia and do not penetrate the media. The media is bound on the luminal side by an internal elastic lamina. The number of smooth muscle cell layers varies with vessel diameter, ranging from six layers in 300 μm vessels to a monolayer in arterioles (Mulvany & Aalkjaer, 1990). The smooth muscle cells are circumferentially arranged and are mechanically connected mainly through membranous contacts. This arrangement permits cell contraction to reduce the lumen diameter and increase blood flow resistance. Vascular smooth muscle cells (VSMCs) are relatively small compared to other myocytes.

When relaxed, they have an elongated spindle shape with a length of about 100 μm and a width of less than 10 μm at the widest central region. Vascular smooth muscle is classified as single-unit smooth muscle since many muscle cells contract together as a single unit. The VSMCs function to generate force as well as to maintain the shape of the vessel. The endothelial cells are squamous and form a continuous layer. In small arteries they frequently project through fenestrations in the internal elastic lamina and may make contact with the vascular smooth muscle cells within the media (Mulvany & Aalkjaer, 1990). The endothelium can release vasoactive substances which mediate either relaxation (endothelium-dependent relaxing and hyperpolarizing factors: EDRF, discovered to be nitric oxide, and EDHF) or contraction (endothelium-dependent contracting factor: EDCF) in smooth muscle (Furchgott, 1984).

(ii) Mechanism of Contraction

Smooth muscle contains both actin and myosin filaments which interact with each other in much the same way that this occurs for actin and myosin derived from skeletal muscle. Large numbers of actin filaments are attached to dense bodies, which anchor the actin to the cell membrane. These filaments overlap a single myosin filament located midway between the dense bodies and operate by the sliding filament mechanism exhibited by skeletal muscle. The dense bodies of smooth muscle serve the same structural role

as the Z-bands in skeletal muscle. Some of the dense bodies bond to those of adjacent cells, thereby transmitting the force of contraction (Schaus *et al.*, 1990).

The initiating event in smooth muscle contraction is an increase in intracellular calcium ions. Energy for contraction is derived from the hydrolysis of ATP. In relaxed muscle, the internal [Ca^{2+}] is below 10^{-7} M. Ca^{2+} must rise to a threshold of 0.1 to 0.3 μM in order for contraction to occur (Saida & Nonomura, 1978; Iino, 1981).

Smooth muscle does not contain troponin, the regulatory protein in skeletal muscle that allows interaction of actin and myosin filaments when Ca^{2+} binds to it as part of the actin-troponin-tropomyosin complex. Instead, smooth muscle cells contain large quantities of calmodulin, another regulatory protein. Calmodulin initiates contraction by activating the myosin cross-bridges. Calcium ions bind to calmodulin to form a calmodulin-calcium complex. This complex then joins with and activates myosin light chain kinase (MLCK), which enables the interaction of actin and myosin. This interaction ceases with the dephosphorylation of myosin by myosin phosphatase. As the [Ca^{2+}] falls to a lower steady-state level, a "latch state" is established whereby the degree of activation of the muscle is far less than the initial level. This enables tonic contractions to develop in which the muscle will still maintain its full strength of contraction at a fraction of the energy (Murphy, 1988). Relaxation occurs by the removal of calcium ions by

a calcium pump, which pumps the calcium ions out of the smooth muscle fibre back into the extracellular fluid or sarcoplasmic reticulum. This transport of Ca^{2+} may involve an exchange of Na^{+} and Ca^{2+} with the Na-electrochemical gradient providing energy to power Ca^{2+} extrusion, since removal of extracellular Na^{+} enhances Ca^{2+} influx and decreases Ca^{2+} efflux in vascular smooth muscle (Kuriyama, 1981).

(II) Excitation-Contraction Coupling

Excitation-contraction coupling is the cellular process by which the excitatory events of the plasma membrane are linked to activation of the contractile proteins. Extensive evidence indicates that this coupling is the result of an increase in the cytoplasmic concentration of ionized calcium. Excitation and contraction in vascular smooth muscle are linked via two coupling mechanisms: electromechanical and pharmacomechanical coupling.

(i) Electromechanical Coupling

Electromechanical coupling refers to contraction elicited by changes in membrane potential. The two pathways of electromechanical coupling are: (1) Ca^{2+} influx through voltage-gated channels and (2) Ca^{2+} release from the sarcoplasmic reticulum (SR). Contraction is initiated when the membrane potential is depolarized to a critical threshold of >-50 mV. This causes the opening of voltage-operated Ca^{2+} channels which allows Ca^{2+} to enter into the

cell. Phasic contractions in several mammalian portal veins are coupled to spontaneous action potentials (Somlyo & Somlyo, 1968; Ito & Kuriyama, 1971), showing definite coupling between electrical and mechanical activities in blood vessels. Contraction is also directly correlated to nerve-evoked action potentials in some large muscular arteries (Cheung, 1982). This is the underlying mechanism behind KCl-induced contractions of vascular tissues. Elevated extracellular K^+ also suppresses the voltage-dependent outward currents thereby limiting their tendency to repolarize. This leads to sustained Ca^{2+} channel opening as well as tension.

When excess K^+ is applied to smooth muscle tissues, the contractures consist of phasic and tonic components. The initial rapidly developed tension (phasic) is followed by a gradual decline to a certain level which is sustained for more than 10 minutes (tonic). The ratio of phasic and tonic responses differ from tissue to tissue, and depend for the greater part on the spike generation and concentration of K^+. It is thought that the phasic component of the contraction follows the release of stored intracellular Ca^{2+} to initiate contraction, while in the case of the tonic component an adequate amount of Ca^{2+} penetrates the membrane to maintain the contraction (Kuriyama, 1981).

(ii) Pharmacomechanical Coupling

Pharmacomechanical coupling is defined (Somlyo & Somlyo, 1968) as

the collective of mechanisms that, in response to a ligand binding to its receptor, can cause contraction or relaxation without a necessary change in surface membrane potential. Agonists that affect contraction include noradrenaline (NA), adrenaline, acetylcholine, angiotensin, vasopressin, oxytocin, serotonin, and histamine. Excitatory receptors for the agonists on the cell membrane cause contraction of smooth muscle, whereas inhibitory receptors cause inhibition of contraction. The three potential pathways of pharmacomechanical coupling are: (1) IP_3-induced Ca^{2+} release from the SR; (2) Ca^{2+} influx through voltage-dependent and ligand-gated channels, and; (3) modulation of the Ca^{2+}-sensitivity of the contractile regulatory apparatus (Somlyo *et al.*, 1991). Examples of "pure" pharmacomechanical coupling, changes in force in the absence of any change in membrane potential, have been observed in some instances in polarized smooth muscles (Somlyo *et al.*, 1991). In several blood vessels, low concentrations of NA are able to elicit contractions in the absence of altered membrane potential. Contractions induced by KCl and high [NA] exhibit a different temporal relationship with membrane depolarization. In the rat tail artery, it has been shown using concurrent electrical and mechanical recordings that membrane depolarization occurs prior to KCl-induced contractions but subsequent to NA-induced contractions (Cheung, 1984). This illustrates the difference in the coupling mechanisms.

(iii) Calcium Channels

In cardiac and smooth muscle, two types of voltage-operated Ca^{2+} channels have been identified, T(transient)- and L(long lasting)-types (Reuter *et al.*, 1982; Benham *et al.*, 1987). Of the two, L-type Ca^{2+} currents appear to predominate in the vasculature. Voltage-operated Ca^{2+} channels in vascular preparations open when the membrane potential is depolarized to a threshold of > -50 mV. The influx of Ca_o^{2+} results in a slow maintained contraction. Smooth muscle contraction evoked by elevated KCl is thought to be due to opening of voltage-operated Ca^{2+} channels. The threshold level of depolarization required to elicit contraction of vascular smooth muscle correlates with that for Ca^{2+} channel opening.

Structurally, the L-type Ca^{2+} channel in muscle cells contains five subunits, α_1, α_2 β, γ, and δ. The α_1 subunit contains the dihydropyridine binding sites and important phosphorylation sites for cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, and Ca^{2+}/calmodulin kinase (Nastainczyk *et al.*, 1987; Rohrkasten *et al.*, 1988; Hosey & Lazdunski, 1988; Tuana & Murphy, 1990). The phosphorylation process has been shown to be involved in the modulation of Ca^{2+} channels by a variety of neurotransmitters which operate through multiple second messengers and G-protein-linked pathways (Miller & Fox, 1990).

(III) Signal Transduction Pathways

(i) G-protein

Many cell-surface receptors use G (GTP-binding) proteins to initiate intracellular signalling chain reactions which regulate a cell's actions. These proteins play a central role in signal transduction and regulate at least six different subtypes of mammalian adenylyl cyclase, many phospholipases A and C, and numerous potassium and calcium channels (Neubig, 1994). G proteins transmit the signal to effectors (enzymes or ion channels) which transduce the binding of a ligand into an intracellular signal, such as changes in the concentration of diffusable second messengers (e.g. cAMP, IP_3) or the gating of ion currents.

G proteins are involved in receptors for several biogenic amines, muscarinic acetylcholine, metabotropic glutamate and $GABA_B$ receptors, purinergic receptors and receptors for a variety of eicosanoids and for numerous peptide hormones and neuromodulators as well as sensory receptors for light and odorants (Offermanns & Schultz, 1994). When these receptors are activated by agonists, they interact with G proteins. G proteins are heterotrimers, composed of α, ß and γ subunits. The ß and γ subunits are a dimer that form a functional unit. Binding of GTP to the α subunit of G proteins is promoted by activated receptors. This binding causes activation and dissociation of α subunits and ßγ complexes. It has recently been

shown that the $\beta\gamma$ complex as well as the α subunit can directly bind and activate numerous effectors (Clapham, 1996).

The functions of G proteins are regulated cyclically by association of GTP with the α subunit, hydrolysis of GTP to GDP and P_i, and dissociation of GDP. Binding to GTP is closely linked with activation of the G protein and consequent regulation of the activity of the appropriate effector. Hydrolysis of GTP initiates deactivation. Dissociation of GDP appears to be rate limiting, and this step is accelerated by interaction between G protein and receptor.

In the guinea-pig portal vein, Ohya & Sprelakis (1988) reported that GTPγS activates the L-type Ca^{2+} channel. Xiong *et al.* (1990) also observed the enhancing effect of GTPγS on the ATP-operated cation channel in the rabbit portal vein. Involvement of G proteins and phospholipase C in second messenger activation of receptor operated channels follows from the observation that thrombin-activated Ca^{2+} influx in platelets was inhibited by phorbol ester stimulated C kinase-mediated phosphorylation of α subunits of G_i and G_p. This type of negative feedback is also present in agonist stimulation of smooth muscle (van Breemen, 1989). The Ca^{2+}-sensitizing effect of excitatory agonists is mediated by a G-protein: the increase in both force and 20 kDa myosin light chain (LC_{20}) phosphorylation are inhibited by GDPγS (Kitazawa & Somlyo, 1990; Kitazawa *et al.*, 1991), and can also be

mimicked by the non-hydrolysable GTP analog, GTPγS (Fujiwara *et al.*, 1989; Kitazawa *et al.*, 1991).

(ii) IP_3

Agonist-induced vascular smooth muscle (VSM) contraction is associated with signal transduction processes linked to elevated intracellular levels of calcium and activation of various endogenous enzymes. The formation of inositol 1,4,5-trisphosphate (IP_3) is the focal point for two major pathways, one initiated by a family of G protein-linked receptors and the other by receptors linked by tyrosine kinases (Berridge, 1993). Both pathways lead to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a form of phospholipase C resulting in the second messengers IP_3 and 1,2-diacylglycerol (DAG). IP_3 binds to an IP_3 receptor in the sarcoplasmic reticulum (SR) to mobilize stored calcium, while DAG activates protein kinase C (PKC). Calcium release by IP_3 has been suggested to be responsible for the initial phasic component of the contraction. IP_3 has met several criteria as a physiological mediator in smooth muscle. The effects of inhibition of G-protein activity with GDPßS (Kobayashi *et al.*, 1988a; Kitazawa *et al.*, 1989), the blockade of agonist-induced Ca^{2+}-release by the IP_3 specific inhibitor, heparin (Kobayashi *et al.*, 1988b) and by the phospholipase C inhibitor, neomycin (Kobayashi *et al.*, 1989), verify that interruption of any step of the phosphatidylinositol cascade prevents

pharmacomechanical Ca^{2+} release. Activation of α_1-adrenoceptors was suggested to increase phosphatidylinositol turnover in rat vas deferens and caudal artery (Fox *et al.*, 1985). Campbell *et al.* (1985) found that PIP_2 hydrolysis may account for the rapid phase of norepinephrine-induced contraction in rabbit aorta while phosphatidic acid (PA) or its immediate precursor diacylglycerol may account for receptor-induced Ca^{2+} influx.

(iii) cAMP

A possible role for intracellular cAMP in the regulation of smooth muscle tone and its responsiveness to physiological stimuli has been suggested as the result of various investigations. It has been shown that β-adrenergic agonists activate the membrane adenylate cyclase, and the resultant increase in cAMP has been implicated in the inhibition of contractility (Kuriyama, 1981). In addition, cAMP-dependent phosphorylation has been shown to modulate several ion channels including Ca^{2+}, K^+, and Cl^- channels (Ewald *et al.*, 1985; Hartzell *et al.*, 1991).

(iv) cGMP

Intracellular cyclic guanosine monophosphate (cGMP) is known to inhibit contraction of vascular smooth muscle. Rapoport (1986) was the first to demonstrate that agents which elevate cGMP inhibit agonist-elevated inositol monophosphate accumulation in smooth muscle. This supports the

idea that cGMP acts through inhibition of phosphatidylinositol breakdown through activation of cGMP-dependent protein kinase (PKG). Cyclic GMP kinases are serine/threonine protein kinases which are most abundant in three cell types in vertebrates: smooth muscle, platelets, and cerebellum (Lincoln & Cornwell, 1993). Sperelakis *et al.* (1994) showed that in VSM cells, both cAMP and cGMP act in the same direction, namely to inhibit Ca^{2+} channel activity and contraction. Activation of sarcolemmal adenylate cyclase or guanylate cyclase by appropriate membrane receptors and their G coupling proteins produces elevation of cAMP and cGMP and activation of PKA and PKG. In addition, nitric oxide free radical or nitroprusside/nitroglycerin can directly stimulate a soluble guanylate cyclase to elevate cGMP levels. This is due to the lipid solubility of organic nitroesters resulting in their penetration of the cell membrane. PKA and PKG can phosphorylate the L-type Ca^{2+} channel to inhibit channel opening and the K^+ channels to stimulate channel opening, resulting in diminished Ca^{2+} influx and lower $[Ca^{2+}]_i$ (Sperelakis *et al.*, 1994). The action of cGMP is terminated when the 3',5'-cyclic phosphoester bond is opened, a process that yields GMP, which is unable to activate PKG. The inactivation of cGMP is catalyzed by phosphodiesterases, one of which is cGMP specific (Torfgård & Ahlner, 1994).

(IV) Tyrosine Kinase Pathway

Protein tyrosine kinases (PTKs) are involved in many different signalling pathways. These include the initiation of mitogenic responses by certain growth factors, and the modulation of ß-adrenergic signal transduction in fibroblasts (Di Salvo *et al.*, 1993b). Tyrosine kinases exhibit a specificity for tyrosine residues and use ATP as the preferred phosphate donor (Hunter & Cooper, 1985). Some substrates for PTKs include growth factor receptors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin and fibroblast growth factor as well as myosin light chain (Sauro & Thomas, 1993). The superfamily of tyrosine kinases consists of three general subclasses: (1) the membrane receptor PTKs, including the receptors for EGF, PDGF and insulin; (2) cytosolic non-receptor PTKs; and (3) the membrane-associated non-receptor PTKs related closely to $p60^{v\text{-}src}$ (Hollenberg, 1994). Several potential substrates for these tyrosine kinases have been identified, such as phospholipase C_{γ} and MAP kinase that are believed to be directly involved in cell signalling.

The family of receptor tyrosine kinases shares similar structural features. A binding domain on the outside of the cell with an NH_2 terminus can specifically recognize its ligand. A stretch of hydrophobic amino acids long enough to cross the plasma membrane once provides the link to the cytoplasm. The intracellular part of receptor tyrosine kinases encodes the catalytic domain. This domain is the most conserved of these receptors and

contains several residues that form a typical ATP-binding site, found in all protein kinases. The kinase domain catalyzes the transfer of the γ-phosphate of ATP to tyrosine residues on substrate proteins, as well as onto the receptor molecules themselves in an autophosphorylation reaction (Panayotou & Waterfield, 1993).

The nonreceptor protein tyrosine kinases represent cellular enzymes grouped together based on their lack of defined extracellular sequences. All of these PTKs are likely to be involved in one or more signalling pathways that modulate growth, differentiation, and mature cell function. The major area of sequence homology between the nonreceptor PTKs is their catalytic domain. The catalytic domains for the nonreceptor PTKs are the SH1 (src homology 1) domains since this is the region between the kinases that shares the greatest sequence similarity with the c-src catalytic domain (Saouaf *et al.*, 1995). With the exception of the Jak and Fak groups, all other nonreceptor PTKs possess one or more SH2 domains and/or an SH3 domain. In most cases, the SH2 and SH3 domains are at the amino-terminal end of the enzyme with respect to the catalytic domain. Individual SH2 domains are capable of specific binding to selected phosphotyrosine-containing proteins and are thought to play critical roles in the interactions between signalling components in PTK-dependent pathways. The SH3 domains recognize proline-rich peptide ligands which in some cases are present in guanine nucleotide exchange factors and GTPase-activating

proteins (Saouaf *et al.*, 1995).

The phosphorylation of proteins on tyrosine residues by tyrosine kinases has been suggested to contribute to signalling processes that lead to smooth muscle contraction. Studies by Tsuda *et al.* (1991) and Molloy *et al.* (1993) reported that protein tyrosine phosphorylation of several substrates occurred when cultured rat aortic vascular smooth muscle cells were stimulated with different agonists, such as NA and angiotensin II. A similar pattern of protein tyrosine phosphorylation elicited by phenylephrine in cultured canine vascular smooth muscle cells was noted by Di Salvo *et al.* (1994).

Compounds that inhibit tyrosine kinases have been the primary tools to study the proposed involvement of tyrosine kinase in agonist-induced VSM contraction. Tyrphostins are a series of synthetic PTK inhibitors which have a structure similar to tyrosine. They inhibit EGFR kinase, insulin receptor kinase, PDGF receptor kinase, and some other PTKs both *in vitro* and *in vivo*. Erbstatin is an antibiotic isolated from the culture medium of *Streptomyces*. Methyl 2,5-dihydroxy cinnamate (2,5-MC) is a stable analogue of erbstatin which inhibits the ligand-induced autophosphorylation of EGF receptors. Erbstatin and 2,5-MC inhibit cell proliferation both *in vitro* and *in vivo*, both agents competing with the substrate and inhibiting PTK. Genistein, which was isolated from the culture broth of *Pseudomonas*, competes with ATP and inhibits the autophosphorylation of EGF receptors, PDGF receptors

and src kinase. None of these PTK inhibitors inhibit PKC or PKA activity (Shimokado *et al.*, 1995).

Tyrphostin, a specific inhibitor of PTKs, was found to inhibit PDGF-induced contraction in rat aorta, but had no effect on contraction induced by phenylephrine or phorbol 12,13-dibutyrate (PDB), agents which mediate contraction through activation of PKC (Sauro & Thomas, 1993). In another study, the PTK inhibitors genistein and tyrphostin caused an inhibition of noradrenaline (NA)-induced contraction in rat aorta. Potassium chloride (KCl)-induced contraction was not affected (Abebe & Agrawal, 1995). Di Salvo *et al.* (1993b) have found that contractions elicited by stimulation of muscarinic receptors in guinea pig taenia coli or alpha-adrenergic receptors in mesenteric microvessels and canine carotid artery were greatly and reversibly inhibited by tyrosine kinase inhibitors. However, only a slight (20%) inhibition occurred in contractions elicited by K^+-induced depolarization. In addition, tyrphostin did not inhibit direct Ca^{2+}-mediated activation cf the contractile apparatus in preparations permeabilized with ß-escin. These results suggest that tyrosine kinase activity is involved in pharmacomechanical coupling more than in either electromechanical coupling or direct activation of the contractile apparatus with Ca^{2+}.

Tyrosine kinase may participate in ion channel activity regulation in vascular smooth muscle cells. In a study by Wijetunge *et al.* (1992), tyrphostin and genistein inhibited voltage-operated calcium channel currents

in rabbit ear artery vascular smooth muscle cells. Xiong *et al.* (1995) demonstrated the modulation of Ca^{2+}-activated K^+ channel activities in vascular smooth muscle cells by tyrosine kinase inhibitors. Cell excitability by membrane hyperpolarization is depressed by activation of Ca^{2+}-activated K^+ channels (Bolzon *et al.*, 1993). Therefore, potentiation of the activity of these K^+ channels by tyrosine kinase inhibitors could contribute to their inhibitory effect on contraction in vascular smooth muscle. The observations of the previous studies suggest that inhibition of PTK activity results in inhibition of contraction. This was further supported by the fact that sodium orthovanadate, a protein tyrosine phosphatase inhibitor, selectively potentiated the NA-induced contraction (Abebe & Agrawal, 1995). The inhibitory response of genistein observed both in the presence and absence of extracellular calcium was found to be more pronounced on the phasic contraction. This suggests a greater role of tyrosine kinases in mediating the responses associated with the release of intracellular calcium (Abebe & Agrawal, 1995).

(V) MAPK Signalling Cascade

The mitogen-activated protein (MAP) kinase (MAPK) signalling cascade is a prominent cellular pathway used by many growth factors, hormones and neurotransmitters to regulate physiological responses. MAPKs, also known as extracellular-signal-regulated kinases (ERK), are activated through

threonine/tyrosine phosphorylation by a dual specificity protein kinase, known as a MAPK kinase (MAPKK), which is itself activated by serine phosphorylation by a third protein kinase, known as MAPKK kinase (MAPKKK) (Hunter, 1995). In smooth muscle, the role of MAPK may be to phosphorylate h-caldesmon, thereby altering a mechanical property of the muscle (Adam *et al.*, 1995). H-caldesmon is the high molecular weight isoform of caldesmon and is an abundant, smooth muscle-specific, actin-binding protein. Adam *et al.* (1995) used porcine carotid arteries to show that only the tyrosine-phosphorylated form of the MAPK is active in contracted smooth muscle and exhibits phosphotransferase activity. They also suggested that MAPK activation in arterial smooth muscle is controlled by a combination of both mechanical load and pharmacological manipulation. In another study involving porcine carotid arteries, Katoch & Moreland (1995) demonstrated that both the 42 and 44 kDa isoforms of MAPK are activated in response to stimulation of intact carotid artery by either a receptor and G protein-mediated pathway (using the agonists NA, endothelin-1, and histamine) or by membrane depolarization (using KCl). They suggest that MAP kinases may be involved in the fundamental mechanisms regulating smooth muscle contraction and that protein kinase C-dependent activation of MAP kinase catalyzes caldesmon phosphorylation. Phosphorylation of caldesmon prevents its inhibitory effect on actin and myosin interaction, thereby allowing the expression of activity.

In a study using ferret aortic cells (Khalil *et al.*, 1995), tyrosine phosphorylation was revealed to be an important signalling event during Ca^{2+}-independent activation. A finding of the study was that MAP kinase initially translocates to the surface membrane but then undergoes a second redistribution to the cytoskeleton. PKC activation appears to regulate the initial translocation of MAP kinase to the surface membrane, while tyrosine phosphorylation appears to regulate the delayed localization of MAP kinase to the cytoskeleton.

(VI) Model of tyrosine kinase-mediated signal transduction pathways based on the literature

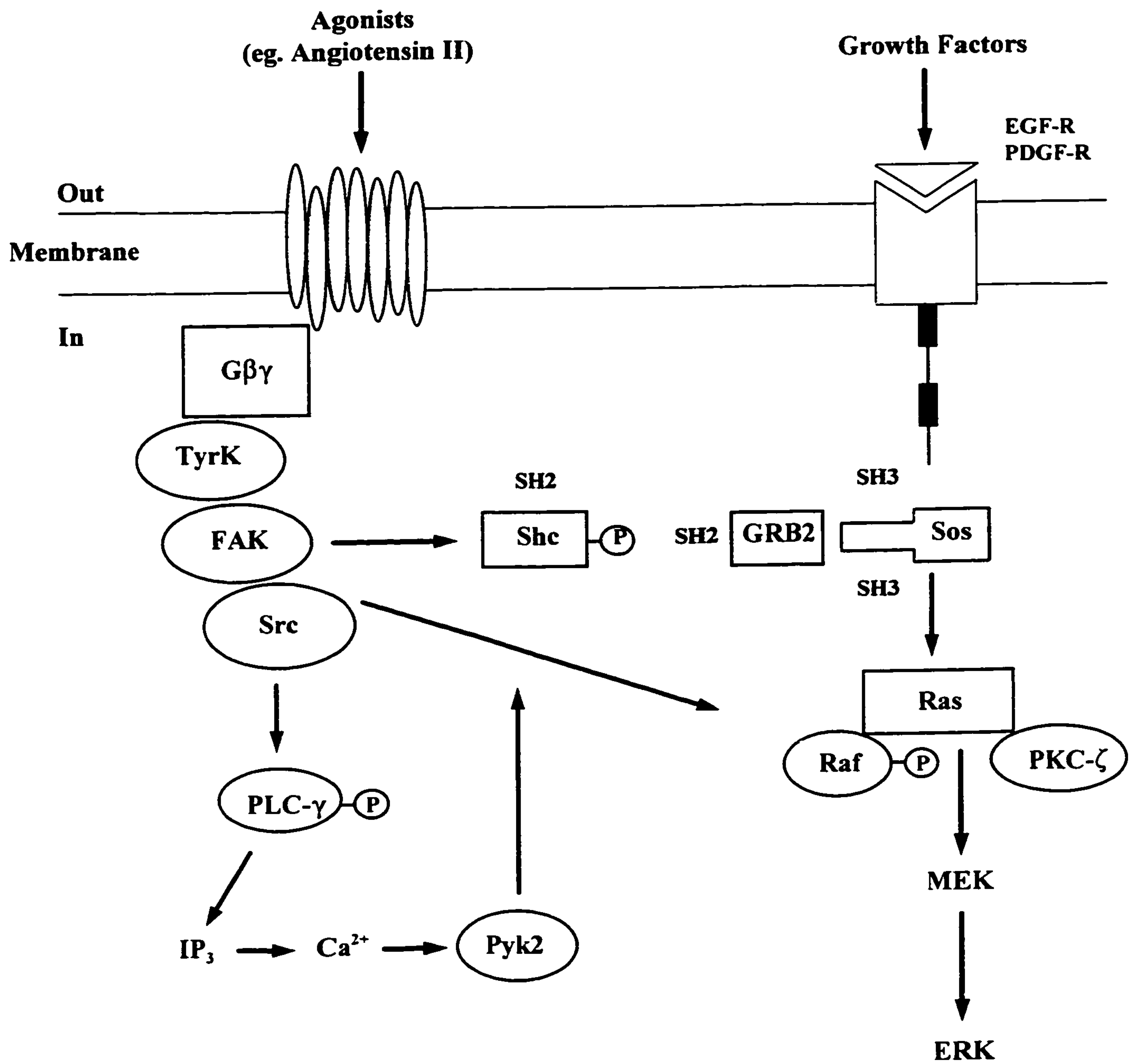
It has become apparent that tyrosine phosphorylation plays an important role in the signalling pathway of angiotensin II. Thrombin and several vasoactive peptides including angiotensin II, arginine vasopressin (AVP), bombesin, bradykinin and endothelin lead to increased intracellular tyrosine phosphorylation after binding to G protein-coupled receptors (Marrero *et al.*, 1995). The tyrosine kinase-mediated signal transduction pathways stimulated by agonists such as angiotensin II in VSMCs (Figure 1) will be discussed to illustrate the involvement of tyrosine kinase. The signal transduction events stimulated by angiotensin II binding to the AT_1 receptor are similar to those stimulated by growth factors and cytokines and include activation of PLC, induction of proto-oncogenes, protein tyrosine

phosphorylation, and activation of the 42- and 44-kD MAP kinases (Berk & Corson, 1997, and references cited).

The structure of the AT_1 receptor shows that it is a member of the seven transmembrane-spanning, G protein-coupled receptor family. These receptors share the property that they bind to heterotrimeric G proteins and lack intrinsic tyrosine kinase activity, in contrast to growth factor receptors. However, many angiotensin effects require tyrosine phosphorylation. Release of G-protein $\beta\gamma$ subunits from G protein-coupled receptors stimulates downstream events leading to tyrosine phosphorylation of a linker protein called Shc. This tyrosine phosphorylation of Shc appears to be an important mechanism used by all G protein-coupled receptors (Berk & Corson, 1997, and references cited), and as shown in Figure 1, is hypothesized to be mediated by Src, FAK, and other unidentified tyrosine kinases (TyrKs). The phosphorylated Shc then binds GRB2 via SH2 domain interactions. In addition, GRB2 binds to the guanine nucleotide exchange factor Sos via SH3 domains. Sos induces the exchange of GDP for GTP on Ras, which then recruits Raf to the plasma membrane for activation. Active Raf phosphorylates and activates MEK, which can then phosphorylate and activate ERK1/2 (Berk & Corson, 1997). In a related pathway, angiotensin II binding to the AT_1 receptor stimulates PLC-γ to hydrolyze PIP_2, generating the second messengers IP_3 and diacylglycerol. DAG activates protein kinase

Figure 1. Summary of tyrosine kinase-mediated signal transduction pathways stimulated by agonists such as angiotensin II in VSMCs

Depicted are several cytosolic and transmembrane protein kinases potentially activated by agonists in VSMCs.


Agonists
(eg. Angiotensin II)
Growth Factors
EGF-R
PDGF-R
Out
Membrane
In
Gβγ
TyrK
FAK
Src
SH2
Shc
P
SH2
GRB2
SH3
Sos
SH3
Ras
Raf
P
PKC-ζ
PLC-γ
P
MEK
ERK
IP3
Ca2+
Pyk2

C, while IP_3 stimulates the release of Ca^{2+} from intracellular stores, which leads to the increase of cytoplasmic calcium levels and the contraction of the smooth muscle cell. The elevation of intracellular calcium will stimulate tyrosine kinase activity. Shc may also be phosphorylated in a calcium-dependent manner by a second focal adhesion kinase (FAK) family member, denoted Pyk2. Pyk2 has been postulated as a potential link between calcium-dependent signalling pathways and protein tyrosine kinase pathway. An additional link is that angiotensin II signal transduction is suggested to be mediated in part by growth factor receptor transactivation (Berk & Corson, 1997, and references cited). Growth factor binding stimulates autophosphorylation of the receptor which then binds the SH2 domain of GRB2, continuing with the previously described pathway.

Early signal transduction events activated by the angiotensin II type I receptor resemble those events activated by tyrosine kinase-coupled receptors, such as the PDGF receptor. These events now include activation of phospholipase C-γ, Janus kinase and TYK kinases, src kinase, H-Ras, Raf, and MAP kinase (Liao *et al.*, 1996, and references cited).

(VII) Neuropeptide Y

Neuropeptide Y (NPY) is one of the most abundant neuropeptides involved in the autonomic neural control of cardiovascular function. It exists in the heart, in peripheral tissues, and in many central nervous structures

where it was first identified. NPY is one of the most highly conserved peptides known. All mammalian forms of neuropeptide Y consist of 36 amino acid residues, with five tyrosine residues in each molecule and a C terminal amide structure. The forms of NPY from humans, rats, rabbits, and guinea pigs are all identical and possess a readily oxidized methionine residue at position 17 (McDermott *et al.*, 1993). NPY is believed to be released from nerve endings in the non-oxidized form, and its oxidation may be important in determining the biological activity of the peptide.

The presence of NPY has been demonstrated in most parts of the vascular tree. NPY released at nerve endings influences sympathetic cardiovascular control by having both direct and indirect (prejunctional and postjunctional) effects (McDermott *et al.*, 1993). Postjunctional effects of NPY on blood vessels in mammals differ according to localization of the vessel and mammalian species, and were either found to be vasoconstrictive or non-existent, but not vasorelaxant. In coronary and cerebral arteries the peptide exerts a direct vasoconstrictive effect, while in other vascular beds NPY remains ineffective when given alone but potentiates the effect of other vasoconstrictive agents. This potentiating behaviour has been observed in saphenous vein from dog (Hieble *et al.*, 1989), in ear (Glover, 1985; Daly & Hieble, 1987), pulmonary (Wahlestedt *et al.*, 1985), and femoral arteries from rabbit (Edvinsson *et al.*, 1984), and in tail arteries (Neild, 1987; MacLean & McGrath, 1990) and mesenteric arteries from rat (Westfall *et al.*, 1987). It

has been shown that NPY is able to potentiate the effect of vasoconstricting agents including agonists at α adrenergic receptors, histamine, angiotensin II, 5-hydroxytryptamine, prostaglandin $F_{2\alpha}$, and endothelin (Edvinsson *et al.*, 1984). In many sympathetic nerve endings, NPY can act prejunctionally by reducing the release of noradrenaline or other transmitters. Inhibitory prejunctional effects of NPY may function as a feedback mechanism preventing excessive depletion of transmitter reserves during prolonged sympathetic stimulation (Glover, 1985).

Data collected regarding the signal transduction systems in vascular smooth muscle include a study by Mihara *et al.* (1989) in which the possible coupling of receptors for NPY on cultured porcine aortic smooth muscle cells to intracellular calcium concentration ($[Ca^{2+}]_i$) was investigated. NPY increased $[Ca^{2+}]_i$ in a dose dependent manner by a mechanism involving a G_i protein. NPY-induced mobilization of Ca^{2+} from internal storage sites was suggested to be the cause of the increase in $[Ca^{2+}]_i$ in these cultured smooth muscle cells. The intracellular messenger substances, inositol 1,4,5 triphosphate (IP_3) or cyclic adenosine 3',5' monophosphate (cAMP), seemed not to be involved. In a study in which NPY was given to dogs by intracoronary injection, the vasoconstrictive action of NPY was attenuated by Ca^{2+} channel blocking agents, indicating that the contraction of vascular smooth muscle cells in the coronary artery is dependent on the influx of Ca^{2+} from the exterior space (Aizawa *et al.*, 1989). In rat mesenteric arterioles,

NPY markedly enhanced Ca^{2+} entry dependent responses elicited either by the addition of Ca^{2+} to depolarized vessels or by the addition of the calcium agonist, BAY K 8644 (Andriantsitohaina & Stoclet, 1988). These effects are consistent with partial depolarization and subsequent influx through voltage dependent calcium channels.

Different mechanisms of the positive contractile effect of NPY may work in vascular smooth muscle cells from different origins. It appears that the effects of NPY on some vascular tissues causing mobilization of cytosolic calcium, and promotion of calcium influx into the cell, or eliciting contraction are mediated by a pertussis toxin sensitive G protein (McDermott *et al.*, 1993). This may be an inhibitory G protein coupled to an ion channel rather than coupled to adenylate cyclase.

AIM

To further determine the role of tyrosine kinase in the contraction of rat vascular smooth muscle.

HYPOTHESIS

Tyrosine kinase inhibitors inhibit NA- and KCl-induced contractions in rat vascular smooth muscle independently of the endothelium and act via calcium influx through the L-type calcium channel.

OBJECTIVES

i) To study the effect of tyrosine kinase inhibitors on vascular contraction induced by NA, KCl and NPY.

ii) To study the effect of sodium orthovanadate on contraction.

iii) To study the role of endothelium dependence in contraction.

iv) To study the role of L-type Ca^{2+} channels in the tyrosine kinase-related response.

MATERIALS AND METHODS

(A) Materials

NPY, sodium orthovanadate, nifedipine and tyrphostin 25 were purchased from Sigma Chemical Company (St. Louis, MO), while genistein, daidzein, lavendustin A and erbstatin analog were purchased from Research Biochemicals International (Natick, MA). All other chemicals were of analytical or comparable grade.

(B) Stock Solutions

Table 1 shows details of the stock solutions used, including stock concentration, solvent, storage temperature, and final working concentration.

(C) Buffers

The composition of buffers used in experiments is shown in Table 2. The majority of experiments were performed using Ringer's solution, with equimolar KCl replacing NaCl in potassium depolarization studies.

(D) Preparation of the Arterial Ring Segment

The effects of NPY, nifedipine, sodium orthovanadate and various tyrosine kinase inhibitors were tested in intact ring segments of rat

Table 1. Stock Solutions

	Stock	*Solvent*	*Storage*	*Final*
Genistein	10 mM	DMSO[1]	-80 °C	10 μM
Daidzein	10 mM	DMSO[1]	-80 °C	10 μM
Neuropeptide Y	100 μM	ddH_2O	-80 °C	0.1 μM
Erbstatin analog	10 mM	NaOH[1]	-80 °C	10 μM
Tyrphostin 25	10 mM	DMSO[1]	-80 °C	50 μM
Lavendustin A	10 mM	DMSO[1]	-80 °C	10 μM
Nifedipine	10 mM	DMSO[1]	-20 °C	1 μM
Sodium Orthovanadate	100 mM	ddH_2O	4 °C	1 mM

[1]Final concentration <0.1%; no effect on contraction.

Table 2. Buffer Composition (mM)

	Ringer's	*Ca^{2+}-free Ringer's*
NaCl	120	120
KCl	5	5
$CaCl_2$	2.5	
NaH_2PO_4	1	1
$MgSO_4$	1	1
$NaHCO_3$	25	25
Glucose	11	11

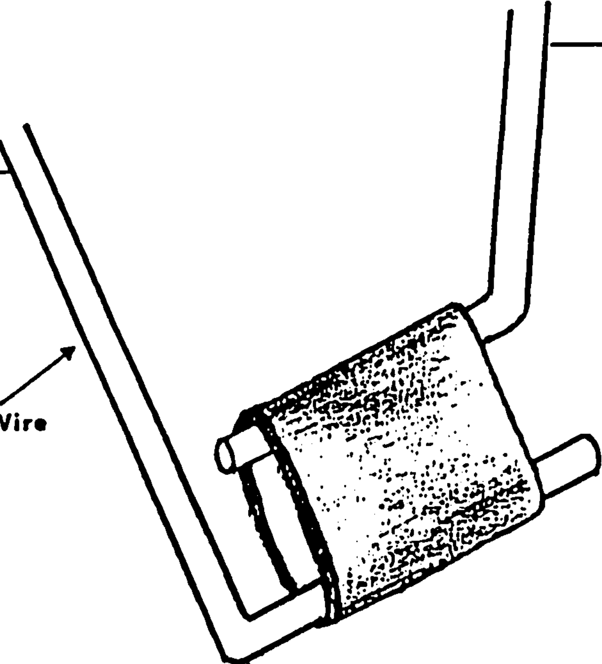
mesenteric and tail arteries. Ring segments 3-5 mm in length were obtained from male Wistar rats 10-12 weeks of age. Tension was measured by two tungsten wires inserted through the lumen and connected to Grass FT 03C transducers (Figure 2). The ring segment was submerged in a 2.0 mL bath of physiological salt solution (PSS) maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The PSS contained (in mM) NaCl 120, $NaHCO_3$ 25, glucose 11, KCl 5, $CaCl_2$ 2.5, NaH_2PO_4 1 and $MgSO_4$ 1, and was changed every 15 minutes. In potassium depolarization studies, NaCl was replaced by equimolar KCl. Phentolamine (1 μM) was added to prevent activation of α-adrenoceptors by NA released by depolarization of nerve terminals. A resting tension of 0.5 g was applied to the ring segment using the micromanipulator. Following an equilibration period of 30 minutes, 10 μM noradrenaline (NA) was added to the bath intermittently until a stable maximal tension was established. Similar methods have been used by other researchers (Evéquoz *et al.*, 1994; Filipeanu *et al.*, 1995). Experiments of a similar time frame were performed by Abebe & Agrawal (1995), in which no alterations in responsiveness with time were observed.

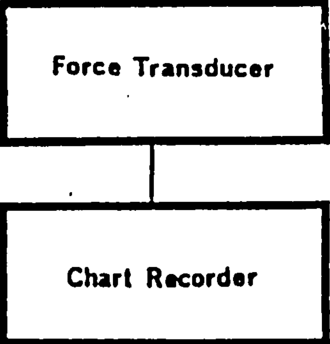
Denuded arterial rings were prepared by gently scraping away the endothelial layer with the blunt head of a pin. The removal of the endothelium was verified by the absence of acetylcholine-induced relaxation in arterial rings precontracted with NA.

Figure 2. Setup of the arterial ring segment

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

Micromanipulator


Tungsten Wire


Force Transducer
Chart Recorder

In studies examining the absence of external calcium, some tissues were used in which calcium was always present, and other tissues were used in which calcium was absent after the establishment of a stable maximal tension. The calcium-free tissues were washed for 20 minutes in a calcium-free solution prior to the addition of NA 10^{-6}M. Tissues were again washed for 20 minutes in a calcium-free solution prior to a second addition of NA 10^{-6}M. The values of these two contractions were then averaged. The tissue was then washed for 20 minutes in a calcium-free solution prior to the addition of the reagent in question, followed by NA 10^{-6}M. The reference value for the calculated percentages was the stable maximal contraction of NA 10^{-5}M in the presence of external calcium.

(E) Statistics

Paired student's t-tests were used to evaluate statistical differences, with a value of $P<0.05$ considered to be statistically significant. Statistical significance in the figures was denoted by the following: * $P<0.05$; ** $P<0.01$; ***$P<0.001$.

RESULTS

(I) Effect of NA on mesenteric and tail arteries

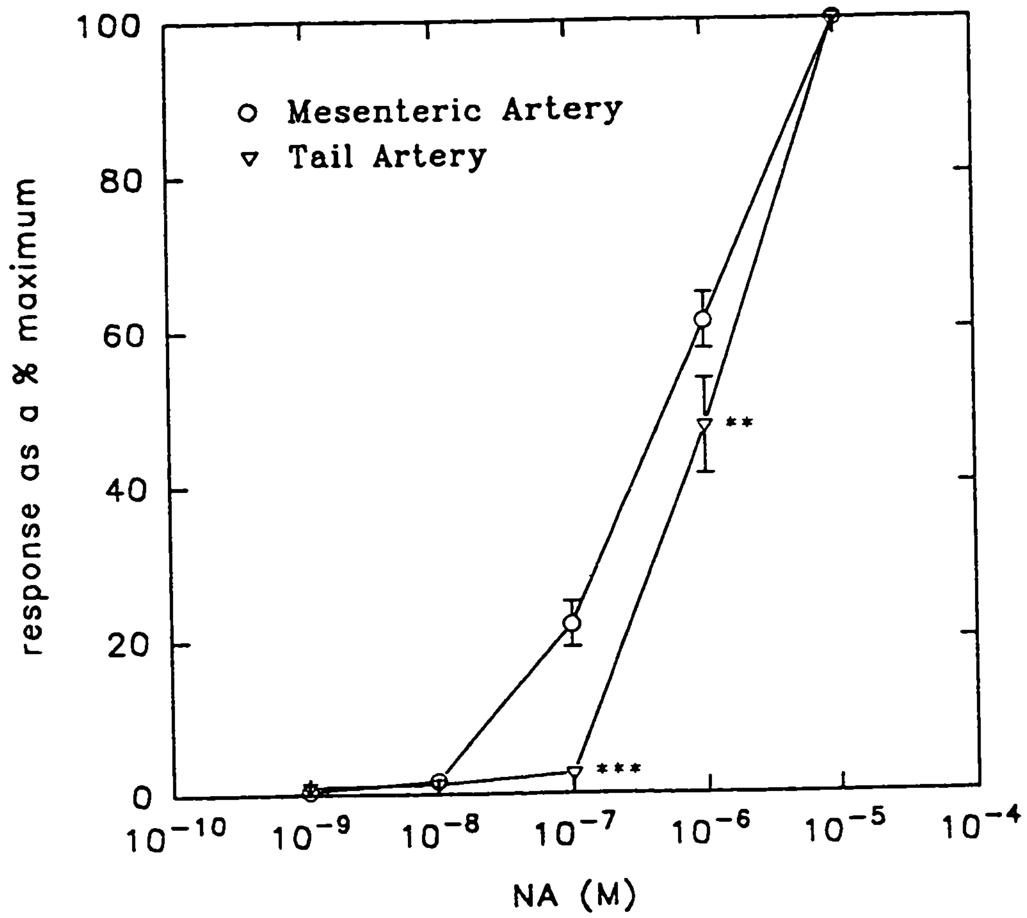
The relative sensitivities of the mesenteric and tail arteries to NA were compared. A dose response curve, in which increasing doses of NA were added to the tissue, was obtained. The maximal contraction obtained for each tissue was assigned as 100%. Submaximal contractions were then calculated as a percentage of the maximum. In comparing the dose response curves, the mesenteric artery showed greater sensitivity to NA than the tail artery (Figure 3). The ED_{50} for the mesenteric artery was 5.2×10^{-7}M, while the ED_{50} for the tail artery was 2×10^{-7}M.

(II) Effect of pre-incubation time of genistein on inhibition of contraction in rat mesenteric and tail artery

Previous studies using genistein have used varying pre-incubation times. It was therefore deemed relevant to determine the necessary pre-incubation time of genistein which would best inhibit contractions induced by NA. Genistein (10^{-5}M) was pre-incubated for either 20, 30 or 40 minutes prior to an NA dose-dependent contraction. In the mesenteric artery (Figure 4), the results seem to indicate that a pre-incubation time of greater than 20 minutes was sufficient to produce a maximal inhibition by genistein. There

Figure 3. Contraction in mesenteric versus tail arteries

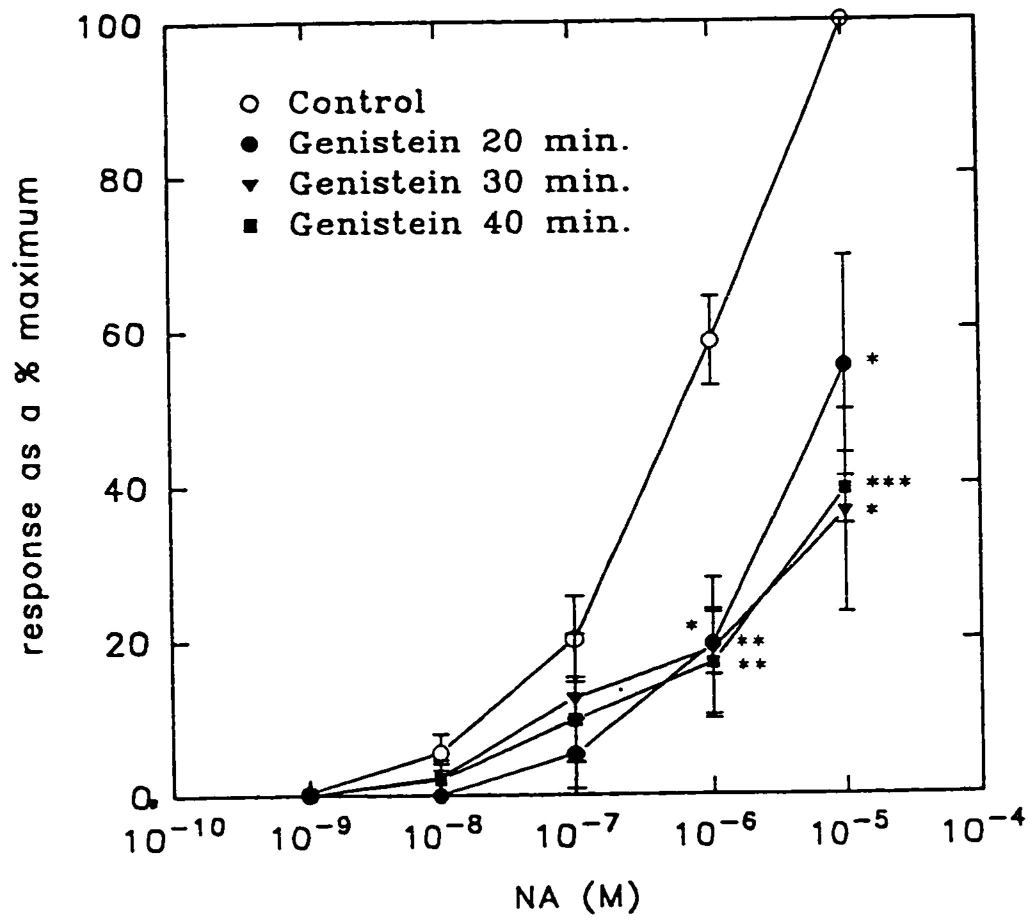
Comparison of control NA-induced contraction in the rat mesenteric (○) versus tail (▿) arteries; n=9; **P<0.01, ***P<0.001.



Mesenteric Artery
Tail Artery
response as a % maximum
0
20
40
60
80
100
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
NA (M)
***
**

Figure 4. Effect of varying pretreatment times of genistein on the contraction in the mesenteric artery

Effect of different pretreatment times (● 20 minutes, n=5; ▼ 30 minutes, n=4; ■ 40 minutes, n=5) of genistein 10^{-5}M on NA-induced contraction in the rat mesenteric artery; (○ Control, n=14); *$P<0.05$, **$P<0.01$, ***$P<0.001$.



Control
Genistein 20 min.
Genistein 30 min.
Genistein 40 min.
response as a % maximum
NA (M)
100
80
60
40
20
0
10−10
10−9
10−8
10−7
10−6
10−5
10−4

appeared to be little further inhibition when pre-incubation times of 30 and 40 minutes were used. Consequently, further experiments with genistein used a pre-incubation time of 30 minutes.

In the tail artery, genistein was not able to significantly inhibit the NA dose-dependent contractions except for the 40 minute pre-incubation at the NA dose of 10^{-6}M (Figure 5). This illustrates a difference in sensitivity to genistein of the tail artery compared to the mesenteric artery.

The reversibility of genistein was not tested since its effect has been shown in patch clamp studies to be reversible upon washing with physiological salt solution (Xiong *et al.*, 1995).

(III) Effect of the endothelium and genistein on NA-induced contraction in rat mesenteric artery

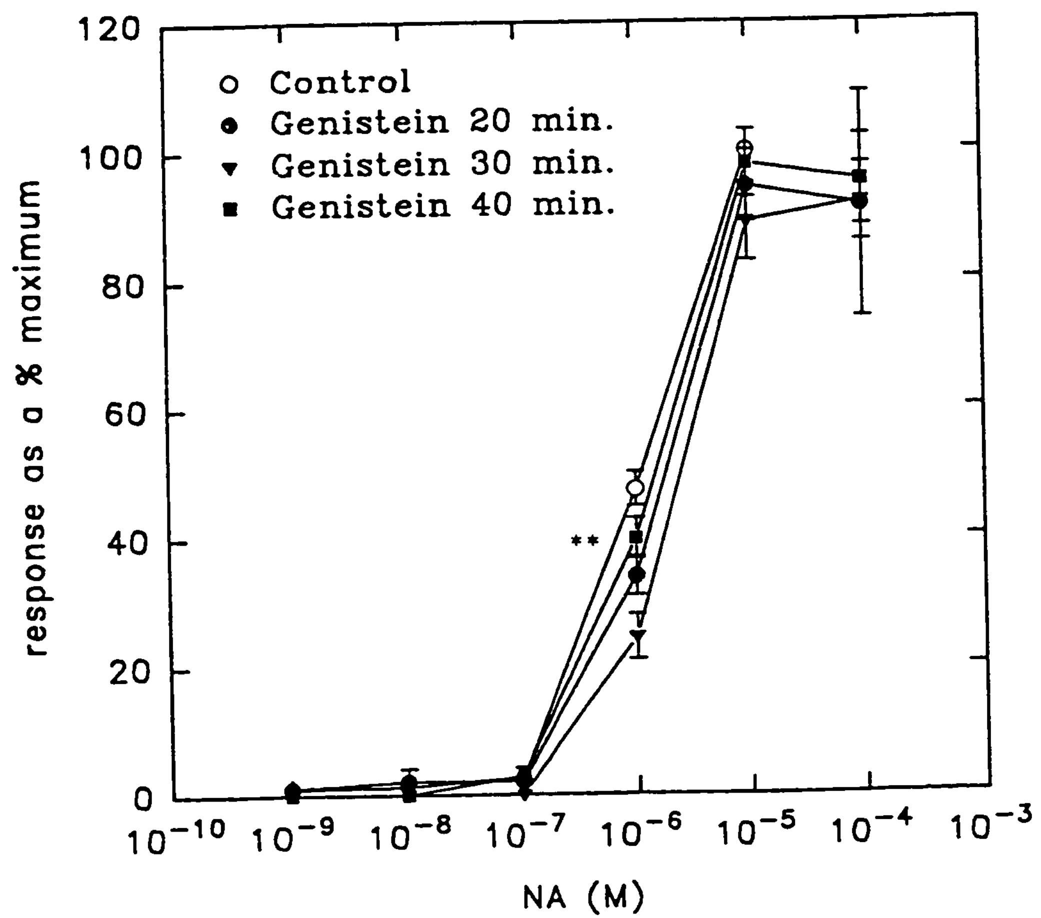
To determine whether genistein produces an endothelium-dependent effect, the effect of genistein on contractions in denuded mesenteric arterial rings was tested. Genistein (10^{-5}M) significantly inhibited the NA-induced contractions in the absence of the endothelium (Figure 6). This would suggest that genistein acts independently of the endothelium.

(IV) Effect of genistein and NPY, alone and combined, on NA- and KCl-induced contraction in rat mesenteric artery

NPY (10^{-7}M) significantly potentiated the response to NA in intact

Figure 5. Effect of varying pretreatment times of genistein on the contraction in the rat tail artery

Effect of different pretreatment times (● 20 minutes, n=2; ▼ 30 minutes, n=2; ■ 40 minutes, n=5) of genistein 10^{-5}M on NA-induced contraction in the rat tail artery; (○ Control, n=9); $^{**}P<0.01$.



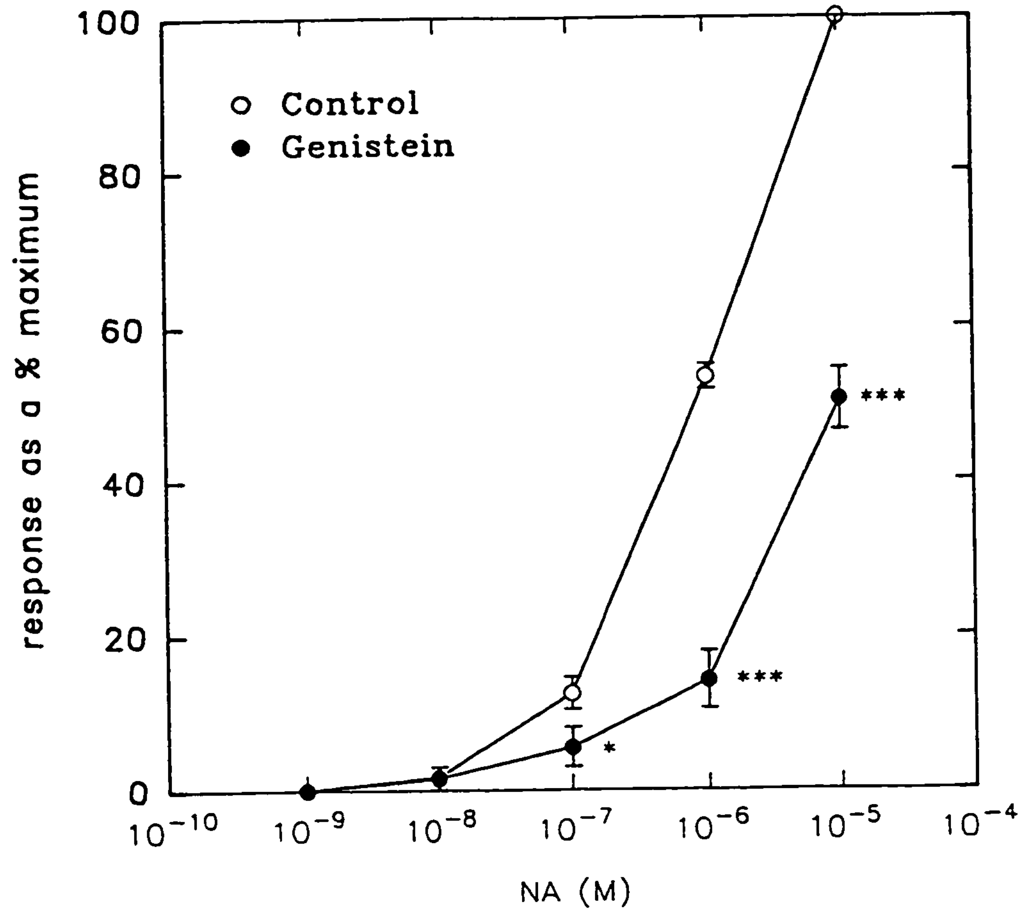
Control
Genistein 20 min.
Genistein 30 min.
Genistein 40 min.
response as a % maximum
120
100
80
60
40
20
0
**
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
NA (M)

Figure 6. Effect of genistein on the contraction in denuded mesenteric artery

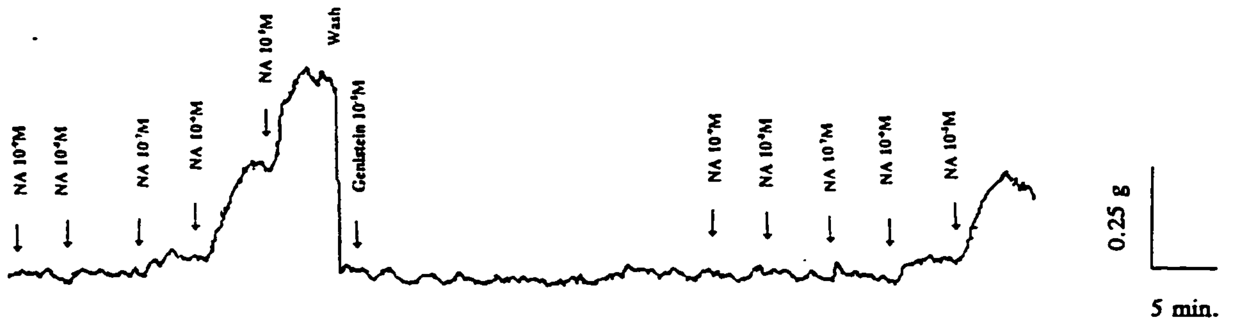
(A) Effect of genistein 10^{-5}M (● 30 minutes, n=6) on NA-induced contraction in the denuded rat mesenteric artery; (○ Control, n=6); *P<0.05, ***P<0.001.

(B) Sample trace illustrating the effect seen in (A).

A



B



mesenteric arteries (Figure 7A). In contrast, when the artery was pre-incubated 30 minutes with genistein (10^{-5}M), the NA-induced contraction was not potentiated by NPY and remained at the same level as when treated with genistein alone. Genistein (10^{-5}M) pre-incubation significantly inhibited the KCl-induced contraction in the rat mesenteric artery, while NPY (10^{-7}M) potentiated the KCl-induced dose-dependent contractions (Figure 7B). Genistein (10^{-5}M) pre-incubation inhibited the potentiating effect of NPY on KCl-induced contraction. The inhibition of contraction relative to the control was to the same extent as with genistein alone.

(V) Effect of daidzein and tyrosine kinase inhibitors on NA- and KCl-induced contraction in rat mesenteric artery

Daidzein (10^{-5}M), the inactive analog of genistein, did not inhibit the NA-induced contractions (Figure 8A) or the KCl-induced contractions in the rat mesenteric artery (Figure 8B). This is in contrast to the active analog genistein which produced an inhibitory effect. A lack of inhibition of both the NA-induced and the KCl-induced contractions was also observed with tyrosine kinase inhibitors lavendustin A (10^{-5}M) (Figure 9) and tyrphostin 25 ($5x10^{-5}$M) (Figure 10). Another tyrosine kinase inhibitor, erbstatin analog, was studied more extensively. Figure 11A shows that erbstatin analog (10^{-5}M) did not produce an inhibition of the NA-induced contractions, but rather an increase in contractions. The potentiation of NA-induced contraction by NPY (10^{-7}M)

Figure 7. Effect of genistein, NPY, and genistein + NPY on contraction

(A) Comparison of the effect of genistein 10^{-5}M (● 30 minutes, n=3), NPY 10^{-7}M (▼ 5 minutes, n=6), and genistein 10^{-5}M + NPY 10^{-7}M (■ n=5) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=14); *$P<0.05$, **$P<0.01$, ***$P<0.001$.

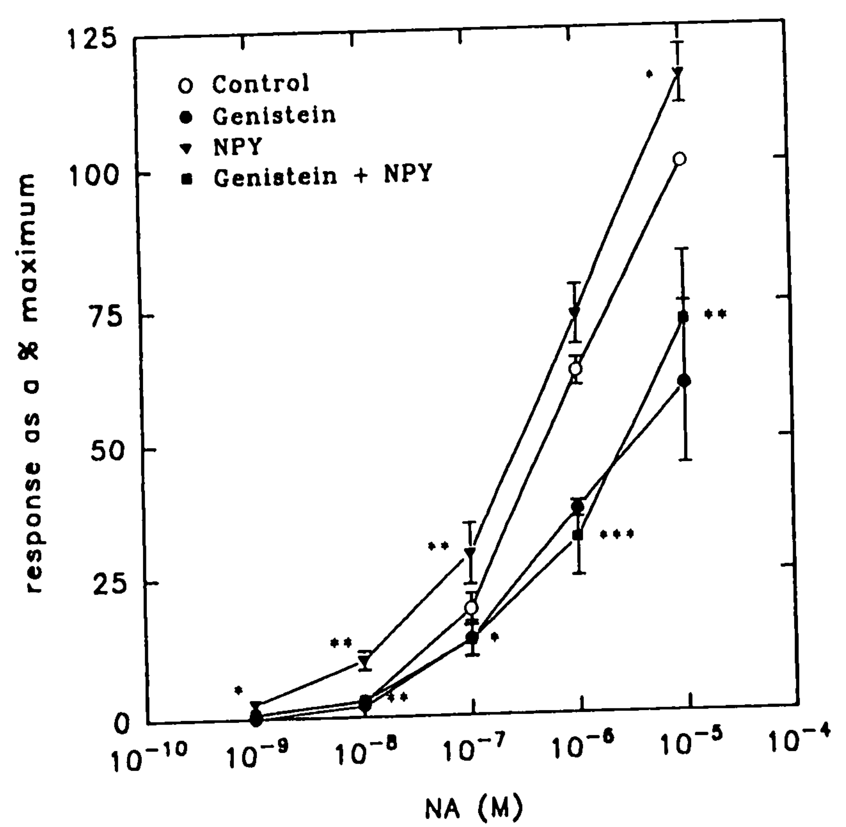
(B) Comparison of the effect of genistein 10^{-5}M (● 30 minutes, n=4), NPY 10^{-7}M (▼ 5 minutes, n=5), and genistein 10^{-5}M + NPY 10^{-7}M (■ n=4) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=13); *$P<0.05$, **$P<0.01$, ***$P<0.001$.

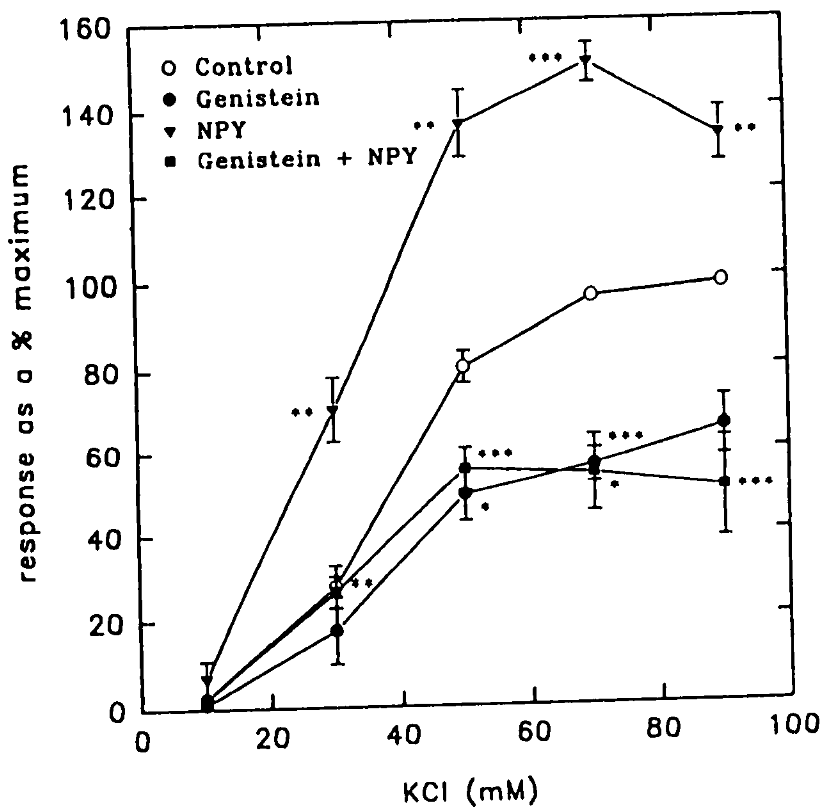
(C) Sample trace illustrating the effect of NPY on KCl-induced contraction.

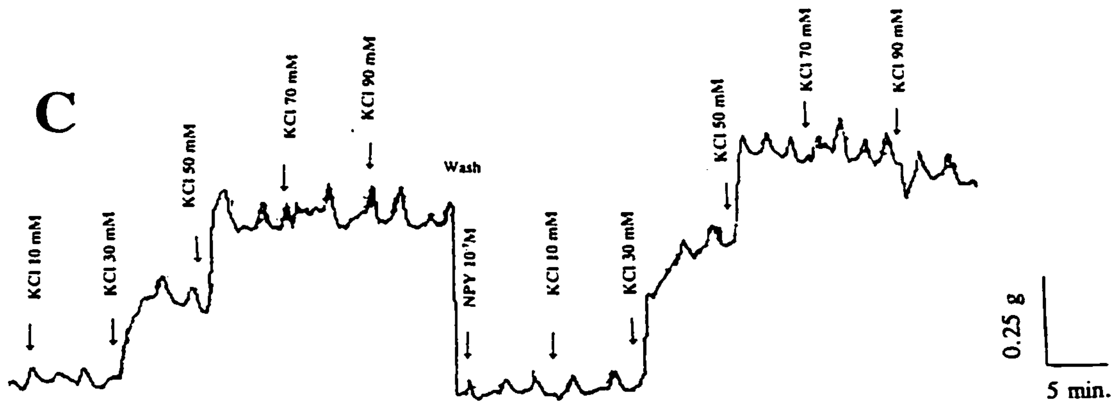
(D) Sample trace illustrating the effect of genistein + NPY on KCl-induced contraction.

(E) Sample trace illustrating the effect of NPY on NA-induced contraction.

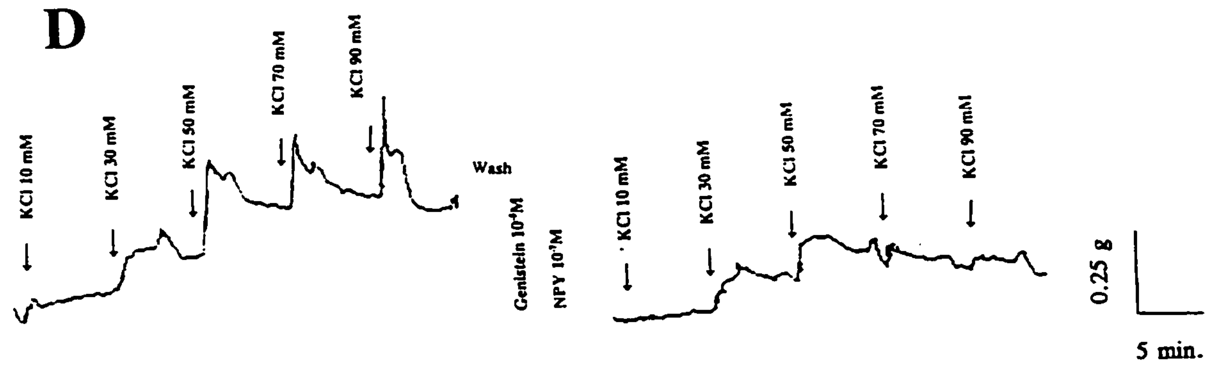
(F) Sample trace illustrating the effect of genistein + NPY on NA-induced contraction.


A
Control
Genistein
NPY
Genistein + NPY
response as a % maximum
NA (M)
0
25
50
75
100
125
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}

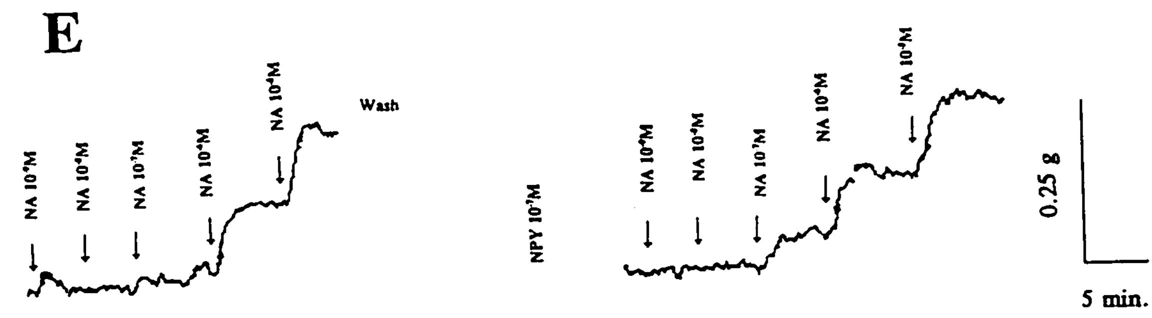

B
Control
Genistein
NPY
Genistein + NPY
response as a % maximum
KCl (mM)
0
20
40
60
80
100
120
140
160
0
20
40
60
80
100



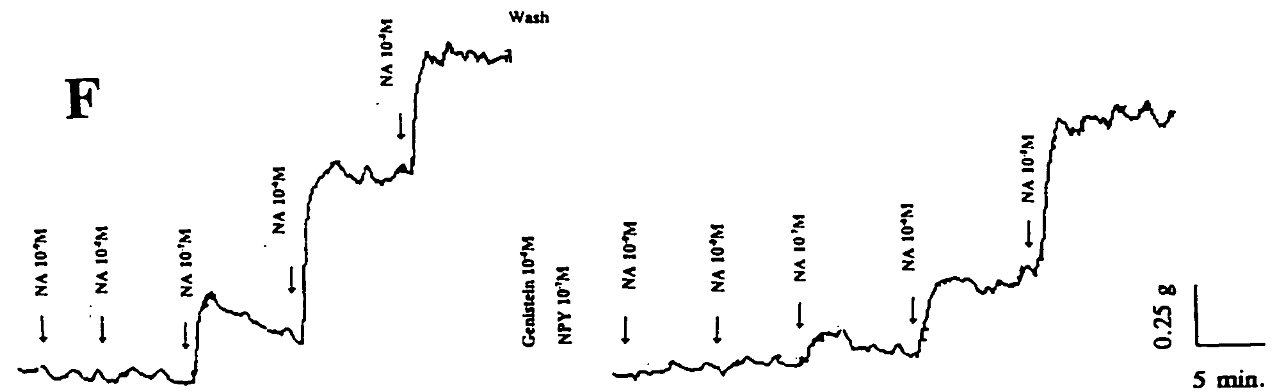
C
KCl 10 mM
KCl 30 mM
KCl 50 mM
KCl 70 mM
KCl 90 mM
Wash
KCl 10 mM
KCl 30 mM
KCl 50 mM
KCl 70 mM
KCl 90 mM
0.25 g
5 min.



D
KCl 10 mM
KCl 30 mM
KCl 50 mM
KCl 70 mM
KCl 90 mM
Wash
KCl 10 mM
KCl 30 mM
KCl 50 mM
KCl 70 mM
KCl 90 mM
0.25 g
5 min.



E
Wash
0.25 g
5 min.



F
Wash
0.25 g
5 min.

Figure 8. Effect of daidzein on contraction

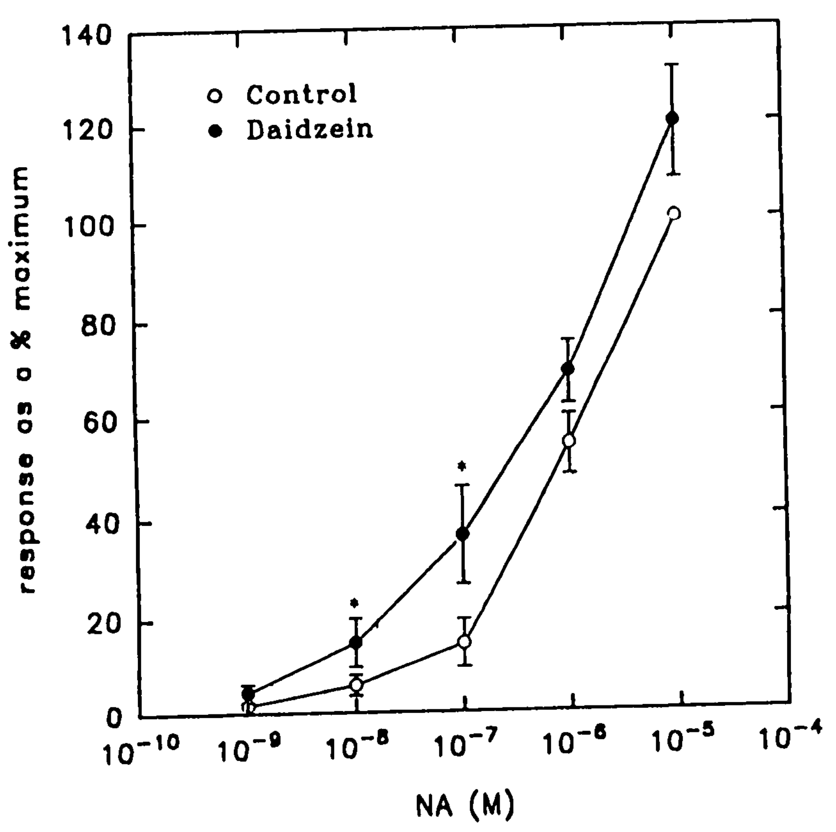
(A) Effect of daidzein 10^{-5}M (● 30 minutes, n=6) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=6); *P<0.05.

(B) Effect of daidzein 10^{-5}M (● 30 minutes, n=6) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=6).

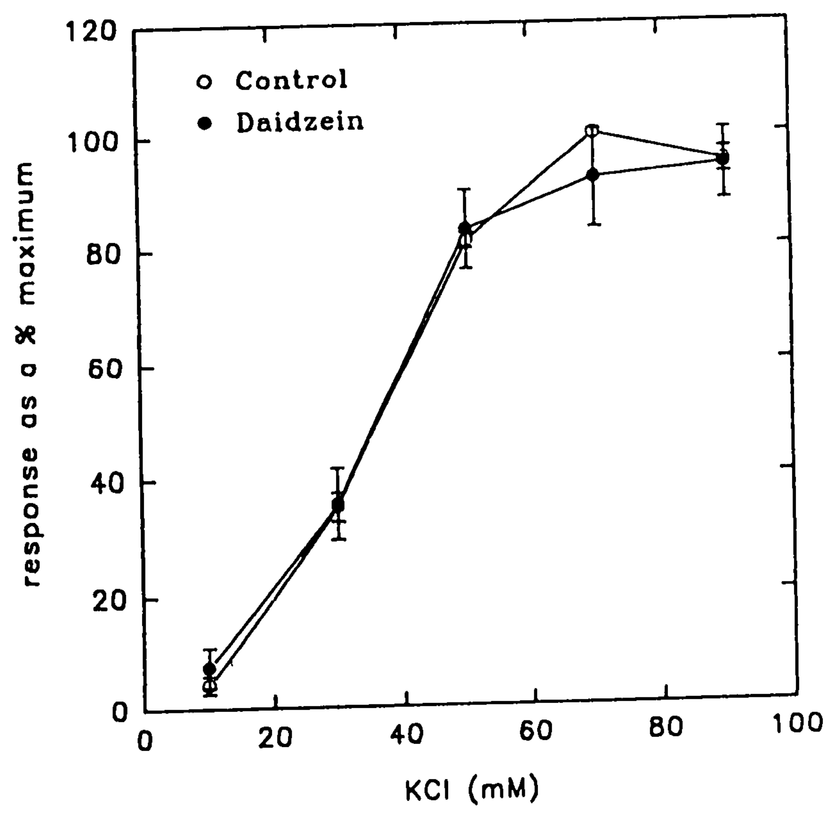
(C) Sample trace illustrating the effect seen in (A).

(D) Sample trace illustrating the effect seen in (B).

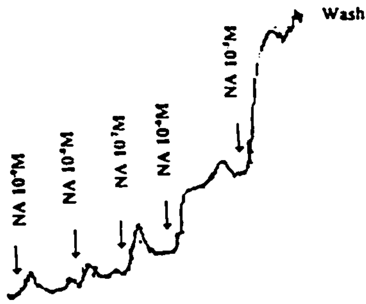
A



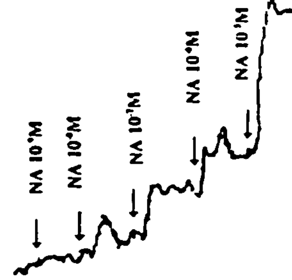
B

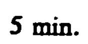


C

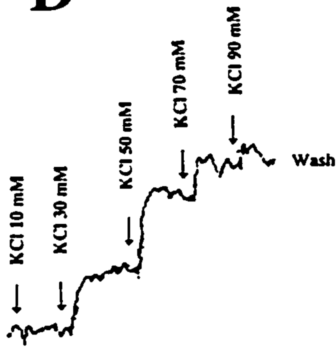


Daidzein 10⁻⁵M





D



Daidzein 10⁻⁵M

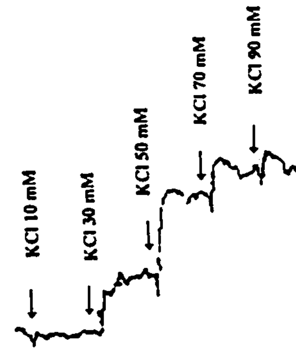
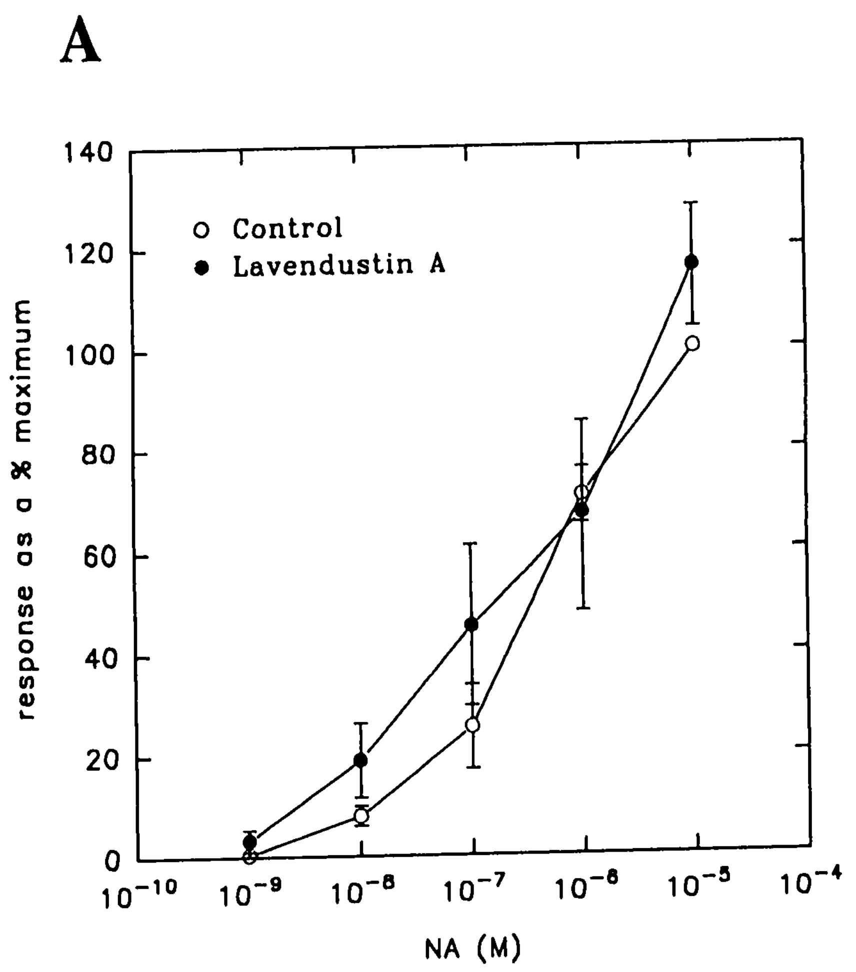


Figure 9. Effect of lavendustin A on contraction

(A) Effect of lavendustin A 10^{-5}M (● 30 minutes, n=5) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=5).

(B) Effect of lavendustin A 10^{-5}M (● 30 minutes, n=4) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=4).

A



B

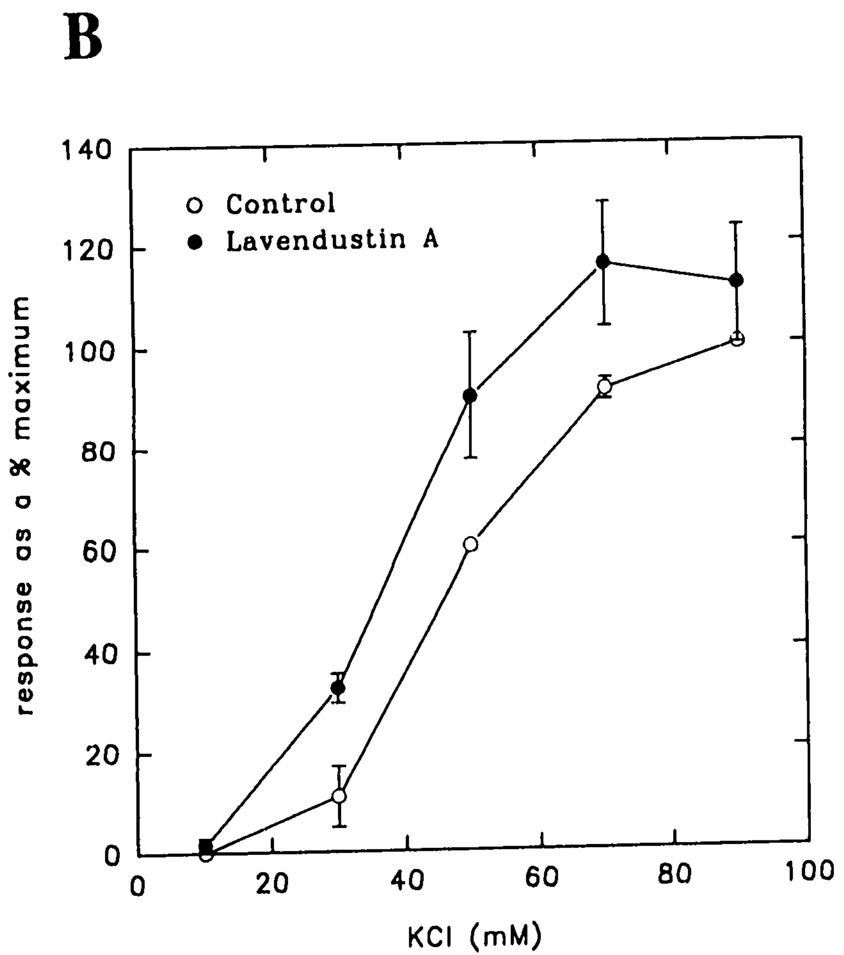
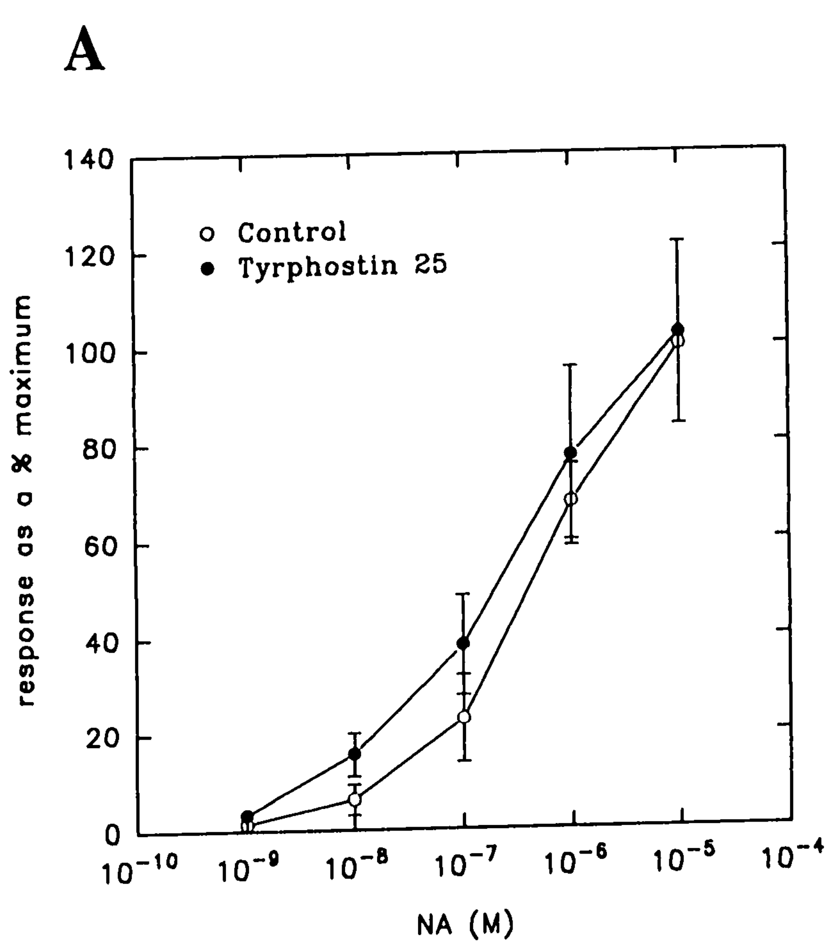


Figure 10. Effect of tyrphostin 25 on contraction

(A) Effect of tyrphostin 25 $5x10^{-5}$M (● 30 minutes, n=5) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=5).

(B) Effect of tyrphostin 25 $5x10^{-5}$M (● 30 minutes, n=4) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=4).


A
response as a % maximum
140
120
100
80
60
40
20
0
Control
Tyrphostin 25
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
NA (M)

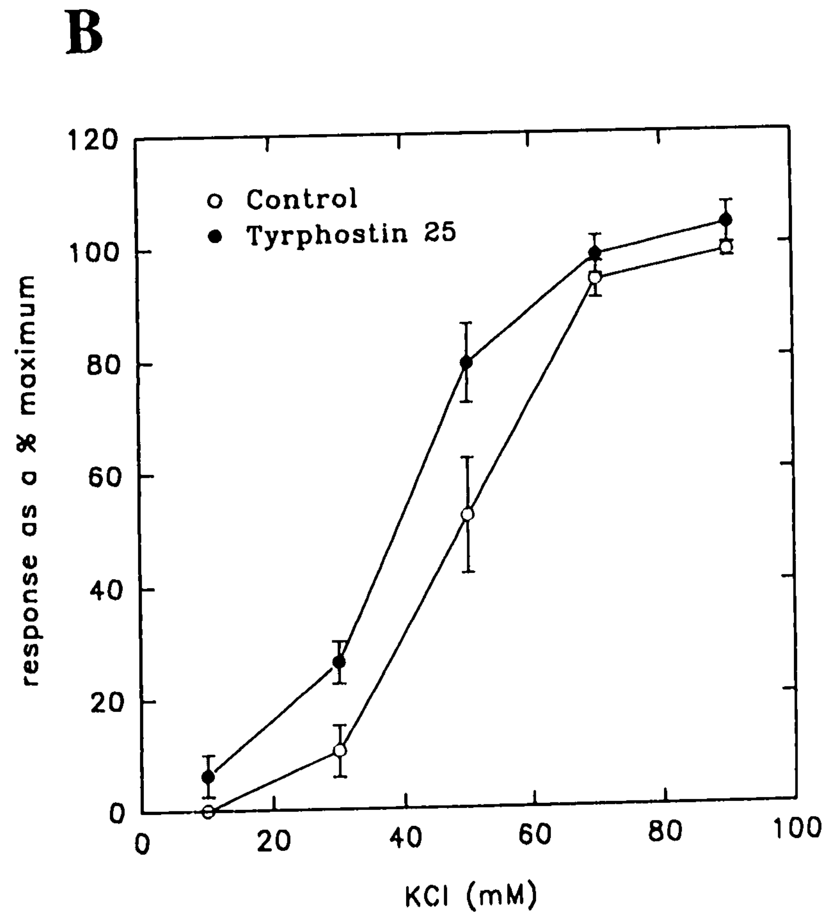
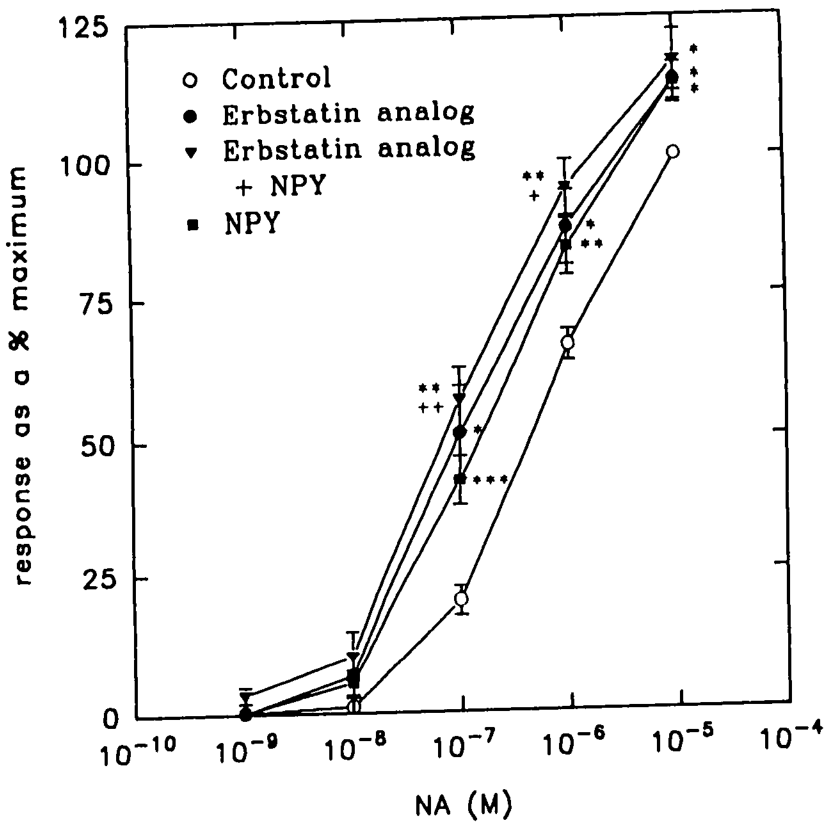
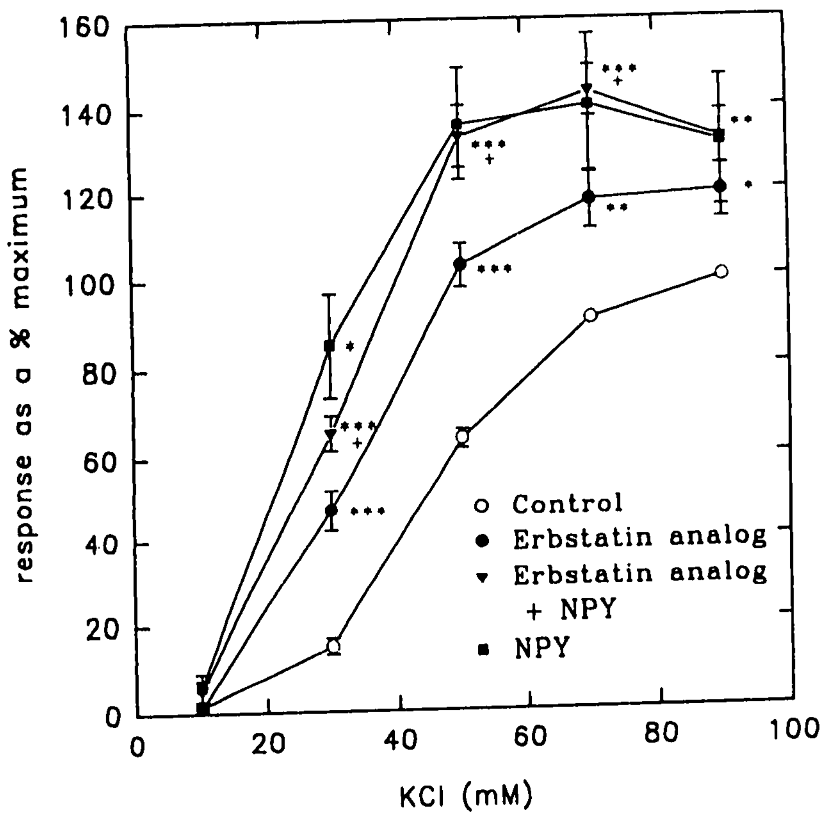

B
response as a % maximum
120
100
80
60
40
20
0
Control
Tyrphostin 25
0
20
40
60
80
100
KCl (mM)

Figure 11. Effect of erbstatin analog, NPY, and erbstatin analog + NPY on contraction

(A) Comparison of the effect of erbstatin analog 10^{-5}M (● 30 minutes, n=4), NPY 10^{-7}M (■ 5 minutes, n=4), and erbstatin analog 10^{-5}M + NPY 10^{-7}M (▼ n=4) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=12); *P<0.05, **P<0.01, ***P<0.001, +P<0.05, ++P<0.01, where * is versus control and + is ▼ versus ■.

(B) Comparison of the effect of erbstatin analog 10^{-5}M (● 30 minutes, n=10), NPY 10^{-7}M (■ 5 minutes, n=3), and erbstatin analog 10^{-5}M + NPY 10^{-7}M (▼ n=10) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=23); *P<0.05, **P<0.01, ***P<0.001, +P<0.05, where * is versus control and + is ▼ versus ●.


A
response as a % maximum
125
100
75
50
25
0
Control
Erbstatin analog
Erbstatin analog
+ NPY
NPY
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
NA (M)


B
response as a % maximum
160
140
120
100
80
60
40
20
0
Control
Erbstatin analog
Erbstatin analog
+ NPY
NPY
0
20
40
60
80
100
KCl (mM)

was compared to the effect of a pretreatment combination of NPY and erbstatin analog. The combination produced a greater contraction compared to NPY alone. Figure 11B shows that erbstatin analog had a similar effect on KCl-induced contractions. When combined with NPY, the contractions were greater than with erbstatin analog alone.

(VI) Effect of different doses of Na_3VO_4 on KCl-induced contraction in rat mesenteric artery

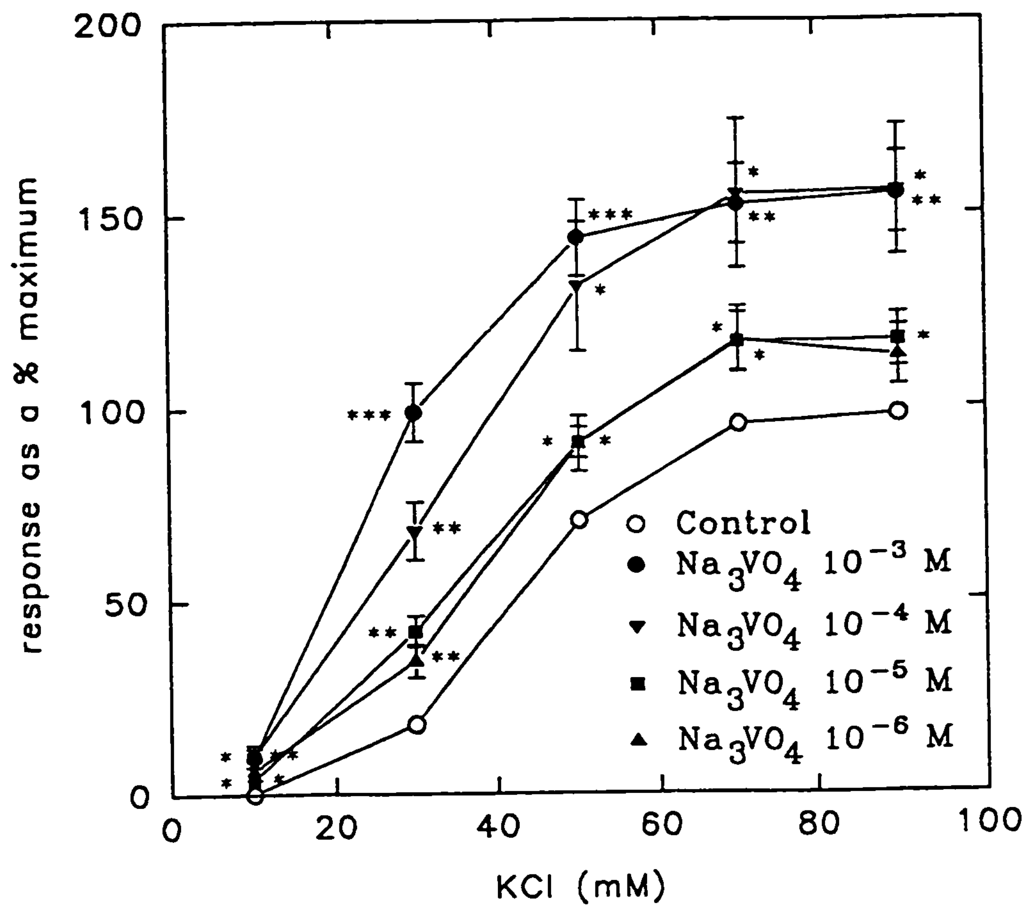
Tissues were pre-exposed to sodium orthovanadate for 5 minutes in order to ensure phosphatase inhibition. Doses of 10^{-3}M and 10^{-4}M both produced the maximum potentiating effect of Na_3VO_4 (Figure 12). While doses of 10^{-5}M and 10^{-6}M were able to produce a potentiation of KCl-induced contraction, the level of potentiation was not as great as that seen by the higher doses.

(VII) Effect of Na_3VO_4 and NPY on NA- and KCl-induced contraction in rat mesenteric artery

Na_3VO_4 (10^{-3}M) significantly potentiated the NA-induced contraction in the rat mesenteric artery (Figure 13A). Na_3VO_4 in combination with NPY (10^{-7}M) potentiated the contraction to the same level as with Na_3VO_4 alone. Na_3VO_4 (10^{-3}M) also significantly potentiated the KCl-induced contraction (Figure 13B). When NPY was combined with Na_3VO_4, the potentiation of

Figure 12. Effect of varying concentrations of Na_3VO_4 on contraction

Effect of different concentrations (● 10^{-3}M, n=6; ▼ 10^{-4}M, n=6; ■ 10^{-5}M, n=7; ▲ 10^{-6}M, n=7) of Na_3VO_4 (5 minutes) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=26); *P<0.05, **P<0.01, ***P<0.001.



200
150
100
50
0
0
20
40
60
80
100
KCl (mM)
response as a % maximum
Control
Na_3VO_4 10^{-3} M
Na_3VO_4 10^{-4} M
Na_3VO_4 10^{-5} M
Na_3VO_4 10^{-6} M

Figure 13. Effect of Na_3VO_4 10^{-3}M and Na_3VO_4 10^{-3}M + NPY on contraction

(A) Comparison of the effect of Na_3VO_4 10^{-3}M (● 5 minutes, n=7), and Na_3VO_4 10^{-3}M + NPY 10^{-7}M (▼ 5 minutes, n=5) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=12); *$P<0.05$, **$P<0.01$, ***$P<0.001$.

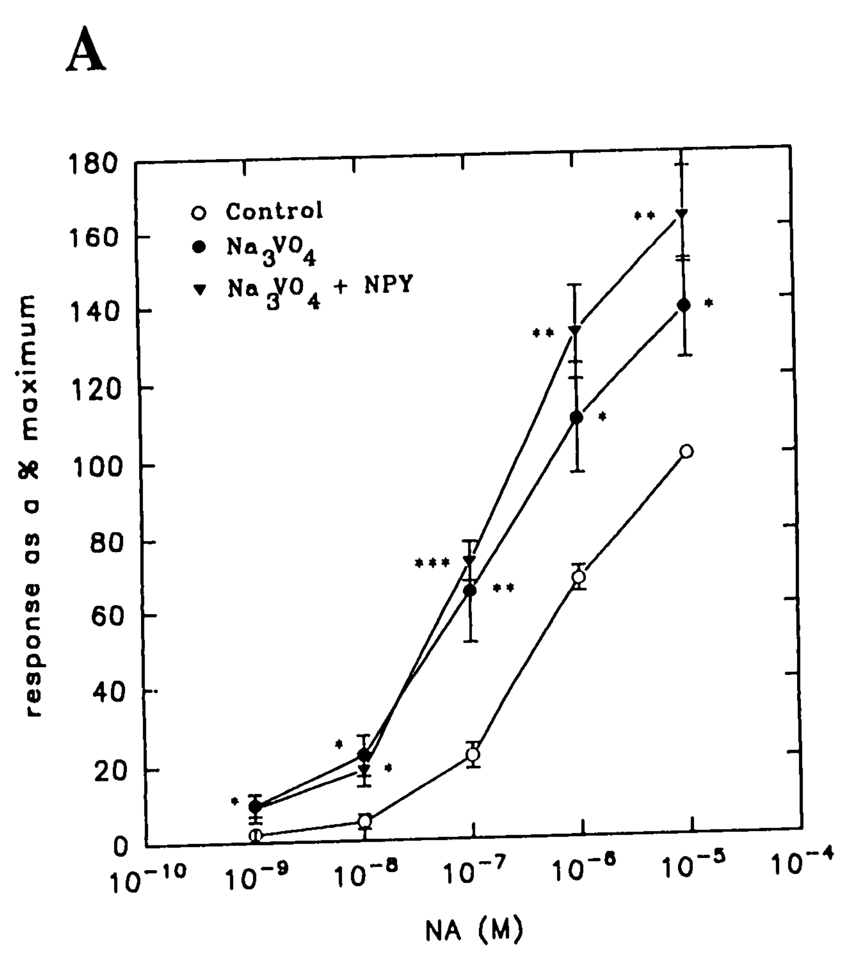
(B) Comparison of the effect of Na_3VO_4 10^{-3}M (● 5 minutes, n=5), and Na_3VO_4 10^{-3}M + NPY 10^{-7}M (▼ 5 minutes, n=6) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=11); ***$P<0.001$, +$P<0.05$, ++$P<0.01$, +++$P<0.001$, where * is versus control and + is ● versus ▼.

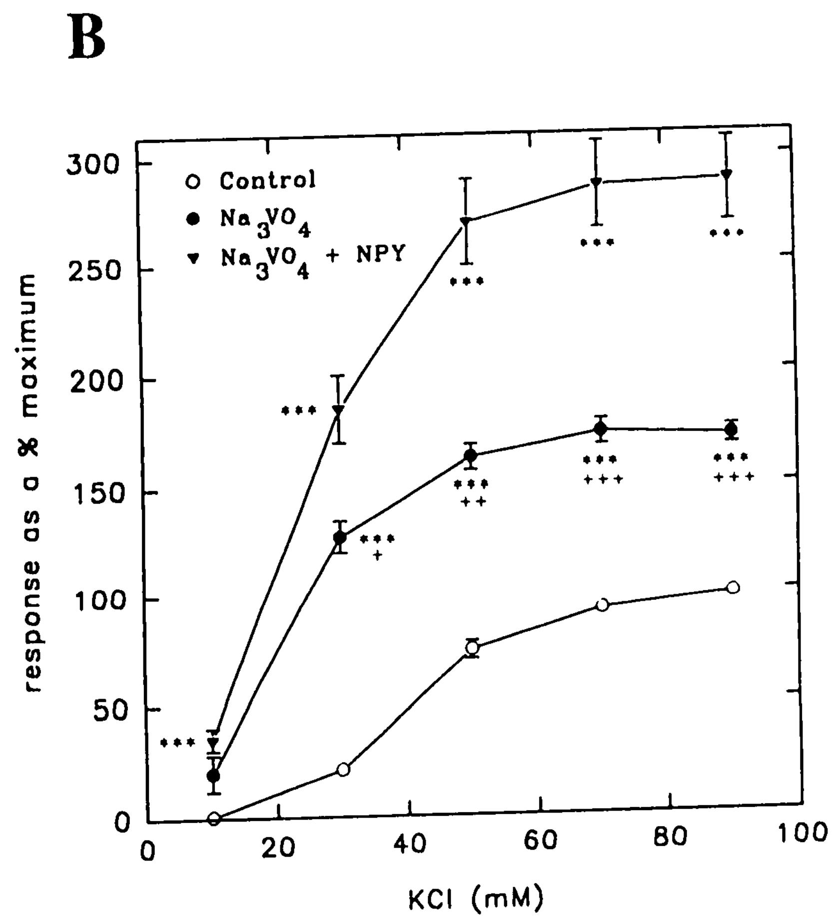
(C) Sample trace illustrating the effect of Na_3VO_4 on NA-induced contraction.

(D) Sample trace illustrating the effect of Na_3VO_4 + NPY on NA-induced contraction.

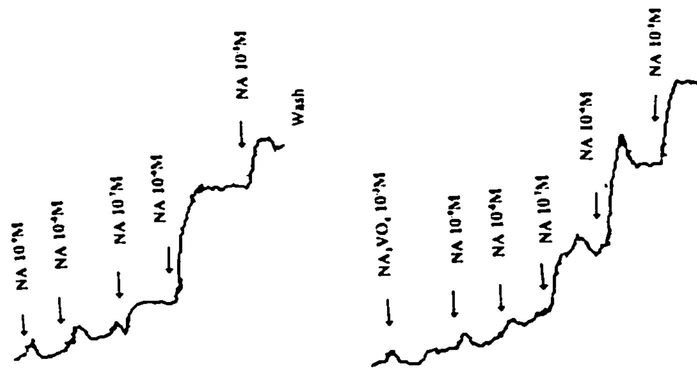
(E) Sample trace illustrating the effect of Na_3VO_4 on KCl-induced contraction.

(F) Sample trace illustrating the effect of Na_3VO_4 + NPY on KCl-induced contraction.


A
Control
Na_3VO_4
Na_3VO_4 + NPY
response as a % maximum
0
20
40
60
80
100
120
140
160
180
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
NA (M)


B
Control
Na_3VO_4
Na_3VO_4 + NPY
response as a % maximum
0
50
100
150
200
250
300
0
20
40
60
80
100
KCl (mM)

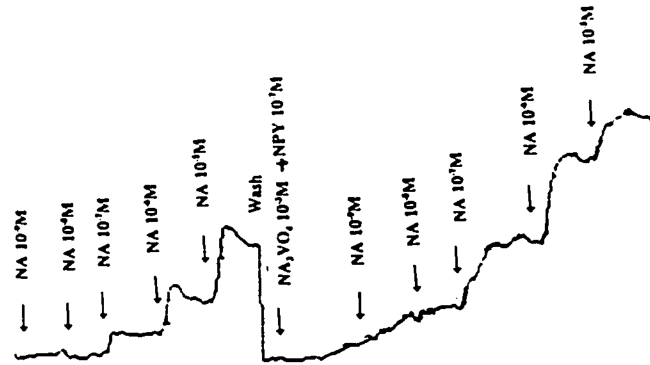
C



0.25 g

5 min.

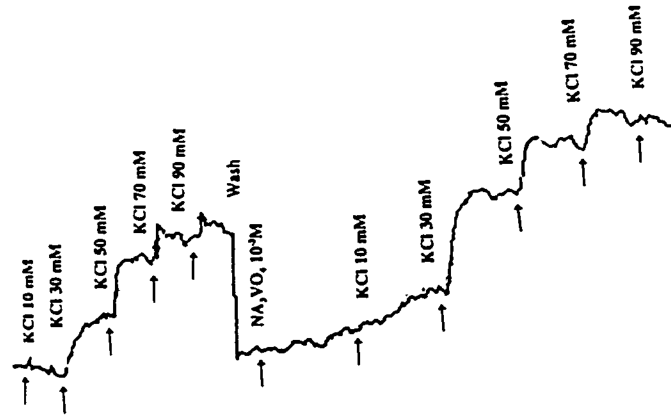
D



0.25 g

5 min.

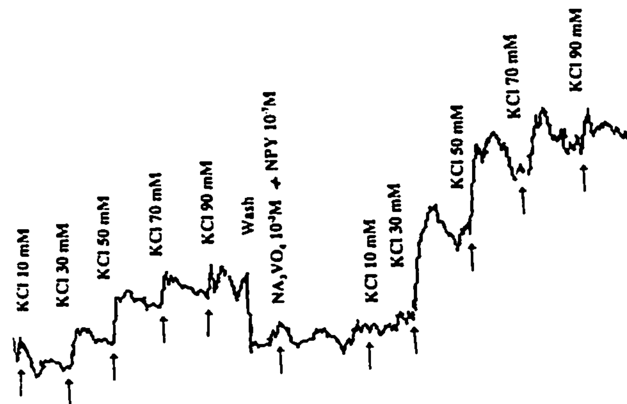
E



0.25 g

5 min.

F



0.25 g

5 min.

KCl-induced contraction was significantly greater than that seen by Na_3VO_4 alone.

(VIII) Effect of genistein and Na_3VO_4 on NA- and KCl-induced contraction in rat mesenteric artery

Genistein (10^{-5}M) in combination with Na_3VO_4 (10^{-3}M) did not alter the NA-induced contraction in rat mesenteric artery (Figure 14A). This combination prevented the potentiating effect seen by Na_3VO_4 alone and prevented the inhibiting effect seen by genistein alone. Genistein (10^{-5}M) in combination with Na_3VO_4 (10^{-3}M) potentiated the KCl-induced contraction and was only able to inhibit the potentiating effect of Na_3VO_4 (10^{-3}M) at a KCl dose of 30 mM (Figure 14B). In Figure 15, Na_3VO_4 was used at a lower concentration of 10^{-4}M. When this was combined with genistein (10^{-5}M), a potentiation of the NA-induced contraction resulted.

(IX) Effect of genistein, Na_3VO_4 and NPY on KCl-induced contraction in rat mesenteric artery

The combination of genistein (10^{-5}M) and Na_3VO_4 (10^{-3}M) potentiated the KCl-induced contraction in rat mesenteric artery (Figure 16). When NPY (10^{-7}M) was added to this combination, the potentiating effect was significantly greater at a KCl dose of 50 mM.

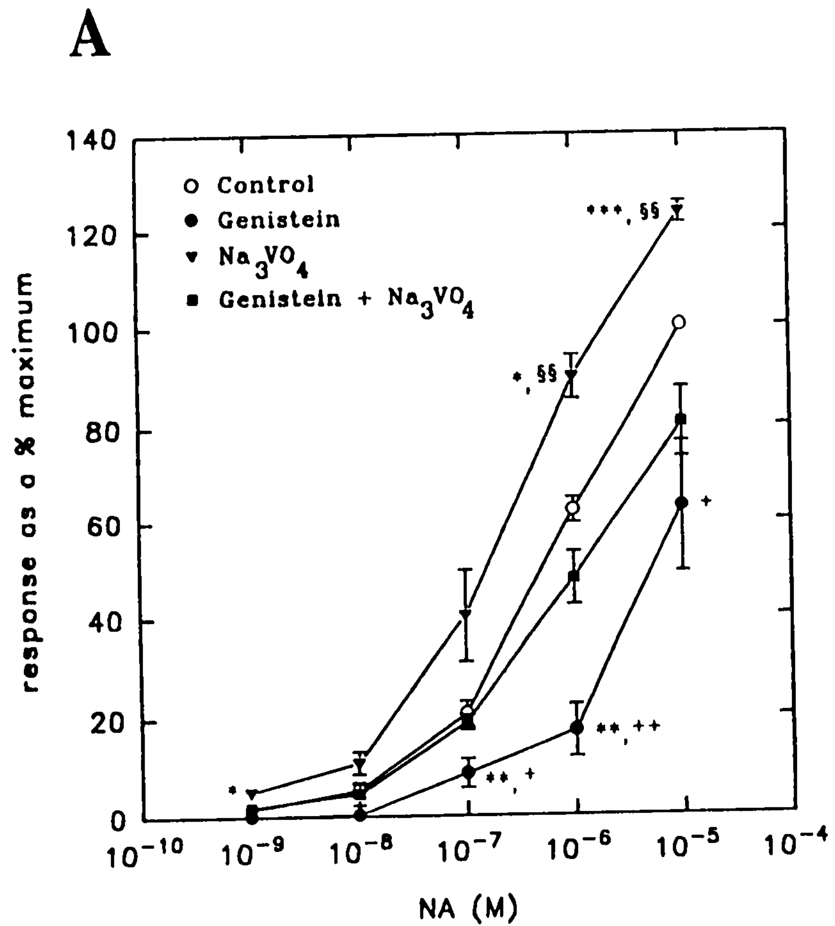
Figure 14. Effect of genistein, Na_3VO_4 10^{-3}M, and genistein + Na_3VO_4 10^{-3}M on contraction

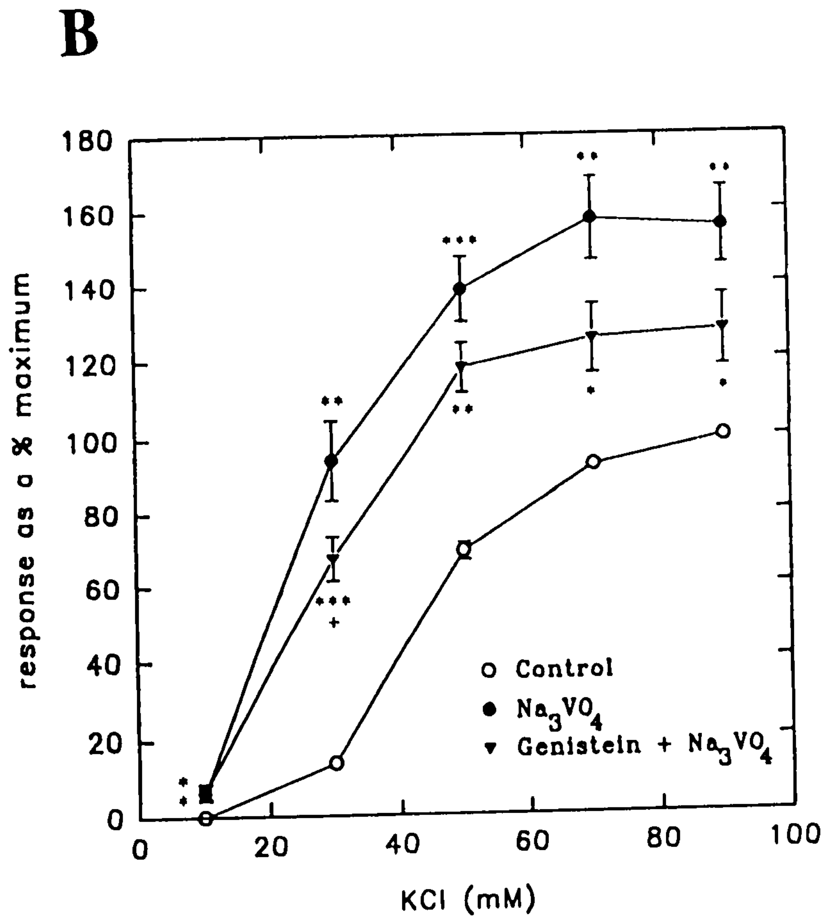
(A) Comparison of the effect of genistein 10^{-5}M (● 30 minutes, n=4), Na_3VO_4 10^{-3}M (▼ 5 minutes, n=5), and genistein 10^{-5}M + Na_3VO_4 10^{-3}M (■ n=5) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=14); *P<0.05, **P<0.01, ***P<0.001, +P<0.05, ++P<0.01, §§P<0.01, where * is versus control, + is ● versus ■, and § is ▼ verus ■.

(B) Comparison of the effect of Na_3VO_4 10^{-3}M (● 5 minutes, n=6), and genistein 10^{-5}M (30 minutes) + Na_3VO_4 10^{-3}M (5minutes) (▼ n=6) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=12); *P<0.05, **P<0.01, ***P<0.001, +P<0.05, where * is versus control and + is ● versus ▼.

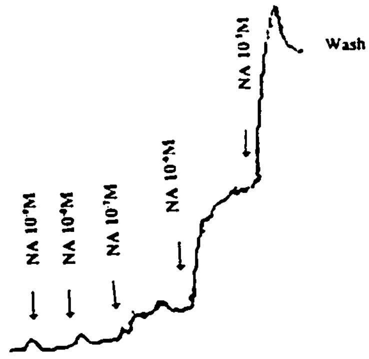
(C) Sample trace illustrating the effect of genistein + Na_3VO_4 on NA-induced contraction.

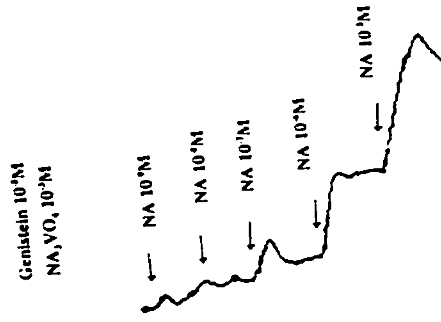
(D) Sample trace illustrating the effect of genistein + Na_3VO_4 on KCl-induced contraction.


A
Control
Genistein
Na_3VO_4
Genistein + Na_3VO_4
response as a % maximum
NA (M)


B
Control
Na_3VO_4
Genistein + Na_3VO_4
response as a % maximum
KCl (mM)

C

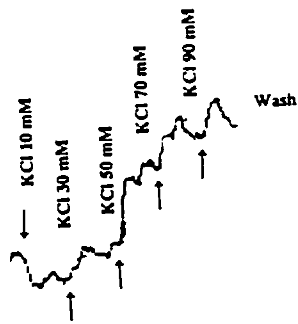




0.5 g

5 min.

D



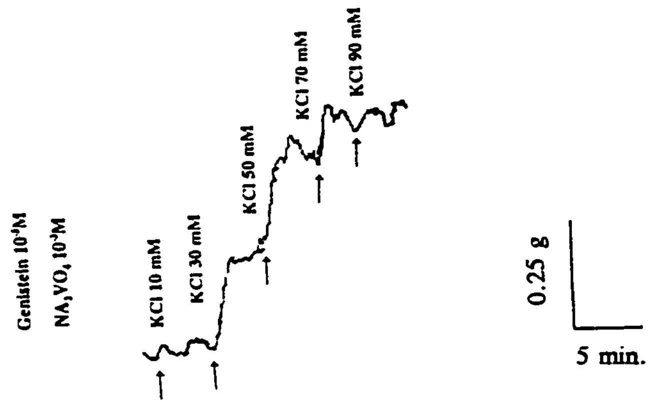
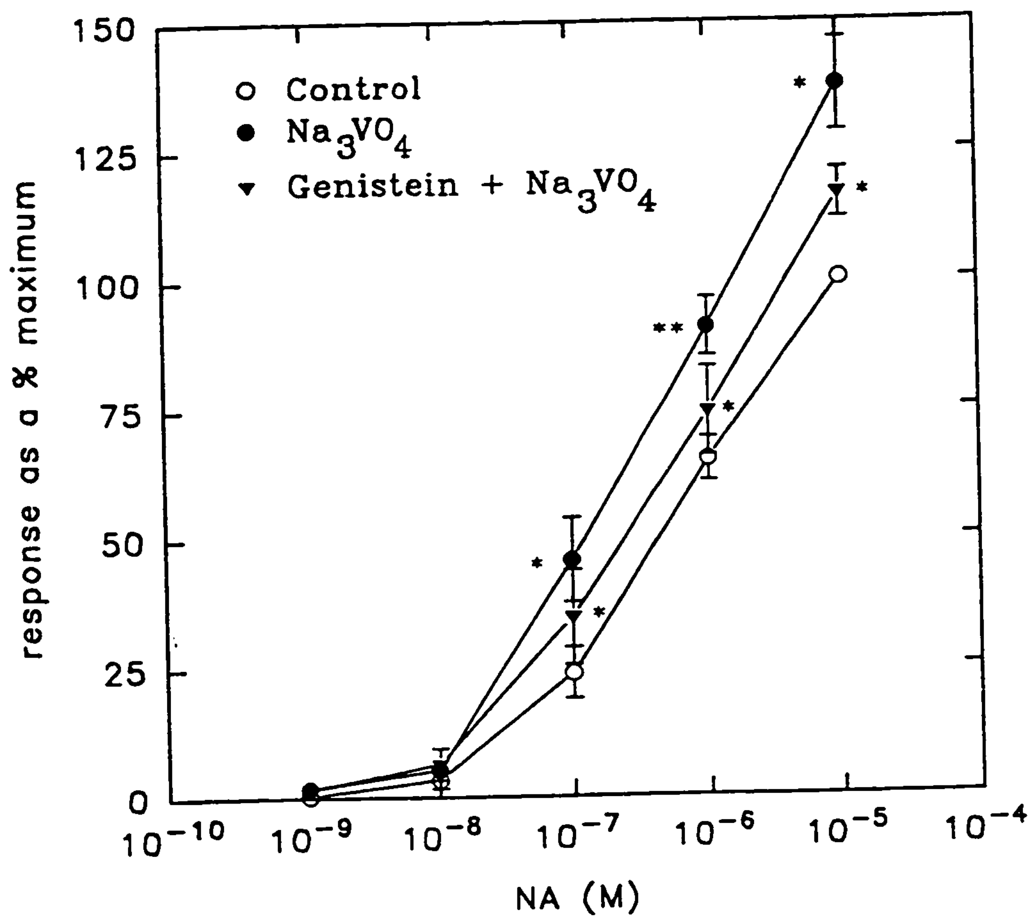


Figure 15. Effect of Na_3VO_4 10^{-4}M and genistein + Na_3VO_4 10^{-4}M on NA-induced contraction

Comparison of the effect of Na_3VO_4 10^{-4}M (● 5 minutes, n=4), and genistein 10^{-5}M (30 minutes) + Na_3VO_4 10^{-4}M (5 minutes) (▼ n=6) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=10); *P<0.05, **P<0.01.



Control
Na_3VO_4
Genistein + Na_3VO_4
response as a % maximum
NA (M)
0
25
50
75
100
125
150
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}

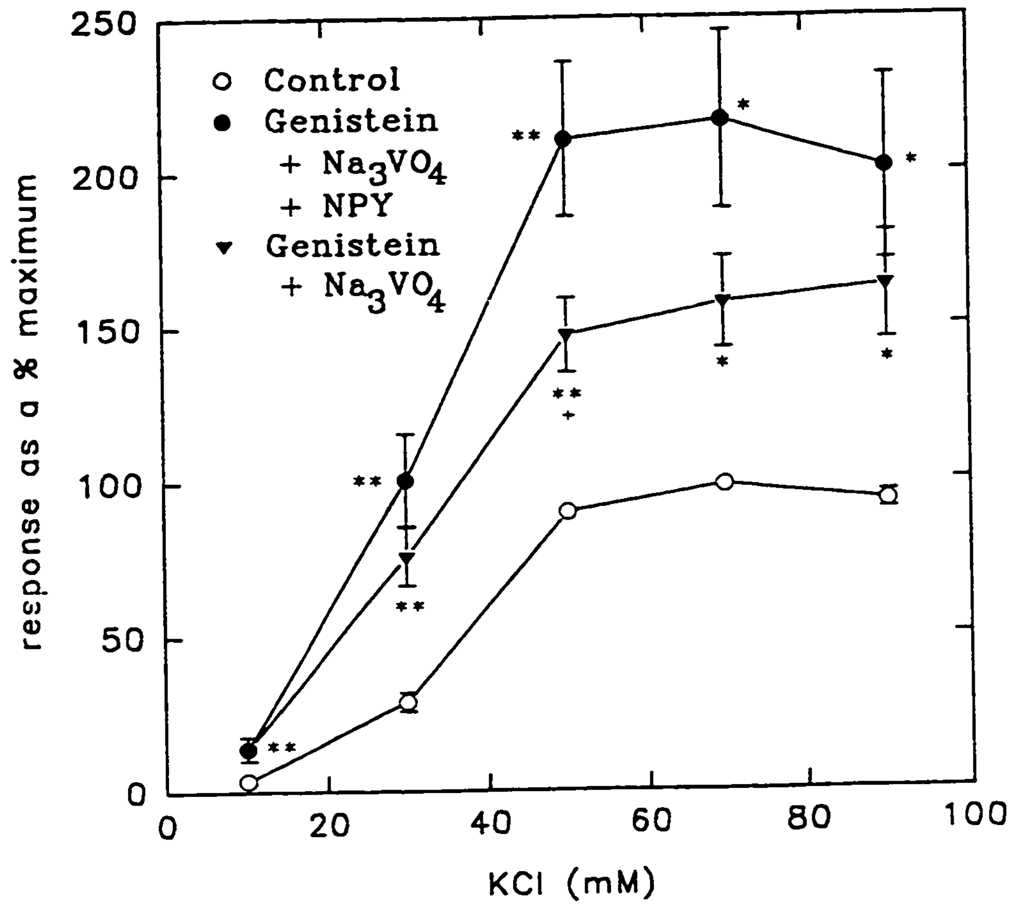
Figure 16. Effect of genistein + Na_3VO_4 10^{-3}M + NPY and genistein + Na_3VO_4 10^{-3}M on KCl-induced contraction

(A) Comparison of the effect of genistein 10^{-5}M (30 minutes) + Na_3VO_4 10^{-3}M (5 minutes) + NPY 10^{-7}M (5 minutes) (● n=5), and genistein 10^{-5}M (30 minutes) + Na_3VO_4 10^{-3}M (5minutes) (▼ n=6) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=11); *P<0.05, **P<0.01, +P<0.05, where * is versus control and + is ● versus ▼.

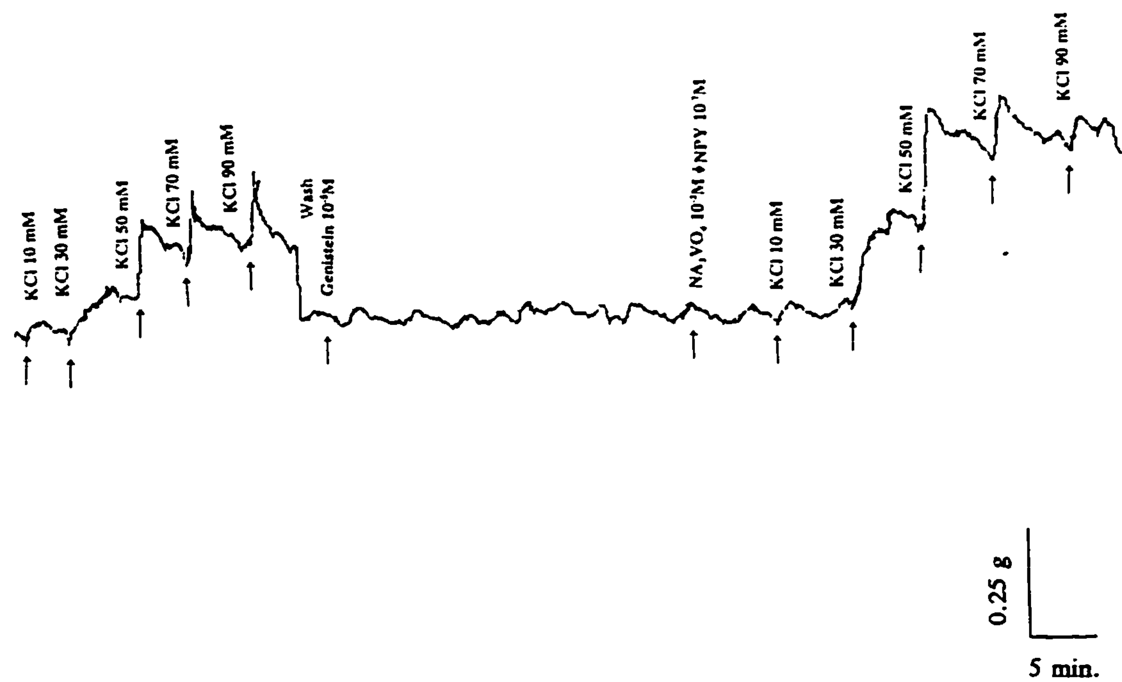
(B) Sample trace illustrating the effect of genistein + Na_3VO_4 + NPY on KCl-induced contraction.

(C) Sample trace illustrating the effect of genistein + Na_3VO_4 on KCl-induced contraction.

A



B



C

(X) Effect of nifedipine, Na_3VO_4 and NPY on NA- and KCl-induced contraction in rat mesenteric artery

Nifedipine (10^{-6}M) alone and in combination with either NPY (10^{-7}M) or Na_3VO_4 (10^{-3}M), significantly inhibited the NA-induced contraction in rat mesenteric artery (Figure 17A). The inhibitions caused by the various combinations were not significantly different from each other. The combination of nifedipine (10^{-6}M) and Na_3VO_4 (10^{-3}M) significantly inhibited the KCl-induced contraction (Figure 17B). When NPY (10^{-7}M) was added to the combination, the KCl-induced contraction was inhibited to the same level as with nifedipine and Na_3VO_4 in the absence of NPY.

(XI) Effect of genistein, NPY, and Na_3VO_4 on NA-induced contraction in the presence and absence of calcium in rat mesenteric artery

The NA (10^{-6}M)-induced contraction in rat mesenteric artery was reduced when a calcium-free bath solution was used compared to a calcium-containing solution. When genistein (10^{-5}M) was used as pretreatment in the absence of calcium, there was no inhibition of NA-induced contraction (Figure 18). NPY (10^{-7}M) (Figure 19) and Na_3VO_4 (10^{-3}M) (Figure 20) were still able to produce a potentiating effect on NA-induced contraction in the absence of calcium.

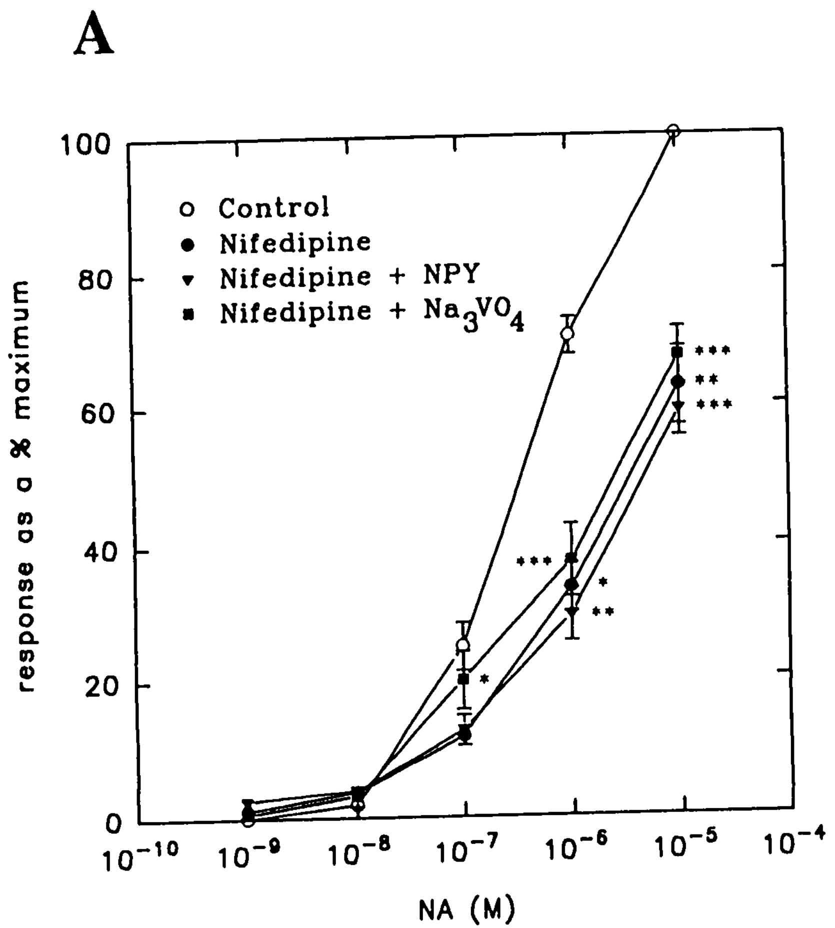
Figure 17. Effect of combinations of nifedipine, NPY, and Na_3VO_4 10^{-3}M on contraction

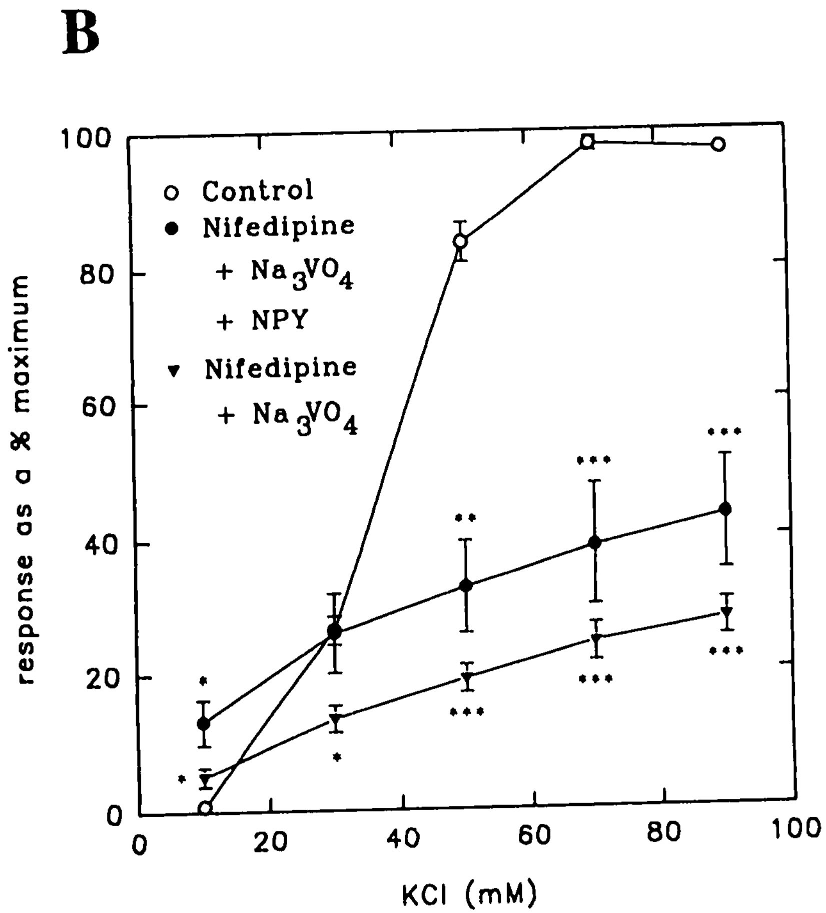
(A) Comparison of the effect of nifedipine 10^{-6}M (● 5 minutes, n=4), nifedipine 10^{-6}M + NPY 10^{-7}M (▼ 5 minutes, n=5), and nifedipine 10^{-6}M + Na_3VO_4 10^{-3}M (■ 5 minutes, n=6) on NA-induced contraction in the rat mesenteric artery; (○ Control, n=15); *$P<0.05$, **$P<0.01$, ***$P<0.001$.

(B) Comparison of the effect of nifedipine 10^{-6}M + Na_3VO_4 10^{-3}M + NPY 10^{-7}M (● 5 minutes, n=7), and nifedipine 10^{-6}M + Na_3VO_4 10^{-3}M (▼ 5 minutes, n=6) on KCl-induced contraction in the rat mesenteric artery; (○ Control, n=13); *$P<0.05$, **$P<0.01$, ***$P<0.001$.

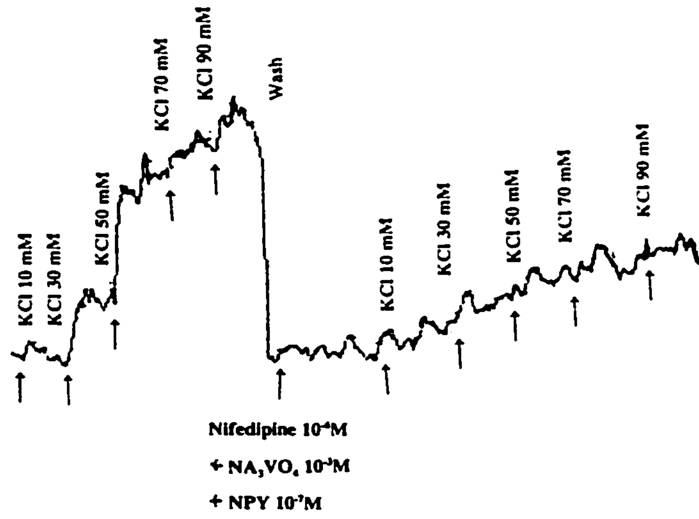
(C) Sample trace illustrating the effect of nifedipine + Na_3VO_4 + NPY on KCl-induced contraction.

(D) Sample trace illustrating the effect of nifedipine + Na_3VO_4 on KCl-induced contraction.


A
Control
Nifedipine
Nifedipine + NPY
Nifedipine + Na_3VO_4
response as a % maximum
NA (M)
0
20
40
60
80
100
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}


B
Control
Nifedipine
+ Na_3VO_4
+ NPY
Nifedipine
+ Na_3VO_4
response as a % maximum
KCl (mM)
0
20
40
60
80
100

C



0.25 g

5 min.

D

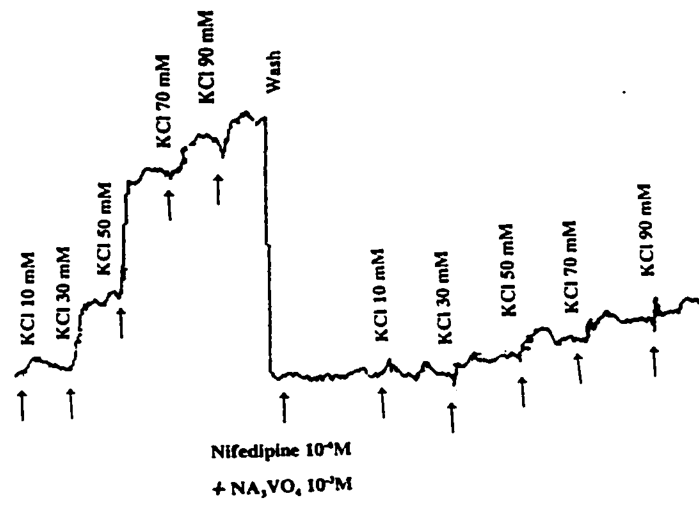


Figure 18. Effect of genistein on contraction with and without calcium

Comparison of the effect of genistein (10^{-5}M, 30 minutes) on NA (10^{-6}M)-induced contraction in the presence and absence of extracellular calcium; □ +Ca^{2+} n=3; ■ Ca^{2+}-free n=7; +++P<0.001, where + is NA (+Ca^{2+}) versus NA (Ca^{2+}-free).

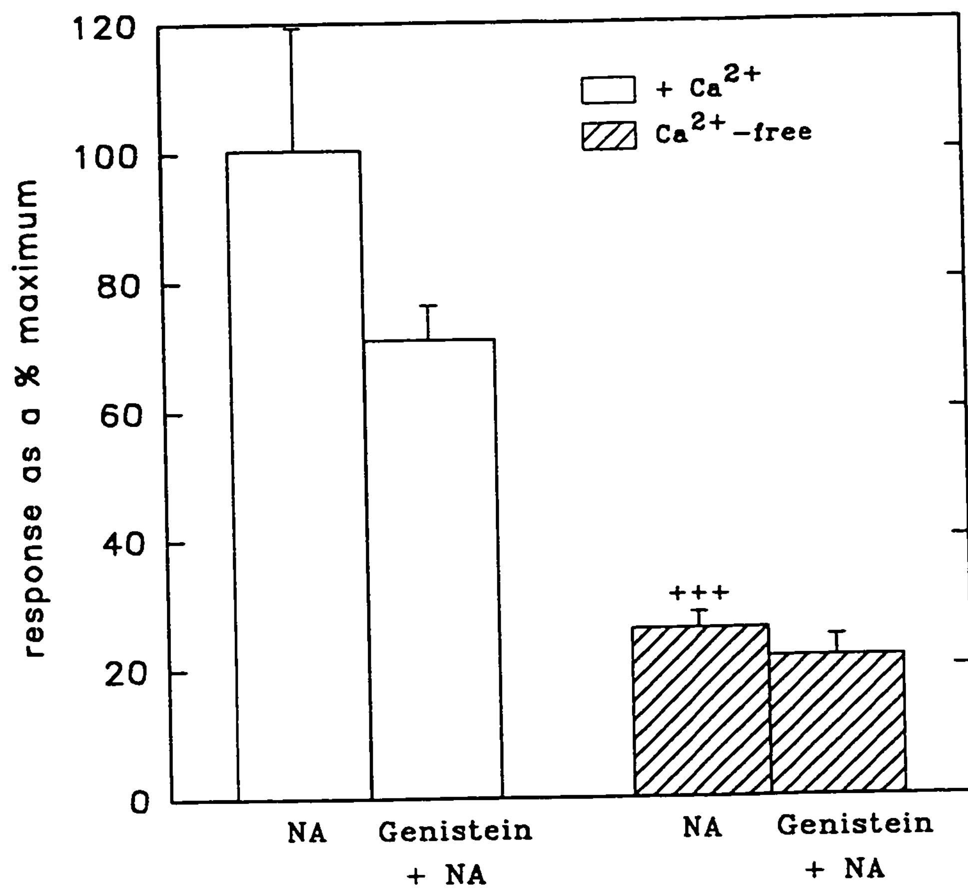
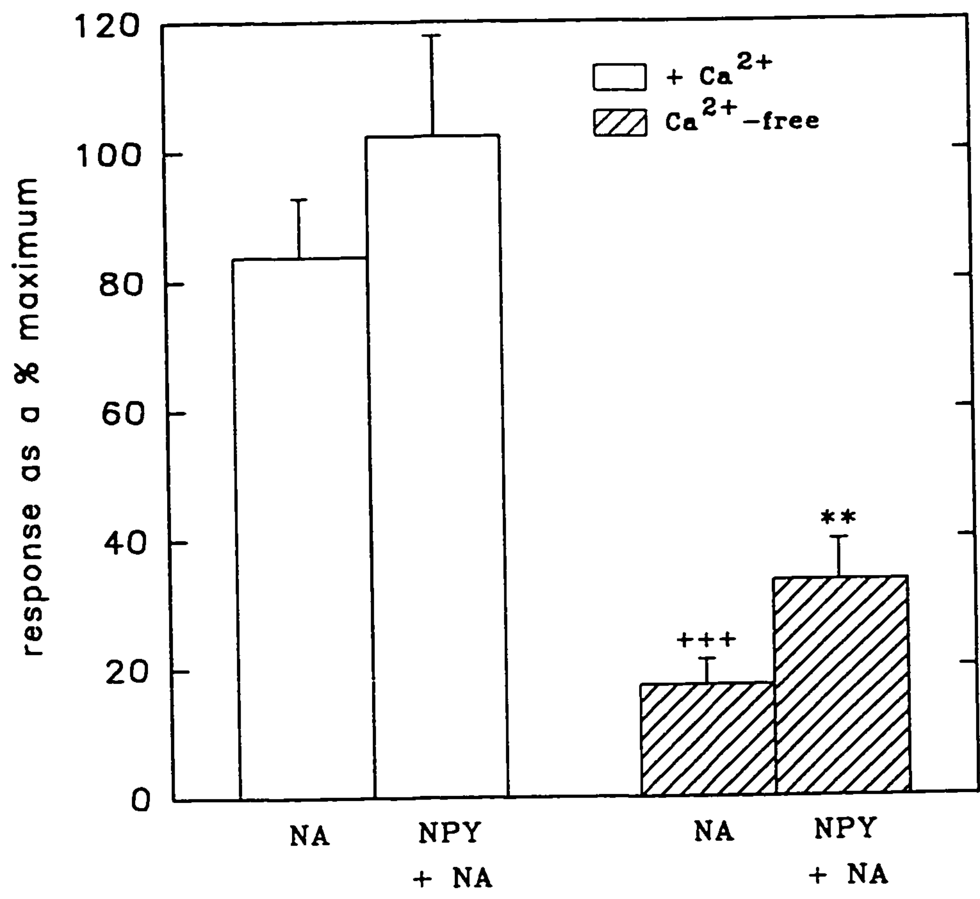

120
100
80
60
40
20
0
response as a % maximum
+ Ca2+
Ca2+-free
+++
NA
Genistein
+ NA
NA
Genistein
+ NA

Figure 19. Effect of NPY on contraction with and without calcium

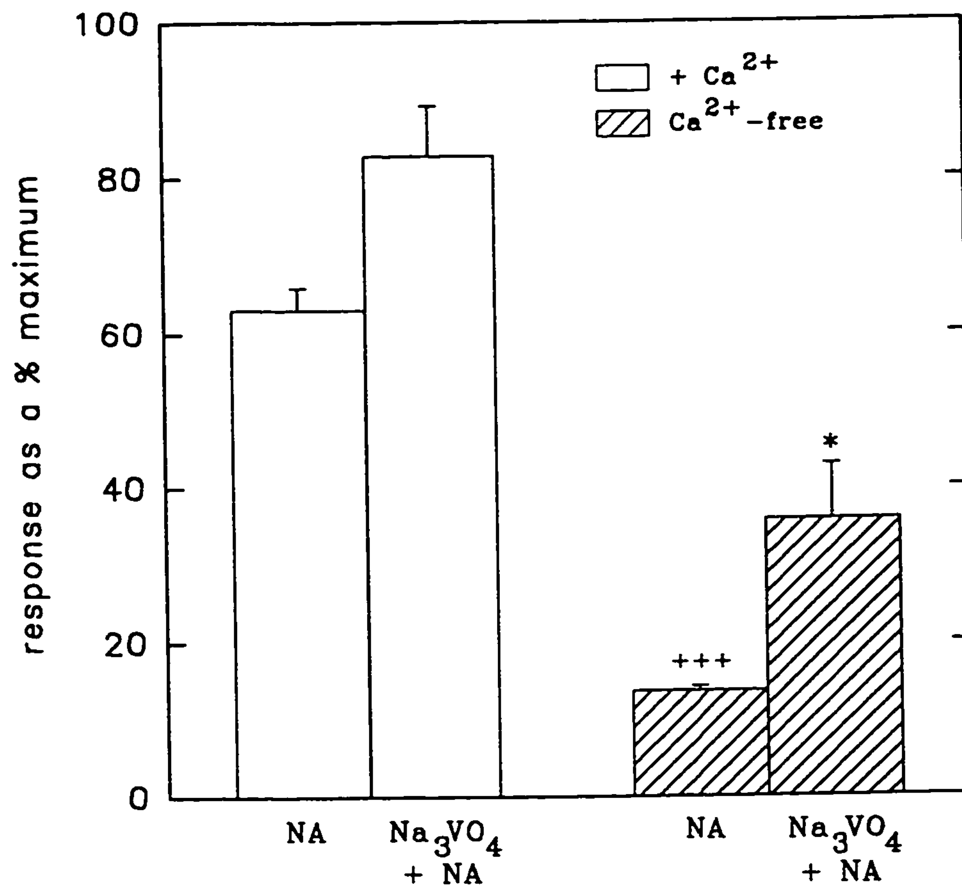
Comparison of the effect of NPY (10^{-7}M, 5 minutes) on NA (10^{-6}M)-induced contraction in the presence and absence of extracellular calcium; □ $+Ca^{2+}$ n=3; ■ Ca^{2+}-free n=6; **P<0.01, +++P<0.001, where * is NA (Ca^{2+}-free) versus NPY (Ca^{2+}-free) and + is NA ($+Ca^{2+}$) versus NA (Ca^{2+}-free).



120
100
80
60
40
20
0
response as a % maximum
+ Ca2+
Ca2+-free
**
+++
NA
NPY
+ NA
NA
NPY
+ NA

Figure 20. Effect of Na_3VO_4 $10^{-3}M$ on contraction with and without calcium

Comparison of the effect of Na_3VO_4 ($10^{-3}M$, 5 minutes) on NA ($10^{-6}M$)-induced contraction in the presence and absence of extracellular calcium; □ $+Ca^{2+}$ n=4; ■ Ca^{2+}-free n=4; *$P<0.05$, +++$P<0.001$, where * is NA (Ca^{2+}-free) versus Na_3VO_4 (Ca^{2+}-free) and + is NA ($+Ca^{2+}$) versus NA (Ca^{2+}-free).



100
80
60
40
20
0
response as a % maximum
+ Ca2+
Ca2+-free
+++
*
NA
Na3VO4 + NA
NA
Na3VO4 + NA

DISCUSSION

(I) Comparison of results with previous findings

The overall purpose of these experiments was to further determine the role of tyrosine kinase in the contraction of rat vascular smooth muscle. The tissue of choice for the majority of experiments performed in this study was the rat mesenteric artery. Before choosing between the rat mesenteric and tail arteries, a couple of preliminary experiments were done. Firstly, each tissue was examined in its response to NA applied in a dose-dependent manner (Figure 3). Secondly, varying pretreatment times of the tyrosine kinase inhibitor genistein were used to determine the necessary pre-incubation time which would best inhibit contractions induced by NA (Figures 4 and 5). The mesenteric artery showed a greater sensitivity to NA than the tail artery, producing a superior dose-response curve. A difference in the sensitivity to genistein of the two preparations was also observed, in which the tail artery showed inferior inhibition of contraction with genistein compared to the mesenteric artery, further indicating variations throughout the vasculature. Consequently, all further experiments were performed using the rat mesenteric artery, since any effects of added substances would show up more clearly using this tissue, thereby giving more accurate results.

Genistein was found to inhibit NA-induced contraction in the mesenteric artery (Figure 3). However, since the endothelium can release vasoactive substances which mediate either relaxation or contraction, it was important to determine the role of the endothelium on the effect of genistein. It was found that genistein was able to inhibit NA-induced contractions in rat mesenteric artery denuded of its endothelium (Figure 6). This suggests that genistein acts via an endothelium-independent mechanism. Patch clamp studies using isolated vascular smooth muscle cells which show that genistein increases Ca^{2+}-activated K^+ channel currents (Xiong *et al.*, 1995) support the observation that genistein can act independently of the endothelium.

Genistein is known to inhibit both soluble and membrane-associated tyrosine kinases by acting at the ATP binding site of tyrosine kinase. By inhibiting the ability of ATP to bind to the tyrosine kinase, phosphorylation of the tyrosine kinase is prevented, resulting in a lack of contraction. As mentioned, genistein inhibited the NA-induced contraction in the mesenteric artery. The dose of genistein used was low enough in order to act as a specific tyrosine kinase inhibitor, with no effect on the activity of serine and threonine kinases (Akiyama *et al.*, 1987). This indicates the presence of tyrosine kinases in the signalling pathway activated as a result of α-adrenoceptor activation.

NPY influences sympathetic cardiovascular control by having both direct and indirect effects. In coronary and cerebral arteries NPY exerts a

direct vasoconstrictive effect, while in other vascular beds NPY remains ineffective when given alone but potentiates the effect of other vasoconstrictive agents. Consistent with this statement, NPY was found to potentiate contractions due to NA (Figure 7A). However, this potentiation was abolished with genistein pretreatment. Evéquoz *et al.* (1994) showed that the direct constrictor effect of NPY in the mesenteric artery is mediated by stimulation of Y_1-receptors and does not depend on the presence of an intact endothelium. Two second messenger responses frequently associated with Y_1 receptors are influx of Ca^{2+} and inhibition of cAMP accumulation (Wahlestedt *et al.*, 1990; Wahlestedt *et al.*, 1992). Since both of these responses lead to increased contraction, this supports the observation of potentiation of contraction seen by NPY. NPY is thought to potentiate only the component of the contraction that is sensitive to calcium channel blockers. In a study by Small *et al.* (1992), NPY had a potentiating effect only on the nifedipine-sensitive component of KCl, mATP and NA contractions, but not on the nifedipine-insensitive component of the NA contraction. This effect was found to be independent of the endothelium. It has been suggested that tyrosine kinase inhibitors block calcium channels (Wijetunge *et al.*, 1992). Their study showed that the tyrosine kinase inhibitors tyrphostin 23 and genistein produced concentration-dependent inhibition of voltage-operated calcium channel currents in vascular smooth muscle cells from rabbit ear artery. Their findings suggest that voltage-

operated calcium channels in vascular smooth muscle may be modulated by endogenous tyrosine kinases. The possibility that the potentiating effect of NPY on nifedipine-sensitive calcium channels may be related to tyrosine kinases would explain the observed inhibition of contraction due to the presence of both NPY and genistein. Since genistein inhibits both NPY and voltage-operated calcium channels, it follows that NPY acts through the activation of PTK.

Genistein also has an effect on the electromechanical coupling mechanism since it significantly inhibited KCl-induced contractions in the mesenteric artery (Figure 7B). This is in contrast to a study by Abebe & Agrawal (1995) which failed to find an inhibition in rat aorta denuded of endothelium. They concluded that genistein did not produce its effects by depolarization-associated mechanisms. However, similar results to the ones in Figure 7B were observed in a study by Di Salvo *et al.* (1993b) on guinea pig mesenteric microvessels, where tyrphostin, another tyrosine kinase inhibitor, inhibited the responses elicited by K^+-induced depolarization. The discrepancy exhibited by the results of the former authors might be an indication of species differences.

Similar to the effect on NA-induced contractions, the KCl contractions were also potentiated by NPY and inhibited by a combination of genistein and NPY. This would suggest involvement of tyrosine kinases in KCl-induced contractions. The fact that the combination of genistein and NPY inhibited

both the NA- and KCl-induced contractions to the same level as seen by genistein alone suggests that genistein acts on the nifedipine-sensitive calcium channel.

In contrast to genistein, daidzein, the inactive analog of genistein, did not inhibit the NA- or KCl-induced contractions (Figure 8). This would further support the suggestion that the inhibition of contraction was due to inhibition of protein tyrosine kinases. In order to provide additional evidence for the role of protein tyrosine kinases in contraction, tyrosine kinase inhibitors other than genistein were also used to examine their effects on contraction. Neither of the tyrosine kinase inhibitors lavendustin A, tyrphostin 25 and erbstatin analog produced an inhibition of NA- and KCl-induced contractions (Figures 9, 10 and 11). Lavendustin A is a potent selective inhibitor of the EGF receptor tyrosine kinase and does not inhibit PKA or PKC. It is 50 times more potent in its inhibition of EGF receptor-associated tyrosine kinase than erbstatin (Onada *et al.*, 1989). It has also been shown to be active *in vitro* but not *in situ* since it does not cross the plasma membrane. Therefore, it would have no effect on the internal tyrosine kinase thought to be involved in smooth muscle contraction. This may also explain why the other tyrosine kinase inhibitors mentioned above did not produce an inhibition of contraction. Onada *et al.* (1990) synthesized a methyl ester derivative of lavendustin A to overcome its problem with penetration. Lavendustin A methyl ester inhibited autophosphorylation and internalization of epidermal growth factor

receptor in cultured A431 cells. It also inhibited phosphatidylinositol kinase *in vitro* and phosphatidylinositol turnover *in situ*.

Tyrphostins are low molecular weight synthetic inhibitors of PTK which block cell proliferation and act at the peptide binding site of tyrosine kinase. Bilder *et al.* (1991) showed that tyrphostins are potent reversible inhibitors of PDGF-induced mitogenesis. They act by inhibiting the tyrosine kinase activity of the PDGF receptor and the subsequent signalling cascade. In a study by Sauro & Thomas (1993), tyrphostin inhibited PDGF-induced contraction in rat aortic smooth muscle over a range of calcium concentrations, suggesting that it may oppose contraction through inhibition of calcium influx via the PDGF-induced receptor-operated channel. They also observed that tyrphostin had no effect on KCl-mediated voltage-operated calcium channels, which agrees with our results. Semenchuk & Di Salvo (1995) studied the effects of genistein and tyrphostin on receptor-activated increases in cellular Ca^{2+} ($[Ca^{2+}]_i$), and protein tyrosine phosphorylation in cultured canine femoral arterial smooth muscle cells. Their experiments in Ca^{2+}-free medium showed that 70-80% of the transient increase in $[Ca^{2+}]_i$ elicited by the agonists serotonin and phenylephrine is due to influx of extracellular Ca^{2+} whereas the plateau phase is only due to Ca^{2+} entry. They determined that tyrosine phosphorylation probably participates in both the influx and release components of the Ca^{2+} transient since both genistein and tyrphostin greatly suppress phosphorylation and essentially abolish the Ca^{2+} transient.

Erbstatin analog is a tyrosine analog that has been shown to inhibit membrane associated tyrosine kinases. Since, as is the case with lavendustin A and tyrphostin 25, erbstatin analog is a specific inhibitor of the membrane-associated tyrosine kinases, this would explain why the internal tyrosine kinase proposed to be involved in contraction was not affected. Since the contractions in these experiments were induced by NA and KCl rather than EGF and PDGF, these particular tyrosine kinase inhibitors had no inhibitory effect. A possible explanation for the potentiation of contraction produced by erbstatin analog could be a gradual change of responsiveness to NA and KCl.

Protein tyrosine phosphatases are integral components of signal transduction cascades, either as terminators or as positive regulators of signalling (Streuli, 1996). The tyrosine phosphatase inhibitor sodium orthovanadate enhances protein tyrosine phosphorylation and therefore stimulates contraction of smooth muscle. The fact that the contraction is potentiated provides further evidence of the role of tyrosine kinase in smooth muscle contraction. NPY and orthovanadate produced a greater potentiation of KCl-induced contraction compared to orthovanadate alone (Figure 13B). This would support the suggestion that NPY promotes membrane depolarization by inhibition of Ca^{2+}-activated K^+ channels coupled to potentiation of Ca^{2+} current (Xiong & Cheung, 1994). This increased membrane depolarization in combination with orthovanadate would produce

a greater contraction. Genistein was combined with sodium orthovanadate in order to provide further information about the role of protein tyrosine kinase in contraction (Figure 14). Since genistein only partially inhibits the potentiation of contraction caused by orthovanadate, this would indicate that orthovanadate contractile effects are not exclusively a consequence of tyrosine phosphatase inhibition. However, the possibility of incomplete inhibition of PTK by genistein was not ruled out since other doses of genistein were not tested, despite the specificity of genistein for PTK at the dose used (Akiyama *et al.*, 1987). Similar results were observed in a study by Filipeanu *et al.* (1995) in which genistein (10^{-4}M) was able to inhibit only 48% of the contraction of rat aorta potentiated by sodium orthovanadate. Other suggested biological activities of vanadate include inhibition of ATPase activity, inhibition of sarcolemmal Ca^{2+} ATPase and inhibition of Na-K^+ ATPase (Di Salvo *et al.*, 1993a). Another possibility for the partial inhibition of genistein is the fact that the effect of orthovanadate has been found to be irreversible (Filipeanu *et al.*, 1995). Yet another possibility would be that the dose of orthovanadate was toxic, resulting in the incomplete inhibitory effect of genistein. Consequently, an analogous experiment on NA-induced contraction with a lower orthovanadate dose of 10^{-4}M was performed (Figure 15). However, genistein was still not able to inhibit the potentiating contractile effects of orthovanadate. Therefore, the dose of orthovanadate used was not a factor.

A similar experiment to that shown in Figure 13B was performed in which genistein was added to sodium orthovanadate and to the combination of NPY and sodium orthovanadate (Figure 16). Genistein was not able to abolish the potentiating effects of orthovanadate alone or in combination with NPY.

To determine whether tyrosine kinase inhibitors act via calcium influx through the L-type calcium channel, the effect of nifedipine, a Ca^{2+}-channel blocker, was studied in combination with NPY and sodium orthovanadate. Figure 17 shows that the potentiating effects of both NPY and sodium orthovanadate were abolished in the presence of nifedipine. Nifedipine produced a larger inhibition of the KCl-induced contractions compared to the NA-induced contractions. This may be due to a smaller contribution of voltage-operated calcium channels to the response with NA. Previous results have shown that the potentiating effect of NPY is more specific to contraction mediated by nifedipine-sensitive calcium channels (Small *et al.*, 1992). Since sodium orthovanadate behaved in the same manner as NPY when combined with nifedipine, this would suggest that sodium orthovanadate acts via the nifedipine-sensitive calcium channel. For further clarification, a series of experiments was performed in which NA-induced contractions were examined in the absence of extracellular calcium compared to the control situation of the presence of calcium. Figure 18 shows that genistein was not able to produce an inhibition of contraction in the absence of calcium, suggesting the

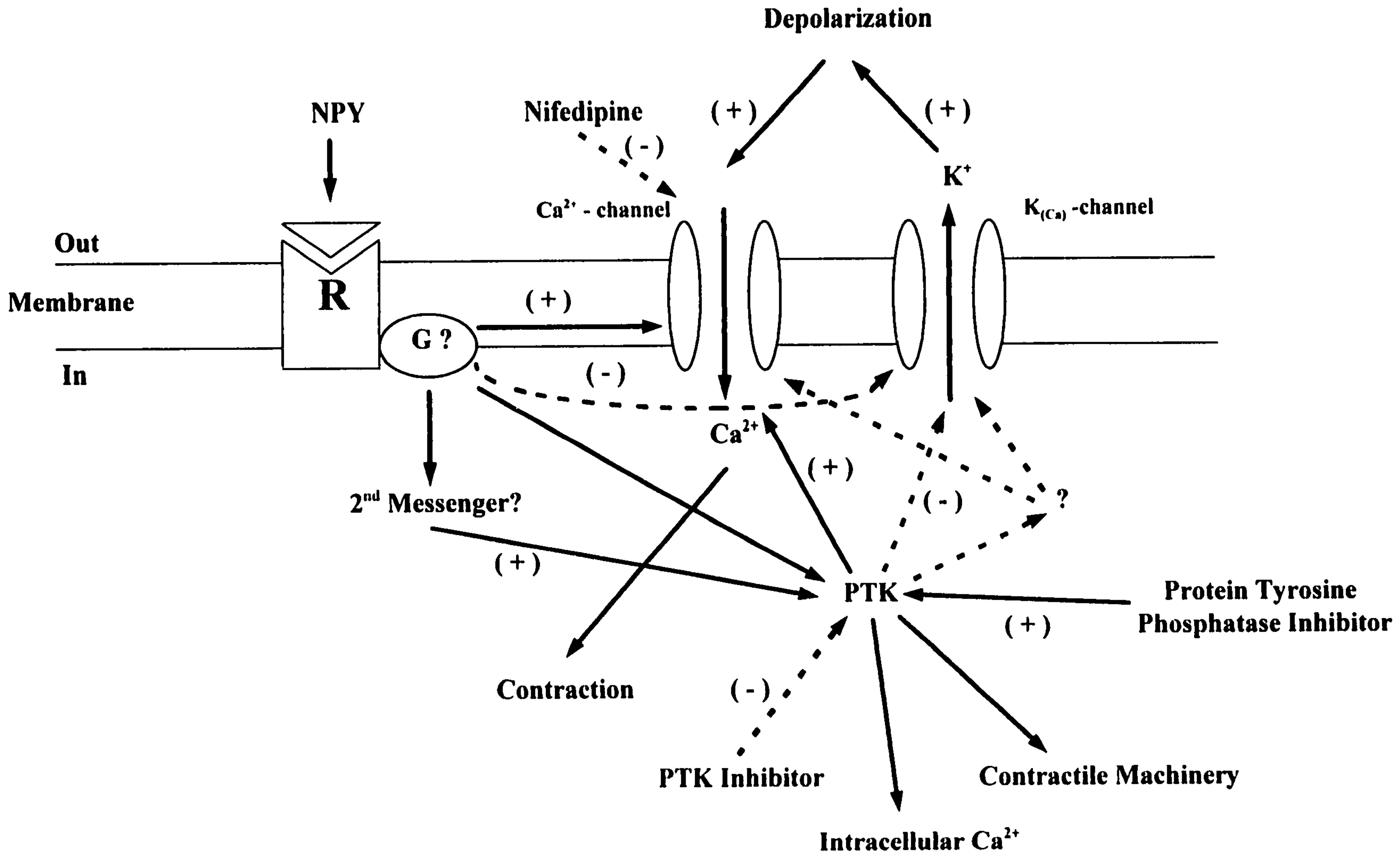
involvement of the L-type calcium channel in its mechanism of action. Both NPY and sodium orthovanadate were still able to potentiate the NA-induced contraction in the absence of calcium (Figures 19 & 20). This would suggest the possibility of the presence of residual calcium, since a calcium chelator such as EGTA was not used. If calcium was not completely eliminated, then NPY and sodium orthovanadate would cause a potentiation of contraction mediated by calcium entry. However, Sunano *et al.* (1988) concluded that the vanadate-induced contraction utilizes both extracellular and intracellularly bound calcium. In addition, Fukuzaki *et al.* (1992) found that vanadate (30 μM) contracts uterine smooth muscle of estrogen-dominated non-pregnant rats in Ca^{2+}-free medium.

(II) Model of smooth muscle cell contraction based on the literature

Schematically, the process of smooth muscle contraction can be seen in Figure 21, which depicts a smooth muscle cell with the components involved in causing contractions. The cell membrane consists of many different channels as well as receptors. Two channels of interest are the Ca^{2+}-channel and the $K_{(Ca)}$-channel. Excitation and contraction in vascular smooth muscle are linked via pharmacomechanical and electromechanical coupling. NA-induced contractions are an example of pharmacomechanical coupling since the binding of NA to its receptor causes contraction without a necessary change in surface membrane potential (Somlyo & Somlyo, 1968).

Figure 21. Schematic diagram of a smooth muscle cell showing components involved in causing contraction

Possible signal transduction pathway linking NPY to Ca^{2+} and $K_{(Ca)}$-channels as well as PTK to contraction.



Depolarization
NPY
Nifedipine
(+)
(+)
(-)
Ca^{2+} - channel
K⁺
$K_{(Ca)}$ -channel
Out
Membrane
R
In
G ?
(+)
(-)
Ca^{2+}
(+)
2nd Messenger?
(-)
?
(+)
PTK
Protein Tyrosine
Phosphatase Inhibitor
(+)
Contraction
(-)
PTK Inhibitor
Contractile Machinery
Intracellular Ca^{2+}

The three potential pathways of pharmacomechanical coupling are: (1) IP_3-induced Ca^{2+} release from the SR; (2) Ca^{2+} influx through voltage-dependent and ligand-gated channels, and; (3) modulation of the Ca^{2+}-sensitivity of the contractile regulatory apparatus (Somlyo *et al.*, 1991). In NA-induced contractions, stimulation by the α-adrenoceptor NA leads to the influx of Ca^{2+} through the Ca^{2+}-channel. Protein tyrosine phosphorylation also occurs with NA stimulation, which, along with Ca^{2+} influx, both lead to contraction of the smooth muscle cell. KCl-induced contractions are an example of electromechanical coupling since the contraction is elicited by changes in membrane potential. The external KCl leads to membrane depolarization, which in turn leads to Ca^{2+} influx, protein tyrosine phosphorylation and subsequently smooth muscle cell contraction. The phosphorylation of proteins on tyrosine residues by tyrosine kinases has been suggested in some cases to contribute to signalling processes that lead to smooth muscle contraction. In a study of gastric smooth muscle by Hollenberg (1994), it was found that angiotensin II requires extracellular Ca^{2+} for its contractile action. It was also suggested that both the agonists angiotensin II and epidermal growth factor (EGF) involved non-receptor tyrosine kinase pathways which may play a key role in both vascular and gastrointestinal smooth muscle pharmacomechanical coupling. Supporting data from cell culture systems indicate (1) a role for intracellular Ca^{2+} in modulating protein tyrosine phosphorylation; and (2) a role for tyrosine kinase activity in the regulation

of peptide-stimulated Ca^{2+} influx (Hollenberg, 1994, and references cited).

The presence of protein tyrosine kinase inhibitors prevents the phosphorylation of protein tyrosine by binding to one of its active sites. This PTK inhibition has the effect of inhibiting smooth muscle cell contraction. In cultured canine femoral arterial smooth muscle cells, pre-incubation with the PTK inhibitors genistein or tyrphostin inhibited the transient rise in $[Ca^{2+}]_i$ elicited by serotonin or phenylephrine (Semenchuk & Di Salvo, 1995). In isolated rat aorta denuded of endothelium, genistein and tyrphostin significantly inhibited the contractile responses of the aorta to NA both in the absence and presence of extracellular calcium, but not to KCl (Abebe & Agrawal, 1995). The use of PTK inhibitors in the rat aorta was found to block a step involved in Ca^{2+} entry and Ca^{2+} store refilling (Filipeanu *et al.*, 1995).

The presence of tyrosine phosphatase inhibitors has the opposite effect of PTK inhibitors in that they increase protein tyrosine phosphorylation, thereby causing an increase in contraction. Processes that are reversibly controlled by protein phosphorylation require a protein phosphatase (PP) in addition to a protein kinase. Target proteins are phosphorylated at specific sites by one or more PKs, and these phosphates are removed by specific PPs (Hunter, 1995). Intrinsic protein tyrosine phosphatase (PTP) activity is generally two or three orders of magnitude greater than that of the PTKs. This increased activity accounts for the low level of protein tyrosine in cellular

proteins and for the transient nature of most protein tyrosine responses. For PTK signalling to be effective, PTP activity is tightly regulated by discrete subcellular localization and serine/threonine or tyrosine phosphorylation (Hunter, 1995, and references cited).

NPY binds to a membrane receptor associated with a G-protein resulting in protein tyrosine phosphorylation, either directly or via a second messenger. This in turn leads to a potentiation of contraction. NPY released at nerve endings influences sympathetic cardiovascular control by having both direct and indirect (prejunctional and postjunctional) effects (McDermott *et al.*, 1993). In coronary and cerebral arteries the peptide exerts a direct vasoconstrictive effect, while in other vascular beds NPY remains ineffective when given alone but potentiates the effect of other vasoconstrictive agents. This potentiating behaviour has been observed in, among others, tail arteries (Neild, 1987; MacLean & McGrath, 1990) and rat mesenteric arteries (Westfall *et al.*, 1987). Ca^{2+} channels participate in the action of NPY since the contraction potentiated by NPY is antagonized by Ca^{2+} channel blockers such as nifedipine (Cheung, 1991; Small *et al.*, 1992), and since NPY has a potentiating effect on L-type Ca^{2+} channel currents in voltage-clamp studies (Xiong *et al.*, 1993). In addition, NPY has an inhibitory effect on Ca^{2+}-activated K^+ channels (Xiong & Cheung, 1994), which, coupled to potentiation of the Ca^{2+} current, would promote membrane depolarization or prolong the duration of the action potential, causing further increase in Ca^{2+} influx.

CONCLUSION

The present studies showed that the tyrosine kinase inhibitor genistein inhibits vascular smooth muscle contraction independently of the endothelium. The inactive analog of genistein, daidzein, failed to show an inhibition of NA- and KCl-induced contraction. It is suggested that genistein acts on the nifedipine-sensitive calcium channel since it produced the same level of inhibition when used alone or in combination with NPY. The tyrosine kinase inhibitors lavendustin A, tyrphostin 25, and erbstatin analog did not produce an inhibition of contraction. This was most likely due to their specificity for membrane-associated tyrosine kinases. The phosphatase inhibitor sodium orthovanadate potentiated contractions due to its effect of prolonged tyrosine kinase phosphorylation as well as other mechanisms. This potentiation was blocked in the presence of the calcium channel blocker nifedipine, suggesting the involvement of calcium influx through the L-type Ca^{2+} channel. When genistein was used as pretreatment in the absence of calcium, there was no inhibition of NA-induced contraction, suggesting the involvement of the L-type calcium channel in its mechanism of action. NPY and sodium orthovanadate were still able to produce a potentiating effect on NA-induced contraction in the absence of calcium, which would suggest the presence of residual calcium, since a calcium chelator such as EGTA was not used.

Possible signal transduction pathways linking NPY to Ca^{2+} and $K_{(Ca)}$-channels are shown in Figure 21. Stimulation of the NPY receptor leads to the production of an unidentified second messenger. The second messenger then activates one or more non-receptor tyrosine kinases which act on the channels. In addition to causing a direct tyrosine phosphorylation on the channels, it is possible that tyrosine kinase may regulate other kinases, which in turn act on the channels. NPY promotes vasoconstriction by increasing influx of Ca^{2+} through Ca^{2+} channels.

FUTURE STUDIES

Future studies could include:

1) Determining whether or not nuclear protein tyrosine kinases are involved in agonist-induced contraction by the use of Western blotting techniques.

2) Determining which of the endogenous protein tyrosine kinases are involved in agonist-induced contraction by the use of Western blotting techniques.

3) Finding a tyrosine kinase inhibitor other than genistein that produces an inhibition of NA- and KCl-induced contractions in mesenteric artery.

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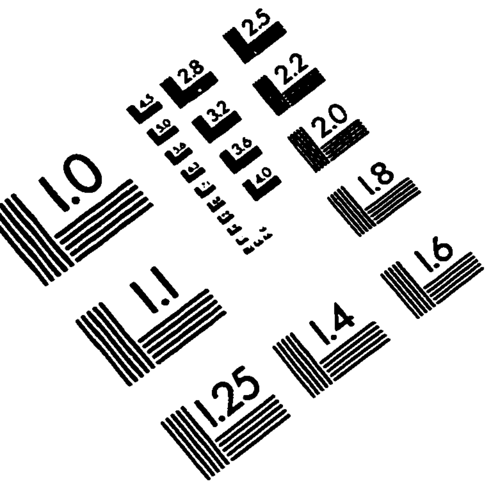
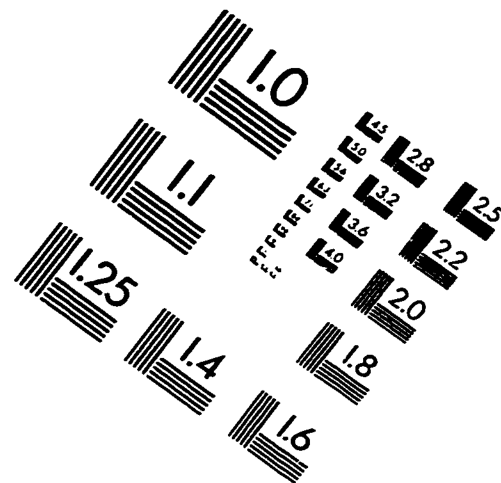
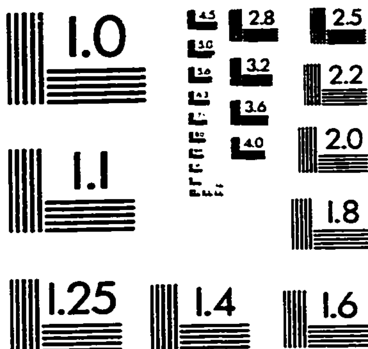
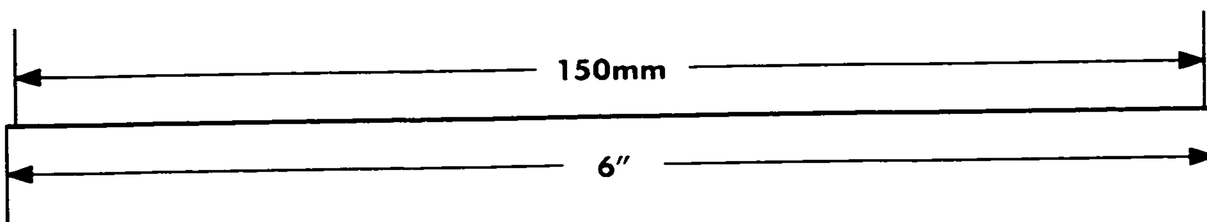
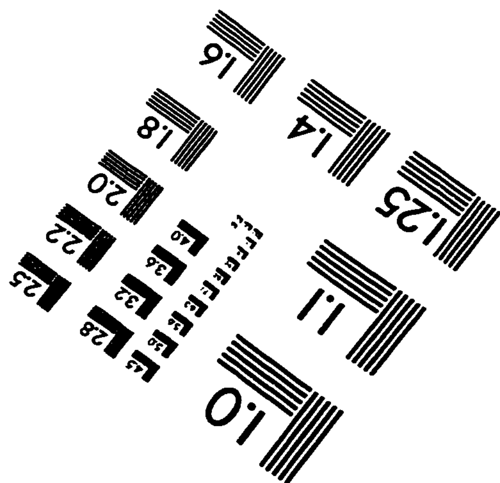
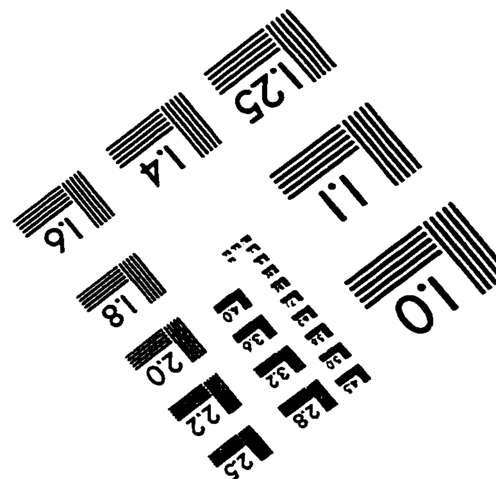
IMAGE EVALUATION TEST TARGET (QA-3)

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