Neuropeptide Y Stimulation of Myosin Light Chain Phosphorylation in Cultured Aortic Smooth Muscle Cells*

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Neuropeptide Y (NPY) is released from an extensive network of postganglionic sympathetic perivascular neurons. NPY has been shown to affect vascular tone postsynaptically by 1) directly stimulating contraction; 2) inhibiting vasorelaxation; and 3) potentiating contraction elicited by exogenous vasoconstrictors. The molecular mechanisms mediating these effects of NPY are undefined. Therefore, we examined the possibility that NPY could stimulate smooth muscle contraction through myosin light chain phosphorylation in cultured porcine aortic smooth muscle cells. NPY (100 nM) caused a rapid, transient increase in myosin light chain (MLC) phosphorylation, an important regulatory event in the initiation of smooth muscle contraction. NPY-stimulated MLC phosphorylation was prevented by preincubation of cells with pertussis toxin and was independent of extracellular Ca++. In parallel studies, NPY alone had no detectable effect on cellular cAMP or cGMP content; however, NPY potentily inhibited forskolin-stimulated CAMP accumulation (IC50 = 0.03 nM) through a pertussis toxin-sensitive pathway. NPY had no detectable effect on basal phosphoinositide hydrolysis or protein kinase C activation but enhanced angiotensin II-stimulated production of inositol phosphates and activation of protein kinase C. These results indicate that NPY-stimulated MLC phosphorylation can occur in the absence of detectable changes in cAMP content, cGMP content, inositol phosphate production, or protein kinase C activation; however, the interactions between NPY and other vasoactive agents may be mediated by the indirect effects of NPY on adenylate cyclase activity and phosphoinositide hydrolysis.

Neuropeptide Y (NPY),1 a 36-amino acid member of the pancreatic polypeptide family, is localized peripherally, primarily in postganglionic sympathetic neurons. NPY-containing neurons are particularly abundant surrounding the vasculature of most major organ systems, including the heart, respiratory tract, gastrointestinal tract, and genitourinary tract (1). In many of these neurons, NPY is co-localized with norepinephrine in large dense core vesicles (2) and is co-released with norepinephrine following sympathetic stimulation (3). Elevated plasma levels of NPY are associated with widespread, long lasting vasoconstriction and an increase in blood pressure which is not responsive to adrenergic blockade (4). The abundance of NPY-containing neurons surrounding blood vessels and the pressor response to NPY administration have prompted much speculation regarding the physiological, and potentially pathological, role of NPY in the regulation of blood pressure.

NPY binds to specific membrane receptors (5, 6) and affects vascular tone through both presynaptic and postsynaptic mechanisms. Presynaptically, NPY has been shown to inhibit the release of norepinephrine (7) and acetylcholine (8). Postsynaptic effects of NPY on vascular tone in vitro include 1) direct stimulation of vasoconstriction (9); 2) inhibition of vasorelaxation (10); and 3) potentiation of contraction elicited by transmural nerve stimulation (11) or exogenously applied vasoconstrictors (7, 12). These responses cannot be demonstrated in every arteriolar or venous preparation, and marked species and tissue differences in NPY responsiveness have been reported.

The cellular signal transduction mechanisms that underlie the effects of NPY on smooth muscle contraction have not yet been defined. NPY has been shown to inhibit forskolin-stimulated adenylate cyclase in cat cerebral arteries (13) and cultured rabbit pulmonary artery cells (14) as well as in several nonvascular smooth muscle preparations (15, 16); however, a direct effect of NPY on basal adenylate cyclase activity has been demonstrated only in neural cells and tissues (17, 18). Similarly, NPY stimulation of phosphoinositide hydrolysis has been reported only in cultured dorsal root ganglion cells (19) and at high doses of NPY (10 μM) in rat brain (20). The effects of NPY on smooth muscle cell Ca++ homeostasis are also poorly defined. NPY-mediated vasoconstriction has been shown to be inhibited by nifedipine, a Ca++ entry blocker, implying a NPY-mediated stimulation of extracellular Ca++ influx (16). NPY activation of voltage-gated Ca++ channels has been postulated based on recent reports of NPY-mediated membrane depolarization in rat tail artery (21, 22). However, a direct effect of NPY on intracellular Ca++ was not found in cultured rabbit pulmonary artery cells that were loaded with the Ca++ indicator, fura (14).

This study was designed to examine the molecular mechanisms underlying NPY-stimulated smooth muscle contraction. Cultured porcine aortic smooth muscle cells (PSMCs)
were chosen as a useful model system in which to study NPY receptor/effector coupling in the absence of complications introduced by an intact endothelium. The results demonstrate the existence of functional NPY receptors on PSMCs linked to the existence of functional NPY receptors on PSMCs linked with confluent PSMCs for 5-7 days at 37°C. In this study, PSMCs were incubated overnight in DMEM supplemented with 6 mM glucose and 1% (w/v) BSA and then for 3 h with 0.2 mM [3H]orthophosphate (Du Pont New England Nuclear) as described previously (31). Labeled cells were treated for 30 s with 0.1 mM NPY, 0.1 mM angiotensin II or for 1 min with 1.6 μM phorbol 12-myristate 13-acetate before rinsing and homogenizing in 0.5 mL of radiolabeled immunoprecipitation assay buffer (in mM: 150 NaCl, 50 Tris/HCl pH 8.3, 5 EDTA; 1% Nonidet P-40, 1% bovine serum albumin (BSA), 1% (w/v) BSA). Samples containing equivalent trichoroacetic acid precipitable radioactivity were incubated with normal rabbit serum for 1 h at 4°C and then with Pansorbin cells (Calbiochem) for 30 min before pelleting for 5 min at 10,000 x g. Supernatants were precipitated on the same culture. The cell pellet was boiled, and subjected to electrophoresis on a 7.2% sodium dodecyl sulfate-polyacrylamide gel as described previously (31). MARCKS protein phosphorylation was quantitated by scanning densitometry using an autoradiograph of the gel. 

**Statistics and Data Analysis—**Data were evaluated statistically

\[ \text{Cyclic Nucleotide Content—Total cell cAMP and cGMP content was measured using a modification of the method of Rozengurt et al. (27) as described previously (18). Briefly, cells were equilibrated for approximately 1 h at 37°C in HEPES-buffered DMEM and then incubated for an additional 10 min in the presence of LiCl. Inositol phosphate and cyclic AMP or cGMP was measured in the neutralized cell extracts using commercially available radiomiminoassays (The Prompt-New England Nuclear, Boston). Values were normalized to total cell protein/plate (Bio-Rad).} \]

**Inositol Phosphate Metabolism—**The effect of NPY on cellular inositol phosphate levels was determined as described previously (18, 28). Briefly, cells were incubated for approximately 4 h in low inositol medium (medium 199 (GIBCO), 1% (w/v) BSA (globulin-free fraction VI, Sigma); 6 mM glutamine; 0.1% conditioned medium, 5 μM LiCl/mL myo-[2-3H]inositol (Amersham Corp.). Cells were then rinsed and incubated for 20 min in HEPES-buffered medium 199 (4 mM NaHCO₃; 1% (w/v) BSA; 20 mM HEPES (pH 7.4)) with 10 mM LiCl added for an additional 10 min. Additions were made in the continued presence of LiCl. Inositol phosphates were extracted into 10% (w/v) perchloric acid on ice. Solutions were added to glass wool and precipitated on ice for 15-20 min at 0-4°C. Precipitated fractions were dried under nitrogen, re-suspended in 60% (v/v) ethanol, and peroxidase-conjugated goat anti-rabbit IgG affinity-isolated antibodies (1:50 dilution in TBS/T: Tago, Inc., Burlingame, CA) were chosen as a useful model system in which to study NPY receptor/effector coupling in the absence of complications introduced by an intact endothelium. The results demonstrate the existence of functional NPY receptors on PSMCs linked with confluent PSMCs for 5-7 days at 37°C. In this study, PSMCs were incubated overnight in DMEM supplemented with 6 mM glucose and 1% (w/v) BSA and then for 3 h with 0.2 mM [3H]orthophosphate (Du Pont New England Nuclear) as described previously (31). Labeled cells were treated for 30 s with 0.1 mM NPY, 0.1 mM angiotensin II or for 1 min with 1.6 μM phorbol 12-myristate 13-acetate before rinsing and homogenizing in 0.5 mL of radiolabeled immunoprecipitation assay buffer (in mM: 150 NaCl, 50 Tris/HCl pH 8.3, 5 EDTA; 1% Nonidet P-40). Samples containing equivalent trichoroacetic acid precipitable radioactivity were incubated with normal rabbit serum for 1 h at 4°C and then with Pansorbin cells (Calbiochem) for 30 min before pelleting for 5 min at 10,000 x g. Supernatants were precipitated on the same culture. The cell pellet was boiled, and subjected to electrophoresis on a 7.2% sodium dodecyl sulfate-polyacrylamide gel as described previously (31). MARCKS protein phosphorylation was quantitated by scanning densitometry using an autoradiograph of the gel. 

**Statistics and Data Analysis—**Data were evaluated statistically
using one-way analysis of variance (with appropriate a posteriori multiple range comparison procedures). Data are expressed as mean ± S.E. (n refers to the number of individual determinations).

Materials—Neuropeptide Y (human) was obtained from Peninsula Laboratories, Inc., Richmond, CA. Angiotensin II, HEPES, forskolin, pertussis toxin, caffeine, hydrogen peroxide (30%, w/v) and isobutylmethylxanthine (IBMX) were purchased from Sigma.

RESULTS

MLC Phosphorylation—We assessed the ability of NPY to regulate smooth muscle cell contraction by determining the effect of NPY on the phosphorylation state of MLC. Exposure of cells to 0.1 μM NPY caused a transient increase in MLC phosphorylation which was maximal after 30 s and decreased to basal levels by 5 min (Fig. 1A). A graded increase in MLC phosphorylation was seen following a 30-s exposure of cells to concentrations of NPY ≥ 1 nM (data not shown).

Because of the high basal phosphorylation in PSMCs, we were able to observe a decrease in MLC phosphorylation following exposure of cells to 10 μM forskolin (Fig. 1D). Forskolin has been shown to activate adenylate cyclase directly in a wide variety of cells (32) and has been shown to cause relaxation of smooth muscle, presumably through a mechanism involving an increase in cellular cAMP content (23). Following a 30-s delay, forskolin caused MLC phosphorylation to decrease to approximately 15%. Simultaneous exposure to 0.1 μM NPY resulted in the characteristic increase in MLC phosphorylation at 30 s followed by a slowing of forskolin-stimulated MLC dephosphorylation (Fig. 1B). The NPY-stimulated increase in MLC phosphorylation could be completely prevented by prior incubation of cells with 100 ng/ml pertussis toxin (Table I), implying that a guanine nucleotide regulatory protein is involved in coupling NPY receptor activation to stimulation of MLC phosphorylation.

The inclusion of IBMX, a phosphodiesterase inhibitor, in the incubation medium had no effect on the initial NPY-mediated increase in MLC phosphorylation but accelerated the forskolin-stimulated MLC dephosphorylation observed between 30 s and 5 min (data not shown). As phosphodiesterase inhibition should enhance the forskolin-stimulated increase in cAMP, these data support the involvement of cAMP in the forskolin-stimulated MLC dephosphorylation in these cells. In addition, 0.1 μM NPY was unable to stimulate MLC phosphorylation when cells were exposed to a 10-fold higher forskolin concentration (percent MLC phosphorylated = 11 ± 4 and 13 ± 1 in the presence and absence of 0.1 μM NPY, respectively (30 s; n = 3)), suggesting that an excess of cAMP can inhibit NPY-mediated MLC phosphorylation.

Effect of NPY on Cellular cAMP Content—Because of the implied involvement of cAMP in NPY-mediated MLC phosphorylation described above, we determined the effect of NPY on cellular cAMP content under similar experimental conditions (Fig. 2A). NPY alone had no detectable effect on cell cAMP content between 30 s and 10 min. However, 0.1 μM NPY prevented the forskolin-stimulated increase in cAMP content at 30 s and inhibited the increase in cAMP content throughout the 10-min incubation period. NPY inhibition of

![Fig. 1. Effect of NPY on the phosphorylation state of myosin light chain.](image)

![Fig. 2. Effect of NPY on cellular cAMP content. A, cells were exposed to either 0.1 μM NPY, 10 μM forskolin, or both for the period of time indicated on the abscissa (n = 3). B, cells were incubated with 10 μM forskolin and the indicated NPY concentration for 10 min. IBMX (50 μM) was included in all solutions to inhibit phosphodiesterase activity (n = 3).](image)
forskolin-stimulated cAMP accumulation was dose dependent, with a half-maximally effective concentration between 0.01 and 0.1 nM (Fig. 2B), well within the range of reported NPY binding affinities (5, 6, 17).

As reported above for NPY stimulation of MLC phosphorylation, NPY-mediated inhibition of forskolin-stimulated cellular cAMP accumulation could be inhibited completely by a 3-h exposure of cells to 100 ng/ml pertussis toxin (Table II). These data are consistent with previous reports showing the involvement of a pertussis-sensitive G-protein in coupling NPY receptor activation to inhibition of adenylate cyclase (17, 33).

Effect of NPY on Cellular cGMP Content—NPY has been reported to be a potent inhibitor of smooth muscle relaxation (10). Because vasodilation is often associated with increased guanylate cyclase activity and a rise in cGMP content (34), we determined the effects of NPY on basal and stimulated cellular cGMP accumulation in PSMCs. Basal levels of cGMP were stable throughout the 10-min incubation period and were unaffected by exposure of cells to NPY (Fig. 3). Atrial natriuretic peptide, a stimulator of particulate guanylate cyclase (34), caused a 60-fold increase in cGMP content; however, neither the peak level nor the time course of cGMP degradation was affected by simultaneous exposure of cells to NPY (Fig. 3). These data imply that guanylate cyclase is not involved in NPY signal transduction in PSMCs.

Effect of NPY on Inositol Phosphate Accumulation—Receptor-activated phospholipase C-mediated IP3 production and subsequent release of Ca2+ from the sarcoplasmic reticulum (SR) are a well characterized signal transduction pathway for many vasoactive hormones and neurotransmitters (35). Therefore, we examined the effects of NPY on total inositol phosphate accumulation in PSMCs. Angiotensin II, a vasoactive peptide shown to activate phosphoinositide hydrolysis in these cells (36), was used as a positive control. NPY failed to stimulate a consistent increase in total cell inositol phosphate content after a 15- or 60-s incubation in the presence of 10 mM LiCl, despite a 3-4-fold stimulation by angiotensin II (Fig. 4). However, in the absence of an effect of NPY alone, NPY potentiated the response to angiotensin II when added simultaneously (Fig. 4). Consistent with the other cellular effects of NPY on PSMCs reported above, NPY potentiation of phosphoinositide hydrolysis could be inhibited by preincubation of cells with 100 ng/ml pertussis toxin (Table III). These data suggest an interaction between NPY receptor activation and the phosphoinositide signal transduction pathway despite the absence of a demonstrable direct stimulation of inositol phosphate accumulation.

Effect of NPY on Protein Kinase C Activation—The presence of NPY-induced potentiation of phosphoinositide hydrolysis in these cells suggested that NPY may also activate protein kinase C through production of diacylglycerol. Therefore, we assessed the ability of NPY to activate protein kinase C by determining the effect of NPY on the phosphorylation of the MARCKS protein (29, 30). This cellular protein has been well characterized as a protein kinase C substrate (31). The combined results from two experiments like that
shown in Fig. 5 indicated that incorporation of $^{32}$P into immunoprecipitated MARCKS protein was not significantly increased by a 30-s exposure of cells to NPY. In contrast, angiotensin II increased MARCKS phosphorylation 7-fold, consistent with the ability of angiotensin II to stimulate inositol phosphate accumulation in these cells. Simultaneous exposure of cells to angiotensin II and NPY caused a further increase in MARCKS phosphorylation (n = 4; p < 0.05 compared with cells treated with angiotensin II alone), which could reflect the ability of NPY to potentiate Ca$^{2+}$ stimulation of this signaling pathway. Finally, NPY had no effect on phorbol 12-myristate 13-acetate-mediated MARCKS phosphorylation, implying the absence of a direct effect of NPY on protein kinase C activity.

Ca$^{2+}$ Requirement for NPY-stimulated MLC Phosphorylation—Smooth muscle contraction is initiated by an increase in intracellular Ca$^{2+}$ through activation of the Ca$^{2+}$/calmodulin-activated myosin light chain kinase (23). Consequently, we examined the Ca$^{2+}$ requirements for NPY stimulation of MLC phosphorylation in PSMCs. Incubation of cells for 3 min in Ca$^{2+}$-free buffer containing 0.1 mM EGTA caused a slight decrease in basal MLC phosphorylation but had no effect on the incremental increase in MLC phosphorylation stimulated by either NPY or angiotensin II (Fig. 6). In an attempt to deplete intracellular Ca$^{2+}$ stores, cells were incubated for 10 min with 10 mM caffeine in Ca$^{2+}$-free buffer containing 0.1 mM EGTA and then for 50 additional min in the same solution without caffeine. Caffeine is known to mobilize Ca$^{2+}$ from the sarcoplasmic reticulum (SR) in smooth muscle (37). Despite causing a marked decrease in basal MLC phosphorylation, this treatment had no effect on the incremental increase in MLC phosphorylation stimulated by either NPY or angiotensin II (Fig. 6).

**DISCUSSION**

MLC Phosphorylation—Phosphorylation of the 20,000 M₉ myosin light chain by the Ca$^{2+}$/calmodulin-activated myosin light chain kinase allows activation of myosin Mg$^{2+}$-ATPase by actin and is an important regulatory step in the initiation of smooth muscle contraction (23). A transient increase in MLC phosphorylation can be stimulated in vascular smooth muscle preparations by a variety of vasoactive hormones and neurotransmitters, including angiotensin II (38-40), histamine (39, 41, 42), and the α-adrenergic agonist, phenylephrine (41, 42). The results of this study indicate that NPY can also stimulate a transient increase in MLC phosphorylation in PSMCs, consistent with its reported ability to cause smooth muscle contraction in a variety of vascular preparations (43).

MLC phosphorylation thus serves as a biochemical index of contraction which can be used to study the cellular signal transduction mechanisms underlying NPY-induced smooth muscle contraction.

In this study, basal levels of MLC phosphorylation routinely fell within the range of 40–50% of total MLC phosphorylated. Although control levels of smooth muscle MLC phosphorylation are generally reported to average 10% (39), higher basal phosphorylation levels have been reported in both intact arterial muscle (44) and cultured vascular smooth muscle cells (45). High basal phosphorylation of MLC has been attributed to tissue dissection damage and tissue processing in intact tissues (23). However, in cultured vascular cells, increased MLC phosphorylation appears to be related to time in culture, being highest in postconfluent cells equivalent to those used in the present study (46). The underlying mechanism(s) are not known.

Ca$^{2+}$ Requirement for NPY-stimulated MLC Phosphorylation—Both direct NPY-stimulated contraction and NPY-mediated potentiation of contraction in vascular smooth muscle preparations have been shown to be inhibited by the calcium channel blocker nifedipine, implying a requirement for extracellular Ca$^{2+}$ influx (47). In contrast, the NPY-stimulated rise in intracellular Ca$^{2+}$ measured in fura2-loaded cultured cells (19, 48) was unaffected by removal of extracellular Ca$^{2+}$ and addition of Ca$^{2+}$ chelators to the medium. In PSMCs, the absence of an effect of extracellular Ca$^{2+}$ depletion on NPY-stimulated MLC phosphorylation suggests that release of Ca$^{2+}$ from an intracellular pool, most likely the SR, may be involved. Smooth muscle SR contains two types of Ca$^{2+}$ release channel; one type is sensitive to Ca$^{2+}$, ATP, and caffeine; and the other is activated by IP$_3$ (49). In this study, the inability of caffeine depletion of SR Ca$^{2+}$ to inhibit NPY-mediated MLC phosphorylation and the absence of an effect of NPY on IP$_3$ production imply that NPY is not releasing SR Ca$^{2+}$ through either of these channels. These data suggest either that we are unable to deplete the appropriate intracellular Ca$^{2+}$ pool with caffeine using this protocol or that a Ca$^{2+}$-independent mechanism is responsible for NPY-mediated MLC phosphorylation in PSMCs.

Effect of NPY on Cellular cAMP Content—In PSMCs, NPY alone had no detectable effect on basal cellular cAMP content in the absence or presence of a phosphodiesterase inhibitor,
NPY-stimulated Light Chain Phosphorylation

Effect of NPY on Phosphoinositide Hydrolysis—Our studies indicate that NPY alone has no detectable effect on phosphoinositide hydrolysis or on vascular smooth muscle preparations, NPY-mediated inhibition of agonist-stimulated adenylate cyclase may be one mechanism underlying the previously reported ability of NPY to inhibit vasorelaxation by acetylcholine, adenosine, norepinephrine, and substance P in preconstricted arteries (10, 22). As alluded to above, the action of many vasorelaxing agents is accompanied by a rise in CAMP or cGMP and decrease in Ca\(^{2+}\) (34). By analogy with the previously reported ability of NPY to inhibit vasorelaxation, NPY may inhibit adenylate cyclase indirectly by receptor-activated dissociation of a pertussis toxin-sensitive G-protein releasing G\(_{\alpha}\) complexes that bind to and inactivate G\(_{\alpha}\) (50). This mechanism could explain the demonstrated pertussis toxin sensitivity of adenylate cyclase inhibition in PSMCs.

Although NPY-mediated inhibition of adenylate cyclase may not explain the ability of NPY to increase MLC phosphorylation in PSMCs or contraction in vascular smooth muscle preparations, NPY-mediated inhibition of agonist-stimulated adenylate cyclase may be one mechanism underlying the previously reported ability of NPY to inhibit vasorelaxation. NPY-mediated inhibition of stimulat ed adenylate cyclase in these tissues (recently reviewed by Fredholm et al. (50)). It has been suggested that NPY may inhibit adenylate cyclase indirectly by receptor-activated dissociation of a pertussis toxin-sensitive G-protein releasing G\(_{\alpha}\) complexes that bind to and inactivate G\(_{\alpha}\) (50). This mechanism could explain the demonstrated pertussis toxin sensitivity of adenylate cyclase inhibition in PSMCs.

Effect of NPY on Phosphoinositide Hydrolysis—Our studies indicate that NPY alone has no detectable effect on phosphoinositide hydrolysis in PSMCs. However, simultaneous exposure of cells to angiotensin II and NPY increased angiotensin II-stimulated inositol phosphate production by 40–60% through a pertussis toxin-sensitive mechanism and angiotensin II-stimulated protein kinase C activation by 25%. Thus, NPY potentiation of phosphoinositide hydrolysis may be one mechanism underlying the ability of NPY to potentiate contraction mediated by other vasoactive agonists independent of Ca\(^{2+}\) influx (12). Similar effects of NPY to enhance phosphoinositide hydrolysis stimulated by phenylephrine have been noted in rat vas deferens (15) and pig spleen (50). An effect of NPY alone on phosphoinositide hydrolysis has been reported only in cultured dorsal root ganglion cells (19) and at high doses (10 \(\mu\)M) in rat brain minipreparations (20).

In the present study, the enhancement of phosphoinositide hydrolysis by angiotensin II was not affected by pertussis toxin, which implies that NPY potentiation of phosphoinositide hydrolysis occurs through a pathway that is somewhat different from angiotensin II-activated hydrolysis. Fredholm et al. (50) have suggested that NPY-mediated membrane depolarization, observed previously in rat tail artery (21) and guinea pig basilar artery (22), may activate a membrane potential-sensitive phospholipase resulting in enhanced agonist-stimulated phosphoinositide hydrolysis. Alternatively, an NPY-mediated decrease in cellular CAMP content may increase phospholipase C activity, in agreement with a recent report that an increase in CAMP inhibits histamine-stimulated phosphoinositide hydrolysis in canine tracheal smooth muscle (52).

In conclusion, the ability of NPY to enhance agonist-stimulated phosphoinositide hydrolysis and to decrease agonist-stimulated CAMP accumulation may be involved in NPY potentiation of vasoconstriction and inhibition of vasorelaxation, respectively. Through these molecular mechanisms, NPY may influence the contractile state of vascular smooth muscle in vivo, where the vasculature is continuously bathed by a changing milieu of vasoactive hormones and neurotransmitters. Future studies will explore the effects of NPY on Ca\(^{2+}\) and attempt to clarify the cellular mechanism(s) underlying NPY enhancement of agonist-stimulated phosphoinositide hydrolysis.

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