Interactions between neuropeptide Y and the adenylate cyclase pathway in rat mesenteric small arteries: role of membrane potential

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1. Simultaneous measurements of membrane potential and tension were performed to investigate the intracellular mechanisms of neuropeptide Y (NPY) in rat mesenteric small arteries.

2. NPY (0·1 μM) depolarized arterial smooth muscle cells from −55 to −47 mV and increased wall tension by 0·22 N m^{-1}, representing 11% of the contraction elicited by a high-potassium solution. Isoprenaline (1 μM) and acetylcholine (1 μM) evoked hyperpolarizations of 11 and 17 mV, respectively. NPY inhibited the isoprenaline-induced effects on membrane potential without affecting those of acetylcholine.

3. Forskolin evoked sustained concentration-dependent hyperpolarizations of small mesenteric arteries. NPY (0·1 μM) inhibited the responses to 1 μM forskolin, but did not alter the stable hyperpolarization elicited by the specific activator of protein kinase A (PKA) S_P-5,6-DCl-cBIMPS (0·1 mM). Forskolin increased the cyclic AMP (cAMP) content of the arteries 21-fold, and NPY inhibited the forskolin-evoked increase in cAMP levels by 91%.

4. The hyperpolarization produced by 1 μM forskolin was not affected by either charybdotoxin (0·1 μM) or 4-aminopyridine (0·5 mM), but glibenclamide (5 μM) inhibited the hyperpolarization by 70%. Glibenclamide also inhibited the hyperpolarization evoked by S_P-5,6-DCl-cBIMPS by 59%.

5. Neither depolarization nor contraction caused by NPY were significantly affected by either glibenclamide (5 μM) or nifedipine (1 μM), but they were reduced by gadolinium (10 μM). However, the blocking effect of NPY on forskolin-elicited hyperpolarization was not affected by gadolinium.

6. Charybdotoxin (0·1 μM) and 4-aminopyridine (0·5 mM) strongly enhanced the depolarization and contraction caused by NPY (0·1 μM), and nifedipine (1 μM) prevented the enhanced responses to NPY in the presence of charybdotoxin.

7. These findings suggest that NPY acts through at least two different intracellular mechanisms in mesenteric small arteries: a depolarization of arterial smooth muscle which is probably due to activation of non-selective cation channels, and a marked inhibition of adenylate cyclase activity, which in turn inhibits the hyperpolarization produced by cAMP accumulation in these arteries.

Neuropeptide Y (NPY), a thirty-six amino acid peptide belonging to the pancreatic polypeptide (PP) family, plays an important role in the regulation of cardiovascular function by acting at both central and peripheral targets (Edvinsson, Håkanson, Wahlestedt & Uddman, 1987; McDermott, Millar & Piper, 1993; Wahlestedt & Reis, 1993). In the periphery, where the peptide is widely distributed in nerve fibres to the heart and around blood vessels and often colocalized with noradrenaline, NPY acts through both pre- and postsynaptic mechanisms (McDermott *et al.* 1993). The presynaptic mechanisms are related to the inhibition of neurotransmitter release. At postsynaptic sites, NPY mainly exerts vasopressor actions either through a direct contractile effect on arterial smooth muscle (Neild, 1987; Prieto, Simonsen & Nyborg, 1995), or through indirect mechanisms involving both potentiation of the responses of other vasoconstrictors (Edvinsson *et al.* 1987; Prieto, Benedito, Simonsen & Nyborg, 1991; Adriantsitohaina, Bian, Stoclet & Bukoski, 1993) and inhibition of vasodilatation (Abel & Han, 1989; McDermott *et al.* 1993).

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NPY acts on vascular smooth muscle by binding to specific membrane receptors, the majority of which belong to the Y_1 subtype (Wahlestedt & Reis, 1993; Prieto *et al.* 1995) that has been cloned from several species and is a member of the superfamily of G protein-coupled receptors (Larhammar, Blomqvist, Yee, Jazin, Yoo & Wahlestedt, 1992). Several signal transduction mechanisms have been suggested to explain the ability of NPY to potentiate the contractile responses to electrical field stimulation and exogenously added agonists. NPY mobilizes intracellular Ca^{2+} in vascular smooth muscle (Mihara, Shigeri & Fujimoto, 1989), and there is evidence that the peptide specifically potentiates transmembrane Ca^{2+} influx through voltage-gated L-type channels (Xiong, Bolzon & Cheung, 1993). In mesenteric resistance arteries, NPY potentiates noradrenaline responses through a mechanism which involves extracellular Ca^{2+} entry via a pertussis toxin-sensitive G protein (Adriantsitohaina *et al.* 1993). On the other hand, it is well established that NPY is a potent inhibitor of adenylate cyclase in cardiac muscle (Kassis, Olasmaa, Terenius & Fishman, 1987), brain conduit arteries (Fredholm, Jansen & Edvinsson, 1985) and cultured vascular smooth muscle cells (Mihara *et al.* 1989; Lobaugh & Blackshear, 1990). NPY has also been shown to increase myosin light chain phosphorylation (Lobaugh & Blackshear, 1990) and potentiate the agonist-stimulated inositol phosphate production (Lobaugh & Blackshear, 1990; Duckles & Buxton, 1994). Finally, recent patch-clamp studies have demonstrated that NPY inhibits Ca^{2+}-activated K^+ (K_{Ca}) channels (Xiong & Cheung, 1994), and this has been suggested as a mechanism of membrane depolarization which might account for the potentiating effect of the peptide in vascular smooth muscle cells.

In an attempt to understand how some of these signalling mechanisms interact, we have investigated rat mesenteric small arteries in which we have made simultaneous measurements of membrane potential and tension, and measurements of intracellular cAMP, under clearly defined physiological conditions. The work has therefore allowed us to evaluate the interactions between NPY, membrane potential and adenylate cyclase through both a biochemical and an electrophysiological approach.

METHODS

Dissection and mounting

Adult male Wistar rats (12–14 weeks old) were killed by CO_2 inhalation and exsanguination. The mesenteric vascular bed was removed and placed in physiological salt solution (PSS) (see below for composition). Third-order branches of the superior mesenteric artery (one vessel per animal) were dissected free from the surrounding tissue, cut into 2 mm long segments and mounted as ring preparations in a myograph for isometric force recording (J. P. Trading, Denmark). The vessels were allowed to equilibrate for 30 min at 37 °C in PSS bubbled with a mixture of 5% CO_2–95% O_2 to maintain pH at 7·4. The relation between resting wall tension and internal circumference was determined, and from this the internal circumference L_{100}, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ*, was calculated (Mulvany & Halpern, 1977). The vessels were set to the normalized internal circumference $L_1 = 0{\cdot}9L_{100}$, where the artery develops maximal active tension. The effective internal lumen diameter was determined as $l_1 = L_1/\pi$.

Simultaneous measurements of force and membrane potential

Simultaneous measurements of membrane potential and force were performed as described previously (Mulvany, Nilsson & Flatman, 1982; Nilsson, Jensen & Mulvany, 1994). Briefly, the arteries were mounted in a microvascular myograph set to L_1 and continuously superfused with PSS (37 °C) at a flow rate of 3 ml min^{-1}. Intracellular recordings of the membrane potential were obtained from the adventitial side by using glass capillary microelectrodes mounted on a hydraulic micromanipulator (Narishige MW-3, Japan) and connected to a WPI M-707 amplifier. The electrodes were pulled from aluminosilicate glass (AS 100F-4, WPI; o.d., 1·0 mm; i.d., 0·5 mm), filled with 3 M KCl and had resistances between 50 and 90 MΩ. Electrical signals were continuously monitored on an oscilloscope and recorded on a pen recorder. The criteria used for acceptance of the intracellular microlectrode impalements were: (1) a sudden negative change in voltage upon impalement of the cells, followed by a stable negative voltage for at least 2 min; (2) return to zero voltage on dislodgement of the microelectrode; (3) less than 10% change in tip resistance before and after impalement.

Experimental procedure

At the beginning of each experiment, the viability of the arteries was tested by activation with a high-potassium solution (125 mM K^+, K-PSS). NPY (0·1 μM) was applied only once in each vessel due to the development of tachyphylaxis. Arteries were exposed to NPY (0·1 μM) or forskolin (1 μM) for at least 5 min, which was the minimal period of time required to obtain steady effects with these drugs. The effects of charybdotoxin (0·1 μM), 4-aminopyridine (0·5 mM), glibenclamide (5 μM), nifedipine (1 μM) and gadolinium (10 μM) were tested by superfusing the arteries for 20–30 min with the drugs, which were present in the superfusate during further application of either 0·1 μM NPY or 1 μM forskolin. Since the hyperpolarization elicited by 1 μM forskolin could be repeated twice in the same artery without a loss of response, the effects of the different K^+ channel blockers were evaluated by first exposing the artery to 1 μM forskolin (control response). After 30 min of perfusion with drug-free PSS, the preparation was incubated with the blocker for the required time, and then exposed to forskolin in a second stimulation.

Determination of cyclic AMP content

Total content of cAMP was measured by $^{125}I^-$ radioimmunoassay. Arterial segments 8 mm long were mounted on single wires in a tissue bath, equilibrated in 5% CO_2–95% O_2-aerated PSS at 37 °C for 30 min and then stimulated for 8 min with 1 μM forskolin, 0·1 μM NPY, or 0·1 μM NPY (8 min) plus subsequent addition of 1 μM forskolin (8 min). At the time of measurement, arteries were quickly frozen in liquid nitrogen, transferred to 500 μl tubes containing a melting solution of 10% (w/v) trichloroacetic acid (TCA) and stored at −80 °C until analysis. Arterial segments were homogenized in the TCA solution at 4 °C and proteins precipitated by centrifugation (2500 *g*, 15 min) and stored for determination. TCA from the supernatants (2 ml) was extracted 5 times with 5 ml water-saturated diethyl ether, and the final water phase was lyophilized in a Speedvac concentrator. The lyophilized supernatant was dissolved in 150 μl assay buffer and analysed using kits for the acetylated radioimmunoassay protocol obtained from DuPont NEN® Research Products (Boston, MA, USA). cAMP

content was related to the protein content of each artery. Total protein content was determined according to the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as standard.

Solutions and drugs

PSS had the following composition (mmol l^{-1}): NaCl, 119; KCl, 4·7; KH_2PO_4, 1·18; $MgSO_4$, 1·17; $CaCl_2$, 2·5; EDTA, 0·026; and glucose, 5·5. In high-potassium solutions (K-PSS), NaCl was replaced by KCl on an equimolar basis giving a final concentration of 125 mM K^+. Experiments with gadolinium were performed in bicarbonate-free Hepes-buffered solution of the following composition (mmol l^{-1}): Hepes, 5; NaCl, 140; $MgSO_4$, 1·17; $CaCl_2$, 2·5; KCl, 4·6; glucose, 10; EDTA, 0·026; and KH_2PO_4, 1·18; bubbled with O_2 and pH adjusted to 7·4 with 1 N NaOH. Acetylcholine HCl, 4-aminopyridine, forskolin, gadolinium HCl, glibenclamide, 3-isobutyl-L-methyl xanthine (IBMX), isoprenaline HCl, nifedipine, porcine neuropeptide Y, human NPY and norepinephrine HCl were purchased from Sigma, charybdotoxin from Latoxan (Rosans, France), S_p-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate (S_p-5,6-DCl-cBIMPS) from Biolog-Life Science Institute (Bremen, Germany) and phentolamine HCl from Ciba-Geigy (Basel, Switzerland). Stock solutions of forskolin (10 mM), glibenclamide (10 mM) and S_p-5,6-DCl-cBIMPS (100 mM) were made in dimethyl sulphoxide and those of nifedipine (10 mM) in 96% ethanol. These drugs were used at the following concentrations: forskolin, 1 μM; glibenclamide, 5 μM; S_p-5,6-DCl-cBIMPS, 0·1 mM; nifedipine, 1 μM. Therefore, the final concentration of DMSO applied in the tissue bath was 0·02% when using forskolin and 0·1% when using glibenclamide or cBIMPS. The final concentration of ethanol when using nifedipine was 0·02%. Preliminary experiments showed that at the concentrations applied, the solvents were ineffective on either membrane potential or tension. Experiments with nifedipine were performed in subdued light in order to minimize light-induced degradation.

Statistics

Mechanical responses of the vessels were measured as force and expressed as active wall tension (ΔT) which is the increase in force above baseline (ΔF) divided by twice the segment length. The results are expressed as means ± S.E.M., where n represents the number of animals studied in each experiment. One vessel was studied per animal. Statistical differences between groups were tested by the use of Student's paired or unpaired t test when appropiate. When multiple comparisons were made, values were analysed by one-way analysis of variance (ANOVA), using the Bonferroni method as a *post hoc* test. Probability levels less than 5% were considered as significant.

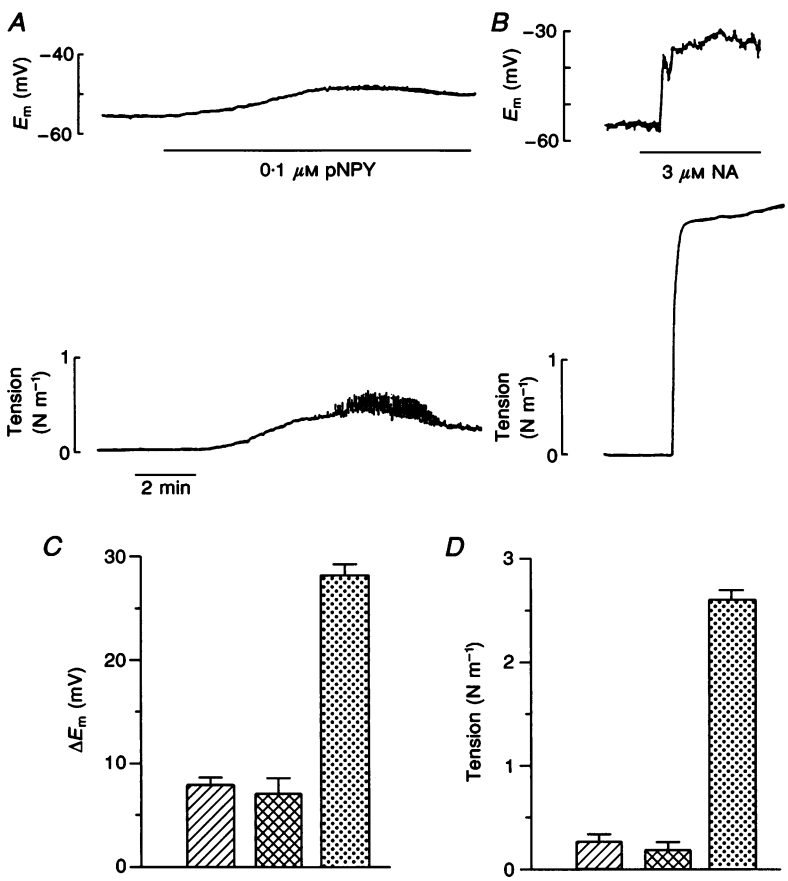

Figure 1. Depolarization and contraction evoked by NPY

A and *B*, simultaneous measurements of membrane potential (upper traces) and tension (lower traces) showing the differential responses of two small mesenteric arteries to porcine neuropeptide Y (pNPY, 0·1 μM) (*A*) and noradrenaline (NA, 3 μM) (*B*). Note in *A* oscillations in tension, usually accompanied by oscillations in membrane potential, which were evoked by NPY in 28% of the arteries. Average depolarizations (*C*) and contractions (*D*) evoked by pNPY (0·1 μM, ▨), human neuropeptide Y (hNPY, 0·1 μM, ▩) and NA (3 μM, ⊡). Here, and in subsequent figures, columns and bars represent means ± S.E.M.

RESULTS

Effects of NPY on membrane potential and tension

Rat mesenteric small arteries with l_1 of 223 ± 3 μm ($n = 86$) had a mean resting membrane potential of −55·2 ± 0·3 mV ($n = 86$). Figure 1 shows simultaneous measurements of membrane potential and tension and illustrates the effects of porcine and human NPY compared with those of noradrenaline in mesenteric small arteries. Porcine NPY (0·1 μM) depolarized the smooth muscle from −55·3 ± 1·1 to −46·8 ± 1·1 mV ($n = 18$) (Fig. 1*A* and *C*) and increased tension by 0·22 ± 0·05 N m^{-1} ($n = 18$) (Fig. 1*A* and *D*), equal to 11 ± 3% of the K-PSS-induced contraction, whereas noradrenaline depolarized the arteries to −25·8 ± 0·3 mV ($n = 5$) (Fig. 1*B* and *C*) and induced a contraction of 2·7 ± 0·1 N m^{-1} ($n = 5$) (Fig. 1*B* and *D*), representing 93 ± 9% ($n = 5$) of the K-PSS response. Human NPY also induced depolarization and contraction of mesenteric arteries which were not significantly different from those elicited by porcine NPY (Fig. 1*C* and *D*). All subsequent experiments were performed with porcine NPY, which will be referred to in the following text just as NPY. Both depolarization and contraction in response to NPY were slower in onset and of lesser magnitude than those elicited by noradrenaline (Fig. 1). The depolarization induced by NPY was variable ranging from 3 to 15 mV.

Effects of NPY on the adenylate cyclase pathway

The β-adrenoceptor agonist isoprenaline (1 μM) elicited a sustained hyperpolarization of mesenteric resistance arteries, which averaged 10·9 ± 1·0 mV ($n = 9$). NPY (0·1 μM) inhibited the isoprenaline-induced responses by 73 ± 8% ($n = 5$; Fig. 2*A*). Thus, 1 μM isoprenaline hyperpolarized smooth muscle to −67·0 ± 1·1 mV ($\Delta E_m = 10·2 \pm 1·3$ mV, $n = 5$) in the absence, and to −55·8 ± 1·1 mV ($\Delta E_m = 3·0 \pm 0·9$ mV, $P < 0·001$ *vs.* controls, $n = 5$) in the presence of 0·1 μM NPY. Acetylcholine (1 μM) evoked a hyperpolarization quicker in onset and larger than that evoked by 1 μM isoprenaline (Fig. 2*B*). However, in contrast to its inhibitory effect on the β-agonist-evoked hyperpolarization, NPY did not significantly alter the responses to the cholinergic agonist (Fig. 2*B*). Thus, 1 μM ACh hyperpolarized the arteries to −71·3 ± 1·5 mV ($\Delta E_m = 17·0 \pm 1·5$, $n = 6$) in the absence, and to −69·3 ± 0·8 mV ($\Delta E_m = 17·3 \pm 0·9$, $n = 4$) in the presence of 0·1 μM NPY.

The adenylate cyclase activator forskolin evoked a sustained concentration-dependent hyperpolarization (Fig. 3). Forskolin effects on the membrane potential of mesenteric resistance arteries were markedly inhibited by NPY (Fig. 4). Thus, the membrane potential after addition of 1 μM forskolin was −66·5 ± 0·8 mV ($n = 8$) in the absence and −51·8 ± 1·7 mV ($n = 8$) ($P < 0·01$) in the presence of 0·1 μM NPY, respectively (Fig. 4*A* and *B*). Moreover, in arteries hyperpolarized by 1 μM forskolin from −56·8 ± 1·2 ($n = 5$) to −66·8 ± 1·1 mV ($n = 5$), 0·1 μM NPY rapidly repolarized smooth muscle to −54·4 ± 0·8 mV ($n = 5$).

In order to investigate whether the inhibitory action of NPY on forskolin-hyperpolarizing responses is exerted on

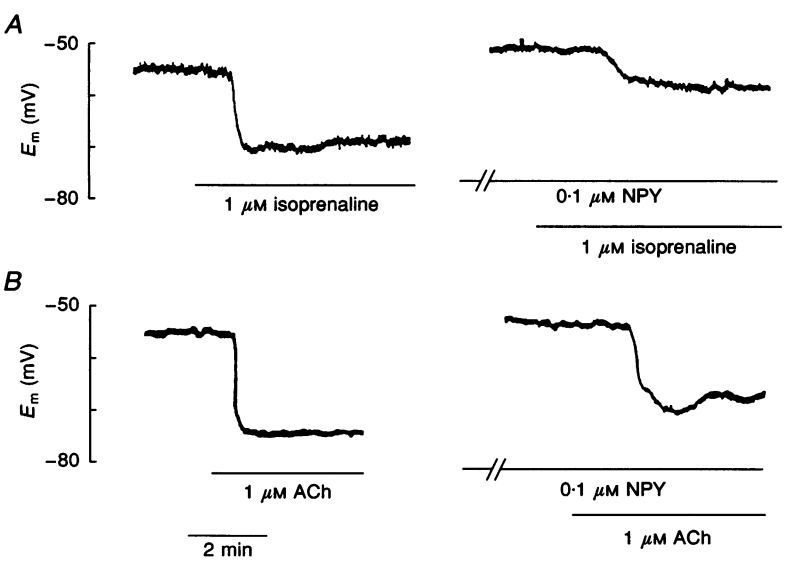

Figure 2. NPY inhibits isoprenaline- but not acetylcholine-elicited hyperpolarization

Original recordings of membrane potential showing the differential effects of NPY on the hyperpolarizations elicited by isoprenaline (*A*) and acetylcholine (ACh) (*B*) in two different small mesenteric arteries mounted in a microvascular myograph and superfused with PSS. In both *A* and *B*, recordings on the left show the control hyperpolarizations in response to 1 μM isoprenaline and 1 μM acetylcholine, respectively, applied during the period indicated by the bars. Recordings on the right show the effects of NPY on these responses. After superfusing with drug-free PSS for 30 min, 0·1 μM NPY was given 5–8 min before a second stimulation with either isoprenaline (*A*) or acetylcholine (*B*). NPY inhibited the hyperpolarization elicited by isoprenaline, without affecting that elicited by acetylcholine.

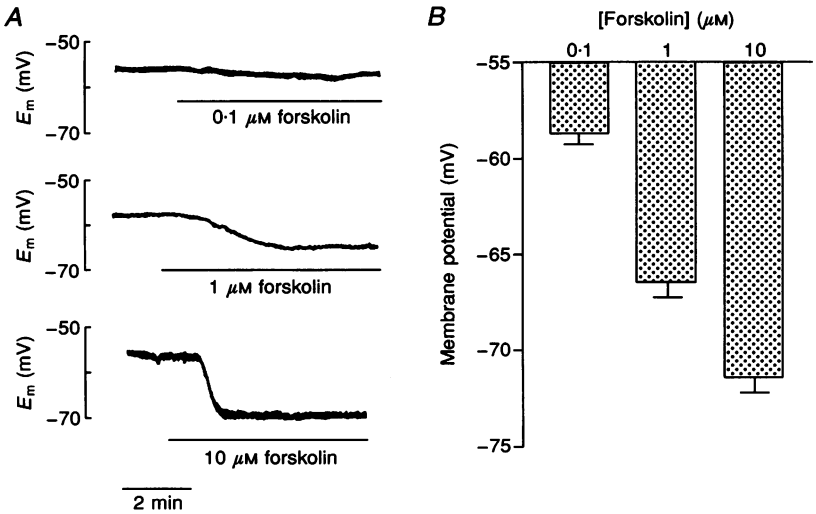

Figure 3. Forskolin hyperpolarizes mesenteric small arteries

A, original recordings of membrane potential showing that forskolin (0·1–10 μM) evokes slow and sustained concentration-dependent hyperpolarizations of three different arterial segments. *B*, mean concentration-dependent responses evoked by forskolin on membrane potential of small mesenteric arteries. Forskolin at 0·1, 1 and 10 μM hyperpolarized smooth muscle to −58·7 ± 0·5 mV ($n = 4$), −66·5 ± 0·8 mV ($n = 8$) and −71·5 ± 0·8 mV ($n = 4$), respectively.

adenylate cyclase, we examined the effects on membrane potential of the membrane permeant cyclic nucleotide analogue S_P-5,6-DCl-cBIMPS which is a potent and specific activator of the cAMP-dependent protein kinase (protein kinase A, PKA) (Sandberg *et al.* 1991). S_P-5,6-DCl-cBIMPS (0·1 mM) elicited a 10·1 ± 0·4 mV ($n = 7$) hyperpolarization, which started after a delay of 2–5 min and reached steady values of membrane potential (−64·9 ± 0·3 mV, $n = 7$) after 10–15 min. In contrast to its inhibitory effects on isoprenaline and forskolin responses, NPY did not affect the hyperpolarization elicited by S_P-5,6-DCl-cBIMPS (Fig. 4*C*); 0·1 mM S_P-5,6-DCl-cBIMPS hyperpolarized mesenteric

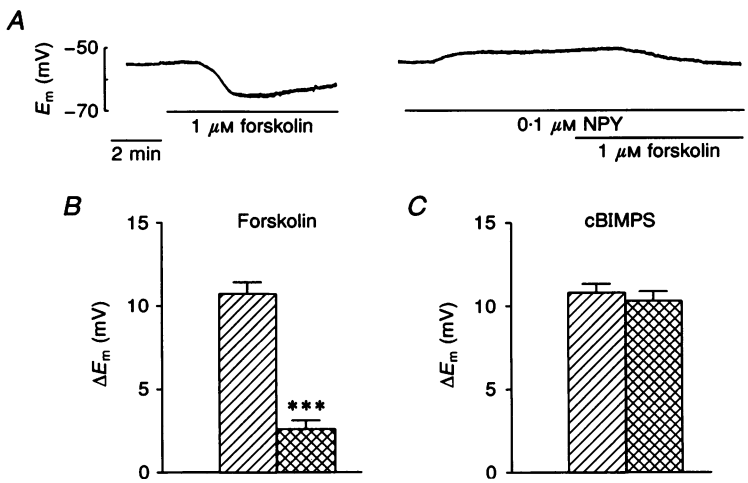

Figure 4. NPY inhibits the hyperpolarization evoked by forskolin but not that by S_P-5,6-DCl-cBIMPS

A, original traces showing the responses of a mesenteric small artery to 1 μM forskolin in control conditions (left panel) and after 8–10 min stimulation with 0·1 μM NPY (right panel). After the first exposure to forskolin, the artery was superfused with drug-free PSS for 30 min and then NPY was given 8–10 min before a second exposure to forskolin. *B*, mean hyperpolarizing responses to 1 μM forskolin in the absence (▨) and presence (▩) of 0·1 μM NPY. *C*, mean hyperpolarizations evoked by S_P-5,6-DCl-cBIMPS (cBIMPS, 0·1 mM) in the absence (▨) and presence (▩) of 0·1 μM NPY. ***$P < 0·001$ *vs.* controls (Student's unpaired *t* test).

Table 1. Effect of NPY on basal and forskolin-stimulated cAMP levels

	Basal (fmol (μg protein)$^{-1}$)	1 μM forskolin (fmol (μg protein)$^{-1}$)
Control	28·0 ± 9·9 (6)	596·1 ± 42·5 (4)[a]
0·1 μM NPY	17·3 ± 3·8 (6) n.s.	46·2 ± 8·4 (6)[b]

Numbers in parentheses represent the number of determinations (from three different animals). [a]Significantly different from basal cAMP levels ($P < 0·001$). [b]Significantly different from 1 μM forskolin-stimulated arteries ($P < 0·001$). n.s., not significantly different from basal cAMP levels (ANOVA followed by Bonferroni test).

arteries to −65·3 ± 0·5 mV ($n = 4$) in control conditions, and to −63·3 ± 1·3 mV ($n = 4$) after 8–10 min incubation with 0·1 μM NPY.

The basal cAMP content of rat mesenteric small arteries averaged 28 fmol (μg protein)$^{-1}$ and treatment for 8 min with 1 μM forskolin increased cAMP levels 21-fold (Table 1). Pre-incubation with 0·1 μM NPY did not significantly affect the mean basal cAMP content, whereas it inhibited the forskolin-stimulated cAMP accumulation by 91 ± 2% (Table 1). In two separate determinations on arteries from two animals, in the presence of the phosphodiesterase inhibitor IBMX (0·1 mM) NPY inhibited the forskolin-induced cAMP increase by 94 and 86%.

Mechanisms of forskolin-elicited hyperpolarization

To investigate the mechanisms underlying cAMP effects on membrane potential of mesenteric small arteries, the effects of different K^+ channel blockers were examined on the hyperpolarization elicited by forskolin. The blocker of large- and medium-conductance K_{Ca} channels, charybdotoxin, did not affect either resting membrane potential (Table 2) or the hyperpolarization elicited by forskolin. Thus, 1 μM forskolin hyperpolarized smooth muscle to −70·0 ± 1·3 mV ($\Delta E_m = 13·8 \pm 1·4$ mV, $n = 4$), and to −69·3 ± 2·5 mV ($\Delta E_m = 12·8 \pm 0·6$ mV, $n = 4$) in the absence and the presence of 0·1 μM charybdotoxin, respectively. Similarly, incubation with 0·5 mM 4-aminopyridine did not significantly alter the resting membrane potential nor did it inhibit forskolin responses. Forskolin (1 μM) hyperpolarized mesenteric arteries to −64·3 ± 0·7 mV ($\Delta E_m = 9·3 \pm 0·5$ mV,

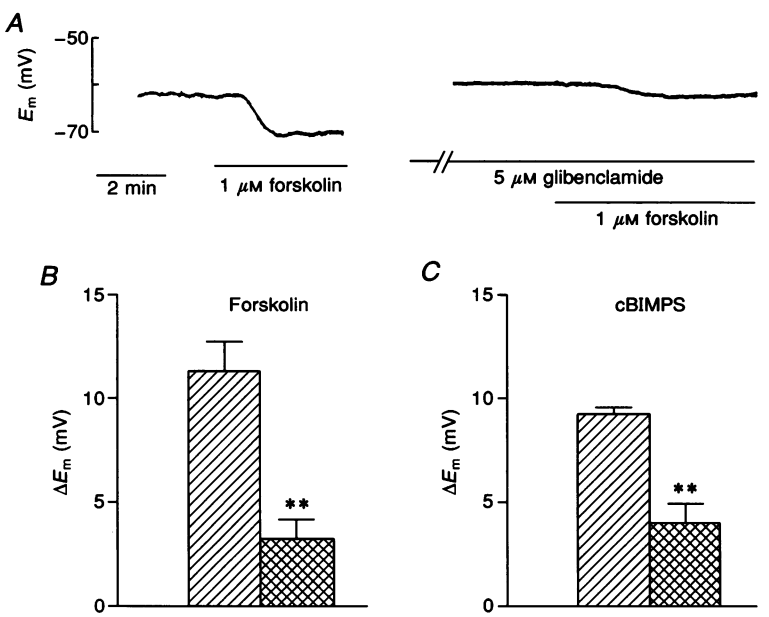

Figure 5. Glibenclamide markedly inhibits the hyperpolarizations evoked by both forskolin and S_P-5,6-DCl-cBIMPS

A, original recordings of membrane potential in a mesenteric small artery showing the hyperpolarization evoked by 1 μM forskolin in control conditions (left panel) and after 20 min superfusion with PSS containing the K_{ATP} channel inhibitor, glibenclamide (right panel). The artery was superfused with drug-free PSS for 30 min after the first stimulation with forskolin, and before incubation with glibenclamide. *B*, mean responses to forskolin in the absence (▨) and presence (▩) of 5 μM glibenclamide. In control conditions, 1 μM forskolin hyperpolarized the membrane to −68·5 ± 2·3 mV ($n = 4$), and in the presence of 5 μM glibenclamide to −58·6 ± 1·2 mV ($n = 5$) ($P < 0·01$ *vs.* controls). *C*, mean hyperpolarizations evoked by S_P-5,6-DCl-cBIMPS in the absence (▨) and presence (▩) of 5 μM glibenclamide. S_P-5,6-DCl-cBIMPS (0·1 mM) hyperpolarized mesenteric arteries to −64·3 ± 0·2 mV ($n = 4$) in control conditions and to −57·5 ± 0·9 mV ($n = 4$) after incubation with 5 μM glibenclamide. **$P < 0·01$ *vs.* controls (Student's unpaired *t* test).

Table 2. Effects of K^+ channel blockers on resting membrane potential (E_m) of rat mesenteric resistance arteries

	E_m		
	Control (mV)	Treated (mV)	n
0·1 μM charybdotoxin	−56·1 ± 0·5	−54·7 ± 0·8	6
0·5 mM 4-aminopyridine	−53·0 ± 1·2	−53·0 ± 1·2	8
5 μM glibenclamide	−57·1 ± 1·1	−55·0 ± 0·9**	7

** $P < 0·01$ *vs.* controls (Student's paired *t* test).

$n = 4$) and to −63·3 ± 1·5 mV ($\Delta E_m = 9·5 \pm 1·3$ mV, $n = 4$), in the absence and the presence of 4-aminopyridine, respectively. However, the selective blocker of ATP-sensitive K^+ (K_{ATP}) channels glibenclamide (5 μM) slightly depolarized mesenteric small arteries (Table 2) and caused a 70 ± 9% ($n = 5$) inhibition of the forskolin-evoked hyperpolarization (Fig. 5*A* and *B*). Glibenclamide also caused a 59 ± 9% ($n = 4$) inhibition of the response to the PKA activator S_P-5,6-DCl-cBIMPS (Fig. 5*C*).

Mechanisms of NPY-induced depolarization: interactions with the adenylate cyclase pathway?

Having demonstrated that K_{ATP} channels are apparently involved in the hyperpolarization elicited by cAMP accumulation, the effects of glibenclamide on both depolarization and contraction produced by NPY were examined in order to investigate whether interactions with adenylate cyclase could account for the membrane depolarization elicited by NPY in mesenteric small arteries. Unlike its inhibitory effects on forskolin-elicited hyperpolarization, blockade of K_{ATP} channels with 5 μM glibenclamide did not significantly reduce the effects of 0·1 μM NPY on membrane potential and tension of mesenteric resistance arteries (Fig. 6*A* and *B*), even though this inhibitor slightly depolarized arterial smooth muscle (Table 2).

Treatment with the L-type Ca^{2+} channel blocker, nifedipine (1 μM), did not alter significantly the effect of NPY on either membrane potential or tone of mesenteric resistance arteries (Fig. 6*A* and *B*). However, these responses were reduced by the inhibitor of stretch-activated cation channels gadolinium (10 μM) (Sachs, 1992), in the presence of which 0·1 μM NPY depolarized the arteries from −53·4 ± 0·8 to −50·6 ± 0·9 mV ($n = 5$) ($P < 0·01$) (Fig. 6*A*) and increased tension by 0·03 ± 0·01 N m^{-1} ($n = 5$) ($P < 0·05$) (Fig. 6*B*). In contrast to its inhibitory effect on NPY depolarization, gadolinium did not prevent the inhibition by NPY of forskolin-elicited hyperpolarization, and thus, in the presence of 10 μM gadolinium, 0·1 μM NPY blocked the responses induced by 1 μM forskolin by 68 ± 7%.

Incubation with the blockers of K_{Ca} and voltage-gated K^+ channels, charybdotoxin (0·1 μM) and 4-aminopyridine

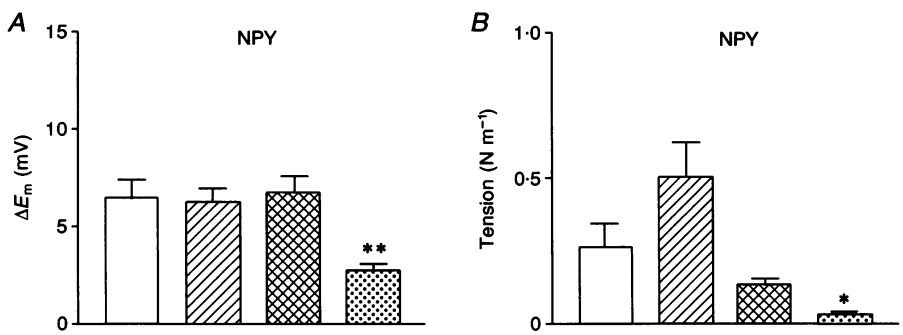

Figure 6. NPY-evoked depolarization and contraction were not decreased by either glibenclamide or nifedipine but were reduced by gadolinium

Mean responses to 0·1 μM NPY in control conditions (□), and effects of glibenclamide (▨), nifedipine (▩) and gadolinium (⊡) on depolarization (*A*) and contraction (*B*) produced by 0·1 μM NPY in rat mesenteric small arteries. The treated arteries were superfused for 20 min with either PSS containing glibenclamide (5 μM) or nifedipine (1 μM), or bicarbonate-free Hepes containing 10 μM gadolinium, respectively, before exposure to NPY (0·1 μM) for 8–10 min. NPY (0·1 μM) depolarized arterial smooth muscle from −55·1 ± 1·4 to −48·6 ± 1·8 mV ($n = 6$) in control arteries, and from −54·8 ± 0·8 to −49·0 ± 0·5 mV ($n = 4$), from −53·2 ± 2·1 to −46·4 ± 1·7 mV ($n = 5$) and from −53·4 ± 0·8 to −50·6 ± 0·9 mV ($n = 5$) ($P < 0·01$ *vs.* controls), in arteries treated with glibenclamide, nifedipine and gadolinium, respectively. * $P < 0·05$; ** $P < 0·01$ (Student's unpaired *t* test).

(0·5 mM), respectively, greatly enhanced depolarization and tension elicited by NPY, in contrast to their lack of effect on responses to forskolin. In the presence of charybdotoxin, NPY (0·1 μM) caused peak values of membrane potential and tension of −24·7 ± 1·7 mV ($n = 4$) and 2·4 ± 0·2 N m^{-1} ($n = 4$) (Fig. 7*A*, *C* and *D*), respectively, during the first 4–6 min, which then decreased after 10 min to −34·0 ± 2·8 mV ($n = 4$) and 0·91 ± 0·26 N m^{-1} ($n = 4$), respectively. Both membrane potential and tone exhibited fluctuations with a maximum amplitude of 10–18 mV and 0·3–0·8 N m^{-1}, respectively. However, whereas blockade of voltage-gated Ca^{2+} channels did not affect NPY elicited responses, this procedure abolished the enhancement of NPY depolarization and contraction elicited by charybdotoxin. Figure 7 depicts original tracings illustrating the effects of the blockade of large and medium conductance K_{Ca} channels in the absence (Fig. 7*A*) and in the presence (Fig. 7*B*) of nifedipine. In the presence of both charybdotoxin (0·1 μM) and nifedipine (1 μM), 0·1 μM NPY depolarized smooth muscle from −53·3 ± 0·5 to −44·0 ± 1·9 mV ($n = 4$) (Fig. 7*C*) and increased tension by 0·13 ± 0·01 N m^{-1} ($n = 4$) (Fig. 7*D*), values not significantly different from those elicited by the peptide in control conditions.

Treatment with 0·5 mM 4-aminopyridine also enhanced depolarization and contraction elicited by NPY in mesenteric resistance arteries. In the presence of 4-aminopyridine, 0·1 μM NPY elicited peak values of membrane potential and tension of −17·5 ± 2·8 mV ($n = 4$) and 2·4 ± 0·2 N m^{-1} ($n = 4$), respectively, after 2–5 min, falling then to −36·8 ± 4·9 mV ($n = 4$) and 0·89 ± 0·13 N m^{-1} ($n = 4$) after 8–10 min. The arteries also exhibited fluctuations in membrane potential and tension with a variable maximum amplitude of 10–20 mV and 0·5–1·5 N m^{-1}, respectively.

DISCUSSION

The present study demonstrates different intracellular mechanisms underlying NPY actions on rat mesenteric small arteries. Thus, NPY inhibits adenylate cyclase and antagonizes the functional effects of cAMP accumulation on

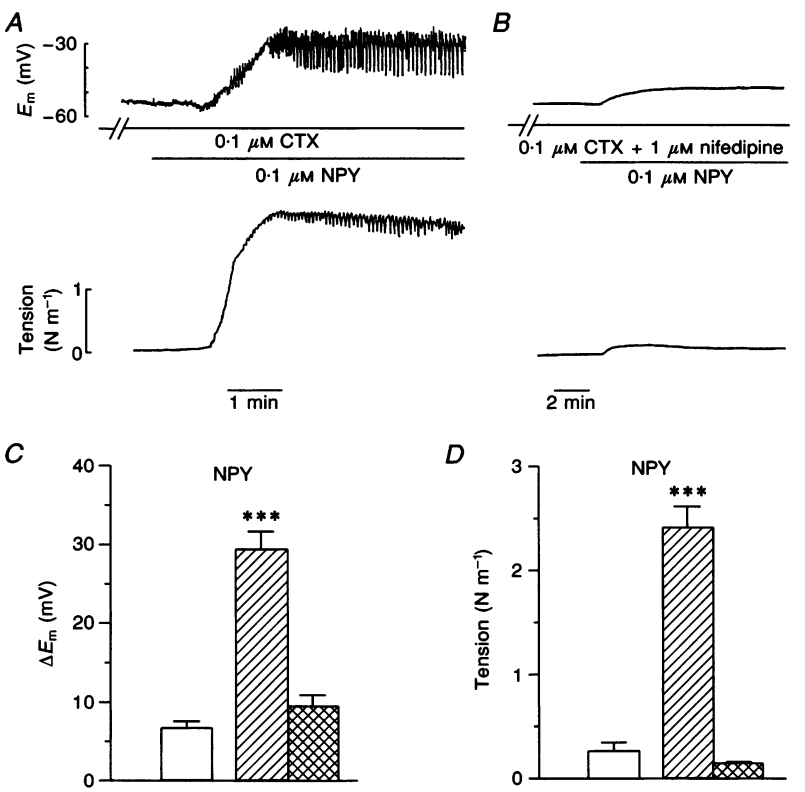

Figure 7. Charybdotoxin enhances both depolarization and contraction evoked by NPY and nifedipine prevents this enhancement

A and *B*, original traces showing simultaneous measurements of membrane potential (upper traces) and tension (lower traces) in two different mesenteric small arteries and illustrating the effects of charybdotoxin alone (*A*) and charybdotoxin with nifedipine (*B*) on the depolarization and contraction elicited by 0·1 μM NPY. The arteries were superfused for 20 min with PSS containing 0·1 μM charybdotoxin (CTX; *A*) or 0·1 μM charybdotoxin plus 1 μM nifedipine (*B*), before stimulation with 0·1 μM NPY for 8–10 min. Representative traces showing the electrical and mechanical responses to NPY in control conditions are illustrated in Fig. 1*A*. Mean depolarizations (*C*) and contractions (*D*) in response to 0·1 μM NPY in control conditions (□) and in the presence of either charybdotoxin (0·1 μM) alone (▨) or charybdotoxin (0·1 μM) plus nifedipine (1 μM) (▩).

membrane potential. Furthermore, the peptide also directly depolarizes arterial smooth muscle, mainly through an activation of gadolinium-sensitive cation channels. These mechanisms appear to represent two independent signalling pathways which might account for the postjunctional vasopressor actions of NPY in resistance arteries and would explain both the inhibition of vasodilatation and the potentiation of vasoconstriction, respectively, reported for the peptide (McDermott *et al.* 1993; Wahlestedt & Reis, 1993).

As pointed out in the Introduction, several signalling mechanisms have been proposed to mediate the potentiating effect of NPY on the responses to nerve stimulation, noradrenaline and other vasoconstrictors (Mihara *et al.* 1989; Lobaugh & Blackshear, 1990; Xiong *et al.* 1993; Duckles & Buxton, 1994). In resistance arteries, NPY has been suggested to enhance noradrenaline contractions by promoting an influx of extracellular Ca^{2+} (Adriantsitohaina *et al.* 1993), which is consistent with the present results showing that in the same preparation NPY causes a moderate depolarization of the smooth muscle, which would enhance Ca^{2+} entry. This may explain the potentiating effect of the peptide, since slight depolarizations caused either by raising extracellular K^+ (Mulvany *et al.* 1982) or by denervation (Neild, 1987) are known to increase the sensitivity of arterial smooth muscle to several vasoconstrictors. NPY has been shown earlier to elicit depolarization in brain large arteries (Abel & Han, 1989) and pial arterioles (Xia, Neild & Kotecha, 1992) and in rat caudal artery (Neild, 1987). In these studies, as in the present experiments, the effects of the peptide on membrane potential were slow in onset and sustained, and NPY also caused a weak contraction but potentiated the vasoconstriction elicited by other agonists.

Evidence for NPY inhibiting adenylate cyclase

The enhancing effect of NPY on peripheral resistance *in vivo* has been ascribed not only to its vasoconstrictor and potentiating properties, but also to the inhibition of the relaxant effects of several vasodilators (Abel & Han, 1989; McDermott *et al.* 1993). Thus, despite the moderate depolarizing effect exerted by NPY in rat mesenteric small arteries, it markedly inhibited the hyperpolarization elicited by isoprenaline without significantly affecting that of acetylcholine. This effect of isoprenaline on membrane potential confirms earlier evidence in large arteries (Somlyo, Somlyo & Smiesko, 1972) and veins (Nakashima & Vanhoutte, 1995), and suggests that hyperpolarization of arterial smooth muscle may underlie the vasodilatation elicited by agonists which increase intracellular cAMP in resistance arteries. Relaxation of vascular smooth muscle by β-agonists is conventionally held to occur via a G protein-dependent stimulation of adenylate cyclase with a subsequent accumulation of cAMP (Murray, 1990). Accordingly, NPY also inhibited the hyperpolarizations evoked by the direct activator of adenylate cyclase, forskolin. This inhibitory effect of NPY on forskolin responses appears to be exerted specifically on adenylate cyclase for two reasons. First, at a concentration which inhibited forskolin and isoprenaline hyperpolarizations, NPY did not affect the stable hyperpolarization evoked by S_p-5,6-DCl-cBIMPS, a potent nucleotide-permeant analogue which activates PKA types I and II, without affecting cGMP-dependent protein kinase (Sandberg *et al.* 1991). Second, the biochemical experiments showed that NPY almost completely prevented the 21-fold increase in basal cAMP content induced by forskolin in mesenteric small arteries, both in the absence and presence of the phophodiesterase inhibitor, IBMX. Therefore, our findings demonstrate that inhibition of adenylate cyclase is a signal transduction mechanism of NPY which is also present in resistance arteries and might account for the inhibition of vasodilatation reported for the peptide (Abel & Han, 1989; McDermott *et al.* 1993).

In contrast to its marked inhibitory effect on forskolin-stimulated cAMP accumulation, NPY did not have a significant effect on basal cAMP levels, which in principle suggests no direct inhibition by the peptide of basal adenylate cyclase activity, and confirms previous evidence in cardiac muscle, cerebral vessels and cultured vascular smooth muscle cells (Fredholm *et al.* 1985; Kassis *et al.* 1987; Mihara *et al.* 1989; Lobaugh & Blackshear, 1990).

Mechanisms of hyperpolarization in response to adenylate cyclase activation

Several types of K^+ channels have been suggested to mediate the hyperpolarization of vascular smooth muscle in response to cAMP accumulation, usually through a PKA-dependent phosphorylation of the channels (Minami, Fukuzawa, Nakaya, Zeng & Inoue, 1993; Quayle & Standen, 1994; Aiello, Walsh & Cole, 1995). The lack of effect of 4-aminopyridine and charybdotoxin on forskolin responses indicates that neither K_{Ca} nor voltage-gated K^+ channels appear to play a role in the hyperpolarizations to cAMP in small mesenteric arteries. Both large and small conductance K_{ATP} channels have been demonstrated in arterial smooth muscle cells including those of mesenteric arteries (Standen, Quayle, Davies, Brayden, Huang & Nelson, 1989; Quayle & Standen, 1994), and are involved in the hyperpolarization elicited by certain vasodilators such as adenosine, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) (Standen *et al.* 1989; Nelson, Huang, Brayden, Hescheler & Standen, 1990; Quayle & Standen, 1994). The present results demonstrate that the membrane hyperpolarization evoked by activation of adenylate cyclase in mesenteric resistance arteries seems to be due preferentially to an activation of K_{ATP} channels, since forskolin responses are largely inhibited by the sulphonylurea, glibenclamide. Furthermore, this activation may be at least in part mediated by PKA, since S_p-5,6-DCl-cBIMPS, the specific activator of PKA, elicited a stable hyperpolarization of mesenteric small arteries which was also effectively inhibited by glibenclamide. Therefore, the current experiments reinforce previous evidence, including patch-clamp analyses which demonstrate that the signal

transduction pathway for the hyperpolarization and vasodilatation to agonists that stimulate adenylate cyclase involves activation of PKA and opening of K_{ATP} channels (Quayle, Bonev, Brayden & Nelson, 1994; Klepisch & Nelson, 1995).

Evidence for NPY causing adenylate cyclase-independent depolarization through non-selective cation channels

As discussed above, the present study demonstrates a strong inhibitory effect of NPY on stimulated cAMP accumulation, and also an involvement of K_{ATP} channels in the effect of cAMP on membrane potential of mesenteric resistance arteries. However, our data suggest that inhibition of K_{ATP} channels, either directly or via an inhibition of adenylate cyclase, is not the mechanism by which NPY depolarizes arterial smooth muscle. First, biochemical experiments showed a lack of effect of NPY on basal cAMP levels. Second, if blockade of K_{ATP} channels was involved in NPY depolarization, a reduction of this depolarizing effect would be expected in the presence of glibenclamide. This blocker depolarized rat mesenteric arterial smooth muscle, thus confirming a basal activity of K_{ATP} channels in this vascular bed which may play a role in the maintenance of resting membrane potential and basal tone (Nelson *et al.* 1990; Quayle & Standen, 1994). However, glibenclamide did not affect the depolarization elicited by NPY. This excludes an inhibition of K_{ATP} channel activity as a potential mechanism contributing to the peptide-elicited membrane depolarization, in contrast to that reported for other peptides with vasopressor actions such as angiotensin II, endothelin and vasopressin (Miyoshi *et al.* 1992; Quayle & Standen, 1994).

As an alternative to a role for K_{ATP} channels in the depolarization evoked by NPY, our results suggest a role for other ionic conductances, viz. an involvement of non-selective cation channels. Both depolarization and contraction evoked by NPY in rat mesenteric resistance arteries were significantly reduced by gadolinium, a trivalent lanthanide which at concentrations of 10–50 μM blocks non-selective stretch-activated cation channels in several tissues, although it may block Ca^{2+} channels at even lower concentrations (Sachs, 1992). However, in combination with the effect of gadolinium, the lack of effect of nifedipine on the membrane potential responses to NPY suggests that the peptide-elicited depolarization is due to an influx of cations through non-selective channels. This is in agreement with previous reports demonstrating that a number of contractile peptides, including vasopressin and endothelin, can promote a Ca^{2+}-permeable non-selective cation conductance in vascular smooth muscle cells, even though they act at distinct receptors (Van Renterghem, Romey & Lazdunski, 1988; Chen & Wagoner, 1991). This cation current is insensitive to nifedipine but probably modulated by extracellular Ca^{2+} (Chen & Wagoner, 1991; Isenberg, 1993).

In contrast to its inhibitory effect on the depolarization evoked by NPY, gadolinium did not alter the NPY-elicited inhibition of forskolin hyperpolarization, thus adding further support to the notion that NPY acts through at least two separate mechanisms: depolarization of smooth muscle and inhibition of adenylate cyclase. Whether these two intracellular signalling systems are coupled to a single or multiple receptor types remains to be clarified. However, coupling of a single receptor to two different signalling mechanisms (i.e. influx of extracellular Ca^{2+} and inhibition of adenylate cyclase) has been confirmed previously by heterologous expression of the Y_1 receptor (Larhammar *et al.* 1992), and rat mesenteric small arteries recently have been reported to possess Y_1 receptors (Chen, Fetscher, Schäfers, Wambach, Philipp & Michel, 1996).

A possible role for K_{Ca} channels in NPY-elicited depolarization

Recent patch-clamp studies have demonstrated that NPY inhibits K_{Ca} channels in smooth muscle cells of rat caudal artery (Xiong & Cheung, 1994), and this has been proposed as a mechanism which may contribute to the excitatory action of the peptide on vascular smooth muscle. In contrast, the current experiments show that the K_{Ca} channel blocker, charybdotoxin, did not inhibit NPY effects on membrane potential but rather induced a large transient increase in both depolarization and contraction elicited by the peptide, which was abolished in the presence of nifedipine. This suggests that NPY indirectly activates K_{Ca} channels by causing Ca^{2+} influx through voltage-dependent Ca^{2+} channels. In the absence of charybdotoxin, nifedipine also tended to reduce the contractile effect of NPY, which is consistent with an enhanced Ca^{2+} entry through voltage-dependent channels as a consequence of NPY-elicited depolarization. However, after treatment with nifedipine, a significant contraction remained which may be due either to enhanced Ca^{2+} sensitivity, to release of intracellular Ca^{2+} or to Ca^{2+} influx through non-selective ion channels. The fact that gadolinium inhibited not only the depolarization but also the contraction elicited by NPY is consistent with Ca^{2+} influx also through non-selective cation channels. However, in view of the similar membrane potentials after nifedipine alone and after combined application of charybdotoxin and nifedipine, any such Ca^{2+}influx seems insufficient to cause a significant activation of K_{Ca} channels.

The large inhibitory effect of nifedipine on the depolarization to NPY in the presence of charybdotoxin suggests that Ca^{2+} influx, directly or indirectly, has a large depolarizing influence which is normally masked by a hyperpolarizing influence due to enhanced K^+ conductance. Therefore, as indicated above, it is suggested that the main membrane effect of NPY is to activate a gadolinium-sensitive cation channel. The ensuing, moderate depolarization promotes Ca^{2+} entry through voltage-dependent channels, which during the steady state response is balanced by K^+ efflux

due to the simultaneous activation of K_{Ca} channels. The present findings thus confirm the well established ability of K_{Ca} channels to function as a negative feed-back mechanism which regulates membrane depolarization and vasoconstriction (Nelson & Quayle, 1995). The same would be the case for the blockade of the voltage-dependent K^+ channels with 4-aminopyridine, which also increased the effects of NPY on membrane potential and tension of the small mesenteric arteries. These channels act mainly to limit membrane depolarization in many arterial smooth muscles which do not generate action potentials, but respond to stimulation with graded membrane potential changes (Nelson & Quayle, 1995).

In conclusion, the present findings suggest that NPY acts through at least two different intracellular mechanisms in mesenteric small arteries: a depolarization of arterial smooth muscle which is likely due to activation of non-selective cation channels, and a marked inhibition of adenylate cyclase activity, which in turn inhibits the hyperpolarization produced by cAMP accumulation in these arteries.

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