Inhibition of Vasoconstriction and Ca^{2+} Currents Mediated by Neuropeptide Y Y2 Receptors

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Abstract

The effects of neuropeptide Y (NPY) and agonists selective for NPY Y1 and Y2 receptors were studied on contraction and Ca^{2+} currents in arterial smooth muscle. In isolated arterioles from the guinea-pig small intestine, small brief constrictions were evoked by depolarising the arteriolar smooth muscle using high K^+ solution applied from a micropipette. The constrictions were reduced in amplitude by the Y2-selective agonists PYY(13-36) and *N*-acetyl[Leu²⁸, Leu³¹]NPY-(24-36) in concentrations from 20-100 nM. NPY or the Y1-selective agonist [Leu³¹ Pro³⁴] NPY in concentrations from 50 pM to 100 nM increased the amplitude of the constrictions, with a maximum effect at 10 nM. Smooth muscle cells were isolated from rat small mesenteric arteries, and voltage-activated Ca^{2+} currents measured by whole cell patch clamping. The peak amplitude of the Ca^{2+} currents was decreased by *N*-acetyl[Leu²⁸, Leu³¹]NPY-(24-36), and by NPY (100 nM). [Leu³¹, Pro³⁴]NPY either had no effect or slightly increased the Ca^{2+} currents. We conclude that Y2 receptors on vascular smooth muscle can reduce Ca^{2+} currents induced by depolarisation, and thus oppose constriction caused by smooth muscle depolarisation.

Key words: arteries, smooth muscle, neuropeptide Y, vasodilator

Introduction

Neuropeptide Y (NPY) is found in a variety of neurones in the autonomic and central nervous systems. It is prominent in areas concerned with cardiovascular control, and in the peripheral nervous system it is found in almost all sympathetic vasoconstrictor nerves. In experiments on isolated arteries NPY causes contraction of vascular smooth muscle, and an increase in vasoconstrictor effect of many other vasoconstrictors (potentiation); actions which are consistent with the vasoconstrictor role of sympathetic nerves.

However, NPY is also found co-localised with VIP (vasoactive intestinal peptide) or ChAT (choline acetyl transferase) in some vasodilator nerves (Gibbins and Morris, 1988, Suzuki *et al.*, 1990). The functional significance of a potent vasoconstrictor such as NPY in these

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vasodilator nerves is not obvious, but our previous experiments (Neild and Lewis, 1995) showed that activation of the Y2 subtype of NPY receptor on arterial smooth muscle could *inhibit* vasoconstriction caused by high external K^+ . We have now extended this study to investigate the effects of selective agonists for both Y1 and Y2 receptors, and effects of simultaneous activation of both receptors by NPY.

There have been few previous studies of Y2 receptors on arterial smooth muscle, but in neuronal tissues Y2 activation has been shown to inhibit voltage-activated Ca^{2+} currents (Wiley *et al.*, 1993, Qian *et al.*, 1997). In our experiments, the constriction of the submucosal arterioles caused by K^+ was due to smooth muscle depolarisation and Ca^{2+} influx through voltage-gated channels. Here we present evidence from patch clamp studies on isolated vascular smooth muscle cells that Y2 receptor activation inhibits Ca^{2+} influx in vascular smooth muscle, thus providing a possible mechanism for the Y2-mediated inhibition of vasoconstriction.

Methods

Guinea-pig submucosal arterioles

All experiments were performed in accordance with the National Health and Medical Research Council of Australia guidelines, and approved by the Flinders University Animal Welfare Committee. Guinea-pigs of either sex and weighing 200–300 g were killed by a heavy blow to the head followed by decapitation. A sheet of connective tissue containing the submucosal arterioles and nerve plexus was removed from the ileum and pinned out in a small chamber with a transparent base. The preparation was viewed using an inverted compound microscope equipped with a television camera, and arteriole diameter was monitored by computer analysis of the television images (Neild, 1989).

The preparation was superfused continuously with warmed oxygenated physiological saline, composition (mM) NaCl 120, KCl 5.0, $CaCl_2$ 2.5, $MgCl_2$ 2.0, $NaHCO_3$ 25, NaH_2PO_4 1.0, glucose 11, and was equilibrated with 95% O_2 /5% CO_2 . High potassium solution was made by replacing 95 mmol/l of NaCl with KCl, to give a final K^+ concentration of 100 mM. It was applied to the arteriole by pressure ejection from a micropipette using pulses 100–400 ms in duration, or in the superfusing solution to determine the maximum constriction for the arteriole (Neild and Kotecha, 1989).

The potentiating effect of NPY was calculated using an index S (Xia *et al.*, 1992; Neild and Xia, 1997) defined as:

$$S = \log_{10} \left[\left(\frac{n}{\max - n} \right) / \left(\frac{c}{\max - c} \right) \right]$$

where c is the amplitude of the control constriction, n is the amplitude of the constriction to the same stimulus in the presence of NPY or a selective agonist for Y1 or Y2 receptors, and $\max$ is the maximum constriction of which the arteriole is capable. S gives an index of the potentiating effect; $S=0$ for no effect, $S>0$ for potentiation and $S<0$ for depression. Differences between S values were analysed by ANOVA followed by a Bonferroni test. Student's t test was used to determine whether individual values differed from 0. A probability

value <0.05 was considered significant.

Rat mesenteric artery smooth muscle cells

Male Wistar rats (250–300 g) were killed by stunning, cervical dislocation and carotid exsanguination. The mesentery was removed and second and third order arteries were dissected out and cleaned of connective tissue in a low calcium solution with the following composition (mM) NaCl 137, KCl 5.4, HEPES 10, $MgCl_2$ 1, Na_2HPO_4 0.4, NaH_2PO_4 0.4, $NaHCO_3$ 4.1, glucose 10, $CaCl_2$ 0.1, (pH 7.4 with NaOH). Acutely dissociated smooth muscle cells were prepared by enzymatic digestion using a combination of papain with dithioerythritol (DTE) and collagenase Type F with hyaluronidase (Kubo *et al.*, 1997).

Cells were plated onto glass cover slips, stored at 4°C and used on the day of preparation. The smooth muscle cells were superfused at 2 ml min⁻¹ with a solution of the following composition (mM): NaCl 130, KCl 5.4, glucose 10, HEPES 10, and either $BaCl_2$ 10 with EGTA 0.1 or $CaCl_2$ 10, adjusted to pH 7.3 with NaOH. The barium solution was used most often because currents evoked in this solution had a larger amplitude and were more resistant to rundown. Patch electrodes (2–5 MΩ) were filled with a solution of the following composition (mM): CsCl 150, HEPES 10, EGTA 10, GTP 0.3, phosphocreatine 8.8, $MgCl_2$ 2.1, $CaCl_2$ 0.1 (pH 7.3 with CsOH). Whole cell recordings were made with an Axopatch 200 A amplifier and data was collected using pClamp6 software (Axon Instruments USA).

Smooth muscle cells had a capacitance of 12.8 ± 0.5 pF ($n=70$) and series resistance was always less than 10 MΩ. Holding potential was -60 mV and peak calcium currents were elicited once every minute by a 100 ms voltage step to $+10$ mV. Similar current voltage relationships were determined using either barium or calcium as the main charge carrying ion and in both cases the peak calcium current was evoked at $+10$ mV, therefore results using both solutions were pooled. Amphotericin permeabilised patch recordings were also carried out ($n=14$) and there was no significant difference between the stability of calcium currents recorded using this method and whole cell recordings (only whole cell recording data was used for analysis). All recordings were made at room temperature (20–25°C). Drugs were added to the superfusate and effects on calcium currents were recorded 2 minutes after complete bath solution exchange (approx 1 minute). Data was sampled at 5 kHz and subsequently filtered at 800 Hz using clampfit (pClamp6 software, Axon Instruments). Leak and capacitive currents have been subtracted from the whole cell currents in all the traces shown in this paper using the P/4 protocol in pClamp6.

To determine if enzymatic dissociation affected G-protein coupled mechanisms the effect of noradrenaline (3–10 μM) on calcium currents was tested. Noradrenaline evoked a $31.5 \pm 6.8\%$ potentiation of calcium currents in 4 cells, had no effect on calcium currents in 10 cells, and showed an inhibition in 2 cells. It has been shown that there is a degree of variability in the α-adrenoceptor mediated modulation of calcium currents in vascular smooth muscle cells and it is common not to get modulation in dissociated smooth muscle cells in some preparations and in only a percentage of cells in a responsive preparation (Benham and Tsien, 1988). From these results it can be seen that G-protein coupling was evident in 40% of the mesenteric smooth muscle cell population. Due to this variability in response within the population the

mean percentage of effect $\pm$ standard error of the mean of any given drug was calculated only from the responsive cells (Wellman *et al.*, 1998).

Drugs used were neuropeptide Y (porcine sequence, synthesised in the Department of Biochemistry, Monash University); PYY-(13-36), [Leu³¹, Pro³⁴]NPY, *N*-acetyl[Leu²⁸, Leu³¹]NPY-(24-36) (Auspep, Melbourne, Australia); ω -conotoxin GVIA (Sapphire Bioscience, Alexandria, NSW, Australia); tetrodotoxin, noradrenaline bitartrate, papain, DTE, collagenase type F and hyaluronidase (SIGMA).

Results

Submucosal arterioles

Transient constrictions of the arterioles were evoked every 5 minutes by the application of 100 mM K⁺ solution from a micropipette. The duration of the application and the position of the micropipette was varied to give an amplitude of around 20% of the maximum constriction possible. The constrictions were confined to a region 200 μ m either side of the application pipette and their amplitudes were not altered by 30 nM ω -conotoxin GVIA or 300 nM tetrodotoxin, so it was concluded that the K⁺ was acting by depolarization of the smooth muscle of the arterioles and not by release of neurotransmitter from perivascular nerves.

Following at least 5 control constrictions of repeatable amplitude, NPY or related compounds were added to the superfusing solution in known concentrations. The compounds were added for two minutes before a K⁺ application, and washed off immediately afterwards (Figure 1). This protocol has been shown to maximise the potentiating effects of Y1 receptor stimulation in this preparation by giving time for the NPY to act but minimising desensitization (Van Riper and Bevan, 1991, Xia *et al.*, 1992). However, only one application of an NPY receptor agonist was made on a preparation in the current experiments to avoid any possibility of

Fig. 1. Records of arteriole diameter from two arterioles to show the effects of agonists selective for Y1 ([Leu³¹ Pro³⁴]NPY 50 nM, upper trace) and Y2 (PYY(13-36) 50 nM, lower trace) receptors on constrictions caused by brief applications of high K⁺ solution by pressure ejection from a micropipette. At the start of each experiment a maximum constriction was evoked by superfusion with 100 mM K⁺ solution (unfilled bar). High K⁺ was then applied every five minutes (filled circles) until stable responses were obtained, and the NPY receptor agonist was added in the superfusing solution for 2 minutes prior to a constriction (filled bar).

Fig. 2. Potentiation and depression of constrictions caused by brief applications of K^+ , expressed in terms of the parameter S as defined in the Methods. $S > 0$ represents potentiation, $S < 0$ represents depression. Effects of NPY (●, solid line), the Y1 agonist $[Leu^{31}, Pro^{34}]NPY$ (■, dashed line), and the Y2 agonists PYY(13-36) (▲, dotted line) and N -acetyl $[Leu^{28}, Leu^{31}]NPY$ -(24-36) (△) are shown. Standard error ($n=6$) bars are shown on two points; 20 nM NPY and PYY(13-36). The lines drawn through the points are arbitrary.

variation caused by desensitization.

The selective Y2 agonists PYY-(13-36) (Wahlestedt and Hakanson, 1986) and N -acetyl $[Leu^{28}, Leu^{31}]NPY$ -(24-36) (Potter *et al.*, 1994; Modin *et al.*, 1996) both reduced the constrictions caused by K^+ . PYY-(13-36) was used in concentrations from 50 pM–100 nM, and was effective in concentrations of 10 nM or greater. N -acetyl $[Leu^{28}, Leu^{31}]NPY$ -(24-36) was used at 50 and 100 nM. The results are summarized in figure 2. Higher concentrations of the peptides were not used in an attempt to ensure that the Y2-selective agonists acted mainly on Y2 receptors with minimal interaction with Y1 receptors.

$[Leu^{31}, Pro^{34}]NPY$ caused potentiation as previously reported (Xia *et al.*, 1992). The maximum effect occurred at 10 nM, with lesser effects at higher concentrations. $[Leu^{31}, Pro^{34}]NPY$ is selective for Y1 and the structurally similar Y5 receptors in preference to Y2 receptors (Fuhlendorff *et al.*, 1990; Michel *et al.*, 1998), and its effects on blood vessels are generally considered to be mediated via Y1 receptors. The effects of NPY were similar and not significantly different (ANOVA/Bonferroni, $p > 0.05$) from those of $[Leu^{31}, Pro^{34}]NPY$, suggesting that the major effect of both agonists was mediated via the same receptors. The effects of PYY-(13-36) were significantly different from those of NPY and $[Leu^{31}, Pro^{34}]NPY$.

These results indicate that Y2 receptor activation reduced the constrictor effect of high K^+ . The constrictions were due to depolarization and Ca^{2+} entry through voltage activated Ca^{2+} channels, and we therefore investigated the effect of Y2 receptor activation on Ca^{2+} currents in vascular smooth muscle cells. It was not practical to isolate cells from the submucosal arterioles of the guinea-pig small intestine due to the small size of the tissue sample and the low proportion of arterioles in the tissue, so the experiments were carried out on mesenteric arterioles from rats. Rat mesenteric cells have been used in previous studies (Kubo *et al.*, 1997) and their basic properties have been well characterised, whereas at the time of this study there

Fig. 3. Voltage dependent calcium currents from isolated rat mesenteric artery cells were blocked by nifedipine. Current record obtained from a cell in the absence of nifedipine (control), 3 minutes after superfusion with nifedipine ($1\ \mu\text{M}$) and 5 minutes after washout of nifedipine (wash).

was no comparable baseline data from guinea-pig mesenteric artery cells.

Effects of NPY and selective Y1 and Y2 agonists on vascular smooth muscle cell calcium currents

Acutely dissociated smooth muscle cells were prepared from small mesenteric arteries of rats as described above, and voltage clamped using whole cell patch clamp. Calcium currents were evoked by a voltage step to $+10\ \text{mV}$ in 129 cells. The calcium channel blockers cadmium ($1\ \text{mM}$) or nifedipine ($1\ \mu\text{M}$) reduced calcium currents by $94.4 \pm 2.17\%$ ($n=7$) and $91 \pm 0.3\%$ ($n=4$, Figure 3) respectively. The time course and the dihydropyridine sensitivity of the calcium currents indicate that the currents were initiated through L-type calcium channels (Benham and Tsien, 1988).

NPY ($100\ \text{nM}$) inhibited peak calcium current amplitude in 6 of 9 cells ($26.4 \pm 5.3\%$ Figure 4A). The effect was reversed on washout. NPY acts through Y1 and Y2 receptors, so to determine which of the subtypes were involved in this inhibitory effect the more subtype selective NPY analogues were used. The selective Y1 receptor agonist [Leu³¹, Pro³⁴] NPY (50 – $100\ \text{nM}$) potentiated the calcium current amplitude in 2 of 8 cells and this effect was reversed on washout (Figure 4B). The rest of the cells showed no effect. The selective Y2 receptor agonist *N*-acetyl[Leu²⁸, Leu³⁴]NPY-(24–36) ($100\ \text{nM}$) inhibited peak calcium current amplitude in 5 of 10 cells ($36.9 \pm 9.3\%$ Figure 4C). This effect was reversed on washout. This suggests that Y2 receptors are coupled to L-type calcium channels in these cells and activation of these receptors results in an inhibition of calcium currents.

Discussion

Our results suggest that NPY may have a functionally inhibitory effect on the smooth muscle of mesenteric resistance vessels and intestinal arterioles when muscle contraction is caused by depolarization and subsequent Ca^{2+} influx. This inhibitory effect was mediated by

Fig. 4. Effect of NPY and selective agonists on calcium channel currents. A. NPY (100 nM) was applied for 3 minutes and caused an inhibition of peak current amplitude. B. [Leu³¹Pro³⁴] NPY (50 nM), a Y1 receptor selective agonist, was applied for 3 minutes and caused a weak potentiation of the current amplitude. C. N-Acetyl[Leu²⁸Leu³¹] NPY 24-36 (100 nM), a Y2 receptor selective agonist, was applied for 3 minutes and caused an inhibition of peak current.

Y2 receptors, as it could be produced by two different Y2-selective agonists, and is distinct from the vasoconstriction or potentiation of the effects of other vasoconstrictors that are mediated by Y1 receptors in the same vessels (Andriantsitohaina and Stoclet, 1988, Xia *et al.*, 1992). Y2 receptor activation has also been reported to inhibit vasoconstriction caused by noradrenaline in the hamster cheek pouch (Boric *et al.*, 1995). This may not be the same phenomenon that we observe, however, as in the guinea-pig submucosal arterioles constriction in response to noradrenaline is not affected by Y2 agonists (Xia *et al.*, 1992), only responses to K⁺ are reduced.

The inhibition that we have seen could be explained by reduction of depolarization-induced Ca²⁺ influx, and our observations of a reduction of Ca²⁺ currents by PYY(13-36) support this view. Reduction of Ca²⁺ currents by NPY has been observed in several other tissues (Xenopus oocytes, Sun *et al.*, 1997; hippocampal neurones, Qian *et al.*, 1997; cardiac myocytes, Bryant and Hart, 1996; adrenal chromaffin cells, Lemos *et al.*, 1997; sympathetic neurones, Foucart *et al.*, 1993, Wiley *et al.*, 1993). The effect is not confined to one type of Ca²⁺ channel, with N and P/Q channels affected in rat hippocampal neurones (Qian *et al.*, 1997) and L channels in cardiac myocytes (Bryant and Hart, 1996) and PC12 cells (McCullough and Westfall, 1996). In contrast

activation of Y1 receptors has increased L channel currents in some studies on vascular smooth muscle (Xiong *et al.*, 1993), and we observed a small effect of this type in 25% of cells in which it was sought.

In the majority of vascular tissues Y2 agonists have little effect on smooth muscle unless they are used at concentrations high enough to activate Y1 receptors (Morris and Sabesan, 1994), although in the pig spleen (Modin *et al.*, 1991) and the rat femoral artery (Tessel *et al.*, 1993) they cause constriction. When a vasodilator effect of a Y2 agonist was sought in rat iridial arterioles (Newhouse and Hill, 1997) it was not found, and it is clear that the phenomena that we have observed are not common to all arteries. Further studies in which contractile properties and Ca^{2+} currents are measured in the same artery are needed.

The natural ligand for both Y1 and Y2 receptors on arteriolar smooth muscle is probably nerve-released NPY, and the reduction of Ca^{2+} currents and constriction that we have observed could form that basis of a vasodilator action of NPY. NPY acting on Y2 receptors would reduce vasoconstrictor influences that relied on smooth muscle depolarization such as ATP released from sympathetic nerves, but would have little effect on processes that relied mainly on release of Ca^{2+} from internal stores. Rather than acting against all vasoconstrictor influences, Y2 activation would specifically antagonise depolarization, which is probably the main source of smooth muscle tone in resistance vessels (Hirst and Edwards, 1989). Thus NPY in vasodilator nerves may have a unique physiological role, acting against constriction caused by alterations of membrane potential while not affecting constrictor processes that work through modulation of release from the internal Ca^{2+} stores.

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