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Mechanism of interaction between neuropeptide Y and angiotensin II in the rabbit femoral artery

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Abstract

Neuropeptide Y has direct vasoconstrictor actions and potentiates the effects of other vasoconstrictor agents. To find out whether both effects of neuropeptide Y are mediated via the same receptor and intracellular mechanism, the interaction between neuropeptide Y and angiotensin II was studied in rabbit femoral arteries. In this preparation, neuropeptide Y, but not its 13–36 fragment, induced constriction. Only neuropeptide Y potentiated the vasoconstrictor response to angiotensin II and the associated rise in inositol-1-phosphate. These potentiating effects of neuropeptide Y were totally prevented by removal of extracellular Ca^{2+}, partially prevented by a Ca^{2+}-channel blocker and mimicked by a Ca^{2+}-channel activator. Pharmacological modulation of adenylate cyclase had no effect. These results suggest that the direct and indirect vascular effects of neuropeptide Y are mediated via Y_1 receptors and depend on the influx of extracellular Ca^{2+}. The rise in inositol-1-phosphate seems to be secondary to an increase in intracellular Ca^{2+}, while modulation of adenylate cyclase is apparently not involved.

Keywords: Neuropeptide Y; Angiotensin II; Vasoconstriction; Second messenger; Femoral artery

1. Introduction

Neuropeptide Y, a 36-amino acid peptide first described by Tatemoto in 1982 (Tatemoto, 1982; Tatemoto et al., 1982), is widely distributed in the mammalian central and peripheral nervous system. It is co-released with noradrenaline from sympathetic nerve endings in the vasculature, where it exerts various effects (Michel, 1991; Wahlestedt and Reis, 1993). Via postjunctional receptors of the Y_1 subtype, neuropeptide Y acts as a direct vasoconstrictor. Presumably via the same receptor subtype, it may potentiate the action of other endogenous vasoconstrictors. Finally, neuropeptide Y inhibits the release of noradrenaline via prejunctional receptors of the Y_2 subtype. Through these various actions neuropeptide Y might play an important role as a modulator of sympathetic activity in the vascular system (Michel, 1991; Wahlestedt and Reis, 1993). However, the vasoconstrictor response to neuropeptide Y varies widely in different segments of the vascular tree and between species. Moreover, it is still not clear whether the direct and indirect vascular effects of neuropeptide Y are mediated by the same intracellular messenger systems.

In the present experiments we studied the vascular effects of neuropeptide Y in the rabbit femoral artery. In contrast to previous observations (Adamsson and Edvinsson, 1991) we found that, under our experimental conditions, neuropeptide Y exerts a direct and an indirect vasoconstrictor action in this preparation. This made it possible to investigate these functional responses and the underlying intracellular mechanisms in the same experimental model. In a first series of experiments we studied the direct vasoconstrictor effects of neuropeptide Y and its potentiating effects on the vasoconstriction induced by angiotensin II. In a second series we assessed the possible involvement of inositol phosphates (IP) as an intracellular messenger system. In these experiments we measured the accumulation of inositol phosphates under different experimental conditions. Pharmacological agents known to stimulate or inhibit adenylate cyclase were used to estimate the possible role of cAMP in modulating the effects of neuropeptide Y on IPs. Changes in extracellular Ca^{2+}

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and pharmacological modulators of Ca^{2+} channels were used to analyse the importance of this intracellular messenger and to identify its possible sources.

2. Materials and methods

Male Chinchilla rabbits were obtained from Dr. Thomae AG, Biberach an der Riss, Germany. They were fed a standard laboratory diet and allowed free access to tap water. Animals weighing 2.5–3 kg were used. The rabbits were killed by a blow to the neck. Both femoral arteries were removed, transferred into physiological salt solution, carefully dissected free of all connective tissue, and cut into rings.

2.1. Studies of contraction

For studies of contraction, 2-mm-wide rings were suspended between two parallel silver hooks in 10-ml organ baths containing a physiological salt solution (NaCl 112.0 mM, KCl 5.0 mM, $NaHCO_3$ 25.0 mM, NaH_2PO_4 1.0 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, glucose 11.2 mM) according to Criscione et al. (1989, 1990). This solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2 to obtain a pH of 7.4. Isometric tension was measured with a force-displacement transducer (Biegestab K30, Hugo Sachs Elektronik, Freiburg im Breisgau, Germany) coupled to a potentiometric pen recorder (Lineacord Mark VII, WR 3101, Hugo Sachs Elektronik). The rings were adjusted to a tension of 500 mg. After an equilibration period of 60 min, the rings were maximally contracted with 10 μM noradrenaline. The preparations were then washed and, 30 min later, stimulated with 0.1 μM noradrenaline. The presence of an intact endothelium was checked by the addition of 0.1 μM acetylcholine, which normally induces total relaxation of noradrenaline-precontracted rings. Preparations showing incomplete relaxation were rejected. The rings were then washed and equilibrated for a further 45 min. This phase of the protocol was the same in all experiments.

Even when derived from the same animal, the rings showed considerable variability in sensitivity and amplitude of contraction in response to neuropeptide Y. When the contraction induced by neuropeptide Y was compared with that induced by noradrenaline, no significant correlation was demonstrable. For this reason the results are expressed as absolute values (i.e., mg tension), rather than as percentages of the maximum noradrenaline-induced contraction.

2.2. Studies with inositol phosphates

For the measurement of inositol phosphates according to Legan (1989) and Berridge (1984), the femoral arteries were cut into rings 3–4 mm wide (8–9 rings per vessel), which were incubated in 1 ml of a physiological solution of the following composition: NaCl 119.0 mM, KCl 4.8 mM, $NaHCO_3$ 24.8 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM, glucose 10.0 mM. The solution was gassed with a mixture of 95% O_2 and 5% CO_2 and was maintained at 37°C. After 30 min preincubation, 10 μCi [^{3}H]myo-inositol was added per ml of solution. After incubation for 2 h the rings were carefully washed 3 times to remove free [^{3}H]myo-inositol. Each ring was then transferred to a small tube containing 480 μl of the physiological solution, placed in an incubator at 37°C and gassed. A solution of LiCl, 10 μl, was added to each tube to obtain a final concentration of 10 mM. Ten minutes later, the agonists were added in a volume of 10 μl each. After 60 min incubation, the reaction was stopped by addition of 300 μl of an ice-cold mixture of chloroform/methanol (1:2). The tubes were vortexed for 30 s, then 300 μl of chloroform was added, followed by 300 μl H_2O, the tubes being vigorously vortexed after each addition. Thereafter, they were centrifuged at $1000 \times g$ for 5 min. An aliquot of 900 μl of the upper aqueous phase was loaded on Dowex columns prepared as follows. Small pieces of glass fibre were inserted into Pasteur pipettes and topped with a layer of 200 μl of a sand suspension, followed by 1 ml of Dowex (1X8, Fluka). The resin was then charged with 10 ml of ammonium formate 1 M. The charged columns were stored at 4°C. Before use, they were washed with 10 ml of water. The columns loaded with the extract were first washed with 10 ml water to eliminate the free [^{3}H]myo-inositol. Then the inositols were eluted in the following sequence: glycerol phosphorylinositol with 6 ml of 5 mM sodium tetraborate/60 mM sodium formate; inositol monophosphate (IP_1) with 6 ml of 200 mM ammonium formate/100 mM formic acid; inositol bisphosphate (IP_2) with 8 ml of 400 mM ammonium formate/100 mM formic acid; inositol triphosphate (IP_3) with 8 ml of 800 mM ammonium formate/100 mM formic acid. From the eluate of each fraction, 6 ml was mixed with 15 ml Irgascint and vigorously agitated. After at least an hour, the vials were counted in a beta counter (Packard, Tri-Carb 1500).

2.3. Drugs

Porcine neuropeptide Y (Sigma) and neuropeptide Y-(13–36) (Ciba-Geigy, Basel) were dissolved in water as 500 μM stock solution and stored at −20°C. Angiotensin-II amide (Hypertensin, Ciba) and acetylcholine HCl (Roche) were dissolved in water as 1 mg/ml stock solution and stored at −20°C. Noradrenaline HCl was prepared as 1 mg/ml stock solution containing ascorbic acid 0.1 mM and stored in the dark at 4°C. Nitrendipine (1,4-dihydro-2,6-dimethyl-4-

(3-nitrophenyl)-pyridin-3,5-dicarboxylic acid ethyl-methyl ester) (synthesized at Ciba-Geigy) was dissolved in DMSO as 10 mM stock solution and stored at −20°C. D,L-Isoprenaline(4-[1-hydroxy-2-[(1-methyl-ethyl)-amino] ethyl]-1,2-benzenediol) (Sigma, St. Louis, MO, USA) solutions were diluted with saline containing 1% ascorbic acid and were kept in the dark before use. Clonidine HCl (2,6-dichloro-*N*-2-imidazolidinylidenebenzenamine) (synthesized at Ciba-Geigy) was dissolved in water as 1 mM stock solution and kept in the dark at −20°C. BHT 933 Cl2 (2-amino-6-ethyl-5,6,7,8-tetrahydro-4*H*-oxazolo[4,5-*d*]azepine) (Boehringer Ingelheim) was dissolved in water as 1 mM stock solution and stored at −20°C. Prazosin HCl (1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarboxyl) piperazine) (synthesized at Ciba-Geigy) was dissolved in ethanol as 1 mM stock solution; dilutions were made with water. BayK 8644 (2,6-dimethyl-5-nitro-4-[2-trifluoromethyl-phenyl]-1,4-dihydro-pyridine-3-carboxylic acid methyl ester) (Bayer) was dissolved in ethanol as 1 mM stock solution; dilutions were made with water.

2.4. Experimental protocol

2.4.1. Studies of contraction

In an initial series of experiments, a dose-response curve for neuropeptide Y was established. Most rings were exposed to cumulative concentrations of neuropeptide Y; single concentrations were only used in a few cases. Further studies were performed to determine whether extracellular Ca^{2+} played a role in the contraction induced by neuropeptide Y: in a first series, a Ca^{2+}-free medium was used. In some of these experiments, EGTA (2 mM) was added to this medium. In a second series, the Ca^{2+}-channel blocker, nitrendipine, was used at concentrations of 1 μM to determine whether neuropeptide Y-induced Ca^{2+} entry occurred via voltage-dependent, L-type Ca^{2+} channels. The rings were incubated with the drug 10–15 min before the addition of neuropeptide Y.

To test the indirect effect of neuropeptide Y, angiotensin II was given after 5 min preincubation with neuropeptide Y or its 13–36 fragment. Because of tachyphylaxis we used only single concentrations of angiotensin II and measured the development of tension over 20 min. Control and neuropeptide Y-pretreated rings were always tested at the same time.

2.4.2. Studies with inositol phosphates

Neuropeptide Y was added to the preparations 2 min before angiotensin II, and incubation was continued for a further 60 min. To inhibit adenylate cyclase, we used the α_2-adrenoceptor agonists, clonidine or BHT 933, and to stimulate it, the β-adrenoceptor agonist, isoprenaline. Both α_2- and β-adrenoceptors are present in our preparation (Satake and Shibata, 1987; Demirel et al., 1989; Li and Zimmerman, 1991). We preincubated the rings with either of these drugs for 5–10 min before the addition of angiotensin II.

For the modulation of Ca^{2+} the rings were incubated in Ca^{2+}-free medium, and 2 mM EGTA was added 1 min before the administration of the agonist. In pilot experiments, we had observed that the responses to the agonists were abolished with longer preincubation times. Angiotensin II was tested at the concentration at which we had observed the strongest potentiation of IP_1 formation (1.0 μM). To find out whether neuropeptide Y promoted the influx of extracellular Ca^{2+} via the L-type channels, we used nitrendipine at the same concentration (1.0 μM) as in the studies on contraction. Preincubation lasted 10 min. To find out whether an influx of Ca^{2+} from the

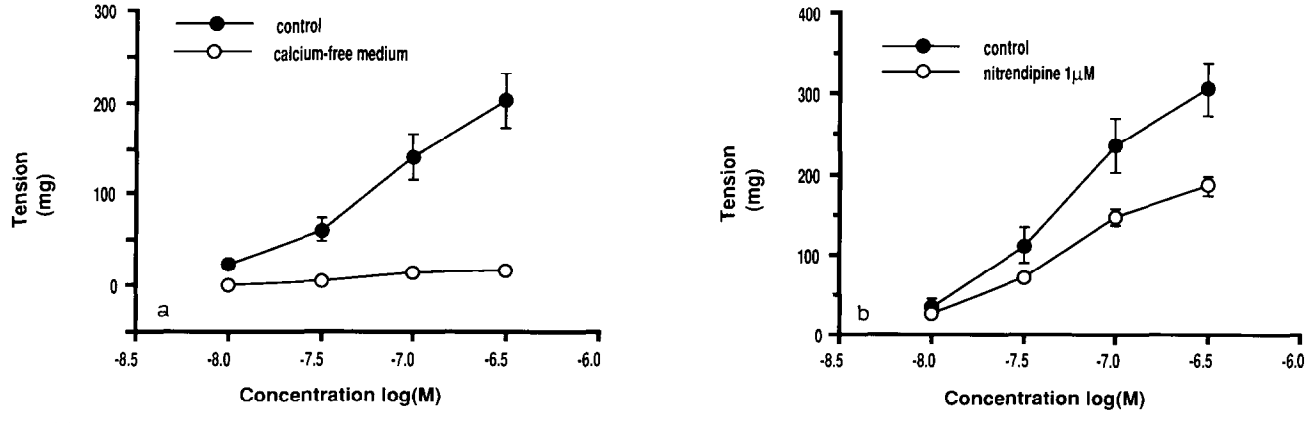

Fig. 1. (a) Effects of neuropeptide Y in the rabbit femoral artery in the presence (filled circles) or the absence (open circles) of extracellular Ca^{2+}. Responses are expressed in mg tension. Each point represents the mean of at least 6 observations ± S.E.M. (b) Effects of neuropeptide Y in the rabbit femoral artery in the absence (filled circles) or the presence (open circles) of the Ca^{2+}-channel blocker, nitrendipine (1 μM). Responses are expressed in mg tension. Each point represents the mean of at least 6 observations ± S.E.M.

extracellular space affects the IP_1 formation, we used the Ca^{2+}-channel opener BayK 8644. As with neuropeptide Y, preincubation lasted 2 min.

All results are expressed as percentages of IP_1 formation. No changes in IP_2 or IP_3 were observed. This can be explained by the fact that, during the long incubation time, all inositol phosphates are transformed to IP_1.

2.5. Statistics

The results are expressed as means ± S.E.M. The significance of differences was evaluated using Student's *t*-test, *P* values less than 0.05 being considered significant.

3. Results

3.1. Studies of contraction

Neuropeptide Y induced concentration-dependent contractions (Fig. 1a). The threshold concentration was between 1 and 10 nM and maximal contraction was obtained with 300 nM. The EC_{50} of neuropeptide Y was 39 nM, while that of noradrenaline in this preparation was 273 nM. The maximum tension obtained with neuropeptide Y (300 nM) was 224 ± 18 mg ($n = 32$ rings); noradrenaline (300 nM) induced a much greater increase in tension (1368 ± 100 mg). The response to neuropeptide Y showed a marked tachyphylaxis: even 2 h after the first administration of neuropeptide Y (300 nM) a second administration at the same concentration induced either no change or only a very slight increase in tension.

After 15 min preincubation in Ca^{2+}-free medium, neuropeptide Y was completely inactive, even at the highest concentration, irrespective of the presence or absence of the Ca^{2+} chelator, EGTA (2 mM) (Fig. 1a). Nitrendipine diminished the effect of neuropeptide Y, but did not totally abolish it. Maximal contraction was reduced to 185 ± 12 mg, as compared to 305 ± 32 mg in control preparations (Fig. 1b).

In response to 1 nM angiotensin II, contraction reached its maximum (529 ± 56 mg) within 8 min (Fig. 2a). In the presence of neuropeptide Y (50 nM), a maximum of 1010 ± 74 mg was also reached at 8 min. Under both conditions, the contraction was sustained for up to 20 min, and the difference between control and neuropeptide Y-pretreated rings remained significant during the whole period. At 10 nM angiotensin II, the magnitude of contraction was more pronounced. It reached its maximum earlier than at the lower concentration of angiotensin II (1 nM), the maximal value, 1868 ± 59 mg, being observed after 3 min. Thereafter, tension decreased towards the resting levels, but was still 578 ± 126 mg after 20 min. In the presence of 50 nM neuropeptide Y, the initial phase of angiotensin II-induced contraction was similar; the maximal increase in tension was also reached within 3 min and amounted to 1843 ± 59 mg. However, the tension decreased more slowly and remained at a higher level than in the controls. The differences between control and treated rings were significant only between 16 and 20 min. At the end of the experiment, the residual tension in neuropeptide Y-pretreated rings was 1030 ± 68 mg, about twice as high as in the control.

To test the effect of neuropeptide Y-(13–36) on angiotensin II-induced contraction, we used 1 nM angiotensin II, a concentration at which significant poten-

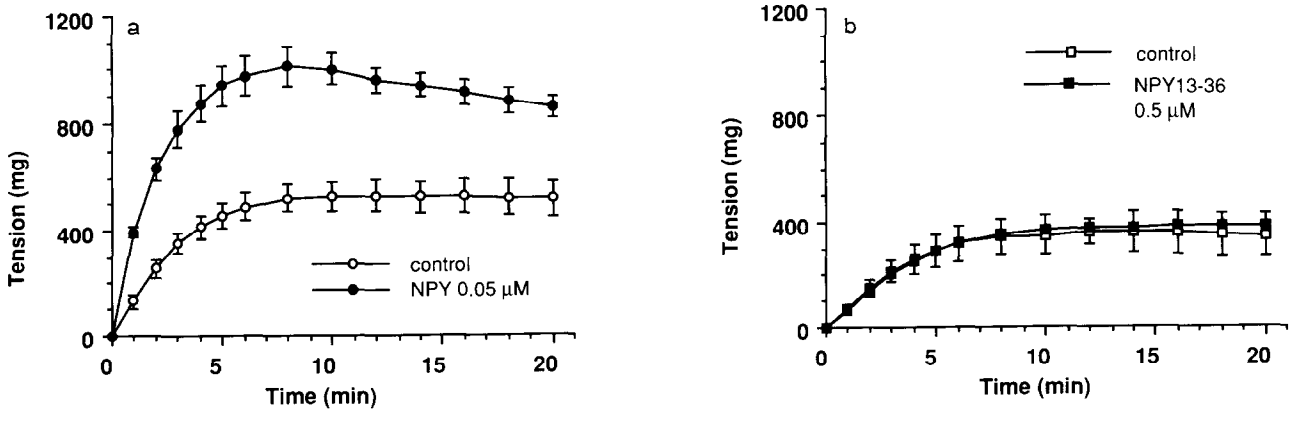

Fig. 2. (a) Time course of the contraction induced by angiotensin II (ANGII) (1 nM) in the rabbit femoral artery in the absence (open circles) or the presence (filled circles) of neuropeptide Y (NPY) (50 nM). Responses are expressed in mg. Each point represents the mean of at least 5 observations ± S.E.M. (b) Time course of the contraction induced by angiotensin II (ANGII) (1 nM) in the rabbit femoral artery in the absence (open squares) or the presence (filled squares) of neuropeptide Y fragment 13–36 (NPY13–36) (500 nM). Responses are expressed in mg. Each point represents the mean of at least 5 observations ± S.E.M.

tiation was observed with the whole neuropeptide Y peptide. When we preincubated the rings with the neuropeptide Y-(13–36) fragment at 500 nM, a concentration 10 times higher than that used with neuropeptide Y 1-36, we observed no difference in the effect of angiotensin II (Fig. 2b). Maximal effects were 357 ± 83 mg and 382 ± 22 mg in control and pretreated rings. Neuropeptide Y-(13–36) alone, up to 1 μM, did not induce any change in tension.

3.2. Studies with inositol phosphates

Incubation of the rings with 1 μM neuropeptide Y for 1 h induced no change in the production of inositol phosphates. Angiotensin II (0.01–1.0 μM) caused a concentration-dependent increase in IP_1 (Fig. 3). In the presence of neuropeptide Y (1 μM), a substantial increase in angiotensin II-induced formation of IP_1 was observed. The difference between control and neuropeptide Y-treated rings reached significant levels at the two higher concentrations of angiotensin II (0.1 and 1.0 μM).

Neither neuropeptide Y (1 μM) nor neuropeptide Y-(13–36) (1 μM) alone had an effect on the basal production of IP_1. The respective values of 110 ± 8% and 102 ± 8% were not significantly different from the control (Fig. 3). As in the preceding experiment, neuropeptide Y (1 μM) more than doubled the effect of angiotensin II (1 μM); the values were 576 ± 52% in the presence of neuropeptide Y and 252 ± 37% in the controls. In contrast, the value obtained with the fragment was the same as for the control rings (224 ± 25%).

Clonidine was tested at 0.01, 0.1, and 1.0 μM; only the highest concentration induced a significant increase in the basal production of IP_1, to 146 ± 12.5%.

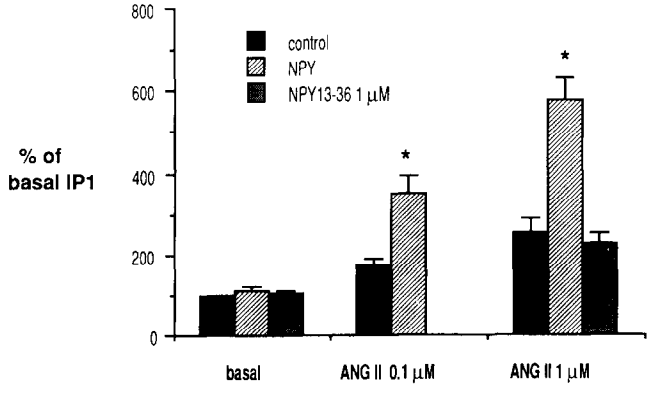

Fig. 3. Changes in basal or angiotensin II (ANGII)-induced (1 μM) formation of IP_1 in the rabbit femoral artery, in the absence (solid bars) or the presence of neuropeptide Y (NPY) (1 μM, hatched bars) or neuropeptide Y-(13–36) (NPY13–36) (1 μM, stippled bars). Results are expressed in percents of basal formation of IP_1. Each bar represents the mean of 6–9 observations. Error bars show S.E.M. * Significant difference ($P < 0.01$) from control.

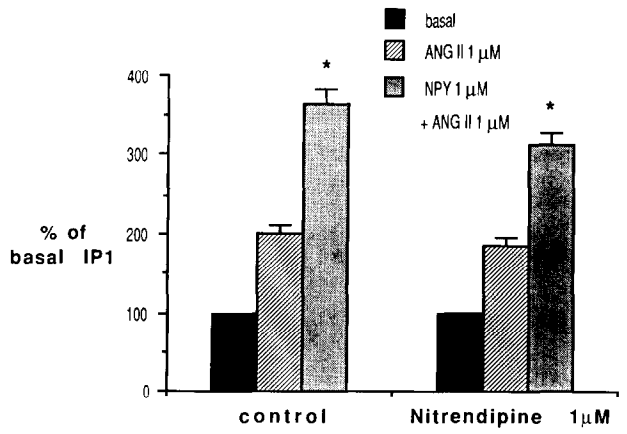

Fig. 4. Effect of the Ca^{2+}-channel blocker, nitrendipine (1 μM), on the changes in IP_1 formation in the rabbit femoral artery, under basal conditions (solid bars), in the presence of angiotensin II (ANGII) (1 μM) (hatched bars) or the presence of neuropeptide Y (NPY) (1 μM) and angiotensin II (ANGII) (1 μM) (stippled bars). Results are expressed in percents of basal formation of IP_1. Each bar represents the mean of 6–9 observations, error bars show S.E.M. * Significant difference ($P < 0.05$) from ANGII-treated group.

When the rings were pretreated with prazosin (1 μM) this effect was no longer seen. Angiotensin II (1 μM) alone increased IP_1 production by up to 225 ± 8.8%. In the presence of 0.01 μM and 0.1 μM of clonidine, no significant additional effect was observed (249 ± 24% and 270 ± 12.6% respectively), but at 1 μM the difference was statistically significant (301 ± 21.1%, $P < 0.05$). However, the more specific α_2-adrenoceptor agonist, BHT 933 (Kobinger, 1986), at a concentration of 1 μM induced no change in IP_1 production under either basal or stimulated conditions.

The β-adrenoceptor agonist, isoprenaline, at two concentrations (0.2 and 2.0 μM) affected neither the basal production of IP_1 nor the angiotensin II (1 μM)-induced increase in IP_1 formation.

In Ca^{2+}-free medium, neuropeptide Y (1 μM) did not potentiate the effects of angiotensin II (1 μM): 145 ± 8% in the absence and 145 ± 8% in the presence of neuropeptide Y (1 μM). The action of angiotensin II on basal IP_1 formation was reduced by approximately 50% in Ca^{2+}-free medium.

The IP_1 values in control and nitrendipine-pretreated rings were similar (Fig. 4). Nitrendipine affected neither angiotensin II-induced stimulation of IP_1 formation, nor the potentiating effect of neuropeptide Y. The values for control preparations (199 ± 11.4% for angiotensin II and 363 ± 19.4% for neuropeptide Y + angiotensin II) were not significantly different from those in nitrendipine-pretreated rings (184 ± 10.5% for angiotensin II and 311 ± 15.7% for neuropeptide Y + angiotensin II).

BayK 8644 (1 μM) did not induce any change in the basal formation of IP_1 (93 ± 5.0%) (Fig. 5). However, it

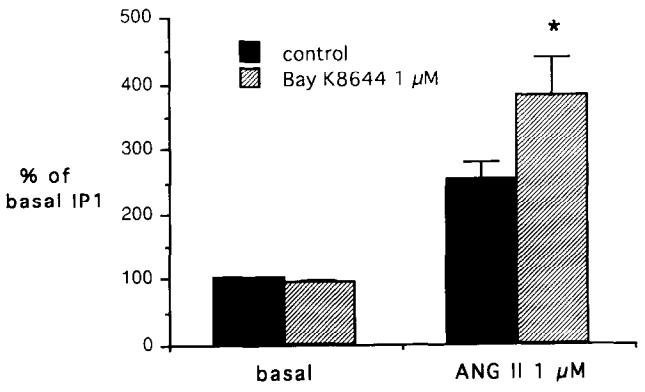

Fig. 5. Changes in basal or angiotensin II (ANGII) (1 μM)-induced formation of IP_1 in the rabbit femoral artery, in the absence (solid bars) or the presence (hatched bars) of the Ca^{2+}-channel opener, BayK 8644 (1 μM). Results are expressed in percents of basal formation of IP_1. Each bar represents the mean of 6–9 observations, error bars show S.E.M. *Significant difference ($P < 0.05$) from the respective control.

increased the angiotensin II-induced formation of IP_1 from 252 ± 27.5% to 379 ± 58.7%. This potentiating effect was not as pronounced as that of neuropeptide Y, but was statistically significant ($P < 0.05$).

4. Discussion

The magnitude of the direct vasoconstrictor effects of neuropeptide Y depends on the species, on the localization of the vessel, and on its calibre. In our experimental model, the rabbit femoral artery, neuropeptide Y induced a slowly developing, sustained contraction. The response of individual preparations was variable, even among rings cut from the same femoral artery, and correlated with neither sensitivity to noradrenaline, nor the integrity of the endothelium. Abel and Han (1989) found that, in cerebral arteries of rabbits, neuropeptide Y also had a weak direct contractile effect; the EC_{50} was about 3 nM, 10 times lower than in our experiments, but the coronary artery gave values similar to those in the present study (Han and Abel, 1987). Edvinsson et al. (1984) observed no effect of the peptide in several other rabbit vessels. In the rat femoral artery, neuropeptide Y elicited a comparable, slowly developing and sustained contraction, but its efficacy and potency were lower than in our preparation. Tachyphylaxis to neuropeptide Y, like that observed in our experiments, has also been reported in human resistance vessels (Hughes et al., 1988), guinea-pig arteries, and guinea-pig vena cava (Morris, 1991). This phenomenon may be due to desensitization of neuropeptide Y receptors, as demonstrated by Daniels et al. (1989) in human erythroleukaemia (HEL) cells.

As the neuropeptide Y-induced contraction was totally abolished in Ca^{2+}-free medium, it appears that an influx of extracellular Ca^{2+} is required for the direct vasoconstrictor action of neuropeptide Y. The fact that high concentrations of the Ca^{2+}-channel blocker, nitrendipine, only partially reduced neuropeptide Y-induced contractions indicates that Ca^{2+} enters the cell not only via nitrendipine-sensitive L-type channels, but also via other types of channel. Our results are in agreement with data obtained with human coronary arteries, in which nifedipine induced only 60% relaxation of neuropeptide Y-precontracted arteries (Franco-Cereceda, 1989). The relevance of extracellular Ca^{2+} was also demonstrated by Pernow et al. (1986) in the rat femoral artery, but in their experiments pretreatment with nifedipine totally abolished the neuropeptide Y-induced contraction.

Potentiation of the effects of various vasoconstrictors by neuropeptide Y has consistently been observed in different experimental models in vitro and in vivo (Waeber et al., 1988). This indirect effect of neuropeptide Y, which occurs at concentrations much lower than those needed for a direct action, can even be demonstrated in vessels that do not show a direct response. In various in vitro preparations, neuropeptide Y potentiated the effects of noradrenaline (Oshita et al., 1989; Abel and Han, 1989; Han and Abel, 1987; Wong-Dusting and Rand, 1988; Saville et al., 1990; Andriantsitohaina and Stoclet, 1988; Lundberg et al., 1985; Neild, 1987; Vu et al., 1989) or electrical nerve stimulation (Wong-Dusting and Rand, 1988; Saville et al., 1990; Daly et al., 1988). Neuropeptide Y also potentiated other vasoconstrictors such as histamine in rabbit cerebral (Abel and Han, 1989) and coronary arteries (Han and Abel, 1987).

The capacity of neuropeptide Y to potentiate the effects of other vasoconstrictors was also observed in our experiments with rabbit femoral artery. The potentiation was greater at low concentrations of angiotensin II than at high concentrations, at which neuropeptide Y merely prolonged the contraction, without augmenting the maximal increase in tension. In our preparations, as in others (Wahlestedt et al., 1986), the postjunctional direct and indirect contractile effects of neuropeptide Y seem to be mediated by a neuropeptide Y Y_1 receptor, since the 13–36 fragment was totally ineffective. In an early paper Wahlestedt et al. (1987) suggested that the neuropeptide Y Y_1 receptor subtype may be directly coupled to phospholipase C. Later they argued that the small increase in inositol phosphate that can sometimes be observed after neuropeptide Y must be secondary to an effect of Ca^{2+} on phospholipase C (Wahlestedt et al., 1990). Hence, we investigated whether the direct or indirect vasoconstrictor effects of neuropeptide Y were associated with changes in the intracellular concentrations of inositol phosphates. In our experiments we obtained no evidence for involvement of the inositol phosphate mes-

senger system in the direct vasoconstrictor action of neuropeptide Y: neuropeptide Y had no effect on the levels of IP_1, whereas angiotensin II induced marked and dose-dependent increases. This is consistent with the negative observations of other authors in porcine aortic (Mihara et al., 1989) and in rabbit pulmonary artery vascular smooth-muscle cells (Reynolds and Yokota, 1988). In contrast, stimulation of phospholipase C by neuropeptide Y has been shown in non-vascular tissues such as rat vas deferens (Haggblad and Fredholm, 1987) and cultured rat sensory neurons (Perney and Miller, 1989). In HEL cells, Daniels et al. (1989) demonstrated an increased activity of phospholipase C and showed that the neuropeptide Y-induced rise in intracellular Ca^{2+} followed an increase in IP_3. These data are at variance with the observations made with the same model by Motulsky and Michel (1988), who found no correlation between the Ca^{2+} signal and the subsequent small increase in IP_1.

In contrast, when added before angiotensin II, neuropeptide Y markedly potentiated the IP_1 response to the vasoconstrictor. This observation might suggest that these indirect effects of neuropeptide Y are due to stimulation of phospholipase C. However, the fact that neuropeptide Y had not shown any effect of its own on IP_1 levels is not consistent with the assumption that the inositol phosphate pathway is the primary mediator of the potentiation. Even though this effect appears to be mediated by neuropeptide Y Y_1 receptors, it seems rather likely that neuropeptide Y enhanced the angiotensin II-induced rise in inositol phosphates via intracellular mechanisms other than stimulation of phospholipase C. To identify the primary signal we assessed the role of cyclic AMP and Ca^{2+} in further experiments.

Adenylate cyclase has been reported to be inhibited by neuropeptide Y. In human neuroepithelioma (SK-N-MC) cells, neuropeptide Y caused a concentration-dependent, pertussis toxin-sensitive inhibition of forskolin-stimulated cAMP accumulation (Lobaugh and Blackshear, 1990). Aakerlund et al. (1990) suggested that the neuropeptide Y receptor in these cells was a Y_1 subtype and demonstrated that its stimulation led to both inhibition of adenylate cyclase and an increase in intracellular Ca^{2+}. In HEL cells, Motulsky and Michel (1988) demonstrated that neuropeptide Y inhibits adenylate cyclase and mobilizes intracellular Ca^{2+}. In these cells, α_2-adrenoceptor stimulation evokes the same response as neuropeptide Y, but no synergism has been demonstrated (Motulsky and Michel, 1989).

In our experiments we used α_2- or β-adrenoceptor agonists as pharmacological tools to modulate the activity of adenylate cyclase. Inhibition of adenylate cyclase through stimulation of α_2-adrenoceptors with clonidine affected neither the basal nor the angiotensin II-induced formation of IP_1. A slight effect seen at the highest concentration of clonidine was probably due to the activation of α_1-adrenoceptors, because it was blocked by the α_1-adrenoceptor antagonist, prazosin. Moreover a selective α_2-adrenoceptor agonist, BHT 933 (Kobinger and Pichler, 1981; Kobinger, 1986), had no effect on either basal or angiotensin II-stimulated adenylate cyclase. The β-adrenoceptor agonist, isoprenaline, which stimulates adenylate cyclase, would be expected to decrease IP_1 formation. However, we observed no effects under basal conditions and after angiotensin II. Since neither α_2- nor β-adrenoceptors produced any change in the formation of IP_1 in our preparation, it is unlikely that neuropeptide Y acted via inhibition of adenylate cyclase. We therefore refrained from measuring concentrations of cAMP in our experiments.

In our investigations of the role of Ca^{2+} we studied the neuropeptide Y-induced potentiation of the IP_1 response to angiotensin II either in the absence of external Ca^{2+} or after blockade of L-type Ca^{2+} channels with nitrendipine. After treatment with EGTA, potentiation by neuropeptide Y was no longer observed. In contrast, pretreatment of the arteries with nitrendipine had no influence on the IP_1 response to angiotensin II and neuropeptide Y, which suggests that a receptor-operated channel is involved. This finding corresponds to our observation that the contractile responses to neuropeptide Y were entirely dependent on the availability of external Ca^{2+}, but only partly on its influx through L-type Ca^{2+} channels. Our results are not consistent with the observations of Wahlestedt et al. (1985), that the potentiation of noradrenaline-induced contraction was independent of extracellular Ca^{2+}, whereas the intracellular Ca^{2+} pool played a critical role. In experiments examining the potentiating effect of neuropeptide Y on vasoconstriction in rat resistance arteries, Andriantsitohaina and Stoclet (1988) arrived at the conclusion that neuropeptide Y exerted its action via partial depolarization of the arterioles and an increase in Ca^{2+} entry. These observations are consistent with ours, except that they suggest that Ca^{2+} enters the cell through voltage-dependent channels.

Finally, we investigated whether the effects of neuropeptide Y could be mimicked by augmenting Ca^{2+} influx by way of other mechanisms. In these studies we used the Ca^{2+}-channel opener, BayK 8644. This compound had no intrinsic effects on IP_1 levels, but, like neuropeptide Y, significantly enhanced the angiotensin II-induced rise in IP_1, albeit to a lesser extent than neuropeptide Y. The lower efficacy of BayK 8644 could be explained by its acting on different Ca^{2+} channels, or by the concentration used not being maximally effective in our preparation. In any case, the observation that stimulation of Ca^{2+} influx with BayK 8644 induced the same response as neuropeptide Y

strongly supports our assumption that the changes in IP_1 concentration after neuropeptide Y are secondary to changes in intracellular Ca^{2+}.

In conclusion, the results of the present study with the rabbit femoral artery demonstrate that neuropeptide Y, in addition to its direct vasoconstrictor effect, strongly potentiates the contractile responses to angiotensin II. Both the direct and indirect effects of neuropeptide Y appear to be mediated by a neuropeptide Y Y_1 receptor subtype. Neuropeptide Y has no effects of its own on the intracellular concentration of inositol phosphates but potentiates the angiotensin II-induced increase in IP_1. However, accumulation of inositol phosphates does not seem to be the effect primarily responsible for the indirect vasoconstrictor action of neuropeptide Y. In contrast to the original receptor classification, according to which neuropeptide Y Y_1 receptors were thought to be coupled to phospholipase C, it seems rather likely that the IP_1 response is secondary to a rise in intracellular Ca^{2+}, as assumed later by Wahlestedt et al. (1990). This action of neuropeptide Y appears to be mediated by activation of receptor-operated channels, while cAMP-dependent mechanisms do not appear to be involved.

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