Neuropeptide Y-induced intracellular Ca\(^{2+}\) increases in vascular smooth muscle cells

Shin-ichi Mihara, Yasushi Shigeri and Masafumi Fujimoto

Shionogi Research Laboratories, Shionogi & Co. Ltd, 5-12-4 Sagisu Fukushima-ku, Osaka 553, Japan

Received 27 October 1989

The effect of neuropeptide Y (NPY) on cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)r]) was studied in cultured smooth muscle cells from porcine aorta (PASMC) and compared with the effect of bradykinin (BK) and angiotensin II (ATII) on [Ca\(^{2+}\)r]. All peptides induced dose-dependent and transient rises in [Ca\(^{2+}\)r], which were not blocked by extracellular EGTA, but the NPY response was different from the others as follows. First, the [Ca\(^{2+}\)r] rise induced by NPY was not as rapid as that induced by BK or ATII. Second, pertussis toxin abolished the [Ca\(^{2+}\)r] rise induced by NPY, but not by BK or ATII. Third, following initial treatment with BK, PASMC were able to respond to NPY, but not to ATII. Finally, BK and ATII, but not NPY, significantly increased inositol 1,4,5-trisphosphate (Ins(1,4,5)P\(_3\)) generation. Although NPY attenuated forskolin-induced accumulation of cyclic AMP, forskolin- and 3-isobutyl-1-methyl-xanthine-induced alterations in intracellular cyclic AMP did not affect the NPY-induced [Ca\(^{2+}\)r] rise. These results suggest that NPY increases [Ca\(^{2+}\)r], by a pertussis toxin-sensitive GTP binding protein-involved mechanism which is not mediated by the intracellular messengers such as Ins(1,4,5)P\(_3\) and cyclic AMP.

1. INTRODUCTION

Neuropeptide Y (NPY) coexists with noradrenaline in a population of sympathetic neurons and modulates noradrenergic responses [1,2]. Vascular smooth muscle is one of such tissues that are under the control of noradrenaline and NPY [2-5]. For example, NPY potentiates noradrenaline-evoked vasoconstriction post-junctionally and suppresses the release of noradrenaline from sympathetic nerve endings upon stimulation [3,4]. In some isolated blood vessels, in addition, NPY itself is known to be a potent vasoconstrictor [1,2,6].

The transmembrane signalling mechanism of NPY action has been demonstrated in neural cells [7-10]. Perney and Miller have found that NPY can stimulate the synthesis of inositol trisphosphate (Ins(1,4,5)P\(_3\)) and increase cytosolic free calcium ([Ca\(^{2+}\)r]) in cultured rat sensory neurons [9]. On the other hand, studies with vascular smooth muscle cells have been confined to suggesting the possibility that inhibition of cyclic AMP accumulation is causally related to vasoconstriction [11,12].

Since the primary trigger for contraction of smooth muscle is thought to be a rise in [Ca\(^{2+}\)r] [13], we attempted to examine the possible coupling of NPY receptors on porcine aortic smooth muscle cells (PASMC) to intracellular calcium regulating systems. We show here that NPY may elevate PASMC [Ca\(^{2+}\)r] by a different mechanism from bradykinin (BK) or angiotensin II (ATII). The NPY response seems to involve a pertussis toxin-sensitive GTP binding protein and not to be mediated by Ins(1,4,5)P\(_3\) or cyclic AMP.

2. MATERIALS AND METHODS

2.1. Materials

Porcine NPY, BK, and ATII were purchased from the Peptide Institute (Osaka, Japan). Fura-2-AM was obtained from Dojin (Kumamoto, Japan). 3-Isobutyl-1-methyl-xanthine (IBMX) and forskolin were from Sigma. Nisordipine, verapamil and diltiazem were donated by Dr Ikuo Adachi of our laboratories.

2.2. Cell culture

PASMC were isolated from explants of aortic media as described by Ross [14]. Cells were grown in Medium 199 supplemented with 10% fetal calf serum (Gibco), 10 mM Hepes (pH 7.4) and antibiotics in a 5% CO\(_2\)/95% air incubator at 37°C. The cells were subcultured at confluency, and only cells in passages 3-8 were used for experiments.

2.3. Determination of [Ca\(^{2+}\)r]

The cytosolic Ca\(^{2+}\) concentration was fluorometrically measured using the Ca\(^{2+}\)-sensitive fluorescent dye fura-2. PASMC were grown to reach confluency in 75-cm\(^2\) flasks and trypsinized (0.05% trypsin with 0.2% EDTA, 3 min, 37°C). Cell suspensions were washed once with growth medium. The single cells were counted and resuspended in Hepes (20 mM)-buffered Hanks' solution so that a final concentration of 1 x 10\(^6\) cells/ml was obtained. The cell suspensions were incubated with 2 \(\mu\)M fura-2-AM at 37°C for 30-60 min. The fura-2-loaded cells thus obtained were washed twice and resuspended in Hepes (20 mM)-buffered Hanks'-solution (pH 7.4) at 5 x 10\(^3\) cells/ml, and 0.5 ml of the suspension was placed in each cuvette. During experiments in the spectrofluorometer (CAF-100, Japan Spectroscopy Inc., Tokyo, Japan), the cell suspensions were continuously stirred with a small magnetic bar within the cuvettes. Ex-
citation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were used. $[Ca^{2+}]_{i}$ was calculated according to the following formula [15]

$$[Ca^{2+}]_{i} = K(F - F_{min})/(F_{max} - F)$$

In this formula, $K$ is the affinity of fura-2 for $Ca^{2+}$ ions. We set $K = 224$ nM, as previously determined by Grynkiewicz et al. [15]. $F$, $F_{min}$, and $F_{max}$ are the fluorescence at a time of interest, at zero, and saturated $[Ca^{2+}]_{i}$, respectively, after correction for extracellular fura-2. Fluorescence dye to extracellular fura-2 was determined by adding 100 $\mu$M MnCl$_2$ to the cells at the beginning and the end of each experiment. The cells were lysed with Triton X-100 followed by the addition of EGTA plus Tris to obtain $F_{max}$ and $F_{min}$, respectively.

2.4. Measurement of cyclic AMP

Single cells at $1 \times 10^6$/ml were preincubated in a total volume of 200 $\mu$l Medium 199 containing 10 mM Hepes (pH 7.4) in the presence or absence of 0.1 mM IBMX for 10 min at 37°C, and then incubated with 1 $\mu$M NPY with or without 10 $\mu$M forskolin. 40 $\mu$l of 50% trichloroacetic acid was added to stop the incubation. After extracting trichloroacetic acid with water-saturated ether, media were subjected to radioimmunoassay for cyclic AMP (Amersham).

2.5. Measurement of inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$)

Ins(1,4,5)P$_3$ was determined using the protein binding assay kit (Amersham) as described by Fu et al. [16]. In brief, single cells at 5 x $10^6$/ml were preincubated in 200 $\mu$l Medium 199 containing 10 mM LiCl and 10 mM Hepes (pH 7.4) for 10 min at 37°C, and then incubated with 1 $\mu$M NPY, BK, or ATII. At the end of the incubation, 37 $\mu$l of 20% HClO$_4$ was added. After neutralizing the media with 13.5-14$\mu$l 10 M KOH, supernatants were subjected to Ins(1,4,5)P$_3$ assay.

3. RESULTS

The effects of NPY on $[Ca^{2+}]_{i}$ in PASMC are documented in fig.1. NPY caused a dose-dependent and transient increase in $[Ca^{2+}]_{i}$, which reached maximal level at $10^{-7}$ M; the ED$_{50}$ estimated at approximately $3 \times 10^{-9}$ M (fig.1A). To study whether the increase in $[Ca^{2+}]_{i}$ could come either from intracellular stores or from outside the cell, we added 2 mM EGTA to a cell suspension to chelate all extracellular $Ca^{2+}$ 2 min before adding NPY (fig.1B). The treatment only slightly diminished the increase in $[Ca^{2+}]_{i}$ induced by NPY. It was the case with those induced by BK and ATII (data not shown). The calcium channel blocker, nisodipine, did not inhibit the NPY response at all (fig.1B). Verapanil and diltiazem had no effect either (results not shown).

PASMC responded to not only NPY, but also to BK or ATII, as shown in fig.1C. Once a maximal dose of each agonist increased $[Ca^{2+}]_{i}$, in PASMC, the cells did not respond to an additional exposure to the same agonist (fig.1C). A first stimulation with a maximal dose of BK strongly suppressed the $[Ca^{2+}]_{i}$ response to a subsequent challenge with a maximal dose of ATII. In contrast, after BK challenge, a maximal dose of NPY still induced a $[Ca^{2+}]_{i}$ rise. A first stimulation with a

Fig.1. NPY-, ATII- and BK-induced increases in $[Ca^{2+}]_{i}$ in PASMC. $[Ca^{2+}]_{i}$ was measured with a fluorescent indicator, fura-2. Calculated values of $[Ca^{2+}]_{i}$ are shown on the ordinates. (A) Concentration–response relationship of NPY-induced $Ca^{2+}$-fura-2 fluorescence. (B) Effect of pretreatment of cells with EGTA or nisodipine (NS) for 2 min on the NPY response. (C) Effect of sequential stimulations on the $[Ca^{2+}]_{i}$, rises induced by NPY, ATII and BK. Cells were stimulated by $10^{-7}$ M NPY, $10^{-6}$ M ATII, or $10^{-6}$ M BK at arrows. (D) Effect of pretreatment of cells with pertussis toxin on NPY-, ATII and BK induced increases in $[Ca^{2+}]_{i}$. Cells were cultured without (i) or with 100 ng/ml pertussis toxin (IAP) (ii) for 3 h before preparing fura-2-loaded cell suspensions. Cells were challenged with $10^{-6}$ M of each agonist. Dotted lines represent the initial velocity of the $[Ca^{2+}]_{i}$ rises. The traces were obtained from one experiment, but are representative of 3 other experiments.
maximal dose of NPY hardly influenced the BK- or ATII-response (results not shown).

As shown in fig.1C and D, the maximal NPY response was always smaller than the maximal BK response, but nearly equal to or a little greater than the maximal ATII response. The [Ca\(^{2+}\)]\(_i\) rise induced by NPY occurred after a lag period of a few seconds and was not as rapid as those induced by ATII and BK, as shown by the dotted lines in fig.1D. When PASMC were pretreated with 100 ng/ml pertussis toxin for 3 h and then challenged with the agonists, only the NPY-induced increase in [Ca\(^{2+}\)]\(_i\) was prevented (fig.1D).

Ins(1,4,5)P\(_3\), a product of phosphoinositide hydrolysis, is believed to act as a second messenger mediating the release of Ca\(^{2+}\) from intracellular store sites [17]. The resting level of Ins(1,4,5)P\(_3\) after 10-min incubation with 10 mM LiCl was 3.90 ± 0.98 pmol/10\(^6\) cells. As shown in fig.2, BK and ATII induced 220% and 63% increases above basal levels for 15-s incubation, respectively. The potency of BK and ATII to generate Ins(1,4,5)P\(_3\) seemed to correlate to that of each peptide to induce the [Ca\(^{2+}\)]\(_i\) rise (fig.1C and D). However, NPY did not significantly increase Ins(1,4,5)P\(_3\) above basal levels.

Next, we investigated the involvement of cyclic AMP in the NPY-induced [Ca\(^{2+}\)]\(_i\) rise. NPY did not have any effect on the basal cyclic AMP level in PASMC (fig.3B). Preincubation with IBMX (0.1 mM) increased the cyclic AMP level in PASMC by 16-fold, but it had no effect on the NPY-induced [Ca\(^{2+}\)]\(_i\) rise (fig.3). Furthermore, forskolin had no effect on resting [Ca\(^{2+}\)]\(_i\) and NPY-induced [Ca\(^{2+}\)]\(_i\) rise, whereas NPY significantly suppressed forskolin-induced accumulation of cyclic AMP, as described by others [11,12] (fig.3).

4. DISCUSSION

Reynolds and Yokota have demonstrated that NPY did not stimulate phosphoinositide hydrolysis or the elevation of [Ca\(^{2+}\)]\(_i\) in cultured smooth muscle cells from rabbit pulmonary artery [12]. In PASMC, in contrast, NPY dose-dependently caused a rapid and transient [Ca\(^{2+}\)]\(_i\) rise. Increases in [Ca\(^{2+}\)]\(_i\) in the smooth muscle cells are induced from two main sources; one is influx of extracellular Ca\(^{2+}\) and the other is release of Ca\(^{2+}\) from store sites. The NPY-stimulated increase in [Ca\(^{2+}\)]\(_i\) was only slightly diminished by extracellular excess EGTA and was not affected by voltage dependent Ca\(^{2+}\) channel blockers. Therefore, the main source of NPY-induced Ca\(^{2+}\) mobilization seems to be the intracellular Ca\(^{2+}\) storage site. Besides, NPY may induce a small influx of Ca\(^{2+}\) via the receptor-operated Ca\(^{2+}\) channel. Alternatively, apparent partial dependency of the NPY response upon extracellular Ca\(^{2+}\) may be because EGTA may facilitate the leak of Ca\(^{2+}\) from cells leading to the decrease in the intracellular Ca\(^{2+}\).
stores or because replenishment of the intracellular Ca\(^{2+}\) pool following agonist-induced emptying of the pool is partially due to a pathway from the extracellular space to the pool [18].

Although ATII- and BK-induced increases in [Ca\(^{2+}\)]\(_i\) were mainly dependent upon intracellular Ca\(^{2+}\) storage in the same way as the NPY response, some differences were observed between NPY- and the other peptide-induced [Ca\(^{2+}\)]\(_i\) rises. First, the [Ca\(^{2+}\)]\(_i\) rise induced by NPY was not as rapid as that induced by BK or ATII. Second, pertussis toxin abolished the [Ca\(^{2+}\)]\(_i\) rise induced by NPY, but not by BK or ATII. Third, following initial treatment with BK, PASMC were able to respond to NPY, but not to ATII. Finally, BK and ATII, but not NPY, significantly increased Ins\((1,4,5)P_3\) generation. These results suggest that NPY increases [Ca\(^{2+}\)]\(_i\) in PASMC by a pertussis toxin-sensitive GTP binding protein-involved and Ins\((1,4,5)P_3\)-independent signal-transducing system, whereas the effects of ATII and BK are at least partially common, as judged from their hetero-desensitization (fig.1C), and possibly mediated through the elevation of Ins\((1,4,5)P_3\) which releases Ca\(^{2+}\) from intracellular store sites, as described previously [19,20]. The reason the NPY response is not as rapid as the BK or ATII response is not clear at present, but must be closely related to the mechanism of NPY action.

IBMX- and forskolin-induced changes in intracellular cyclic AMP did not have any effect on the NPY-induced [Ca\(^{2+}\)]\(_i\) rise, although NPY attenuated the forskolin-induced accumulation of cyclic AMP (fig.3). These results indicate that the Ca\(^{2+}\) mobilization is not secondary to inhibition of adenylate cyclase.

Activation of many types of receptors mobilizes Ca\(^{2+}\) through generation of Ins\((1,4,5)P_3\) [17]. In rat dorsal root ganglion neurons, NPY stimulates Ins\((1,4,5)P_3\) generation leading to an increase in [Ca\(^{2+}\)]\(_i\) through a pertussis toxin-sensitive GTP binding protein- and Ins\((1,4,5)P_3\)-independent signal-transducing system, whereas the effects of ATII and BK are at least partially common, as judged from their hetero-desensitization (fig.1C), and possibly mediated through the elevation of Ins\((1,4,5)P_3\) which releases Ca\(^{2+}\) from intracellular store sites, as described previously [19,20]. The reason the NPY response is not as rapid as the BK or ATII response is not clear at present, but must be closely related to the mechanism of NPY action.

The mechanism of NPY-induced Ca\(^{2+}\) mobilization in PASMC also remains obscure. NPY receptors may be linked to the generation of an as yet unknown messenger leading to mobilization of Ca\(^{2+}\). Alternatively, like receptors for glucagon, insulin and oxytocin [22-24], they may be negatively linked to the plasma membrane-Ca\(^{2+}\) pump (Ca\(^{2+}\).ATPase) leading to an increase in [Ca\(^{2+}\)]\(_i\).

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