Neuropeptide Y modulates ATP-induced increases in internal calcium via the adenylate cyclase/protein kinase A system in a human neuroblastoma cell line

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The modulatory effects of neuropeptide Y (NPY) on ATP-induced increases in cytosolic free-calcium concentration ($[Ca^{2+}]_i$) were investigated in the CHP-234 human neuroblastoma cell line. Pretreatment of cells with 100 nM NPY potentiated the increase in $[Ca^{2+}]_i$ evoked subsequently by 20 μ M ATP, compared with initial application of ATP in a control experiment, whereas a similar pretreatment with 1 μ M NPY attenuated the subsequent response to ATP. Both actions of NPY were completely blocked by H-89 (*N*-[2-((3-(4-bromo-phenyl)-2-propenyl)-amino)-ethyl]-5 isoquinoline sulphonamide dihydrochloride), a selective an-

tagonist of protein kinase A. The effects of 100 nM NPY were mimicked by H-89, while forskolin and 8-Br-cAMP mimicked the effects of 1 μ M NPY. Both basal and forskolin-stimulated cAMP levels were inhibited by 100 nM NPY and by 100 nM NPY(13-36), a selective agonist of the NPY Y_2 -receptor subtype. In contrast, at 1 μ M such inhibition was not observed for either NPY or NPY(13-36). It is concluded that NPY has a biphasic modulatory effect on increases in $[Ca^{2+}]_i$ produced by ATP, which probably involves the cAMP/protein kinase A cascade.

INTRODUCTION

Neuropeptide Y (NPY) is widely distributed in the central [1] and peripheral nervous systems [2]. It is stored and released as a co-transmitter together with noradrenaline and ATP [3]. NPY activates at least three different receptor subtypes (named Y_1 , Y_2 and Y_3), which have been subclassified based notably upon differences in ligand-binding studies (for reviews, see [4–7]). Both the Y_1 [8,9] and Y_2 [10,11] receptor subtypes have been cloned, and recently the cloning of two other novel receptors sensitive to NPY has also been reported: a Y_4 receptor subtype [12] and the so-called PP_1 (pancreatic polypeptide) receptor [13].

Stimulation of NPY receptors leads to diverse actions (for reviews, see [4–6,14]), including vasoconstriction [15], inhibition of catecholamine release from bovine chromaffin cells [16,17] and inhibition of neurotransmitter release from sensory neurones [18]. These effects can be elicited directly via specific postsynaptic NPY receptors, or indirectly by NPY acting presynaptically to inhibit its own release and that of catecholamines. In addition, NPY modulates the response of other neurotransmitters. For example, NPY attenuates bradykinin- and angiotensin II-induced Ca^{2+} mobilization in a human neuroblastoma cell line by inhibiting the activation of phospholipase C [19]. In bovine chromaffin cells, NPY-induced inhibition of nicotinic current [20] appears to be mediated by the cAMP/protein kinase A system [17]. Potentiation of the contractile response to vasoconstrictors such as noradrenaline [21–23] and ATP [22,24,25] has been widely reported. Contractile potentiation is accompanied by increases in cytosolic free-calcium concentration ($[Ca^{2+}]_i$) [23,26]. The molecular basis of this potentiating effect is not clear yet, but mechanisms, including facilitation of internal Ca^{2+} mobilization [14,26], upregulation of voltage-dependent Ca^{2+} channel activity [27], inhibition of Ca^{2+} -sensitive K^+ channels [28] and potentiation of phospholipase C activation by noradrenaline [29], have been proposed.

We have recently reported that in CHP-234 human neuroblastoma cells, NPY Y_2 -receptor stimulation causes an increase in $[Ca^{2+}]_i$ by a mechanism which involves a G-protein-independent Ca^{2+} influx and also inhibition of forskolin-stimulated cAMP accumulation [30,31]. In these cells, ATP also increases $[Ca^{2+}]_i$, but via a different mechanism, which includes Ca^{2+} influx plus mobilization of internal stores following $InsP_3$ production [31]. In the present study, we investigated the interaction between NPY and ATP in relation to their capacities to increase $[Ca^{2+}]_i$, using the same cell type. We found that NPY can positively or negatively couple to ATP-induced $[Ca^{2+}]_i$ increases, depending upon the concentration of NPY. This effect appears to be mediated by NPY-induced decreases or increases in cAMP levels.

METHODS

Cell culture

The CHP-234 cell line was derived from a human neuroblastoma and was obtained from Dr. J. L. Biedler (Sloan-Kettering Cancer Center, New York, NY, U.S.A.). Cells were cultured in T25 or T65 Nunclon culture flasks (Nunc, Roskilde, Denmark) in a medium containing a 1:1 (v/v) mixture of Eagle's minimal essential medium and Ham's F-12 (Eurobio, Paris, France), supplemented (v/v) with 15 % fetal calf serum, 1 % glutamine and 1 % non-essential amino acids (Gibco BRL, Cergy Pontoise, France). Cells were maintained at 37 °C in a humidified incubator gassed with 5 % CO_2 in air, until confluence was reached. Cells were then detached with 0.15 % (w/v) trypsin in Ca^{2+} -free PBS containing 0.4 mM EDTA and, after centrifugation, pelleted cells were resuspended in the same culture medium as above. For $[Ca^{2+}]_i$ measurements, cells were plated at $(8–10) \times 10^3$ cells/cm² in 35-mm Petri dishes in which a 2 cm-diam. hole had been cut in the base and replaced by a thin (0.07 mm) glass coverslip. For cAMP measurements, cells were plated at a density of $(1–2) \times 10^5$

Abbreviations used: NPY, neuropeptide Y; $[Ca^{2+}]_i$, cytosolic free-calcium concentration; fura 2/AM, fura 2/acetoxymethyl ester; H-89, *N*-[2-((3-(4-bromo-phenyl)-2-propenyl)-amino)-ethyl]-5 isoquinoline sulphonamide dihydrochloride.

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cells/35-mm Petri dish, and maintained in culture for 3–4 days until confluence was reached. Cells were passaged once or twice, before another culture was established from aliquots stored in liquid N₂.

Measurement of intracellular Ca²⁺

[Ca²⁺]_i levels were determined as previously described [30]. Briefly, cells (plated on Petri dishes as described above) were incubated for 30 min at 37 °C in normal external solution (in mM: NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 2, glucose 11, Hepes 10, pH 7.3 with NaOH) containing 2.5 μM fura 2/acetoxymethyl ester (fura 2/AM). Cells were then placed on an inverted microscope (Nikon Diaphot) and perfused continuously for 15 min. Fluorescence measurements were performed at 30 °C on small groups of dispersed cells viewed with a Nikon UV-Fluor 40 × (numerical aperture 1.3) oil-immersion objective. Fura-2 fluorescence was excited alternatively at 340 and 380 nm with illumination provided by a 100 W halogen lamp. Emitted fluorescence was filtered by a 490–530 nm bandpass filter (Nikon) and measured with a Darkstar-800 intensified CCD camera (Photonics Sciences, Millham, U.K.). Video images were digitized (IMSTAR, Paris, France) onto a computer and analysed using IMSTAR software. Ratiometric Ca²⁺ images were generated at 2 s intervals, using four averaged images at each wavelength. For each cell, [Ca²⁺]_i was averaged from pixels within manually outlined cell areas. The [Ca²⁺]_i was calculated, after background compensation, as previously described [30], using values determined *in situ* for the limiting ratios for saturating (10 mM Ca²⁺ in the bath) and minimal (no Ca²⁺ and 10 mM EGTA in the bath) [Ca²⁺]_i, both in the presence of 10 μM ionomycin.

Drug application

For [Ca²⁺]_i measurements, NPY, NPY(13–36) and ATP were locally applied by pressure-ejection (10 kPa) from puffer pipettes placed ~100–200 μm from the cell. When different drugs were applied to the same cell, two or more such pipettes were used. NPY was usually applied for 40–60 s, and after intervals of 10–20 s (after which time Ca²⁺ had returned to basal levels) or 1–2 s for cells that, respectively, responded initially to NPY or not; ATP was subsequently applied for 30–40 s. H-89 {N-[2-((3-(4-bromo-phenyl)-2-propenyl)-amino)-ethyl]-5 isoquinoline sulphonamide dihydrochloride}, forskolin and 8-Br-cAMP were directly added to the bath. Ca²⁺-free external solution contained 2 mM EGTA with no added CaCl₂.

Measurement of cAMP production

The method used for measurement of cAMP production was as described previously [30]. Briefly, cells were washed with 1 ml of PBS containing (in mM): NaCl 137, KCl 2.7, CaCl₂ 0.9, MgCl₂ 0.5, Na₂HPO₄ 6.5, pH 7.2. They were then incubated at 37 °C for two periods of 10 min with 1 ml of assay buffer (in mM: NaCl 150, KCl 5, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, Hepes 10, BSA 10 mg/ml), pH 7.4. After removal of the buffer, 950 μl of fresh assay buffer containing in addition 0.5 mM 3-isobutyl-1-methyl-xanthine was added, and the cells were incubated for 10 min. After this time, 50 μl of the respective drugs were added, as indicated. After a further 10 min, the reaction was stopped by removal of the buffer and immediate addition of 1 ml of cold 0.1 M HCl followed by sonication. After centrifugation, the supernatant was collected for measurement of cAMP by radioimmunoassay. Cells from some dishes were counted to determine the cell number at the time of the experiment, allowing normalization of cAMP level/10⁶ cells.

Data analysis

Data are presented as means ± S.E.M. for *n* cells or experiments as indicated. Statistical analysis was made using unpaired Student's *t*-test or the Mann–Whitney U-test, where appropriate. For results expressed as a percentage of corresponding control values, the law of error propagation was respected in calculating S.E.M. values. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to the method of Bonferroni [52]. A level of *P* < 0.05 was considered significant.

Drugs

Fura-2/AM, ATP, 8-Br-cAMP and 3-isobutyl-1-methyl-xanthine were from Sigma (L'Isle d'Abeau Chesnes, France). Ionomycin and H-89 were from Calbiochem (Meudon, France). hNPY, pNPY(13–36) and p[Leu³¹,Pro³⁴]NPY (where h is human and p is porcine) were from Peninsula (Belmont, CA, USA) or Neosystem (Strasbourg, France).

RESULTS

Effect of NPY on ATP-induced [Ca²⁺]_i increase

As we have described [30,31], a 40 s long puffer application of NPY (100–1000 nM) in normal external solution caused a transient increase in [Ca²⁺]_i in CHP-234 cells (Figures 1B and 2B). The percentage of responsive cells again was generally low

Figure 1 Potentiating action of prior exposure to 100 nM NPY on ATP-induced [Ca²⁺]_i increases is not dependent on a previous rise in [Ca²⁺]_i

Representative traces of [Ca²⁺]_i signals for control stimulation with 20 μM ATP (A and D) and for the potentiating effect of 100 nM NPY to a subsequent challenge with 20 μM ATP for individual fura-2-loaded CHP-234 neuroblastoma cells which responded (B; *n* = 3–36) or not (E; *n* = 50–80) to NPY initially. Average values (means ± S.E.M.) for cells from 3–4 different cultures are shown in (C) and (F). Cells were bathed in normal external solution and agonists were locally applied using pressurized puffer pipettes. **P* < 0.05, ***P* < 0.01.

Figure 2 Inhibitory effect of 1 μM NPY on subsequent ATP-induced $[\text{Ca}^{2+}]_i$ signals

(A and D) Representative traces of increases in $[\text{Ca}^{2+}]_i$ for control applications of 20 μM ATP from individual fura-2-loaded CHP-234 neuroblastoma cells. (B and E) The inhibitory effect of a prior application of 1 μM NPY on subsequent challenges to 20 μM ATP was observed both for cells responsive (B; $n = 15$ –75) or not (E; $n = 50$ –70) to NPY. Cells were bathed in normal external solution and agonists were locally applied using pressurized puffer pipettes. (C and F) Average values (means $\pm$ S.E.M.) for cells from 3–4 different cultures. *** $P < 0.001$.

and varied between 20 and 30 % depending on the cell culture. Similarly, when applied in the same way, 20 μM ATP also induced an increase in $[\text{Ca}^{2+}]_i$ (Figure 1, A and D and Figure 2, A and D), with the responsiveness of cells to ATP varying markedly among cultures, as reported previously [31]. For example, between 30 and 70 % of cells responded to ATP in 11 different cultures, with extremes in peak $[\text{Ca}^{2+}]_i$ signals ranging between 300 ± 30 nM and 1200 ± 180 nM depending on the culture; note, however, that in any one culture, both the percentage of responsive cells and the maximal response were fairly similar. After a first application of ATP, ~ 30 min wash-out was required to completely recover the response. Control applications of 200 μM adenosine had no effect on $[\text{Ca}^{2+}]_i$ ($n = 106$).

It has been reported in some systems that NPY potentiates the response elicited by other agonists, including ATP [22,24,25]. It was therefore of interest to test in our cells whether NPY also modulates ATP-induced responses. To test this possibility, the cells were challenged with 20 μM ATP after a 40–60 s long application of either 100 nM or 1 μM NPY. The responses were then compared with control ATP-stimulated cells.

As shown in Figure 1(B), 100 nM NPY significantly potentiated subsequent ATP-induced $[\text{Ca}^{2+}]_i$ signals. Interestingly, even cells which did not respond with an initial increase in $[\text{Ca}^{2+}]_i$ when challenged by 100 nM NPY alone, had significantly greater responses to ATP after NPY (Figure 1E). This suggests that the potentiating effect of NPY was not related to a previous rise in

Figure 3 The effects of NPY on ATP-induced $[\text{Ca}^{2+}]_i$ signals are no longer seen when cells are bathed in Ca^{2+} -free external solution

Representative traces of increases in $[\text{Ca}^{2+}]_i$ in individual fura-2 loaded CHP-234 neuroblastoma cells bathed in Ca^{2+} -free external solution stimulated with 20 μM ATP before (A and D) and after challenge with 100 nM NPY (B; $n = 50$ –60) or 1 μM NPY (E; $n = 60$ –90). Cells were washed with Ca^{2+} -free external solution for 10 min, and then used for up to 40 min. (C and F) Average values (means $\pm$ S.E.M.) for cells from two different cultures in normal external solution (two histograms on the left) and in Ca^{2+} -free external solution (two histograms on the right). * $P < 0.05$.

$[\text{Ca}^{2+}]_i$. When a higher concentration (1 μM) of NPY was used, no potentiation of the subsequent ATP response was seen. Instead, a significant inhibition of the ATP-induced response was observed, both for cells which responded (Figure 2B), or not (Figure 2E), to 1 μM NPY alone. Because of the long time necessary for the recovery of ATP responses after a first application, we could not compare the effects of ATP with and without a previous application of NPY in the same cells. Thus, average values from a population of cells were used and compared under the two different conditions (Figure 1, C and F, and Figure 2, C and F).

Effect of NPY on ATP-induced Ca^{2+} mobilization

When cells bathed in Ca^{2+} -free external solution for 10–40 min were stimulated with ATP, a transient increase in $[\text{Ca}^{2+}]_i$ was seen (Figures 3A and 3D), as found before [31]. In other experiments, this intracellular Ca^{2+} mobilization induced by ATP was observable after up to 2 h in Ca^{2+} -free solution (results not shown), indicating that intracellular Ca^{2+} stores were not depleted under the experimental conditions used. Prior exposure of cells to 100 nM (Figure 3B) or 1 μM NPY (Figure 3E) caused no significant modification of subsequent ATP-induced Ca^{2+} responses when compared with those obtained in the absence of NPY (Figures 3C and 3F). This is in contrast to the modulatory effects of NPY on ATP-induced Ca^{2+} signals observed in normal Ca^{2+} -containing external solution (Figures 1, 2 and 3, C and F). As found previously [30,31], no increases in $[\text{Ca}^{2+}]_i$ were observed for stimulation with NPY in Ca^{2+} -free external solution.

Table 1 Effect of H-89 on the potentiating and inhibitory effects of NPY on ATP-induced $[Ca^{2+}]_i$ increases

Pretreatment with 300 nM H-89 completely abolished both the potentiating effect of 100 nM NPY ($n = 85-150$) and the inhibitory effect of 1 μ M NPY ($n = 50-100$) on 20 μ M ATP-induced $[Ca^{2+}]_i$ signals. Note the differences between 'a' and 'b' for the peak values of the control responses to ATP and for the responses to ATP in the presence of H-89. Maximal control responses to ATP were obtained at 100 μ M, with peak $[Ca^{2+}]_i$ corresponding to 1200 ± 300 nM. Cells from three different cultures were used. Value given $\pm$ S.E.M.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (significantly different from control ATP).

Condition	$[Ca^{2+}]_i$ (nM)
Control ATP ^a	500 ± 38
ATP after 100 nM NPY	$898 \pm 83^{***}$
ATP + H-89	$733 \pm 49^{***}$
ATP after 100 nM NPY + H-89	$613 \pm 38^{**}$
Control ATP ^b	800 ± 58
ATP after 1 μ M NPY*	$396 \pm 150^*$
ATP + H-89	757 ± 47
ATP after 1 μ M NPY + H-89	1098 ± 316

Protein kinase A is involved in the effects of NPY

As we reported earlier [30], in CHP-234 cells NPY decreased forskolin-induced cAMP production up to 100 nM in a concentration-dependent manner; such an inhibition by NPY was initially described by Fredholm et al. [32]. Since there is a large literature on interactions between cellular cAMP levels and $[Ca^{2+}]_i$, the possible role of this second messenger in ATP-induced $[Ca^{2+}]_i$ increases was investigated. When cells were first incubated for 20 min with 300 nM H-89, a selective inhibitor of cAMP-dependent protein kinase A, in all five cultures in which cells were less sensitive to ATP (peak $[Ca^{2+}]_i = 522 \pm 33$ nM; $n = 160$), responses to ATP were significantly potentiated (peak $[Ca^{2+}]_i = 677 \pm 32$ nM, $n = 250$; $P < 0.05$) in a manner similar to that for 100 nM NPY (Figures 1B and 1E). The basal $[Ca^{2+}]_i$ (52 ± 1.8 nM, $n = 160$) was also significantly increased after treatment with H-89 (60 ± 1.8 nM; $n = 250$; $P < 0.05$). Note that in three other cultures where peak Ca^{2+} responses to ATP were elevated (886 ± 81 nM), no significant effect of H-89 preincubation on peak $[Ca^{2+}]_i$ increases in response to ATP (757 ± 47 nM; $n = 130$) was seen (results not shown).

After pretreatment with H-89, an additional potentiating action of a prior 100 nM NPY application on ATP-induced $[Ca^{2+}]_i$ signals was not observed (Table 1), suggesting that protein kinase A is involved in the effect of 100 nM NPY.

The inhibitory effect of 1 μ M NPY on ATP-induced $[Ca^{2+}]_i$ increases was also completely abolished after preincubation with 300 nM H-89 (Table 1). This suggests that at 1 μ M, NPY negatively modulates ATP responses, apparently also via a mechanism which involves protein kinase A.

Effect of forskolin and 8-Br-cAMP on ATP-induced $[Ca^{2+}]_i$ increase

Both basal $[Ca^{2+}]_i$ levels and the peak increase in $[Ca^{2+}]_i$ induced by ATP were strongly and significantly diminished by 10 μ M forskolin (Table 2). Similar results were found with 100 μ M 8-Br-cAMP, a permeant analogue of cAMP (Table 2). These observations are in agreement with our working hypothesis, whereby inhibition of cAMP accumulation potentiates $[Ca^{2+}]_i$ responses induced by ATP with, conversely, high cellular levels of cAMP inhibiting ATP-induced $[Ca^{2+}]_i$ increases.

Table 2 Effects of forskolin and 8-Br-cAMP on ATP-induced $[Ca^{2+}]_i$ levels

Cells were preincubated with 10 μ M forskolin for 20 min and then challenged with 20 μ M ATP ($n = 80$). 8-Br-cAMP was used at 100 μ M ($n = 100$). Control stimulation with 20 μ M ATP on cells ($n = 60-100$) bathed in normal external solution was carried out first. Both basal and ATP-induced $[Ca^{2+}]_i$ signals were significantly reduced by the two treatments. Values given $\pm$ S.E.M.; * $P < 0.05$, *** $P < 0.001$.

Condition	$[Ca^{2+}]_i$ (nM)
Basal	75 ± 5.4
Basal in the presence of forskolin	$48 \pm 2.6^{***}$
Control ATP	655 ± 73
ATP in the presence of forskolin	$388 \pm 22^{**}$
Basal	74 ± 2.7
Basal in the presence of 8-Br-cAMP	$65 \pm 1.9^*$
Control ATP	709 ± 53
ATP in the presence of 8-Br-cAMP	$555 \pm 55^*$

Effect of NPY on cAMP accumulation

Increased cAMP production induced by 3 μ M forskolin was inhibited by 100 nM NPY (Table 3), as already reported [30]. A similar inhibition was observed (Table 3) with 100 nM NPY(13-36), a reportedly selective agonist of Y_2 -type receptors [7,33,34]. However, surprisingly, at 1 μ M, neither NPY nor NPY(13-36) had a significant effect on cAMP levels produced by 3 μ M forskolin (Table 3). These results were invariably reproduced in all cultures tested. The same pattern of response was observed for resting levels of cAMP: an inhibitory effect at 100 nM NPY, with in contrast, basal cAMP being increased in the presence of 1 μ M NPY (Table 3). NPY(13-36) also diminished the resting level of cAMP at 100 nM with, however, no significant effect of NPY(13-36) at 1 μ M (Table 3). Up to 100 nM [Leu³¹,Pro³⁴]NPY had no inhibitory effect on cAMP levels, for cells stimulated or not with 3 μ M forskolin (results not shown).

Table 3 Effect of NPY and NPY(13-36) on forskolin-induced cAMP accumulation and basal cAMP levels

Cells were exposed to 3 μ M forskolin for 10 min in the absence or presence of the peptides NPY and NPY(13-36) at 100 nM and 1 μ M respectively. Data are from triplicate radioimmunoassay determinations from 3-4 separate experiments and are expressed as a percentage of the basal, unstimulated or FSK-induced cAMP values. In untreated cells, basal levels of cAMP were 10.59 ± 0.75 pmol/10 min per 10^6 cells, and after forskolin alone, cAMP levels were 298.61 ± 18.86 pmol/10 min per 10^6 cells. For results expressed as a percentage of corresponding control values, the law of error propagation was respected in calculating S.E.M. values. * $P < 0.05$, ** $P < 0.01$.

Treatment	cAMP accumulation (%)
Basal	100 ± 2.4
Basal + 100 nM NPY	$79.9 \pm 5.4^*$
Basal + 100 nM NPY(13-36)	$80.5 \pm 7.9^*$
Basal + 1 μ M NPY	$129.9 \pm 9.0^{**}$
Basal + 1 μ M NPY(13-36)	92.5 ± 7.7
Forskolin	100.0 ± 6.3
Forskolin + 100 nM NPY	$52.6 \pm 6.6^{**}$
Forskolin + 100 nM NPY(13-36)	$76.3 \pm 5.3^*$
Forskolin + 1 μ M NPY	87.1 ± 15.1
Forskolin + 1 μ M NPY(13-36)	138.8 ± 24.1

These data strongly suggest that, by an as yet unknown mechanism, NPY at high concentrations increases cAMP production. Furthermore, this stimulation of cAMP production may be a possible mechanism by which 1 μM NPY negatively modulates ATP-induced $[\text{Ca}^{2+}]_i$ increases, in agreement with the inhibitory effects of forskolin and 8-Br-cAMP described above (Table 2).

Effect of ATP on cAMP production

When CHP-234 cells were incubated in the presence of ATP (0.01–100 μM) for 10 min, no significant changes in cAMP content were found. Basal unstimulated cAMP levels were 15.6 ± 2.9 pmol/10 min per 10^6 cells, and the following values (in pmol/10 min per 10^6 cells) were found after stimulation with ATP (at the indicated concentrations): 14.2 ± 2.1 (0.01 μM), 14.2 ± 2.0 (0.1 μM), 18.2 ± 3.8 (1 μM), 14.6 ± 2.3 (10 μM) and 16.9 ± 4.1 (100 μM). Data are triplicate determinations from three separate experiments.

DISCUSSION

The present results show that NPY modulates ATP-induced $[\text{Ca}^{2+}]_i$ signals in CHP-234 cells in a biphasic manner: at 100 nM, NPY potentiated the effect of ATP, while at 1 μM , the response to ATP was inhibited. Several lines of evidence suggest that both modulatory actions of NPY are mediated by a mechanism which involves cAMP/protein kinase A. Firstly, NPY also had a biphasic effect on both basal and forskolin-stimulated cAMP levels at the same concentrations for which ATP responses were modulated. In addition, H-89, a selective inhibitor of protein kinase A, completely abolished both actions of NPY. Finally, the effect of 100 nM NPY was mimicked by H-89, while forskolin and 8-Br-cAMP mimicked the effect of 1 μM NPY.

Such a concentration-dependent, biphasic effect on adenylate cyclase activity has been reported previously for NPY(17-36) in rat cardiac tissue, with inhibitory and stimulatory effects being observed at concentrations of < 1 nM and > 1 nM respectively, in a G-protein-dependent manner [35]. In this latter preparation, NPY at all concentrations tested had only inhibitory effects on adenylate cyclase activity, while the proposed antagonist NPY(18-36), while clearly competitively inhibiting NPY binding, was alone without effect on adenylate cyclase [36]. Similar agonist-mediated, concentration-dependent biphasic effects on adenylate cyclase activity, mediated by G-proteins following activation of cloned α_2 -adrenergic receptor isoforms, have also been described [37]. Other similar examples include biphasic regulation of adenylate cyclase activity and of Ca^{2+} pump activity in hepatocytes by glucagon [38] and mini-glucagon [39] respectively.

We propose that resting adenylate cyclase activity in our cells is high enough so that the well-known inhibitory action of NPY on adenylate cyclase [32] is sufficient to decrease basal cAMP levels, thereby potentiating ATP-induced $[\text{Ca}^{2+}]_i$ increases. Indeed, resting levels of cAMP were significantly decreased by 100 nM NPY (Table 3). This hypothesis is also in agreement with the observed potentiating effects of H-89 alone on basal $[\text{Ca}^{2+}]_i$ and on responses to ATP (Table 1).

It has been reported that NPY can attenuate or potentiate the $[\text{Ca}^{2+}]_i$ increase induced by other agonists via inhibition or augmentation of phospholipase C activity [19,29]. However, ATP-stimulated phospholipase C activity in CHP-234 cells [31] appears unlikely to be modified by NPY, since no changes in ATP-induced Ca^{2+} mobilization were seen for cells pretreated with either 100 nM or 1 μM NPY in the absence of extracellular

Ca^{2+} (Figure 3). For the same reason, modulation of the degree of phosphorylation of the InsP_3 receptor, and thus its sensitivity to InsP_3 [40], via the concentration-dependent effects of NPY on cAMP levels, also seems unlikely. Taken together, these data might also suggest that NPY modifies only the Ca^{2+} influx component of ATP responses.

Extracellular ATP has been shown to induce both inhibition [41] and stimulation [42] of cAMP accumulation. Here, ATP had no effect on cAMP levels. This appears to rule out the possibility that 100 nM NPY positively modulates ATP responses by decreasing putative cAMP accumulation induced by ATP, as it does with forskolin-stimulated cells (Table 3). In the same way, it is unlikely that 1 μM NPY acts synergistically with ATP in increasing cAMP levels to diminish ATP-induced $[\text{Ca}^{2+}]_i$ responses. That NPY modulates the actions of adenosine (which may be present as a metabolite of ATP) can be excluded, as adenosine had no effect on $[\text{Ca}^{2+}]_i$.

The mechanism by which NPY might modify the Ca^{2+} influx component of the ATP response, and which involves the cAMP pathway, is not clear, but some hypotheses can be eliminated. Firstly, if cAMP-dependent potentiation of Ca^{2+} -activated K^+ -channel activity [43] and membrane hyperpolarization occurred here, the driving force for Ca^{2+} entry would increase, perhaps thereby causing potentiation of the $[\text{Ca}^{2+}]_i$ signal in response to ATP. Note that we have previously shown that 100 nM NPY increases Ca^{2+} -activated K^+ -channel activity in these cells [31]; however, at this concentration, NPY decreases cAMP levels. Taken together with the inhibitory action of NPY on Ca^{2+} -activated K^+ -channel activity in myocytes from rat tail artery [28], this possibility therefore appears to be unlikely. On the other hand, at high NPY concentrations, stimulation of Ca^{2+} efflux via activation of Ca^{2+} -ATPase or increased $\text{Na}^+/\text{Ca}^{2+}$ exchange by elevated cAMP levels, as proposed for smooth muscle cells [44], might contribute to the decreased $[\text{Ca}^{2+}]_i$ response to ATP. Finally, it may also be possible that NPY, via the cAMP/protein kinase A system, modulates $\text{P}_{2\text{U}}$ -type purinergic receptors in our cells, in a similar manner to the proposed effects of NPY on nicotinic cholinergic receptors in bovine chromaffin cells [17]. This would be in agreement with previous reports which show that in pig saphenous artery [25] and rabbit ear artery [24], NPY has a post-junctional modulatory effect on the contractile response elicited by α, β -methylene ATP. It has also been found that elevated cAMP levels modulate ATP-induced oscillations in $[\text{Ca}^{2+}]_i$ in single hepatocytes from rats [45]. However, our results do not allow one to distinguish amongst these possibilities, and further experiments are required to clarify this point.

In binding studies, CHP-234 cells were found to express only, or almost exclusively, Y_2 -type receptors [33,34,46], and we found that the Y_1 -selective agonist [Leu³¹,Pro³⁴]NPY [46] was ineffective in producing increases in $[\text{Ca}^{2+}]_i$ in these cells [30]. In addition, in the present study we found that [Leu³¹,Pro³⁴]NPY at concentrations < 100 nM had no inhibitory effect on adenylate cyclase activity. Together, these data are consistent with the absence of Y_1 -type receptors in these cells [33,34,46]. Thus, the biphasic effect of NPY found here may indicate the presence of another as yet undefined NPY Y_2 -receptor subtype, as suggested by others [11,47]; clearly, the development of truly subtype-specific receptor antagonists would aid in the resolution of this possibility.

Another possibility is receptor-independent activation of G-proteins [48] by NPY and related peptides [49], as initially proposed to explain the NPY-induced, receptor-independent release of histamine from mast cells and the associated paradoxical fall in systemic arterial pressure observed for high NPY concentrations [50,51]. Such an effect might account for the

stimulatory action of high concentrations of NPY on cAMP levels seen in the present study (Table 3). In any case, to our knowledge, there have been no other reports of NPY, or its analogues, stimulating cAMP production via any of the NPY receptor subtypes identified to date, with the exception of the, perhaps receptor-independent, biphasic effects of NPY(17-36) on rat cardiac adenylate cyclase [35] described above.

In conclusion, the results presented here suggest that NPY modulates the effects of ATP on $[Ca^{2+}]_i$ levels in CHP-234 cells by a mechanism which involves the cAMP/protein kinase A pathway. Diminution of cAMP levels, which potentiates the action of ATP, appears to be mediated by activation of Y_2 -type receptors at low NPY concentrations. In contrast, increases in cAMP content are correlated with decreases in ATP-induced $[Ca^{2+}]_i$ signals for high concentrations of NPY. These latter effects may perhaps be receptor-independent, involving direct activation of G-proteins, and/or indicative of the presence of another as yet undefined NPY Y_2 -receptor subtype. Note that both of the recent reports of cloned Y_2 receptors were based on libraries constructed from neuronal tissue (SMS-KAN neuroblastoma [11], human hippocampus [10]). It is interesting that no hybridization was detected with RNA from peripheral tissues using Northern analysis [11], and it was suggested that perhaps another subtype of Y_2 receptor exists [11].

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