British Journal of Pharmacology (2002) 135, 1701–1706

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Neuropeptide Y increases 4-aminopyridine-sensitive transient outward potassium current in rat ventricular myocytes

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1 The modulation of 4-aminopyridine sensitive transient outward potassium current (4-AP I_{to}) by neuropeptide Y (NPY) (100 nM) in rat ventricular myocytes was examined using the whole cell voltage-clamp technique.

2 Continuous exposure to NPY (100 nM) for 3–6 h significantly increased 4-AP I_{to} density. The stimulation of 4-AP I_{to} density by NPY was concentration-dependent ($EC_{50} = 10$ nM).

3 In the presence of BIBP 3226, an NPY receptor antagonist that binds selectively to NPY Y1-receptors, the effect of NPY on 4-AP I_{to} density was maintained. However, in the presence of BIIE 0246, a highly selective non-peptide NPY Y2-receptor antagonist, NPY was unable to increase 4-AP I_{to} density.

4 The effect of NPY on 4-AP I_{to} density was prevented by pretreatment with 500 ng ml^{-1} pertussis toxin (PTX) and by the specific protein kinase C (PKC) inhibitor, calphostin C (100 nM).

5 Thus, short term exposure to NPY induces an increase of 4-AP I_{to} density in rat ventricular myocytes mediated by Y2-receptors and involving the action of PKC *via* a PTX-sensitive signalling cascade.

British Journal of Pharmacology (2002) **135,** 1701–1706

Keywords: Neuropeptide Y; K^+ channels; transient outward; potassium; current; protein kinase C; heart

Abbreviations: 4-AP I_{to}, 4-aminopyridine sensitive transient outward potassium current; NPY, neuropeptide Y; PKC, protein kinase C; PTX, pertussis toxin

Introduction

Neuropeptide Y (NPY) is a 36-amino-acid peptide that is widely distributed through the central and peripheral nervous system of many species including man (Wahlestedt & Reis, 1993). In the peripheral nervous system NPY is mainly stored along with norepinephrine in some sympathetic nerve endings (Dumont *et al*., 1992). A variety of actions have been attributed to NPY in the control of cardiovascular function which include its being a potent vasoconstrictor of arteries and veins (Edvinsson *et al*., 1987). In the periphery NPY can modulate cardiovascular function by acting at pre- or postsynaptic level. In the heart, three types of postjunctional receptors named Y1, Y2 and Y3 have been described (Balasubramaniam *et al*., 1990; McDermott *et al*., 1997). Postsynaptic NPY receptors act through pertussis sensitive G proteins and intracellular signalling pathways (Michel, 1991; Michel *et al*., 1998). Although the signal-transducing mechanism most frequently associated with NPY receptors is the inhibition of adenylate cyclase (Michel, 1991), phospholipase C (PLC) dependent pathways have also been reported (Bell *et al*., 1997; Goldberg *et al*., 1998).

Regarding the heart, the direct effect of NPY on cardiac contraction may vary depending on the species and tissues used (McDermott *et al*., 1993). Thus, NPY has been shown to exert positive, negative or no effect on cardiac muscle strips. Inotropy was not affected by NPY in human atrial strips (Franco-Cereceda *et al*., 1987; Michel *et al*., 1989). Negative inotropic effects of NPY were demonstrated in adult rat cardiomyocytes and the opposite was shown in those of adult guinea-pig (Millar *et al*., 1991). To explain this discrepancy Millar *et al*. (1991) have postulated that the negative inotropic effect of NPY in rat cardiomyocytes may be coupled to activation of a transient potassium outward current (I_{to}) given that a selective blocker of this current, 4-AP, abolishes the negative inotropic effect and can even unmask a small positive inotropic effect. Since guinea-pig cardiomyocytes lack the above I_{to}, a positive inotropic effect of NPY might be observed in this species (Millar *et al*., 1991).

Therefore, the aim of this study is to analyse the modulation of NPY on 4-AP I_{to} in adult ventricular rat myocytes including the NPY receptor subtype involved and the possible participation of an intracellular pathway that comprises a PTX sensitive G protein and PKC mechanisms. For this purpose we used the non-peptide selective Y1-receptor antagonist (BIBP 3226) (Rudolf *et al*., 1994; Doods *et al*., 1996) and the selective Y2-receptor antagonist (BIIE 0246) (Doods *et al*., 1999; Dumont *et al*., 2000). Finally we used PTX and calphostin C to ascertain the possible intracellular pathway involved in the effect of NPY on 4-AP I_{to}.

Methods

Isolation of ventricular myocytes

Adult male Wistar rats weighing 250–300 g were heparinized (4 IU g^{-1} i.p.) and anaesthetized with pentobarbitone (50 mg kg^{-1}) in accordance with the European rules. The

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hearts were removed and mounted on a Langendorff-perfusion apparatus. The ascending aorta was cannulated, and a retrograde perfusion was set up. The heart was first perfused for 2–3 min at 36–37°C with a nominally calcium-free Tyrode solution containing 0.2 mM EGTA and then for 3–4 min approximately with the same Tyrode solution containing 251 IU of collagenase type II (Worthington) and 0.1 mM $CaCl_2$. At the end of the perfusion period, the heart was removed from the Langendorff apparatus and the apical part of the heart was cut off, chopped into small pieces, and gently stirred in the above-mentioned solution containing 1 mg ml^{-1} of bovine serum albumin (BSA, Sigma). Subsequently, isolated cells were filtered, centrifuged and suspended in the Tyrode solution containing 2 mg ml^{-1} of bovine serum albumin and 0.5 mM $CaCl_2$. Finally cells were again centrifuged and re-suspended in a store solution containing 2 mg ml^{-1} of bovine serum albumin and 1 mM $CaCl_2$. We used only cells from the apical part of the heart because in a previous study we demonstrated that 4-AP I_{to} density was more homogeneous in this region (Benitah *et al*., 1993). All experiments were performed at room temperature (24–26°C) on Ca^{2+} tolerant rod-shaped myocytes. The yield was over 80%. Cells were used within 10 h of being isolated.

Solutions and drugs

The nominally Ca^{2+} free tyrode solution for isolation of myocytes contained (mM): NaCl 130, KCl 5.4, NaH_2PO_4 0.4, $MgCl_2$ 0.5, HEPES 25, $NaHCO_3$ 5, Glucose 22; pH adjusted to 7.4 with NaOH. The solution for I_{to} current recordings contained (mM): Choline chloride 135, Glucose 10, HEPES 10, $MgCl_2$ 1, $CaCl_2$ 1, KCl 5.4, Atropine sulphate 0.01 and $CoCl_2$ 2 (pH adjusted to 7.4 with KOH). The intracellular recording pipette solution contained (mM): KCl 150, TEA 20, EGTA 10, HEPES 5, $MgCl_2$ 1, Na_2ATP 5, Na_2GTP 0.4 and Glucose 10; pH adjusted to 7.2 with KOH.

Porcine NPY (Tocris Co.) and PTX (Research Biochemical Inc.) were dissolved in bidistilled water to make stocks solutions. Calphostin C (Sigma Chemical Co.) was dissolved in dimethylsulphoxide (final DMSO concentration less than 0.01%). BIBP 3226 and BIIE 0246 (Boehringer Ingelheim, Pharma, Germany) were dissolved in dimethylsulphoxide and further diluted in bidistilled H_2O.

Recording techniques

Cell membrane currents were recorded using the whole-cell of the patch-clamp technique. The voltage-clamp circuit was provided by an Axopatch-1D amplifier with a 100 MΩ feedback resistance (Headstage CV-4 1/100, Axon Instruments) controlled by a computer which was equipped with pClamp (version 6.0, Axon Instruments) and interfaced to the amplifier with a 125 kHz Labmaster board. The recording pipettes were made from 1.5 mm-OD soft-glass capillary tubing with a microprocessor-based patch-clamp puller (P97/PC, Sutter Instruments) and, when filled with internal solution, had tip resistances ranging from 0.9 to 2 MΩ. The series resistance range from 3 to 5 MΩ before compensation (60–80% of the series resistance was compensated). Whole-cell recordings were started 5 min after seal disruption, to allow for cell dialysis. I_{to} was obtained by applying depolarizing pulses from a holding potential of −80 mV to voltage tests from −40 to +60 mV in 10 mV increments for 1000 ms at a frequency of 0.2 Hz. I_{to} amplitude was taken as the difference between the peak outward current and the current at the end of the pulse. The 4-AP I_{to} was obtained by the subtraction of the currents obtained before and after 3 mM 4-AP application.

Current density was calculated from the current amplitude normalized by the membrane capacitance. Membrane capacitance (C_m) was elicited by applying ±10 mV voltage steps from the resting potential and C_m was calculated according to the equation $C_m = \iota_c\ I_0/\Delta E_m\ [1-(I_\infty/I_0)]$.

Where ι_c is the time constant of the membrane capacitance, I_0 is the maximum capacitance current value, ΔE_m is the amplitude of the voltage step, and I_∞ is the amplitude of the steady-state current.

Statistical analysis

Data are presented as mean±s.e.mean. Statistical comparisons were performed by Student's *t*-test for unpaired data. A value of $P<0.05$ was considered significant.

Results

Increase of 4-AP I_{to} after 3–6 h NPY incubation

In the present study we investigated the effect of a short-term exposure of ventricular myocytes to 100 nM NPY on I_{to}. NPY concentration was chosen because this dose has been proved to induce activation of I_{to} in rat ventricular myocytes (Millar *et al*., 1991). Figure 1A shows the family of current traces obtained from one control (left panel) and one 100 nM NPY-treated (3–6 h) ventricular cell (right panel). Upper panels show two examples of total I_{to} traces evoked by 1 s pulses from −10 to +60 mV from a holding potential of −80 mV. Total I_{to} was higher in the cell treated with NPY (right panel) than in the non-treated cell (left panel). When both cells were perfused with 3 mM 4-AP, total I_{to} was dramatically decreased (middle panels). Lower panels also showed 4-AP sensitive current, obtained by subtracting current in the presence of 4-AP from that under control conditions. Traces clearly showed that 4-AP I_{to} was higher in the cell treated with NPY. Similar results were obtained in 17 control and 16 NPY-treated cells. To avoid error in pooling data from different-sized myocytes, we normalized the I_{to} amplitude by the cell capacitance to obtain I_{to} density. Mean current density-voltage relations are illustrated in Figure 1B and show that at any potential studied 4-AP I_{to} density was significantly higher in cells incubated 3–6 h with 100 nM NPY compared with control cells. No significant differences were obtained when membrane capacitances from control (118.3±6.2 pF; $n=17$) and NPY-treated cells (114.4±8.2 pF; $n=16$) were compared.

Concentration-dependence of the action of NPY on 4-AP I_{to}

The NPY-induced increase of 4-AP I_{to} was concentration-dependent (Figure 2). In these experiments, 4-AP I_{to} was obtained on depolarization from −80 to +60 mV at 0.2 Hz. The threshold of the NPY-induced increase of 4-AP I_{to} was

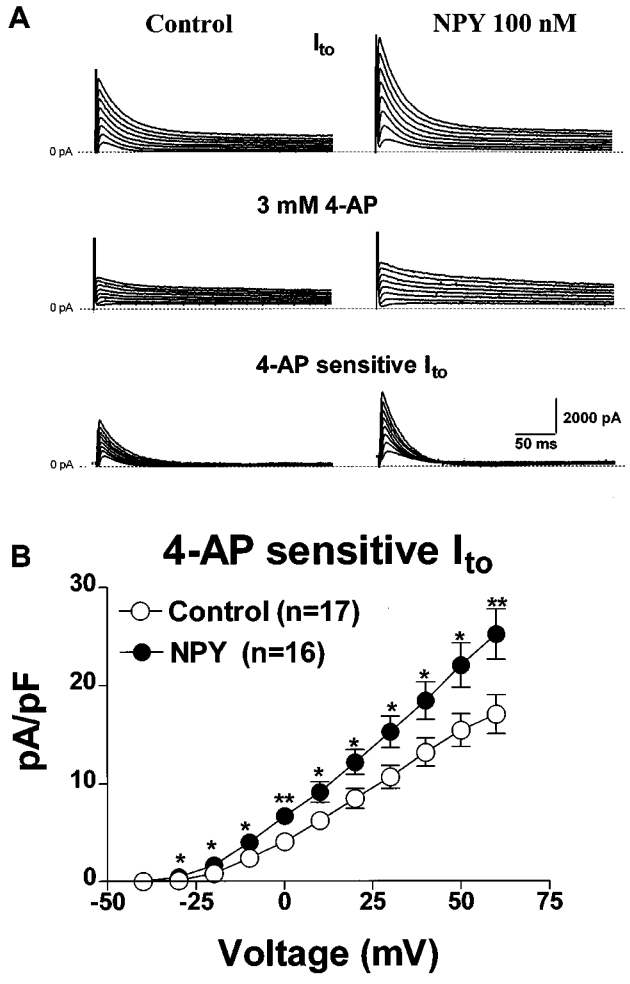

Figure 1 Tracings (A) and graphs (B) showing the increase of I_{to} and 4-AP-sensitive I_{to} after 3–6 h incubation with NPY 100 nM. (A) Left panel shows outward potassium currents in one control cell and right panel shows outward potassium currents in a different cell treated with 100 nM NPY. Upper panels show I_{to} traces obtained during step depolarizations from −10 to +60 mV, middle panel shows I_{to} traces recorded in the same control and NPY-treated cell but after the addition of 3 mM 4-AP and bottom panel shows 4-AP-sensitive I_{to} traces obtained by the subtraction of the currents before and after 3 mM 4-AP application. (B) Graph showing mean current density-voltage relation of 4-AP-sensitive I_{to} in control and NPY-treated cells. Data are given as mean values±s.e.mean. $^*P<0.05$, $^{**}P<0.01$.

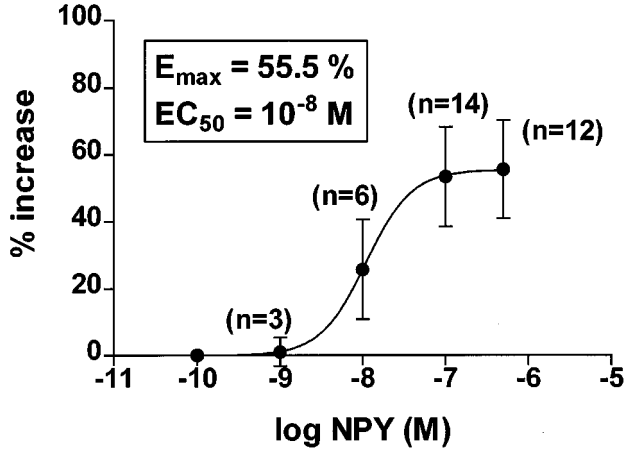

Figure 2 Concentration-response curve of the NPY-induced increase of 4-AP I_{to} in rat ventricular myocytes. Increase of 4-AP I_{to} over basal current is shown in per cent. Fit was obtained by using Graph Pad software (version 3.0). The numerical values derived for E_{max} and EC_{50} were 55.5% and 10^{-8} M, respectively. Data are given as mean values±s.e.mean, with the number of tested cells indicated in parentheses.

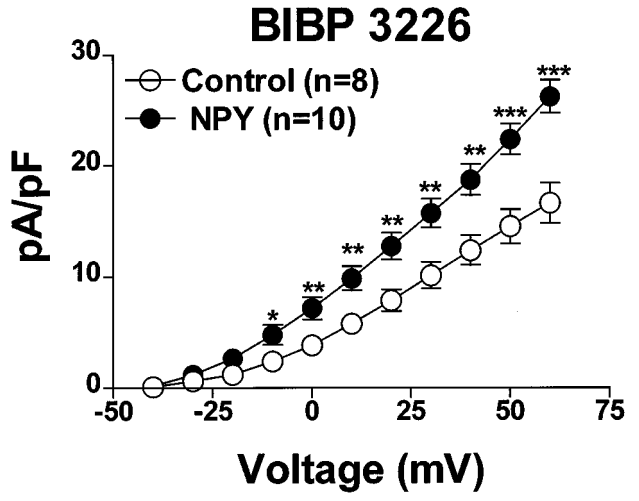

Figure 3 Effect of 100 nM NPY on 4-AP I_{to} in the presence of selective Y1-receptor antagonist BIBP 3226. Graph showing mean current density-voltage relation of 4-AP I_{to} in control and NPY-treated cells. All cells were pretreated with the Y1-receptor antagonist BIBP 3226 (1 μM). Data are given as mean values±s.e.mean. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

10^{-9} and the maximum increase of 4-AP I_{to} was obtained with 5×10^{-7} M NPY. The EC_{50} of the concentration-response curve was 10^{-8} M and E_{max} was 55.5% over basal 4-AP I_{to}.

NPY receptor involved

In order to establish the type of NPY receptor involved in the increase of 4-AP I_{to} by NPY, we used a selective antagonist of Y1-receptors (BIBP 3226, 1 μM) (Rudolf *et al*., 1994) and a selective antagonist of Y2-receptors (BIIE 0246, 1 μM) (Doods *et al*., 1999; Dumont *et al*., 2000). Figure 3 shows mean current density-voltage relations of 4-AP I_{to} density obtained in eight cells treated during 30 min with the selective antagonist of Y1-receptor, BIBP 3226 (open circles), and 10 cells treated previously with BIBP 3226 and exposed to NPY (100 nM) for 3–6 h (closed circles). Figure 3 shows that NPY was able to induce an increase of 4-AP I_{to} that was even more significant than the increase illustrated in Figure 1B in absence of any NPY receptor antagonists. Figure 4 shows mean current density-voltage relations of 4-AP I_{to} obtained in 14 cells treated during 30 min with the selective antagonist of Y2-receptor BIIE 0246 (open circles) and eight cells treated previously with BIIE 0246 and exposed to NPY (100 nM) for 3–6 h (closed circles). Figure 4 shows that in the presence of Y2-receptor antagonist, NPY was unable to induce an increase of 4-AP I_{to} in rat ventricular myocytes. Thus, these results suggest that the effect of NPY on 4-AP I_{to} is mediated through Y2-receptors.

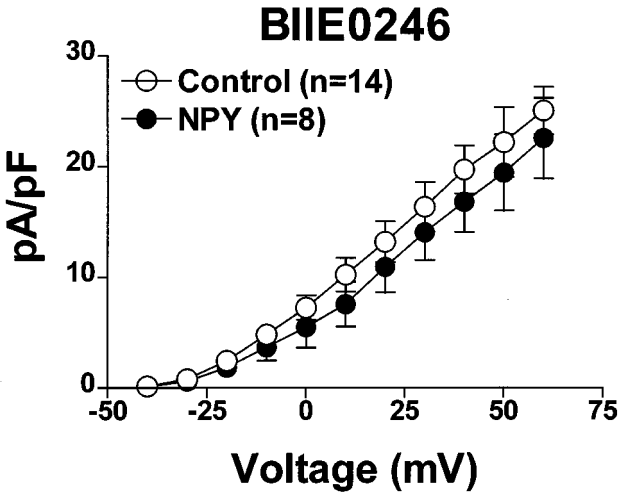

Figure 4 Effect of 100 nM NPY on 4-AP I_{to} in the presence of selective Y2-receptor antagonist BIIE 0246. Graph showing mean current density-voltage relation of 4-AP I_{to} in control and NPY-treated cells. All cells were pretreated with the Y2-receptor antagonist BIIE 0246 (1 μM).

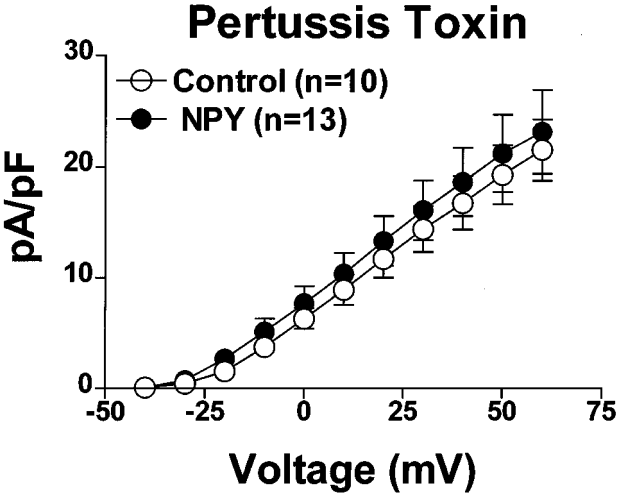

Figure 5 Effect of 100 nM NPY on 4-AP I_{to} in cell pretreated with PTX (500 ng ml^{-1}). Graph showing mean current density-voltage relation of 4-AP I_{to} in control and NPY-treated cells. All cells were pretreated for more than 5 h with PTX.

Involvement of G-proteins/potential signal transduction pathway

Nowadays it is accepted that the NPY receptors are coupled to G_i proteins (Michel *et al*., 1998). In order to establish whether the increase of 4-AP I_{to} by NPY, was mediated or not *via* a pertussis-sensitive G protein, a group of experiments were performed on cells treated with PTX (500 ng ml^{-1}) during at least 5 h prior to incubation with 100 nM NPY. Figure 5 shows mean current density-voltage relations obtained in control cells, treated with PTX (open circles, $n=10$) or with PTX plus NPY (closed circles, $n=13$). In cells pretreated with PTX, NPY was unable to induce an increase of 4-AP I_{to} density, suggesting an involvement of PTX-sensitive G proteins on this effect.

Although the typical signalling responses of NPY receptors is the inhibition of adenylate cyclase (Michel *et al*., 1998), activation of PLC has also been involved in some functional responses after stimulation of NPY receptors (Bell *et al*., 1997; Goldberg *et al*., 1998). In addition, several studies have demonstrated that PKC can modulate Kv4 channels (Nakamura *et al*., 1997) which may encode native cardiac I_{to} (Nerbonne, 2000). In order to establish whether PKC might be involved in the increase of I_{to} induced by NPY, we incubated the cells for 30 min with 100 nM calphostin C an specific PKC blocker (Kobayashi *et al*., 1989). Figure 6 shows mean current density-voltage relations obtained in 13 control cells treated with calphostin C and in 12 cells treated with calphostin C and NPY. In the presence of calphostin C, NPY was not able to induce an increase of 4-AP I_{to}. These results suggest that PKC is, in some way, involved in the cascade of events associated with the increase of 4-AP I_{to} density by NPY in rat ventricular myocytes.

Discussion

The present study was designed to investigate the effect of NPY (100 nM) on 4-AP I_{to} in rat ventricular myocytes. Our results demonstrate that NPY induces a significant increase in I_{to} density in rat ventricular myocytes mediated by Y2-receptors and involving the action of PKC *via* a PTX-sensitive signalling cascade.

In rat ventricular myocytes, three distinct K^+ currents are usually recorded. The inward rectifier K^+ current (I_{k1}), the transient outward potassium current (I_{to}); and the delayed rectifier K^+ current (I_K) (Barry & Nerbonne, 1996).

I_{to} is a voltage-dependent potassium current that plays an important role in the early repolarization phase of several cardiac tissues including rat (Apkon & Nerbonne, 1991), rabbit (Hiraoka & Kawano, 1989), dog (Tseng & Hoffman, 1989) and human (Wettwer *et al*., 1994), but not guinea-pig (Hume & Uehara, 1985). Our results demonstrate that

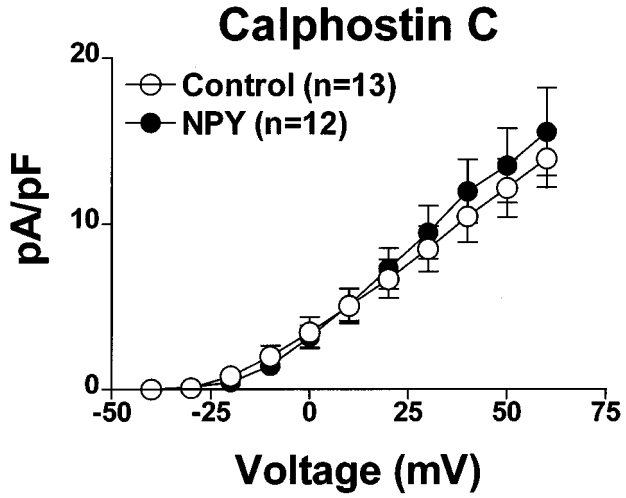

Figure 6 Effect of 100 nM NPY on 4-AP I_{to} in cell pretreated with the selective PKC inhibitor calphostin C. Graph showing mean current density-voltage relation of 4-AP I_{to} in control and NPY treated cells. All cells were pretreated with calphostin C (100 nM) for 30 min.

continuous exposure to NPY (100 nM) for 3–6 h significantly increased 4-AP I_{to} density in rat ventricular myocytes (Figure 1). These results are in concordance with a previous report by Millar *et al*. (1991) which showed electrophysiological recordings of I_{to} activation by NPY in rat ventricular myocytes. In addition, NPY-induced activation of K^+ currents have been reported in the arcuate nucleus of rat brain (Rhim *et al*., 1997), and in bullfrog sympathetic neurons (Zidichouski *et al*., 1990). Moreover, our results also demonstrate that the effect of NPY on 4-AP I_{to} involves a pertussis-sensitive G protein pathway (Figure 5) which is in concordance with the characteristics of NPY receptors that have been demonstrated to be coupled to G_i proteins (Michel *et al*., 1998). G-protein-mediated regulation of potassium channels in the heart may occur either directly through interaction of a G protein subunit with the ion channel, which is the case of the inward rectifier channel I_{KACh} (Wickman *et al*., 1994) or indirectly *via* the production of second messengers, by an enzyme that is directly modulated by the G protein subunit such as adenylyl cyclases (Babenko & Vassort, 1997) or phospholipases C (Lee *et al*., 2001). Until now no evidence has been found of a direct regulation of I_{to} by any G protein (Oudit *et al*., 2001). However, there is experimental evidence of a direct modulation of PKC on the I_{to} channel. Experiments carried out on Kv4 channels, which have been established to encode native cardiac I_{to} (Nerbonne, 2000), showed a reduction of I_{to} when PKC was activated by phorbol 12-myristate 13 acetate (PMA) (Nakamura *et al*., 1997). In addition, activation of PKC was also postulated to be involved in the increase of I_{to} by noradrenaline in chagasic canine epicardium (Han *et al*., 1997). In our experiments, I_{to} was increased after 3–6 h treatment with 100 nM NPY, and this effect was prevented by the specific PKC inhibitor calphostin C (Figure 6), suggesting an involvement of PKC intracellular pathway in the effect of NPY on I_{to}. One possible explanation of the opposing effects related to the activation of PKC on I_{to} channel might be the existence of two different phosphorylation sites involved in the modulation of I_{to}. One site would mediate an increase while the phosphorylation of the other site would downregulate the current. Biphasic regulation of K^+ channels by PKC activators has been reported previously. It was postulated that two different phosphorylation sites were involved in the modulation of the Aplysia Kv1.1a channel (Furukawa *et al*., 1995) and in the minK-KvLQT1 channel (Lo & Numann, 1998).

To date six subtypes of NPY receptors have been identified and designated as Y1, Y2, Y3, Y4, Y5 and Y6 (Michel *et al*., 1998). In the heart only the Y1, Y2 and Y3 subtypes have been recognized although Y3 has not been cloned and no specific agonists or antagonists of the Y3 subtype have been described (McDermott *et al*., 1993; Michel *et al*., 1998). The results reported in this paper (Figures 3 and 4) using the non-peptide selective Y1 receptor antagonist (BIBP 3226) (Rudolf *et al*., 1994) and the selective Y2-receptor antagonist (BIIE 0246) (Doods *et al*., 1999; Dumont *et al*., 2000) demonstrate that the effect of NPY on 4-AP I_{to} is mediated through Y2-receptors. Although the functional roles of the Y1- and Y2-receptors in the heart have yet to be clearly established, it has been postulated that Y1-receptors might mediate positive inotropic effects whereas, Y2-receptors would mediate negative inotropic effects (McDermott *et al*., 1997). With regard to this our finding will be in concordance with this hypothesis, given that an increase in I_{to} density mediated through NPY binding to Y2-receptors could shorten the action potential duration, reducing the time available to calcium influx and thus reducing the amount of intracellular calcium available for contraction.

Although the typical signalling responses of NPY receptors is the inhibition of adenyl cyclase (Michel *et al*., 1998), activation of PLC has also been involved in some functional responses after stimulation of NPY receptors. In rat cardiomyocytes, the attenuate response of NPY on isoprenaline-stimulated contraction was mediated by activation of PLC-dependent pathways (Bell *et al*., 1997).

In conclusion, our results support the idea that the Y2-receptor is coupled *via* the PTX-sensitive G_1 to activate a signalling pathway that involves activation of PKC and stimulation of the I_{to} channel. A similar signalling mechanism has recently been postulated to explain the stimulation of the mitochondrial K_{ATP} channel involved in the cardioprotective role of adenosine on A1-receptors (Lee *et al*., 2001).

Further experiments will be necessary to prove the functional role of the NPY-induced increase of 4-AP I_{to} in rat ventricular myocytes.

This study was supported by the Comisión Interministerial de Ciencia y Tecnología of Spain (SAF99-0068). We thank Boehringer Ingelheim for kindly having given us the two selective NPY receptor antagonists, BIBP 3226 and BIIE 0246. We are grateful to Manuel Bas, Fernando Ortego and Maria Luisa Hidalgo for their excellent technical assistance. MP Heredia is a graduate research fellow of the Comunidad Autónoma de Madrid and M Fernández-Velasco is a graduate research fellow of the Ministerio de Ciencia y Tecnología of Spain. Authors thank Dr Ana Gonez for assistance.

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(Received January 11, 2002
Accepted January 28, 2002)