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# The direct stimulation of Gi proteins by neuropeptide Y (NPY) in the rat left ventricle☆

Laura Raimondi*, Grazia Banchelli, Rosanna Matucci, Francesca Stillitano, Renato Pirisino

*Department of Pharmacology, University of Florence, Florence, Italy*

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**Abstract**

Neuropeptide Y (NPY) is a neuropeptide with high distribution in the cardiovascular system of mammals, where it modulates heart and vessel contractility. In the rat heart, the presence of at least three different NPY receptor subtypes has been hypothesised. Notwithstanding this, receptor activation might not be the only mechanism responsible for the complex cardiac effects of the peptide. In this study, we investigated the effect of NPY on the GTPase activity of G-proteins in the rat left ventricle as a possible alternative mechanism of action for the peptide in the rat heart. Our results show that NPY, but also the neuropeptide fragment (18–36) (NPY (18–36)), stimulated the basal, spontaneous GTPase activity of ventricle membranes only when it was measured under the condition of an absence of $Mg^{2+}$. This stimulation was resistant to BIBP3226 a non-peptidergic antagonist at Y1 receptors, but it was significantly reduced in membranes treated with selective antibodies against the Giα subunits. NPYs effect was concentration-dependent with a maximum of activity at 10 nM. At this concentration, NPY (and NPY 18–36) was able to inhibit forskolin (FSK)-induced cyclic adenosine-5′-monophosphate (cAMP) elevation in rat left ventricle slices. Our results assess that NPY in the rat heart is able to activate the GTPase activity of Gi proteins, in a receptor-independent way. 

*Keywords:* Heart metabolism; G-proteins; Second messengers; Signal transduction

## 1. Introduction

NPY is a highly conserved, 36-aminoacid residue peptide belonging to the family of structurally-related peptides that includes the peptide YY (PYY) and the pancreatic polypeptide (PP) [1,2]. NPY is widely distributed throughout the central nervous system, where it is co-stored with different transmitters. NPY deeply affects food and water intake, produces anxiolytic and sedative effects and seems implicated in memory retention [3]. These central effects of NPY have been studied extensively because of their involvement in metabolic diseases, such as diabetes and obesity [4,5] or in neurological syndromes that are characterised by alterations of mood and behaviour [6]. NPY (and its receptors) is also highly distributed in the periphery and is co-localised with norepinephrine in synaptic terminals of post-ganglionic neurones innervating the cardiovascular system. It represents, together with the atrial natriuretic peptide one of the most abundant peptides found in the heart [7], where it modulates sympathetic transmission [8] and tissue growth [9]. NPY and NPY fragment effects on rat heart mechanical activity are complex [10]. In particular, NPY plays an anti-adrenergic role affecting both isoprenaline-dependent contractility and adenosine-5′-monophosphate (cAMP) increases in isolated cardiomyocytes, whereas, in the same cells, in the presence of 4-aminopirydine, NPY induces stimulation of cardiomyocyte contractility [11,12]. The negative inotropic effect is sensitive to the pertussis toxin and to the partial peptide NPY (18–36), whereas the positive effect is not [13]. From this it was proposed that at least three different receptor subtypes (Y1, Y2 and Y3) linked to the opposite effects might exist in the rat heart [14]. However, the relationship

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* Corresponding author. Tel.: +39-55-427-1210; fax: +39-55-427-1280.
*E-mail address:* raimondi@server1.pharm.unifi.it (L. Raimondi).

*Abbreviations:* NPY, neuropeptide Y (1–36); NPY (18–36), neuropeptide fragment (18–36); FSK, forskolin; cAMP, cyclic adenosine-5′-monophosphate; BIBP3226, (*N*2[diphenylacetyl]-*N*-[(4-hydroxyphenyl)-D-arginine amide; GTP, guanosine-5′-triphosphate; GTPase activity, guanosine-5′-triphosphate hydrolysing activity; Giα, α subunit of Gi protein.

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between specific receptor activation and the biological effects of the peptide is still lacking. To clarify this point, to date, neither the measurement of the second messengers nor the use of NPY fragments have been of much help. In fact, the links between individual NPY receptors and second messenger substances have not been conclusively established [15], probably because NPY receptors (Y1 and Y2) do not differ completely in second messenger activation [16,17]. Moreover, most of the NPY fragments lack in specificity for the different receptor subtypes, and often possess their own pharmacological profile [18]. NPY (18–36), for example, behaves as an agonist or antagonist in the rat cardiovascular system depending on the experimental model used [19,20].

Several pieces of evidence confirm the ability of NPY to modulate adenylate cyclase activity in the rat heart too, whereas its effect on phospholipase activation remains to be conclusively demonstrated [11,21,22]. Different approaches, based on the use of toxins, guanosine-5′-triphosphate binding and GTP analogues, have suggested the coupling of rat heart NPY receptors to G-protein activation [10,13].

Because of the complex effects of the peptide described in the rat heart and because of the difficulty to ascribe the stimulation of a specific receptor to each effect, we wondered whether NPY, in the rat heart, could have other mechanisms of action. In particular, because the ability of NPY to directly stimulate G-protein activity in brain [23] and rat mast cells [24] has already been documented, we aimed at verifying whether this mechanism could occur in the rat heart as well.

G-proteins play a pivotal role as transducers or as amplifiers of extracellular signals recognised by receptors. G-proteins are a family of heterotrimer proteins composed of α, β and γ subunits. Activation of G-proteins by agonist–receptor complexes facilitates the dissociation of GDP from the α subunits and the subsequent binding of GTP. The activated α subunits of the G-proteins dissociate from the βγ complex to modulate the activity of second messengers generating enzymes, such as adenylate cyclase and phospholipase. The Gα subunit has intrinsic high affinity GTP hydrolysing activity (GTPase activity) which blunts the signal induced by the agonist binding to the receptor. Thus, measuring the GTPase activity of G-proteins represents an indirect mode to assess G-protein activation.

It is already known that a number of compounds, such as cationic amphiphilic peptides can also activate G-proteins directly in a receptor-independent manner [25,26]. Both NPY and NPY (18–36) share the same amphiphilic structure retained essential for both receptor activation and receptor-independent effects.

Thus, our aim was to investigate the effect of NPY on GTPase activity in rat left ventricle membranes as an index of G-protein (essentially Go/Gi) activation; a possibility that had not yet been explored.

The fact that NPY can have additional mechanisms other than classical receptor activation could open up new perspectives in understanding the complex role the peptide plays in the rat heart.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Porcine NPY and NPY (18–36) were obtained from NovaBiochem. [γ-$^{32}$P]GTP (25 Ci/mol) was obtained from ICN, GTP, bovine serum albumin (BSA), cAMP, ethylendiamminetetracetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), creatine phosphokinase, phosphocreatine, adenosine-5′-triphosphate (ATP), 5′-adenylylimidodiphosphate, dithiothreitol, 3-isobutyl-1-methyl xanthine (IBMX) (*N*2[diphenylacetyl]-*N*-[(4-hydroxyphenyl)-D-arginine amide (BIBP3226), and monoclonal peroxidase-conjugate goat anti-rabbit antibodies were all obtained from Sigma–Aldrich.

The enzyme immunoassay system for cAMP level determination and the ECL detection system were obtained from Amersham. Polyclonal antibodies against the carboxyterminal portion (COOH-terminal) of α subunit of Gi protein (Giα) were obtained from Calbiochem.

### 2.2. Animals

Male Wistar rats (180–200 g) were obtained from Charles-River (Italy) breeding colonies. The animals were housed at 23 ± 1° with a 12 hr light–dark cycle, light on at 7 a.m., and were fed a standard laboratory diet with water *ad libitum*. All the experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) for experimental animal care.

### 2.3. Left ventricle membrane preparation

Rats were killed by cervical dislocation and the hearts were removed and immediately placed in cold oxygenated Krebs-bicarbonate buffer (pH 7.4) solution (KRB) of the following composition (mM): $NaHCO_3$ (25), glucose (11.1), NaCl (11.8), KCl (4.7), $KH_2PO_4$ (1.2), $MgSO_4$ (0.6).

Left ventricles were homogenised (1:5 w/v) in cold Tris–HCl (50 mM, pH 7.4) using an Ultra-Turrax. Homogenates were centrifuged at 1000 *g* for 20 min to remove cell debris and nuclei. The supernatant was subjected to further centrifugation at 40,000 *g* for 40 min at 4°. The corresponding pellet was resuspended in Tris buffer, subjected to the same centrifugation step as above and then homogenised in Tris–HCl buffer (pH 7.4) to obtain a final concentration of 1–1.5 mg of proteins/mL. The membrane homogenate was divided into small fractions and quickly frozen at −80° until used.

### 2.4. GTPase activity

GTP hydrolysing activity was evaluated by measuring the radioactivity of the $^{32}P_i$ released from [$\gamma$-$^{32}$P]GTP (25 Ci/mmol, ICN) derived from the enzymatic conversion of GTP to guanosine-5′-diphosphate, GDP and inorganic orthophosphate by the incubated membranes.

Membranes (3–8 μg of proteins/tube) were incubated at 30° for 15 min in 100 μL of 50 mM Tris–HCl buffer (pH 7.5) containing ATP (0.5 mM), 5′-adenylylimidodiphosphate (0.5 mM), phosphocreatine (5 mM), creatine phosphokinase (50 U/mL), BSA (50 μg/mL), EDTA (0.1 mM), EGTA (0.2 mM), dithiothreitol (2 mM), cAMP (0.5 mM), IBMX (0.5 mM) and NaCl (100 mM). In some experiments, $MgCl_2$ (2 mM) was also added.

The reaction started by adding [$\gamma$-$^{32}$P]GTP (from 0.05 to 0.5 μM) to the incubation medium in the absence (total GTPase activity) or in the presence of cold GTP 100 mM (low-affinity GTPase activity). GTP was incubated with membranes for 15 min at 30°.

The enzymatic reaction was terminated by transferring the tubes to an ice bath followed by the addition of 700 μL of 5% (w/v) activated charcoal in 20 mM phosphoric acid. The tubes were kept chilled for about 30 min and then centrifuged at 13,000 *g* for 10 min. An aliquot (200 μL) from the supernatant fraction was counted in a liquid scintillator. The GTPase activity (''high affinity'') was expressed as pmoles of $^{32}$P released/mg of proteins/15 min and it represents the difference between the total and the ''low-affinity'' hydrolysing activity measured.

### 2.5. The effect of BIBP3226 and of an anti-Giα subunit (isoforms 1–3) antibody on NPY-induced GTPase activity

Rat left ventricle membranes were incubated for 1 hr at 30° in 50 mM Tris buffer (pH 7.4) with polyclonal antibodies raised against the carboxyterminal Giα (isoforms 1–3, final dilution 1:10) or with an equivalent amount of non-immunised rabbit serum (as control).

When used, BIBP3226 (10 μM) was incubated at 30° with membranes 10 min before the NPY addition. GTPase activity was then measured using the procedure described above.

### 2.6. cAMP measurement in rat left ventricle slices

Left ventricles, isolated as described above, were cut into transverse slices (200 μm) using a McIlwain tissue chopper. These slices were then distributed in KRB buffer containing IBMX (0.5 mM), adenosine deaminase (0.5 mU/mL), and incubated at 37° for 15 min. FSK, 10 μM, was then added and further incubated for 10 min at 37°. NPY, when used, was pre-incubated 10 min before the FSK addition. The reaction was stopped by immersing the tubes in ice and by the addition of EDTA (4 mM). Slices were then homogenised at 4° and the proteins denatured at 100° for 10 min. Samples were centrifuged for 10 min at 10,000 *g* and cAMP was estimated in the clear supernatant by using a cAMP enzyme immunoassay method, according to the manufacturer's instructions. Results are expressed as pmol of cAMP/mg of tissue/10 min.

### 2.7. Data analysis

All results presented are the mean ± SE of at least four different experiments run in duplicate or triplicate. Statistical analysis was performed by using or Student's *t*-test or one-way ANOVA with Dunnett's post-test, considering significant a $P < 0.05$. $K_m$, $V_{max}$ and $EC_{50}$ values were calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, copyright, 1994–1999 by GraphPad Software).

## 3. Results

### 3.1. High affinity GTPase activity of rat left ventricle membranes

Spontaneous high affinity GTPase activity of G-proteins was measured in rat left ventricle membranes under the condition of a high cAMP (0.5 mM) concentration, in the presence or in the absence of $Mg^{2+}$.

High affinity basal enzymatic activity (0. 3 μM GTP) was low in the absence of $Mg^{2+}$ and it increased significantly in the presence of $Mg^{2+}$ (2 mM) (Fig. 1).

### 3.2. The effect of NPY on high affinity GTPase activity in rat left ventricle membranes

When rat left ventricle membranes were pre-incubated with NPY (10 nM) or NPY (18–36, 1 μM) 10 min before

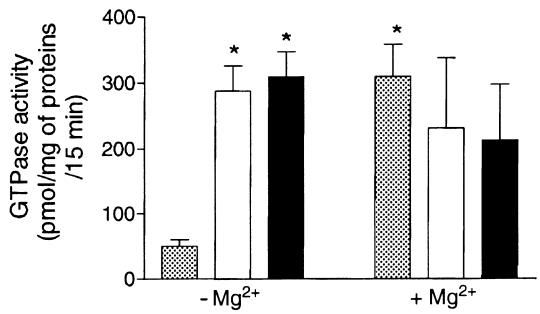


Fig. 1. NPY and NPY (18–36) stimulate the basal GTPase activity of rat left ventricle membranes. Basal GTPase activity of left ventricle membranes was evaluated as $^{32}$P release from 0.3 μM [$\gamma$-$^{32}$P]GTP hydrolysis in the absence or in the presence of $Mg^{2+}$ ions (2 mM). Basal high affinity activity was evaluated as the difference between the total hydrolysis (labelled GTP 0.3 μM) and the low-affinity hydrolysis of the nucleotide (GTP 100 mM). Results are the mean ± SE of six different experiments performed in duplicate. (*) Significantly different from basal activity ($P < 0.05$). (▨) Basal, (□) NPY 10 nM, (■) NPY (18–36) 1 μM.

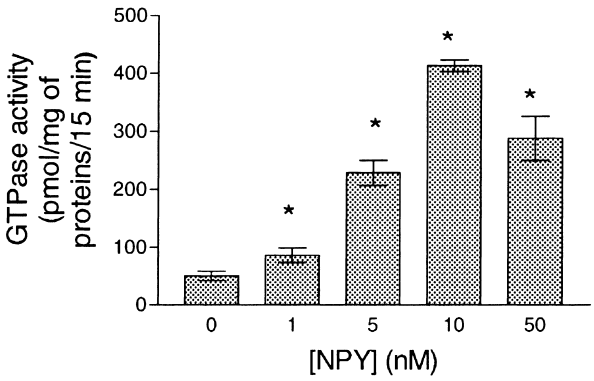


Fig. 2. NPY modifies in a concentration-dependent fashion the GTPase activity of rat left ventricle membranes. NPY at different concentrations (from 1 to 50 nM) was pre-incubated with ventricle membranes for 10 min before the addition of labelled GTP (0.3 μM). The reaction was stopped after 15 min and the radioactivity released measured as described in Section 2. Results are the mean ± SE of four different experiments run in duplicate. (∗) Significantly different from basal values ($P < 0.05$, Student's $t$-test).

the addition of GTP, a significant increase in high affinity GTPase activity was measured (Fig. 1). This stimulation was the result of an increase in "total GTPase activity" and was evident only when enzymatic activity was measured in the absence of $Mg^{2+}$ ions.

In the presence of $Mg^{2+}$, GTPase activity (high affinity) was not increased further by the presence of NPY or NPY (18–36) (Fig. 1).

### 3.3. NPY effect on GTP hydrolysis

The stimulation of GTP hydrolysis by NPY was evident at 1 up to 10 nM of concentration ($EC_{50}$ 5.06 ± 0.15 nM). At higher NPY concentrations, enzymatic activity did not increase further (Fig. 2). Moreover, at 50 nM or higher concentrations of NPY, activation induced by the peptide became lower and lower (data not shown). NPY (18–36) was not able to stimulate GTPase activity at concentrations lower than 0.5 μM (data not shown).

### 3.4. NPY affects apparent kinetic constants for GTP hydrolysis

The stimulating effect of NPY measured in the absence of $Mg^{2+}$ was associated with a significant change in the apparent $K_m$ for GTP hydrolysis which shifted from 0.34 ± 0.02 to 0.07 ± 0.01 μM. $V_{max}$ did not changed significantly (393 ± 50 in basal and 388 ± 31 pmol/mg of proteins/15 min when NPY was present; Fig. 3A and B).

### 3.5. The effect of NPY on GTPase activity in rat left membranes treated with antibodies against Giα subunits and with BIBP3226

GTPase activity was measured in membranes treated with antibodies against the COOH-terminal portion of the Giα (1–3 subunits). Under our conditions (high cAMP and

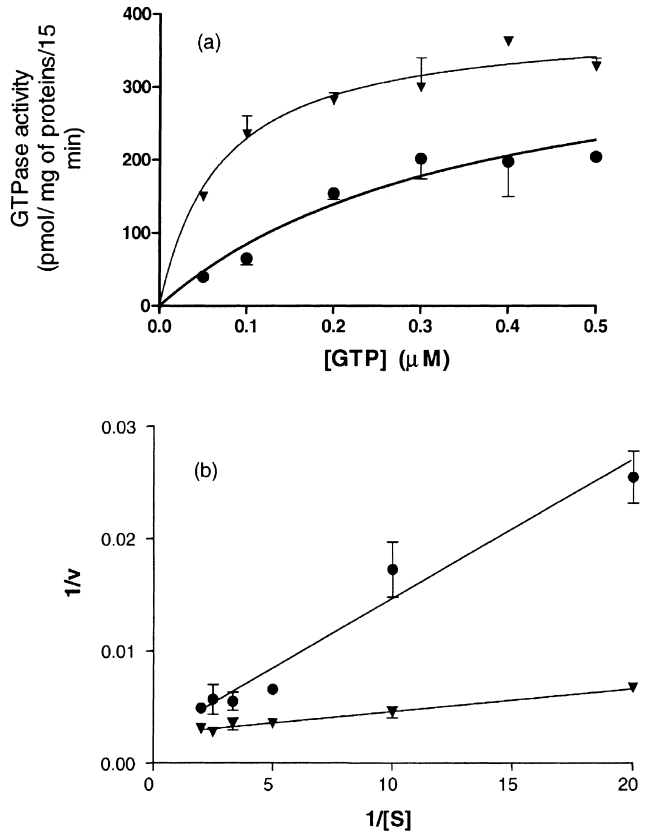


Fig. 3. NPY affects the apparent kinetic constants for GTP hydrolysis in rat left ventricle membranes. Apparent kinetic constants for high affinity GTPase-dependent hydrolysis of $^{32}$P-γ-GTP in a nominally free $Mg^{2+}$ buffer were calculated in the absence or in the presence of NPY (10 nM) as described in Section 2. Panel A: Michaelis–Menten plot: (●) control, (▼) NPY (10 nM). Panel B: Lineweaver–Burk plot: (●), control, (▼) NPY (10 nM).

in the absence of $Mg^{2+}$), a reduction in both basal and NPY-stimulated GTPase activity was measured. However, in the antibody-treated membranes, the activation produced by NPY was not completely prevented (Table 1).

Table 1
The effect of NPY on the GTPase activity is modified by treating membranes with a selective antibody against the C-terminal portion of the Giα subunits ($Gi\alpha_{1-3}$)

| | GTPase activity pmol/mg of proteins/15 min | |
|---|---|---|
| | −Giα Antibodies | +Giα antibodies |
| Basal | 40 ± 3.7 | Not measurable |
| Basal + NPY (10 nM) | 154 ± 8.03 | 61.6 ± 3.5[a] |
| NPY (10 nM) + BIBP3226 (10 μM) | 168 ± 28 | |

The GTPase activity of the left ventricle was measured in membranes pre-treated for 1 hr with a 1:10 dilution of an anti-Giα antibody (non-selective for the different subunits) or with a corresponding amount of non immunised rabbit serum (control). BIBP3226 (10 μM) was instead pre-incubated for 10 min at 30°. GTPase activity was then measured as described in Section 2 using GTP concentration of 0.05 μM.

[a] Significantly different from the value obtained in the absence of antibody treatment.

On the contrary, the stimulating activity of NPY did not change in the presence of BIBP3226 (10 μM).

### 3.6. NPY modified FSK-induced cAMP elevation in rat left ventricle slices

NPY concentration-dependently reduced the FSK-induced cAMP elevation in ventricular slices ($\log EC_{50}$ M $-8.04 \pm 0.22$). NPY (10 nM) and NPY (18–36; 1 μM) reduced FSK-stimulation of cAMP accumulation from $3.88 \pm 0.22$ (FSK) to $2.54 \pm 0.27^*$ and $2.71 \pm 0.04^*$ pmol/mg of tissue, respectively ($*$ significantly different from FSK value, $P < 0.05$, Student's *t*-test).

## 4. Discussion

At physiological pH value, the polycationic amphiphilic peptide, NPY, presents an a helix structure, the same one that is shared by the fragment NPY (18–36). The spatial conformation of these peptides is responsible for both receptor activation and for the direct activation of G-proteins, an alternative mechanism of action described for all of them [25,27]. In particular, the activation of high affinity GTPase activity in the Gα subunits of G-proteins (Gi/Go) by NPY has been documented in rat mast cells and in the central nervous system [23,28,29]. To date, no evidence that NPY also has this mechanism of action in the rat heart has been found.

We now demonstrate that NPY can directly activate basal GTPase activity (high affinity) of rat left ventricle membranes. This stimulation occurs at NPY concentrations usually considered appropriate for receptor activation. It is also obtained using the fragment NPY (18–36) and is evident only in the absence of $Mg^{2+}$, a condition that allows for discrimination between the receptor-dependent and receptor-independent mode of activation of GTPase activity. In fact, the GTP hydrolysing activity stimulated by receptor agonists, such as dopamine and acetylcholine, was reported as absolutely dependent on the presence of $Mg^{2+}$ in the reaction medium. In the presence of $Mg^{2+}$, total and non-specific GTPase activity in membranes, increased in parallel, producing a net positive increase in "high-affinity" activity. As already described, under these conditions, receptor agonists can further increase total GTPase activity, leaving the "low affinity" portion of the hydrolysis unaffected only when $Mg^{2+}$ is present in the incubation medium. From this, the agonist-induced high affinity GTPase activity from receptor-coupled G-proteins was defined as being $Mg^{2+}$-dependent [30]. Under our conditions, we found that non-stimulated GTPase activity (basal, "high affinity") was also increased in the presence of $Mg^{2+}$ (Fig. 1). However, NPY and NPY (18–36) increased total GTPase activity, leaving the "low affinity" portion of the activity unaffected only when a nominally-free $Mg^{2+}$ buffer was used. This condition is typical for substances that act as direct activators of GTPase activity. To confirm a receptor-independent mechanism of NPY on G-proteins, we found that NPY-induced stimulation of "high affinity" GTPase activity was not antagonised by BIBP3226, a non-peptidergic antagonist at NPY Y1 receptors [31]. This compound, did not show any effect on GTPase activity, confirming that only peptidergic, polycationic structures can activate it directly.

The NPY-stimulating effect on G-proteins is concentration-dependent in the range from 1 to 10 nM. Higher NPY concentrations did not produce a parallel increase in the stimulation of the GTPase activity. Regarding the mechanism of activation, our results show that the peptide mainly modifies the apparent $K_m$ for GTP hydrolysis, producing a strong increase in the affinity for the substrate.

We found that NPY stimulates GTPase activity in the rat left ventricle at the same concentrations that are used when NPY receptor activation is studied. This finding might support the hypothesis that the mechanism studied can be of more physiological relevance than previous findings reporting the NPY-induced stimulation of GTPase activity in rat mast cells and brain. In these latter models, G-protein stimulation by NPY was found to occur at high peptide concentrations (μM) and, for this reason, considered an alternative to NPY receptor activation. On the contrary, under our conditions, the stimulating effect of GTPase activity by NPY occurs in the nanomolar range.

Having ascertained that NPY stimulates G-protein activity in the absence of $Mg^{2+}$, we then tried to verify if, among the different G-proteins, NPY could also activate GTPase activity of Gi proteins. Gi proteins are highly expressed in rat left ventricle membranes, essentially in the $Gi\alpha_2$ and $Gi\alpha_3$ isoforms (data not shown). Our results, using antibodies against Giα subunits, confirm that the stimulating effect of NPY also involves Gi proteins.

The ability of NPY and some NPY fragments to modulate, either by inhibiting or stimulating, adenylate cyclase activity in the rat heart has been previously described [11]. The reduction in myocyte adenylate cyclase activity produced by NPY was reported to be pertussis toxin sensitive, an experimental condition that does not allow to conclude which type of receptor(s) is involved in the negative effect of NPY on adenylate cyclase. Our results show that NPY and NPY (18–36) both reduce FSK-induced cAMP elevation. This finding is in agreement with the modulatory role of NPY on adenylate cyclase activity and suggest that NPY (18–36), at the concentration used, acts at the same target as NPY. This conclusion seems to be further supported by the fact that NPY (18–36) demonstrates the same behaviour on both adenylate cyclase and GTPase activity.

In conclusion, our results produce evidence that, in the rat heart, NPY can have an alternative mechanism to receptor activation. This conclusion could account for the many and sometimes controversial, biological effects of the peptide.

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