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Intracellular signaling mechanisms mediating catecholamine release upon activation of NPY Y_1 receptors in mouse chromaffin cells

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Abstract

The adrenal chromaffin cells synthesize and release catecholamine (mostly epinephrine and norepinephrine) and different peptides, such as the neuropeptide Y (NPY). NPY stimulates catecholamine release through NPY Y_1 receptor in mouse chromaffin cells. The aim of our study was to determine the intracellular signaling events coupled to NPY Y_1 receptor activation that lead to stimulation of catecholamine release from mouse chromaffin cells. The stimulatory effect of NPY mediated by NPY Y_1 receptor activation was lost in the absence of extracellular Ca^{2+}. On the other hand, inhibition of nitric oxide synthase and guanylyl cyclase also decreased the stimulatory effect of NPY. Moreover, catecholamine release stimulated by NPY or by the nitric oxide donor (NOC-18) was inhibited by mitogen-activated protein kinase (MAPK) and protein kinase C inhibitors. In summary, in mouse chromaffin cells, NPY evokes catecholamine release by the activation the NPY Y_1 receptor, in a Ca^{2+}-dependent manner, by activating mitogen-activated protein kinase and promoting nitric oxide production, which in turn regulates protein kinase C and guanylyl cyclase activation.

Keywords: adrenal mouse chromaffin cells, catecholamine release, mitogen-activated protein kinase, neuropeptide Y, nitric oxide, NPY Y_1 receptor.
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Chromaffin cells are neuroendocrine cells specialized for synthesis, storage and release of catecholamine [mainly epinephrine (EP) and norepinephrine (NE)]. Neuropeptide Y (NPY) is a 36-aminoacid peptide co-stored and co-released with catecholamine, which also has a modulatory effect on the secretory activity of chromaffin cells (Cavadas *et al.* 2001, 2006). There are six NPY receptors coupled to G-proteins: NPY Y_1, Y_2, Y_3, Y_4, Y_5, and y_6 receptors (Michel 1991; Krause *et al.* 1992; Silva *et al.* 2002). Recently, we have demonstrated that mouse adrenal glands contain mRNA for Y_1, Y_2, and Y_5 NPY receptors (Cavadas *et al.* 2006). Moreover, NPY stimulates catecholamine release from chromaffin cells isolated from adrenal glands of wild-type mice, but not in chromaffin cells of NPY Y_1 knockout mice (Cavadas *et al.* 2006). The NPY Y_1 receptor is coupled to a variety of second messenger systems, including the increase of intracellular [Ca^{2+}], and the inhibition of adenylate-cyclase, resulting in a decrease of protein kinase A (PKA) activity (Mihara *et al.* 1989; Aakerlund *et al.* 1990; Strosberg 1991; Herzog *et al.* 1992; Cavadas *et al.* 2001; Silva *et al.* 2002). Other intracellular mechanisms coupled to this receptor have been described. Some studies show that the activation of NPY Y_1 receptor stimulates phospholipase C that will induce an increase both in inositol 1,4,5 triphosphate production, mobilizing Ca^{2+} from intracellular stores, and in diacylglycerol production, the endogenous activator of protein kinase C (PKC) (Daniels *et al.* 1989; Michel *et al.* 1992; Nishizuka 1992; Misra *et al.* 2004; Heredia Mdel *et al.* 2005). Moreover, in transfected Chinese Hamster Ovary cells, it was demonstrated that NPY Y_1 receptor is coupled to mitogen-activated protein kinases (MAPKs) phosphorylation involving different kinases such as PKC, Ras, PI3K, and Akt (Nakamura *et al.* 1995; Mannon and Raymond 1998; Nie and Selbie 1998; Mannon and Mele 2000).

Intracellular mechanisms coupled to NPY Y_1 receptor that stimulate catecholamine release from mouse chromaffin were not yet investigated. Several intracellular mechanisms involved in the stimulation of catecholamine secretion in chromaffin cells are already known. The Ca^{2+} entry through

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Abbreviations used: EP, epinephrine; GC, guanylyl cyclase; MAPK, mitogen-activated protein kinase; NAME, nitroarginine-methyl-ester; NE, norepinephrine; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; NPY, neuropeptide Y; ODQ, 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12–myristate 13 acetate; TH, tyrosine hydroxylase.

voltage-operated channels of the plasma membrane is essential for triggering the secretory response (Douglas 1968; Smith *et al.* 1998). Protein kinases, such as MAPK and PKC, also participate in the regulation of secretion in chromaffin cells (Sena *et al.* 1995; Cox *et al.* 1996; Cox and Parsons 1997; Smith *et al.* 1998; Teschemacher and Seward 2000). Nitric oxide (NO) is synthesized from L-arginine by the enzyme NO synthase (NOS), which is reported to exist in three major isoenzymatic forms: neuronal NOS (nNOS), endothelial NOS, and inducible NOS. In chromaffin cells from adrenal medulla, the presence of nNOS was already demonstrated by several techniques (Oset-Gasque *et al.* 1994, 1998; Schwarz *et al.* 1998). Moreover, using imunohistochemical methods, NOS-containing fibers closely associated with chromaffin cells were observed (Afework *et al.* 1994; Heym *et al.* 1994; Tanaka *et al.* 1996). Of the three isoforms of NOS, nNOS has received the most attention as a potential modulator of catecholamine secretion. However there are some contradictory reports concerning the role of NO on basal and evoked catecholamine secretion in chromaffin cells (Oset-Gasque *et al.* 1994, 1998; Torres *et al.* 1994; Schwarz *et al.* 1998; Vicente *et al.* 2002; McNeill and Perry 2005). Recently, it was shown, in other systems, that NPY induces NO production, suggesting that NO is a potential mediator of NPY function (Dimitrijevic *et al.* 2006).

The aim of the present study was to investigate the signaling pathways involved in catecholamine release stimulated by NPY Y_1 receptor activation, including the role of NO, in mouse chromaffin cells.

Materials and methods

Materials

Neuropeptide Y was purchased from Novabiochem (Laufelfingen, Switzerland); MAPK inhibitor (PD 98059) was from RBI (Natick, MA, USA); H89 (PKA inhibitor), bisindolylmaleimide I (PKC inhibitor), forskolin, phorbol 12–myristate 13 acetate (PMA), L-NAME (L-nitroarginine-methyl-ester; NOS inhibitor), collagenase solution (Type H), penicillin, streptomycin were obtained from Sigma Chemical Co. (St Louis, MO, USA); ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; guanylate cyclase inhibitor) was from Tocris (Bristol, UK); NOC-18 was from Alexis Corporation (Lausanne, Switzerland), Dulbecco's Modified Eagle's Medium/Ham's F12 was purchased from Invitrogen (Eugene, Oregon, USA) and fetal calf serum from Biochrom (Berlin, Germany).

Primary cultures of mouse chromaffin cells

Cultures of mouse chromaffin cells were prepared as previously described (Rosmaninho-Salgado *et al.* 2007). Adrenal glands were obtained from 10 to 12 weeks old mice. Mice were sacrificed and the adrenal glands rapidly removed, cut into small pieces, and digested during 30 min at 37ºC in a 0.2% collagenase solution in Hanks Balanced Salt Solution with 100 UI/mL penicillin and 100 μg/mL streptomycin. The digested tissue was washed twice by resuspension in the culture medium: Dulbecco's Modified Eagle's Medium/Ham's F12, 10% fetal calf serum, 100 UI/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained during 3–5 days at 37°C with 95% O_2 and 5% CO_2.

Release experiments

The release experiments were performed as previously described (Rosmaninho-Salgado *et al.* 2007). Cells were plated on 48 multi-well plates (100 000 chromaffin cells/well) and release experiments were performed 3 days after plating. Culture medium was aspirated and cells washed three times in Krebs buffer (111 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 24.8 mmol/L $NaHCO_3$, 11.1 mmol/L glucose, and 15 mmol/L HEPES, pH 7.4). To study the effects of drugs on catecholamine release, cells were incubated for 10 min with Krebs buffer (basal release) with or without drugs. To evaluate the role of extracellular Ca^{2+}, cells were incubated during 10 min with Krebs buffer (basal release) or in a Ca^{2+}-free Krebs buffer (111 mmol/L NaCl, 0.38 mmol/L $CaCl_2$, 0.5 mmol/L EGTA, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 24.8 mmol/L $NaHCO_3$, 11.1 mmol/L glucose, and 15 mmol/L HEPES, pH 7.4) in the presence or absence of NPY (100 nmol/L). Samples were acidified with $HClO_4$ (0.4 mmol/L) and catecholamine were extracted on alumina and quantified by HPLC with electrochemical detection (ESA, Coulochem II, Chelmsford, MA, USA) (Cavadas *et al.* 2001). The release of catecholamine and NPY was expressed as the percentage of intracellular content and the effect of drugs in each experiment expressed as the percentage of change relatively to the basal release. Drugs used: NPY; NPY Y_1 receptor antagonist, BIBP 3226; MAPK inhibitor, PD 98059; PKA inhibitor, H89, PKC inhibitor, bis-indolylmaleimide I; NOS inhibitor, L-NAME; inactive L-NAME stereoisomer, D-NAME; guanylyl cyclase (GC) inhibitor, ODQ; NO donor, NOC-18; PKA stimulator, forskolin; PKC stimulator, PMA.

Statistical analysis

The data were analyzed using a two-factor ANOVA tests. Data expressed as mean ± SEM.

Results

Neuropeptide Y stimulates catecholamine release via NPY Y_1 receptors in a Ca^{2+}-dependent manner

When mouse chromaffin cells were incubated with NPY (100 nmol/L) we observed an increase of catecholamine release: 243.4 ± 13.9% and 228.9 ± 15.7% of NE and EP release, respectively, compared to basal release (Fig. 1). The NPY Y_1 receptor antagonist, BIBP 3226 (1 μmol/L), inhibited this NPY stimulatory effect. The release of catecholamine from chromaffin cells induced by NPY was completely abolished in the Ca^{2+}-free Krebs buffer (Fig. 1). The absence of Ca^{2+} did not alter the basal catecholamine release (data not shown).

Protein kinase C and mitogen-activated protein kinase, but not protein kinase A, mediate neuropeptide Y-stimulated catecholamine release

To investigate which signaling pathways are involved in catecholamine release stimulated by NPY Y_1 receptor, we

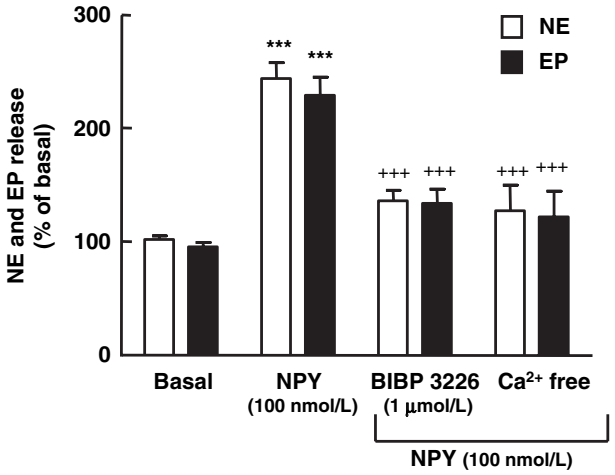

Fig. 1 NPY induces catecholamine release through NPY Y_1 receptor in a Ca^{2+}-dependent manner in mouse chromaffin cells. Cells were incubated with NPY (100 nmol/L) for 10 min in the presence of the NPY Y_1 antagonist BIBP 3226 (1 μmol/L) or in Ca^{2+}-free buffer. Catecholamine were measured by HPLC as described in Materials and methods. Data expressed as percentage of basal release; mean ± SEM; three to eight independent cultures of mice chromaffin cells; each condition was performed in triplicate. $^{***}p < 0.001$ compared to basal release; $^{+++}p < 0.001$ compared to NPY (100 nmol/L). Two-way ANOVA *post hoc* test was used as statistical test.

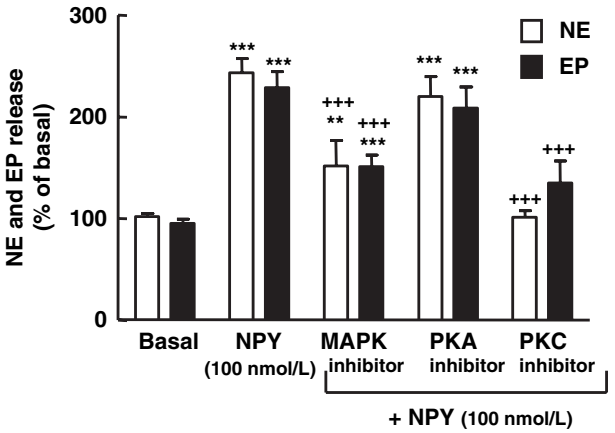

Fig. 2 Signal transduction pathways involved in catecholamine release stimulated by NPY in mouse chromaffin cells. Cells were incubated for 10 min with NPY (100 nmol/L) alone or with MAPK inhibitor (PD 98059, 50 μmol/L), PKA inhibitor (H89, 1 μmol/L), or PKC inhibitor (bisindolylmaleimide I, 1 μmol/L). Catecholamine were measured by HPLC as described in Materials and methods. Data expressed as percentage of basal release; mean ± SEM; three to eight different mice chromaffin cells culture; each condition was performed in triplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to basal release; $^{+++}p < 0.001$ compared to NPY (100 nmol/L). Two-way ANOVA *post hoc* test was used as statistical test.

tested PKA, PKC, and MAPK inhibitors (Fig. 2). The MAPK inhibitor (PD 98059, 50 μmol/L) reduced 64.7 ± 17.9% and 59.1 ± 8.5% the stimulatory effect of NPY on NE and EP release, respectively. The PKC inhibitor also inhibited the stimulatory effect of NPY: 100.0 ± 4.6% and 71.0 ± 15.9% inhibition of the stimulatory effect of NPY on NE and EP release, respectively. In contrast, the PKA inhibitor did not change catecholamine release evoked by NPY (Fig. 2).

The NO-cGMP pathway mediates neuropeptide Y-stimulated catecholamine release in a protein kinase C-dependent manner

The role of NO on catecholamine release stimulated by NPY was studied by using a specific NOS inhibitor (L-NAME, 500 μmol/L). L-NAME inhibited 40.5 ± 14.9% and 42.3 ± 20.3% the stimulatory effect of NPY on NE and EP release, respectively (Fig. 3). D-NAME (500 μmol/L), an inactive stereoisomer of L-NAME, did not change catecholamine release evoked by NPY (data not shown). The direct effect of NO on catecholamine release was investigated by incubating the cells with an exogenous NO donor (NOC-18, 100 μmol/L). This NO donor increased up to 255.3 ± 17.5% and 251.1 ± 21.1% the NE and EP release compared to basal release (Fig. 3). Moreover, the GC inhibitor (ODQ, 50 μmol/L) significantly decreased the NPY and NOC-18 stimulatory effect on catecholamine release (Fig. 3). The percentage of

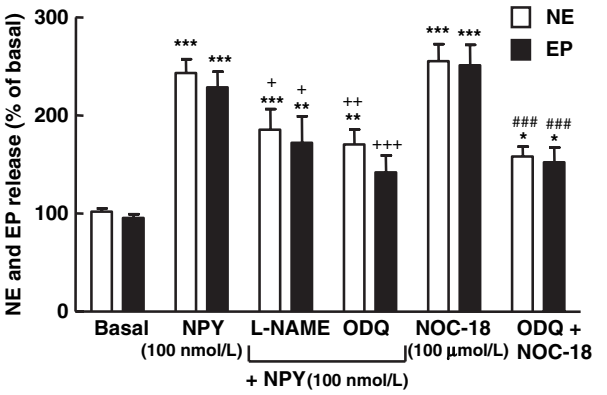

Fig. 3 Role of NO and cyclic GMP on catecholamine release stimulated by NPY. Cells were pre-incubated for 15 min with a NOS inhibitor (L-NAME, 500 μmol/L) or a GC inhibitor (ODQ, 50 μmol/L), followed by the stimulation with NPY (100 nmol/L) for 10 min in the presence of the inhibitors . Cells were stimulated with an exogenous NO donor (NOC-18, 100 μmol/L) with or without ODQ (50 μmol/L). Catecholamine were measured by HPLC as described in Materials and methods. Data expressed as percentage of basal release; mean ± SEM, three to eight different mice chromaffin cells culture; each condition was performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to basal release; $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$ compared to NPY (100 nmol/L); $^{\#\#\#}p < 0.001$ compared to NOC-18 (100 μmol/L). Two-way ANOVA *post hoc* test was used as statistical test.

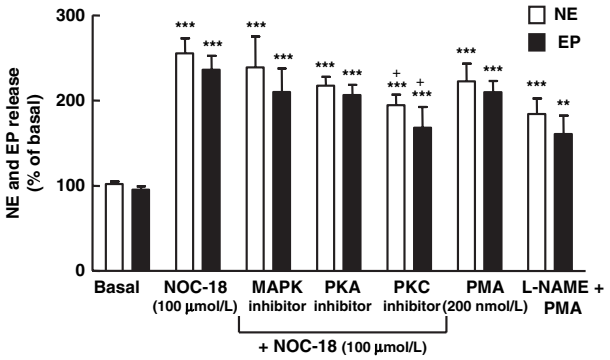

Fig. 4 Intracellular pathways involved on catecholamine release stimulated by NO. Cells were incubated 10 min with NOC-18 (100 μmol/L) alone or with the MAPK inhibitor (PD 98059, 50 μmol/L), or the PKA inhibitor (H89, 1 μmol/L) or the PKC inhibitor (bis-indolylmaleimide I, 1 μmol/L). Cells were incubated with the phorbol ester (PMA, 200 nmol/L) with or without L-NAME (500 μmol/L) during 10 min. Catecholamine were measured by HPLC as described in Materials and methods. Data expressed as percentage of basal release; mean ± SEM, two to eight different mice chromaffin cells culture; each condition was performed in triplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to basal release; $^{+}p < 0.05$ compared to NOC-18 (100 μmol/L). Two-way ANOVA *post hoc* test was used as statistical test.

inhibition by ODQ on NPY-evoked release was 37.4 ± 11.5% and 39.8 ± 11.1% (NE and EP, respectively). ODQ also inhibited the stimulatory effect of NOC-18: 63.3 ± 6.7% and 59.7 ± 10.7% inhibition of NE and EP release evoked by NOC-18, respectively. L-NAME or ODQ alone did not change non-stimulated catecholamine release (data not shown).

Subsequently, we studied the involvement of MAPK, PKA, and PKC on catecholamine release evoked by NO. MAPK or PKA inhibitors did not change the NOC-18 stimulatory effect (Fig. 4). However, the PKC inhibitor decreased the stimulatory effect of NOC-18 on NE and EP release (Fig. 4). The PKC stimulator, PMA (200 nmol/L), induced an increase up to 219.1 ± 18.6% and 209.8 ± 13.2% the NE and EP release compared to basal release, and this stimulatory effect was not changed by the presence of L-NAME (Fig. 4).

Discussion

NPY Y_1 receptor mediates the effect of neuropeptide Y on catecholamine release in mouse chromaffin cells

In the present study, we observed that the NPY Y_1 antagonist, BIBP 3226, inhibited the NPY stimulatory effect on catecholamine release from mouse adrenal chromaffin cells. This result is in agreement with a previous study from our group (Cavadas *et al.* 2006), showing that NPY stimulates catecholamine release from chromaffin cells of wild-type mice, but not in chromaffin cells isolated from NPY Y_1 knockout mice (Cavadas *et al.* 2006). Together, these studies show that NPY stimulates catecholamine release through activation of the NPY Y_1 receptor in mouse chromaffin cells. Moreover, by comparing the activity of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) in the adrenal glands of wild-type and NPY Y_1 knockout mice we have shown that NPY Y_1 receptor regulates catecholamine synthesis (Cavadas *et al.* 2006). The interaction between NPY Y_1 and TH was confirmed using Y_1 expressing cells transfected with the TH promoter, and it was shown that NPY acts by a PKA pathway which activates the cAMP response element-binding elements promoting the cAMP response element site phosphorylation and, consequently, the induction of TH transcription (Cavadas *et al.* 2006). As catecholamine synthesis and release are coupled, these studies suggest a role for NPY Y_1 receptor in intracellular catecholamine homeostasis in mouse adrenal gland.

Other studies, using bovine chromaffin cells, demonstrate that NPY regulates catecholamine homeostasis in a different manner. In fact, in these cells, NPY was reported to have an inhibitory effect on catecholamine secretion and to decrease TH activity (Zheng *et al.* 1997). On the other hand, also in bovine chromaffin cells, NPY inhibits PKA activity, subsequent to adenylate cyclase inhibition and decreases cAMP production (Zhu *et al.* 1992).

Differential protein kinase dependency of neuropeptide Y-stimulated catecholamine release

Another aim of the present study was to investigate the signaling pathways coupled to NPY Y_1 receptor activation that evoke catecholamine release from mouse chromaffin cells. It is well established that Ca^{2+} entry through voltage-operated Ca^{2+} channels of the plasma membrane is essential for triggering the chromaffin secretory response (Garcia *et al.* 2006) For example, leptin, angiotensin II, and histamine activate different types of voltage-sensitive Ca^{2+} channels with a consequent increase of catecholamine release from chromaffin cells (O'Farrell and Marley 1999; Takekoshi *et al.* 2001; Cavadas *et al.* 2003). In the present work, we also observed that the removal of extracellular Ca^{2+} decreased the stimulatory effect of NPY through NPY Y_1 receptor activation. Although we did not further investigated the coupling of the NPY Y_1 receptor and the increase in intracellular $[Ca^{2+}]$ in these cells, there are several studies showing that in other cells types the activation of Y_1 receptor increases intracellular $[Ca^{2+}]$ (Mihara *et al.* 1989; Aakerlund *et al.* 1990; Daniels *et al.* 1992; van Biesen *et al.* 1996; Vanderheyden *et al.* 1998; Cavadas *et al.* 2001). Besides its role on catecholamine release, the increase of intracellular $[Ca^{2+}]$ may regulates catecholamine synthesis, acting on Calmodulin-Dependent Protein Kinase II, which phosphorylates TH on Ser^{19} residue (Toska *et al.* 2002).

Based on the knowledge of the pathways coupled to the NPY Y_1 receptor in other systems, in the present study we investigated the effect of different kinases inhibitors on catecholamine release. Our results demonstrate that NPY stimulated catecholamine release in mouse chromaffin cells by a MAPK- and a PKC-dependent pathway. However, there was no significant inhibition of NPY stimulatory effect by the PKA inhibitor, suggesting that PKA is not involved in NPY-evoked catecholamine release.

Although NPY Y_1 receptor activation causes the inhibition of adenylate cyclase (Aakerlund *et al.* 1990; Michel 1991), it was described that NPY, *per se*, has no effect on cAMP accumulation (Prieto *et al.* 1997). In fact, in human and bovine chromaffin cells, NPY only inhibited cAMP accumulation stimulated by forskolin, having no effect on non-stimulated cells (Zheng *et al.* 2000; Cavadas *et al.* 2001). These observations might explain why in our experimental conditions, with non-stimulated adenylate cyclase, the PKA inhibitor did not change catecholamine release induced by NPY.

The coupling of NPY Y_1 receptor to MAPK activation was also demonstrated by others in different cell types (Mannon and Raymond 1998; Nie and Selbie 1998; Keffel *et al.* 1999; Hansel *et al.* 2001; Chan *et al.* 2005). Moreover, MAPK activation is involved in catecholamine synthesis and has been implicated in the secretory response of adrenal chromaffin cells (Ely *et al.* 1990; Cox and Parsons 1997). In fact, in bovine chromaffin cells, the MAPK inhibitor partially inhibited nicotine- and KCl-induced catecholamine release (Cox and Parsons 1997). These studies are in agreement with the present work, where we observed a role of MAPK on catecholamine release by NPY Y_1 receptor activation in mouse chromaffin cells.

The present study demonstrates, for the first time, that NPY Y_1 receptor modulates catecholamine release by a PKC-dependent pathway in mouse chromaffin cells. It has been shown that NPY Y_1 receptor activates PKC in different cell types, including mouse macrophages, Y_1-receptor transfected CHO cells, and rat postnatal neuronal precursor cells (De la Fuente *et al.* 1993; Selbie *et al.* 1995; Hansel *et al.* 2001). Although some contradiction was found in the literature regarding the involvement of PKC in promoting secretory responses in chromaffin cells, NPY modulates catecholamine release in rat, bovine, and porcine chromaffin cells and also in rat pheochromocytoma cells, by a PKC-dependent pathway promoting an increase of Ca^{2+} influx and triggering exocytosis (Malhotra *et al.* 1989; Sena *et al.* 1995; McCullough and Westfall 1996; Tanaka *et al.* 1996; Warashina 1997; Takekoshi *et al.* 2001; Shoji-Kasai *et al.* 2002). Furthermore, it was shown that activation of PKC promotes vesicle recruitment to the release-ready state prior to Ca^{2+}-triggered fusion in chromaffin cells (Kumakura *et al.* 2004).

Role of nitric oxide in catecholamine release following activation of NPY Y1 receptors

Additionally, in this work we demonstrate that NO is an intracellular messenger that links NPY Y_1 receptor activation and PKC. Our results show that the stimulatory effect of NPY Y_1 receptor on catecholamine release is inhibited by L-NAME and by the GC inhibitor ODQ, suggesting that the activation of the NOS-GC pathway and the downstream events are important factors leading to NPY induced-catecholamine secretion. As NO production is largely Ca^{2+}-dependent, and NPY can lead to Ca^{2+} entry by causing the opening of voltage-dependent Ca^{2+} channels, as discussed above, the involvement of NO in NPY-induced catecholamine release is very likely to occur. In fact, inhibition of NOS partially prevented NPY-stimulated catecholamine release. Furthermore, stimulation of chromaffin cells with a NO donor (NOC-18) had a similar effect on catecholamine release to that of NPY. Similarly to what it was observed with NPY, inhibition of PKC blocked the NOC-18-evoked catecholamine release. Direct activation of PKC with PMA caused catecholamine release that was not sensitive to inhibition of NO production by L-NAME, suggesting that the NO pathway is an upstream activator of PKC. On the other hand, inhibition of MAPK did not abolish the effect of NOC-18 on catecholamine release, therefore suggesting that

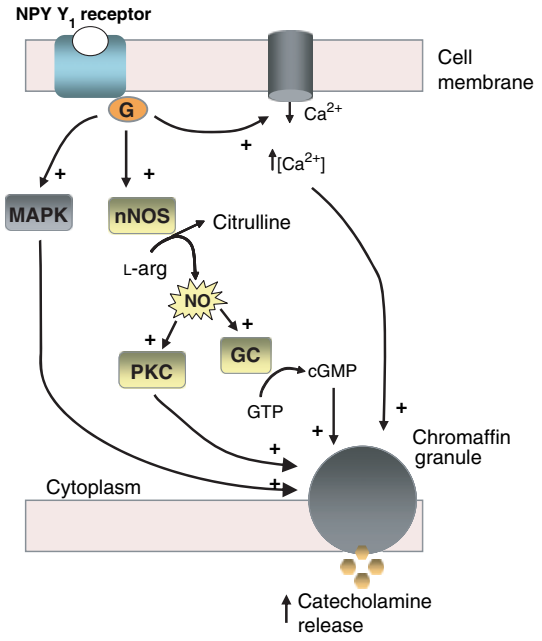

Fig. 5 Scheme representing the intracellular pathways involved in catecholamine release upon NPY Y_1 receptor stimulation in mouse chromaffin cells. NPY evokes catecholamine release by the activation of the NPY Y_1 receptor, in a Ca^{2+}-dependent manner, either by activating MAPK or leading to production of NO, which is linked to the activation of PKC and GC.

the MAPK pathway is independent of the PKC-NO-cGMP pathway.

The role of NO on PKC activation was also demonstrated in other systems, namely in heart models, where NO activates the PKC signaling pathway, being implicated in the development of cardioprotection (Ping *et al.* 1999; Liu *et al.* 2001). Other studies suggest that NO is a mediator of the effects of NPY Y_1 receptor activation. For instance, in a rat model of focal ischemia with reperfusion, NPY intracerebroventricular injection increased the number of nNOS positive neurons, the nNOS activity and the NO production, whereas these effects were inhibited by the Y_1 receptor antagonist, BIBP 3326 (Chen *et al.* 2002; Chen and Cheung 2005). Moreover, although NPY is known as a potent vasoconstrictor of peripheral blood vessels, Nilsson *et al.* (2000) show that NPY induces vasodilatation via the NPY Y_1 receptor and this effect is inhibited by the NOS inhibitor L-NAME. Other studies also describe that NO activates GC, regulating catecholamine secretion in bovine and rainbow trout chromaffin cells (Oset-Gasque *et al.* 1994, 1998; Torres *et al.* 1994; Schwarz *et al.* 1998; Vicente *et al.* 2002; McNeill and Perry 2005).

In summary, our data show that in mouse chromaffin cells, NPY evokes catecholamine release by the activation of the NPY Y_1 receptor, in a Ca^{2+}-dependent manner, either by activating MAPK or leading to production of NO, which is linked to the activation of PKC and GC (Fig. 5). These intracellular signaling mechanisms are also coupled to other different receptors/systems. The knowledge of these signaling pathways is relevant in pathophysiological conditions where occur an increase of NPY release from adrenal gland and an increase of circulating NPY, such as stress or hypertensive crises (Castagne *et al.* 1987; Edvinsson *et al.* 1991; Wocial *et al.* 1995; Bernet *et al.* 1998). The increased NPY activates NPY receptor that induces further adrenal catecholamine release that putatively aggravates pathological conditions related to adrenergic receptor activation.

Acknowledgements

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