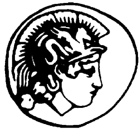

Pergamon

Neuropharmacology 44 (2003) 282–292

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

Functional interaction between neuropeptide Y receptors and modulation of calcium channels in the rat hippocampus

Ana P. Silva [a], Arsélio P. Carvalho [a], Caetana M. Carvalho [a], João O. Malva [a,b,*]

[a] *Center for Neuroscience of Coimbra, Department of Zoology, University of Coimbra, 3004-517 Coimbra, Portugal*
[b] *Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal*

Received 20 May 2002; received in revised form 24 October 2002; accepted 14 November 2002

Abstract

We investigated the functional interaction between neuropeptide Y (NPY) receptors using nerve terminals and cultured rat hippocampal neurons, and we evaluated the involvement of voltage-gated Ca^{2+} channels (VGCCs) in NPY receptors-induced inhibition of Ca^{2+} influx and glutamate release. The KCl-evoked release of glutamate from hippocampal synaptosomes was inhibited by 1 μM NPY and this effect was insensitive to either BIBP3226 (Y1 receptor antagonist) or L-152,804 (Y5 receptor antagonist), but was sensitive to BIIE0246 (Y2 receptor antagonist). We could also pharmacologically dissect the NPY receptors activity by using Y1, Y2 and Y5 receptor agonists ([Leu^{31},Pro^{34}]NPY, NPY13-36, NPY (19-23)-(Gly^{1},Ser^{3},Gln^{4},Thr^{6},Ala^{31},Aib^{32},Gln^{34})-pancreatic polypeptide (PP), respectively), and in all the cases we observed that these agonists could inhibited the KCl-induced release of glutamate. However, the selective and specific co-activation of both Y1 and Y2 or Y2 and Y5 receptors resulted in non-additive inhibition, and this effect was prevented in the presence of the Y2 antagonist, but was insensitive to the Y1 or Y5 receptor antagonist. Moreover, as we previously showed for Y1 receptors, we also observed that the activation of Y5 receptors inhibited the glutamate release in the dentate gyrus and CA3 subregion, without significant effect in the CA1 subregion of the hippocampus. The same qualitative results were obtained when we investigated the role of NPY Y1 and Y2 receptors in modulating the changes in $[Ca^{2+}]_i$ due to KCl depolarisation in cultured hippocampal neurons. The inhibitory effect of nitrendipine (L-type VGCC blocker) or ω-conotoxin GVIA (ω-CgTx; N-type VGCC blocker) was not potentiated by the simultaneous activation of Y1 or Y2 receptors. Moreover, the exocytotic release of glutamate was inhibited by ω-agatoxin IVA (ω-Aga; P-/Q-type VGCC blocker), and this VGCC blocker did not potentiate Y1, Y2 or Y5 receptor-mediated inhibition of glutamate release. Also, the effect of ionomycin in inducing the exocytotic release of glutamate from hippocampal synaptosomes was insensitive to the activation of NPY receptors. In the present paper, we identified a role for NPY Y1, Y2 and Y5 receptors in modulating the exocytotic release of glutamate and the $[Ca^{2+}]_i$ changes in the rat hippocampus. In conditions of co-activation, there appears to exist a physiological cross-talk between Y1 and Y2 and also between Y2 and Y5 receptors, in which Y2 receptors play a predominant role. Moreover, we also show that Y1 and Y2 receptors exert their inhibitory action by directly modulating L-, N-, and P-/Q-type VGCCs, whereas the inhibition of glutamate release mediated by the Y5 receptors seems to involve P-/Q-type VGCCs.
© 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Neuropeptide Y; Glutamate release; Intracellular calcium; Voltage-gated Ca^{2+} channel; Synaptosomes; Hippocampus

1. Introduction

Neuropeptide Y (NPY) is one of the most abundant and widely distributed neuropeptides in both central and peripheral nervous system (Wahlestedt and Reis, 1993). Moreover, it is known that NPY plays an important role in the regulation of neuronal activity: inhibits calcium currents (Toth et al., 1993), modulates transmitter release in a highly selective manner (Silva et al., 2001) and reduces hippocampal epileptiform activity in vitro and in vivo (Colmers and Bleakman, 1994; Woldbye et al., 1997). However, many other functions have been suggested for NPY, including regulation of feeding, anxiety, memory, cardiovascular function (Grundemar and Håkanson, 1994), and NPY can also act as a neuroproliferative factor (Hansel et al., 2001).

Studies in which agonist fragments and analogues of

* Corresponding author. Tel.: +351-239-833369; fax: +351-239-822776.
E-mail address: jomalva@cnc.cj.uc.pt (J.O. Malva).

0028-3908/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0028-3908(02)00382-9

NPY were tested indicate that at least three subtypes of NPY receptor can be distinguished pharmacologically in the central nervous system (Michel et al., 1998). The NPY Y1 receptor has a high affinity for [Leu^{31},Pro^{34}]NPY, whereas Y2 binds COOH-terminal fragments like NPY13-36 (Gerald et al., 1995). More recently, Cabrele et al. (2000) developed the first Y5 receptor-selective analogue of NPY, [Ala^{31},Aib^{32}]NPY. Also, the non-peptide antagonist, BIBP3226, binds and inhibits specially the Y1 receptor subtype (Wieland et al., 1995), whereas T_4-[NPY(33-36)]$_4$ is a competitive inhibitor of Y2 receptors (Grouzmann et al., 1995). Recently, Doods et al. (1999) have also characterized a more potent and selective Y2 receptor antagonist, BIIE0246, and Kanatani et al. (2000) a new Y5 receptor antagonist, L-152,804.

The hippocampus is particularly enriched with NPY and NPY receptors, with high expression of Y1 receptors in dentate gyrus, expression of Y2 receptors in all of the other hippocampal regions and relatively high content of mRNA for Y5-receptor throughout the CA regions and dentate gyrus (Parker and Herzog, 1999). Indeed, the seizure-related increase in NPY expression is accompanied by modified levels of NPY subtypes Y1, Y2 and Y5 in the hippocampus (Kopp et al., 1999). The NPY Y5 receptor is the most recently discovered NPY receptor, and has been proposed to mediate the anticonvulsant actions of NPY in adult rats in vivo (Woldbye et al., 1997).

NPY is able to inhibit excitatory neurotransmission in both slices as well as in hippocampal cell cultures (Bleakman et al., 1992). The activation of Y2 receptors inhibits potassium-stimulated glutamate release in hippocampal slices (Greber et al., 1994), and NPY can also reduce activity-dependent calcium increases in rat dentate granule cells, predominantly by activating an Y1 receptor subtype (McQuiston et al., 1996). There is evidence that presynaptic inhibition produced by NPY is associated with a reduction in Ca^{2+} influx caused by selective inhibition of neuronal N-type Ca^{2+} channels (Toth et al., 1993; McQuiston et al., 1996). NPY also inhibits both N- and L-type VGCCs of frog melanotrophs (Valentijn et al., 1994). Moreover, it is known that ω-conotoxin GVIA-sensitive (N-type) and ω-agatoxin IVA-sensitive (P-/Q-type) calcium channels are involved in synaptic transmission at the CA3-CA1 synapse (Wu and Saggau, 1994), and unidentified types of calcium channels, resistant to both ω-CgTx GVIA and ω-Aga IVA, are also significantly involved in the process of transmitter release. Indeed, activation of Y2 receptors inhibits N-type, P-/Q-type, and unidentified presynaptic VGCC (Qian et al., 1997).

We have recently identified Y1 and Y2 receptors in glutamatergic nerve terminals, and both receptors inhibited the KCl-evoked release of glutamate (Silva et al., 2001). However, the functional interplay between Y1 and Y2 receptors, and the selective role of Y5 receptors on the modulation of glutamate release and on the $[Ca^{2+}]_i$ changes were never addressed. The aim of this study was to investigate the involvement of NPY Y1, Y2 and Y5 receptors in the modulation of the exocytotic release of glutamate and the $[Ca^{2+}]_i$ changes in the rat hippocampus. We show here a physiological cross-talk between Y1 and Y2 receptors and also between Y2 and Y5 receptors, with Y2 receptor playing a predominant role. Moreover, we could also show a direct involvement of L-, N-, and P-/Q-type VGCCs, on the inhibitory effects mediated by NPY receptors.

2. Methods

2.1. Materials

Neuropeptide Y, [Leu^{31},Pro^{34}]neuropetide Y, neuropetide Y13-36, neuropeptide Y (19-23)-(Gly^1,Ser^3,Gln^4,Thr^6,Ala^{31},Aib^{32},Gln^{34})-Pancreatic Polypeptide and ω-conotoxin GVIA were obtained from Bachem, Bubendorf, Switzerland. ω-Agatoxin IVA was obtained from Peptide Institute, Inc., Osaka, Japan, whereas nitrendipine was a kind gift of Dr G. Terstappen, from Bayer AG, Germany. (R)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl] argininamide (BIBP3226) was purchased from Peninsula Laboratories, Belmont, CA, USA, and (S)-N^2-[[1-[2-[4-[(R,S)-5,11-Dihydro-6(6h)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl] cylopentyl] acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamid (BIIE0246) was kindly supplied by Dr Henri Doods (Boehringer Ingelheim Pharma KG, Germany). L-152,804 (5,5-dimethyl-2-(2,3,4,9-tetrahydydro-3,3-dimethyl-1oxo-1*H*-xanthen-9-yl)-1,3-cyclohexanedione) was obtained from Tocris, Bristol, UK. Ionomycin was purchased from Calbiochem-Boehringer, San Diego. Neurobasal medium, B27 supplement, gentamicin and trypsin (USP grade) were purchased from GIBCO BRL, Life Technologies, Scotland, UK. Glutamate, DNase (DN-25) and L-glutamic acid dehydrogenase type II were obtained from Sigma Chemical, St. Louis, MO, USA. The acetoxymethyl ester of Fura-2 (Fura-2/AM) and Pluronic F-127 were purchased from Molecular Probes, Leiden, The Netherlands. All the other reagents were from Sigma Chemical, St Louis, MO, USA or from Merck-Schuchardt, Germany.

2.2. Preparation of synaptosomes

A partially purified synaptosomal fraction (P_2) was isolated from hippocampi or from hippocampal subregions (CA1, CA3 and dentate gyrus) (Silva et al., 2001) of male Wistar rats (2 month-old), essentially as

described previously for brain cortex (McMahon et al., 1992), with some modifications (Malva et al., 1996). The hippocampi were homogenized in 0.32 M sucrose, 10 mM HEPES–Na, pH 7.4, and centrifuged at 3000 ***g*** for 2 min. The pellet obtained was resuspended, followed by sedimentation at the same speed. The combined supernatants were spun for 12 min at 14,600 ***g***, and a P_2 pellet was obtained. The upper, whiter layer of this pellet was resuspended in the sucrose medium.

Coronal slices of the hippocampus (800 μm thick) were prepared for the isolation of synaptosomes from hippocampal subregions (CA1, CA3 and dentate gyrus). In each slice, the fimbria and the subiculum were separated from the rest of each slice, under stereomicroscopic observation. CA3 sub-slices were obtained by separation from CA1 and dentate gyrus, and, the last separation (CA1 from dentate gyrus) was performed through the hippocampal sulcus. The pooled sub-slices were homogenized in the sucrose medium indicated above, transferred to Eppendorf tubes and centrifuged as described for the isolation of whole hippocampal synaptosomes. The P_2 pellet of each subregion was resuspended in buffered sucrose medium (Malva et al., 1996; Silva et al., 2001). The protein concentration was determined by the Biuret method (Layne, 1957). For glutamate release experiments the synaptosomes were stored as drained pellets, containing 1 mg of protein.

2.3. Cell culture

Hippocampal neurons were dissociated from hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (2.0 mg/ml, 15 min, 37 °C) and deoxyribonuclease I (0.20 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4). The cells were cultured in serum-free Neurobasal medium, supplemented with B27 supplement, glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml), as described previously (Brewer et al., 1993). Cultures were kept at 37 °C in a humidified incubator in 5% CO_2/95% air, for 7–8 days, the time required for maturation of hippocampal neurons. Cells were plated on poly-D-lysine-coated (0.1 mg/ml) glass coverslips at a density of 0.2×10^6 cells/cm^2 for Fura-2 fluorescence.

2.4. Measurement of glutamate release

The release of endogenous glutamate was followed using a continuous fluorimetric assay as previously described (Nicholls et al., 1987), with some modifications (Malva et al., 1996). Synaptosomes (1 mg protein) were incubated for 20 min at 37 °C in the following medium (in mM): 132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 0.1 $CaCl_2$, 10 glucose, 10 HEPES-Na, pH 7.4, with 0.1% fatty acid-free bovine serum albumin (BSA). After this period, the NPY receptor agonists/antagonists, and/or ω-Aga IVA were added to the incubation medium for more 10 min. Then, synaptosomes were centrifuged at 15,800 g_{max}, and resuspended in 1 ml of the same medium, without bovine serum albumin and with 1 mM $CaCl_2$. The suspension was transferred to a stirred cuvette at 37 °C, followed by the addition of 1 mM $NADP^+$, 50 U of purified glutamate dehydrogenase, and again NPY receptor agonists/antagonists and/or ω-Aga IVA. Fluorescence was measured by using a Perkin Elmer model LS-5B luminescence spectrometer at the excitation and emission wavelengths of 340 and 460 nm, respectively, with excitation and emission slits of 5 and 10 nm, respectively. The data were collected at 0.5 s intervals and the quantification of glutamate was performed at the end of each experiment by adding 2.5 nmol of L-glutamate, for calibration.

2.5. $[Ca^{2+}]_i$ measurements

Measurement of $[Ca^{2+}]_i$ were carried out in populations of hippocampal neurons cultured on glass coverslips at a density of 0.2×10^6 cells/cm^2. The cells were incubated with 5 μM Fura-2/AM and 0.2% Pluronic F-27 in Krebs buffer (132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 1.2 mM H_3PO_4, 10 mM glucose and 10 mM HEPES–Na, pH 7.4) with 0.1% fatty acid-free bovine serum albumin for 40 min at 37 °C, and further incubated for 10 min in Krebs buffer without the dye. When NPY receptor agonists and antagonist, ω-Aga IVA and ω-CgTx GVIA were tested, they were present both in the last ten minutes incubation and in reaction medium. The coverslips were rinsed with Krebs buffer and mounted, with a special holder (Perkin-Elmer L2250008), in a temperature-controlled cuvette. The fluorescence of Fura-2-loaded cells was monitored using a computer-assisted Spex Fluoromax spectrofluorometer, at 510 nm emission and double excitation at 340 and 380 nm, using 5 nm slits. The calibration was made in the presence of 6 μM ionomycin (1 mM $CaCl_2$; R_{max}), at 400 s, and 8 mM EGTA (R_{min}), at 700 s.

2.6. Data analysis

The data are expressed as means ± SEM. Statistics were performed using an analysis of variance (ANOVA), followed by Dunnett's or Bonferroni's post-tests, as indicated in the figure legends.

3. Results

3.1. Modulation of glutamate release by NPY Y1, Y2 and Y5 receptors in rat hippocampal nerve terminals: functional interaction between presynaptic NPY receptors

Excitatory synaptic transmission in the nervous system is mostly mediated by glutamate. Under pathological conditions leading to massive glutamate release the hyperactivation of glutamate receptors causes deregulation of the $[Ca^{2+}]_i$, which activates a large number of Ca^{2+}-dependent processes that cause neuronal damage. We have previously examined the modulation of glutamate release by Y1 and Y2 receptors, and observed that these receptors have a differential effect in the three main hippocampus subregions (CA1, CA3 and dentate gyrus) (Silva et al., 2001).

Our first experiments revealed that the endogenous agonist, NPY (1 μM), inhibited the KCl-evoked total glutamate release in hippocampal nerve terminals to 48.2±2.2% of the control (Fig. 1). As we showed before (Silva et al., 2001), this inhibitory effect was insensitive to 1 μM BIBP3226, a NPY Y1 receptor antagonist (52.3 ± 3.7% of the control) (Fig. 1B), and was partially prevented by the competitive antagonist of Y2 receptor, T_4-$[NPY(33\text{-}36)]_4$ (1 μM) (72.6 ± 3.1% of the control). However, in the present study we show that the more selective Y2 receptor antagonist, BIIE0246 (1 μM), completely prevents the inhibition caused by NPY (98.6 ± 3.0% of the control) (Fig. 1B), whereas the Y5 receptor antagonist did not have a significant effect (61.2 ± 2.3% of control). This finding indicates that Y2 receptors play a predominant role in mediating NPY-induced inhibition of glutamate release in the hippocampus.

Since NPY is able to activate all NPY receptors, we have now pharmacologically dissected the activity of Y1, Y2 and Y5 receptors. We observed that 1 μM $[Leu^{31},Pro^{34}]$NPY, 300 nM NPY13-36 or 1 μM NPY(19-23)-(Gly^1,Ser^3,Gln^4,Thr^6,Ala^{31},Aib^{32},Gln^{34})-PP inhibited the glutamate release to 71.6 ± 1.8%, 60.1 ± 2.1% or 68.2 ± 2.5% of the control, respectively (Fig. 2).

The inhibitory effect induced by Y1 receptor activation was completely abolished by 1 μM BIBP3226 (93.4 ± 2.3% of the control), but not by the Y2 (72.17 ± 1.1% of the control) or Y5 (64.5 ± 2.3% of the control) receptor antagonist (Fig. 2). Moreover, 1 μM BIIE0346 completely prevented the inhibition of glutamate release induced by 300 nM NPY13-36 (109.7 ± 4.3% of the control), and this effect was not altered by the Y1 (61.0 ± 2.9% of the control) or Y5 (59.8 ± 3.7% of the control) receptor antagonist (Fig. 2). Concerning the Y5 receptor subtype, the inhibitory effect induced by 1 μM of the agonist was not significantly abolished by 1 μM BIIE0246 (69.2 ± 1.3% of the

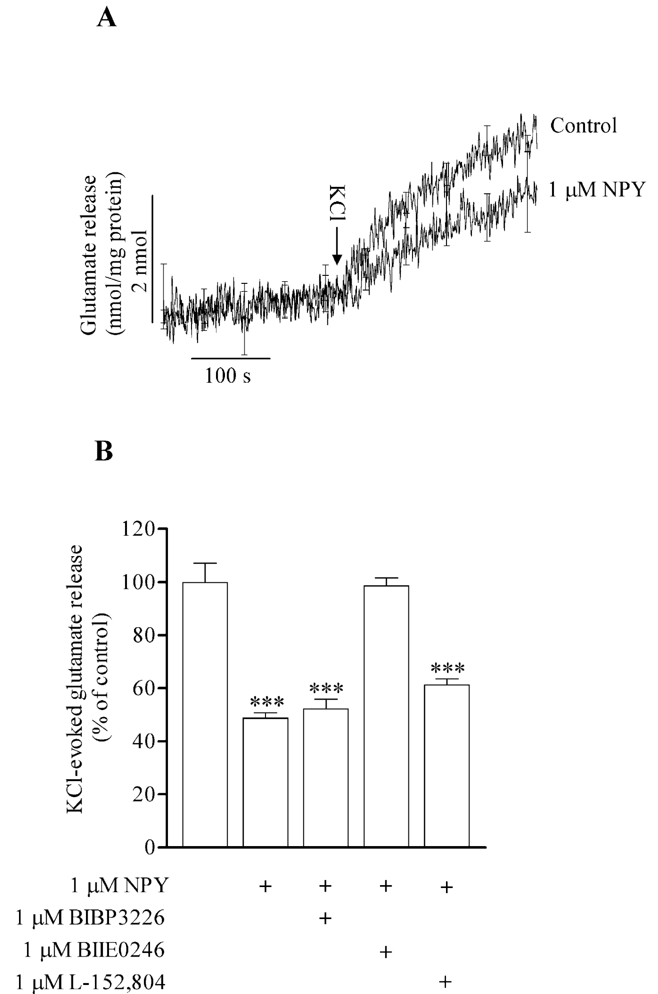


Fig. 1. (A) Inhibitory effect of NPY (1 μM) on the 15 mM KCl-evoked glutamate release in hippocampal synaptosomes (1 mM Ca^{2+} present in the external medium). Total glutamate release in control conditions was 1.1 ± 0.1 nmol glutamate/mg protein/min, while in the presence of 1 μM NPY was 0.6 ± 0.1 nmol glutamate/mg protein/min. (B) Quantitative analysis of the effect of Y1, Y2 or Y5 receptor antagonists on KCl-evoked glutamate release inhibition induced by 1 μM NPY. The synaptosomes were incubated with the NPY receptor agonists or/and antagonists for 10 min before starting the measurement of glutamate release, which was followed for 240 s after KCl depolarisation. The results are expressed as mean percentage of control ± SEM of 5–10 independent experiments, in different synaptosomal preparations. $^{***}P < 0.001$—Dunnett's post-test; statistical significance when compared to the control (KCl stimulation).

control), nor by 1μM BIBP3226 (79.1 ± 1.4% of the control), but was prevented in the presence of the Y5 receptor antagonist (95.0 ± 3.8% of the control). Furthermore, the inhibition of glutamate release induced by 1 μM NPY(19-23)-(Gly^1,Ser^3,Gln^4,Thr^6,Ala^{31},Aib^{32},Gln^{34})-PP was due to the activation of Y5 receptors in the dentate gyrus and CA3 subregion of the hippocampus, since the glutamate release was 55.3 ± 2.8% of the control in the dentate gyrus and 70.8 ± 1.8% of the control in the CA3 subregion, without an effect in the CA1

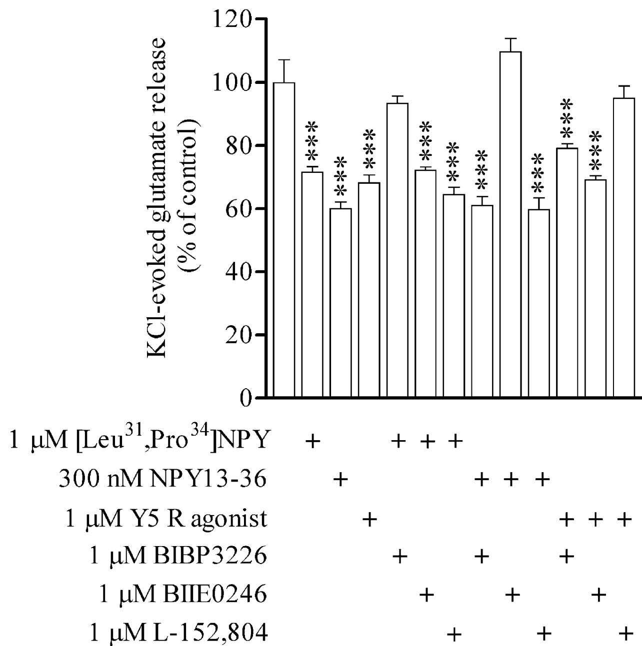


Fig. 2. Quantitative analysis of the effect of NPY Y1, Y2 and Y5 receptor agonists/antagonists on the total glutamate release evoked by 15 mM KCl depolarisation in hippocampal synaptosomes. Glutamate release in control conditions was on average 1.1 ± 0.1 nmol glutamate/mg protein/min. The results are expressed as mean percentage of control ± SEM of 5–14 independent experiments, in different synaptosomal preparations. $^{***}P < 0.001$—Dunnett's post-test; statistical significance when compared to the control (KCl stimulation).

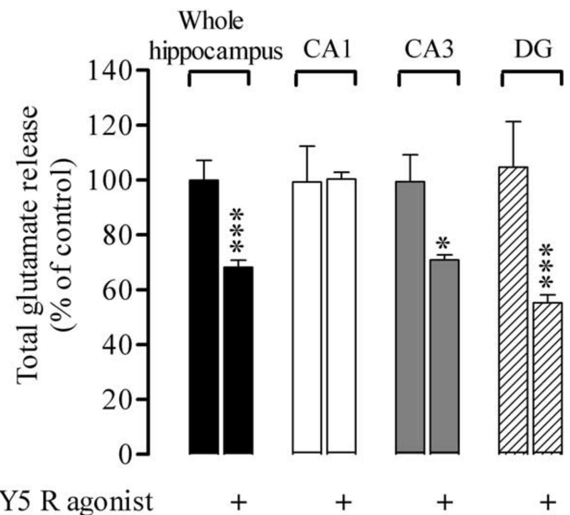


Fig. 3. Quantitative analysis of the effect of 1 μM NPY (19-23)-(Gly[1],Ser[3],Gln[4],Thr[6],Ala[31],Aib[32],Gln[34])-PP on the total glutamate release evoked by 15 mM KCl in synaptosomes obtained from the whole hippocampus and from the subregions, CA1, CA3 and dentate gyrus (DG). The results represent the mean ± SEM of 3–12 independent experiments, in different synaptosomal preparations. $^{*}P <$ 0.05; $^{***}P < 0.001$—Dunnett's post-test; statistical significance when compared to the control (KCl stimulation in each subregion).

subregion of the hippocampus (100.3 ± 2.5% of control) (Fig. 3).

The addition of the selective agonists for Y1 and Y2 or for Y2 and Y5 receptors did not result in a potentiation of the inhibitory effect of each agonist added separately (67.6 ± 2.2% or 65.7±3.6% of the control, respectively) (Fig. 4A,B). Moreover, the inhibition of the glutamate release induced by the simultaneous presence of 1 μM [Leu[31],Pro[34]]NPY and 300 nM NPY13-36 (Fig. 4A) or 300 nM NPY13-36 and 1 μM NPY (19-23)-(Gly[1],Ser[3],Gln[4],Thr[6],Ala[31],Aib[32],Gln[34])-PP (Fig. 4B) was prevented by the blockade of only Y2 receptors with BIIE0246 (94.0 ± 1.8% or 91.7 ± 2.2% of the control, respectively), and not by the inhibition of Y1 or Y5 receptors with BIBP3226 (72.0±1.8% of the control) (Fig. 4A) or L-152,804 (67.0 ± 2.7% of the control) (Fig. 4), respectively. In contrast, the co-activation of both Y1 and Y5 receptors resulted in a potentiation of the inhibitory effect (52.0 ± 2.1% of the control) of each agonist added separately (Fig. 4C). This effect was only partially prevented by 1 μM BIBP3226 (83.5 ± 1.8% of the control) or 1 μM L-152,804 (81.8 ± 1.3% of the control), but the presence of both antagonists completely blocked the inhibitory effect of the agonists (93.3 ± 1.3% of the control) (Fig. 4C).

3.2. Modulation of the $[Ca^{2+}]_i$ response by NPY Y1, Y2 and Y5 receptor activation in hippocampal neurons: functional interaction between NPY Y1 and Y2 receptors

Calcium is a mediator of many physiological functions, and deregulation of the $[Ca^{2+}]_i$ leads to cell dysfunction and ultimately to cell death. Therefore, we investigated the modulatory effect of NPY receptors on the KCl-evoked increase in the $[Ca^{2+}]_i$ measured in a whole population of hippocampal neurons.

In the presence of the endogenous agonist, 1 μM NPY, the $[Ca^{2+}]_i$ response induced by 30 mM KCl was significantly inhibited (79.9 ± 1.3% of the control) (Figs. 5B and 6A), and this effect was completely prevented by the Y2 receptor antagonist, BIIE0246, (110.7 ± 9.2% of the control), while the Y1 receptor antagonist, BIBP3226, did not have any effect (70.1 ± 2.8% of the control) (Fig. 6A). Moreover, the selective activation of Y1 or Y2 receptors also resulted in an inhibitory effect (76.9 ± 4.0% or 76.4 ± 2.8% of the control, respectively) (Figs. 5C,D and 6A), in contrast to what happened in the presence of the Y5 receptor agonist (93.4 ± 1.6% of the control), which did not inhibit the KCl-stimulated $[Ca^{2+}]_i$ changes (Figs. 5E and 6A).

We further investigated the selectivity of Y1 and Y2 receptors-mediated increase in the $[Ca^{2+}]_i$ by using Y1 and Y2 receptor antagonists. The Y1 receptor antagonist completely prevented the inhibitory effect induced by 1 μM [Leu[31],Pro[34]]NPY (104.5 ± 2.7% of the control)

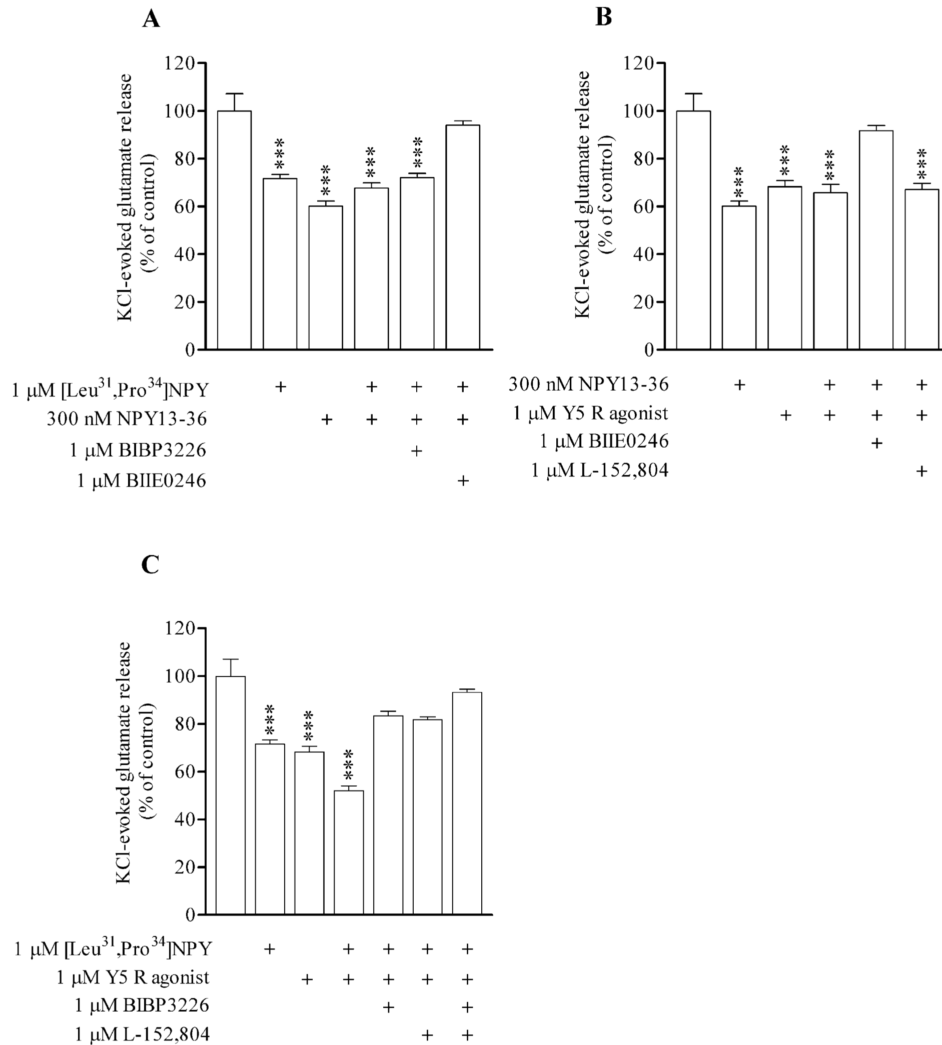


Fig. 4. Qualitative analysis of the effect of (A) NPY Y1 and Y2, (B) Y2 and Y5 or (C) Y1 and Y5 receptor agonists and antagonists on the total glutamate release evoked by 15 mM KCl depolarisation in hippocampal synaptosomes. The results represent the mean ± SE.M of 3–12 independent experiments, in different synaptosomal preparations. $^{***}P < 0.001$—Dunnett's post-test; statistical significance when compared to the control (KCl stimulation).

(Fig. 6A), while the Y2 receptor antagonist did not have any significant effect (74.8 ± 4.8% of the control) (Fig. 6A). In contrast, inhibition caused by 300 nM NPY13-36 was prevented by the selective Y2 receptor antagonist (115.0 ± 4.7% of the control) (Fig. 6A), but was not altered by the Y1 receptor antagonist (74.9 ± 5.6% of the control) (Fig. 6A).

As in glutamate release studies, we observed that the addition of both selective agonists did not result in a potentiation of the independent inhibitory effect of the agonists added separately (79.2 ± 2.0% of the control) (Fig. 6B). Moreover, again the inhibitory effect induced by 1 μM [Leu^{31},Pro^{34}]NPY plus 300 nM NPY13-36 was completely prevented by the Y2 receptor antagonist (101.1 ± 3.3% of the control) (Fig. 6B), but not by the Y1 receptor antagonist (74.0 ± 8.0% of the control) (Fig. 6B).

3.3. Characterization of the inhibitory effect mediated by NPY Y1 and Y2 receptors activation

To further understand the effect of Y1 and Y2 receptors activation on Ca^{2+} influx, we compared the effects of VGCC blockers and NPY receptor agonists on both the KCl-evoked $[Ca^{2+}]_i$ increase and glutamate release.

As we shown before, 1 μM [Leu^{31},Pro^{34}]NPY (Figs. 5B and 7A) or 300 nM NPY13-36 (Figs. 5C and 7B) reduced the $[Ca^{2+}]_i$ increase induced by 30 mM KCl to 76.9 ± 4.0% or 76.4 ± 2.8% of the control, respectively. Co-application of 1 μM [Leu^{31},Pro^{34}]NPY (Fig. 7A) or 300 nM NPY13-36 (Fig. 7B) with 1 μM nitrendipine, an L-type VGCC inhibitor (Bean, 2001), did not further inhibit the $[Ca^{2+}]_i$ response to 30 mM KCl (71.4 ± 3.9% or 72.0 ± 1.6% of the control, respectively), as compared to the effect of either

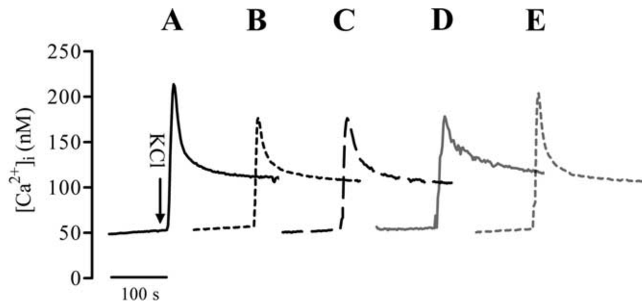


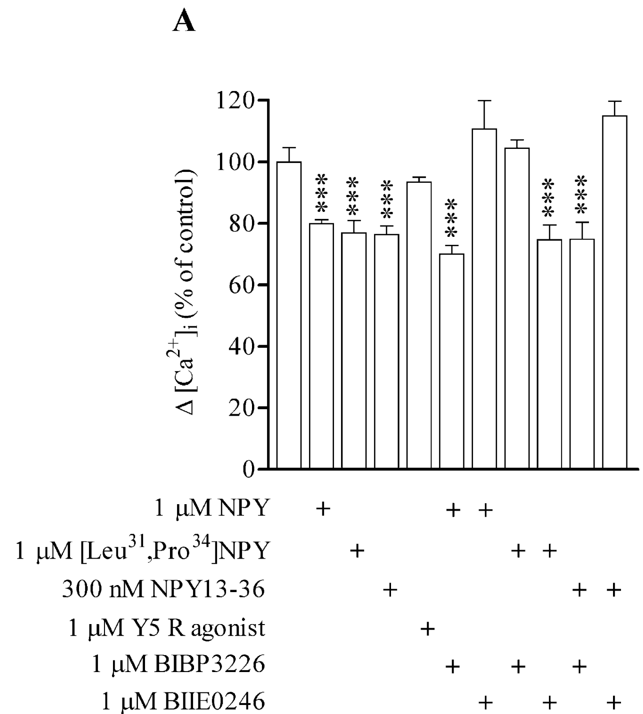
Fig. 5. Representative recordings showing the increase in $[Ca^{2+}]_i$ stimulated by 30 mM KCl in a control situation (A), and in the presence of 1 μM NPY (B), 1 μM [Leu^{31},Pro^{34}]NPY (C), 300 nM NPY 13-36 (D) or 1 μM NPY (19-23)-(Gly^{1},Ser^{3},Gln^{4},Thr^{6},Ala^{31},Aib^{32},Gln^{34})-PP (E), in cultured hippocampal neurons. In control conditions the basal $[Ca^{2+}]_i$ was 51.4 ± 1.6 nM and the KCl-evoked $[Ca^{2+}]_i$ was 214.0 ± 8.6 nM ($n = 11$). The cells were incubated with the NPY receptor agonists for 10 min before starting the $[Ca^{2+}]_i$ measurements. In (B–D) is possible to observe the inhibition of KCl-stimulated $[Ca^{2+}]_i$ changes.

[Leu^{31},Pro^{34}]NPY (Fig. 7A) or NPY13-36 (Fig. 7B). Nitrendipine alone had an effect (74.7 ± 2.2% of the control) similar to that obtained in the presence of Y1 (Fig. 7A) or Y2 (Fig. 7B) receptor agonists, and did not affected the basal $[Ca^{2+}]_i$ (110.0 ± 9.9% of the control, $n = 6$, $P > 0.05$, Dunnett's test).

The same qualitative results were obtained in the presence of the N-type VGCC blocker ω-conotoxin GVIA (ω-CgTx GVIA), since the simultaneous application of this toxin and [Leu^{31},Pro^{34}]NPY (Fig. 7A) or NPY13-36 (Fig. 7B) (74.3 ± 1.9% or 76.7 ± 2.7% of the control, respectively), did not potentiate the $[Ca^{2+}]_i$ inhibition induced by Y1 or Y2 receptor agonists alone. Also, the inhibitory effect induced by 500 nM ω-CgTx GVIA (74.4 ± 2.7% of control) was similar to that obtained with Y1 (Fig. 7A) or Y2 (Fig. 7B) receptor agonists, and again this toxin had no significant effect on the basal $[Ca^{2+}]_i$.

Contrary to what happened in the presence of nitrendipine, or ω-conotoxin GVIA, the P-/Q-type VGCC blocker ω-Aga IVA, did not show a strong inhibitory effect by itself (88.3 ± 3.3% of control), and co-application of [Leu^{31},Pro^{34}]NPY or 300 nM NPY13-36 with 100 nM ω-Aga IVA did not further inhibited $[Ca^{2+}]_i$ response to 30 mM KCl, as compared to the effect of either Y1 or Y2 receptor agonists (75.9 ± 3.9% or 78.1 ± 3.1% of control, respectively).

We showed previously that Y1 and Y2 receptors activation inhibits both Ca^{2+}-dependent and Ca^{2+}-independent glutamate release (Silva et al., 2001), and we have now investigated the pathway between NPY receptor activation and the inhibition of glutamate release. For this, we triggered the exocytotic release of glutamate with ionomycin in the presence of Ca^{2+}. This Ca^{2+} ionophore promotes Ca^{2+} influx and glutamate release inde-



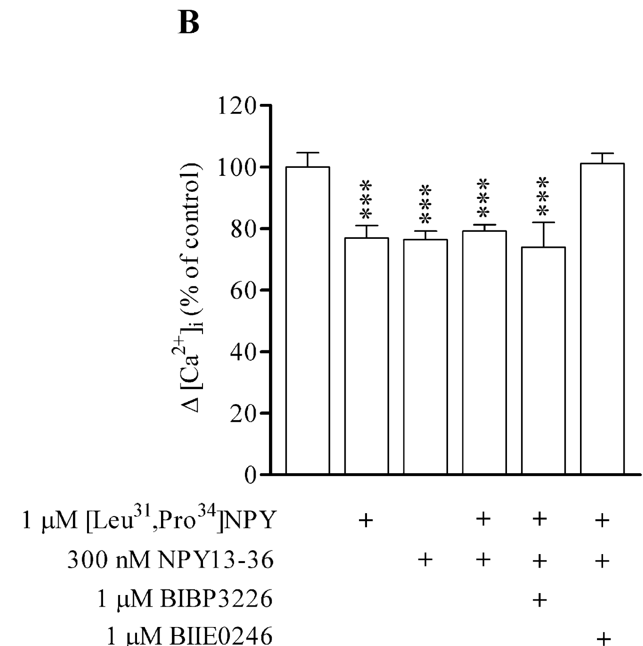


Fig. 6. (A) Quantitative analysis of the effect of NPY Y1, Y2 and Y5 receptor agonists/antagonists and (B) effect of co-activation of NPY Y1 and Y2 receptors on the initial rise in $[Ca^{2+}]_i$ stimulated by 30 mM KCl, in cultured rat hippocampal neurons. The changes in $[Ca^{2+}]_i$ (△$[Ca^{2+}]_i$) were calculated as the difference between the peak values following depolarisation and the basal values acquired just prior to the stimulus. The cells were incubated with the NPY receptor agonists or/and antagonists for 10 min before starting the $[Ca^{2+}]_i$ measurements. The results are expressed as mean percentage of control ± SEM of 5–13 independent experiments. $^{***}P < 0.001$—Dunnett's post-test; statistical significance when compared to the control (KCl stimulation).

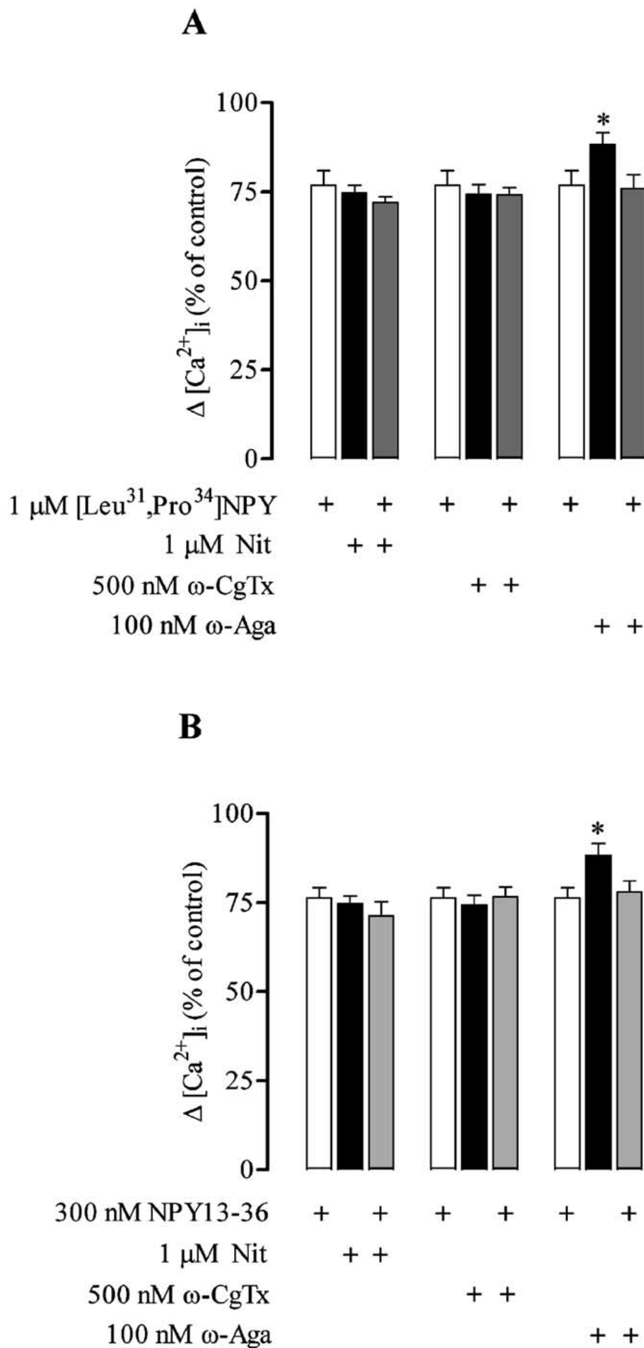


Fig. 7. Comparison of the inhibitory effects of Y1 (A) or Y2 (B) receptor agonists and of Ca^{2+} channel blockers ω-Conotoxin GVIA (ω-CgTx, 500 nM), ω-Agatoxin IVA (ω-Aga, 100 nM) or nitrendipine (Nit, 1 μM) on the $[Ca^{2+}]_i$ increase stimulated by 30 mM KCl, in cultured hippocampal neurons. The cells were incubated with the Ca^{2+} channels blockers and the NPY receptor agonists for 10 min before starting the $[Ca^{2+}]_i$ measurements. In control conditions the basal $[Ca^{2+}]_i$ was 51.4 ± 1.6 nM and the KCl-evoked $[Ca^{2+}]_i$ was 214.0 ± 8.6 nM. The results are expressed as mean percentage of control ± SEM of 6–9 independent experiments. $^*P < 0.05$—Bonferroni's post-test; statistical significance when compared to the effect of [Leu^{31},Pro^{34}]NPY (A) or NPY13-36 (B) alone.

pendently of VGCC activity. We observed that none of the NPY receptor agonists was able to reduce the ionomycin-stimulated glutamate release (Fig. 8), in contrast to what happened when we stimulated glutamate release with KCl (Figs. 1B and 2).

We have also used nerve terminals to investigate whether Y1, Y2 or Y5 receptor activation inhibit glutamate release by blocking P-/Q-type channels, the predominant VGCC at the nerve terminals. In the presence of 100 nM ω-Aga IVA, the total glutamate release was inhibited to 52.9 ± 3.0% of the control (Fig. 9). Moreover, co-application of [Leu^{31},Pro^{34}]NPY, 300 nM NPY13-36 or 1 μM NPY (19-23)-(Gly^1,Ser^3,Gln^4,Thr^6,Ala^{31},Aib^{32},Gln^{34})-PP with 100 nM ω-Aga IVA did not further inhibited the glutamate release in response to 15 mM KCl, as compared to the effect of ω-Aga IVA by itself (48.9 ± 1.1%, 50.5 ± 1.9% or 41.2 ± 2.6% of control, respectively) (Fig. 9).

4. Discussion

Our results suggest that the selective co-activation of both NPY Y1 and Y2 or Y2 and Y5 receptors inhibits the activity of Y1 or Y5 receptors, respectively, by an undetermined mechanism. Moreover, we also show that the inhibitory mechanism triggered by NPY receptor activation involves the activity of VGCCs.

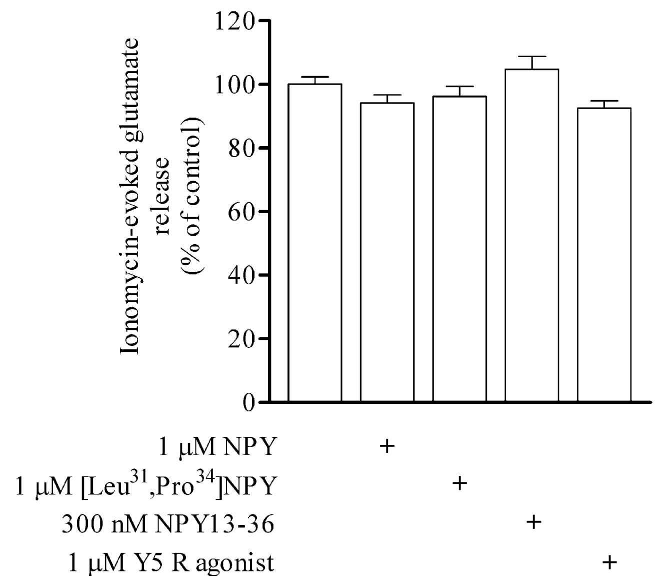


Fig. 8. Lack of effect of NPY and Y1, Y2 and Y5 receptor agonists on the glutamate release evoked by 5 μM ionomycin, in rat hippocampal synaptosomes. The synaptosomes were incubated with the NPY receptor agonists for 10 min before starting the measurement of glutamate release. Total glutamate release in control conditions was 1.0 ± 0.02 nmol glutamate/mg protein/min The results are expressed as mean percentage of control ± SEM of 7–14 independent experiments, in different synaptosomal preparations.

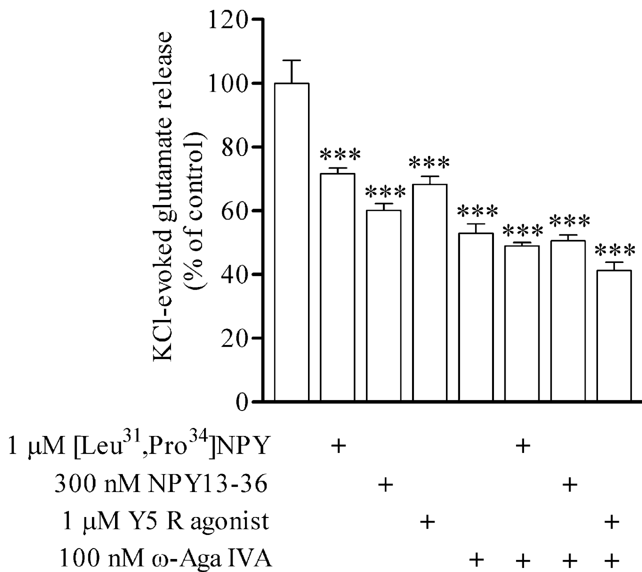


Fig. 9. Comparison of the inhibitory effects of Y1, Y2 or Y5 receptor agonists and Ca^{2+} channel blocker ω-Agatoxin IVA (ω-Aga, 100 nM) on the total glutamate release stimulated by 15 mM KCl, in rat hippocampal synaptosomes. The synaptosomes were incubated with ω-Aga IVA and the NPY receptor agonists for 10 min before starting the measurement of glutamate release. Glutamate release in control conditions was on average 1.1 ± 0.1 nmol glutamate/mg protein/min. *** $P < 0.001$—Dunnett's post-test; statistical significance when compared to the control (KCl stimulation).

Presynaptic inhibition induced by NPY is mostly mediated by Y2 receptors (Colmers et al., 1991). Indeed, in the adult rat hippocampus, the inhibitory effect of NPY on glutamate-mediated excitatory transmission appears to be mediated by presynaptic Y2 receptors, possibly by reducing Ca^{2+} influx through N-type Ca^{2+} channels (Bleakman et al., 1992; Klapstein and Colmers, 1992). However, Y1 receptors play also a critical role in the rat hippocampus. McQuiston et al. (1996) demonstrated that NPY inhibits an N-type I_{Ca} in rat dentate granule cells by activating Y1 receptors. Accordingly, we also determined that Y1 and Y2 receptors play an important role in the modulation of glutamate release, since when selectively activated they both inhibit Ca^{2+}-dependent glutamate release in hippocampal nerve terminals (Silva et al., 2001). However, the Y2 receptor subtype seems to play a predominant role in synaptic excitation when activated by the endogenous agonist, NPY, (McQuiston and Colmers, 1996), which is in agreement with the present results. Indeed, we show here that the Y1 or Y5 receptor antagonists did not prevent the inhibition of glutamate release induced by NPY. This effect is not due to the absence of functional Y1 and Y5 receptors since, in the presence of $[Leu^{31},Pro^{34}]$NPY or NPY (19-23)-$(Gly^{1},Ser^{3},Gln^{4},Thr^{6},Ala^{31},Aib^{32},Gln^{34})$-PP, we observed a significant inhibition of the glutamate release. Therefore, in spite of the presence of the three receptors in hippocampal nerve terminals, the endogenous agonist, NPY, inhibits glutamate release by activating Y2 receptors instead of Y1 and Y5 receptors.

We also show in this study that Y5 receptor activation inhibits glutamate release in hippocampal nerve terminals, and that this effect is due to the activation of Y5 receptors in dentate gyrus and CA3 subregion of the hippocampus. These results are in agreement with the idea that Y5 receptors play an important role in the control of the glutamatergic mechanism in the hippocampus, and that Y5-like receptors inhibit kainic acid seizures (Woldbye et al., 1997; Marsh et al., 1999). Recently, Guo et al. (2002) also showed that Y5 receptor subtype plays a critical role in modulation of hippocampal excitatory transmission at the hilar-to-CA3 synapse in the mouse.

Using hippocampal neurons in culture, we also observed that Y1 and Y2 receptors inhibited KCl-evoked $[Ca^{2+}]_i$ changes in a very selective way, in contrast to what happened in the presence of the Y5 receptor agonist, which failed to significantly modulated the $[Ca^{2+}]_i$ changes. Moreover, as we showed in the glutamate release studies, the inhibitory effect induced by NPY is also mediated by Y2 receptors. Thus, if these three NPY receptors subtypes (Y1, Y2 and Y5) coexist in the hippocampus, and have an important role in mediating neuronal excitability, it is critical to understand the putative functional interaction between different NPY receptors when selectively and simultaneously activated.

In the presence of both selective agonists for Y1 and Y2 or Y2 and Y5 receptors, we did not observe a potentiation of the inhibitory effect when compared to the independent inhibition induced by each agonist, in glutamate release or $[Ca^{2+}]_i$ changes studies. This observation is in agreement with the co-expression of different NPY receptors in the same glutamatergic terminals of the dentate gyrus and CA3 subregion. In a previous study we showed that the selective activation of Y1 receptors in the CA1 subregion of the hippocampus did not inhibit the KCl-evoked glutamate release (Silva et al., 2001), and in the present study we show the same effect for the Y5 receptor. Moreover, the inhibition of glutamate release and $[Ca^{2+}]_i$ changes induced by $[Leu^{31},Pro^{34}]$NPY plus NPY13-36 was sensitive only to the presence of the Y2 receptor antagonist. Also, the inhibition of glutamate release induced by the co-activation of both Y2 and Y5 receptors was only prevented by the Y2 receptor antagonist. Surprisingly, the same was not observed when the Y1 and Y5 receptors were simultaneously activated. Therefore, it seems that Y2 is functionally coupled to Y1 and Y5 receptors, and when Y2 receptors are activated the Y1 and Y5 receptors stay 'silent'.

Functional interaction between different metabotropic receptors has been described (Bouvier, 2001). Oligomerization of $GABA_B$ receptors (Marshall et al., 1999), somatostatin receptors (Rocheville et al., 2000), opioid

receptors (Jordan and Devi, 1999), and even oligomerization of metabotropic receptors for different neurotransmitters like β-adrenergic and opioid (Jordan et al., 2001) can profoundly affect the expected pharmacology of each receptor individually (Bouvier, 2001). The presently described interaction between Y1 and Y2 or Y2 and Y5 receptors suggest the formation of oligomers of Y1 and Y2 or Y2 and Y5 receptors, and the activation of such a complex would predominantly activate the NPY Y2 receptors over Y1 and Y5 receptors.

In the present study we also show that the inhibitory effect induced by Y1 or Y2 receptors in $[Ca^{2+}]_i$ changes is mainly due to the inhibition of N- and L-type, in both cases. Since in this work we measured the $[Ca^{2+}]_i$ changes in the whole cell, these results are compatible with the presence of Y1 receptors in the cell body, dendrites and in the nerve terminal, which is in agreement with the pre- and post-synaptic localization of these receptors (Silva et al., 2001). Concerning the results that we obtained for the Y2 receptors, the inhibition of $[Ca^{2+}]_i$ changes through L-type VGCCs, may suggest the presence of these receptors in dendrites. We also used nerve terminals to better understand the involvement of Ca^{2+} channels on the glutamate release inhibition induced by presynaptic Y1, Y2 and Y5 receptors. We firstly observed that the inhibitory effect induced by NPY receptors was mediated by VGCCs, and then that the P-/Q-type channels are clearly involved in the inhibition of glutamate release. So, these results strongly suggest the involvement of L-, N- and P-/Q-type channels in the inhibition of $[Ca^{2+}]_i$ changes and glutamate release mediated by NPY receptors in the hippocampus. In agreement, other studies in rat dentate granule cells also show that NPY inhibited Ca^{2+} currents mainly via Y1 receptors, inhibiting an N-type calcium channel (McQuiston et al., 1996). However, presynaptic Y2 receptors activation by NPY also inhibits N-type, P-/Q-type and unidentified presynaptic VGCCs (Qian et al., 1997), or even L-type VGCCs can be involved in the inhibitory effects mediated by NPY (Valentijn et al., 1994).

The cross-talk between Y1, Y2 and Y5 receptors, described for the first time in this study, allow us to better understand the functional interaction and the role of each NPY receptor in the hippocampus. Since these receptors play an important role in regulating excitatory neurotransmission under physiological and pathological conditions, these results may be of particular interest for future studies concerning the anticonvulsant properties of NPY receptors.

Acknowledgements

We acknowledge Dr Henri Doods (Boehringer Ingelheim Pharma KG) for generously provide us the BIIE0246. We would also like to thank Ms Elisabete Carvalho for assisting us in hippocampal cell cultures. This work was supported by the Foundation for Science and Technology, PRAXIS XXI Program, Portugal (Project PRAXIS XXI/2/2.1/SAU/1348/95 and Grant PRAXIS 18261/98).

References

Bean, B.P., 2001. Calcium channels. In: Encyclopedia of Life Science. Nature Publishing Group, pp. 1–7.

Bleakman, D., Harrison, N.L., Colmers, W.F., Miller, R.J., 1992. Investigations into neuropeptide Y-mediated presynaptic inhibition in cultured hippocampal neurones of the rat. British Journal of Pharmacology 107, 334–340.

Bouvier, M., 2001. Oligomerization of G-protein-coupled transmitter receptors. Nature Reviews Neuroscience 2, 274–286.

Brewer, G.J., Torricelli, J.R., Evege, E.K., Price, P.J., 1993. Optimized survival of hippocampal neurons in B27-supplemented neurobasal™, a new serum-free medium combination. Journal of Neuroscience Research 35, 567–576.

Cabrele, C., Langers, M., Bader, R., Wieland, H.A., Doods, H.N., Zerbe, O., Beck-Sickinger, A.G., 2000. The first selective agonist for the neuropeptide YY5 receptor increase food intake in rats. Journal of Biological Chemistry 46, 36043–36048.

Colmers, W.F., Klapstein, G.J., Fournier, A., St-Pierre, S., Treherne, K.A., 1991. Presynaptic inhibition by neuropeptide Y in rat hippocampal slice in vitro is mediated by a Y_2 receptor. British Journal of Pharmacology 102, 41–44.

Colmers, W.F., Bleakman, D., 1994. Effects of neuropeptide Y on the electrical properties of neurons. Trends in Neurosciences 17, 373–379.

Doods, H., Gaida, W., Wieland, H.A., Dollinger, H., Schnorrenberg, G., Esser, F., Engel, W., Eberlein, W., Rudolf, K., 1999. BIIB0246: a selective and high affinity neuropeptide Y Y2 receptor antagonist. European Journal of Pharmacology 384, R3–R5.

Gerald, C., Walker, M.W., Vaysse, P.J.J., He, C., Branchek, T.A., Weinshank, R.L., 1995. Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y2 receptor subtype. Journal of Biological Chemistry 270, 26758–26761.

Greber, S., Schwarzer, C., Sperk, G., 1994. Neuropeptide Y inhibits potassium-stimulated glutamate release through Y_2 receptors in rat hippocampal slices in vitro. British Journal of Pharmacology 113, 737–740.

Grouzmann, E., Buclin, T., Martire, M., Cannizzaro, C., Dörner, B., Razaname, A., Mutter, M., 1995. Characterization of a selective antagonist of neuropeptide Y at the Y2 receptor. Journal of Biological Chemistry 272, 7699–7706.

Grundemar, L., Håkanson, R., 1994. Neuropeptide Y effector systems: perspectives for drug development. Trends in Pharmacological Sciences 15, 153–159.

Guo, H., Castro, P.A., Palmiter Barbaran, S.C., 2002. Y5 receptors mediate neuropeptide Y actions at excitatory synapses in area CA_3 of the mouse hippocampus. Journal of Neurophysiology 87, 558–566.

Hansel, D.E., Eipper, B.A., Ronnett, G.V., 2001. Neuropeptide Y functions as a neuroproliferative factor. Nature 410, 940–944.

Jordan, B.A., Devi, L.A., 1999. G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399, 697–700.

Jordan, B.A., Trapaidze, N., Gomes, I., Nivarthi, R., Devi, L.A., 2001. Oligomerization of opioid receptors with β_2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proceeding of the National Academy of Sciences of the United States of America 98, 343–348.

Kanatani, A., Ishibara, A., Iwaasa, H., Nakamura, K., Okamoto, O., Hidaka, M., Ito, J., Fukuroda, T., MacNeil, D.J., Van der Ploeg, L.H., Ishii, Y., Fukami, T., Ihara, M., 2000. L-152,804: orally active and selective neuropeptide Y Y5 receptor antagonist. Biochemistry Biophysics Research Communications 272, 169–173.

Klapstein, G.J., Colmers, W.F., 1992. 4-Aminopyridine and low Ca^{2+} differentiate presynaptic inhibition mediated by neuropeptide Y, baclofen and-chloroadenosine in rat hippocampal CA1 in vitro. British Journal of Pharmacology 105, 470–474.

Kopp, J., Nanobashvili, A., Kokaia, Z., Lindvall, O., Hökfelt, T., 1999. Differential regulation of mRNAs for neuropeptide Y and its receptor subtypes in widespread areas of the rat limbic system during kindling epileptogenesis. Molecular Brain Research 72, 17–29.

Layne, E., 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Colowick, S.P., Kaplan, N.O. (Eds.), Methods in Enzimology, vol. 3. Academic Press, New York, NY, pp. 447–451.

Malva, J.O., Carvalho, A.P., Carvalho, C.M., 1996. Domoic acid induces the release of glutamate in the rat hippocampal CA3 subregion. Neuroreport 7, 1330–1344.

Marshall, F.H., Jones, K.A., Kaupmann, K., Bettler, B., 1999. $GABA_B$ receptors-the first 7TM heterodimers. Trends in Pharmacological Sciences 20, 396–399.

Marsh, D.J., Baraban, S.C., Hollopeter, G., Palmiter, R.D., 1999. Role of the Y5 neuropeptide Y receptor in limbic seizures. Proceeding of the National Academy of Sciences USA 96, 13518–13523.

McMahon, T.H., Foran, P., Dolly, J.O., Verhage, M., Wiegant, V.M., Nicholls, D.G., 1992. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, -aminobutyric acid, asparate, and met-enkephalin release from synaptosomes. Journal of Biological Chemistry 267, 21338–21343.

McQuiston, A.R., Colmers, W.F., 1996. Neuropeptide Y_2 receptors inhibit the frequency of spontaneous but not miniature EPSCs in CA3 pyramidal cells of rat hippocampus. Journal of Neurophysiology 76, 3159–3168.

McQuiston, A.R., Petrozzino, J.J., Connor, J.A., Colmers, W.F., 1996. Neuropeptide Y_1 receptors inhibit N-type calcium currents and reduce transient calcium increases in rat dentate granule cells. Journal of Neuroscience 16, 1422–1429.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., Westfall, T., 1998. XVI. International union of pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacological Reviews 50, 143–150.

Nicholls, D.G., Sihra, T.S., Sanchez-Prieto, J., 1987. Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. Journal of Neurochemistry 49, 50–57.

Parker, R.M.C., Herzog, H., 1999. Regional distribution of Y-receptor subtype mRNA in rat brain. European Journal of Neuroscience 11, 1431–1448.

Qian, J., Colmers, W.F., Saggau, P., 1997. Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: modulation of presynaptic Ca^{2+} entry. Journal of Neuroscience 17, 8169–8177.

Rocheville, M., Lange, D., Kumar, U., Sasi, R., Patel, R.C., Atel, Y.C., 2000. Subtypes of the somatostatin receptor assemble as functional homo and heterodimers. Journal of Biological Chemistry 275, 7862–7869.

Silva, A.P., Carvalho, A.P., Carvalho, C.M., Malva, J.O., 2001. Modulation of intracellular calcium changes and glutamate release by neuropeptide Y1 and Y2 receptors in the rat hippocampus: differential effects in CA1, CA3 and dentate gyrus. Journal of Neurochemistry 79, 286–296.

Toth, P.T., Bindokas, V.P., Bleakman, D., Colmers, W.F., Miller, R.J., 1993. Mechanism of presynaptic inhibition by neuropeptide Y at sympathetic nerve terminals. Nature 364, 635–639.

Valentijn, J.A., Vaudry, H., Kloas, W., Cazin, L., 1994. Melanostatin (NPY) inhibited electrical activity in frog melanotrophs through modulation of K^+, Na^+ and Ca^{2+} currents. Journal of Physiology (London) 475 (185-19), 5.

Wahlestedt, C., Reis, D.J., 1993. Neuropeptide Y-related peptides and their receptors-are the receptors potential therapeutic drug targets? Annual Reviews of Pharmacology and Toxicology 32, 309–352.

Wieland, H.A., Willim, K.D., Doods, H.N., 1995. Receptor binding profile of NPY analoques and fragments in different tissues and cell lines. Peptides 16, 1389–1394.

Woldbye, D.P.D., Larsen, P.J., Mikkelsen, J.D., Klemp, K., Madsen, T.M., Bolwig, T.G., 1997. Powerful inhibition of kainic acid seizures by neuropeptide Y via Y5-like receptors. Nature Medicine 3, 761–764.

Wu, L.G., Saggau, P., 1994. Pharmacological identification of two types of presynaptic voltage-dependent calcium channels at CA3-CA1 synapses of the hippocampus. Journal of Neuroscience 14, 5613–5622.