Volume 260, number 1, 73–78 FEB 08011 January 1990

Y_1 receptors for neuropeptide Y are coupled to mobilization of intracellular calcium and inhibition of adenylate cyclase

Lars Aakerlund, Ulrik Gether, Jannie Fuhlendorff, Thue W. Schwartz and Ole Thastrup°

Laboratory for Molecular Endocrinology, University Department of Clinical Chemistry's Rigshospitalet and °*Thrombosis Group, Copenhagen Science Park Symbion and University Department of Clinical Chemistry's Rigshospitalet, Copenhagen, Denmark*

Received 16 October 1989; revised version received 22 November 1989

Two types of binding sites have previously been described for neuropeptide Y (NPY), called Y_1 and Y_2 receptors. The intracellular events following Y_1 receptor activation was studied in the human neuroblastoma cell line SK-N-MC. Both NPY and the specific Y_1 receptor ligand, [Leu^{31},Pro^{34}]-NPY, caused a rapid and transient increase in the concentration of free calcium in the cytoplasm as measured by the fluorescent probe, Fura-2. The effect of both peptides was independent of extracellular calcium as addition of EGTA or manganese neither changed the size nor the shape of the calcium response. The calcium response to NPY was abolished by pretreatment with thapsigargin, which can selectively deplete a calcium store in the endoplasmic reticulum. Y_1 receptor stimulation, by both NPY and [Leu^{31},Pro^{34}]NPY, also inhibited the forskolin-stimulated cAMP production with an EC_{50} of 3.5 nM. There was a close relation between the receptor binding and the cellular effects as half-maximal displacement of [^{125}I-Tyr^{36}]monoiodoNPY from the receptor was obtained with 2.1 nM NPY. The Y_2-specific ligand NPY(16–36)peptide had no effect on either intracellular calcium or cAMP levels in the SK-N-MC cells. It is concluded that Y_1 receptor stimulation is associated with both mobilization of intracellular calcium and inhibition of adenylate cyclase activity.

Peptide receptor; Calcium intracellular; Fura-2; cAMP; pH, intracellular

1. INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid regulatory peptide which is widely distributed throughout the central and peripheral nervous systems [1–3]. The primary structure of NPY is evolutionarily well conserved [1,4] and the peptide belongs together with peptide YY and pancreatic polypeptide to the so-called pancreatic polypeptide-fold peptide family. This family of peptides is characterized by a common tertiary structure which is stable even in dilute aqueous solutions [5].

In the peripheral nervous system, NPY is often found to colocalize with noradrenaline in the sympathetic nerve terminals. NPY is released together with noradrenaline and acts together with noradrenaline in regulation of the vascular tone. The neurophysiology of NPY has recently been reviewed in a symposium volume [6].

It was recognized early that NPY receptors are coupled to G-proteins [7,8]. However, a rather diffuse picture of the secondary messenger systems for NPY has since appeared [9]. NPY has been shown to inhibit adenylate cyclase in several tissues and some cell lines [10–16]. Mobilization of intracellular calcium has also been associated with NPY in human erythroleukemia cells and in cultured neurons from rat dorsal root ganglia [16,17]; whereas this effect was not found in cultured vascular smooth muscle cells from the rabbit lung artery [15]. Furthermore, NPY has been shown to be able to inhibit voltage-sensitive calcium channels [18].

Recently, two different types of NPY receptors, called Y_1 and Y_2, have been defined on the basis of their different binding profile for long C-terminal fragments of NPY and of the new, specific Y_1 receptor ligand [Leu^{31},Pro^{34}]NPY [5,19–22]. Presently, we have studied the second messengers coupled to the activation of the Y_1 type receptor in a human neuroblastoma cell line (SK-N-MC), which only expresses Y_1 receptors. It is found that Y_1 receptor activation leads both to mobilization of intracellular calcium and to inhibition of adenylate cyclase.

2. MATERIALS AND METHODS

2.1. *Peptides*

Porcine NPY and [Leu^{31},Pro^{34}]NPY were synthesized by automatic stepwise solid phase synthesis on an Applied Biosystems model 430A peptide synthesizer, as previously described in detail [22]. The peptides were quantified and amino acid composition controlled by amino acid analysis according to [22]. NPY(16–36) was obtained from Peninsula Laboratories, St. Helens, England. The peptides were stored at −20°C in stock solutions of 1.0 mM in 10^{-3} M acetic acid. The peptides were added in 1% (v/v) to the cell suspensions from adequate solutions in Hepes buffer.

Correspondence address: L. Aakerlund or U. Gether, Lab. Molecular Endocrinology, Rigshospitalet 6321, Blegdamsvej 9, DK-2100, Copenhagen Ø, Denmark

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.2. *Cell culture*

SK-N-MC human neuroblastoma cells [23] were kindly provided by Drs June Biedler and Barbara A. Spengler, Sloan Kettering Memorial Institute, NY, USA. The cells were cultured in 800 ml culture flasks (Costar, Cambridge, MA, USA) in a 1:1 mixture of Ham F12 medium and Dulbecco's modified Eagles medium with 10% fetal calf serum, 1% non-essential amino acids, 2 mM glutamin and penicillin/streptomycin (100 IU and 100 μg/ml, respectively), all purchased from Gibco, Uxbridge, England.

2.3. *Cytosolic free calcium concentration ($[Ca^{2+}]_i$)*

Confluent monolayer cultures of SK-N-MC cells were harvested 3–7 days after seeding by washing with medium and gently scraping with a rubber policeman. The cells were centrifuged for 5 min at 200 × *g* and resuspended in cell medium to a concentration of 3×10^6 cells/ml. Fura-2 acetoxymethyl ester (Molecular Probes, OR, USA) was added at a final concentration of 2.5 μM from a stock solution of 1 mM in dimethyl sulfoxide (DMSO). The cells were incubated for 30 min in the dark at room temperature. After centrifugation (200 × *g* for 5 min), the cells were resuspended at a density of 2.0×10^6 cells/ml in 3 ml Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$ and 10 mM glucose in 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), with 150 mg/l bovine serum albumin, adjusted to pH 7.4). The cells were used within 30 min and kept in suspension by gentle magnetic stirring at 37°C. The Fura-2 fluorescence was measured in a Hitachi Spectrofluorometer (F-2000) with a monochromatic setting of 340 nm excitation and 500 nm emission (slit = 10 nM). Calculation of cytoplasmic calcium concentrations were done according to Grynkiewicz et al. [24] using the formula:

$$[Ca^{2+}]_i = K(F - F_{min})/(F_{max} - F)$$

K = 224 nM represents the apparent dissociation constant for Fura-2-Ca^{2+}. F_{max}, constituting the fluorescence of Fura-2 when saturated with Ca^{2+}, was determined by adding 2.5 μM ionomycin (Sigma) in the presence of 1 mM extracellular Ca^{2+}. F_{min}, fluorescence of Fura-2 in absence of Ca^{2+}, was measured by chelating external Ca^{2+} with 10 mM EGTA following the addition of ionomycin. F, F_{max} and F_{min} were corrected for extracellularly present Fura-2 by subtracting corresponding fluorescence values determined in the supernatant of centrifuged (200 × *g* for 10 min) cell suspension.

2.4. *Cytosolic pH measurements*

SK-N-MC cells in suspension (density 3×10^6 cells/ml) were loaded with the pH-sensitive fluorescent probe, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) together with Fura-2 under conditions as described above. pH and $[Ca^{2+}]_i$ could then be measured simultaneously by alternating the monochromatic setting between 340 nM excitation/500 nM emission for Fura-2 measurements and 495 nM excitation/530 nM emission for BCECF measurements. At the end of each experiment, the protonophore nigericine (Sigma) was added in a final concentration of 5 μM in order to equalize pH_i and pH_o, after pH was determined as a function of fluorescence by measurement of corresponding pH and fluorescence values.

2.5. *Binding experiments*

Binding experiments were performed as previously described in detail [18–21]. Shortly, SK-N-MC cells (1.2×10^6) were grown in Petri wells, 6-well culture plates (Costar, Cambridge, MA, USA) for 1–2 days before incubation at 37°C for 60 min with 25 pM [^{125}I-Tyr^{36}]monoiodoNPY and cold peptide as indicated. All determinations in the experiments were done in triplicate.

2.6. *cAMP measurements*

The cells were harvested as described above and resuspended in Hepes buffer at a cell density of 2.5×10^6 cells/ml and divided into vials containing 1 ml of cell suspension each. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Sigma) was added to a concentration of 0.5 mM. The cells were preincubated for 10 min at 37°C before incubation for 20 min with 5 μM forskolin (Sigma) and peptides as indicated. The incubations were terminated and the cells extracted by the addition of 0.2 ml 20% perchloric acid and vigorous vortexing, followed by a 15-min incubation on ice and centrifugation for 15 min at 2000 × *g*. The amount of generated cAMP was measured in the supernatants after 50-fold dilution in 0.1 M acetate buffer by using Amersham's single range cAMP assay kit, RPA.508.

3. RESULTS

In SK-N-MC cells, NPY (100 nM) caused a rapid, transient elevation of $[Ca^{2+}]_i$ from a resting level between 90 and 100 nM to a peak value of 205 nM ± 8.7 nM (mean ± SE, n = 15) (fig.1). The signal returned to baseline within 60 s (fig.1). The specific Y_1 receptor agonist, [Leu^{31},Pro^{34}]NPY similarly raised $[Ca^{2+}]_i$ with 107 nM ± 15 nM (mean ± SE, n = 4) (fig.1), whereas stimulation of the cells with the Y_2 specific ligand, NPY(16–36) peptide, caused no measurable increases in the Fura-2 fluorescence (fig.1).

The effect of NPY was found to be independent of extracellular calcium. It thus appeared that removal of extracellular calcium (addition of 1 mM EGTA) neither changed the shape nor the size of the $[Ca^{2+}]_i$ response to NPY or to [Leu^{31},Pro^{34}]NPY; the increase during NPY stimulation in the absence of extracellular calcium was 117 nM ± 11 nM (mean ± SE, n = 13) and during [Leu^{31},Pro^{34}]NPY stimulation 90 nM ± 14 nM (mean ± SE, n = 3) (fig.1). Furthermore, when using manganese (Mn^{2+}) as a marker for Ca^{2+} influx [25], we were not able to detect any Mn^{2+}-induced quenching of the Fura-2 signal.

Stimulation of cholinergic, muscarinic receptors induced a $[Ca^{2+}]_i$ signal that was different from the NPY response. It was thus found that optimal concentrations (100 μM) of carbacholine (CCh) only induced a minor $[Ca^{2+}]_i$ rise (47 nM ± 3 nM, mean ± SE, n = 10), and that $[Ca^{2+}]_i$ stayed elevated in the presence of extracellular calcium for an extended period (fig.2, upper panel). On removal of external Ca^{2+}, the CCh signal became transient (fig.2, lower panel). This pattern of events corresponds to an initial discharge of intracellular stored Ca^{2+} followed by an influx from the external medium. Pretreatment with CCh did not diminish the $[Ca^{2+}]_i$ response to NPY (fig.2). Although the NPY response following CCh appears larger than the response to NPY alone, this was not a general observation.

In order to characterize the intracellular Ca^{2+} pool sensitive to NPY, we used the Ca^{2+}-mobilizing agent, thapsigargin. This agent releases Ca^{2+} from a discrete intracellular pool by a direct, highly selective inhibition of Ca^{2+}-activated ATPase, the presumptive Ca^{2+} pump, situated in the membranes of the endoplasmic reticulum [26]. Taking into account that thapsigargin easily permeates the plasma membrane, this compound can be used to study intracellular Ca^{2+} release in intact

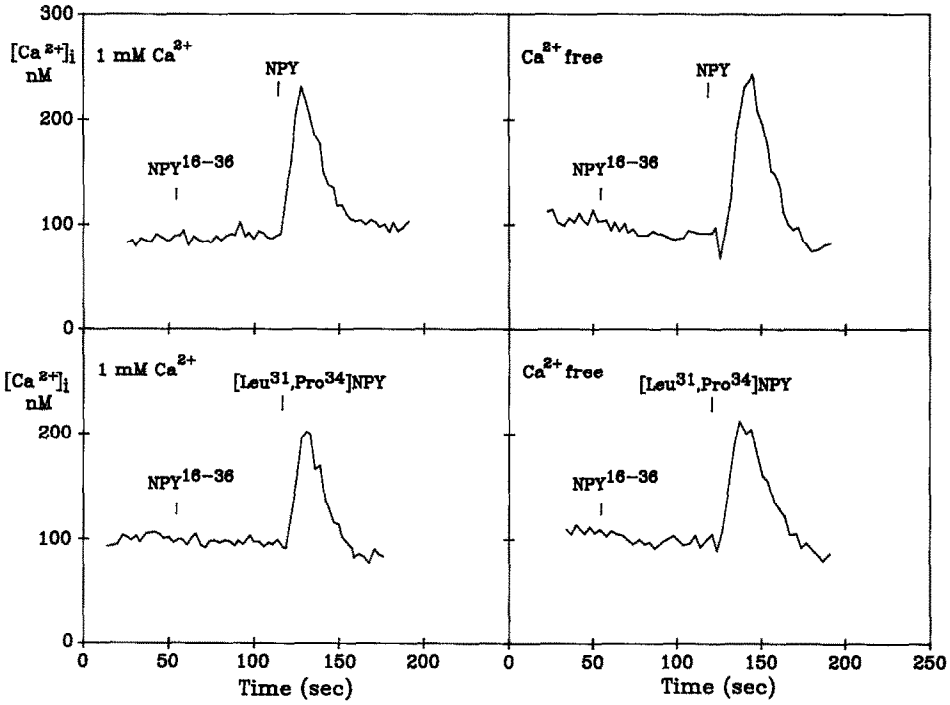

Fig.1. Effect of NPY, NPY(16–36) peptide, and [Leu^{31},Pro^{34}]NPY on $[Ca^{2+}]_i$ in human SK-N-MC cells. The experiments were performed with 100 nM of the peptides on cells in suspension; in the left panels in the presence of 1 mM Ca^{2+} and in the right panels without calcium and in the presence of 1 mM of EGTA. The calcium chromophore Fura-2 was used and the calculated cytoplasmic free calcium concentration is plotted against time. The traces shown are representative of 3–15 experiments.

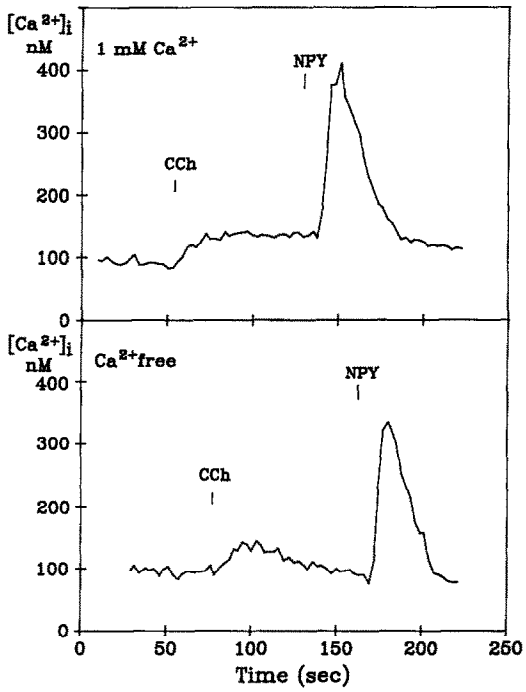

Fig.2. The effect of CCh and NPY on $[Ca^{2+}]_i$ of SK-N-MC cells in suspension in the presence and absence of extracellular calcium. The upper panel shows the response to 0.1 mM CCh followed by 100 nM NPY in the presence of 1 mM Ca^{2+} and the lower panel in the presence of 1 mM EGTA. Calculated calcium concentration is plotted against time. The traces shown are representative of 4 experiments.

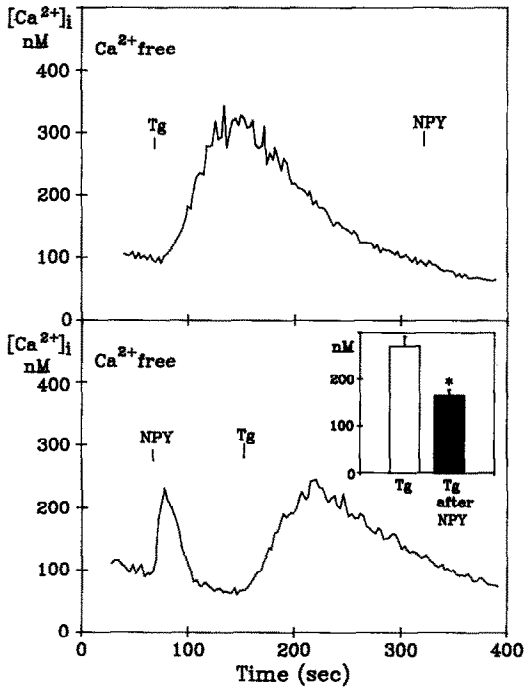

Fig.3. Effect of NPY and thapsigargin (Tg) on $[Ca^{2+}]_i$ of SK-N-MC cells in absence of extracellular calcium. The upper panel shows the stimulation with 50 nM Tg followed by 100 nM NPY; the lower panel, the stimulation with NPY followed by Tg. In the insert, the peak increase in cytoplasmic calcium concentration following Tg alone (50 nM) and with prior stimulation with 100 nM NPY (* $P <$ 0.05, n = 3–4, t-test) is shown.

cells by a mechanism not involving production of inositol polyphosphates or activation of protein kinase C [27,28]. In the presence of extracellular Ca^{2+}, thapsigargin raised $[Ca^{2+}]_i$ to a new elevated steady state level (data not shown); on removal of external Ca^{2+}, the thapsigargin signal became transient (fig.3, upper panel). NPY given shortly before thapsigargin significantly reduced this rise (fig.3, lower panel). The reverse administration of the two agonists showed that thapsigargin completely abolished the response to NPY (fig.3, upper panel), indicating that the stores from which NPY mobilizes calcium in the SK-N-MC cells are included in the intracellular stores that are sensitive to thapsigargin.

NPY and [Leu^{31},Pro^{34}]NPY also potently attenuated the forskolin-stimulated accumulation of cAMP (fig.4A), whereas the NPY(16–36) peptide had no detectable effect on the accumulation of cAMP in agreement with the binding properties of the Y_1 receptor found on these cells. The inhibition of the cAMP accumulation was dose dependent with an EC_{50} of 3.5 nM (fig.4B). Probably the attenuation of the cAMP accumulation is due to inhibition of adenylate cyclase rather than to stimulation of the phosphodiesterase, as the experiments were performed in the presence of the cAMP-phosphodiesterase inhibitor IBMX. A close relation between the receptor binding and the cellular effects was found as NPY displaced [^{125}I-Tyr^{36}]monoiodoNPY bound to the SK-N-MC cells with a half-maximal concentration of 2.1 nM (fig.4C).

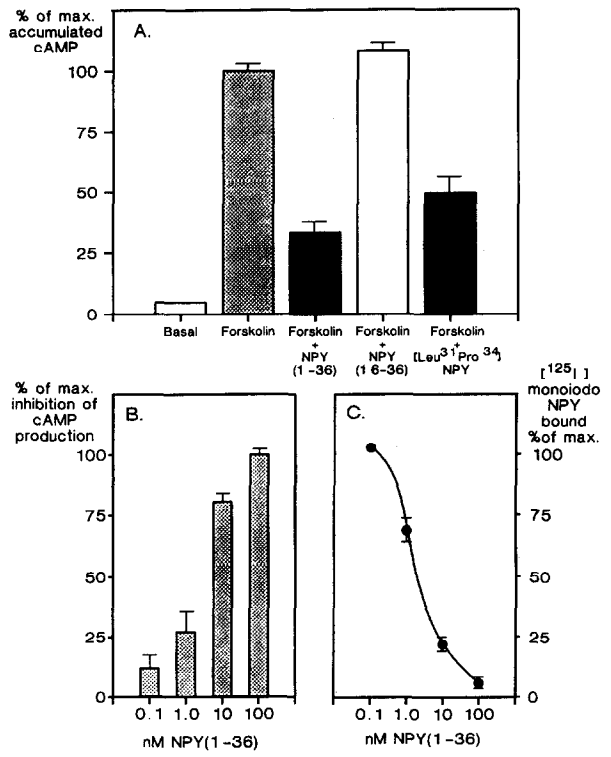

Fig.4. The effect of NPY, NPY(16–36) peptide, and [Leu^{31},Pro^{34}]NPY on forskolin-induced cAMP production in SK-N-MC cells, compared with peptide binding. (A) The effect of 100 nM of the indicated peptides. Data are expressed as percent of maximal accumulated cAMP following 5 μM forskolin in 20-min incubations (mean ± SE of 6–9 determinations in 3 different experiments). Maximal accumulated cAMP varied between 223 pmol/10^6 cells and 381 pmol/10^6 cells. (B) Dose response of the effect of NPY. Data are expressed as percent of the maximal inhibitory effect (10^{-7} M NPY) (mean ± SE of 6 determinations in 2 different experiments). (C) Inhibition of [^{125}I-Tyr^{36}]monoiodoNPY binding by NPY to the SK-N-MC cells (n = 13).

To exclude that the observed rise in $[Ca^{2+}]_i$ was secondary to a fall in cAMP level, the cells were stimulated with 10 μM forskolin 3 min prior to the stimulation with NPY. In the present study, forskolin raised the intracellular level of cAMP 12-fold above the basal level. Forskolin did not influence $[Ca^{2+}]_i$ itself, nor did it alter the response to NPY. In a paired experiment, NPY raised $[Ca^{2+}]_i$ to 131 nM ± 13 nM (n = 3) above the basal level in cells pretreated with forskolin, compared to 140 nM ± 4 nM (n = 3) in untreated cells.

Some receptors which are linked to an inhibition of adenylate cyclase also stimulate the Na^+/H^+ exchange, thereby elevating the intracellular pH [29,30]. NPY did not elevate the intracellular pH, as assessed by the pH-sensitive probe BCECF. On the contrary, NPY slightly, but significantly lowered the pH of the SK-N-MC cells 0.021 ± 0.008 (n = 10, $P < 0.02$, t-test). The basal pH level was 7.45 ± 0.02 (n = 10). The small decrease in pH may be secondary to the rise in $[Ca^{2+}]$ [31] as the calcium ionophore ionomycin lowered the intracellular pH by 0.073 ± 0.009 (n = 10, $P < 0.001$, t-test).

4. DISCUSSION

NPY receptors have been both physiologically [19] and biochemically [20–22] divided into two types (Y_1 and Y_2). The Y_1 receptor appears in most species and tissues to be the major postsynaptic receptor which is responsible for the vasoconstrictory action of NPY. By use of a human neuroblastoma cell line (SK-N-MC), which only expresses Y_1 type receptors for NPY [20–22], and by using a newly characterized specific ligand for this receptor type, we find that stimulation of Y_1 receptors is associated both with an inhibition of adenylate cyclase activity and with an increase in $[Ca^{2+}]_i$. The latter caused by mobilization of calcium from the thapsigargin depletable store, conceivably the endoplasmic reticulum.

NPY-induced mobilization of intracellular calcium has previously been shown in human erythroleukemia cells (HEL) and in cultured neurons from dorsal root ganglia of rats (DRG) [16,17]. In these cases, the NPY receptor type was not characterized. However, in the HEL cells, the calcium response was similar to the response found in the SK-N-MC cells as it was transient, independent of extracellular calcium and combined with an inhibition of cAMP accumulation [16].

Thus, the NPY receptor on the HEL cells is probably also of the Y_1 type.

The mechanism by which calcium is mobilized by NPY is not known but at least it seems to involve a G_0 or G_1-like protein [16,17]. Whether this coupling to a G-protein leads to activation of phospholipase C and generation of inositol polyphosphates is uncertain. In the HEL cells, no increase in phosphoinositide turnover could be observed following stimulation with NPY and following activation of α_2 receptors [16,32]. In addition, the monophasic calcium response to NPY differs from the classical biphasic response observed following stimulation of receptors coupled to phosphoinositide breakdown as seen, e.g. following the activation of muscarinic receptors [28] (fig.2). The apparent inability of NPY to induce Ca^{2+} influx could be explained by a lacking generation of inositol polyphosphates. Inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P_4) together with Ins 1,4,5-P_3 has thus been proposed to be involved in the regulation of Ca^{2+} entry (for review see [33]). Thapsigargin, however, induced Ca^{2+} influx, even though it does not cause production of inositol polyphosphates [27]. The mechanism by which this occurs is believed to be emptying an Ins 1,4,5-P_3-sensitive pool which then functions as a signal for Ca^{2+} entry [26,28]. It is tempting to speculate that this discrepancy is due to the fact that NPY is targeting a discrete pool in the endoplasmic reticulum that does not take part in the signalling of Ca^{2+} entry.

Inhibition of adenylate cyclase by NPY has been shown in a variety of cell types [10–16] and, although no direct study has been performed, this effect appears also to be associated with the Y_2 receptor. In, e.g. the hippocampus, which only contains Y_2 type receptors [20], the inhibition of adenylate cyclase is very clear [10]. Multiple receptors inhibiting adenylate cyclase activity are independently coupled to other cellular events including opening of K^+ channels, inhibition of voltage-dependent calcium channels, Na^+/H^+ exchange, and calcium mobilization (for review see [34]). Thus, it is likely to assume that the inhibition of adenylate cyclase and the calcium mobilization represent two independent effects coupled to one receptor, i.e. the Y_1 receptor. Whether the Y_2 receptor subtype is associated with other cellular events than the inhibitory effect on adenylate cyclase remains to be determined. Possibly in some species it may be linked to voltage-sensitive calcium channels which are believed to mediate the prejunctional Y_2 receptor effect, i.e. inhibition of noradrenaline and substance P release [6,18,19].

Acknowledgements: During the work, L.A. was the recipient of a scholarship from the Danish Biotechnology Center through the Biotechnology Center for Neuropeptide Research and U.G. was the recipient of a scholarship from the NOVO Foundation. T.W.S. was the recipient of a professorship in Molecular Endocrinology from the Danish Medical Research Council and the Weimann Foundation. The work of O.T. was supported by the Danish State Biotechnology Program 1987–1990. The work was also supported by grants from the NOVO and Carlsberg Foundations.

REFERENCES

[1] Tatemoto, K. (1982) Proc. Natl. Acad. Sci. USA 79, 5485–5489.
[2] O'Donohue, T.L., Cornwall, B.M., Pruss, R.M., Mezey, E., Kiss, J.Z., Eiden, L.E., Massari, V.J., Tessel, R.E., Pickel, V.M. and DiMaggio, D.A. (1985) Peptides 6, 55–76.
[3] Allen, Y.S., Adrian, T.E., Allen, J.M., Tatemoto, K., Crow, T.J., Bloom, S.R. and Polak, J.M. (1983) Science 221, 877–879.
[4] O'Hare, M.M.T., Tenmuko, S., Aakerlund, L., Hilsted, L., Johnsen, A. and Schwartz, T.W. (1988) Regul. Pept. 20, 293–304.
[5] Schwartz, T.W., Fuhlendorff, J., Langeland, N., Thøgersen, H., Jørgense, J.C. and Sheikh, S.P. (1989) in: Neuropeptide Y, XIV Nobel Symposium (Mutt, V., Høkfelt, T., Lundberg, J. and Fuxe, K. eds) pp.143–151, Raven, New York.
[6] Mutt, V., Høkfelt, T., Lundberg, J. and Fuxe, K. (eds) (1989) Neuropeptide Y, XIV Nobel Symposium, Raven, New York.
[7] Unden, A. and Bartfai, T. (1984) FEBS Lett. 177, 125–128.
[8] Chang, R.S.L., Lotti, V.L., Chen, T.B., Cerino, D.J. and Kling, P.J. (1985) Life Sci. 37, 2111–2122.
[9] Fredholm, B.B., Haggblad, J., Harfstrand, A. and Larsson, O. (1989) in: Neuropeptide Y, XIV Nobel Symposium (Mutt, V., Høkfelt, T., Lundberg, J. and Fuxe, K. eds) Raven, New York.
[10] Lundberg, J.M., Hemsen, A., Larsson, O., Rudehill, A., Saria, A. and Fredholm, B.B. (1988) Eur. J. Pharmacol. 145, 21–29.
[11] Westlind-Danielson, A., Unden, A., Abens, J., Andell, S. and Bartfai, T. (1987) Neurosci. Lett. 74, 237–242.
[12] Petrenko, S., Olianas, M.C., Onali, P. and Gessa, G.L. (1987) Eur. J. Pharmacol. 136, 425–428.
[13] Kassis, S., Olasmaa, M., Terenius, L. and Fishman, P.H. (1987) J. Biol. Chem. 262, 3429–3431.
[14] Olasmaa, M., Påhlman, S. and Terenius, L. (1987) Neurosci. Lett. 83, 161–166.
[15] Reynolds, E.E. and Yokata, S. (1988) Biochem. Biophys. Res. Commun. 151, 919–925.
[16] Motulsky, H.J. and Michel, M.C. (1988) Am. J. Physiol. 255, E880–885.
[17] Perney, T.M. and Miller, R.J. (1989) J. Biol. Chem. 264, 7317–7327.
[18] Walker, M.W., Ewald, D.A., Perney, T.M. and Miller, R.J. (1988) J. Neurosci. 8, 2438–2446.
[19] Wahlestedt, C., Edvinsson, L., Ekblad, E. and Håkanson, R. (1986) Regul. Pept. 13, 307–318.
[20] Sheikh, S.P., Håkanson, R. and Schwartz, T.W. (1989) FEBS Lett. 245, 209–214.
[21] Sheikh, S.P., O'Hare, M.M.T., Tortora, O. and Schwartz, T.W. (1989) J. Biol. Chem. 264, 6648–6654.
[22] Fuhlendorff, J., Gether, U., Aakerlund, L., Langeland Johansen, N., Thøgersen, H., Melberg, S.G., Olsen, U.B., Thastrup, O. and Schwartz, W.T. (1990) Proc. Natl. Acad. Sci. USA, in press.
[23] Biedler, J.L., Helson, L. and Spengler, B.A. (1973) Cancer Res. 33, 2643–2652.
[24] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[25] Hallam, T.J. and Rink, T.J. (1985) FEBS Lett. 186, 175–179.
[26] Thastrup, O., Dawson, A.P., Scharff, O., Foder, B., Cullen, P.J., Drøbak, B.K., Bjerrum, P.J., Christensen, S.B. and Hanley, M.R. (1989) Agents Actions 27, 18–23.
[27] Jackson, T.R., Patterson, S.I., Thastrup, O. and Hanley, M.R. (1988) Biochem. J. 253, 81–86.

[28] Takemura, H., Hughes, A.R., Thastrup, O. and Putney, J.W. (1989) J. Biol. Chem. 264, 12266–12271.
[29] Isom, L.L., Cragoe, E.J. and Limbird, L.E. (1987) J. Biol. Chem. 262, 17504–17509.
[30] Pouyssegur, J. (1986) Trends Biochem. Sci. 10, 453–455.
[31] Zavoico, G.B., Cragoe, E.J. and Feinstein, M.B. (1986) J. Biol. Chem. 261, 13160–13167.
[32] Michel, M.C., Brass, L.F., Williams, A., Bokoch, G.M., LaMorte, V.J. and Motulsky, H.J. (1989) J. Biol. Chem. 264, 4986–4991.
[33] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–205.
[34] Limbird, L.E. (1988) FASEB J. 2, 2686–2695.