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Neuropeptide Y Y_2 receptor and somatostatin sst_2 receptor coupling to mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells

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1 In this study we have investigated neuropeptide Y (NPY) and somatostatin (SRIF) receptor-mediated elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the human neuroblastoma cell line SH-SY5Y.

2 The Ca^{2+}-sensitive dye fura 2 was used to measure $[Ca^{2+}]_i$ in confluent monolayers of SH-SY5Y cells. Neither NPY (30–100 nM) nor SRIF (100 nM) elevated $[Ca^{2+}]_i$ when applied alone. However, when either NPY (300 pM–1 μM) or SRIF (300 pM–1 μM) was applied in the presence of the cholinoceptor agonist carbachol (1 μM or 100 μM) they evoked an elevation of $[Ca^{2+}]_i$ above that caused by carbachol alone.

3 The elevation of $[Ca^{2+}]_i$ by NPY was independent of the concentration of carbachol. In the presence of 1 μM or 100 μM carbachol NPY elevated $[Ca^{2+}]_i$ with a pEC_{50} of 7.80 and 7.86 respectively.

4 In the presence of 1 μM carbachol the NPY Y_2 selective agonist peptide YY(3–36) (PYY(3–36)) elevated $[Ca^{2+}]_i$ with a pEC_{50} of 7.94, the NPY Y_1 selective agonist [Leu^{31},Pro^{34}]-NPY also elevated $[Ca^{2+}]_i$ when applied in the presence of carbachol, but only at concentrations >300 nM. The rank order of potency, PYY(3–36) $\geqslant$ NPY $>>$ [Leu^{31},Pro^{34}]-NPY indicates that an NPY Y_2-like receptor is involved in the elevation of $[Ca^{2+}]_i$.

5 In the presence of 1 μM carbachol, SRIF elevated $[Ca^{2+}]_i$ with a pEC_{50} of 8.24. The sst_2 receptor-preferring analogue BIM-23027 (c[*N*-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe]) elevated $[Ca^{2+}]_i$ with a pEC_{50} of 8.63, and the sst_5-receptor preferring analogue L-362855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]) elevated $[Ca^{2+}]_i$ with a pEC_{50} of approximately 6.1. Application of the sst_3 receptor-preferring analogue BIM-23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2, 1 μM) to SH-SY5Y cells in the presence of carbachol neither elevated $[Ca^{2+}]_i$ nor affected the elevations of $[Ca^{2+}]_i$ caused by a subsequent coapplication of SRIF. The rank order of potency, BIM-23026 $\geqslant$ SRIF $>>$ L-362855 $>>>$ BIM-23026 suggests that an sst_2-like receptor is involved in the elevation of $[Ca^{2+}]_i$.

6 Block of carbachol activation of muscarinic receptors with atropine (1 μM) abolished the elevation of $[Ca^{2+}]_i$ by the SRIF and NPY.

7 Muscarinic receptor activation, not a rise in $[Ca^{2+}]_i$, was required to reveal the NPY or SRIF response. The Ca^{2+} channel activator maitotoxin (2 ng ml^{-1}) also elevated $[Ca^{2+}]_i$ but subsequent application of either NPY or SRIF in the presence of maitotoxin caused no further changes in $[Ca^{2+}]_i$.

8 The elevations of $[Ca^{2+}]_i$ by NPY and SRIF were abolished by pretreatment of the cells with pertussis toxin (200 ng ml^{-1}, 16 h). This treatment did not significantly affect the response of the cells to carbachol.

9 NPY and SRIF appeared to elevate $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores. Both NPY and SRIF continued to elevate $[Ca^{2+}]_i$ when applied in nominally Ca^{2+}-free external buffer. Thapsigargin (100 nM), an agent which discharges intracellular Ca^{2+} stores, also blocked the NPY and SRIF elevations of $[Ca^{2+}]_i$.

10 δ-Opioid receptor agonists applied in the presence of carbachol also elevate $[Ca^{2+}]_i$ in SH-SY5Y cells. When NPY (30 nM) or SRIF (100 nM) was applied together with a maximally effective concentration of the δ-opioid receptor agonist DPDPE ([D-Pen^{2,5}]-enkephalin) (1 μM), the resulting elevations of $[Ca^{2+}]_i$ were not greater than those caused by application of DPDPE alone.

11 Thus, in SH-SY5Y cells, NPY and SRIF can mobilize Ca^{2+} from intracellular stores via activation of NPY Y_2 and sst_2-like receptors, respectively. Neither NPY nor SRIF elevated $[Ca^{2+}]_i$ when applied alone. The requirements for the elevations of $[Ca^{2+}]_i$ by NPY and SRIF are the same as those for δ- and μ-opioid receptor and nociceptin receptor mobilization of $[Ca^{2+}]_i$ in SH-SY5Y cells.

Keywords: SH-SY5Y; somatostatin; neuropeptide Y; intracellular calcium; muscarinic receptors; calcium mobilization

Introduction

Neuropeptide Y (NPY) and somatostatin (SRIF) interact with receptors that are members of the seven transmembrane domain-, Gi/Go-coupled receptor family to affect the activity of a wide variety of cells. In neuronal cells, both NPY and SRIF receptors couple to a similar range of cellular effectors including inhibition of voltage-dependent Ca^{2+} currents (Lewis *et al*., 1986; Walker *et al*., 1988), inhibition of adenylyl cyclase activity (Heisler *et al*., 1982; Westlind-Danielsson *et al*., 1987), stimulation of Ca^{2+} entry (Miyoshi *et al*., 1989; Lynch *et al*., 1994) and mobilization of intracellular Ca^{2+} (Perney & Miller, 1989; Okajima & Kondo, 1992). SRIF receptor activation also modulates a variety of K^+ currents (Mihara *et al*., 1987; Wang *et al*., 1989). Where examined, virtually all the effects of NPY and SRIF in neuronal cells have been shown to be mediated by pertussis toxin-sensitive G-proteins.

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A variety of subtypes of NPY receptor have been proposed, with the classification primarily based on the rank order of potencies of agonist peptide analogues of NPY (reviewed in Wahlestedt & Reis, 1993). In general, the classification for NPY receptors have been based on the *in vitro* pharmacology of the native receptors, and the recent cloning of 3 different NPY receptors, Y_1 (Krause *et al*., 1992; Larhammer *et al*., 1992), Y_2 (Gerald *et al*., 1995; Rose *et al*., 1995) and Y_4 (Bard *et al*., 1995), that exhibit pharmacological profiles similar to previously characterized native receptors, confirms the utility of such an approach.

In contrast, data from radioligand binding studies suggested the existence of two major classes of SRIF receptor (e.g. SS_1/SS_2, $SRIF_1$/$SRIF_2$, SOM_A/SOM_B) before the cloning of 5 different SRIF receptor genes (sst_1-sst_5, reviewed in Hoyer *et al*., 1995a). Extensive pharmacological profiles of the recombinant receptors have been determined (e.g. Raynor *et al*., 1993a,b; Patel & Srikant, 1994; Hoyer *et al*., 1995b; Schoeffter *et al*., 1995; Castro *et al*., 1996) and these data, when combined with structural information about the receptors, have led to the proposal of 2 classes of SRIF receptor, $SRIF_1$ and $SRIF_2$ (Hoyer *et al*., 1995a). The $SRIF_1$ class comprises sst_2, sst_3 and sst_5 receptors, and appears to correspond to the SS_1 binding site (Hoyer *et al*., 1995a,b), while the $SRIF_2$ class comprises sst_1 and sst_4 receptor and corresponds to the SS_2 binding site (Hoyer *et al*., 1995a; Schoeffter *et al*., 1995). Recently, attempts have been made to correlate the pharmacological profiles determined for the recombinant receptors with those of native receptors in isolated preparations, for example to determine the SRIF receptor involved in SRIF inhibition of ion transport in rat colon (sst_2-like McKeen *et al*., 1995), SRIF inhibition of firing rates in rat locus coeruleus neurones (sst_2-like, Chessell *et al*., 1996) and SRIF contraction of human saphenous veins (also sst_2-like, Dimech *et al*., 1995).

SH-SY5Y cells are a human neuroblastoma cell line that has been used as a system for the investigation of the signal transduction mechanism of many human neurotransmitter receptors (reviewed in Vaughan *et al*., 1995). Both SRIF (Friederich *et al*., 1993) and NPY (McDonald *et al*., 1995) have been shown to inhibit the voltage-dependent Ca^{2+} currents in SH-SY5Y cells via pertussis toxin-sensitive mechanisms. It was proposed that NPY acted via NPY Y_2 receptors to inhibit the Ca^{2+} currents (McDonald *et al*., 1995). The type(s) of SRIF receptor present on SH-SY5Y cells is not known. It is also not known whether either NPY or SRIF receptors couple to cellular effectors other than Ca^{2+} currents in SH-SY5Y cells. Both δ and μ opioid receptors (Seward *et al*., 1990; 1991), as well as receptors for the neuropeptide nociceptin (Connor *et al*., 1996b), have been shown to inhibit voltage-dependent Ca^{2+} channels in SH-SY5Y cells. We have recently shown that δ and μ opioid receptors and receptors for nociceptin also couple to the mobilization of intracellular Ca^{2+} in SH-SY5Y cells, via a novel pathway that requires the simultaneous activation of Gq-coupled receptors such as muscarinic receptors (Connor & Henderson, 1996; Connor *et al*., 1996b). In this study we have sought to determine whether the native receptors for NPY and SRIF can couple to effectors in addition to voltage-dependent Ca^{2+} channels in SH-SY5Y cells. We find that both NPY and SRIF, when applied in the presence of the cholinoceptor agonist carbachol, can couple to the mobilization of intracellular calcium in SH-SY5Y cells; and further, that this Ca^{2+} mobilization is mediated via NPY Y_2-like receptors and sst_2-like receptors, respectively. A preliminary account of this work has been presented to the British Pharmacological Society (Connor *et al*., 1996a; Yeo & Henderson, 1996).

Methods

Cell culture

The studies presented here were carried out on SH-SY5Y cells obtained from the European Collection of Animal Cell Cultures. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine (4 mM), penicillin (100 i.u. ml^{-1}), streptomycin (100 μg ml^{-1}) and foetal bovine serum (12.5%) in a humidified incubator with 5% CO_2. Cells used for Ca^{2+} measurements were seeded onto plastic slides and cultured in Leighton tubes (Costar) until confluent. Cells were passaged every week; cells from passages 9–42 were used in these experiments.

Intracellular calcium measurements

Intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ was measured in confluent monolayers of SH-SY5Y cells with the fluorescent Ca^{2+}-sensitive dye fura 2. The cells were washed 3 times with buffer before loading and then incubated with the methoxy-ester of fura-2 (3 μM) for 1 h at 37°C. Unless stated otherwise experiments were carried out in buffer containing (mM): NaCl 140, KCl 2, $CaCl_2$ 2.5, $MgCl_2$ 1, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) 10, glucose 10, sucrose 40 and bovine serum albumin 0.05%, pH 7.3. 'Ca^{2+}-free' buffer was the same as the above except $MgCl_2$ was substituted for $CaCl_2$ and EGTA (ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid) (10 μM) was added to the buffer. After fura 2 loading the plastic slips were cut into quarters and one piece was placed on a specially constructed block fitted inside a quartz cuvette; the cuvette was then placed in an LS-5B Perkin-Elmer spectrofluorimeter and perfused with buffer (4 ml per min at 37°C). Drugs were added to the perfusion buffer in known concentrations. The spectrofluorimeter was controlled by a computer running a Perkin-Elmer software package. For more details of the recording set up see Pickles and Cuthbert (1991). Generally all four quarters of a slip were used for experiments, each quarter was considered to be the same population in statistical analysis. Data are presented as mean ± s.e.mean; statistical comparisons were made by use of unpaired Student's t test, a P value <0.05 was considered significant.

The fura 2 loaded cells were alternately exposed to light at 340 nm and 380 nm and the emission of the cells at 510 nM was recorded. The autofluorescence of unloaded cells was determined, subtracted from the recorded values and the corrected ratio of 340/380 emissions was converted to $[Ca^{2+}]_i$ by use of the equation given in Grynkiewicz *et al*. (1985). Maximum and minimum values of fura 2 fluorescence was determined by lysing the cells with digitonin in the presence of 20 mM Ca^{2+} or 10 mM EGTA, respectively.

Drugs and chemicals

The DMEM and foetal bovine serum were purchased from GIBCO, buffer salts from BDH. Atropine methylbromide, bovine serum albumin, carbamylcholine chloride (carbachol), DPDPE ([D-Pen^{2,5}]-enkephalin), fura 2-AM, [Leu^{31},Pro^{34}]-neuropeptide Y (human), pertussis toxin, somatostatin (1–14) and thapsigargin were obtained from Sigma U.K. Deltorphin II, neuropeptide Y (human) and peptide YY(3–36) (human) were from Peninsula Laboratories. Maitotoxin was from Calbiochem. BIM-23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2), BIM-23027 (c[*N*-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe]) and L-362855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]) were kind gifts of Dr Pat Humphrey (Glaxo Institute for Applied Biology, Cambridge) (Abu is aminobutyric acid; Aha is 7-aminoheptanoic acid; Nal is β-(2-napthyl)alanine).

Results

NPY and SRIF only elevate $[Ca^{2+}]_i$ in the presence of muscarinic agonists

Application of NPY (30–100 nM, $n=6$) or SRIF (100 nM, $n=6$) alone never altered the $[Ca^{2+}]_i$ of the SH-SY5Y cells. However, when either NPY (Figure 1) or SRIF (Figure 2) was

applied to the cells during continued exposure of the cells to carbachol (1 μM) there was a rapid elevation of $[Ca^{2+}]_i$ in addition to that caused by carbachol alone (Figures 1 and 2). In the continued presence of carbachol, repeated, short duration (30 s) applications of NPY spaced at least 15 min apart evoked reproducible increases in $[Ca^{2+}]_i$ for up to 90 min. In contrast, even a short application of SRIF (30 s) resulted in a profound desensitization to subsequent exposures to the drug (see below). We have not examined the desensitization of the responses to NPY or SRIF in this study. However, preliminary experiments have shown that the desensitization does not appear to be heterologous between NPY, SRIF and opioid receptors in SH-SY5Y cells (Connor *et al*., 1995). All populations of cells tested responded to SRIF, NPY and the δ-opioid receptor agonists, DPDPE and deltorphin II (data not shown), in a qualitatively similar manner. However, the mag-

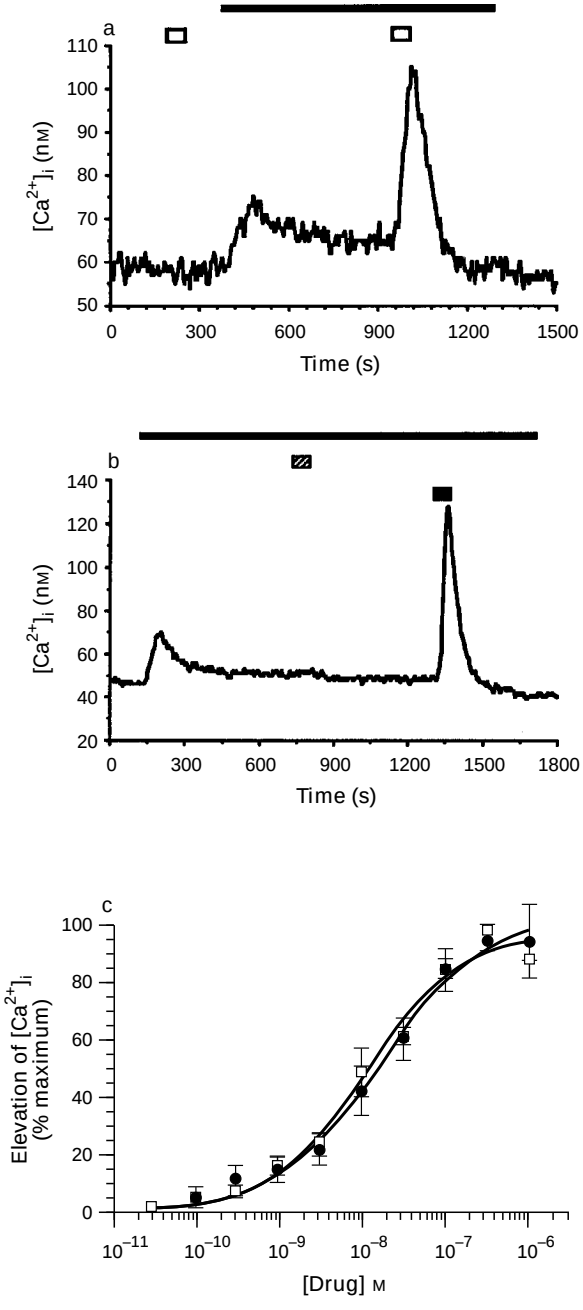

Figure 1 NPY and PYY(3–36) but not [Leu^{31},Pro^{34}]-NPY elevated $[Ca^{2+}]_i$ in the presence of carbachol. In (a) and (b) the traces represent continuous records of $[Ca^{2+}]_i$ in single populations of cells, determined as described in Methods. Drugs were perfused for the duration indicated by the bars. (a) NPY (100 nM, open box) alone did not elevate $[Ca^{2+}]_i$ but when it was applied in the presence of carbachol (1 μM, solid bar) there was a further elevation of $[Ca^{2+}]_i$ above that caused by carbachol. (b) PYY(3–36) (100 nM, solid box), but not [Leu^{31},Pro^{34}]-NPY (300 nM, hatched box), caused a robust increase in $[Ca^{2+}]_i$ when applied in the presence of carbachol (1 μM, solid bar). (c) Concentration-response relationships for NPY (●) and PYY(3–36) (□)-induced elevation of $[Ca^{2+}]_i$ in the presence of carbachol (1 μM). The curves represent pooled data obtained from at least 7 populations, the data were fitted to the Hill equation.

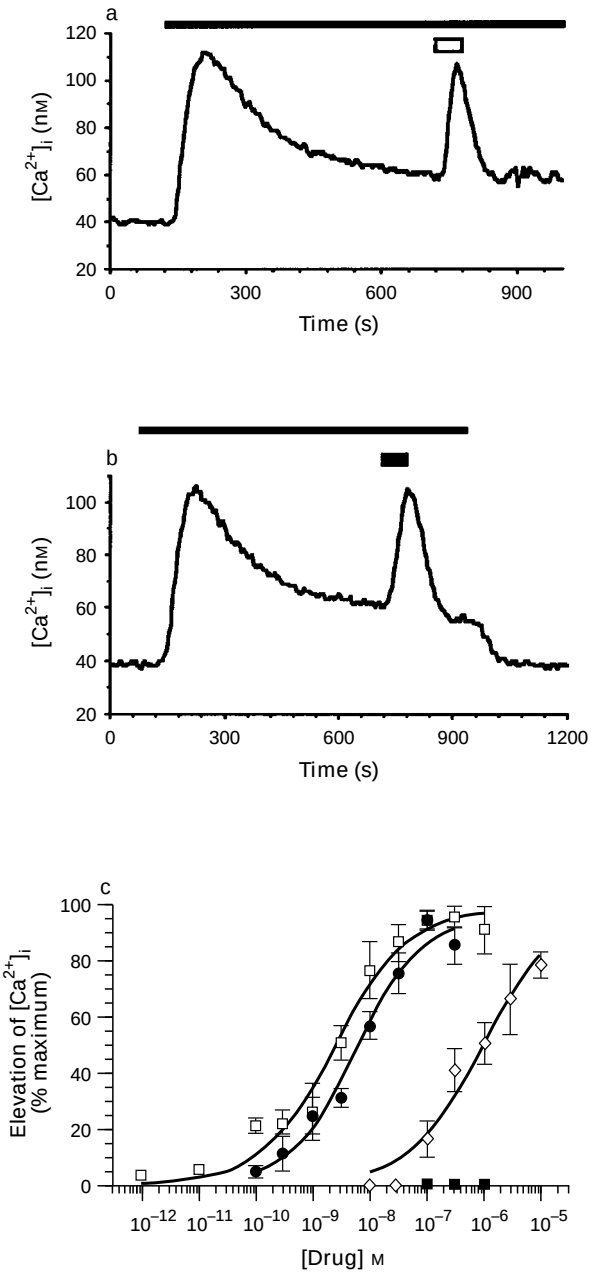

Figure 2 Somatostatin (SRIF) and analogues elevated $[Ca^{2+}]_i$ in the presence of carbachol. In (a) and (b) the traces represent continuous records of $[Ca^{2+}]_i$ in single populations of cells, determined as described in Methods. Drugs were perfused for the duration indicated by the bars. (a) SRIF (100 nM, open box) elevated $[Ca^{2+}]_i$ when it was applied in the presence of carbachol (1 μM, solid bar). (b) The sst_2 receptor-preferring analogue BIM-23027 (10 nM, solid box) also increased in $[Ca^{2+}]_i$ when applied in the presence of carbachol (1 μM, solid bar). (c) Concentration-response relationships for SRIF (●), BIM-23027 (□), L-362855 (◇) and BIM-23056 (■) elevations of $[Ca^{2+}]_i$ in the presence of carbachol (1 μM). The curves represent pooled data obtained from at least 2–16 determinations for each point, as outlined in the Methods. The data was fitted to the Hill equation.

nitude of the elevations of $[Ca^{2+}]_i$ to the agonists varied considerably between populations and between cells of the same passage examined on different days. Accordingly, control experiments were performed on cells of the same passage on the same day.

The NPY and SRIF induced elevations of $[Ca^{2+}]_i$ were concentration-dependent. Concentration-response curves for NPY, in the continued presence of carbachol (1 μM), were generated by applying various concentrations of NPY to individual monolayers for 30 s every 15 min (Figure 1). The order in which the different concentrations of NPY were applied was randomized and varied between each experiment. Data from individual experiments were normalized to the elevation of $[Ca^{2+}]_i$ caused by the maximally effective concentration of NPY in each experiment. The pooled concentration-response data gave a pEC_{50} for NPY elevating $[Ca^{2+}]_i$ of 7.80 ± 0.08 with a Hill slope of slope 0.7 ± 0.1 ($n=7$). When NPY was applied in the presence of a higher concentration of carbachol (100 μM) the pEC_{50} for NPY was 7.86 ± 0.09 and the Hill slope 0.8 ± 0.1 ($n=4$). The magnitude of the elevation of $[Ca^{2+}]_i$ by maximally effective concentrations of NPY was not significantly different at the two concentrations of carbachol, despite the much greater elevations of $[Ca^{2+}]_i$ by 100 μM carbachol. In the presence of 1 μM carbachol the maximum elevation of $[Ca^{2+}]_i$ by NPY was 52 ± 7 nM, the maximum elevation of $[Ca^{2+}]_i$ by NPY in the presence of 100 μM carbachol was 45 ± 4 nM ($P > 0.37$). Carbachol (1 μM) elevated $[Ca^{2+}]_i$ from 52 ± 8 nM to 78 ± 11 nM; when applied at a concentration of 100 μM carbachol elevated $[Ca^{2+}]_i$ from 63 ± 6 nM to 364 ± 36 nM.

In order to determine the type of NPY receptor responsible for the elevations of intracellular $[Ca^{2+}]_i$, we examined the effects of the NPY Y_1 receptor-preferring agonist [Leu^{31},Pro^{34}]-NPY and the NPY Y_2 receptor-preferring agonist PYY(3–36). When applied in the presence of carbachol (1 μM), concentrations of [Leu^{31},Pro^{34}]-NPY up to 300 nM caused barely detectable elevations of $[Ca^{2+}]_i$ (Figure 1). Higher concentrations of [Leu^{31},Pro^{34}]-NPY further elevated $[Ca^{2+}]_i$. However, it was impractical to construct a full concentration-response curve. In contrast, when PYY(3–36) was applied in the presence of carbachol (1 μM), it potently elevated $[Ca^{2+}]_i$, with a pEC_{50} of 7.94 ± 0.09 and a Hill slope 0.8 ± 0.1 ($n=7$) (Figure 1). PYY(3–36) (100 nM, $n=4$) did not elevate $[Ca^{2+}]_i$ when applied to SH-SY5Y cells in the absence of carbachol.

Because of the profound desensitization of the SRIF elevations of $[Ca^{2+}]_i$, it was not possible to construct concentration-response curves in the same manner as for NPY. Instead, each quarter of a cover slip was exposed to a single concentration of SRIF in the continued presence of carbachol (1 μM) for 60 s. Preliminary experiments determined that the maximum response to SRIF was obtained at a concentration of 100 nM (see Figure 2c) and so in all subsequent experiments 100 nM SRIF was applied to one area of the cover slip, with other concentrations applied to the 3 other areas. The elevations of $[Ca^{2+}]_i$ were then normalized to that caused by the 100 nM SRIF and the data pooled. The concentration-response curve for SRIF elevation of $[Ca^{2+}]_i$ gave a pEC_{50} of 8.20 ± 0.12 and Hill slope 0.7 ± 0.1, ($n=5-9$ for each concentration).

In order to determine the type of SRIF receptor responsible for elevating $[Ca^{2+}]_i$, we determined the effect of subtype selective SRIF analogues on $[Ca^{2+}]_i$ by comparing the elevations of $[Ca^{2+}]_i$ by these agents on three quarters of a cover slip with those caused by 100 nM SRIF on the other quarter of the slip. When applied in the presence of carbachol (1 μM) the sst_2 receptor-preferring analogue BIM-23027 potently elevated $[Ca^{2+}]_i$, with a pEC_{50} of 8.6 ± 0.1 and Hill slope of 0.7 ± 0.1 ($n=2-11$ for each concentration). The sst_5-receptor-preferring analogue L-362855 also elevated $[Ca^{2+}]_i$ when applied in the presence of carbachol. However, it was much less potent than SRIF or BIM-23027. A scarcity of material precluded the application of concentrations of L-362855 greater than 10 μM, and at this concentration the elevations of $[Ca^{2+}]_i$ were clearly not maximal when compared with those caused by 100 nM SRIF (Figure 2). If it is assumed that L-362855 is a full agonist then from the computer generated curve the estimated pEC_{50} was 6.1, and the Hill slope 0.7. The sst_3-receptor preferring agonist BIM-23056 was ineffective at elevating $[Ca^{2+}]_i$ at concentrations up to 1 μM ($n=6$). The rank order of potency of SRIF and the analogues in elevating $[Ca^{2+}]_i$ in SH-SY5Y cells was BIM-23027 $\geqslant$ SRIF $>>$ L-362855 $>>>$ BIM-23056. We tested whether BIM-23056 was an antagonist at the SRIF receptors in SH-SY5Y cells by applying a submaximally effective concentration of SRIF (10 nM) to cells in the absence and presence of BIM-23056 (1 μM). The elevations of $[Ca^{2+}]_i$ caused by SRIF in the presence of BIM-23056 were $108 \pm 20\%$ of control responses obtained from the same population of cells ($n=6$).

Carbachol will activate both muscarinic and nicotinic cholinoceptors on SH-SY5Y cells. Blockade of muscarinic receptors with atropine (1 μM) prevented the carbachol-induced elevations of $[Ca^{2+}]_i$ and completely prevented the NPY- and SRIF-evoked increases in $[Ca^{2+}]_i$ in the presence of carbachol (1 μM) ($n=5$, Figure 3b).

Pertussis toxin blocks the increase in $[Ca^{2+}]_i$ produced by NPY and SRIF

The increases in $[Ca^{2+}]_i$ caused by NPY (100 nM) or SRIF (100 nM) in the presence of carbachol (1 μM) were abolished by pretreatment of the cells with pertussis toxin (200 ng ml^{-1}) for 16 h ($n=4$) (Figure 3). The pertussis toxin treatment did not alter the peak elevation of $[Ca^{2+}]_i$ of the cells to carbachol

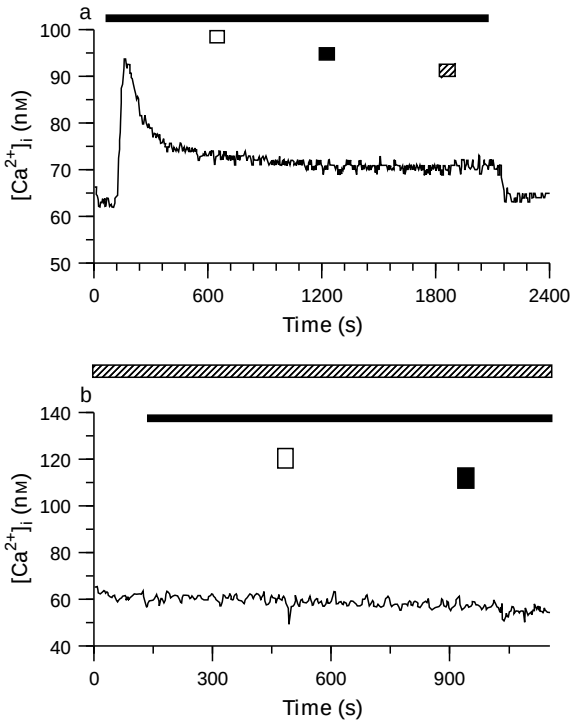

Figure 3 NPY and somatostatin (SRIF)-induced elevations in $[Ca^{2+}]_i$ were pertussis toxin-sensitive and required muscarinic receptor activation. The traces represent a continuous record of $[Ca^{2+}]_i$ in a single population of cells, determined as described in Methods. Drugs were perfused for the duration indicated by the bars. (a) In cells pretreated for 16 h with pertussis toxin (200 ng ml^{-1}), NPY (100 nM, open box), SRIF (100 nM, solid box) and the δ-opioid agonist deltorphin II (100 nM, hatched box) failed to elevate $[Ca^{2+}]_i$ in the presence of carbachol (1 μM, solid bar). (b) Carbachol (1 μM, solid bar) applied in the presence of atropine (1 μM, hatched bar) failed to elevate $[Ca^{2+}]_i$ and subsequent co-application of NPY (100 nM, open box) or SRIF (100 nM, solid box) also failed to elevate $[Ca^{2+}]_i$ further.

when compared with cells of the same passage number tested on the same day (data not shown).

NPY and SRIF mobilize intracellular Ca^{2+}

The increases in $[Ca^{2+}]_i$ caused by NPY and SRIF in the presence of carbachol reflected the mobilization of Ca^{2+} from internal stores, not Ca^{2+} entry across the plasma membrane. When the cells were bathed in nominally Ca^{2+}-free external media, carbachol still caused a rapid elevation of $[Ca^{2+}]_i$ but the plateau phase of the response was abolished (Figure 4). Application of NPY (100 nM) in the presence of carbachol but in the absence of external Ca^{2+}, caused an increase in $[Ca^{2+}]_i$ of 76 ± 19 nM, which was not different from the control increase in $[Ca^{2+}]_i$ (82 ± 7 nM, $n=4$) when NPY was applied in the presence of carbachol and 2.5 mM extracellular Ca^{2+}. Similarly, when SRIF (100 nM) was applied in the presence of carbachol but in the absence of extracellular Ca^{2+} it caused an increase of $[Ca^{2+}]_i$ of 32 ± 5 nM, which was not different from the control elevations of 32 ± 5 nM ($n=4$).

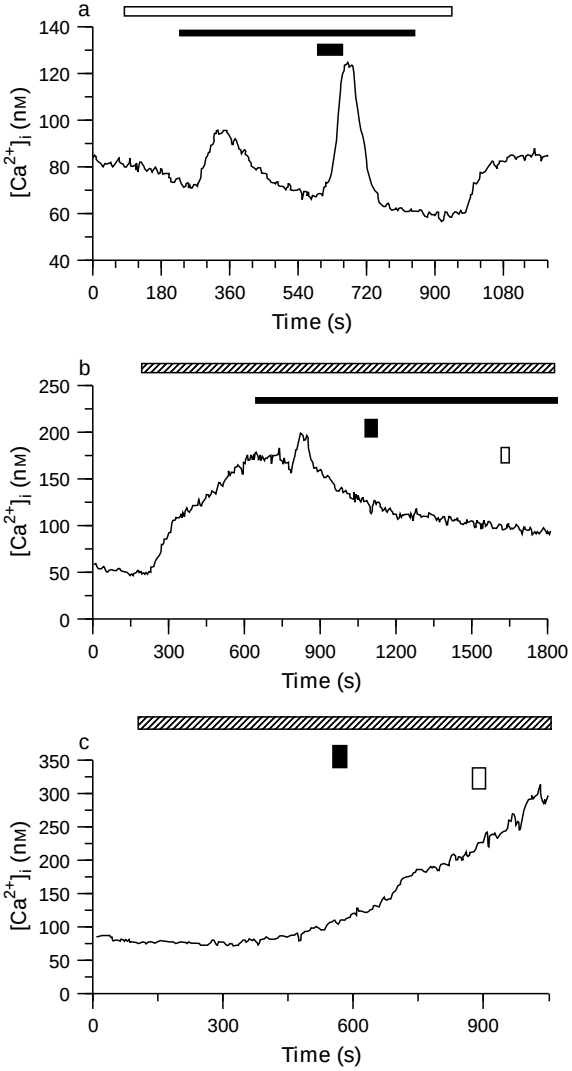

Figure 4 In the presence of carbachol, NPY and somatostatin (SRIF) mobilized Ca^{2+} from intracellular stores. The traces represent continuous records of $[Ca^{2+}]_i$ in single populations of cells, determined as described in Methods. Drugs were perfused for the duration indicated by the bars. (a) In cells exposed to Ca^{2+} free buffer (containing 100 μM EGTA, open bar) for 2 min before drug addition carbachol (1 μM, solid bar) elevated $[Ca^{2+}]_i$ briefly, as did subsequent co-application of NPY (solid box). Note the absence of any plateau of elevated $[Ca^{2+}]_i$ following the initial carbachol-induced spike. This experiment is typical of 4 carried out with NPY and SRIF. (b) Thapsigargin (100 nM, hatched bar) elevated $[Ca^{2+}]_i$ and blocked subsequent elevation of $[Ca^{2+}]_i$ by carbachol (1 μM, solid bar) or NPY (100 nM, solid box) and SRIF (100 nM, open box) in the presence of carbachol ($n=3$). (c) Maitotoxin (2 ng ml^{-1}, hatched bar) elevated $[Ca^{2+}]_i$, neither NPY (100 nM, solid box) nor SRIF (100 nM, open box) added during the period of elevated $[Ca^{2+}]_i$ raised $[Ca^{2+}]_i$ any further.

NPY and SRIF appeared to mobilize Ca^{2+} from IP_3-sensitive stores

Thapsigargin, an irreversible inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al*., 1990), occludes inositol (1,4,5)trisphosphate (IP_3)-induced Ca^{2+} release by promoting the emptying of intracellular Ca^{2+} stores. When cells were exposed to thapsigargin (100 nM) there was a gradual increase of $[Ca^{2+}]_i$ from 77 ± 16 nM to 341 ± 81 nM ($n=3$). When carbachol (1 μM) was added after 10 min of thapsigargin exposure there was a small, transient increase in $[Ca^{2+}]_i$ (see Figure 4b), but when NPY (100 nM) or SRIF (100 nM) was then added in the presence of carbachol there was no further increase in $[Ca^{2+}]_i$ ($n=3$ for each).

Elevation of $[Ca^{2+}]_i$ alone was not sufficient to promote NPY or SRIF mobilization of intracellular Ca^{2+}

In order to determine whether elevation of $[Ca^{2+}]_i$ *per se* was sufficient to permit the further elevation of $[Ca^{2+}]_i$ by NPY and SRIF we used a potent activator of plasma membrane Ca^{2+} channels, maitotoxin, to elevate $[Ca^{2+}]_i$. Maitotoxin has been shown to promote Ca^{2+} flow across the plasma membrane without directly affecting the intracellular Ca^{2+} stores (Soergel *et al*., 1992). Application of maitotoxin (2 ng ml^{-1}) to SH-SY5Y cells elevated $[Ca^{2+}]_i$

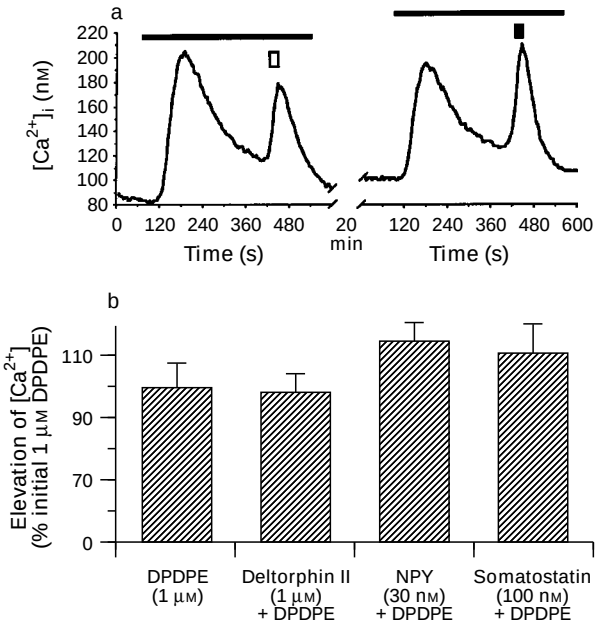

Figure 5 Elevations of $[Ca^{2+}]_i$ by NPY and somatostatin (SRIF) were not additive with those of a δ-opioid agonist. The traces represent continuous records of $[Ca^{2+}]_i$ in a single population of cells, determined as described in Methods. Drugs were perfused for the duration indicated by the bars. (a) In the presence of carbachol (1 μM, solid bar), application of a high concentration of SRIF (100 nM, solid box) together with a high concentration of the δ-opioid agonist DPDPE (1 μM) caused an elevation of $[Ca^{2+}]_i$ that was only slightly larger than that caused by DPDPE alone (1 μM, open box). (b) Co-application of NPY, SRIF or the δ-opioid receptor agonist deltorphin II with DPDPE (1 μM) did not cause a significantly greater increase in $[Ca^{2+}]_i$ than a previous application of DPDPE alone to the same cells.

from 71 nM to 291 nM ($n=2$). Application of NPY (100 nM) or SRIF (100 nM) at any time during the period of elevated $[Ca^{2+}]_i$ caused by maitotoxin did not result in any further elevation of $[Ca^{2+}]_i$ (Figure 4c). Maitotoxin did not deplete intracellular Ca^{2+} stores because carbachol (100 μM) still evoked a robust increase in $[Ca^{2+}]_i$ when applied in the presence of maitotoxin (data not shown).

NPY and SRIF mobilize Ca^{2+} by a similar mechanism to δ opioids

In order to determine whether NPY and SRIF receptor activation mobilized intracellular Ca^{2+} in a manner similar to δ-opioid receptor activation the elevations of $[Ca^{2+}]_i$ following co-application of NPY or SRIF and the δ-opioid receptor agonist DPDPE were examined (Figure 5). In the continued presence of carbachol (1 μM), application of a maximally effective concentration of DPDPE (1 μM) for 30 s caused an elevation of $[Ca^{2+}]_i$ of 38 ± 3 nM ($n=12$), a second application of DPDPE (1 μM) 30 min later caused an elevation of $[Ca^{2+}]_i$ that was $99\pm8\%$ of the first. When a high concentration of another δ-opioid receptor agonist, deltorphin II (1 μM), was co-applied with the second bolus of DPDPE there was no difference between the second elevation of $[Ca^{2+}]_i$ and that caused by DPDPE alone ($99\pm5\%$ of initial, $n=8$), indicating that 1 μM DPDPE was sufficient to saturate the mechanism for δ-opioid receptor mobilization of $[Ca^{2+}]_i$. When high concentrations of NPY (30 nM) or SRIF (100 nM) were applied with the second bolus of DPDPE the resulting elevations of $[Ca^{2+}]_i$ were, respectively, $110\pm10\%$ ($n=7$) and $114\pm4\%$ ($n=7$) of the first DPDPE elevation (Figure 5b). The absence of clear additivity suggests that activation of NPY-Y_2, sst_2-like and δ-opioid receptors resulted in mobilization of intracellular Ca^{2+} by similar mechanism(s).

Discussion

The principle findings of this study are that SRIF, via an sst_2-like receptor, and NPY, via an NPY Y_2-like receptor, can couple to the mobilization of intracellular Ca^{2+} in SH-SY5Y cells. Both SRIF (Freiderich *et al*., 1993) and NPY (McDonald *et al*., 1995) have been previously shown to inhibit a voltage-dependent, N-type, calcium current in SH-SY5Y cells through pertussis toxin (PTX) sensitive G-proteins. Similarly, the mobilization of $[Ca^{2+}]_i$ by sst_2-like and NPY Y_2-like receptors seen in this study was also dependent on the presence of functional pertussis toxin-sensitive G-proteins.

The identification of the NPY receptor subtype involved in the mobilization of intracellular Ca^{2+} was based on the rank order of potency of a series of NPY analogues. PYY(3–36), an N-terminally truncated PYY analogue that is at least 200 fold more selective for the rat (Dumont *et al*., 1994) and human (Gerald *et al*., 1995) Y_2 receptor than Y_1 receptor, was the most potent compound tested. In contrast, the C-terminally modified NPY analogue [Leu^{31},Pro^{34}]NPY, which has at least 1000 fold greater affinity for the cloned human Y_1 receptor than the cloned human Y_2 receptor (Gerald *et al*., 1995), was much less potent than PYY(3–36) or NPY, and the expense of the material precluded construction of a full concentration-response curve. This rank order of potency: PYY(3–36)$\geqslant$NPY$>>$[Leu^{31},Pro^{34}]NPY, is consistent with an NPY Y_2 receptor-mediated effect. If a Y_1 receptor were involved the rank order of potency of the compounds should have been reversed (Wahlestedt & Reis, 1993). If an NPY Y_3-like receptor were involved, PYY(3–36) would have been expected to be inactive (Wahlestedt & Reis, 1993); if the response had been mediated by an NPY Y_4-like receptor, [Leu^{31},Pro^{34}]NPY would have been expected to be approximately as potent as PYY(3–36) or NPY itself (Bard *et al*., 1995). Recently, an NPY Y_1-receptor antagonist has been developed (BIBP3226, Rudolf *et al*., 1994), unfortunately we were unable to obtain a sample of the compound for this study. The EC_{50} for NPY mobilization of intracellular Ca^{2+} in SH-SY5Y cells was about 15 nM, which is similar to the EC_{50} obtained for inhibition by NPY of voltage-dependent Ca^{2+} currents in these cells (EC_{50} approximately 50 nM; McDonald *et al*., 1995), a response also attributed to Y_2 receptor activation.

In the absence of potent SRIF receptor antagonists (see Hoyer *et al*., 1994), the identification of the SRIF receptor type involved in the mobilization of intracellular Ca^{2+} was also based on the rank order of potency of SRIF analogues. This rank order: BIM-23027$\geqslant$SRIF$>>$L-362855$>>>$BIM-23056, is consistent with activation of an sst_2-like receptor. In mouse fibroblasts expressing recombinant $hsst_2$, the rank order of potency of the above compounds for inhibition of [^{125}I]-[Tyr^{11}]-SRIF binding and stimulation of extracellular acidification was identical to that obtained here (Castro *et al*., 1996). In contrast, BIM-23027 was inactive in binding assays performed on fibroblasts expressing the $hsst_1$, while L-362855 and BIM-23056 displayed similar potencies to displace [^{125}I]-[Tyr^{11}]-SRIF binding (Castro *et al*., 1996). In a previous study of $hsst_1$ expressed in Chinese hamster ovary (CHO) cells, BIM-23027, BIM-23056 and L-362855 had IC_{50}s of greater than 1 μM against [^{125}I]-CGP 23996 binding (Raynor *et al*., 1993a). Similarly, in CHO cells expressing $hsst_4$ the compounds used in this study displace [^{125}I]-CGP 23996 with a rank order SRIF$>$L-362855$>$BIM-23056$>>$BIM-23027 (Raynor *et al*., 1993b), quite different from that obtained here for mobilization of Ca^{2+}. In the present study, BIM-23056 was inactive as either an agonist or antagonist at concentrations up to 1 μM, given that BIM-23056 has affinities of 11 nM and 6 nM, respectively, for $hsst_3$ and $hsst_5$ receptors expressed in CHO-K1 cells (Patel & Srikant, 1994), its lack of effect in SH-SY5Y cells suggests that neither sst_3 nor sst_5 receptors were involved in the mobilization of Ca^{2+}. There is at present no information regarding the SRIF binding sites present on SH-SY5Y cells, nor has the expression of SRIF receptors in these cells been examined by use of molecular biological techniques. It is possible that SH-SY5Y cells express more than one kind of SRIF receptor, and in the absence of potent and selective agonists and antagonists for sst_1 and sst_4 receptors it is impossible to rule out completely a contribution of these receptor types to the mobilization of $[Ca^{2+}]_i$ observed in this study. Nevertheless, the rank order of agonist potency seen in this study indicates a predominant involvement of sst_2-like receptors in the mobilization of Ca^{2+} in SH-SY5Y cells.

The mobilization of intracellular Ca^{2+} by NPY and SRIF applied in the presence of carbachol is indistinguishable from that mediated by δ and μ-opioid receptor agonists in SH-SY5Y cells (Connor & Henderson, 1996). We never observed an elevation of $[Ca^{2+}]_i$ in the presence of any of the agonists alone, and the elevations of $[Ca^{2+}]_i$ were blocked when carbachol and either SRIF or NPY were applied in the continued presence of atropine. As with the δ-opioid induced elevations of $[Ca^{2+}]_i$, the extent to which muscarinic receptors were activated did not seem to be critical for the agonist-induced Ca^{2+} mobilizations, because NPY elevated $[Ca^{2+}]_i$ by the same amount and with identical potency when applied in the presence of either 1 μM or 100 μM carbachol. Finally, when either NPY or SRIF was applied together with a maximally effective concentration of the δ-opioid agonist DPDPE, the elevations of $[Ca^{2+}]_i$ were not additive, which suggests that the 3 receptors were acting via a common signal transduction pathway to mobilize intracellular Ca^{2+}.

The precise mechanism by which NPY and SRIF mobilize Ca^{2+} in SH-SY5Y cells is not clear. Direct coupling of NPY or SRIF receptors to phospholipose C (PLC) is unlikely. There is no evidence that NPY or SRIF receptors can couple to G proteins of the Gq family, whose α subunits directly activate PLC (Exton, 1996). There are isoforms of PLC that can be directly activated by the $\beta\gamma$ subunits of Gi/Go proteins (Exton, 1996), and SH-SY5Y cells contain PLC-β3, one of the $\beta\gamma$ responsive isoforms (Yeo, Kelly and Henderson, unpublished observations), but neither NPY nor SRIF elevated $[Ca^{2+}]_i$ by themselves. Concomitant muscarinic receptor activation is

clearly necessary for the Gi/Go-coupled receptor mobilization of Ca^{2+}, as we have previously shown that simultaneous application of a muscarinic antagonist with a δ opioid agonist was sufficient to block the opioid-induced rise in $[Ca^{2+}]_i$, regardless of whether the carbachol concentration was 1 μM or 100 μM (Connor & Henderson, 1996). The precise nature of the link between muscarinic receptor occupancy and the NPY or SRIF receptor mobilization of $[Ca^{2+}]_i$ is not clear. However, it is possible that for some types of PLC, $\beta\gamma$ stimulation of the enzyme requires prior activation by αq; in a manner analogous with the $\beta\gamma$ stimulation of some forms of adenylyl cyclase, which requires prior priming with αs (Tang & Gilman, 1991).

A similar signal transduction pathway to that described here (i.e. Gi/Go coupling to elevation of intracellular Ca^{2+} in the presence of a permissive Gq-coupled receptor activation) has been observed in the neuroblastoma X glioma hybrid cell line NG108-15 (Okajima & Kondo, 1992; Okajima *et al*., 1993). In these cells δ-opioids, SRIF and noradrenaline mobilized intracellular Ca^{2+} only when applied in the presence of bradykinin or ATP as the 'permissive' Gq-coupled receptor agonist. The mobilization of Ca^{2+} by δ-opioids, SRIF and noradrenaline was mediated via pertussis toxin-sensitive G-proteins and appeared to be accompanied by an increase in the amount of IP_3 produced following bradykinin receptor activation (Okajima *et al*., 1993). The identification of this novel signal transduction pathway in two cell types suggests that it may be a mechanism common to many cell types for the coupling of Gi/Go-coupled receptors to intracellular Ca^{2+} stores.

NPY receptors have not previously been shown to be coupled to the mobilization of intracellular Ca^{2+} via a pathway that requires the concomitant activation of another receptor. NPY receptor activation has been shown to mobilize intracellular Ca^{2+} in cultured dorsal root ganglion cells (Perney & Miller, 1989), human erythroleukaemia cells (Motulsky & Michel, 1988), SK-N-MC neuroblastoma cells (Aakerlund *et al*., 1990) and cultured vascular smooth muscle cells (Mihara *et al*., 1989). These mobilizations of Ca^{2+} by NPY were also mediated by pertussis toxin-sensitive G-proteins. However, where examined, the receptors responsible were of the Y_1 type (Aakerlund *et al*., 1990; Shigeri *et al*., 1991; Feth *et al*., 1992). In the dorsal root ganglion, human erythroleukaemia and vascular smooth muscle cells an NPY-stimulated increase in IP_3 production has been shown to accompany the elevations of intracellular Ca^{2+}, suggesting that NPY receptors can couple directly to PLC (Daniels *et al*., 1989; Perney & Miller, 1989; Shigeri *et al*., 1995), which is clearly not the case in the present study. It is possible that in cell types other than SH-SY5Y, Y_1 receptors are activating isoforms of PLC that can be stimulated by the $\beta\gamma$ subunits of pertussis toxin-sensitive G-protein heterotrimers (Exton, 1996). Alternatively, it is possible that under the conditions in which some of the previous studies were performed (i.e. cells in suspension, Motulsky & Michel, 1988; Aakerlund *et al*., 1990), 'priming' agents such as ATP could have been released from damaged cells in the cuvette and interacted with the Y_1 agonists added subsequently. It was in these conditions that δ-opioid mobilization of Ca^{2+} in NG108-15 cells was first noted (Tomura *et al*., 1992). Nevertheless, this study, taken together with those described above, indicates that there are several possible pathways for NPY to mediate mobilization of intracellular Ca^{2+}.

In contrast to NPY receptor activation, native SRIF receptors have previously been shown to mobilize Ca^{2+} only in NG-108 cells (Okajima & Kondo, 1992), although there is some evidence that SRIF can stimulate phosphoinositide hydrolysis in various brain regions (Lachowicz *et al*., 1994), and all 5 subtypes of cloned human sstr activate PLC when heterologously expressed in COS-7 cells (Akbar *et al*., 1994). The receptor type(s) responsible for the mobilization of intracellular Ca^{2+} in neuronal cell lines have not been identified, and as outlined above, the precise mechanism by which SRIF mobilizes Ca^{2+} in neuronal cells is not known.

This study demonstrates that in SH-SY5Y cells NPY Y_2 receptors and native sst_2-like receptors can interact with muscarinic cholinergic systems to promote the mobilization of intracellular Ca^{2+}. This interaction is the same as between μ and δ opioid receptor agonists and carbachol in SH-SY5Y cells, and may represent a common signal transduction pathway for Gi/Go-coupled receptors. As previously shown for μ and δ opioid receptors and receptors for nociceptin (Seward *et al*., 1990; 1991; Connor & Henderson, 1996; Connor *et al*., 1996b); this study shows that NPY Y_2 receptors and SRIF receptors can couple to more than one effector in SH-SY5Y cells (Freiderich *et al*., 1993; McDonald *et al*., 1995). Careful investigation of the interactions between NPY and SRIF receptors and other plasma membrane receptors may lead to new insights into the cellular consequences of SRIF and NPY receptor activation, such interactions may be common in an *in vivo* situation, where cells are exposed to a many neurotransmitters and neuromodulator substances.

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