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A Pertussis Toxin-insensitive Calcium Influx Mediated by Neuropeptide Y_2 Receptors in a Human Neuroblastoma Cell Line*

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Stimulation of neuropeptide Y (NPY) Y_2 receptors induced an intracellular free Ca^{2+} ($[Ca^{2+}]_i$) increase in a human neuroblastoma cell line, CHP-234. When NPY in a Ca^{2+}-free solution was applied, this increase was abolished. Depolarization with high KCl evoked no response, suggesting that the responses were not mediated by voltage-gated Ca^{2+} channels. There was no evidence that the NPY response consisted of a capacitative Ca^{2+} entry sensitive to internal Ca^{2+} store levels. The $[Ca^{2+}]_i$ elevation was diminished by Ni^{2+}, a blocker of Ca^{2+} entry. Mn^{2+} induced a quench of the fura-2 fluorescence, which ceased promptly upon the removal of NPY, indicating that Ca^{2+} entry was linked tightly to receptor activation. Although thapsigargin- and ryanodine-sensitive Ca^{2+} stores were present, NPY-induced responses were not impaired by pretreatment with either drug. Furthermore, NPY had no effect on the thapsigargin-sensitive store. Pertussis toxin did not affect the NPY-stimulated $[Ca^{2+}]_i$ increase, although it abolished the NPY-dependent inhibition of cAMP production. It is concluded that the Y_2 receptors couple directly to receptor-operated Ca^{2+} channels without the involvement of intracellular Ca^{2+} stores. The results also indicate that Y_2 receptors can activate both pertussis toxin-sensitive and -insensitive mechanisms in the same cell.

Neuropeptide Y (NPY)[1] is a 36-amino acid polypeptide containing a carboxyl-terminal amide, which was first purified from the porcine brain (Tatemoto *et al.*, 1982). It is widely distributed throughout the nervous system and is one of the most abundant neuropeptides in the brain (Walker *et al.*, 1991). In the central nervous system, NPY stimulates food intake, reduces blood pressure, causes neuroendocrine alterations, and modifies circadian rhythm and memory processing (for reviews see Danger *et al.*, 1990; Heilig and Widerlov, 1990; Lehmann, 1990). In the peripheral nervous system, NPY is coreleased from sympathetic nerves with norepinephrine and plays a pivotal role in cardiovascular regulation (for reviews see Edvinnson *et al.*, 1987; Walker *et al.*, 1991).

Two major subtypes of NPY receptors (Y_1 and Y_2) have been identified on the basis of their differing sensitivities to carboxyl-terminal NPY fragments (Wahlestedt *et al.*, 1986) and the analog [Leu^{31},Pro^{34}]NPY (Fuhlendorff *et al.*, 1990). A third receptor subtype (Y_3) has also recently been proposed (Wahlestedt *et al.*, 1992). To date, only the Y_1 subtype has been cloned (Herzog *et al.*, 1992; Larhammar *et al.*, 1992), and the degree of structural or functional similarity among the receptor subtypes is unknown.

NPY receptors couple to a range of second messenger systems via both pertussis toxin-sensitive and -insensitive G proteins. In various cell types, its effects include inhibition of adenylate cyclase (Fredholm *et al.*, 1985), increase in inositol 1,4,5-trisphosphate ($InsP_3$) synthesis (Daniels *et al.*, 1989; Perney and Miller, 1989), increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Motulsky and Michel, 1988), inhibition of voltage-gated Ca^{2+} currents (Walker *et al.*, 1988; Bleakman *et al.*, 1991), and inhibition of nicotinic cholinergic currents (Norenberg *et al.*, 1991). The extent to which these effects are transduced through receptor subtype-specific mechanisms is unclear.

Numerous studies have concluded that NPY induces a mobilization of Ca^{2+} from intracellular stores (Motulsky and Michel, 1988; Daniels *et al.*, 1989, 1992; Mihara *et al.*, 1989; Perney and Miller, 1989; Aakerlund *et al.*, 1990; Feth *et al.*, 1991, 1992; Michel *et al.*, 1992). In studies using cell lines expressing the Y_1 receptor subtype, Aakerlund *et al.* (1990) and Daniels *et al.* (1992) found that a thapsigargin-sensitive Ca^{2+} store was involved, which is consistent with the previously observed stimulatory effect of NPY on $InsP_3$ production (Berridge, 1993). In a search for a possible subtype-dependent mechanism, we examined the coupling between Y_2 receptors and $[Ca^{2+}]_i$ increases. The human neuroblastoma cell line CHP-234 was chosen as a model system for the Y_2 receptor (Sheikh *et al.*, 1989). In these experiments, NPY-induced $[Ca^{2+}]_i$ increases in single cells were monitored with a digital imaging system using fura-2-based microfluorometry. Contrary to previous studies, we found that NPY induced a Ca^{2+} entry through receptor-operated channels, and we could find no evidence that it induced a Ca^{2+} release from intracellular stores. Furthermore, the effect was insensitive to pertussis toxin, whereas the coupling of NPY to adenylate cyclase in the same cells was inhibited by pertussis toxin. Thus, this study not only demonstrates a novel mechanism linking NPY receptor activation to $[Ca^{2+}]_i$ increases, but it also shows the receptor can couple to both pertussis toxin-sensitive and -insensitive mechanisms in the same cell.

EXPERIMENTAL PROCEDURES

Cell Culture—The CHP-234 cell line, derived from a human neuroblastoma, was obtained from Dr. J. L. Biedler (Sloan-Kettering Cancer Center, New York) and cultured in 260-ml flasks (Nunc, Roskilde, Den-

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[1] The abbreviations used are: NPY, neuropeptide Y; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular free Ca^{2+} concentration; $InsP_3$, inositol 1,4,5-trisphosphate.

mark) using a medium containing a mixture of Ham's F-12 and Eagle's minimal essential medium (1:1, v/v), supplemented with 15% (v/v) fetal calf serum and 1% (v/v) nonessential amino acids and a 0.2% antibiotic/antimyotic solution (1,000 IU/ml penicillin, 10 mg/ml streptomycin, and 25 μg/ml Fungizone). Tissue culture media were purchased from Eurobio (Paris, France) and fetal calf serum and other culture supplements from Life Technologies, Inc. (Cergy Pontoise, France). Confluent monolayer cultures were harvested 3–4 days after seeding by enzymatic dissociation with 0.15% trypsin in Ca^{2+}-free phosphate-buffered saline containing 0.4 mM EDTA. After centrifugation, cells were resuspended in the same culture medium and plated into 35-mm diameter culture dishes at the densities indicated below.

Measurement of cAMP Production—Cells were plated at a density of 1–2 × 10^5 cells/dish. After 3–4 days of culture, the cells were treated as described previously for the human SK-N-MC neuroblastoma cell line (Feth *et al.*, 1991) with some modifications. Briefly, cells were washed with 1 ml of phosphate-buffered saline containing (in mmol·liter^{-1}) 137 NaCl, 2.7 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 6.5 Na_2HPO_4, 1.5 KH_2PO_4, pH 7.2. They were then incubated at 37 °C for two periods of 10 min with 1 ml of assay buffer consisting of (in mmol·liter^{-1}) 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 10 HEPES, 10 mg·ml^{-1} bovine serum albumin, pH 7.4. After removal of the buffer, 950 μl of fresh assay buffer containing in addition 0.5 mmol·liter^{-1} of 3-isobutyl-1-methyl-anthine was added, and the cells were incubated for a 10-min period after which time 50 μl of the respective drugs were added. Preliminary experiments had shown that the forskolin-stimulated cAMP levels reached a maximum within 10 min. After a further 10-min incubation period, the reaction was stopped by removal of the buffer and the immediate addition of 1 ml of 0.1 M HCl followed by sonication. After centrifugation, the supernatant was collected for measurement of cAMP by means of radioimmunoassay (Koch and Lutz-Bucher, 1991). In experiments investigating the effects of pertussis toxin, cells were incubated with 200 or 25 ng·ml^{-1} of the toxin for 20 h. Cells from some dishes were counted after enzymatic dissociation to determine the cell number at the time of the experiment to normalize the cAMP level/10^6 cells.

Measurement of $[Ca^{2+}]_i$—For imaging studies, cells were plated at low density (1–2 × 10^4 cells/dish) into culture dishes in which a 2-cm diameter hole had been cut in the base and replaced by a thin (0.07-mm) glass coverslip. Fura-2/AM, taken from a 3 mM stock solution in dimethyl sulfoxide, was loaded into the cells at a final concentration of 2 μM in a Ca^{2+}-containing Ringer solution (see below for composition) for 30 min at 37 °C. Fluorescent Ca^{2+} measurements were made at 37 °C on single isolated cells or on small groups of dispersed cells. Digital imaging was performed using an IMSTAR (Paris, France) imaging system. Cells were viewed using a Nikon Diaphot-TMD microscope (Tokyo, Japan), with a Nikon UV-Fluor 40× (NA 1.3) oil immersion objective. Fura-2 fluorescence was excited alternately at 340 and 380 nm via the microscope epifluorescence port, with illumination provided by a 100-watt mercury lamp (Olympus, Tokyo, Japan). Cellular fluorescence was filtered by a 490–530-nm bandpass filter (Nikon) and measured with a Darkstar-800 CCD camera (Photonics Sciences, Millham, U. K.). Images were digitized onto a IBM486 33 MHz computer and analyzed using IMSTAR software. Ratiometric Ca^{2+} images were generated at 2-s intervals, using two or four averaged images at each wavelength. For each cell, $[Ca^{2+}]_i$ was averaged from pixels within manually outlined cell areas.

$[Ca^{2+}]_i$ Calibration—Background compensation was performed by subtracting the illumination from an area of the image which contained no cells. The $[Ca^{2+}]_i$ was then calculated from the 340/380 nm fluorescence ratio as described by Grynkiewicz *et al.* (1985), using *in situ*-determined values for the limiting ratios in both zero $[Ca^{2+}]_i$ (with 10 mM EGTA, 0 calcium) and in saturating $[Ca^{2+}]_i$ (10 mM calcium, 0 EGTA), both in the presence of a 10 μM concentration of the Ca^{2+} ionophore 4Br-A23187.

Solution Exchange—The bath, which had a volume of ~2 ml, was perfused at approximately 0.5 ml·min^{-1} with either a Ca^{2+}-containing Ringer solution consisting of (in mmol·liter^{-1}) 140 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4, or a Ca^{2+}-free solution containing (in mmol·liter^{-1}) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, 10 glucose, pH 7.4. During solution exchanges, the perfusion flow rate was increased to approximately 5 ml·min^{-1} so that the bath exchange was complete within 2 min. Drugs were applied by pressure ejection from fine tipped glass pipettes (2-μm internal diameter) placed 200–300 μm from the target cells. Ejection pressure was adjusted so that nonadherent debris in the target cell region moved only slightly.

Drugs—The following drugs were used: hNPY, pNPY (13–36), p[Leu^{31},Pro^{34}]NPY (Peninsula, Belmont, CA), 4Br-A32187 (Molecular Probes, Eugene, OR), fura-2/AM, 3-isobutyl-1-methyl-anthine, thapsigargin, and ryanodine (Sigma, L'Isle d'Abeau Chesnes, France).

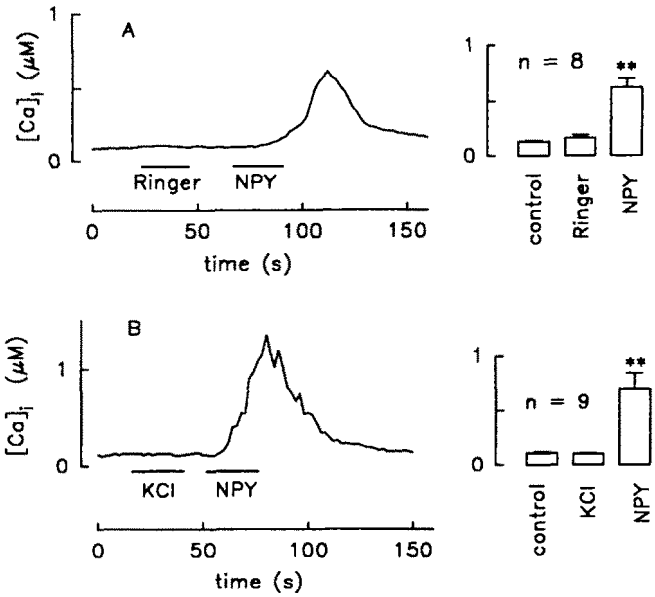

FIG. 1. **Some basic properties of NPY-induced $[Ca^{2+}]_i$ elevations.** Unless otherwise indicated, in this and subsequent figures, all *numbers on vertical axes* represent $[Ca^{2+}]_i$ in μM, and all *error bars* represent ± S.E. *Panel A, left*, a 20-s application of Ca^{2+}-containing Ringer solution from a pressurized micropipette had no effect, whereas a similar 20-s pulsed application of 10 nM NPY dissolved in the same solution elicited a strong $[Ca^{2+}]_i$ increase. *Right*, averaged responses from eight cells, stimulated as shown on the *left*. In this and all other figures, significant differences compared with control were calculated using the Mann-Whitney *U* test (**$p < 0.005$). *Panel B*, no evidence for voltage-gated Ca^{2+} channels. *Left*, a 20-s application of 145 mM KCl elicited no increase in $[Ca^{2+}]_i$, whereas a 20-s application of 10 nM NPY dissolved in a Ca^{2+}-containing solution elicited an increase. The composition of the 145 mM KCl solution was as for normal Ca^{2+}-containing Ringer but with all NaCl replaced by KCl. *Right*, averaged responses from nine cells (**$p < 0.005$).

Data Analysis—Averaged results are presented as means ± S.E. Where appropriate, statistical analysis of the data was performed by the Mann-Whitney *U* test if the Kruskali-Willis indicated a significant difference between multiple groups (Snedecor and Cochran, 1980). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni. Unless otherwise indicated, probability levels with respect to control of 0.05 or less were considered significant.

RESULTS

General Observations—When 10 nM NPY, dissolved in Ca^{2+}-containing vehicle solution, was applied under pressure from a puffer pipette, cells frequently responded with an increase in $[Ca^{2+}]_i$. Such increases were not artifacts of the application method, as control pulses of the same solution without NPY invariably failed to elicit an increase (Fig. 1*A*). Using a pulse application period of 20 s, the percentage of responsive cells in any given preparation was typically 20–50%. With a 20-s pulsed application, cells displayed one transient Ca^{2+} increase, which returned to near the resting level within 60 s of the commencement of the increase. As has been observed previously for NPY-induced Ca^{2+} responses (Daniels *et al.*, 1992; Gimpl *et al.*, 1993), cells did not respond to a second application of NPY (*e.g.* see Fig. 2, *B* and *C*, below), even following a recovery period of up to 30 min.

To test for the presence of voltage-gated Ca^{2+} channels, a 20-s application of a Ca^{2+}-containing 145 mM KCl solution was used to strongly depolarize the cells. As shown in Fig. 1*B*, the KCl application invariably failed to elicit a $[Ca^{2+}]_i$ increase. It was concluded that there are insufficient functional voltage-gated

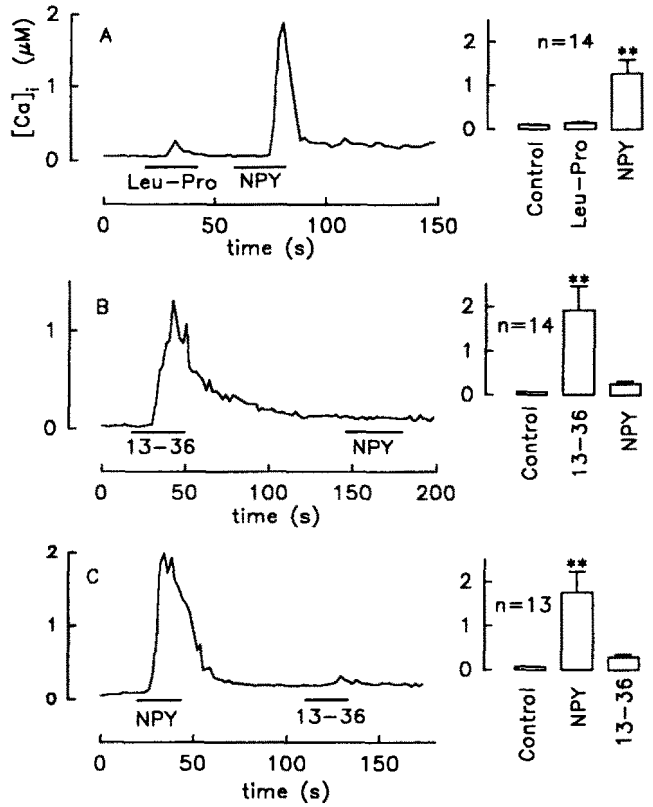

FIG. 2. **Determination of NPY receptor subtype.** The bath contained normal 2.5 mM Ca^{2+} Ringer. All NPY analogs were dissolved in this bath solution. *Panel A, left*, a 20-s pulsed application of 10 nM [Leu^{31},Pro^{34}]NPY did not induce strong $[Ca^{2+}]_i$ augmentation compared with a 20-s pulsed application of 10 nM NPY in the same cell. *Right*, average of 14 similar experiments (**$p < 0.005$). *Panel B, left*, a 20-s pulsed application of 10 nM NPY(13–36) caused a large $[Ca^{2+}]_i$ increase. Because of desensitization, a subsequent 20-s application of 10 nM NPY elicited no increase. *Right*, averages from 14 cells (**$p < 0.005$). *Panel C*, experiment similar to that in *panel B* but with agonists applied in reverse order (**$p < 0.005$).

Ca^{2+} channels to support a rise in $[Ca^{2+}]_i$ induced by strong depolarization and therefore that the Ca^{2+} signal evoked by NPY does not involve activation of voltage-gated Ca^{2+} channels.

CHP-234 cells have been reported to express the Y_2 receptor subtype, which is defined as having the agonist specificity sequence NPY ≥ NPY (13–36) >> [Leu^{31},Pro^{34}]NPY (Sheikh *et al.*, 1989). We sought to confirm this by comparing the magnitude of $[Ca^{2+}]_i$ increases induced by equal (10 nM) concentrations of NPY, NPY(13–36), and [Leu^{31},Pro^{34}]NPY. The complete agonist sequence could not be examined on each cell because of desensitization following an initial response. [Leu^{31},Pro^{34}]NPY, which never elicited a strong response, was much less potent than NPY when tested on the same cell (Fig. 2*A*). Of the 4/14 cells which displayed significant $[Ca^{2+}]_i$ increases with [Leu^{31},Pro^{34}]NPY (*e.g.* Fig. 2*A*), each responded much more strongly with NPY. On the other hand, NPY and NPY(13–16) induced cross-desensitization, which complicated a precise comparison, but indicated at least that receptor specificity for the two agonists was comparable (Fig. 2, *B* and *C*). Thus, the relative potencies of the agonists in evoking $[Ca^{2+}]_i$ increases was NPY ≈ NPY(13–36) >> [Leu^{31},Pro^{34}]NPY, which is characteristic of the Y_2 type receptor.

Ca^{2+} Entry—It has been reported in numerous other systems that NPY-induced $[Ca^{2+}]_i$ increases are the result of the mobilization of an intracellular pool, with little or no dependence on $[Ca^{2+}]_o$. In the present study, the dependence of the NPY-induced response on intracellular and extracellular Ca^{2+} was examined using several approaches. The results of manipulations described in this section are summarized in Table I. When the cells were perfused by a Ca^{2+}-containing bath solution, the pulsed application of Ca^{2+}-free NPY vehicle solution elicited a small $[Ca^{2+}]_i$ augmentation, although in the same cell a much larger increase was always (n = 19) observed following application of Ca^{2+}-containing NPY vehicle (Fig. 3*A*). The consistently observed small responses in the absence of vehicle Ca^{2+} could represent either the incomplete removal extracellular Ca^{2+} by the brief pulsed application, or an NPY-stimulated mobilization of an intracellular Ca^{2+} pool, or both.

In a first attempt to differentiate among these possibilities, the experiment was repeated after exposing the cells for at least 2 min to a Ca^{2+}-free bath, to ensure the absence of extracellular Ca^{2+}. As stated above, when cells were bathed in a Ca^{2+}-containing bath, the pulsed application of Ca^{2+}-free NPY vehicle resulted in a significant $[Ca^{2+}]_i$ increase (Table I, experiment 1). However, in a Ca^{2+}-free bath, the application of Ca^{2+}-free NPY vehicle resulted in no significant increase. Despite this difference, peak Ca^{2+} responses following the pulsed application of Ca^{2+}-containing NPY vehicle were not significantly different whether applied in the presence or absence of bath Ca^{2+} (Table I, experiment 1). This demonstrates that the simultaneous application of NPY and Ca^{2+} is required for the Ca^{2+} response and that in a Ca^{2+}-containing bath, extracellular Ca^{2+} may not be entirely removed by the brief pulsed application of Ca^{2+}-free NPY vehicle.

However, this result does not necessarily rule out the involvement of intracellular Ca^{2+} mobilization. It is possible that either NPY application or the prolonged (2–60 min) removal of extracellular Ca^{2+} may rapidly deplete an intracellular Ca^{2+} pool, leading in turn to a capacitative Ca^{2+} entry pathway (Putney, 1990). No significant $[Ca^{2+}]_i$ increase was observed following the pulsed application of Ca^{2+}-containing, NPY-free vehicle onto cells that had been bathed in Ca^{2+}-free solution for periods from 2 to 60 min, whereas the $[Ca^{2+}]_i$ increase in response to the pulsed application of Ca^{2+}-containing NPY vehicle in the same cells was unimpaired (Table I, experiment 2; Fig. 3*B*). Reapplication of Ca^{2+}-containing NPY-free vehicle after a long (2-min) NPY-induced $[Ca^{2+}]_i$ increase was without effect (Fig. 3*B*). Taken together, these results strongly suggest that the NPY-induced increases in $[Ca^{2+}]_i$ cannot be accounted for by capacitative Ca^{2+} entry linked to depletion of internal stores.

Since depolarization-induced Ca^{2+} entry did not occur in these cells (Fig. 1*B*), it was hypothesized that the Ca^{2+} influx was mediated by either a Ca^{2+} pump mechanism or receptor-operated Ca^{2+} channels. These possibilities can be resolved by examining the effects of Ni^{2+}, which blocks Ca^{2+} entry through receptor-operated channels (for review see Spedding and Paoletti, 1992), and Mn^{2+}, which may be permeant through these channels (Sage *et al.*, 1989; Llopis *et al.*, 1992), whereas neither has been shown to be effective on Ca^{2+} pumps or exchangers. In cells bathed in Ca^{2+}-containing solution, the pulsed application of Ca^{2+}-containing NPY vehicle with 1 mM Ni^{2+} caused a dramatic reduction in the peak $[Ca^{2+}]_i$, as compared with the effect of Ca^{2+}-containing NPY vehicle without Ni^{2+} on the same cell (Table I, experiment 3; Fig. 3*C*). The small but significant residual $[Ca^{2+}]_i$ elevation in the presence of 1 mM Ni^{2+}, which was also observed in Ca^{2+}-free bathing solution (Table I, experiment 4), probably represents incomplete block by Ni^{2+} of the Ca^{2+} influx. Ni^{2+} itself was not responsible for this augmentation, since it had no effect when applied in the absence of NPY (Table I, experiment 5; Fig. 3*D*).

Ion channel permeation by Mn^{2+} can be detected as a rapid diminishment (or quench) of fura-2 fluorescence. Using this

TABLE I

Dependence of NPY-induced $[Ca^{2+}]_i$ elevations on extracellular Ca^{2+} and Ni^{2+}

Summary of results of five different experiments examining the effects of bath Ca^{2+}, and vehicle Ca^{2+}, Ni^{2+}, and NPY on the magnitude of $[Ca^{2+}]_i$ elevations. When present, the bath and vehicle $[Ca^{2+}]_i$ was 2.5 mM, $[Ni^{2+}]_i$ was 1 mM, and [NPY] was 10 nM. Data are means ± S.E. with numbers as displayed for each experiment. Significant differences compared with the first figure in each experiment were calculated with the Mann-Whitney *U* test (* $p < 0.01$; ** $p < 0.005$).

Experiment	Ca^{2+} in bath	Ca^{2+} in vehicle	Ni^{2+} in vehicle	NPY in vehicle	$[Ca^{2+}]_i$
					nM
1 (Fig. 3*A*)	Yes	Yes	No	No	76.4 ± 3.2
$n = 19$	Yes	No	No	Yes	*121.7 ± 21.6
	Yes	Yes	No	Yes	**1,341 ± 255
2 (Fig. 3*B*)	No	No	No	No	74.5 ± 3.3
$n = 12$	No	Yes	No	No	81.1 ± 3.6
	No	Yes	No	Yes	**1,615 ± 297
3 (Fig. 3*C*)	Yes	Yes	No	No	96.4 ± 6.4
$n = 14$	Yes	Yes	Yes	Yes	**247.3 ± 22.7
	Yes	Yes	No	Yes	**3,064 ± 508
4 (not shown)	No	Yes	No	No	55.0 ± 5.7
$n = 7$	No	Yes	Yes	Yes	**158.6 ± 20.3
	No	Yes	No	Yes	**3,395 ± 1,054
5 (Fig. 3*D*)	Yes	Yes	No	No	53.4 ± 10.9
$n = 5$	Yes	Yes	Yes	No	56.4 ± 12.2
	Yes	Yes	Yes	Yes	*121.4 ± 22.2

method, we sought to determine whether NPY could induce a Mn^{2+}-permeant pathway. This was tested on cells bathed in a nominally Ca^{2+}-free bathing solution. The puffer pipettes contained Ca^{2+}-free vehicle solution to which had been added 2.5 mM Mn^{2+}. As shown in the example in Fig. 4, a 60-s control application of this solution elicited no effect. However, when the vehicle solution also contained 10 nM NPY, a diminishing of fluorescent intensity occurred at both excitation wavelengths of 340 and 380 nm. This quench was not accompanied by a Ca^{2+} transient, providing further evidence for the activation of divalent cation permeant pathway without measurable Ca^{2+} mobilization. Similar effects were observed in each of seven cells, where a 60-s application of NPY + Mn^{2+} vehicle reduced the average fluorescence to 68.1 ± 2.1% ($n = 7$) of the original. The quench (or downward slope) in the fluorescence intensity leveled out promptly after the termination of NPY application, even in the continued presence of Mn^{2+} (Fig. 4), suggesting that Mn^{2+} entry was tightly linked to NPY receptor activation and not to a more indirect mechanism, such as the filling state of a Ca^{2+} store.

Thus, the results so far strongly suggest that Ca^{2+} entry through a receptor-operated Ca^{2+} channel is required for the NPY-induced $[Ca^{2+}]_i$ elevation. However, it must also be considered whether Ca^{2+} entry may induce an additional Ca^{2+} mobilization or whether drugs known to interact with the filling state of stores may modulate the NPY response.

Effect of Thapsigargin—Since it has been reported that NPY responses were abolished following thapsigargin pretreatment (Aakerlund *et al.*, 1990; Daniels *et al.*, 1992), the possible involvement of a thapsigargin-sensitive intracellular Ca^{2+} store was investigated. In cells bathed in Ca^{2+}-containing solution, a 2-min pulsed application of 0.1 μM thapsigargin in a Ca^{2+}-containing vehicle solution caused a large increase in $[Ca^{2+}]_i$, after a variable delay (Fig. 5, *A* and *B*). However, following this response, the pulsed application of NPY plus 0.1 μM thapsigargin in the same vehicle solution still elicited a strong augmentation, the magnitude of which showed no obvious dependence on the time following the thapsigargin-induced elevation (Fig. 5, *A* and *B*). The magnitude of NPY responses was not significantly different ($p < 0.05$) from cells that were insensitive to thapsigargin (Fig. 5*C*). Since it has been shown in other systems (Blackmore, 1993; Lo and Thayer, 1993) that thapsigargin may induce a Ca^{2+} influx independently of intracellular stores, it was necessary to ascertain the existence of thapsigargin-sensitive stores. In some cells bathed in Ca^{2+}-free solution for 2–60 min, thapsigargin applied in a Ca^{2+}-free vehicle solution elicited persistent oscillating Ca^{2+} responses (Fig. 6*A*, *top three traces*), whereas in others, no responses were seen (Fig. 6*A*, *bottom two traces*). In the continued presence of thapsigargin, these oscillations frequently continued for at least 5 min (not shown) and were reproducible with repeated applications of thapsigargin (Fig. 6*B*, *center trace*). Thapsigargin applied in a Ca^{2+}-containing vehicle solution induced much larger $[Ca^{2+}]_i$ elevations, with much slower and less robust oscillations (Fig. 6*A*), even in cells that were insensitive to thapsigargin without Ca^{2+} (Fig. 6*A*, *bottom two traces*). Thus, thapsigargin is clearly able to mobilize Ca^{2+} in at least a subset of these cells. In addition, the dependence of the response on extracellular Ca^{2+} suggests that thapsigargin induces a Ca^{2+} entry either directly or as a result of store depletion. Regardless of this exact mechanism, these observations indicate that the presence of thapsigargin-sensitive stores is most clearly discerned in the absence of extracellular Ca^{2+}.

To determine whether NPY can mobilize Ca^{2+} from the thapsigargin-sensitive store, cells were stimulated repeatedly with 60-s pulsed applications of 0.1 μM thapsigargin in a Ca^{2+}-free vehicle solution. Between these applications, NPY dissolved in the same vehicle solution was applied to determine whether it had any effect (*e.g.* stimulation or inhibition) on the thapsigargin-stimulated response. As shown in Fig. 6*B*, in the absence of extracellular Ca^{2+}, NPY was unable to either mimic the store mobilizing effects of thapsigargin or otherwise affect the thapsigargin response in same cell where it caused a large augmentation in the presence of external Ca^{2+}. Similar responses were observed in three cells. By comparison, thapsigargin responses were not different from those recorded in NPY-insensitive cells (Fig. 6*B*, *middle trace*), ruling out the possibility that NPY itself was responsible for the regeneration of the thapsigargin responses. In addition, some NPY-sensitive cells were insensitive to thapsigargin (Fig. 6*B*, *bottom trace*). The three cells illustrated in Fig. 6*B* were recorded concurrently. Thus, NPY responses are unlikely to involve thapsigargin-sensitive stores.

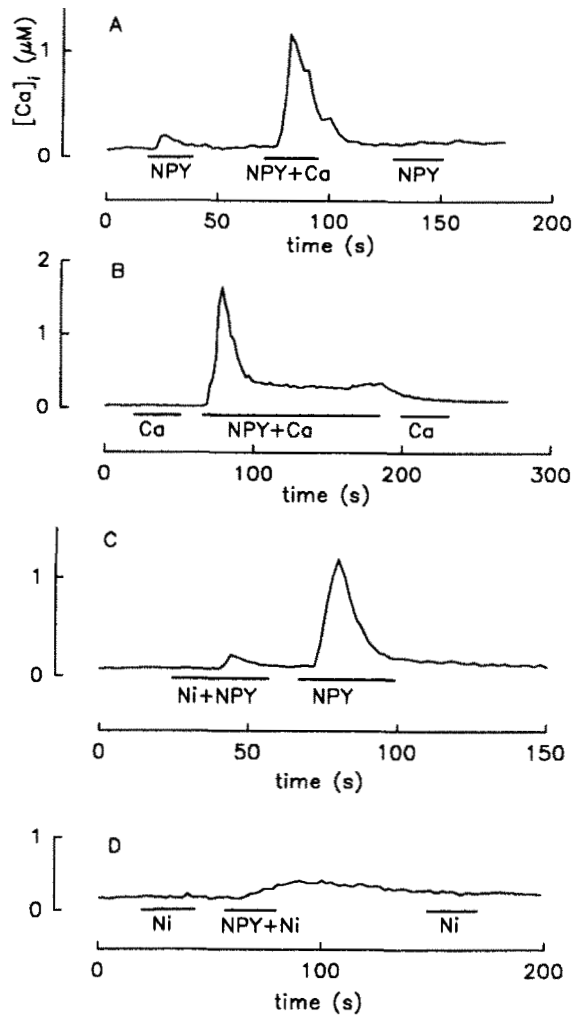

FIG. 3. **Dependence of NPY-induced $[Ca^{2+}]_i$ increase on $[Ca^{2+}]_o$ and $[Ni^{2+}]_o$.** All *vertical scales* represent $[Ca^{2+}]_i$ in μM. *Panel A*, the bath contained normal 2.5 mM Ca^{2+} Ringer. A 20-s pulsed application of 10 nM NPY in Ca^{2+}-containing vehicle produced a strong $[Ca^{2+}]_i$ elevation (*second bar*), whereas when 10 nM NPY was applied in Ca^{2+}-free vehicle (*first* and *third bars*), it produced little or no $[Ca^{2+}]_i$ increase. *Panel B*, the bath contained Ca^{2+}-free Ringer for at least 2 min before commencement of the experiment. A 20-s application of normal Ca^{2+}-containing vehicle solution gave no response (*first* and *third bars*). A 120-s application of 10 nM NPY in Ca^{2+}-containing vehicle gave a strong $[Ca^{2+}]_i$ increase. *Panel C*, the bath contained normal 2.5 mM Ca^{2+} Ringer. A 20-s pulsed application of 10 nM NPY + 1 mM Ni^{2+} in Ca^{2+}-containing vehicle produced a weak $[Ca^{2+}]_i$ elevation (*first bar*), whereas 10 nM NPY applied in the control Ca^{2+}-containing vehicle (*second bar*) caused a large elevation. *Panel D*, the bath contained normal 2.5 mM Ca^{2+} Ringer. A 20-s pulsed application of 1 mM Ni^{2+} in Ca^{2+}-containing vehicle produced no significant response (*first bar*), whereas 10 nM NPY + 1 mM Ni^{2+} in the control Ca^{2+}-containing vehicle (*second bar*) produced a small but significant response.

Effect of Ryanodine—It is possible that the NPY-induced Ca^{2+} influx may enhance $[Ca^{2+}]_i$ by inducing a further Ca^{2+}-activated Ca^{2+} release from a ryanodine-sensitive store (Berridge, 1993). In the presence of extracellular Ca^{2+}, ryanodine at a concentration of 1 μM was able to stimulate large increases in $[Ca^{2+}]_i$. However, responses to NPY following large ryanodine-induced $[Ca^{2+}]_i$ elevations were unimpaired (Fig. 7). Assuming that the large ryanodine response depletes the store, it seems reasonable to conclude that the NPY-induced influx is not activating a significant Ca^{2+} release from this source.

Effect of Pertussis Toxin—Many studies have found that NPY couples to $[Ca^{2+}]_i$ increases through a pertussin toxin-sensitive pathway (*e.g.* Motulsky and Michel, 1988). In this study, NPY-

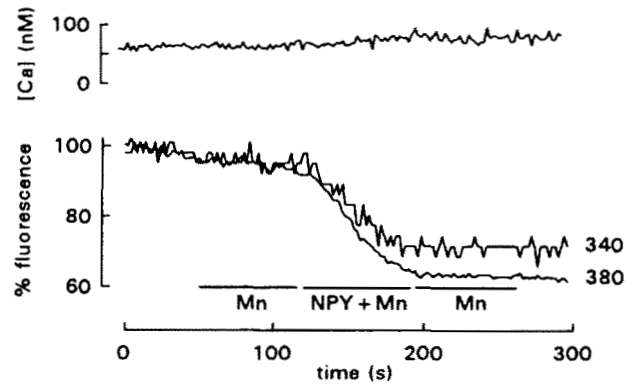

FIG. 4. **Fura-2 fluorescence quench induced by NPY + 2.5 mM Mn^{2+}.** The experiment was performed in nominally Ca^{2+}-free Ringer (*i.e.* no added EGTA) to avoid possible complications caused by Mn^{2+} complexing with EGTA. All traces are from the same cell. The *upper trace* shows $[Ca^{2+}]_i$, which was continuously monitored and did not change dramatically throughout the experiment. The *lower traces* show the relative intensity of the fluorescence stimulated at excitation wavelengths of 340 and 380 nm, expressed as a percentage of the initial intensity. A 60-s pulsed application of 2.5 mM Mn^{2+}, dissolved in nominally Ca^{2+}-free vehicle (*first* and *third bars*), produced no change in fluorescence intensity. A 60-s pulsed application of 10 nM NPY + 2.5 mM Mn^{2+} (*second bar*) induced a quench at both excitation wavelengths. The signal to noise ratio of the *340 nm trace* is lower because at resting $[Ca^{2+}]_i$ (50–100 nM), the fluorescence intensity was about 20% that of 380 nm.

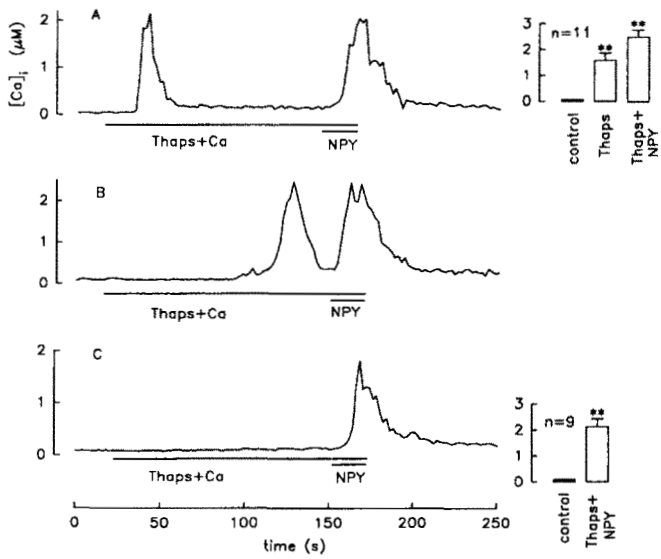

FIG. 5. **NPY responses in the presence of thapsigargin.** Experiments were performed in normal Ca^{2+}-containing Ringer. Thapsigargin (*Thaps*) was applied at a concentration 0.1 μM for 120 s. *Panel A*, an example of a thapsigargin-induced response followed by a response to 10 nM NPY in the maintained presence of thapsigargin. *Right*, average of 11 cells which responded to both thapsigargin and NPY (**$p < 0.005$). *Panel B*, the same experiment as in *panel A* but showing a cell with a longer delay to the initiation of the thapsigargin response. *Panel C*, the same experiment in a cell that was insensitive to 0.1 μM thapsigargin but responsive to 10 nM NPY. *Right*, average of nine cells that responded only to NPY (**$p < 0.005$).

stimulated $[Ca^{2+}]_i$ increases were measured following pertussis toxin preincubation as described under "Experimental Procedures." Positive NPY responses were defined as those in which the maximum stimulated $[Ca^{2+}]_i$ increased to at least twice the resting level. The standard NPY application protocol used in these experiments, together with a typical positive response, is shown in Fig. 8. The percentage of responsive cells was averaged from cells in five pertussis toxin-treated culture dishes

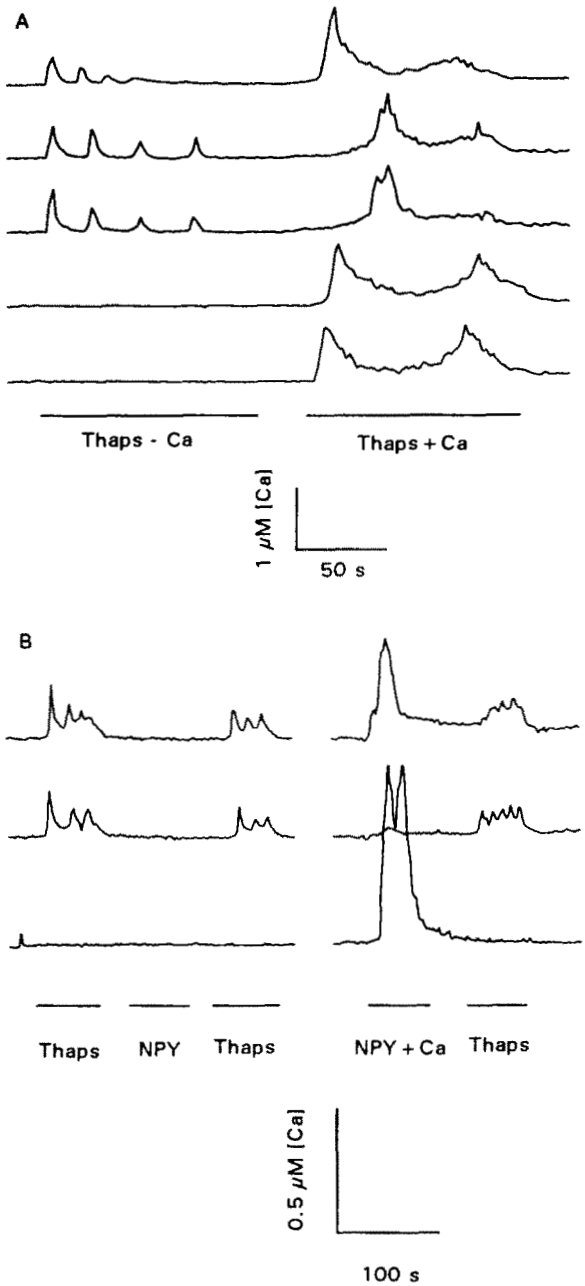

FIG. 6. **Lack of effect of 10 nM NPY on the thapsigargin-sensitive store.** All experiments were performed in Ca^{2+}-free bathing solution. *Calibration bars* in *panels A* and *B* apply to all respective *traces*. Resting $[Ca^{2+}]_i$ in all displayed cells was <100 nM. *A*, The experiment shows 120-s pulsed applications of 0.1 μM thapsigargin (*Thaps*) in Ca^{2+}-free vehicle solution (*first bar*) and normal Ca^{2+}-containing vehicle solution (*second bar*). Five representative traces, all from the same coverslip, are shown. Many cells responded to thapsigargin only in the presence of extracellular Ca^{2+} (*bottom two traces*). *Panel B*, the displayed *traces* are from three cells recorded simultaneously from the same field of view. The discontinuity represents a break in the recording of ~70 s. The *first*, *third*, and *fifth bars* represent repeated 60-s pulsed applications of 0.1 μM thapsigargin in Ca^{2+}-free vehicle solution. The *second bar* represents a 60-s pulsed application of 10 nM NPY in Ca^{2+}-free vehicle solution, and the *fourth bar* represents a 60-s pulsed application of 10 nM NPY in Ca^{2+}-containing vehicle.

and three control dishes. The number of cells tested in each dish ranged between 61 and 68. The mean percentage of responsive cells under control conditions was 42.5 ± 5.0% (n = 3), and the mean percentage of responsive cells following pertussis toxin treatment was 38.3 ± 10.9% (n = 5). These values are not significantly different, indicating that the pertussis toxin treatment was not effective in inhibiting the coupling of NPY to the Ca^{2+} influx.

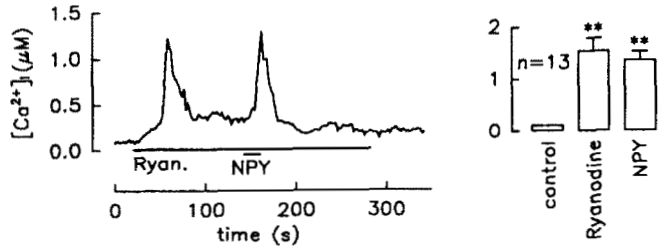

FIG. 7. **Effect of NPY (10 nM) and ryanodine (1 μM) in the presence of Ca^{2+} in both bath and vehicle solutions.** Application of ryanodine (for ~4 min) and a concomitant 20-s application of NPY + ryanodine. A representative example is shown on the *left*, and results averaged from 13 cells are shown on the *right* (**$p < 0.005$).

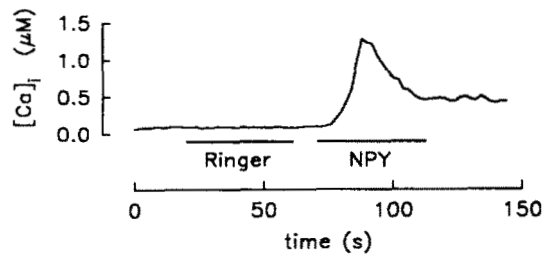

FIG. 8. **Typical example of an NPY-induced Ca^{2+} response in a pertussis toxin-treated cell, incubated with 200 ng·ml^{-1} as described under "Experimental Procedures."** A 40-s control application of Ca^{2+}-containing vehicle (*first bar*) was applied first to rule out the possibility of cell damage caused by pertussis toxin incubation. A 40-s pulse of 10 nM NPY in Ca^{2+}-containing vehicle was then applied.

However, it was necessary to demonstrate the effectiveness of pertussis toxin in this system. One widely-reported property of NPY is that it inhibits the cAMP production stimulated by other agonists (Fredholm *et al.*, 1985). As in other systems, NPY was found to potently inhibit the forskolin-induced cAMP production in CHP-234 cells in a dose-dependent manner (Table II). This effect was completely abolished by pertussis toxin (Table III). A surprising result was that pertussis toxin pretreatment, both at 200 and 25 ng·ml^{-1} also inhibited the forskolin-stimulated cAMP production. These experiments were performed on cells from the same culture, stimulated with the same pertussis toxin incubation protocol as those used in the Ca^{2+} measurements. Accordingly, these results provide a measure of the effectiveness of pertussis toxin in our system. Hence, NPY can couple to both pertussis toxin-sensitive and -insensitive mechanisms in the same cell. The results also indicate that the inhibition of cAMP accumulation is not a prerequisite for the $[Ca^{2+}]_i$ elevation.

DISCUSSION

Receptor-operated Ca^{2+} Entry—These experiments described provide strong evidence that NPY couples directly to a receptor-operated Ca^{2+} entry mechanism without the involvement of intracellular Ca^{2+} stores. The main lines of evidence are summarized as follows. First, the NPY-stimulated $[Ca^{2+}]_i$ increase was critically dependent on the presence of extracellular Ca^{2+} (Fig. 3*A*). The possibility that either the removal of extracellular Ca^{2+} or NPY stimulation may have rapidly depleted an intracellular Ca^{2+} store, inducing a capacitative Ca^{2+} entry pathway (Putney, 1990), can be eliminated because after extracellular Ca^{2+} removal, the application of a Ca^{2+}-containing solution elicited no effect either before or after NPY stimulation (Fig. 3*B*). In addition, the NPY-induced $[Ca^{2+}]_i$ elevation was dramatically inhibited by the addition of 1 mM Ni^{2+}, a blocker

TABLE II
NPY-dependent inhibition of forskolin-stimulated cAMP accumulation

The forskolin (FSK) concentration was 3 μM. Data are means ± S.E., with values from four separate experiments with three determinations/experiment. Basal unstimulated cAMP levels were 16.8 ± 2.6 pmol/10^6 cells/10 min. Significant differences compared with forskolin alone were calculated using the Mann-Whitney *U* test with correction according to Bonferroni (* $p < 0.01$; ** $p < 0.005$).

Treatment	cAMP	%
	pmol/10^6 cells/10 min	
FSK	265.7 ± 24.2	100
FSK + NPY 10^{-11} M	238.2 ± 16.5	89
FSK + NPY 10^{-10} M	195.7 ± 12.2	74
FSK + NPY 10^{-9} M	*147.9 ± 11.7	56
FSK + NPY 10^{-8} M	**138.6 ± 10.6	52
FSK + NPY 10^{-7} M	**139.2 ± 14.5	52

TABLE III
Pertussis toxin (PTX) pretreatment abolishes NPY-induced inhibition of forskolin (FSK)-stimulated cAMP accumulation

[FSK] was 3 μM, and [NPY] was 0.1 μM. Data are means ± S.E. of two separate experiments for each condition with three determinations/experiment. Significant differences compared with FSK alone were calcualted using the Mann-Whitney *U* test (* $p < 0.01$; ** $p < 0.005$).

Treatment	cAMP	%
	pmol/10^6 cells/10 min	
FSK	236.0 ± 10.5	100
FSK + NPY	**120.7 ± 4.7	51
PTX (200 ng/ml) + FSK	90.2 ± 10.3	100
PTX (200 ng/ml) + FSK + NPY	76.9 ± 4.4	85
FSK	360.5 ± 26.0	100
FSK + NPY	*202.6 ± 15.0	56
PTX (25 ng/ml) + FSK	145.0 ± 10.0	100
PTX (25 ng/ml) + FSK + NPY	147.9 ± 10.9	102

of Ca^{2+} influx (Spedding and Paoletti, 1992). Further evidence for an NPY-dependent divalent cation influx was inferred from the fluorescence quench observed in the simultaneous presence of NPY and Mn^{2+} (Fig. 4). This effect was characterized by (i) no accompanying Ca^{2+} mobilization and (ii) cessation of quench upon the removal of NPY but in the maintained presence of Mn^{2+} (Fig. 4). Both of these properties support the hypothesis that the Ca^{2+} entry pathway is linked tightly to receptor activation.

The possible involvement of intracellular stores was tested using the Ca^{2+}-mobilizing agents thapsigargin and ryanodine, which are considered to act on different Ca^{2+} pools (Berridge, 1993). Contrary to previous reports (Aakerlund *et al.*, 1990; Daniels *et al.*, 1992), a 2-min pretreatment with 0.1 μM thapsigargin or 1 μM ryanodine had no effect on the magnitude of NPY-stimulated $[Ca^{2+}]_i$ increases (Figs. 5 and 7). In addition, NPY did not exert any effect on the thapsigargin-sensitive store at the same concentration at which it was able to induce a Ca^{2+} influx (Fig. 6*B*). There was also no evidence for voltage-gated Ca^{2+} entry (Fig. 1*B*). A receptor-operated Ca^{2+} channel, tightly linked to receptor activation, is the only mechanism that is compatible with all of these observations.

Receptor-operated is a generic term encompassing directly agonist-gated, directly G protein-coupled, second messenger-gated, as well as store depletion-activated channels (for review see Putney, 1990; Taylor, 1990; Meldolesi *et al.*, 1991; Neher, 1992). For the channels described here, only the depletion-activated mechanism can be ruled out. However, some consideration of the other possibilities is warranted. A directly NPY-gated ion channel is unlikely because the vast majority of the effects of NPY have been reported to be pertussis toxin-sensitive, and the only NPY receptor sequenced to date belongs to the G protein-coupled receptor superfamily (Herzog *et al.*, 1992; Larhammar *et al.*, 1992). There is as yet no other evidence that NPY acts on any other type of receptor. Thus, a G protein probably also mediates the responses described here. Since NPY receptor activation has been shown to increase $InsP_3$ production (Perney and Miller, 1989; Daniels *et al.*, 1989), products of phosphoinositide turnover are candidates as second messengers. However, it should be noted that $InsP_3$, if produced at a sufficiently high concentration, should also induce Ca^{2+} release from thapsigargin-sensitive stores (Berridge, 1993). On the other hand, it is unlikely that a cAMP increase is involved because cAMP production was actually inhibited by NPY. NPY-induced decreases in the cAMP concentration can also be ruled out as NPY-induced $[Ca^{2+}]_i$ elevations were not impaired when NPY was uncoupled from adenylate cyclase inhibition by pertussis toxin. Thus, it seems likely that NPY couples to channel activation either directly via a pertussis toxin-insensitive G protein, or via a phospholipid-derived second messenger.

These experiments demonstrate that Ca^{2+} influx is required for the initiation of the NPY-induced $[Ca^{2+}]_i$ elevation, but they do not exclude the possibility that this influx may then trigger a Ca^{2+} release from intracellular stores. One possible mechanism is a Ca^{2+}-induced Ca^{2+} release from the ryanodine-sensitive store (Berridge, 1993), although this is unlikely since large NPY responses were recorded directly following ryanodine-stimulated $[Ca^{2+}]_i$ elevations (Fig. 8). Another possibility is that Ca^{2+} influx may have activated Ca^{2+} release from an $InsP_3$-sensitive intracellular store, for example by sensitizing the $InsP_3$ receptor to activation by either ambient or NPY-stimulated $InsP_3$ levels (Missiaen *et al.*, 1992). However, further experiments are required to confirm whether a Ca^{2+}-induced Ca^{2+} release mechanism contributes to NPY-stimulated responses.

Comparison with Other Studies—Following the initial observations by Motulsky and Michel (1988), NPY-stimulated $[Ca^{2+}]_i$ increases have been measured in a variety of cell types. Most studies have found that NPY induces a pertussis toxin-sensitive Ca^{2+} mobilization, which is largely or completely independent of extracellular Ca^{2+} (Motulsky and Michel, 1988; Daniels *et al.*, 1989, 1992; Mihara *et al.*, 1989; Perney and Miller, 1989; Aakerlund *et al.*, 1990; Feth *et al.*, 1991, 1992; Michel *et al.*, 1992). These conclusions were generally based on the observation that responses persisted without noticeable decrement following removal of extracellular Ca^{2+}, although Aakerlund *et al.* (1990) and Daniels *et al.* (1992) reported involvement of a thapsigargin-sensitive store. Ca^{2+} mobilization was shown for both Y_1 (Motulsky and Michel, 1988; Aakerlund *et al.*, 1990; Daniels *et al.*, 1992) and Y_2 (Perney and Miller, 1989) receptor subtypes. However, several studies have also presented evidence for NPY-stimulated Ca^{2+} entry. Using both single cell $[Ca^{2+}]_i$ and electrophysiological measurements, Gimpl *et al.* (1993) concluded that NPY initiated a depolarization, thereby activating Ca^{2+} entry through voltage-gated channels. Indeed, NPY-stimulated Ca^{2+} entry through voltage-gated channels had been inferred previously from pharmacological data (Edvinnson *et al.*, 1987). However, voltage-gated Ca^{2+} entry was eliminated in the present experiments (Fig. 2). Accordingly, this study provides the first evidence for NPY-stimulated Ca^{2+} entry through receptor operated channels. It remains to be investigated whether this pathway is activated specifically by the Y_2 receptor subtype.

Y_1 and Y_2 receptors are sometimes considered to be localized to post- and presynaptic sites, respectively (reviewed in Wahlestedt and Reis, 1993). Y_1 receptors often mediate or potentiate excitatory neurotransmission, whereas Y_2 receptors have been implicated in the suppression of neurotransmitter release via G protein-mediated reduction in voltage-gated calcium currents (Walker *et al.*, 1988; Ewald *et al.*, 1989; Bleakman *et al.*, 1991). The demonstration that Y_2 receptors can also mediate Ca^{2+} increases through either mobilization (Perney and Miller, 1989)

or entry (this study) is not easily reconciled with this simple categorization and suggests that the functional distribution of NPY receptor subtypes may be more complex.

Pertussis Toxin Sensitivity—Pretreatment with pertussis toxin had no effect on the NPY-stimulated $[Ca^{2+}]_i$ elevation, although it completely abolished the NPY-mediated inhibition of adenylate cyclase (Fig. 8). Although NPY effects are generally mediated by pertussis toxin-sensitive G proteins, several studies (Colmers and Pittman, 1989; Foucart and Majewski, 1989; Gimpl *et al.*, 1993) have reported pertussis toxin-insensitive effects. Our results indicate that the activated NPY receptor can couple to pertussis toxin-sensitive and -insensitive mechanisms in the same cell, suggesting a lack of correlation between pertussis toxin sensitivity and receptor subtype. Herzog *et al.* (1992) recently reported that the coupling of a cloned NPY Y_1 receptor to second messenger systems depended upon the type of cell into which it was transfected. From both of these studies, it is clear that the NPY receptor subtype differences do not necessarily underpin the coupling to different cellular signaling systems. It seems likely that the heterogeneity in NPY-induced responses is caused not only by a diversity of receptor subtypes, but also by a diversity of G proteins coupled to a given subtype and possibly also by a diversity of effector mechanisms coupled to given G proteins. Given these complications, the functional characterization of NPY receptor subtypes will not be straightforward.

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