Br. J. Pharmacol. (1991), **103**, 1781–1789

Neuropeptide Y inhibits Ca^{2+} influx into cultured dorsal root ganglion neurones of the rat via a Y_2 receptor

[1]D. Bleakman, *W.F. Colmers, †A. Fournier & R.J. Miller

Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, 947 E58th Street, Chicago, Illinois, U.S.A.; *Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada and †INRS Santé, Pointe-Claire, Québec, Canada

1 The identity of the neuropeptide Y (NPY) receptor associated with the observed inhibition of neuronal Ca^{2+} currents (I_{Ca}) in rat dorsal root ganglion (DRG) cells has been established on the basis of agonist responses to analogues and carboxy terminal (C-terminal) fragments of the NPY molecule.

2 Whole cell barium currents (I_{Ba}) in DRG cells were reversibly inhibited by 100 nM NPY, 100 nM PYY and C-terminal fragments of NPY in a manner that correlated with the length of the NPY fragments (for inhibition of the I_{Ba} NPY = PYY > NPY2–36 > NPY13–36 > NPY16–36 > NPY18–36 ≫ NPY25–36).

3 C-terminal fragments of NPY were also effective in reversibly reducing the I_{Ca}, the associated increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the increased $[Ca^{2+}]_i$ produced by evoked action potentials in the DRG cells. In addition, a Ca^{2+}-activated Cl^- conductance was also reversibly reduced by NPY fragments only when accompanied by a reduction in Ca^{2+} entry.

4 We conclude that the Y_2 receptor for neuropeptide Y is coupled to inhibition of Ca^{2+} influx via voltage-sensitive calcium channels in DRG cells.

Keywords: neuropeptide Y; neuropeptide Y fragments; calcium currents; intracellular calcium; fura-2; voltage clamp; sensory neurones; Y_2 receptor; current clamp

Introduction

Neuropeptide Y (NPY) is one of the most abundant and widely distributed putative neuromodulators in the mammalian nervous system. The actions of NPY include the inhibition of neurotransmitter release at sympathetic neuroeffector junctions (Wahlestedt, 1987) and at glutamatergic synapses in hippocampus (Colmers *et al.*, 1988). Physiological evidence from several neuronal preparations indicate that NPY can inhibit Ca^{2+} influx through voltage-dependent Ca^{2+} channels into neurones such as rat dorsal root ganglion (DRG) neurones in culture (Walker *et al.*, 1988; Ewald *et al.*, 1988). The action of the NPY receptor is mediated through G-proteins, some, but possibly not all, of which are sensitive to the action of pertussis toxin (Ewald *et al.*, 1989; Colmers & Pittman, 1989).

Recent evidence indicates the existence of at least two types of NPY receptors which have been differentiated on the basis of agonist responses to analogues and carboxyl terminal (C-terminal) fragments of the NPY molecule. The Y_1 receptor requires the intact NPY molecule or its natural analogue, PYY, for its activation, whereas the Y_2 receptor was defined on the basis of its sensitivity to C-terminal fragments as short as NPY13–36. The Y_2 receptor has been implicated in the inhibition of transmitter release at both the sympathetic neuroeffector junction (Wahlestedt *et al.*, 1986) and at the stratum radiatum-CA1 glutamatergic synapse in rat hippocampus (Colmers *et al.*, 1991). In the present study, we have examined the action of NPY fragments and analogues on rat DRG cells in culture in order to determine the nature of the NPY receptor mediating the inhibition of Ca^{2+} influx in these cells, using several complementary methodological approaches. The results indicate that the NPY receptor in cultured DRG cells of the rat is identical with the Y_2 receptor which inhibits release of neurotransmitters at sympathetic neuroeffector junctions and hippocampal glutamatergic synapses.

[1] Author for correspondence.

Methods

Cell culture

Neurones were cultured from the dorsal root ganglia (DRG) of neonatal rats essentially as described previously (Thayer *et al.*, 1988). Briefly DRG neurones were dissected from the thoracic and lumbar segments of 1–3 day old Sprague-Dawley rats, incubated for 15 min at 37°C in collagenase/dispase (0.8 and 0.4 u ml^{-1}) and then dissociated into single cells by trituration through a Pasteur pipette. The cells were then plated on laminim-fibronectin-coated coverglasses (25 mm diameter) and incubated in Ham's medium mixture F-12, supplemented (GIBCO, Grand Island, NY, U.S.A.) with 5% heat inactivated rat serum, 4% 17-day embryonic extract, 50 ng ml^{-1} nerve growth factor, 44 mM glucose, 2 mM L-glutamine, 1% MEM 100 × vitamins and penicillin/streptomycin (100 u ml^{-1} and 100 mg ml^{-1} respectively) which was replaced every 2–3 days. Cultures were maintained at 37°C in a water-saturated atmosphere with 5% CO_2. Cells in culture between 4 and 15 days were used for the present experiments.

Whole cell patch clamp

The tight seal whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record the transmembrane I_{Ca} from single cells while simultaneously measuring changes in $[Ca^{2+}]_i$. Cells were mounted in a perfusion chamber and thoroughly rinsed with a buffer solution composed of (in mM), NaCl 138, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, HEPES 10 and glucose 10, adjusted to pH 7.4 with NaOH. Voltage clamp experiments were performed in a solution containing (in mM); tetraethylammonium chloride (TEACl) 143, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 10, pH adjusted to 7.4 with TEAOH. A cell was then approached with a fire-polished pipette containing a solution composed of (in mM): fura-2 pentapotassium salt 0.1, CsCl 135, $MgCl_2$ 1, HEPES 10, diTris phosphocreatinine 14; MgATP 3.6 and creatinine phosphokinase 50 u ml^{-1}; adjusted to pH 7.1 with CsOH. Current clamp experiments on cells were performed

with a pipette solution composed of (in mM): fura-2 pentapotassium salt 0.1, KCl 135, $MgCl_2$ 1, HEPES 10, diTris phosphocreatinine 14, MgATP 3.6 and creatine phosphokinase 50 u ml^{-1}, pH adjusted to 7.1 with KOH. For the current clamp experiments, cells were exposed to (in mM): NaCl 138, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, HEPES 10 and glucose 10, adjusted to pH 7.4 with NaOH. Action potentials were evoked by brief current pulses. The membrane potential aquisition rate was 1 ms^{-1} in order to obtain combined fura-2/electrophysiological records over 4 s periods. Consequently certain action potential traces display variable peak action potential heights due to this slow sampling rate. Background fluorescence was recorded after formation of a gigaseal but before breaking into the cell, thus accounting for fluorescence contributed by the fura-2 in the pipette. Since the pipette approached the cell from above, the objective was focused below the pipette near the middle of the cell, to minimize the pipette fluorescence. Fluorescent recordings were made from the cell soma alone. Full diffusion of the fura-2 into the cell occurs over a period of 1–3 min. Currents were recorded by a List EPC-7 amplifier, filtered by an 8-pole low pass Bessel filter with a cut off frequency of 200 Hz and stored on a computer used for the fluorescence data acquisition. Linear leak corrections were performed by averaging 16, 10 mV hyperpolarizing pulses from the holding potential. The d.c. component of the averaged leak current was then modelled so as to increase the signal to noise ratio. Digital summation of this leak template after appropriate scaling with the current obtained during depolarizing test pulses provided the leak correction. Series resistance compensation of approximately 40% was possible with the uncompensated portion of the series resistance ranged between 1.8 and 3MΩ. Peak I_{Ca} and I_{Ba} values rarely exceeded 1.5 nA or 2.0 nA respectively giving approximate maximum voltage errors of 4.5 mV and 6.0 mV. Cells were discarded when the steady leakage current at the holding potential was greater than 5% of the peak inward current. All experiments were performed at room temperature.

Fura-2 fluorescence techniques

The methods used for this study have been described previously in detail (Thayer *et al.*, 1988). Briefly, for excitation of the fluorescent calcium probe fura-2 (pentapotassium salt), the collimated beam of light from a 200 W Hg arc lamp was passed through a dual spectrophotometer (Phoenix Instruments, Philadelphia, PA, U.S.A.) which alternated wavelengths from 340 to 380 nm by means of a spinning chopper (60 Hz). The light source was placed outside a darkened Faraday cage which enclosed the vibration isolation table supporting a microscope. A fused silica lens was positioned to focus light upon a liquid light guide (3 mm × 1 mm, Oriel, Stratford, CT, U.S.A.) and a similar lens, placed at the terminating end of the guide, was positioned to direct light through the epifluorescent illuminator of the microscope. The light guide eliminated problems associated with vibration from the chopper and electrical noise from the arc lamp. The light was reflected off a dichroic mirror (Nikon, DM 400) and focused through a 70 × oil immersion objective (E. Leitz Inc., Rockleigh, NJ, U.S.A., numerical aperture 1.15). The emission fluorescence was selected for wavelengths with a 480 nm barrier filter and recordings were spatially defined with an adjustable rectangular diaphragm. The fluorescence emission was analyzed with a photomultiplier tube (bialkali) and discriminator (APED II; Thorn EMI Gencom Inc., Plainview, NY, U.S.A.). The discriminator output was converted to pulses which were then integrated by passing the signal through an 8 pole Bessel filter at 500 Hz. The gain on this detection system could be adjusted from 1 to 100 fold by increasing the pulse length. The conversion of light intensity to voltage by this process was confirmed to be linear over the range of the light levels used in these experiments. The signal from the filter was fed into one channel of an analog to digital convertor (PDP-11/73) computer system (Indec Systems, Sunnyvale, CA, U.S.A.). The signals from two photodiodes, each placed in a small portion of the light beam directed toward the monochromators, were connected to two additional channels of the analogue to digital converter.

Sorting the fluorescence output into signals corresponding to excitation at these two wavelengths was performed entirely by software written in BASIC-23 (Cheshire Data, Indec Systems). The photomultiplier output was sorted into signals from 340 and 380 nm excitation by using the photodiode outputs as timing signals and the output observed on-line throughout the experiment.

Cover slips (25 mm diameter), plated with cells, were mounted in the perfusion chamber which was positioned on the opening of the microscope stage. The solution change in the cell superfusion system approximated a step occurring over 10 s. The tubing between the large media reservoirs and the inlet to the chambers delayed the onset of the solution change by an additional 10 s. Figures have been corrected for the perfusion delay.

Calibration and analysis

Records were corrected for experimentally determined background values and the ratio of 340/380 nm fluorescence calculated off-line. Ratios were converted to free Ca^{2+} by the equation, $Ca^{2+} = K(R - R_{min})/(R_{max} - R)$ in which R is the 340/380 nm fluorescent ratio (Grynkiewicz *et al.*, 1985). The maximum ratio (R_{max}), the minimum ratio (R_{min}) and the constant K, the product of the dissociation product for fura-2 and the ratio of the free and bound forms of the dye at 380 nm, were determined from a fit to a standard curve using the above equation with a non-linear least squares analysis computer programme (Fabatio & Fabatio, 1979). The standard curve was determined for the fura-2 salt in calibration buffer (in mM: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 20, KCl 120, NaCl 5, $MgCl_2$ 1, pH 7.1) containing 10 mM ethylene gylcol *bis* (B-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), $K_s = 3.696 \times 10^6\ M^{-1}$ with calculated amounts of Ca^{2+} added to give free calcium concentrations ranging between approximately 0 to 2000 nM. Identical calibration curves were obtained if CsCl was used to replace KCl. Experiments performed over long periods of time (>30 s) were digitally filtered with an algorithm which added 1/2 the value of each datum point to 1/4 of the value of each neighbouring point. The data were cycled through this routine 5 times. The $[Ca^{2+}]_i$ traces in patchclamp experiments were digitally filtered by a single cycle through an 11 point moving average algorithm.

Peptide synthesis

Fragments of NPY were synthesized by the solid phase method using a manual home-made multireactor synthesizer. The syntheses were carried out with a benzhydrylamine resin (Pietta *et al.*, 1974) since the peptides bear an amide C-terminal function. All amino acids were coupled via the BOP/DMF method (Fournier *et al.*, 1988), according to a recently-described protocol (Forest & Fournier, 1990). The Boc amino acids with appropriate side-chain protection were obtained from commercial sources. Completed peptides were cleaved from the resin support and deprotected by a 90 min treatment at 0°C with liquid hydrofluoric acid containing m-cresol and dimethyl sulphide as scavengers (10 : 1 : 1 v/v).

After extraction from the resin and lyophilization, the peptides were purified by reverse-phase chromatography on a Waters Deltapak column, using an eluent of (A) H_2O (0.06% trifluoracetic acid, TFA) and (B) acetronitrile-H_2O (0.06% TFA). Peptides were eluted with successive linear gradients of solvent B. Analytical high performance liquid chromatography (h.p.l.c.) of the individual fractions were carried out and the fractions corresponding to the purified peptide were lyophilized. The purified material was

characterized by analytical h.p.l.c., capillary electrophoresis and amino acid analysis.

Results

Electrophysiological recordings

Experiments in this series were performed on a total of 42 cells. Of these, 19 were studied in voltage clamp mode alone using Ba^{2+} as the charge carrier and in 7 cells Ca^{2+} influx was examined by fura-2 based microfluorimetry under voltage clamp conditions. Of these 26 cells, 21 cells responded to NPY with an inhibition of peak I_{Ca} or I_{Ba}. A response was defined as a reduction in I_{Ca} or I_{Ba} by 20% or more produced by 100 nM NPY. A robust effect in magnitude enabled us to compare reliably the effects of NPY fragments with the native molecule (see below). In 16 cells, Ca^{2+} influx was examined by fura-2 based microfluorimetry under current clamp conditions.

Peptide actions on barium currents

Neuropeptide Y and PYY Tight seal, whole cell recordings of Ba^{2+} currents (I_{Ba}) were made from DRG neurones at a holding potential of −80 mV. Peak whole-cell currents were observed during steps to 0 mV (Figure 1). The initial peak I_{Ba} ranged between 0.3 and 2.0 nA and declined gradually with time. In agreement with earlier observations for I_{Ca}, bath application of 100 nM NPY caused an inhibition of between 20 and 60% of control I_{Ba} amplitudes (Ewald *et al.*, 1988). Mean inhibition of the I_{Ba} by 100 nM NPY was 33.8 ± 2.0% (n = 13). The inhibition was also often accompanied by a slowing of the kinetics of activation of the I_{Ba}, as has been reported for other neurotransmitters (Bean, 1989) although the magnitude of this effect varied and was not always apparent (e.g. compare Figure 1a and b). The inhibition reversed readily upon washout of the peptide (Figure 1a). Higher concentrations (1 μM) of NPY were only slightly more effective than 100 nM NPY, indicating that this concentration produced almost a maximal response (Figure 1, 35.9 ± 6.1% for 100 nM and vs 42.7 ± 5.9% for 1 μM, n = 3). A concentration of 100 nM was

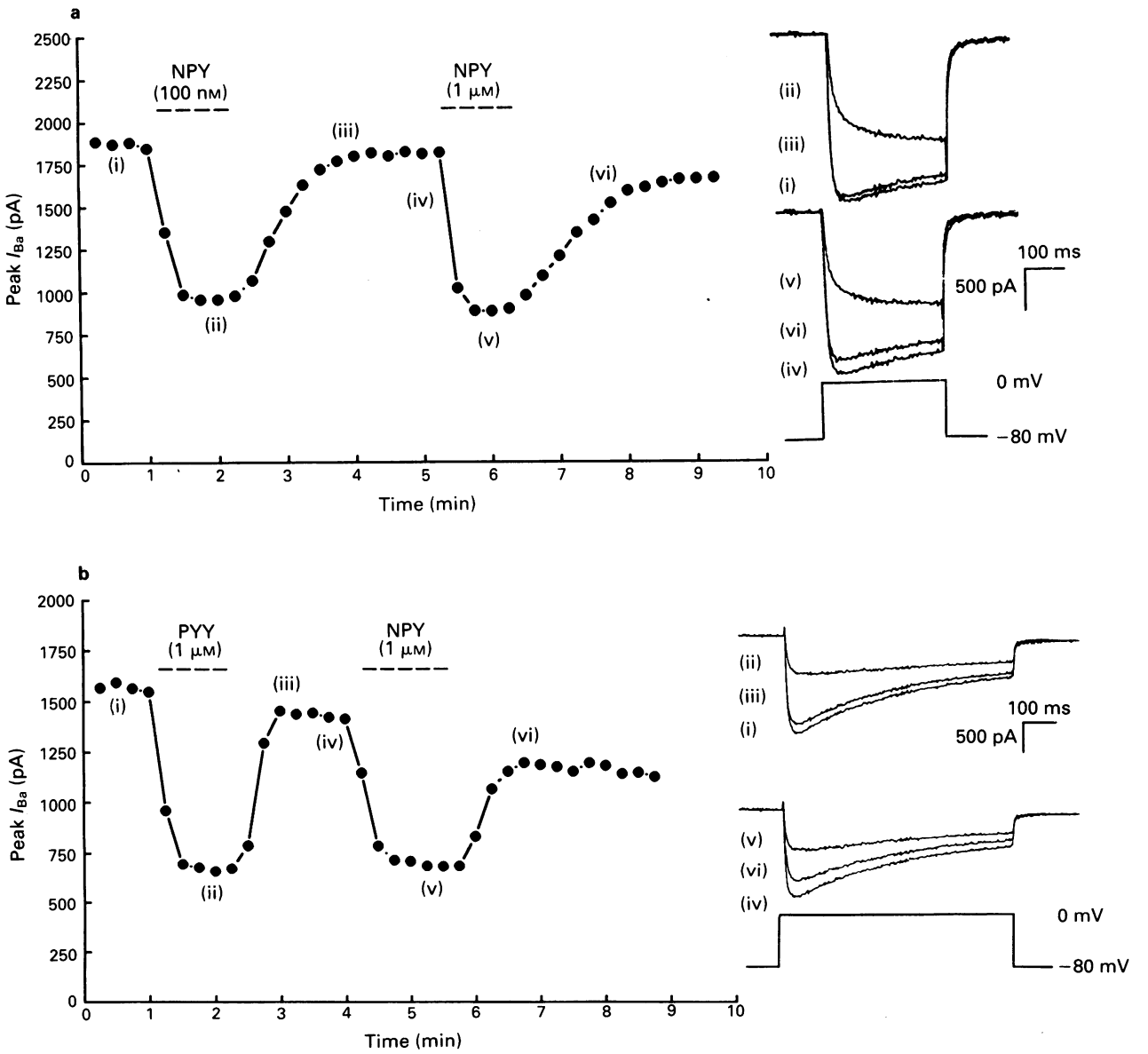

Figure 1 Neuropeptide Y (NPY)- and PYY-mediated inhibition of I_{Ba} in single DRG neurones. (a) The timecourse of inhibition of I_{Ba} by two concentrations of NPY is shown with 20 mM BAPTA in the patch pipette. The insets show superimposed individual current traces evoked from Vh = −80 mV to Vt = 0 mV at the timepoints indicated. The figure is representative of 3 similar experiments. (b) Timecourse of inhibition of I_{Ba} by PYY and NPY with 20 mM BAPTA in the patch pipette. Insets as in (a). The figure is representative of 3 similar experiments.

used for comparison of the actions of NPY analogues and NPY fragments. Application of the full-sequence naturally occurring analogue, PYY (1 μM), also elicited an inhibition of the I_{Ba} which was not significantly different from that observed with NPY (1 μM) (Figure 1b).

Neuropeptide and NPY fragments Application of C-terminal fragments of NPY also elicited reversible inhibitions of the I_{Ba} in DRG neurones (Figure 2). The degree of inhibition of the I_{Ba} was correlated with the length of the fragment, with NPY2–36 being 85% as effective as NPY and PYY (Figure 2a). As observed with NPY, the NPY fragments also appeared to alter the kinetics of the I_{Ba} (Figure 2b). Current-voltage relationships were obtained for NPY and NPY13–36 (Figure 2c and d). A comparison of the relative effects of NPY fragments on the inhibition of the peak I_{Ba} to NPY alone is shown in Figure 3. It may be seen that a significant reduction of the peak I_{Ba} was observed for C-terminal fragments NPY18–36 and longer.

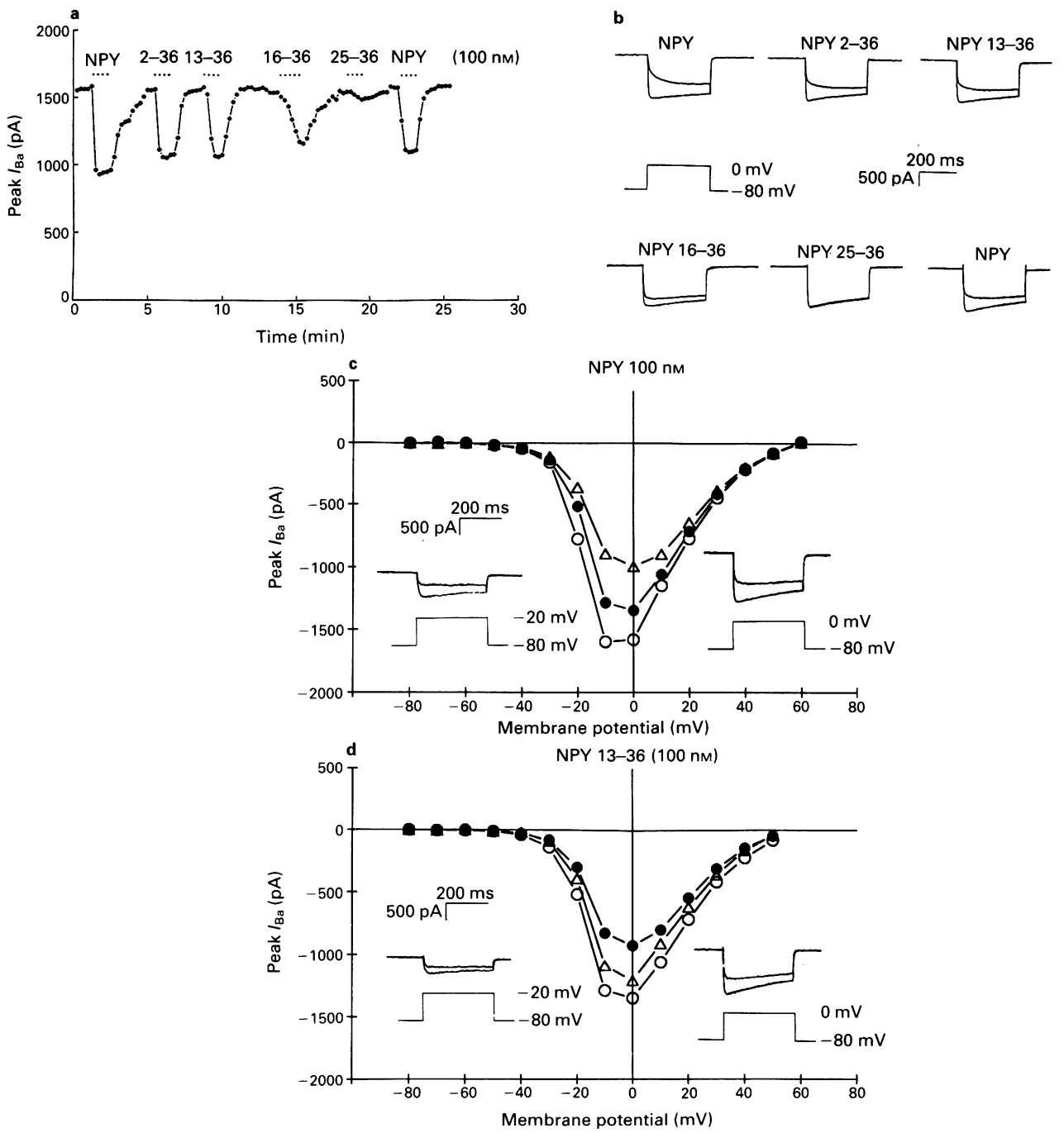

Figure 2 Effect of 100 nM neuropeptide Y (NPY) and 100 nM concentrations of different C-terminal fragments on the I_{Ba} in a DRG neurone. (a) Timecourse of inhibition of peak I_{Ba} by NPY and fragments with 20 mM BAPTA in the patch pipette from Vh = −80 mV to Vt = 0 mV. Fragments were presented in order of decreasing length, while NPY itself was applied both before and after the series of fragments. (b) Effects of NPY and fragments on individual I_{Ba}s in the presence and absence of the peptides as indicated. (c) Current voltage relationship for the effect of 100 nM NPY on the peak I_{Ba} measured in the presence of 20 mM BAPTA in the pipette. The traces show the current-voltage relationship from a Vh of −80 mV to various test potentials in the absence (○) and presence (△) of NPY and following washout of the peptide (●). Insets show individual current traces in the absence and presence of NPY for two different test potentials. (d) Current-voltage relationship for the effect of 100 nM NPY13–36 on the peak I_{Ba} measured in the presence of 20 mM BAPTA in the pipette from the same neurone as shown in (c). The traces show the current-voltage relationship from a Vh of −80 mV to various test potentials in the absence (○) presence (●) of 100 nM NPY13–36 and following washout of the peptide (△). Insets as in (c).

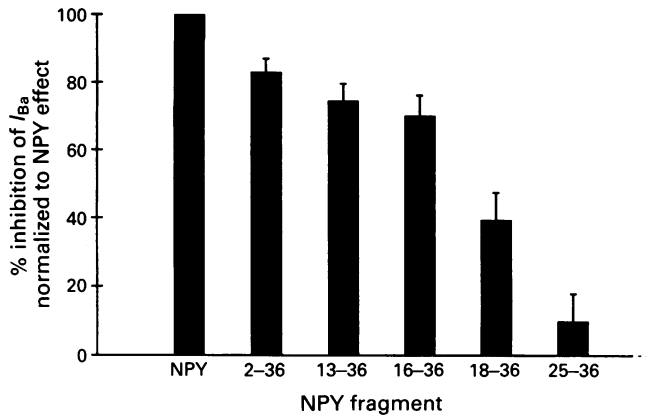

Figure 3 Comparative inhibition of the peak I_{Ba} by neuropeptide Y (NPY) and C-terminal fragments of NPY (all at 100 nM) in DRG neurones. Inhibition of the peak I_{Ba} by C-terminal fragments is normalized to the inhibition produced by 100 nM NPY itself in the same cell. The results are the mean with standard error of the mean for 5 cells in which comparisons were made with full sequence NPY.

Combined voltage clamp/$[Ca^{2+}]_i$ measurements

We further investigated the effects of NPY and C-terminal fragments on I_{Ca} and $[Ca^{2+}]_i$ by simultaneously monitoring the I_{Ca} and $[Ca^{2+}]_i$ in single DRG cells under voltage clamp conditions in which the pipette solution contained 100 μM fura-2 (Figure 4). Brief (80 ms) voltage steps from a holding potential of −80 mV to a test potential of 0 mV were applied every 30 s and the increase in $[Ca^{2+}]_i$ measured. The peak I_{Ca}s in this series of experiments ranged between 0.3 and 1.3 nA. The rate of decay of the inward current carried by Ca^{2+} was significantly more rapid than when Ba^{2+} was the charge carrier in agreement with earlier observations (Bleakman *et al.*, 1990). $[Ca^{2+}]_i$ levels increased in response to the voltage clamp step (Figure 4a). Neither 100 nM NPY nor 100 nM NPY13–36 caused changes in the basal $[Ca^{2+}]_i$, but they did produce a reversible inhibition of the I_{Ca} (28.5 ± 3.5% and 17.7 ± 3.0%, $n = 4$ respectively). The simultaneously recorded increase in $[Ca^{2+}]_i$ was also reduced in the presence of NPY13–36 (100 nM) in parallel with the I_{Ca} and also recovered upon washout (Figure 4b). As with the other experiments, NPY also reversibly reduced the I_{Ca} and

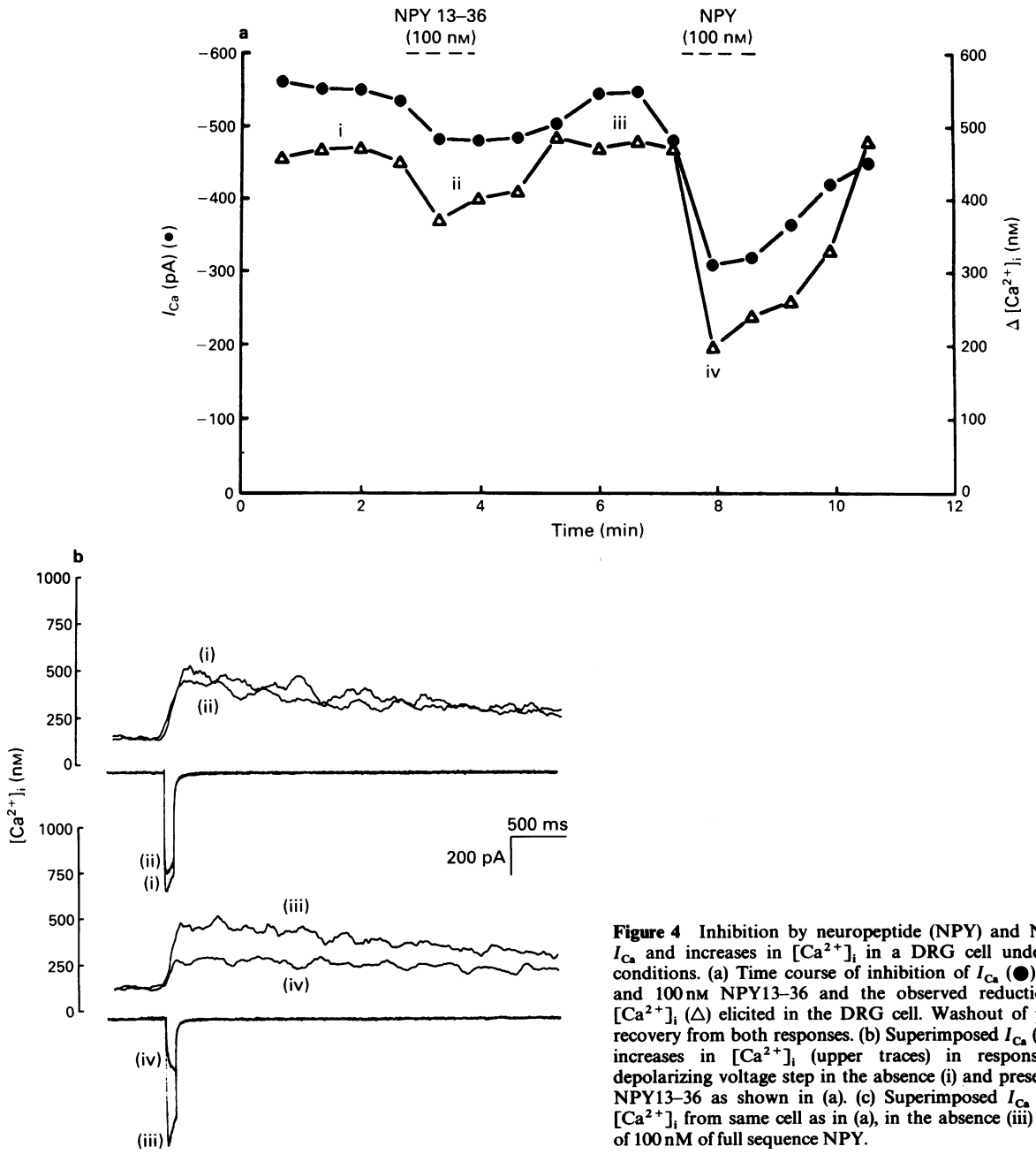

Figure 4 Inhibition by neuropeptide (NPY) and NPY13–36 of the I_{Ca} and increases in $[Ca^{2+}]_i$ in a DRG cell under voltage clamp conditions. (a) Time course of inhibition of I_{Ca} (●) by 100 nM NPY and 100 nM NPY13–36 and the observed reductions in the peak $[Ca^{2+}]_i$ (△) elicited in the DRG cell. Washout of peptides led to a recovery from both responses. (b) Superimposed I_{Ca} (lower traces) and increases in $[Ca^{2+}]_i$ (upper traces) in response to an 80 ms depolarizing voltage step in the absence (i) and presence (ii) of 100 nM NPY13–36 as shown in (a). (c) Superimposed I_{Ca} and increases in $[Ca^{2+}]_i$ from same cell as in (a), in the absence (iii) and presence (iv) of 100 nM of full sequence NPY.

the $[Ca^{2+}]_i$ in DRG cells (Figure 4c). The mean inhibition of the increase in peak $[Ca^{2+}]_i$ in response to an 80 ms voltage clamp step was $32.0 \pm 8.1\%$ and $27.8 \pm 7.0\%$ ($n = 4$) for 100 nM NPY and 100 nM NPY13–36 respectively.

Combined current clamp/$[Ca^{2+}]_i$ measurements

Experiments were also performed on DRG cells under current clamp conditions in which trains of action potentials were elicited by trains of brief depolarizing current pulses (4 ms). The membrane potential was maintained during the course of the experiment to a value of approximately −60 mV. Once stable $[Ca^{2+}]_i$ responses to identical trains of action potentials had been obtained, 100 nM NPY was applied via the bath. As observed with the above experiments under voltage clamp, NPY application itself had no effect on the baseline levels of $[Ca^{2+}]_i$. However, NPY caused a reversible reduction in the rise in $[Ca^{2+}]_i$ which resulted from the evoked action potential train (Figure 5a). In 12 of 16 neurones examined, NPY caused a mean reduction in the peak increase in the $[Ca^{2+}]_i$ of $46 \pm 6\%$. This inhibition reversed upon washout of NPY. In the same 12 neurones, NPY13–36 also caused a reversible inhibition of Ca^{2+} influx in response to a train of action potentials (Figure 5b). At 100 nM, NPY13–36 was only $68.6 \pm 6.7\%$ ($n = 12$ cells) as effective as NPY in inhibiting Ca^{2+} influx elicited in this manner.

Effect of neuropeptide Y and C terminal fragments on $[Ca^{2+}]_i$-dependent membrane events

It has previously been shown that for a subpopulation of DRG cells that were loaded with Cl^-, the action potential was

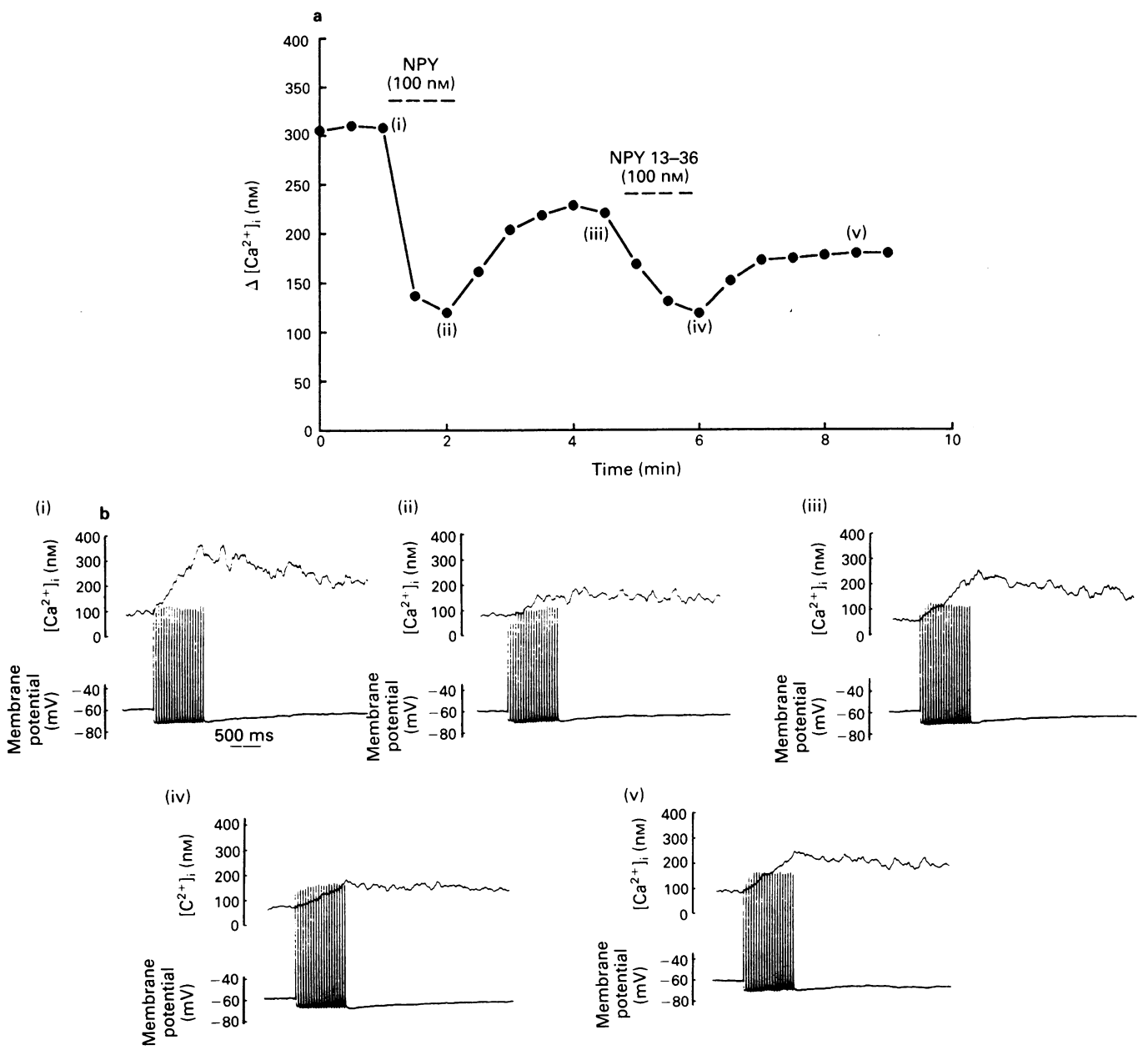

Figure 5 Inhibition by neuropeptide Y (NPY) and NPY13–36 of action potential-evoked increases in $[Ca^{2+}]_i$ in a single DRG cell under current clamp. (a) Time course of inhibition of action potential train-mediated increases (peak-basal) in $[Ca^{2+}]_i$ by 100 nM NPY and 100 nM NPY13–36. Each time point represents the $\Delta[Ca^{2+}]_i$ which results from the firing of a train of 20 action potentials. (b) Representative voltage recording of action potentials evoked once every 30 s in a DRG cell by a train of 4 ms pulses (20 APs, 25 Hz). Increases in $[Ca^{2+}]_i$ in response to train of action potentials were reversibly inhibited by both 100 nM NPY (ii) and 100 nM NPY13–36 (iv).

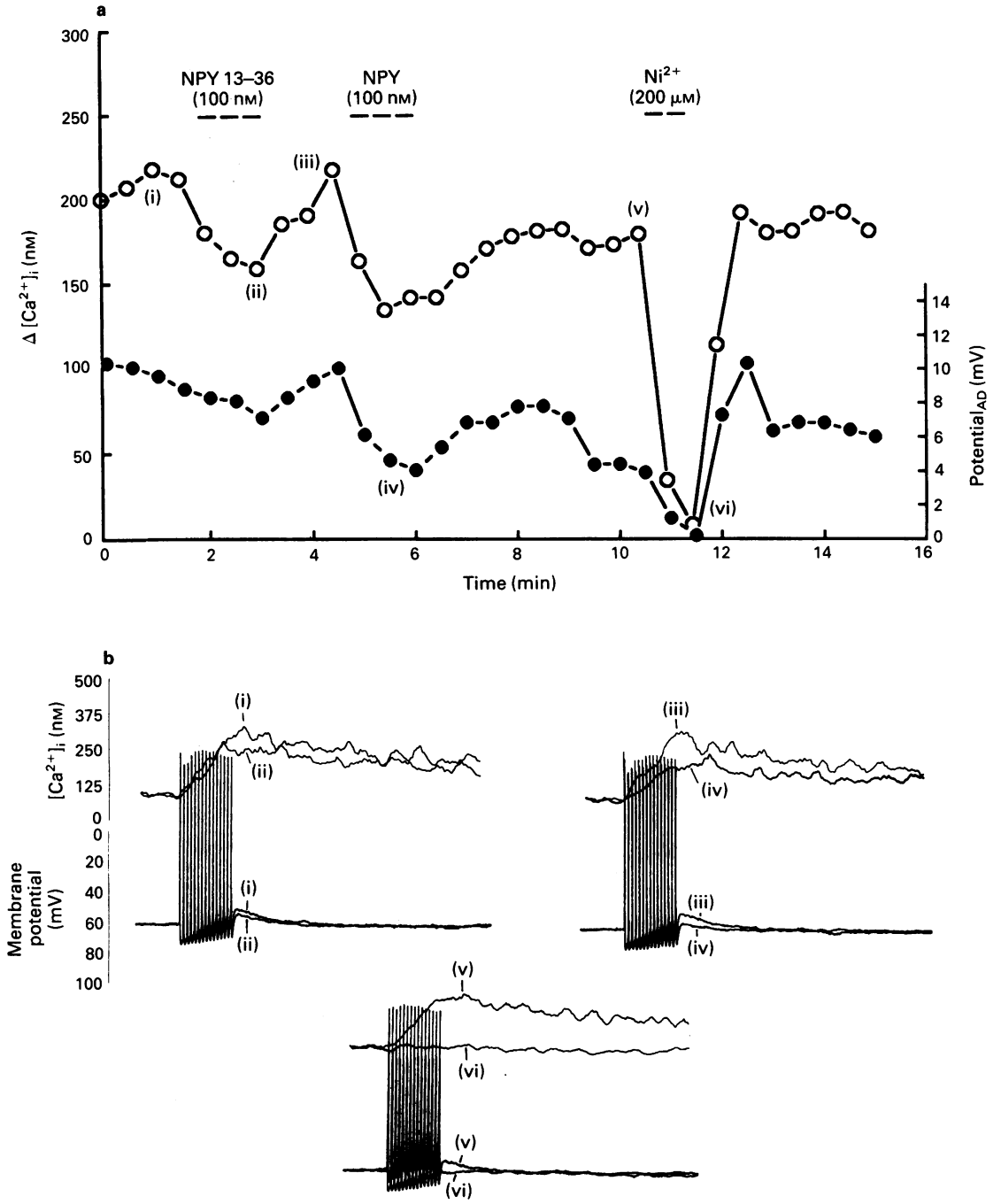

Figure 6 (a) Time course of the rise in $[Ca^{2+}]_i$ due to the firing of 15 action potentials (○) (4 ms, 25 Hz) and the effect of 100 nM neuropeptide Y (NPY), 100 nM NPY13–36 and 200 μM Ni^{2+} on this and on the magnitude of the after-depolarization which resulted. The after-depolarization (AD) (●) was the difference between the resting membrane potential before evoking the train of action potentials and the peak value of the depolarization between the last action potential and 2 s following this point. (b) Individual timepoints of after-depolarizing potentials and their modulation by 100 nM NPY and 100 nM NPY13–36 and 200 μM Ni^{2+} as indicated in (a).

followed by an after-depolarization (Dichter & Fischbach, 1977). In the present experiments, after-depolarizations were also observed which increased in magnitude with longer trains of action potentials and accordingly larger accompanying $[Ca^{2+}]_i$ transients. It is likely that the after-depolarizations are due to activation of a Cl^- conductance by the increase in $[Ca^{2+}]_i$ as described elsewhere (Scott *et al.*, 1988). After-depolarizations were observed in the present series of experiments in 5 of 16 cells and were modulated by agents which reduced Ca^{2+} influx. As can be seen in Figure 6, 100 nM NPY13–36, 100 nM NPY and 200 μM Ni^{2+} reduced the magnitude of the after-depolarization. These changes were always accompanied by decreases in $[Ca^{2+}]_i$.

Under whole-cell voltage clamp conditions the after-depolarization which followed the action potential manifested itself as a slow inward Cl^- tail current ($I_{Cl(Ca)}$) which has previously been described by others (Mayer, 1985; Scott *et al.*, 1988). Tail currents were observed in the DRG cells which lasted between 1 and 4 s following repolarization to −80 mV. Both 100 nM NPY and 100 nM NPY13–36 reduced the magnitude of the tail current and the associated $[Ca^{2+}]_i$ transient (Figure 7). Figure 7c also illustrates the effect of replacing the extracellular Ca^{2+} with Ba^{2+}. Under such conditions the magnitude of the Ca^{2+} activated Cl^- tail current was reduced. A residual fura-2 signal was observed, consistent with the ability of Ba^{2+} to bind to fura-2 (Schilling *et al.*, 1989).

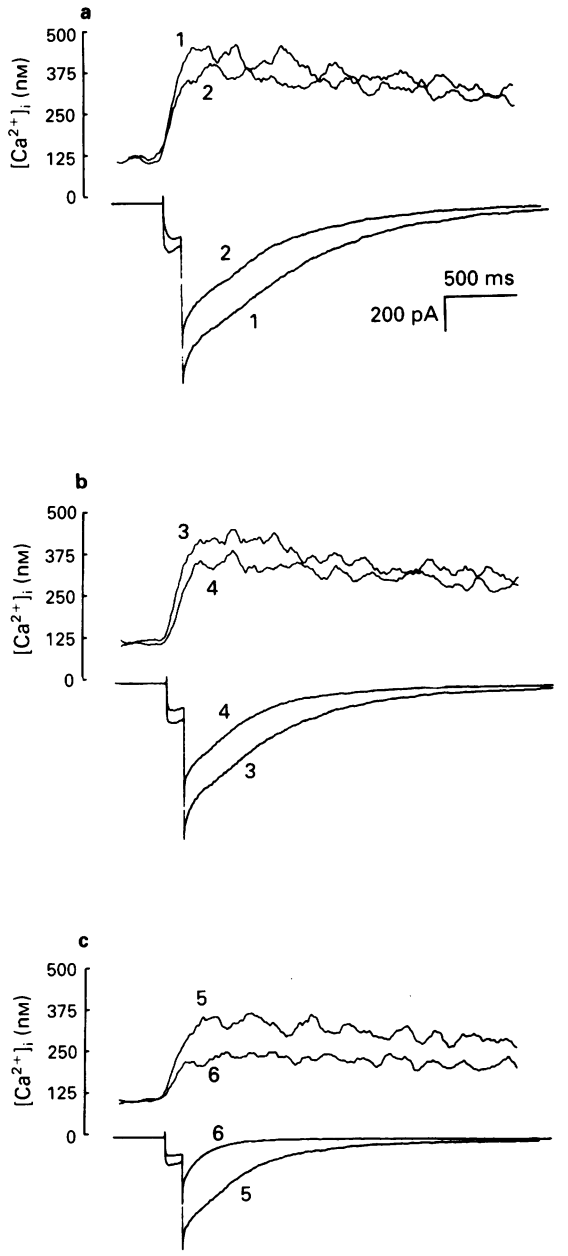

Figure 7 Tail currents and their modulation by 100 nM NPY13–36 ((1) control; (2) NPY13–36) and after recovery from this, 100 nM neuropeptide Y (NPY) ((3) control; (4) NPY). Also shown is the effect of replacement of Ca^{2+} with Ba^{2+} in the same cell as the other traces ((5) 2 mM Ca^{2+}; (6), 2 mM Ba^{2+}).

Discussion

Neuropeptide Y inhibits the influx of Ca^{2+} into rat DRG neurones in culture by inhibition of voltage-operated Ca^{2+} channels (Walker *et al.*, 1988; Ewald *et al.*, 1988). This paper presents evidence that the receptor for NPY on these cells is identical in its agonist profile to the Y_2 receptor present on presynaptic terminals at peripheral sympathetic neuroeffector junctions and on presynaptic glutamatergic terminals in area CA1 of rat hippocampus (Colmers *et al.*, 1987). NPY was without any effect on the holding current or resting membrane potential in the present experiments, and there was no evidence for an effect on cell membrane resistance. Thus the actions of the peptide seem to be manifest only on the Ca^{2+} conductances in these neurones.

The results from three different experimental approaches were congruent. Thus, NPY, PYY and NPY13–36 all inhibited Ba^{2+} influx through voltage-gated Ca^{2+} channels, inhibited Ca^{2+} influx during action potentials and simultaneously inhibited the peak I_{Ca} and the rise in $[Ca^{2+}]_i$ due to the voltage step. In the action potential experiments, the afterdepolarization observed in response to a train of action potentials, which is due to an increase in a Ca^{2+} activated Cl^- conductance (Dichter & Fischbach, 1977; Mayer, 1985) was also reduced by NPY, PYY and NPY13–36 application. In all cases, the short NPY25–36 fragment was without significant activity on any of the parameters measured. Interestingly, the NPY16–36 fragment exhibited clear, but weak, inhibitory effects in all preparations in which it was tested, and the NPY18–36 fragment also weakly inhibited I_{Ba}. A similar, but somewhat smaller inhibitory action of NPY16–36 has been observed on excitatory postsynaptic potentials, but not on population field responses, in area CA1 of rat hippocampal slice *in vitro*; however, unlike the present experiments, no effect of NPY18–36 was seen in this preparation (Colmers *et al.*, 1991).

The Y_2 receptor for NPY has been associated with inhibition of neurotransmitter release from sympathetic (Wahlestedt *et al.*, 1986), parasympathetic (Potter *et al.*, 1989) and sensory neurones (Perney & Miller, 1989). Several second messenger systems have been implicated in mediating the effects of this Y_2 response. For example Y_2 receptors for NPY have been linked to an inhibition of adenylate cyclase (reviewed in McDonald, 1988). However this effect does not appear to be a satisfactory criterion for the subclassification of receptor subtypes for NPY since NPY decreases forskolin-stimulated adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) levels in both SK-N-MC (Y_1-receptor) and SK-N-BE2 (Y_2 receptor) transfected cell lines (Wahlestedt *et al.*, 1990). In addition, in hippocampal slices, the elevation of intracellular cyclic AMP levels with the membrane soluble cyclic AMP analogue, 8(4-chlorophenylthio) cyclic AMP, does not appear to interfere with Y_2 receptor-mediated inhibition of neurotransmitter release (Klapstein *et al.*, 1990). The actions of NPY at the Y_2 receptor have also been associated with elevations in $[Ca^{2+}]_i$ and the generation of IP_3 following activation of PLC in a subpopulation of DRG neurones (Perney & Miller, 1989). However, NPY also increased $[Ca^{2+}]_i$ in neurones which contain Y_1 receptors (Aakerlund *et al.*, 1990). Thus, the characterization of Y_1 and Y_2 receptors for NPY on the basis of intracellular second messenger systems is equivocal.

In the present experiments it is unlikely that the inhibition of the I_{Ca} is secondary to an increase in $[Ca^{2+}]_i$ since inhibition of the I_{Ca} was observed even when the $[Ca^{2+}]_i$ was buffered with BAPTA in the patch pipette. Inhibition of the I_{Ca} by NPY has also been observed in acutely dissociated nodose ganglion cells (Wiley *et al.*, 1990) and in myenteric plexus neurones (Hirning *et al.*, 1990) in both studies the inhibition of the I_{Ca} was found to be sensitive to the action of pertussis toxin (PTX) in agreement with earlier studies on DRG cells in culture (Ewald *et al.*, 1988). However this may not be a universal mechanism for coupling NPY responses since in hippocampal slices the effect of NPY activation on transmitter release, which may be associated with inhibition of the I_{Ca} (Colmers *et al.*, 1987), is insensitive to pretreatment with PTX (Colmers & Pittman, 1989). This suggests that a PTX-insensitive G-protein may be involved in coupling NPY receptors to Ca^{2+} channels in this case. It is interesting to note that the receptor in hippocampal neurones also appears to be of the Y_2 type (Colmers *et al.*, 1991).

Thus the present study provides the first clear evidence that Y_2 receptors are coupled to the inhibition of Ca^{2+} influx by an effect on voltage-gated Ca^{2+} channels. To date there is no evidence that the Y_1 receptor in neurones can be coupled to this response.

D.B. was supported by a Fulbright Travel Fellowship. This work was funded by grants DA 02121, MH-40165 and a Digestive Diseases Research Centre Core Grant IP30-DK42086 to R.J.M. W.F.C. is an Alberta Heritage Foundation for Medical Research Scholar and was supported by an MRC Canada grant MT 10520.

References

AAKERLUND, L., GETHER, U., FUHLENDORFF, J., SCHWARTZ, T.W. & THASTRUP, O. (1990). Y1 receptors for neuropeptide Y are coupled to mobilization of intracellular calcium and inhibition of adenylate cyclase. *FEBS Lett.*, **260**, 73–78.

BEAN, B.P. (1989). Neurotransmitters inhibit neuronal calcium currents by changes in channel voltage dependence. *Nature*, **340**, 153–156.

BLEAKMAN, D., BRORSON, J.R. & MILLER, R.J. (1990). The effect of capsaicin on voltage-gated calcium currents and calcium signals in cultured dorsal root ganglion cells. *Br. J. Pharmacol.*, **101**, 423–431.

COLMERS, W.F., KLAPSTEIN, G.J., FOURNIER, A., ST.-PIERRE, S. & TREHERNE, K.A. (1991). Presynaptic inhibition by neuropeptide Y (NPY) in rat hippocampal slice *in vitro* is mediated by a Y_2 receptor. *Br. J. Pharmacol.*, **102**, 41–44.

COLMERS, W.F., LUKOWIAK, K.D. & PITTMAN, Q.J. (1988). Neuropeptide Y action in the rat hippocampal slice: site and mechanism of presynaptic action. *J. Neurosci.*, **8** 3827–3837.

COLMERS, W.F., LUKOWIAK, K.D. & PITTMAN, Q.J. (1987). Presynaptic action of neuropeptide Y in area CA1 of the rat hippocampal slice. *J. Physiol.*, **383**, 285–299.

COLMERS, W.F. & PITTMAN, Q.J. (1989). Presynaptic inhibition by neuropeptide Y and baclofen in hippocampus; insensitivity to pertussis toxin treatment. *Brain Res.*, **498**, 99–104.

DICHTER. M.A. & FISHBACH, G.D. (1977). The action potential of chick dorsal root ganglion neurons in cell culture. *J. Physiol.*, **267**, 281–298.

EWALD, D.A., MATTHIES, H.J.G., PERNEY, T.M., WALKER, T.M. & MILLER, R.J. (1988). The effect of down regulation of protein kinase C on the inhibitory modulation of dorsal root ganglion neurone Ca currents by neuropeptide Y. *J. Neurosci.*, **8**, 2447–2451.

EWALD, D.A., PANG, I.-H., STERNWEIS, P.C. & MILLER, R.J. (1989). Differential G-protein mediated coupling of neurotransmitter receptors to Ca channels in rat dorsal ganglion neurons *in vitro*. *Neuron*, **2**, 1185–1193.

FABATIO, A. & FABATIO, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)*, **75**, 463–505.

FOREST, M. & FOURNIER, A. (1990). BOP reagent for the coupling of pGlu and BocHis(Tos) in solid phase peptide synthesis. *Int. J. Pept. Res.*, **35**, 89–94.

FOURNIER, A., WANG, C.T. & FELIX, A.M. (1988). Applications of BOP reagent in solid phase synthesis. Advantages of BOP reagent for difficult couplings exemplified by a synthesis of [Ala^{15}]-GRF(1-29)NH_2. *Int. J. Pept. Res.*, **31**, 86–97.

GRYNKIEWICZ, G., PEONIE, M. & TSIEN, R.Y. (1985). A new generation of Ca indicators with greatly improved fluorescent properties. *J. Biol. Chem.*, 260, 3440–3450.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch-clamp technique for high resolution current recording from cells and cell free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HIRNING, L.D., FOX. A.P. & MILLER, R.J. (1990). Inhibition of calcium currents in cultured myenteric neurons by neuropeptide Y: evidence for a direct receptor/channel coupling. *Brain Res.*, **532**, 120–130.

KLAPSTEIN, G.L., TREHERNE, K.A. & COLMERS, W.F. (1990). Neuropeptide Y presynaptic effects in hippocampal slice *in vitro* are Y2 receptor mediated but not via adenylate cyclase. *Proc. N.Y. Acad. Sci.*, **611**, 457–458.

MAYER, M.L. (1985). A calcium dependent chloride current generated after depolarization of rat sensory neurons in culture. *J. Physiol.* (London), **364**, 217–239.

MCDONALD, J.K. (1988). NPY and related substances. *Crit. Rev. Neurobiol.*, **4**, 97–135.

PERNEY, T.M. & MILLER, R.J. (1989). Two different G-proteins mediate Neuropeptide Y and bradykinin-stimulated phospholipid breakdown in cultured rat sensory neurons. *J. Biol. Chem.*, **264**, 7317–7327.

PIETTA, P.G., CAVALLO, P.F., TAKAHASSHI, K. & MARSHALL, G.R. (1974). Preparation and use of benzhydrylamine polymers in peptide synthesis. II. Synthesis of thyrotropin releasing hormone, thyrocalcitonin 26–32 and eledoisin. *J. Org. Chem.*, **39**, 44–48.

POTTER, E.K., MITCHELL, L., MCCLOSKEY, M.J.D., TSENG, A., GOODMAN, A.E., SHINE, J. & MCCLOSKEY, D.I. (1989). Pre- and post-junctional actions of NPY and related substances. *Regul. Pept.*, **25**, 167–177.

SCOTT, R.H., MCGUIRK, S.M. & DOLPHIN, A.C. (1988). Modulation of divalent cation-activated chloride ion currents. *Br. J. Pharmacol.*, **94**, 653–662.

SCHILLING, W.P., RAJAN, L. & STROBL-JAGER, E. (1989). Characterization of the bradykinin-stimulated calcium influx pathway of cultured vascular endothelial cells. *J. Biol. Chem.*, **264**, 12838–12848.

THAYER, S.A., STUREK, M. & MILLER, R.J. (1988). Measurement of neuronal calcium transients using simultaneous microfluorimetry and electrophysiology. *Pflügers Arch.*, **415**, 216–233.

WAHLESTEDT, C. (1987). Neuropeptide Y (NPY): actions and interactions in neurotransmission. *Thesis, University of Lund*, pp. 1–226.

WAHLESTEDT, C., YANAIHARA, N. & HÅKANSON, R. (1986). Evidence for different pre- and post-junctional receptors for neuropeptide Y and related peptides. *Reg. Pept.*, **13**, 307–318.

WAHLESTEDT, C., GRUNDEMAR, L., HÅKANSON, R., HEILEG, M., SHEN, G.H., ZUKOWSKA-GROJEK, Z. & REIS, D.J. (1990) Neuropeptide Y receptor subtypes, Y1 and Y2. *Ann. N.Y. Acad. of Sci.*, **611**, 7–26.

WALKER, M.W., EWALD, D.A., PERNEY, T.M. & MILLER, R.J. (1988). Neuropeptide Y modulates neurotransmitter release and calcium currents in rat sensory neurons. *J. Neurosci.*, **8**, 2438–2446.

WILEY, J.W., GROSS, R.A., LU, Y. & MACDONALD, R.L. (1990). Neuropeptide Y reduces calcium current and inhibits acetylcholine release in nodose neurones via a pertussis toxin-sensitive mechanism. *J. Neurosci.*, **63**, 1499–1507.

(Received November 9, 1990
Revised February 25, 1991
Accepted March 11, 1991)