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Investigations into neuropeptide Y-mediated presynaptic inhibition in cultured hippocampal neurones of the rat

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1 We have examined the effects of neuropeptide Y (NPY) on synaptic transmission and $[Ca^{2+}]_i$ signals in rat hippocampal neurones grown in culture. $[Ca^{2+}]_i$ in individual neurones displayed frequent spontaneous fluctuations often resulting in an elevated plateau $[Ca^{2+}]_i$. These fluctuations were reduced by tetrodotoxin (1 μM) or combinations of the excitatory amino acid antagonists 6-cyano-7-dinitroquinoxaline (CNQX) (10 μM) and aminophosphonovalerate (APV) (50 μM), indicating that they were the result of glutamatergic transmission occurring between hippocampal neurones.

2 $[Ca^{2+}]_i$ fluctuations were also prevented by Ni^{2+} (200 μM), by the $GABA_B$ receptor agonist, baclofen (10 μM) and by NPY (100 nM) or Y_2 receptor-selective NPY agonists. Following treatment of cells with pertussis toxin, NPY produced only a brief decrease in $[Ca^{2+}]_i$ fluctuations which rapidly recovered.

3 Perfusion of hippocampal neurones with 50 mM K^+ produced a large rapid increase in $[Ca^{2+}]_i$. This increase was slightly reduced by NPY or by a combination of CNQX and APV. The effects of CNQX/APV occluded those of NPY. NPY had no effect on Ba^{2+} currents measured in hippocampal neurones under whole cell voltage-clamp even in the presence of intracellular GTP-γ-S. On the other hand, Ba^{2+} currents were reduced by both Cd^{2+} (200 μM) and baclofen (10 μM).

4 Current clamp recordings from hippocampal neurones demonstrated the occurrence of spontaneous e.p.s.ps and action potential firing which were accompanied by increases in $[Ca^{2+}]_i$. This spontaneous activity and the accompanying $[Ca^{2+}]_i$ signals were prevented by application of NPY (100 nM). When hippocampal neurones were induced to fire trains of action potentials in the absence of synaptic transmission, these were accompanied by an increase in cell soma $[Ca^{2+}]_i$. NPY (100 nM) had no effect on these cell soma $[Ca^{2+}]_i$ signals. NPY (100 nM) also had no effect on inward currents generated in hippocampal neurones by micropipette application of glutamate (50 μM).

5 Thus, NPY is able to abolish excitatory neurotransmission in hippocampal cultures through a pertussis toxin-sensitive mechanism. However, no effect of NPY on Ca^{2+} influx into the cell soma of these hippocampal neurones could be discerned. These results are consistent with a localized presynaptic inhibitory effect of NPY on glutamate release in hippocampal neurones in culture.

Keywords: Glutamate; calcium channels; presynaptic inhibition; G-proteins; calcium signals; neuropeptide Y receptors

Introduction

Neuropeptide Y (NPY) is a 36 amino acid neuropeptide which is abundantly distributed in both the central and peripheral nervous systems (Chronwall *et al.*, 1985). The peptide is thought to act as a neurotransmitter at both peripheral neuroeffector junctions and in the brain. NPY-containing fibres and receptors are widely distributed within the central nervous system (Chronwall *et al.*, 1985; Lynch *et al.*, 1989). For example, the CA1 region of the hippocampus is innervated by NPY-immunoreactive axons and terminals which make numerous synaptic contacts with the dendrites of hippocampal neurones (Haas *et al.*, 1987). Previous measurements in brain slices have demonstrated that NPY effectively suppresses transmission at the Schaffer collateral/CA1 synapse (Colmers *et al.*, 1985; 1987; 1988; 1991; Haas *et al.*, 1987). In rat hippocampal slices, NPY acts at a presynaptic site in area CA1, very possibly at the presynaptic terminal itself, as NPY did not affect the antidromic excitability of the presynaptic axons and did not affect the Ca^{2+}-dependent action potential in the presynaptic CA3 pyramidal cells (Colmers *et al.*, 1988). Although the mechanism of its action is unknown, there is no evidence that activation of potassium channels at the presynaptic terminal is involved (Colmers *et al.*, 1988; Klapstein & Colmers, 1992).

NPY is one of a group of agents which has been shown to reduce neurotransmitter release from a variety of peripheral neurones. This is accompanied by a reduction of the neuronal Ca^{2+} current and Ca^{2+} influx into the cell soma (Walker *et al.*, 1988; Schofield & Ikeda, 1988; Thayer & Miller, 1990; Hirning *et al.*, 1990; Bleakman *et al.*, 1991). These effects are mediated by the Y_2 subtype of NPY receptors, as are the effects of NPY in the hippocampal slice (Bleakman *et al.*, 1991; Colmers *et al.*, 1991). Binding studies support the presence of Y_2 receptors for NPY in the hippocampus (Sheikh *et al.*, 1989; Li & Hexum, 1991). The peripheral effects of NPY are mediated by pertussis toxin-sensitive G-proteins (Schofield & Ikeda, 1988; Ewald *et al.*, 1988; Walker *et al.*, 1988; Hirning *et al.*, 1990; Wiley *et al.*, 1990), although in the hippocampal slice preparation the effects of NPY were reported to be resistant to pertussis toxin (PTX) (Colmers & Pittman, 1989). In order to investigate further the mechanism of action of NPY, we have made measurements of its effects on spontaneous synaptic transmission between hippocampal neurones in culture and combined these with measurements of its effects on Ca^{2+} influx into these cells.

Methods

Instrumentation

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, the collimated beam

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of light from a 200W 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 mirro (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 computer system (PDP-11/73, 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 analog to digital convertor.

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 use of the photodiode outputs at 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 occuring 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 $[Ca^{2+}]_i$ by the equation, $[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R)$ 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 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 glyco *bis* (β-aminoethyl)-N,N,N',N'-tetra-acetic acid (EGTA), $Ks = 3696 \times 10^6\ M^{-1}$ with calculated amounts of Ca^{2+} added to give free calcium concentrations ranging between approximately 0 and 2000 mM. 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 data 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 patch-clamp experiments were digitally filtered by a single cycle through an 11 point moving average algorithm.

Cell culture

Hippocampal regions were dissected from the brains of E16-19 rat embryos. Neurones were dissociated with combinations of papain and mechanical trituration (Heuttner & Baughmann, 1986), and plated onto confluent cortical astrocytes on poly-D-lysine coated glass cover slips; cultures were treated with fluorodeoxy uridine (10 μM) after 24 h and were then maintained for up to six weeks in Minimal Eagle's Medium supplemented with 2 mM L-glutamine and 5% horse serum (HyClone).

Whole-cell patch clamp

The tight seal whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record transmembrane I_{Ca} or I_{Ba} alone from single cells and for measuring I_{Ca} while simultaneously measuring $[Ca^{2+}]_i$ transients. Cells were mounted in a perfusion chamber and thoroughly rinsed with buffer. Due to the extensive arborizations of the hippocampal neurones it was difficult to obtain good spatial control of the membrane voltage. Detailed kinetic analysis of the current was therefore not attempted. Indications of poor voltage control were manifest as the all or none activation of the currents in the low membrane potential range (−20 to −30 mV), a slow time-dependent activation in the voltage range −20 to +10 mV and slow deactivation of the currents following repolarization to −80 mV. If any of these characteristics were displayed, experiments were discarded. Acceptable recordings using these criteria represented approximately 20% of the cells from which recordings were made. Background fluorescence was recorded after formation of a giga-seal 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 focussed below the pipette near the middle of the cell to minimize the pipette fluorescence. Fluorescence 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. For the 'perforated patch technique', amphotericin-B (150 μg ml^{-1}) was present in the pipette and access was obtained within 2 min as indicated by an increase in the amplitude of the capacitance transient. If the whole cell configuration and internal perfusion occurred, the cell deteriorated within 2 min. Currents recorded by a List EPC-7 amplifier were filtered by an 8-pole low-pass Bessel filter with a cut-off frequency of 200 Hz and stored on computer. Linear leak corrections were performed by averaging sixteen, 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 40–80% was possible with the uncompensated portion of the series resistance ranging between 1.8 and 3 MOhm. Peak I_{Ca} and I_{Ba} values rarely exceeded 1.5 nA or 2.5 nA respectively, giving approximate maximum voltage errors of 4.5 mV and 7.5 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. Under current clamp conditions action potentials were evoked by brief current pulses (0.5–2 nA; 4–8 ms). Recordings were accepted for analysis if the resting potential exceeded −50 mV, the input resistance

exceeded 200 MOhms and the basal $[Ca^{2+}]_i$ was below 200 nM. All experiments were performed at room temperature (22–25°C).

Fura-2 fluorescence experiments

Experiments in which fura-2 fluorescence alone was measured were performed on coverslips of cells loaded for 30 min at 37°C with 5 μM fura-2 acetoxy methyl ester. Cells were then washed twice with buffer and allowed to incubate for a further 30 min at 37°C. These loading conditions generally resulted in moderate fluorescence similar to that observed for cells dialyzed under whole cell patch clamp with 100 μM fura-2 pentapotassium salt in the patch pipette.

Drugs and chemicals

Extracellular solutions For experiments performed under current-clamp conditions, the solutions were composed of (in mM): NaCl 138, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, HEPES 10, glucose 10 adjusted to pH 7.4 with NaOH. Voltage clamp experiments were performed with cells perfused with solutions containing (in mM): tetraethylammonium chloride (TEACl) 143, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, pH adjusted to 7.4 with TEA hydroxide. $BaCl_2$ 2 mM was substituted for $CaCl_2$ for experiments in which the I_{Ba} was measured. For experiments in which glutamate was applied by micropressure from a pipette (approximately 0.5–1 MOhm tip resistance) using a Picospritzer (6 p.s.i., 30 ms) the extracellular solutions contained no added Mg^{2+}, 3 μM glycine and 0.5 μM tetrodotoxin (TTX). For the micropressure application the pipette was placed approximately 50 μm from the cell.

Pipette solutions Solutions for voltage-clamp experiments containing (in mM): CsCl 135, $MgCl_2$ 1, HEPES 10, diTris phosphocreatinine 14, MgATP 3.6, 50 U ml^{-1} creatinine phosphokinase, adjusted to pH 7.1 with CsOH. For the measurement of I_{Ba}, I_{Ca} or experiments during which micropressure application of glutamate was performed, 10 mM 1,2-bis(2-aminophenoxy)ethane-N',N',N',N''-tetra acetic acid (BAPTA) was also present in the patch pipette. For combined I_{Ca} and $[Ca^{2+}]_i$ transients, 100 μM fura-2 pentapotassium salt was present in the patch pipette. The patch pipettes for current clamp contained (in mM): fura-2 pentapotassium salt 0.1, K gluconate 145, $MgCl_2$ K_2ATP 5, HEPES 5, pH 7.2, osmolality, 310 mosmol kg^{-1}. Amphotericin-B (150 μg ml^{-1}) was included in the patch pipette for the perforated patch experiments.

All reagents were of the highest commercial grade. Fura-2 pentapotassium salt was obtained from Molecular Probes Inc., Eugene, OR. Neuropeptide Y from Richeleau Biochemicals, Quebec and 6-cyano-7-dinitroquinoxaline (CNQX) from Research Biochemicals Inc. All other agents were obtained from Sigma Chemical Company.

Results

Hippocampal neurones form excitatory synapses with one another in culture (Forsythe & Westbrook, 1988; Abele *et al.*, 1990). Under conditions which optimize N-methyl-D-aspartate (NMDA) receptor stimulation, i.e. perfusion in the presence of glycine (10 nM) and in the absence of added Mg^{2+}, hippocampal neurones show frequent large fluctuations in their $[Ca^{2+}]_i$ (Abele *et al.*, 1990). These fluctuations result from spontaneous glutamatergic transmission between the cells. Spontaneous fluctuations in $[Ca^{2+}]_i$, which sometimes fused into a persistent plateau, were also observed in the present experiments. As indicated in Figure 1, fluctuations in $[Ca^{2+}]_i$ were prevented by a combination of CNQX (10 μM) and aminophosphonovalerate (APV) (50 μM), by TTX (1 μM), by Ni^{2+} (200 μM) and reduced by the $GABA_B$ agonist, baclofen (10 μM).

NPY and its homologue PYY (100 nM) were also effective in reducing $[Ca^{2+}]_i$ fluctuations in these hippocampal cultures (Figures 1e and 2a). Peptides which act at the Y_2 receptor for NPY, such as NPY 13-36 (100 nM), were also inhibitory (Figure 2a). We examined these effects further by treating cultures overnight with pertussis toxin (PTX, 150 ng ml^{-1},

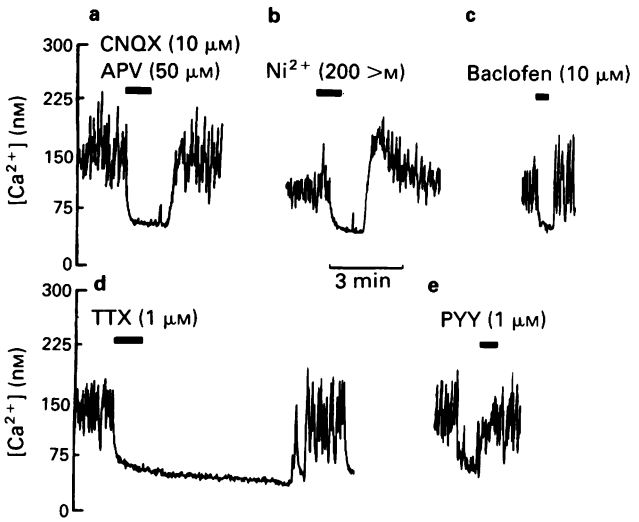

Figure 1 Typical examples of spontaneous fluctuations in $[Ca^{2+}]_i$ observed in single hippocampal neurones in culture. Shown are the effects of perfusion with solutions containing (a) CNQX (10 μM) and APV (50 μM); (b) Ni^{2+} (200 μM); (c) baclofen (10 μM), (d) TTX (1 μM) and (e) PYY (100 nM). For abbreviations, see text.

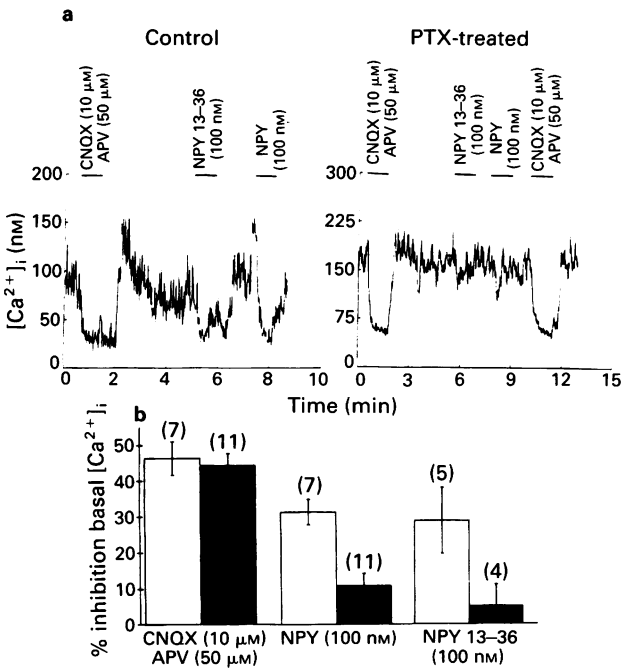

Figure 2 (a) Records of spontaneous $[Ca^{2+}]_i$ fluctuations in hippocampal neurones and the effects of neuropeptide Y (NPY, 100 nM) and NPY 13-36 (100 nM) under control conditions and following pretreatment with pertussis toxin (PTX, 150 ng ml^{-1}, 17–20 h). Also shown is the effect of a combination of CNQX (10 μM) and APV (50 μM). (b) Histogram summarizing the effects of PTX treatment (150 ng ml^{-1}, 17–20 h) on the NPY (100 nM), NPY 13-36 (100 nM) and CNQX/APV-dependent reduction in the mean $[Ca^{2+}]_i$. The reduction in $[Ca^{2+}]_i$ was calculated from an average of thirty, 1 s values prior to and following application of the agents shown. The numbers in parentheses are the number of cells on which the agents were tested. Open columns: controls; solid columns: PTX-treated (*n*). For abbreviations, see text.

17–20 h). Following such treatment, NPY (100 nM) became largely ineffective in reducing fluctuations in $[Ca^{2+}]_i$. In PTX-treated cultures, addition of NPY (100 nM) usually produced a transient reduction in the prevailing $[Ca^{2+}]_i$ which quickly reversed. This contrasted with the control situation where addition of NPY (100 nM) reduced $[Ca^{2+}]_i$ to a greater extent and this persisted throughout the period of application (Figure 2a). Quantitation of these differences indicated that PTX-treatment greatly reduced the inhibitory effects of NPY (100 nM) and NPY 13-36 (100 nM) but not those produced by the combination of CNQX (10 μM) and APV (50 μM) (Figure 2b).

We next examined the ability of NPY (100 nM) to inhibit neuronal voltage-sensitive Ca^{2+} currents in hippocampal neurones. Perfusion of hippocampal neurones with a solution containing 50 mM KCl (iso-osmotically substituted for NaCl) produced large and rapid increases in $[Ca^{2+}]_i$ (Figure 3). Treatment of cells with NPY (100 mM) reduced this increase by 13.6 ± 3.8% ($n = 8$). A combination of CNQX (10 μM)/APV (50 μM), reduced the response by a similar amount (17.3 ± 4.5%, $n = 7$). In the presence of CNQX (10 μM) and APV (50 μM) no additional effect of NPY (100 nM) was discernable ($\Delta = 5.0 \pm 2.9\%$, $n = 7$). We interpret this result as follows. The response to 50 mM K^+ is mostly due to direct depolarization of the cell under observation. A small component of the effect is due to glutamate release from adjoining cells which make synaptic contact with the observed cell. When this minor component is blocked by CNQX/APV, NPY (100 nM) has no further effect.

It has been shown elsewhere that agonists acting at $GABA_B$ or A_1 adenosine receptors and which effectively

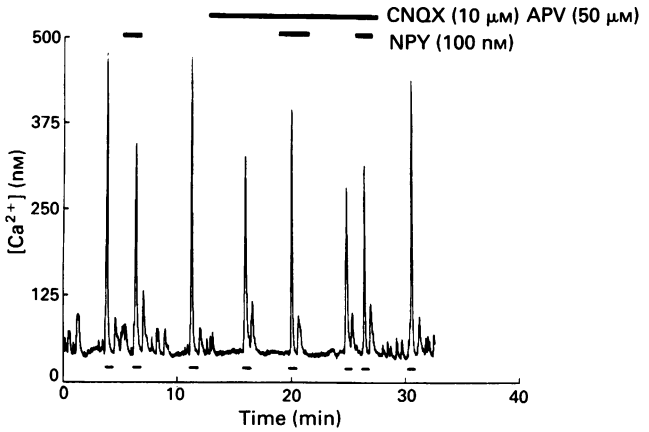

Figure 3 A typical experiment illustrating increases in a single hippocampal neurone $[Ca^{2+}]_i$ resulting from 30 s perfusions with solutions containing 50 mM KCl and showing the inhibitory effects of neuropeptide Y (NPY, 100 nM), CNQX (10 μM) plus APV (50 μM), and CNQX (10 μM) plus APV (50 μM) plus NPY (100 nM). For abbreviations, see text.

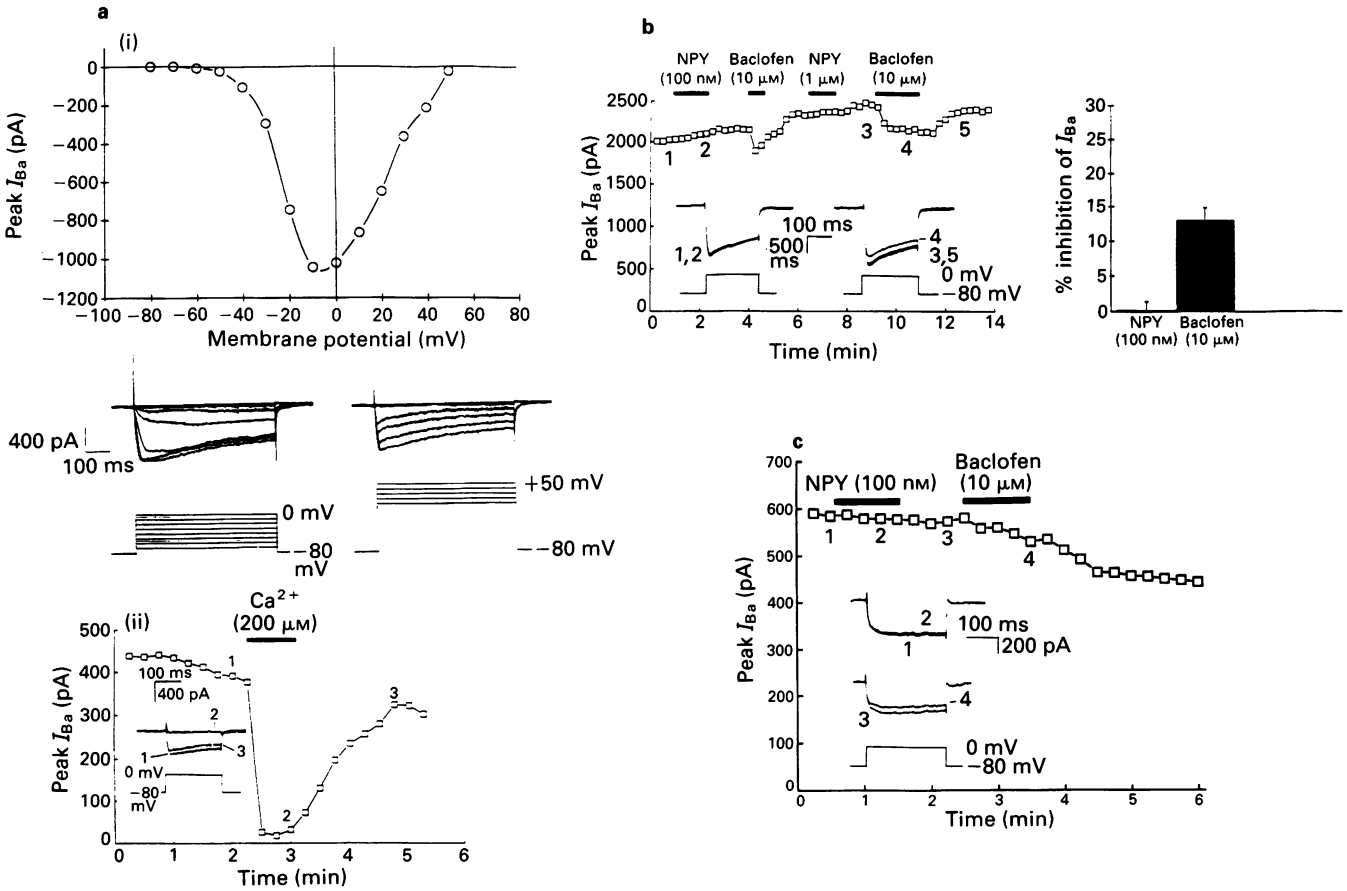

Figure 4 (a) (i) Ba^{2+} current (I_{Ba}) recorded in single hippocampal neuronal cell bodies under voltage-clamp conditions and associated current/voltage relationship in a single hippocampal neurone. $V_h = -80$ mV. (ii) Time course illustrating the inhibitory effect of Cd^{2+} (200 μM) on the I_{Ba}. Insets show the individual current records as denoted in the time course. (b) Time course of the effect of neuropeptide Y (NPY, 100 nM and 1 μM) and baclofen on the I_{Ba} recorded in a hippocampal neurone cell soma. The insets show individual current traces. The histogram summarizes the effects of NPY (100 nM) and baclofen (10 μM) on the recorded I_{Ba}. The inhibition produced was calculated by plotting the peak I_{Ba} as a function of time for at least three data points prior to the addition of the drug. The time course was then plotted as a linear function and the decline in the I_{Ba} extrapolated to the point at which the effects of the drug were stable. The expected magnitude of the I_{Ba} was then calculated and used to obtain the inhibition produced ($n = 5$). (c) Time course showing the effect of NPY (100 nM) and baclofen (10 μM) on the I_{Ba} recorded with GTP-γ-S (150 μM) in the patch pipette. Baclofen produced a 12.9 ± 2.1% inhibition of the I_{Ba} measured 2 min following drug application. NPY produced a 1.9 ± 1.5% inhibition 2 min following application to the same cells ($n = 4$).

block excitatory transmission in the hippocampal slice and in culture, also inhibit the Ca^{2+} current (I_{Ca}) recorded in the cell body of hippocampal neurones (Scholz *et al.*, 1990; Scholz & Miller, 1991a,b). Figure 4a illustrates the I_{Ba} recorded in the cell soma of one of these neurones, as well as its inhibition by Cd^{2+}. Figure 4b shows that the $GABA_B$ agonist, baclofen (10 μM) reduced I_{Ba} in these neurones, whereas NPY (100 nM and 1 μM) was without effect. Inhibition of I_{Ba} was also examined with the non-hydrolyzable analogue of GTP, GTP-γ-S, in the patch pipette (Figure 4c). GTP-γ-S enhances the inhibitory effects of neurotransmitters on neuronal Ca^{2+} currents and renders them irreversible (Dolphin & Scott, 1987). Thus we argued that GTP-γ-S might reveal any small effects of NPY on the I_{Ba} which might not be readily apparent. In the present study inclusion of GTP-γ-S in the patch pipette (150 μM) produced a reduction in the activation rate of the I_{Ba} similar to that reported in other studies (Bean, 1989). However NPY (100 nM) was still without effect whereas baclofen (10 μM) now irreversibly reduced the I_{Ba} in the same cells.

We now tested the possibility that the membrane of an effect of NPY on the I_{Ba} was the result of dialysis of some essential intracellular component necessary for coupling of the NPY receptor to the somatic Ca^{2+} channels. With the pore-forming antibiotic amphotericin-B (150 μg ml^{-1}) in the pipette, electrical access to the cell occurs but dialysis of large intracellular components is prevented (Rae *et al.*, 1991). Under these conditions NPY still had no effect on the I_{Ba} recorded in the cell soma, while in the same neurone, baclofen (10 μM) reduced the I_{Ba} (inhibition for NPY = 0.1 ± 0.7%, baclofen = 18.5 ± 2.4% $n = 3$, mean ± s.d.).

In order to test the possibility that NPY directly blocks postsynaptic glutamate receptors, we also examined the effect of NPY on inward currents recorded in hippocampal neurones generated by 30 ms applications of glutamate (50 μM). Figure 5 shows such an experiment in which the inward current observed was unaffected by NPY (100 nM). In the same neurone it was possible to reduce the glutamate-activated currents using APV (50 μM) and CNQX (10 μM) (not shown).

Current clamp recordings were used to analyze further the effects of NPY. As can be seen in Figure 6a, recordings from cultured hippocampal neurones revealed the presence of excitatory postsynaptic potentials (e.p.s.ps) that sometimes triggered action potentials. In the presence of NPY (100 nM), the spontaneous activity and the resulting increase in $[Ca^{2+}]_i$ was abolished (Figure 6a and b). NPY had no effect on the resting membrane potential of the cells. The observed activity and associated increases in $[Ca^{2+}]_i$ were also prevented by a combination of CNQX (10 μM) and APV (50 μM) (not shown). In addition, spontaneous e.p.s.ps and the accompanying elevated $[Ca^{2+}]_i$ observed in the absence of evoked trains of action potentials could also be prevented by NPY (12 of 15 cells examined). Furthermore, e.p.s.ps could also be abolished by baclofen (10 μM), Ni^{2+} (200 μM), and NPY 13-36 (100 nM) (not illustrated). It should be noted that in 3 cells examined (1 of 15 cells under current clamp conditions and 2 of 32 cells under fura-2 AM recording conditions), NPY produced an increase in the spontaneous activity accompanied by a depolarization or rise in the basal $[Ca^{2+}]_i$ (not shown). Interestingly, this effect was not mimicked by NPY 13-36 which may suggest that it is not due to activation of Y_2 receptors for NPY. Since this effect occurred so infrequently we did not examine it in detail.

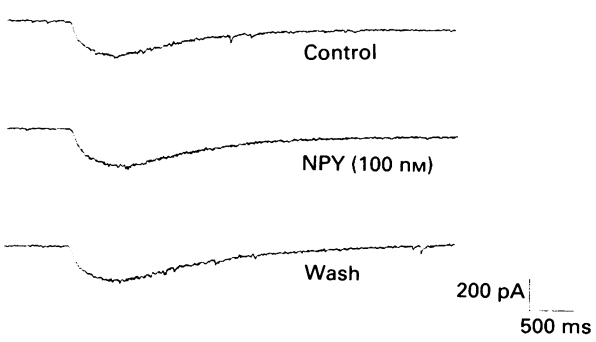

Figure 5 Inward currents associated with pressure ejection application of glutamate (50 μM) for brief periods (ejected under positive pressure of 6 p.s.i., picospritzer, 30 ms) at a holding potentials of −60 mV. Individual examples showing averaged currents (of 5 sweeps of data, 20 s apart) for application of glutamate (50 μM, 30 ms) in the absence of neuropeptide Y (NPY, 100 nM), in the presence of NPY (100 nM) and following washout of the peptide. The data shown are representative of 4 separate experiments.

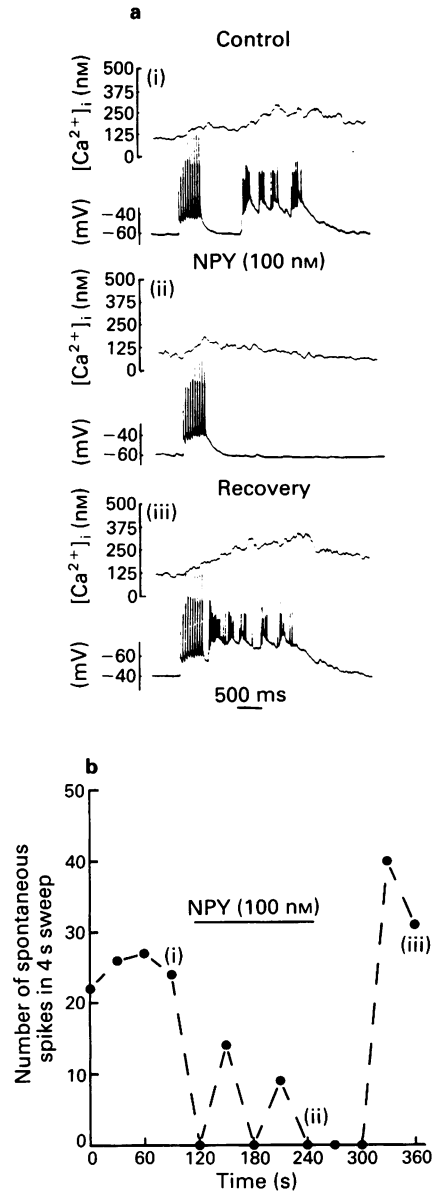

Figure 6 (a) Current clamp records and simultaneous $[Ca^{2+}]_i$ measurements in cells under whole cell patch clamp with 100 μM fura-2 in the patch pipette. Action potentials were evoked by brief current injection (4–8 ms, 100–700 pA). Records show sweeps in the absence (i), presence (ii) and 2 min following washout (iii) of neuropeptide Y (NPY, 100 nM). (b) Plot illustrating the effect of NPY (100 nM) on synaptic activity for the data shown in (a). The total number of action potentials observed in a 4 s sweep of collected data has been plotted for each successive sweep. Sweeps of data were collected every 30 s and NPY (100 nM) included in the perfusion solution as denoted by the horitzontal bar.

Increases in $[Ca^{2+}]_i$ could also be produced when cells were induced to fire trains of action potentials following brief injections of depolarizing current as shown in Figure 6a. NPY (100 nM) had no effect on the magnitude of the action potential-induced increases in $[Ca^{2+}]_i$ in the cell soma. In some experiments, CNQX (10 μM) and APV (50 μM) were included in the perfusing solutions to prevent spontaneous activity; again, NPY (100 nM) had no effect on the magnitude of the action potential-induced increases in $[Ca^{2+}]_i$ in the cell soma. Under these conditions the mean change in the peak increase in $[Ca^{2+}]_i$ in the presence of NPY (100 nM) was $3.5 \pm 6.0\%$ ($n = 6$).

Discussion

The presence observations confirm previous reports showing that NPY can effectively inhibit excitatory glutamate-mediated synaptic transmission in the hippocampus (Colmers *et al.*, 1985; 1987; 1988; 1991; Haas *et al.*, 1987; Abele *et al.*, 1990). Although the precise mechanism by which NPY produces these effects remains unclear, the data suggest several possible explanations, and eliminate others.

NPY is extensively distributed in the peripheral nervous system (Lundberg *et al.*, 1982; Sundler *et al.*, 1983) and is an effective inhibitor of neurotransmitter release at a number of peripheral neuroeffector junctions (Sundler *et al.*, 1983; Wahlestedt *et al.*, 1985; Stretton & Barnes, 1988; Grundemar *et al.*, 1988; Walker *et al.*, 1988; Wiley *et al.*, 1990). NPY has been shown to inhibit the I_{Ca} in the cell bodies of rat DRG neurones (Walker *et al.*, 1988), enteric neurones (Hirning *et al.*, 1990) and sympathetic neurones (Schofield & Ikeda, 1988). These are all tissues in which the peptide also blocks neurotransmitter release (Lundberg *et al.*, 1982; Friel *et al.*, 1986; Walker *et al.*, 1988; Wiley *et al.*, 1990). Furthermore in these peripheral neurones, NPY appears to inhibit selectively the N-type Ca^{2+} channel (Hirning *et al.*, 1990; Wiley *et al.*, 1990; Plummer *et al.*, 1991), which has been associated with providing Ca^{2+} influx associated with neurotransmitter release (Miller, 1990). The coupling of the NPY receptor and the Ca^{2+} channels appears to be mediated by a PTX-sensitive G-protein. In DRG cells, NPY has no effect on other membrane properties, suggesting a single site of action of the Y_2 NPY receptor (Bleakman *et al.*, 1991).

An important question is whether inhibition of the neuronal I_{Ca} underlies the presynaptic inhibitory effects of NPY in the central nervous system as well. A number of neurotransmitters that are capable of inhibiting Ca^{2+} currents in central neurones such as $GABA_B$ agonists, A_1 adenosine agonists and NPY (reviewed in Miller, 1990; see also Chemevskaya *et al.*, 1981; Toselli *et al.*, 1989; Fisher & Johnston, 1990; Pennington & Kelly, 1990; Scholz *et al.*, 1990; Scholz & Miller, 1991a,b) can also inhibit synaptic transmission in cultured hippocampal neurones (Abele *et al.*, 1990; Scholz *et al.*, 1990; Scholz & Miller, 1991a,b). We have shown elsewhere that the inhibitory effects of A_1 adenosine and $GABA_B$ agonists are mediated by pertussis toxin-sensitive G-proteins and are associated with inhibition of Ca^{2+} influx monitored in the soma of these cells (Scholz *et al.*, 1990; Scholz & Miller, 1991a,b). Nevertheless, the latter two receptors can also increase I_K in central neurones (Greene & Haas, 1985). Although NPY also inhibits excitatory synaptic transmission in rat hippocampal slices and cultures, it differs from $GABA_B$ or A_1 adenosine agonists in that it has no effect on postsynaptic ion channels including the Ca^{2+} channels either in the slice (Colmers *et al.*, 1987; 1988) or in culture. In addition no effect of NPY on the I_{Ba} was seen when washout of intracellular components was prevented by the use of the perforated patch technique. All of this contrasts with the effects of NPY on peripheral neurones where it produces large inhibitory effects on the I_{Ca} using precisely the same paradigms (Hirning *et al.*, 1990; Thayer & Miller, 1990; Bleakman *et al.*, 1991; Plummer *et al.*, 1991). It is particularly interesting to note that NPY did not reduce Ca^{2+} influx into the neuronal soma in response to a train of action potentials. In these experiments, no effect on resting membrane potential was observed, indicating that there also appears to be no effect of NPY on resting K^+ conductance in the cell body. Nevertheless a selective activation of a K^+ conductance in the nerve terminal cannot be ruled out entirely. Finally, the possibility that the inhibitory action of NPY was due to an effect on the postsynaptic response to endogenously released glutamate was also examined. We show that glutamate applications to the cell soma, in the absence of synaptic transmission, were unaffected by NPY.

The simplest hypothesis for the mechanism of NPY action is that it inhibits Ca^{2+} influx, but that its receptors and their physiological effects are selectively localized to presynaptic terminals. There is both physiological and anatomical evidence consistent with this hypothesis. In the hippocampal slice, applications of low concentrations of 4-aminopyridine (4-AP) blocked the presynaptic inhibition mediated by NPY. However, lowering the extracellular concentration of Ca^{2+} completely restored the ability of NPY to inhibit transmitter release (Colmers *et al.*, 1988; Klapstein & Colmers, 1992), which is consistent with a mechanism involving regulation of Ca^{2+} influx. Also consistent with this hypothesis is evidence from autoradiographic studies that indicate the presence of NPY receptors in strata oriens and radiatum, where the terminals of the excitatory input to the pyramidal neurones are found. However, few receptors are found in the cell body layer (Martel *et al.*, 1986; 1990). An alternative hypothesis is that NPY blocks glutamate release by a mechanism which does not require it to block Ca^{2+} influx. For example, there is evidence for such a mechanism mediating at least part of the inhibition of transmitter release produced by adenosine at some mammalian neuromuscular junctions (Silinsky, 1986). However one would predict that if NPY interfered with glutamate release at a step subsequent to Ca^{2+} entry, then this inhibition should not be sensitive to manipulations of presynaptic Ca^{2+} presumably caused by 4-AP as observed (Colmers *et al.*, 1988; Colmers *et al.*, 1991). Thus, we conclude that NPY appears to abolish excitatory neurotransmission in cultured hippocampal neurones by preventing glutamate release from presynaptic nerve terminals and that the NPY receptors in the hippocampus are located at or near these terminals.

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