Neuropeptide Y Action in the Rat Hippocampal Slice: Site and Mechanism of Presynaptic Inhibition

William F. Colmers, Ken Lukowiak, and Quentin J. Pittman

Neuroscience Research Group and Department of Medical Physiology, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Neuropeptide Y (NPY), the most abundant peptide in mammalian CNS, has been shown to inhibit excitatory neurotransmission presynaptically at the stratum radiatum–CA1 synapse in the in vitro rat hippocampal slice. We examined the site and mechanism of this inhibition in a series of in vitro intra- and extracellular recordings in areas CA1 and CA3, the source of much of the excitatory synaptic input to the CA1 neurons.

NPY's inhibitory action at the stratum radiatum–CA1 synapse was unaffected by high concentrations of the antagonists bicuculline, theophylline, or atropine, suggesting that it does not act by stimulating the release of the known presynaptic inhibitory transmitters GABA, adenosine, or ACh, respectively.

Bath application of 10^{-6} NPY, a concentration that strongly inhibited the stratum radiatum–CA1 synapse had no effect on CA3 neuron resting potential, input resistance or action potential amplitude, threshold, or duration. NPY also does not alter the amplitude or duration of the prolonged CA3 action potentials evoked in the presence of TTX, tetraethylammonium, and elevated external Ca^{2+} or those evoked in the presence of TTX and Ba^{2+} ions. NPY therefore does not alter the passive or active properties of the somata of the presynaptic CA3 neurons.

Neither the afferent fiber volley of the Schaffer collaterals in stratum radiatum of area CA1 nor the excitability of the CA3 terminals in CA1 was affected by NPY application. However, application of the transient K^+ current blocker, 4-aminopyridine (4-AP) at concentrations of 10 and 50 μM, completely abolished the action of 10^{-6} M NPY on the stratum radiatum–CA1 excitatory synaptic potentials. This action of 4-AP could be reversed by reducing extracellular Ca^{2+} concentrations from a control level of 1.5 to 0.7 mM (in 10 μM 4-AP) and to 0.5 mM (in 50 μM 4-AP).

The evidence suggests that NPY inhibits excitatory synaptic transmission at the Schaffer collateral–CA1 synapse by acting directly at the terminal to reduce a Ca^{2+} influx.

The 36 amino acid peptide, neuropeptide Y (NPY), isolated from porcine CNS by Tatemoto in 1982, is the most abundant peptide yet described in the brain (Chronwall et al., 1985). NPY is distributed widely through the CNS and PNS of mammals (O'Donohue et al., 1985). NPY has been shown to be present within neurons of the hippocampus (Kohler et al., 1986) and to bind specifically and with high affinity to structures in the hippocampus (Martel et al., 1986). Recent studies from our laboratory have examined the effect of NPY on synaptic transmission in the mammalian CNS, using the in vitro rat hippocampal slice preparation. Bath application of NPY reduced the amplitude of the population spike (p.s.), evoked orthodromically in area CA1 of the hippocampus by stimulation of stratum radiatum, in a reversible, concentration-dependent manner (Colmers et al., 1985; Haas et al., 1987). A subsequent study (Colmers et al., 1987) showed that the mechanism of this effect was a reduction in the amplitude of the excitatory postsynaptic potential (EPSP) elicited in the CA1 pyramidal neurons by the stimulus. Because no postsynaptic site of action could be demonstrated, this study strongly suggested that NPY presynaptically inhibited this EPSP.

There are several sites presynaptic to the hippocampal CA1 pyramidal neurons at which NPY might act. The synaptic terminals themselves or the axons or somata that give rise to these terminals (predominantly the CA3 pyramidal neurons and their axons, the Schaffer collaterals; Swanson et al., 1978) may possess NPY receptors. The peptide could act directly at these, possibly by altering the excitability of the terminals, axons, or cell bodies, or by altering the calcium influx at the terminals, their axons, or somata. NPY could also act indirectly, via receptors on inhibitory interneurons, which would then release inhibitory transmitters, such as GABA (Simmonds, 1984; Davidoff and Hackman, 1985), ACh (Hounsgaard, 1978; Valentino and Dingledine, 1980), or adenosine (Dunwiddie, 1980; Dunwiddie et al., 1984) to reduce the excitability or stimulus–secretion coupling mechanisms of the terminals.

This study was designed to elucidate the site and mechanism of NPY's inhibitory action on the stratum radiatum–CA1 excitatory synapse. The results suggest that NPY acts directly on the synaptic terminals and may inhibit excitatory synaptic transmission by modulating a calcium influx at the terminals.

Materials and Methods

Transverse slices of hippocampus, 350–450 μm thick, taken from 75–200 gm male Sprague-Dawley rats, were prepared as described previously (Colmers et al., 1987). Slices were suspended on nylon mesh in a Plexiglas recording chamber. Temperature was maintained at 32 ± 0.2°C. Slices were perfused over both surfaces at a constant rate of between 2 and 3 ml/min with artificial cerebrospinal fluid (ACSF),
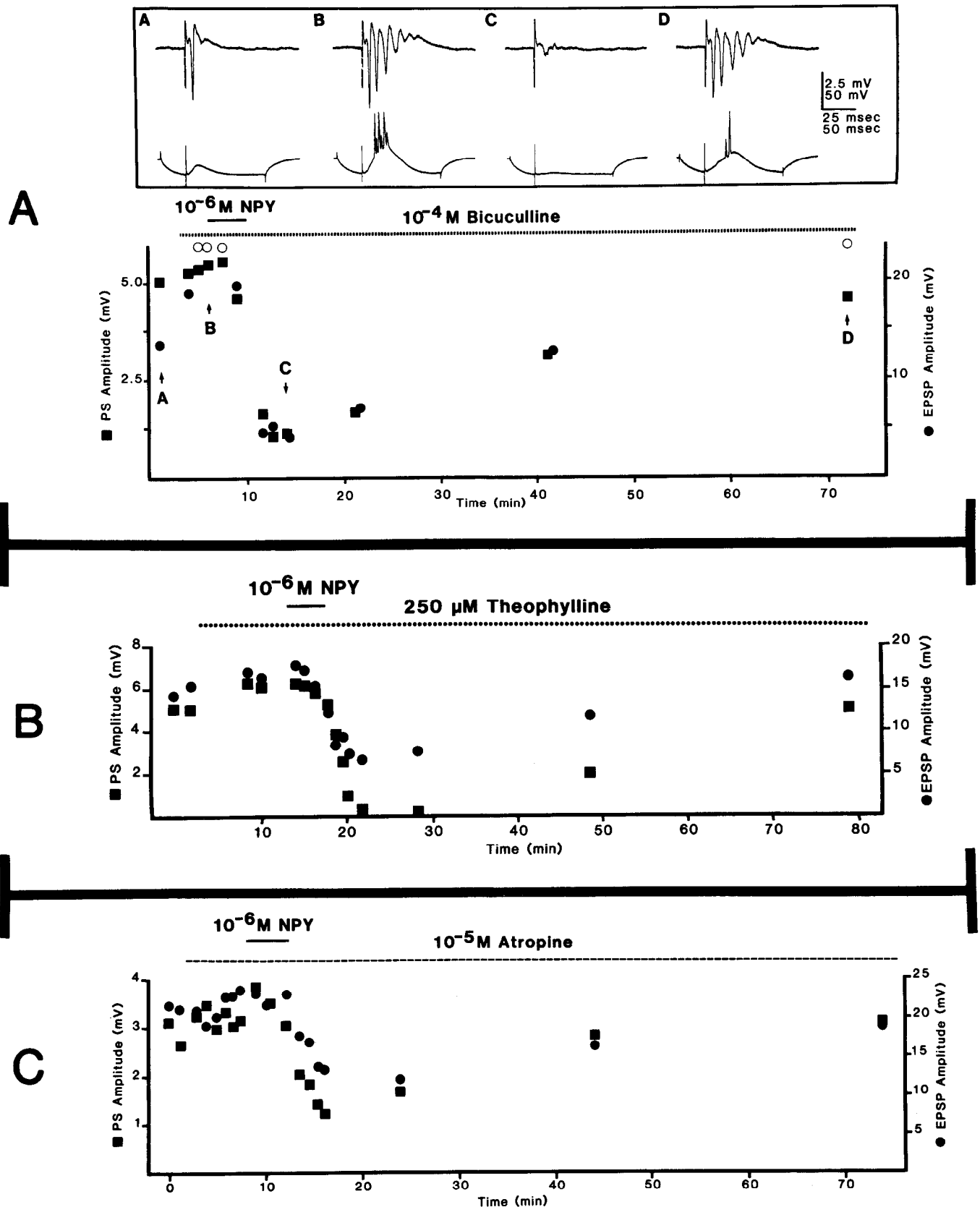
Figure 1. NPY inhibits excitatory transmission at the stratum radiatum–CA1 synapse in the presence of antagonists to receptors for known presynaptic inhibitory neurotransmitters. *A,* Effect of NPY in the presence of 10^{-4} M bicuculline methiodide, an antagonist for $GABA_A$ receptors. *Upper panel,* Extracellular (*upper traces*) and intracellular (*lower traces*) recordings from the cell body layer and pyramidal neurons of CA1, respectively, at times corresponding to those shown by *letters* on lower panel. Each trace is the average of 3 consecutive responses, 10 sec apart, in response to stimulation of stratum radiatum. *Lower panel,* Amplitude of the extracellularly recorded population spike (p.s.; *left-hand vertical axis; solid circles*) and excitatory postsynaptic potential (EPSP; *right-hand vertical axis; solid squares*) plotted against time. After control data were

whose pH and oxygen tension was maintained by continuous bubbling with a gas mixture of 95% O_2, 5% CO_2. ACSF composition was (in mM): NaCl, 124; NaHCO$_3$, 26; MgSO$_4$, 2; KCl, 1.8; CaCl$_2$, 1.5; KH$_2$PO$_4$, 1.25. For experiments where Ca^{2+}-dependent action potentials were studied, 1 μM TTX, to block the transient NA^+-current action potential, and 5 mM tetraethylammonium chloride (TEA), to block K^+ currents and thus to enhance action potential duration, were added to the perfusate. In some experiments, 2 mM BaCl was added to the ACSF, from which the SO_4^{2-} and PO_4^{2-} were omitted and MgCl$_2$ substituted, in order to avoid precipitation of barium salts. TTX, 1 μM, was added to this medium. Bicuculline methiodide, 1,3-dimethylxanthine (theophylline), atropine sulfate, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), TTX, and BaCl$_2$ were obtained from Sigma (St. Louis); all other salts were obtained from Fisher Scientific (Fair Lawn, NJ).

Electrophysiological recordings. Intracellular and extracellular recordings were performed as described previously (Colmers et al., 1987). Briefly, microelectrodes containing 2 M potassium acetate, having resistances of 90–180 MΩ, and connected to a Dagan model 8100 amplifier used in the bridge current-clamp mode were lowered into the cell body layer of the slice. Pyramidal neurons were impaled and identified using standard electrophysiological criteria (Colmers et al., 1987). Neurons were selected whose resting membrane potential was stable at more negative than -50 mV for more than 20 min; to ensure stability, experimental manipulations were generally not initiated until 30 min after a neuron was impaled. Hyperpolarizing current pulses of different amplitudes, injected via the current-clamp bridge circuit of the amplifier, were used to enhance EPSP amplitude and to prevent neurons from reaching action potential threshold during orthodromic synaptic stimulation. Families of hyper- and depolarizing current pulses were used for estimation of input resistance, while depolarizing current pulses were used to evoke action potentials. Extracellular recordings were made with microelectrodes containing 2 M NaCl, with resistances of 2–6 MΩ, which were lowered into the cell body layer or dendritic layer until a maximum field potential response to a given stimulus was recorded.

Orthodromic and antidromic stimuli were provided by means of bipolar nichrome wire stimulating electrodes, using square-wave, monophasic pulses (100 μsec) supplied either by a constant-voltage stimulus isolation unit (Digitimer DS2, 2–25 V) or by a constant-current stimulus isolation unit (WPI 850, 0.1–3.5 mA). The stimulating electrode was placed on stratum radiatum, either near the CA2 or CA3 boundary to evoke orthodromic synaptic potentials in area CA1 or near the middle of area CA1 to evoke antidromic potentials in area CA3. Stimuli were presented at 0.1 Hz. For experiments in which the stratum radiatum–CA1 EPSP was studied, stimulus amplitude was varied between near threshold for a response to the maximum amplitude possible without the neuron achieving action potential threshold. For experiments in which the CA3 field potential response to antidromic stimuli was examined, stimulus amplitude was varied between threshold for the response and a maximum within the relatively linear portion of the stimulus–response relationship. For experiments in which the threshold for antidromic action potential invasion of a CA3 pyramidal neuron's soma was studied, stimulus strength was set to elicit an antidromic action potential approximately 50% of the time.

Once control responses were stable, synthetic porcine NPY (purchased either from Bachem, Torrance, CA, or the I.A.F., Montreal, Quebec, Canada, or kindly supplied to us by Dr. S. St-Pierre, Montreal), previously dissolved in ACSF or distilled water at a concentration of 10^{-4} M and stored in aliquots at -70°C until just prior to use, was diluted to a final concentration of 10^{-6} M in the appropriate ACSF. Ten milliliters of the peptide-containing ACSF was applied through the bath via a switching valve at the same flow rate as the control solution. The peptide was washed out with the control ACSF.

Data were collected and analyzed as described previously (Colmers et al., 1987). A Nicolet 4094 digital oscilloscope was used for current

and voltage measurements, and to average signals. Data were stored either on floppy diskettes or on FM tape (Racal 4 DS). A DC-coupled pen recorder (Gould 2200S) displayed a continuous record of cell membrane potential and injected currents.

Neurons were used as their own controls for statistical purposes. Numerical data are presented as means $\pm$ SEM. Statistical comparisons were performed using a Student's paired t test, unless otherwise noted. Neuron input resistance was calculated from the slope of a least-squares regression line fitted to the data. Slope resistance data were compared using an F test for comparing slopes of regression lines (Sokal and Rohlf, 1981). All data presented are from preparations monitored for at least 30 min after washout was initiated.

Results

The results were based on recordings from 26 CA3 pyramidal neurons and 22 CA1 pyramidal neurons from 41 separate preparations, under different experimental conditions. Intracellular and extracellular recordings in area CA1 were performed as described previously (Colmers et al., 1987).

Indirect action

Three neurotransmitters endogenous to the hippocampus, GABA (Storm-Mathisen, 1977), adenosine (Braas et al., 1986), and ACh (Hounsgaard, 1978; Valentino and Dingledine, 1980) are known to have presynaptic inhibitory actions in mammalian CNS excitatory pathways (Ginsborg and Hirst, 1972; Hounsgaard, 1978; Dunwiddie and Hoffer, 1980; Valentino and Dingledine, 1980; Davidoff and Hackman, 1985). To test the possibility that the observed effects of NPY on the stratum radiatum–CA1 synapse were due to enhanced release of other, endogenous transmitter substances which, in turn, presynaptically inhibit transmitter release at the stratum radiatum–CA1 synapse, the action of NPY was examined following blockade of the receptors mediating the presynaptic inhibition by these substances.

$GABA_A$ receptors

In the first series of experiments, we studied the effect of NPY on the stratum radiatum–CA1 synapse in the presence of a high concentration (10^{-4} M) of the GABA$_A$ receptor blocker bicuculline (Fig. 1A). The amplitude of the extracellularly recorded p.s. (Fig. 1A, upper panel, upper trace) and (in several experiments) intracellularly recorded EPSP (Fig. 1A, upper panel, lower trace), evoked by stratum radiatum stimulation, were recorded simultaneously (Colmers et al., 1987). After control data were taken (Fig. 1 A,A), ACSF containing 10^{-4} M bicuculline was applied to the slice, which rapidly became disinhibited, demonstrating multiple p.s.'s in response to the stimulus and a greatly enhanced EPSP, which often exceeded action potential threshold (Fig. 1 A,B and lower panel). Application of 10^{-6} M NPY in the presence of bicuculline rapidly reduced the amplitudes of both p.s. and EPSP (Fig. 1 A,C and lower panel). Subsequent washout of NPY allowed both p.s. and EPSP to return to near pre-NPY values (Fig. 1 A,D and lower panel). Similar results were obtained to 6 further experiments. The p.s. was

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gathered (A), 10^{-4} M bicuculline in ACSF was applied (*dotted line*), and the response allowed to stabilize (B). The p.s. response became larger, and multiple p.s.'s appeared; the EPSP became greater in amplitude and exceeded action potential threshold (*open circles*). Application of 10^{-6} M NPY in the presence of bicuculline caused a profound inhibition of the p.s. and EPSP (C), which gradually recovered to near pre-NPY values after 1 hr washout with bicuculline ACSF (D). B, Time course of an experiment similar to that in A, testing the effect of NPY in the presence of 250 μM theophylline (*dotted line*), an adenosine receptor antagonist. NPY, 10^{-6} M, strongly inhibited both p.s. and EPSP with theophylline present. Both measures recovered upon subsequent washout of the peptide. C, Time course of an experiment similar to those above, testing NPY's effect in the presence of 10^{-5} M atropine (*dashed line*), an antagonist of muscarinic cholinergic receptors. As in the above experiments, the antagonist did not interfere with the inhibitory action of NPY at the stratum radiatum–CA1 excitatory synapse.

reduced, in the presence of NPY, to $47.0 \pm 9.0\%$ of its pre-NPY value ($p < 0.001$, $n = 7$); the EPSP was reduced to $42.0 \pm 2.0\%$ ($p < 0.001$, $n = 3$).

Adenosine

A similar experimental design was used to test the effects of the adenosine receptor blocker theophylline on the action of NPY at the stratum radiatum–CA1 synapse (Fig. 1B). Application of 250 μM theophylline to the slice (a concentration shown to be effective in blocking the adenosine A_1 receptors responsible for presynaptic inhibition; Dunwiddie et al., 1984) increased both p.s. and EPSP amplitudes; application of 10^{-6} M NPY in the presence of theophylline reduced the p.s. to $13 \pm 1\%$ and the EPSP to $57 \pm 7\%$ of their values just prior to NPY application ($p < 0.001$, $n = 3$). As before, washout returned the values to near pre-NPY values. Experiments conducted at a higher theophylline concentration (1 mM) also failed to prevent the inhibitory effect of NPY.

Acetylcholine

In a third series of experiments, we examined whether NPY might be acting via the release of ACh, which has a presynaptic inhibitory effect via a muscarinic mechanism, in addition to its other, postsynaptic excitatory action (Hounsgaard, 1978; Valentino and Dingledine, 1980). Application of a high concentration of the muscarinic cholinergic blocker atropine (10^{-5} M) to the slice caused a slight enhancement of EPSP and p.s. amplitudes. Application of 10^{-6} M NPY in the presence of atropine (Fig. 1C) again caused a reduction in amplitude of the p.s. to $54 \pm 8\%$ and of the EPSP to $57 \pm 11\%$ of pre-NPY values ($p < 0.001$, $n = 3$). Washout reversed NPY's effects on both measures (Fig. 1C).

Direct action

CA3 somata. To examine whether NPY inhibited synaptic transmission at the Schaffer collateral–CA1 synapse by mechanisms detectable at the soma of the presynaptic neurons, intracellular recordings were made from the somata of CA3 pyramidal cells. Neurons met the following criteria: resting membrane potential more negative than -55 mV (average: -63.7 ± 1.15 mV, $n = 12$), input resistance of greater than 20 MΩ (average: 67.4 ± 6.90 MΩ, $n = 12$), action potential amplitude of >70 mV (average: 85.64 ± 3.14 mV, $n = 13$), and action potential duration, measured at the half-amplitude of the first action potential evoked by a depolarizing current pulse, <1.20 msec (average: 0.85 ± 0.037 msec, $n = 13$). These compare favorably with values obtained for these neurons by others (Brown et al., 1981; Turner and Schwartzkroin, 1984).

The bath application of 10^{-6} M NPY did not significantly alter any of the above active and passive properties of the CA3 pyramidal neurons, based upon paired statistical comparisons of each neuron's properties before and during the period of NPY's greatest effects on the stratum radiatum–CA1 synapse. The resting membrane potential ($p > 0.50$, $n = 18$; Fig. 2A), slope input resistance ($p > 0.50$, $n = 19$; Fig. 2B and C), action potential amplitude ($p > 0.5$, $n = 19$), action potential overshoot ($p > 0.5$, $n = 19$), and action potential duration ($p > 0.5$, $n = 19$) were all unaffected by the application of NPY to the preparation. In addition, the number of action potentials evoked by identical current pulses and the initial membrane voltage trajectories in response to the depolarizing current pulses were unaffected by the peptide.

Of 19 CA3 neurons recorded, 11 demonstrated spontaneous action potential discharge activity. Spontaneous discharge activity varied from 1 to over 100 action potentials per minute, with an average rate of 16.7 ± 7.4/min. Bath application of 10^{-6} M NPY had no significant effect on the rate of spontaneous discharge of these neurons (average: 14.27 ± 6.24, $p > 0.25$, $n = 11$; Fig. 2A).

Ca^{2+} spikes. The possibility remained that NPY might act by interfering with a calcium influx at the terminals, thus reducing transmitter release. Because such an action might have only a small effect at the electrically distant cell body, we sought to isolate and enhance these Ca^{2+} influxes, so these changes could be recorded at the cell soma, as has been seen in other systems (Mudge et al., 1979; Klein et al., 1980; Goldberg et al., 1987). Two experimental approaches were employed to enhance calcium influx into neurons.

In the first series of experiments, CA3 neurons were impaled, then perfused with ACSF containing 1 μM TTX, to block the Na^+-dependent action potential, and 5 mM TEA, to inhibit the time- and voltage-dependent K^+ currents causing repolarization (e.g., Llinás and Yarom, 1981). The concentration of Ca^{2+} was also increased to 4 mM. Under these conditions, the duration of the action potential, measured at 50% repolarization, increased from 0.8–1.0 msec to > 40 msec. The resulting pronounced plateau phase of the action potential made comparisons of its duration at 66% repolarization a more consistent measure than at 50% repolarization. Application of 10^{-6} M NPY to the preparation did not result in any decrease in action potential duration in any of the experiments ($p > 0.25$, $n = 3$, Student's t test for difference in population means).

The second approach was to further enhance calcium currents by adding Ba^{2+} ions to a bathing medium that contained TTX but no TEA. Since Ba^{2+} ions are more permeable in most Ca^{2+} channels than are Ca^{2+} ions, and slow channel inactivation (McClesky et al., 1986), the resulting action potentials were of considerably longer duration than the Ca^{2+} action potentials, sometimes not repolarizing for over 1 sec (Fig. 3A). The Ba^{2+} action potentials often had multiple peaks separated by partial repolarizations, which then often settled into a relatively long plateau potential before repolarizing completely (Fig. 3A). The resting potential would often become unstable in the presence of barium, with large hyperpolarizations and depolarizations occurring if current was not passed to counteract them. Because the duration of the action potential has been reported to be sensitive to the resting potential (McBurney and Neering, 1985; Dolphin et al., 1986), care was taken to ensure that the resting potential remained relatively constant during the experiment. Subsequent analysis showed that there was no significant relationship between the action potential duration and resting potential within the range encountered during the experiment. Despite this, the duration of the action potential was quite variable under control conditions (Fig. 3B). Application of 10^{-6} M NPY to these neurons caused no significant alteration in the duration of the action potential ($p > 0.5$, $n = 3$).

CA3 axons. As NPY had no detectable effects on the passive or active properties of the CA3 somata, we next examined whether the peptide affected the excitability of the axons of CA3 neurons, the Schaffer collaterals. An extracellular microelectrode, placed in stratum radiatum of area CA1 was used to record the field potential evoked by orthodromic stimulation of stratum radiatum. The first component of the field potential after the stimulus artifact is the afferent volley, representing the
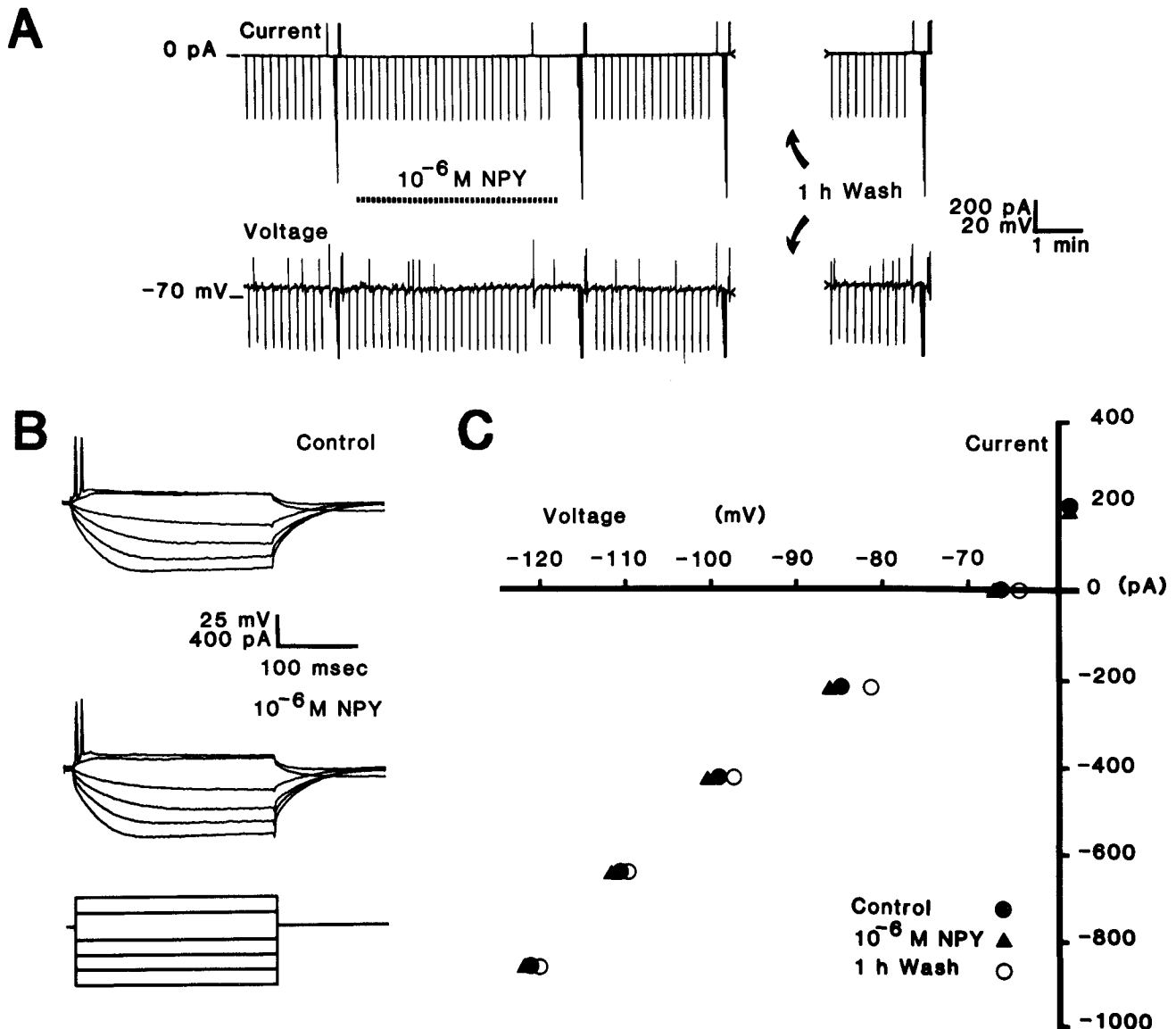
Figure 2. Summary of a typical experiment designed to test possible effects of NPY on the intracellularly recorded properties of a CA3 pyramidal neuron of rat hippocampal slice. *A, Upper trace:* Intracellular current passed through the bridge circuit of the current-clamp amplifier. Downward deflections indicate passage of hyperpolarizing current through the electrode. *Lower trace:* Intracellular voltage. NPY was bath-applied at 10^{-6} M during the time indicated by the *horizontal bar.* Washout with control ACSF began immediately after NPY application ended. Note absence of effect by NPY on resting potential. *B,* Oscilloscope traces of changes in membrane potential of CA3 neuron in response to hyperpolarizing and depolarizing current pulses *(lower trace)* in control *(upper trace)* and after application of 10^{-6} M NPY *(middle trace). C,* Plot of current–voltage relationship derived from records in *B* illustrating absence of effect of NPY on membrane input resistance of neuron. Symbols: control, ●; 10^{-6} M NPY, ▲, 1 hr washout, ○ (not shown in *B*).

discharge of the afferent axons (Fig. 4); the larger, slower component being the population EPSP, upon which inverted, attenuated p.s.'s are superimposed (Andersen et al., 1978). Application of 10^{-6} M NPY to this preparation did not affect the amplitude of the afferent volley, although the population EPSP and p.s. were both reversibly attenuated by the peptide (Fig. 4). The results of this experiment were confirmed in others in which a clear afferent volley was also discernible ($p > 0.5$, $n = 3$).

CA3 terminals. Because neither the axons nor the cell bodies of the CA3 pyramidal neurons appear to be affected by application of concentrations of NPY which produced strong inhibition of both EPSP and p.s. in area CA1 (Colmers et al., 1985, 1987), we next examined whether NPY altered the excitability of the terminals of the CA3 neurons. Two experimental ap-

proaches were used. In the first, CA3 neurons were impaled and stimulated antidromically from stratum radiatum of area CA1, where their axons terminate on the apical dendrites of the CA1 pyramidal cells (Swanson et al., 1978). Action potentials were considered antidromic if they demonstrated constant latency, were initiated suddenly, rather than subsequent to a slower depolarization, and followed high-frequency stimuli (Fig. 5B). Spontaneously active neurons often demonstrated collision cancellation as well (Fig. 5B2). A slow depolarizing potential, which occasionally caused the neuron to achieve threshold (Fig. 5B2), often followed the initial antidromic action potential; this was judged to be synaptic in origin (see below). Care was taken to set stimulus strength so that antidromic action potentials were evoked by approximately 50% of the stimuli. NPY was then
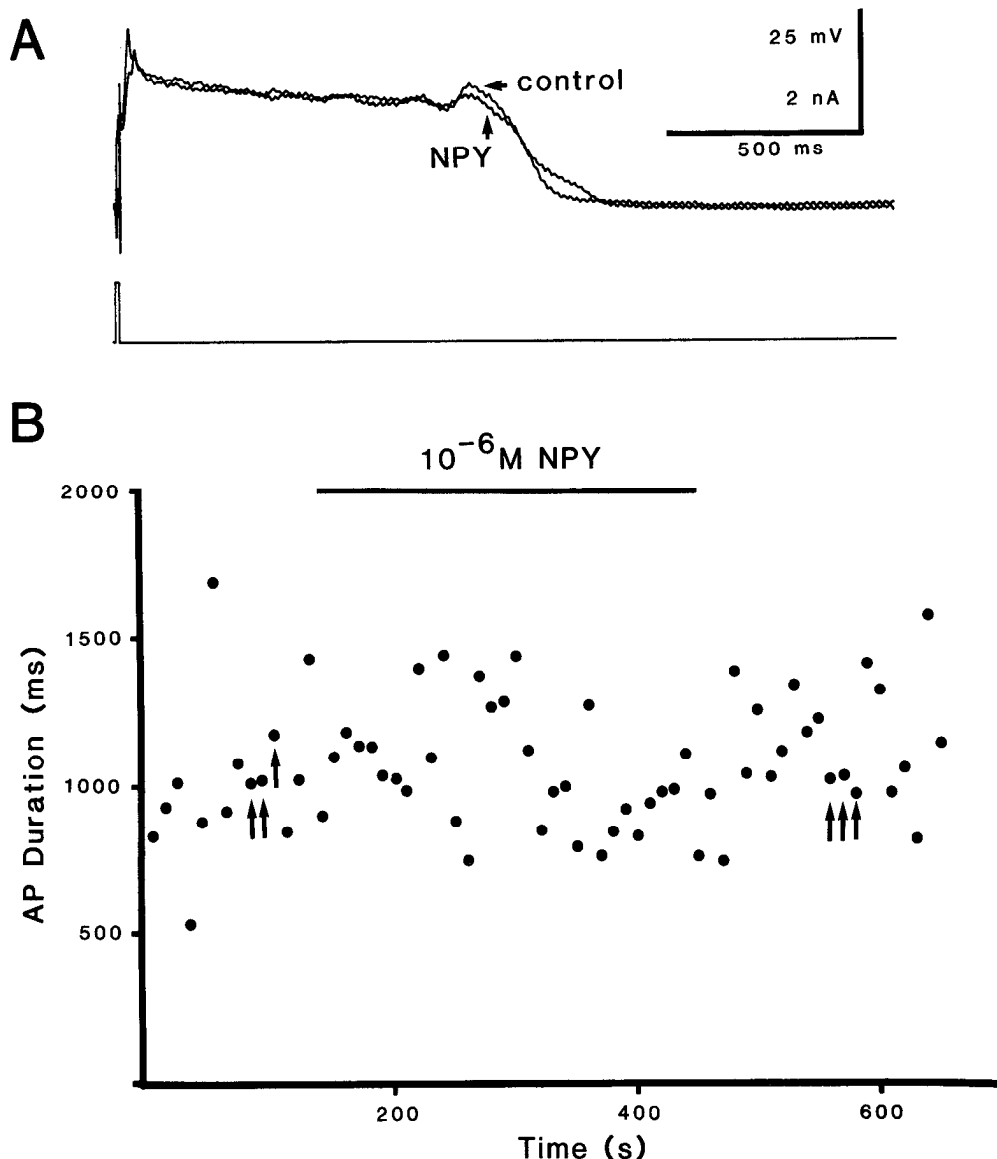
A

B

Figure 3. Ba^{2+}-dependent action potentials in CA3 pyramidal neuron. Na^{+}-dependent potentials were prevented by application of 10^{-6} M TTX prior to NPY application. *A,* Shown superimposed are the averages of 3 successive action potentials each in control and in 10^{-6} M NPY (*arrows* in *B*). *B,* Duration of Ba^{2+}-dependent action potentials of CA3 pyramidal neuron in *A* plotted against time. Average duration was 1.066 ± 0.044 sec (*n* = 20) prior to NPY application. Application of 10^{-6} M NPY (*bar*) had no effect on duration (average = 1.067 ± 0.035 sec, *n* = 20, *p* > 0.5).

applied and the cell monitored for changes in either the probability of antidromic action potential invasion or for a change in latency (Wall, 1958; Carpenter et al., 1963). Figure 5 illustrates the results from one such experiment. Bath application of NPY affected neither the latency (Fig. 5C) nor the probability of antidromic action potential invasion (*p* > 0.5, Student's *t* test for difference in population means). Results from 10 NPY applications to 7 CA3 neurons were similar.

Since the antidromically evoked p.s. represents the excitation of a population of CA3 neurons from their terminals, it could provide a more sensitive index of any small changes in terminal excitability than the behavior of an individual neuron. The second experimental approach was therefore to place an extracellular microelectrode in the cell body layer of area CA3 and stimulate stratum radiatum as before. In this instance, we monitored the amplitude of the antidromically evoked p.s., applied NPY, and determined if there was a change in the p.s. amplitude associated with peptide application (Fig. 6).

At lower stimulus strengths, a single-component, short-latency field potential was evoked; with increasing stimulus strength, a second component emerged. The second component was most

likely synaptic in origin, as it was reversibly abolished by application of ACSF containing 0.5 mM Ca^{2+}, 3.5 mM Mg^{2+} (Colmers et al., 1987); the first component was unaffected by this treatment and was therefore considered antidromic (Fig. 6A). This procedure was carried out with each experimental preparation in this series.

Application of 10^{-6} M NPY to this preparation did not affect the amplitude of the antidromic component of the CA3 field potential (Fig. 6B). This finding was confirmed in other experiments (*p* > 0.50, *n* = 4). In one experiment, the stimulus strength was varied to produce a stimulus–response relationship (Fig. 6C). NPY did not significantly affect the amplitude of the antidromic population spike at any of the stimulus strengths examined. In 2 of the 4 experiments, the synaptic component of the field potential was reversibly attenuated by NPY. The reduction was not as dramatic as was seen in area CA1 (Colmers et al., 1985).

Although NPY did not measurably alter the excitability of the CA3 terminals of stratum radiatum, the possibility remained that it could affect conductances that are not active at rest. To examine this, we studied the effect of NPY on the stratum ra-
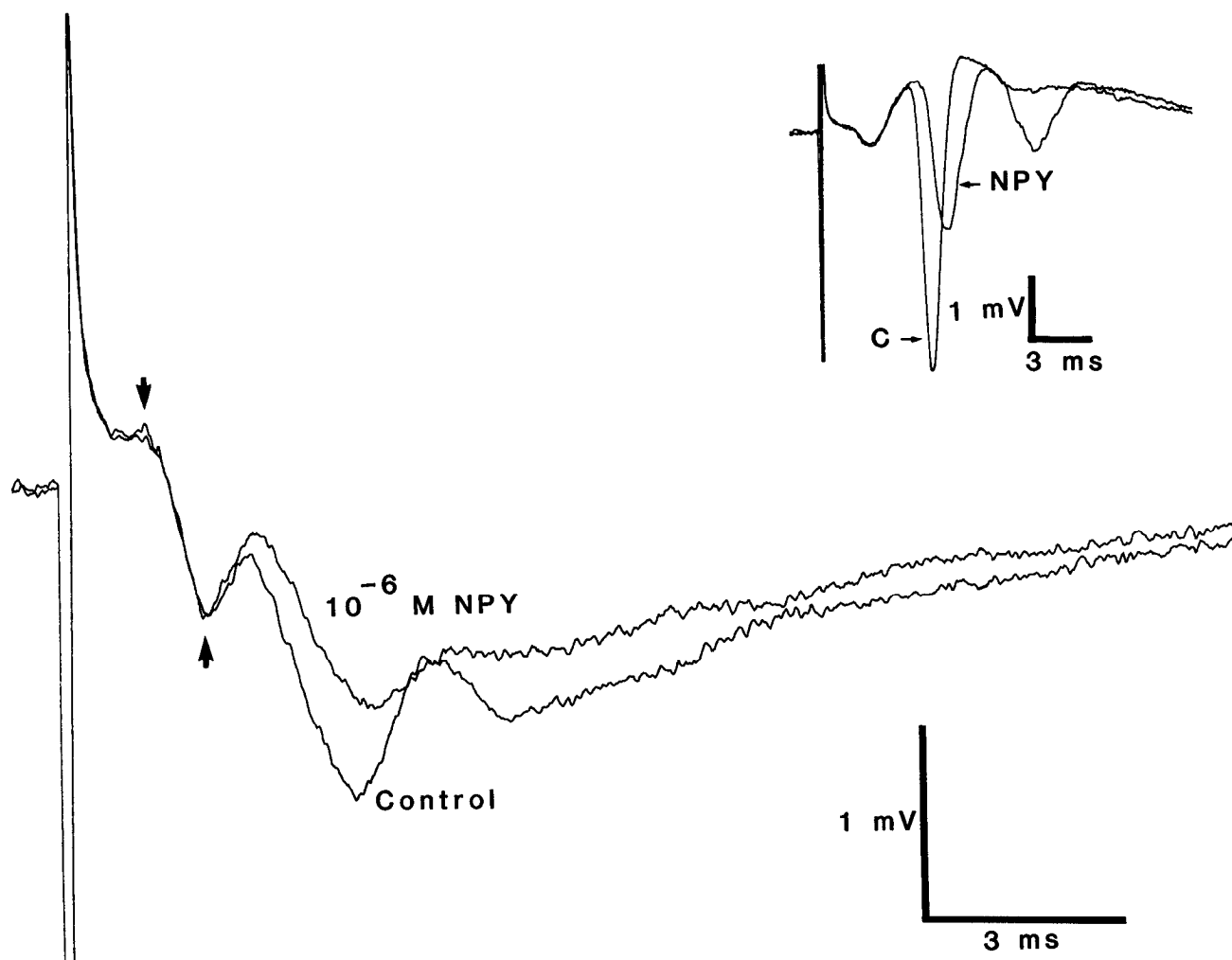
Figure 4. Extracellular field potential records from stratum radiatum and from cell body layer (*inset*) of area CA1 of rat hippocampal slice. Afferent presynaptic volley (*between arrows*) is the first component of the field potential recorded in stratum radiatum, representing the simultaneous discharge of afferent fibers in response to electrical stimulation of stratum radiatum; the larger, slower component of the field potential represents the population EPSP, representing the postsynaptic dendritic response of the CA1 pyramidal neurons. Superimposed on the latter portion of the population EPSP is a positive-going deflection representing the inverted p.s. (see *inset*). Application of 10^{-6} M NPY did not attenuate the amplitude of the afferent volley, although it reduced the amplitude of both the population EPSP and p.s.

diatum–CA1 synapse in the presence of the potassium channel blocker, 4-aminopyridine (4-AP), which is particularly effective at nerve terminal K^+ channels (Katz and Miledi, 1969; Buckle and Haas, 1982; Bartschat and Blaustein, 1985).

Bath application of 50 μM 4-AP enhanced the amplitude and duration of evoked excitatory synaptic transmission, as others have reported (Katz and Miledi, 1969; Buckle and Haas, 1982; Fig. 7). On one occasion, spontaneous epileptiform discharges could be recorded in the extracellular field. This increase in synaptic response was not accompanied by a significant increase in the somatic input resistance of the CA1 pyramidal neurons. In the presence of 50 μM 4-AP, application of 10^{-6} M NPY was without effect on the p.s. (Fig. 7, top left; $n = 3$, $p > 0.5$) or EPSP (not illustrated; $n = 3$, $p > 0.5$). NPY also did not affect the spontaneous epileptiform discharges observed in the one preparation.

Application of 4-AP at the lower concentration of 10 μM also enhanced evoked synaptic transmission, although to a lesser extent than at the higher concentration (Fig. 7, top right); no spontaneous discharges were observed in preparations. There

was also no significant change in somatic input resistance. 4-AP, 10 μM, also completely abolished the inhibition normally seen with 10^{-6} NPY of both p.s. (Fig. 7, top right; $n = 3$; $p > 0.5$) and EPSP ($n = 3$, $p > 0.5$, not illustrated).

Blockade of K^+ channels at the nerve terminal can delay membrane repolarization, consequently enhancing Ca^{2+} influx through voltage-dependent channels (Molgo et al., 1977; Buckle and Haas, 1982). If NPY were to inhibit a Ca^{2+} influx caused by K^+ channel blockade, its action might be overwhelmed by the increased Ca^{2+} influx caused by K^+ channel blockade. To test this possibility, we therefore reduced the Ca^{2+} concentration in the medium to compensate for the increased influx at the terminals and then examined NPY's actions under these conditions.

In separate experiments, it was determined empirically that lowering extracellular Ca^{2+} to 0.7 mM approximately counteracted the effects of 10 μM 4-AP (Fig. 7, center right), such that the p.s. and EPSP were both restored to nearly their control amplitudes and waveforms. Under these conditions, 10^{-6} M NPY caused a long-lasting, reversible reduction in p.s. amplitude to 49.3 $\pm$ 14.3% of control ($n = 4$, $p < 0.001$; Fig. 7) and
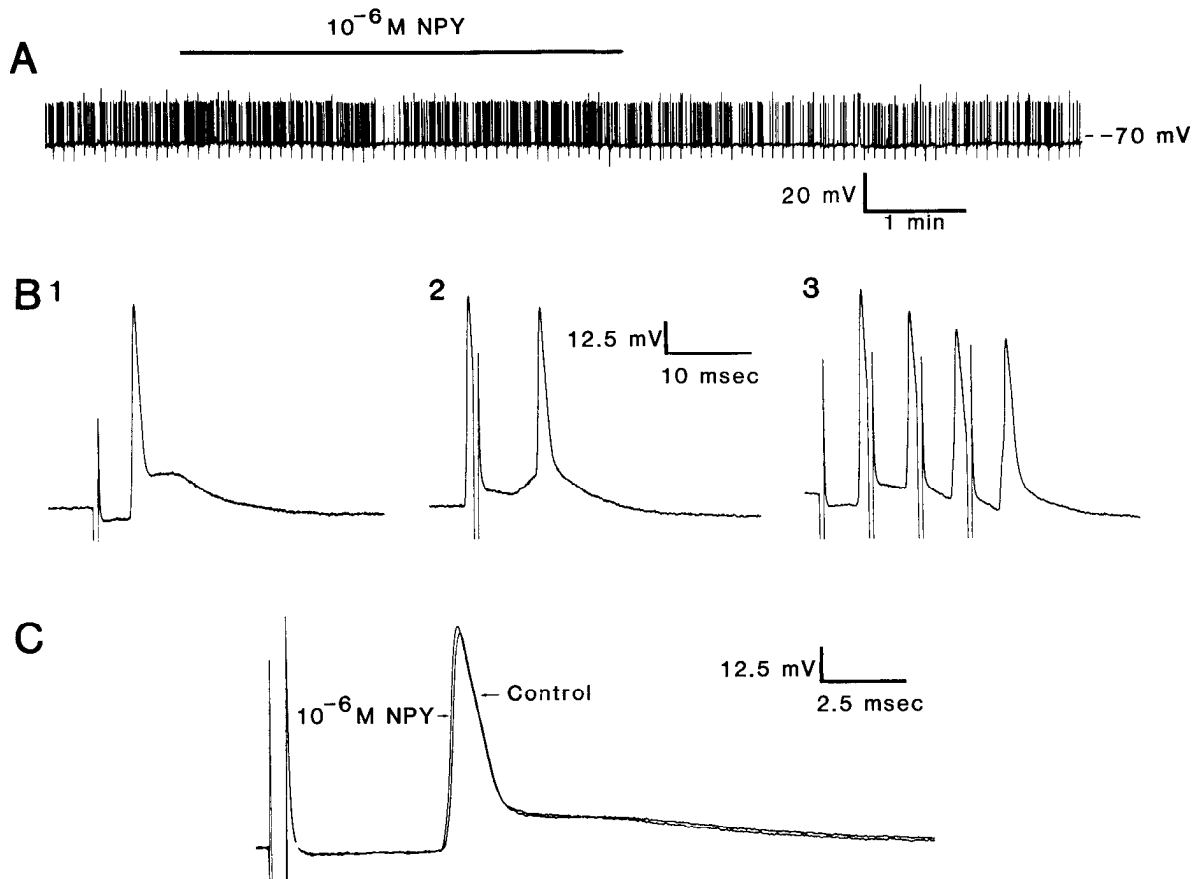
Figure 5. Antidromic activation of a CA3 pyramidal neuron from stratum radiatum of area CA1. Stimulus intensity was set to elicit an action potential in approximately 50% of the stimuli. *A,* Chart record of spontaneous activity and resting potential before, during, and after bath application of 10^{-6} M NPY (*bar*). Downward deflections represent shock artifact from stimulating electrode. NPY application had no significant effect on spontaneous activity. *B,* Action potentials were demonstrably antidromic in origin. *1,* Action potential arises abruptly rest, followed by depolarizing potential, probably synaptic in origin. *2,* Spontaneous action potential prior to stimulus results in collision cancellation of antidromic potential; the synaptic potential than causes neuron to exceed threshold. *B,* Neuron follows antidromic stimuli with constant latency at a frequency of 200 Hz. *C,* Bath application of 10^{-6} M NPY does not affect latency of antidromic action potential. NPY had no effect on the probability of an action potential being elicited antidromically (not shown).

in EPSP amplitude to 66.1 ± 3.51% of control ($n = 3$, $p < 0.001$; Fig. 7). Washout of the peptide allowed recovery of both p.s. and EPSP (not illustrated).

NPY was ineffective in the presence of 50 μM 4-AP and 0.7 mM Ca^{2+} (Fig. 7, center left); however, a further reduction in extracellular Ca^{2+} to 0.5 mM restored NPY's inhibitory action both on the p.s. (to 50.1 ± 10.89% of control, $n = 4$, $p < 0.001$; Fig. 7, bottom left) and on the EPSP (to 51.4 ± 5.06% of control, $n = 3$, $p < 0.001$; Fig. 7).

Discussion

This study confirms previous findings (Colmers et al., 1985, 1987; Haas et al., 1987) that NPY can attenuate excitatory transmission at the Schaffer collateral–CA1 synapse in hippocampus. The present evidence suggests that NPY (1) acts directly, rather than by eliciting the release of another inhibitory substance, and (2) acts at the level of the presynaptic terminal. Furthermore, the evidence here is consistent with an NPY-mediated inhibition of Ca^{2+} influx at the presynaptic terminal.

A number of possible mechanisms for NPY's action were considered in this study. We first examined whether NPY could act indirectly by eliciting the release, from synaptic boutons near the excitatory terminals, of endogenous neurotransmitters which

themselves inhibit transmitter release. Although this could not be tested in a direct way by blocking synaptic transmission, there was no evidence from indirect tests to suggest that NPY required functional $GABA_A$, adenosine A_1, or muscarinic cholinergic receptors for its action. Although we cannot exclude the possibility that another, yet unidentified presynaptic inhibitor of excitatory synaptic transmission is involved, we consider it most likely that NPY acts in a direct manner.

Examination of a number of possible direct mechanisms of NPY action at several sites presynaptic to the CA1 pyramidal neurons showed that NPY did not change the excitability in the cell bodies of the presynaptic neurons by decreasing the membrane input resistance or by changing the resting potential nor did it affect either the Na^+- or the Ca^{2+}-dependent components of the somatic action potential. Because the Ca^{2+}-dependent action potential in these neurons largely involves the noninactivating, "L" type Ca^{2+} channel, these data suggest that NPY action in hippocampus differs from that in cultured rat dorsal root ganglion neurons, where an inhibition by NPY of the L current has recently been reported (Ewald et al., 1987). These data do not exclude the possibility that, in adult hippocampal CA3 neurons, somatic Ca^{2+} conductances not participating in action potential generation could be affected by NPY. NPY also
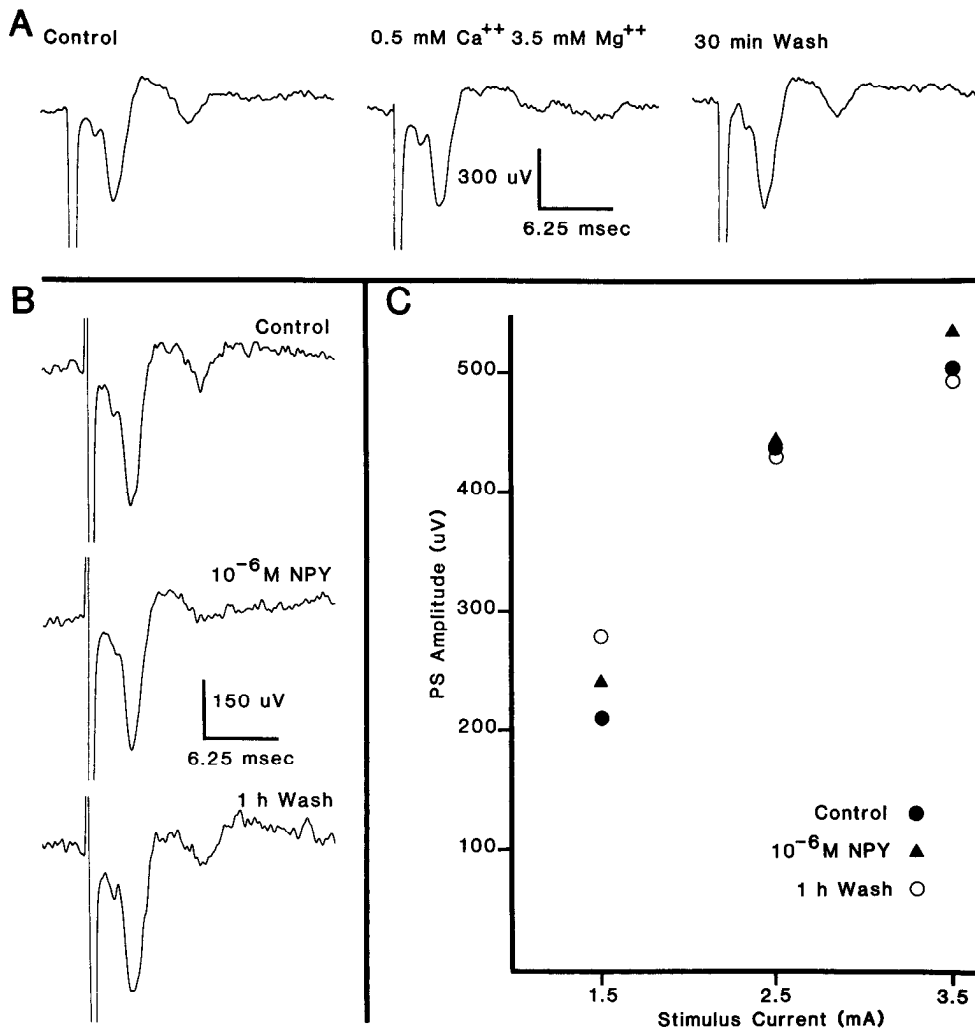
Figure 6. Extracellularly recorded field potential in area CA3 elicited by antidromic stimulation from stratum radiatum of area CA1. *A*, First negative component of field potential after stimulus shock artifact is antidromic in origin, as it is resistant to blockade of synaptic transmission by the bath application of low-Ca^{2+}, high-Mg^{2+} ACSF, while the second component is reversibly inhibited by this treatment. *B*, Bath application of 10^{-6} M NPY does not attenuate antidromic population spike, while attenuating second component reversibly. *C*, Stimulus–response relationship of antidromic component of CA3 field potential in control (●), 10^{-6} M NPY (▲), and after 1 hr washout (○). Note absence of NPY effect at all stimulus intensities.

does not appear to reduce the excitability of the axons of the CA3 pyramids in the stratum radiatum, in agreement with the observations of Haas et al. (1987). Since the postsynaptic CA1 pyramidal neuron is also not affected by NPY (Colmers et al., 1987; Haas et al., 1987), NPY most probably acts upon the presynaptic terminals themselves.

Possible mechanisms by which NPY could affect transmitter release at the synaptic terminal include the activation or enhancement of a K^+ conductance. The blockade of NPY's action by low concentrations of the K^+ channel blocker 4-AP was consistent with the idea that NPY worked via a 4-AP-sensitive conductance. However, even in the presence of 4-AP, NPY's inhibition of the synapse could be restored by lowering the extracellular Ca^{2+} concentration. NPY thus does not appear to modulate a 4-AP-sensitive K^+ conductance. NPY could, however, activate a 4-AP-insensitive K^+ conductance. One of the consequences of 4-AP blockade of K^+ conductances is an increased input resistance at the terminal. However, an NPY-mediated activation of a K^+ conductance under these circumstances would then be expected to lead to a greater hyperpolarization than without 4-AP; therefore, a greater, rather than a reduced, effect of NPY should have been seen. As this was not observed, we conclude that NPY probably does not activate a potassium conductance at the terminal.

The data are consistent, however, with a modulation by NPY

of a Ca^{2+} influx at the presynaptic terminal. We reasoned that the enhanced Ca^{2+} influx into the terminal brought about by 4-AP's action on K^+ currents could overwhelm the normal inhibitory action of NPY on a Ca^{2+} influx. Consistent with this, after reduction of Ca^{2+} levels (and therefore reduction of Ca^{2+} influx), the action of NPY was restored. It would appear that, at this reduced extracellular Ca^{2+} concentrations, NPY inhibition of Ca^{2+} currents again becomes the major factor in the control of transmitter release. An influence of NPY on Ca^{2+} currents, rather than on resting K^+ permeabilities, would also explain our failure to observe changes in terminal excitability in the antidromic stimulation experiments.

Although NPY did not influence the Ca^{2+}- and Ba^{2+}-dependent action potentials in CA3 neurons, the peptide could nonetheless act on such conductances at their terminals. Autoradiographic binding studies show that NPY receptors are not present in the cell body layers of areas CA1 or CA3 but are restricted to strata radiatum and oriens (Martel et al., 1986). If the receptors (and the effectors to which they are coupled) are not present near the soma, changes in Ca^{2+} influx brought about by NPY would not be expected to influence the action potential measured at the soma (e.g., Williams and Ziegelgansberger, 1981). Because the terminals of the CA3 neurons are electrotonically very remote from their somata (up to 100 length constants: D. A. Turner, personal communication), it is possible that NPY's at-
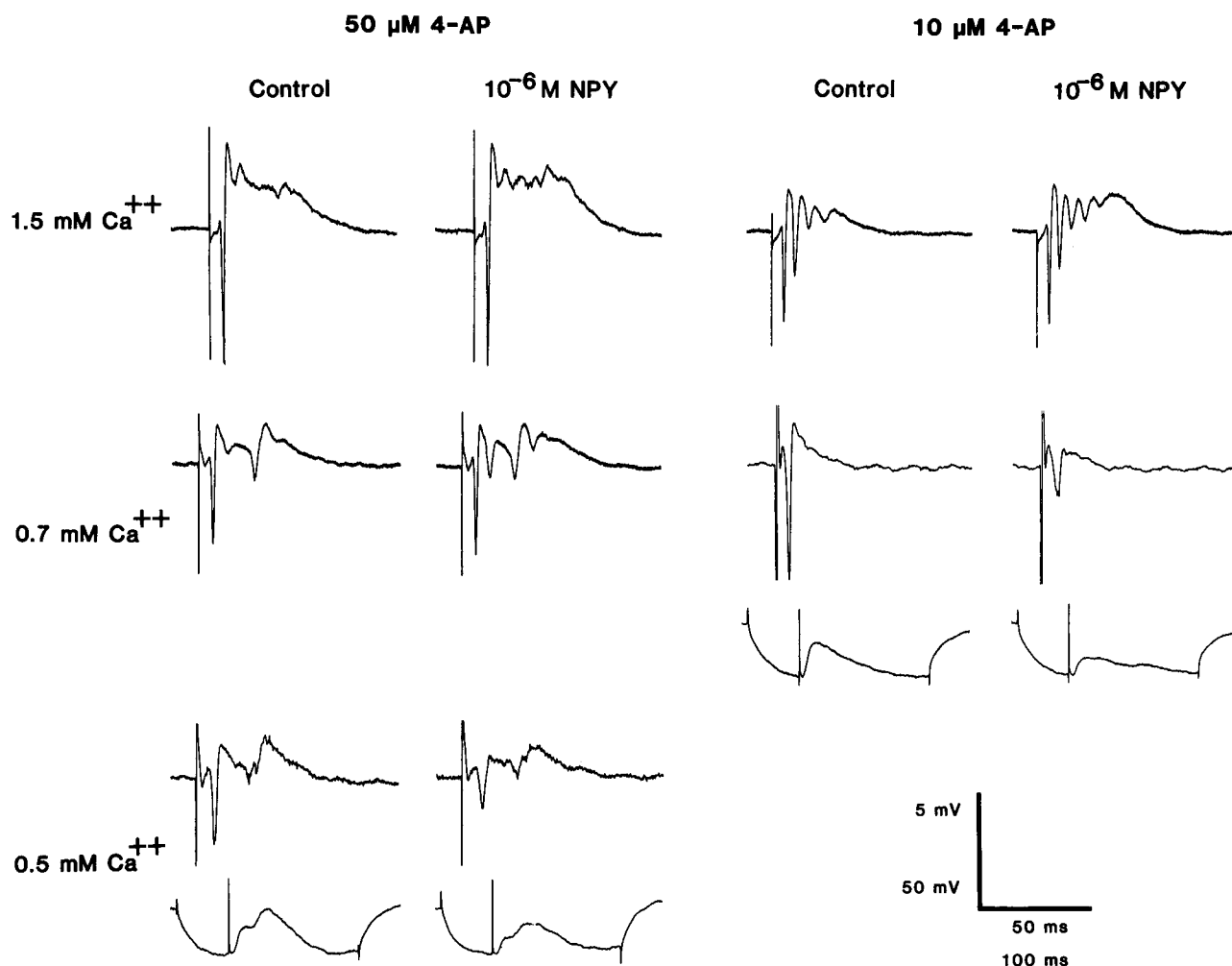
Figure 7. Effects of 4-aminopyridine (4-AP) and reduced extracellular Ca^{2+} concentrations on the NPY-mediated inhibition of excitatory synaptic transmission in CA1. Data for each combination shown were taken from separate preparations. *Left side* of figure shows action of 50 μM 4-AP; *right side,* that of 10 μM 4-AP. *Left-hand column* of each side depicts effects of 4-AP alone; *right columns,* effects after 10^{-6} M NPY has been applied in the presence of 4-AP. Extracellular field potentials only are shown for combinations where NPY was without effect, intracellular records including EPSP are shown where NPY inhibited excitatory synaptic transmission. NPY inhibited synaptic transmission in 10 μM 4-AP with 0.7 mM Ca^{2+} *(center row)* and in 50 μM 4-AP with 0.5 mM Ca^{2+} *(bottom row).* NPY's inhibitory effects were reversible under these circumstances (not illustrated).

tenuation of Ca^{2+} influx at the terminal would not be detectable at the soma. Experiments on hippocampal neurons in culture might permit the resolution of this question, as it is possible that the receptors and effectors may be expressed sufficiently close to the soma to have a measurable influence on somatic properties (Mudge et al., 1980; Williams and Ziegelgansberger, 1981). Indeed, recent evidence from rat dorsal root ganglion cells in culture indicate that NPY does reduce their somatic Ca^{2+} conductances (Ewald et al., 1987).

Since we have not been able to measure Ca^{2+} influx into the presynaptic terminal directly or to examine the calcium sensitivity of transmitter release (Malenka et al., 1986), the present experiments do not constitute an unequivocal demonstration of NPY's mechanism of action. Nonetheless, we consider it most probable that an inhibition of Ca^{2+} influx at the excitatory presynaptic terminals in stratum radiation underlies the reduction in synaptic transmission caused by NPY.

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