4-Aminopyridine and low Ca^{2+} differentiate presynaptic inhibition mediated by neuropeptide Y, baclofen and 2-chloroadenosine in rat hippocampal CA1 *in vitro*

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1 Presynaptic inhibition is mediated by several receptors at the stratum radiatum-CA1 synapse of rat hippocampus. We tested whether the same mechanism is activated by neuropeptide Y (NPY), baclofen and 2-chloroadenosine (2-CA), reasoning that if the receptors all activated the same process, then they should all respond to indirect manipulations of transmitter release in the same manner.

2 The effects on presynaptic inhibition by the potassium channel blocker, 4-aminopyridine (4-AP) and low extracellular concentrations of Ca^{2+} in the presence of 4-AP were compared using evoked population excitatory postsynaptic potentials (p.e.p.s.p.) responses in the rat hippocampal slice *in vitro*.

3 Log concentration-effect relationships for the inhibition of excitatory transmission were constructed for all 3 drugs in normal saline, and in the presence of 30 and 100 μM 4-AP. 4-AP reduced the inhibition mediated by all three substances, 100 μM 4-AP was only slightly more effective than 30 μM .

4 Lowering extracellular Ca^{2+} from 1.5 to 0.75 mM in the presence of 30 μM 4-AP restored the presynaptic inhibition caused by all effective concentrations of NPY and baclofen. By contrast, inhibition caused by 2-CA was not restored by lowering Ca^{2+} , except at concentrations of 2-CA greater than 10 μM .

5 The results are consistent with the hypothesis that presynaptic NPY Y_2 and GABA_B receptors both inhibit transmitter release by the inhibition of voltage-dependent Ca^{2+} influx, but that the A_1 adenosine receptor may activate a different presynaptic mechanism.

Keywords: Neuropeptide Y; GABA_B receptor; rat hippocampal slice; presynaptic inhibition; presynaptic mechanisms; 4-aminopyridine; adenosine A_1 receptor; 2-chloroadenosine; baclofen, NPY Y_2 receptor

Introduction

Several neurotransmitters and neuromodulators are known to mediate the presynaptic inhibition of neurotransmitter release in the mammalian CNS. In hippocampal area CA1, at least 5 receptors are known to mediate inhibition of excitatory amino acid neurotransmitter release, presumably glutamate, from the terminals of stratum radiatum fibres that form synaptic connections with dendrites of CA1 pyramidal neurones. These receptors include: (1) a γ -aminobutyric acid (GABA_A) receptor (Ault & Nadler, 1982); (2) a muscarinic receptor (Hounsgaard, 1978); (3) an unusual glutamate receptor, at which L-2-amino-4-phosphonobutyrate (AP4) is an agonist (Forsythe & Clements, 1990); (4) an adenosine receptor (A_1 -like; Dunwiddie & Fredholm, 1989) and (5) a neuropeptide Y (NPY) Y_2 receptor (Colmers *et al.*, 1988; 1991). Of the known endogenous substances mediating presynaptic inhibition in area CA1 of hippocampus, NPY is the only one that has no postsynaptic effects (Colmers *et al.*, 1987).

Since the first description of presynaptic inhibition, a debate has continued as to the mechanism by which transmitter substances prevent release of transmitter (cf. Starke, 1981). The precise mechanism of presynaptic action in hippocampus is not known for any of these substances although there is evidence that they all activate receptors coupled to guanosine 5'-triphosphate (GTP) binding proteins. Nearly all of these substances have been shown to inhibit calcium influx into cultured or acutely dissociated neurones (Dolphin & Scott, 1986; Macdonald *et al.*, 1986; Wanke *et al.*, 1987; Walker *et al.*, 1988), and most are known to activate potassium conductances in cell bodies (Newberry & Nicoll, 1984; Egan & North, 1986; Greene & Haas, 1985; Zidichouski *et al.*, 1990). Because of technical limitations, it has not been possible to examine the actions of these substances on evoked transmitter release by direct measurements on presynaptic terminals of CNS. The question of how NPY acts at the presynaptic terminal has been indirectly addressed by the use of 4-

aminopyridine (4-AP), which blocks potassium conductances at presynaptic terminals (Buckle & Haas, 1982). 4-AP at concentrations well below those needed to affect the somatic 'A' current has been shown to block the presynaptic actions of NPY in hippocampal CA1 (Colmers *et al.*, 1988); higher concentrations block presynaptic inhibition mediated by adenosine in guinea-pig olfactory cortex (Scholfield & Steel, 1988). Lowering extracellular calcium concentrations in the presence of 4-AP restored NPY-induced inhibition, suggesting the inhibition of a presynaptic terminal calcium conductance by NPY (Colmers *et al.*, 1988).

To examine whether the presynaptic inhibition mediated in hippocampus by NPY, baclofen and adenosine could be via the same mechanism, we compared the effects of 4-AP and low calcium on the concentration-response relationships for all three substances. If inhibition of transmitter release by all three receptors is mediated by the same mechanism, the inhibition by all three should be equally affected by 4-AP and low Ca^{2+} , since neither manipulation should alter the binding of the agonists to their receptors. The data indicate that, although baclofen and NPY are similar in their response to these manipulations, adenosine differs from the others.

Methods

Transverse hippocampal slices (450 μm thick) were prepared from male Sprague-Dawley rats (75–150 g), and incubated at $34 \pm 0.5^\circ\text{C}$, submerged and constantly perfused ($2.5\text{--}3\text{ ml min}^{-1}$) with saline bubbled with 95% O_2 : 5% CO_2 (pH 7.35) (Colmers *et al.*, 1988; 1991). Composition of the saline was (in mM): NaCl 124, KCl 1.8, MgSO_4 2, KH_2PO_4 1.25, CaCl_2 1.5 and glucose 10. Low Ca^{2+} solutions were prepared from Ca^{2+} -free stock solution to which appropriate amounts of CaCl_2 were added. Stimuli were delivered to stratum radiatum of area CA1 via a shielded bipolar, etched-tungsten electrode connected to a stimulus isolation unit (AMPI). Orthodromic population field potentials evoked by stimuli (3–40 V, 50–200 μs , 0.1 Hz) were recorded in stratum radiatum of CA1 with a glass micropipette (4–15 M Ω , 2M NaCl; Figure 1)

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Figure 1 Population e.p.s.p. (digital average of three traces) recorded in stratum radiatum of area CA1. Dashed line indicates slope of p.e.p.s.p., arrow indicates presynaptic volley, which was resolved in most, but not all preparations.

connected to the headstage of an Axoclamp 2A amplifier. For each data point, three successive field potentials were digitized at 50 μ s/point, and averaged using a virtual averaging paradigm by a digital storage oscilloscope (Nicolet 4094; Model 4562 plug-in) and were stored on diskette. The slope of the initial, linear portion of the population e.p.s.p. (p.e.p.s.p.) was measured as the difference in voltage and time between the extremes of the linear portion (determined by inspection) of the p.e.p.s.p., using the oscilloscope. Once stable responses had been obtained, stimulus-response curves were constructed, and a stimulus intensity was chosen to elicit a response on the linear portion of the stimulus-response curve (Andersen *et al.*, 1978). Generally, representative responses were chosen which were of similar size ($\pm 20\%$) to those observed in control for each preparation; however, they were always on the linear portion of the stimulus-response curve. Stimulus-response curves were constructed under each basal condition, and stimulus intensities chosen using the same criteria as for control conditions. In most cases, the responses evoked in a given preparation by a given stimulus in control and in the presence of 4-AP/low Ca^{2+} were similar in slope and amplitude, while 4-AP at both concentrations caused a much greater response, requiring lower stimulus intensities to elicit the same responses. Stimulus-response curves were also obtained during drug effects and upon washout.

Test substances were made freshly in saline of appropriate composition. Synthetic porcine NPY (Richelieu Biotechnologies), was made up at 100 μ M in distilled water and kept frozen until use. 2-Chloroadenosine (2-CA; Research Biochemicals Inc.) and ($\pm$)-baclofen (Lioresal; gift of Ciba-Geigy) were made up as concentrated stock solutions (dilutions of 1:100 or greater in final use) and kept frozen or refrigerated, respectively, in the dark until use.

Data are expressed as percentage inhibition of control values. Preparations served as their own controls; all data are from preparations which showed significant recovery upon washout. Statistical comparisons were performed by a Student's *t* test. Statistical differences were considered significant at $P \leq 0.05$. Concentrations of drugs somewhat above the EC_{50} were selected for comparison in the figures, and for representative statistical comparisons. In addition, ligand concentrations over which significant differences between treatments were observed are given, where appropriate.

Results

Data were obtained from 56 preparations, with a total of 361 drug applications. Initial p.e.p.s.p. slope measurements in control conditions varied from preparation to preparation, and ranged from 0.12 to 1.26 mV ms^{-1} . Although we attempted to perform concentration-response curves on each preparation under each condition, this was not always possible.

Bath application of 1 μ M NPY reversibly reduced the p.e.p.s.p. slope in CA1 by $63.15 \pm 1.94\%$ ($n = 4$; Figure 2). NPY was significantly effective at concentrations above 300 nM (Figure 3); concentrations of NPY higher than 3 μ M were not tested. Baclofen (10 μ M) also reduced the p.e.p.s.p. slope, by $71.53 \pm 2.88\%$ ($n = 6$; Figure 2). Baclofen significantly

Figure 2 Effects of 4-aminopyridine (4-AP) and 4-AP/low Ca^{2+} on inhibition of p.e.p.s.p. by representative concentrations of neuropeptide Y (NPY), baclofen and 2-chloroadenosine (2-CA). Upper row shows superimposed traces in control and during peak drug effects in normal saline (1.5 mM Ca^{2+}) for 1 μ M NPY (left column), 10 μ M baclofen (centre column), and 1 μ M 2-CA (right column). Centre row shows the effects of these drugs, superimposed on controls, in the presence of 30 μ M 4-AP. Bottom row shows the effects of these drugs, relative to controls, in 30 μ M 4-AP with 0.75 mM Ca^{2+} . Data illustrated for a given drug were obtained from the same preparation. All preparations recovered from inhibition upon washout (not illustrated). All traces shown are digital averages of three successive field potentials (0.1 Hz). In all cases, the smaller response represents the effect of the drug tested.

inhibited p.e.p.s.p. slope at concentrations above 300 nM (Figure 3). 2-CA, 1 μ M, inhibited p.e.p.s.p. slope by $65.86 \pm 6.03\%$ ($n = 6$; Figure 2). 2-CA was effective at concentrations of 100 nM or greater (Figure 3).

Figure 3 Dose-response relationships for inhibition of p.e.p.s.p. by neuropeptide Y (a), baclofen (b) and 2-chloroadenosine (c) in control (1.5 mM Ca^{2+}) saline ($\blacktriangle$), and in the presence of 30 μ M 4-aminopyridine (4-AP, $\bullet$), 100 μ M 4-AP ($\circ$), and 30 μ M 4-AP, 0.75 mM Ca^{2+} ($\circ$). Data shown are means for $n \geq 3$ for each point, s.e. mean shown by vertical bars.

Application of $30\text{ }\mu\text{M}$ 4-AP caused a significant increase in orthodromic synaptic responses (Figure 2). At initial stimulus intensities, p.e.p.s.ps were prolonged compared with control, and often demonstrated multiple late components. Recording in the cell body layer revealed multiple population spikes and often spontaneous epileptiform discharges were observed. To study the actions of the presynaptic inhibitors under conditions similar to those of control, stimulus-response relationships were again constructed, and a stimulus amplitude chosen which evoked p.e.p.s.p. of approximately the same slope and amplitude as in control (see Methods). Once stable responses had been obtained, dose-response curves to the inhibitory substances were again constructed.

As reported previously for population spike and intracellularly recorded e.p.s.p. (Colmers *et al.*, 1988), 4-AP also caused a significant ($P < 0.05$) reduction in the inhibition of p.e.p.s.p. slope by NPY at concentrations above 300 nM . At $30\text{ }\mu\text{M}$, 4-AP reduced the inhibition of p.e.p.s.p. by $1\text{ }\mu\text{M}$ NPY from $63.15 \pm 1.94\%$ ($n = 4$) to $30.60 \pm 6.66\%$ ($n = 3$) of control. The inhibition of p.e.p.s.p. slope by $10\text{ }\mu\text{M}$ baclofen was reduced from $71.53 \pm 2.88\%$ ($n = 6$) to $38.78 \pm 8.69\%$ ($n = 6$) of control (Figure 2); the 4-AP effect was significant for baclofen concentrations above $1\text{ }\mu\text{M}$. The inhibition by $1\text{ }\mu\text{M}$ 2-CA was reduced from $65.86 \pm 6.03\%$ ($n = 6$) to $34.82 \pm 6.81\%$ ($n = 6$) of control; 4-AP effects were significant at 2-CA concentrations above 100 nM .

A higher concentration ($100\text{ }\mu\text{M}$) of 4-AP caused only a slight, and generally insignificant, further reduction in p.e.p.s.p. inhibition by all three drugs, indicating that $30\text{ }\mu\text{M}$ 4-AP was near but slightly below the maximal effective concentration for 4-AP at these terminals. However, an interesting effect of increasing concentrations of 4-AP was a significant, progressive depression of the maximal inhibition of p.e.p.s.p. by 2-CA (Figure 3). The maximal inhibition by baclofen was also significantly depressed by $30\text{ }\mu\text{M}$ 4-AP, but was not further reduced by $100\text{ }\mu\text{M}$ 4-AP. It was not possible to observe this effect with NPY, as maximal concentrations could not be tested.

As has been previously observed (Colmers *et al.*, 1988), reduction of extracellular Ca^{2+} in the presence of 4-AP caused a reduction in evoked synaptic responses. Thus, in $30\text{ }\mu\text{M}$ 4-AP/ 0.75 mM Ca^{2+} , p.e.p.s.p. slopes were reduced, as expected from the lower levels of extracellular Ca^{2+} available to the presynaptic terminals. Once responses had stabilized, and the stimulus intensity was re-adjusted so responses were comparable with controls (see Methods), drug effects were again tested. For both NPY and baclofen, low Ca^{2+} /4-AP significantly restored the ability of the ligands to inhibit transmitter release. Indeed, no significant difference was observed between control saline and low Ca^{2+} /4-AP for inhibition mediated by NPY or baclofen. For 2-CA, by contrast, low Ca^{2+} /4-AP caused no significant restoration of inhibition at drug concentrations less than $10\text{ }\mu\text{M}$ (Figure 2). Experiments could not be conducted in the presence of 0.75 mM Ca^{2+} alone since, in the absence of 4-AP, this Ca^{2+} concentration did not support synaptic transmission (not illustrated).

To test whether there was a significant postsynaptic component to the observed reduction in p.e.p.s.p. slope, we applied a supramaximal concentration of the 5-hydroxytryptamine (5-HT) agonist 5-carboxamidotryptamine (5-CT; Beck, 1989). 5-CT activates the postsynaptic 5-HT_{1A} receptors in hippocampus (Beck, 1989) that cause an increase in the same potassium conductance as does baclofen (Andrade *et al.*, 1986). At $1\text{ }\mu\text{M}$, 5-CT had no significant effect on p.e.p.s.p. slope ($n = 5$; Figure 4).

Discussion

NPY, baclofen and 2-CA all inhibit evoked glutamatergic synaptic transmission in area CA1 of the rat hippocampus by presynaptic actions in stratum radiatum. While all of the receptors mediating presynaptic inhibition in hippocampus

Figure 4 5-Carboxamidotryptamine (5-CT; $1\text{ }\mu\text{M}$) does not affect p.e.p.s.p. slope in control (upper traces), in $30\text{ }\mu\text{M}$ 4-aminopyridine (4-AP, middle traces) or in $30\text{ }\mu\text{M}$ 4-AP, 0.75 mM Ca^{2+} . Traces in control and during 5-CT application are shown superimposed. Arrows indicate traces obtained during 5-CT application. Data are all from the same preparation. All traces are digital averages of three successive field potentials.

have also been shown to inhibit Ca^{2+} influx into neuronal cell bodies, an unequivocal demonstration that this is the mechanism whereby they inhibit transmitter release at presynaptic terminals remains elusive. We have asked a related but somewhat different question: does the presynaptic inhibition mediated at the same population of terminals by different receptors respond in the same manner to two manipulations which should only indirectly affect processes inhibiting transmitter release? Because neither of the manipulations should influence the binding of the ligands to their receptors or significantly affect the coupling of the receptors to their G-proteins, these manipulations should have equal effects on responses to all three ligands if they share a common mechanism. The evidence presented here indicates that this is not entirely the case.

All three substances tested inhibit synaptic transmission in hippocampus, with EC_{50} s of between 500 nM and $2.8\text{ }\mu\text{M}$. The physiological measure chosen, the slope of the p.e.p.s.p., is relatively insensitive to the postsynaptic actions of neuromodulators (Harrison *et al.*, 1991). However, it has been demonstrated that postsynaptic GABA_B receptors reduce synaptic NMDA conductances evoked at higher stimulus levels (Morrisett *et al.*, 1991). Here, application of a supramaximal concentration of 5-CT, an agonist at the 5-HT_{1A} receptors (Beck, 1989) that activates the same postsynaptic K⁺ conductances in CA1 pyramidal cells as are activated by GABA_B receptors (Andrade *et al.*, 1986), had no significant inhibitory effect on p.e.p.s.p. slopes when applied either alone or in the presence of $30\text{ }\mu\text{M}$ 4-AP, although a later component of the response was somewhat reduced (Figure 4). The absence of an effect of 5-CT on the slope of the p.e.p.s.p. is in agreement with other observations (Harrison, 1990), and suggests that the postsynaptic potassium conductance activated by both 5-HT_{1A} or GABA_B receptors (Andrade *et al.*, 1986) has no significant effect on the modest levels of excitatory transmission elicited in these experiments. Furthermore, NPY has no demonstrable postsynaptic actions in hippocampus (Colmers *et al.*, 1987; 1988). Therefore, we are confident that the only significant inhibition measured in the present experiments is at a presynaptic site.

The action of 4-AP suggests, at first glance, that the three compounds cause presynaptic inhibition by increasing a K⁺ conductance at the presynaptic terminal, as has been hypothesized for the action of adenosine in guinea-pig olfactory cortex (Scholfield & Steel, 1988). However, the effects of the low- Ca^{2+} /4-AP treatment on NPY and baclofen responses are not consistent with an increase in K⁺ conductance. The inhibition by all effective concentrations tested of both substances was

restored to control levels by lowering extracellular Ca^{2+} in the presence of 4-AP. This restoration militates against either 4-AP or low Ca^{2+} affecting the binding of the drugs to their receptors or the coupling of the receptors to their effectors. In fact, the observed decrease in receptor – G-protein coupling caused by lowered Ca^{2+} concentrations (Gilman, 1987) would be expected to produce the opposite of the results observed here. Because the restoration of inhibition by low Ca^{2+} occurs throughout the concentration-effect curve to both substances, it also argues against a significant component of the observed inhibition by NPY and baclofen being mediated directly by another mechanism, such as an increase of K^+ conductance in the presynaptic terminal (Colmers *et al.*, 1988). Thus, the present evidence is entirely consistent with an inhibitory action by presynaptic NPY and GABA_B receptors on Ca^{2+} influx underlying the effect on synaptic transmission.

However, the response of the 2-CA-mediated inhibition to 4-AP/low Ca^{2+} is not as clearly consistent with an exclusive effect at presynaptic Ca^{2+} channels. Most importantly, lowering extracellular Ca^{2+} did not significantly restore the inhibition, except at concentrations of 2-CA greater than $10\text{ }\mu\text{M}$, (a supramaximal concentration in control; Figure 3). Clearly, this differs significantly from the response of NPY and baclofen; the lack of response to low Ca^{2+} is also not consistent with a mechanism involving predominantly the reduction of Ca^{2+} influx, at least at concentrations of 2-CA lower than $10\text{ }\mu\text{M}$. At the neuromuscular junction, it has been proposed that adenosine inhibits transmitter release at a step subsequent to the entry of Ca^{2+} (Silinsky, 1985). Recent evidence indicates that, in rat hippocampal cultures, adenosine inhibits quantal release of glutamate by a mechanism independent of Ca^{2+} influx (Scholz & Miller, 1991). Although it is difficult to reconcile a mechanism involving a decrease in Ca^{2+} sensitivity by the release process with the present data, it appears

that adenosine receptors are capable of controlling the release process in more than one fashion (Silinsky, 1985). The data admit of the explanation that, at low concentrations, adenosine inhibits transmitter release largely by a process independent of voltage-dependent Ca^{2+} influx, although there may be an additional effect on Ca^{2+} influx at higher concentrations.

The present results suggest that there are at least two different mechanisms of presynaptic inhibition mediated by the three receptors studied here. We consider it unlikely that all three receptors utilize three altogether different mechanisms. Although the nature of the present and similar experiments limits the conclusions about presynaptic processes which can be obtained (Milner *et al.*, 1986) it is nevertheless possible to demonstrate under identical experimental conditions that the events which underlie presynaptic inhibition are not identical for all receptors.

The results indicate that presynaptic inhibitions mediated by NPY and baclofen in stratum radiatum of hippocampal area CA1 *in vitro* respond in a comparable manner to the application of 4-AP and reduced extracellular Ca^{2+} in the presence of 4-AP. The response is consistent with a mechanism of presynaptic inhibition involving the inhibition of Ca^{2+} influx into the presynaptic terminals that release glutamate. However, the inhibition mediated by 2-CA does not respond in the same manner, suggesting that it may thus act by a predominantly different mechanism to inhibit transmitter release.

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