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Possible presynaptic actions of 2-amino-4-phosphonobutyrate in rat olfactory cortex

J. Anson & [1]G.G.S. Collins

Department of Pharmacology, University of Sheffield, Western Bank, Sheffield S10 2TN

1 The effect of 2-amino-4-phosphonobutyrate (APB) on facilitation at the lateral olfactory tract (LOT)-superficial pyramidal cell synapse of the olfactory cortex has been studied by recording the relative changes in amplitude of the N-waves evoked on stimulation of the LOT by pairs of stimuli.

2 Although APB (0.01 to 5 mM) reduced the amplitude of the conditioning response there was an overall increase in facilitation over conditioning intervals of up to 1700 ms which was concentration-dependent and inversely related to the concentration of extracellular calcium (1.25 to 5 mM).

3 The L-(+)-isomer of APB was more potent than the D-(−)-form in increasing synaptic facilitation.

4 The potassium channel blockers 4-aminopyridine (0.25 mM), 3,4-diaminopyridine (0.1 mM), tetraethylammonium (10 mM) and catechol (1 mM) all reduced facilitation but failed to antagonize the increase in facilitation produced by APB (1 mM). In contrast, all 4 drugs antagonized APB-induced reductions in the amplitude of the conditioning response.

5 APB (1 mM) significantly reduced the K^+-evoked release of endogenous aspartate and glutamate but not of γ-aminobutryic acid from slices of olfactory cortex.

6 It is suggested that APB reduces the amplitude of the conditioning response and increases synaptic facilitation by reducing transmitter release from the LOT terminals. The mechanism is unlikely to involve activation of terminal potassium currents.

Introduction

The glutamate analogue 2-amino-4-phosphonobutyrate (APB) blocks excitatory transmission at a number of sites in the mammalian CNS including the spinal cord (Davies & Watkins, 1979; 1982), retina (Slaughter & Miller, 1981) and hippocampus (Koerner & Cotman, 1981; Yamamoto *et al.*, 1983; Lanthorn *et al.*, 1984). When applied to olfactory cortex slices, APB depresses transmission at the lateral olfactory tract (LOT)-superficial pyramidal cell synapse (Collins, 1982; Hori *et al.*, 1982; Hearn *et al.*, 1986), a site at which glutamate and/or aspartate are neurotransmitter candidates (Collins, 1986). Although a potent antagonist of synaptic transmission in the olfactory cortex, APB does not block the postsynaptic actions of glutamate and aspartate (Hori *et al.*, 1982; Surtees & Collins, 1985; Collins & Brown, 1986) suggesting that either the identification of the LOT transmitter is at fault or, perhaps, that APB has a presynaptic site of action (Harris & Cotman, 1983).

Presynaptic drug actions are often difficult to study directly and so indirect approaches are employed. When the LOT of slices of olfactory cortex are stimulated twice in rapid succession, the response evoked by the second test stimulus is greater than that evoked by the first conditioning stimulus (Richards, 1972; Bower & Haberly, 1986). Such synaptic facilitation occurs at a number of sites in the CNS (Andersen, 1960a, b; Eccles *et al.*, 1961; Kuno & Weakly, 1972; Erulker, 1983) and is usually considered to reflect a presynaptic phenomenon involving an increase in transmitter release caused by the persistence of calcium activity triggered by the conditioning stimulus (Katz & Miledi, 1968). However, when transmitter release is high there is a simultaneous underlying reduction in the response to the test stimulus probably caused by depletion of transmitter stores by the conditioning pulse. Thus a reduction in extracellular calcium increases synaptic facilitation whereas in the presence of high extracellular levels of calcium, facilitation is reduced (Creager *et al.*, 1980; Dunwiddie & Haas, 1985). Consequently, if a drug blocks transmission by reducing transmitter release, synaptic facilitation should be potentiated whereas if its site of

[1] Author for correspondence.

action is postsynaptic, facilitation should be unaffected (Harris & Cotman, 1985). The aims of the present study were to ascertain whether APB antagonizes transmission at the LOT-superficial pyramidal cell synapse of the rat olfactory cortex by a presynaptic action and if so, whether the mechanism might involve potentiation of presynaptic potassium currents (Scholfield, 1986).

Methods

Slices of olfactory cortex were prepared from male rats (Wistar origin, University of Sheffield strain) using a bow cutter and perspex guide recessed to 400 μm; Following incubation for 2 h at room temperature in a salt solution (composition mM: NaCl 118.1, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, KCl 2.1, $MgSO_4$ 1.1, KH_2PO_4 0.93), pH 7.3–7.4, continuously bubbled with 95% O_2 and 5% CO_2, slices were mounted cut side down in a perfusion system in which the under surface was perfused at a rate of approximately 2 ml min^{-1} (Pickles & Simmonds, 1976; 1978). The upper pial surface of the slice was exposed to a stream of water-saturated 95% O_2 and 5% CO_2 to prevent drying out. A bipolar platinum stimulating electrode was placed across the LOT and a Ag/AgCl recording electrode located on the pial surface in the region of the prepyriform cortex. Evoked surface field potentials were amplified with a d.c. amplifier and either captured in a Datalab DL1080 transient recorder connected to a Bryans 28000 potentiometric plotter or recorded immediately with a Medelec u.v. system.

Excitatory transmission at the LOT-superficial pyramidal cell synapse was monitored by recording the surface field potential known as the N-wave (Richards & Sercombe, 1968; 1970; Halliwell, 1976) evoked on stimulation of the LOT (50 μs pulse width, 0.1 Hz, various voltages). In all experiments the position of the recording electrode was adjusted so that the evoked N-wave was uncontaminated by population spikes. The amplitudes of the N-wave were measured from the recording baseline to peak negativity, irrespective of latency. Both picrotoxin (25 μM) and theophylline (0.3 mM) were present in the preincubation and perfusion medium to antagonize γ-aminobutyrate (GABA)-mediated transmission (Pickles & Simmonds, 1976; 1978) and the inhibitory effects of endogenous adenosine released on LOT stimulation (Motley & Collins, 1983) respectively.

APB (the racemic mixture unless stated otherwise) was freshly dissolved in the perfusion solution and applied dropwise to the exposed pial surface of slices in 0.1 ml every 90 s for a sufficient time period to produce a sustained plateau effect (10 min) followed, when appropriate, by drug-free solution to allow full recovery (usually 20 min). In experiments in which the effects of APB on the N-wave amplitude evoked by single supramaximal stimuli were measured (see Figure 5), cumulative dose-response curves were constructed over a concentration range of APB of 0.01 to 5 mM. Each slice was used for one study only and then discarded. When the effects of 4-aminopyridine, 3,4-diaminopyridine, catechol, tetraethylammonium chloride and protoveratrine on APB dose-response curves were investigated, each drug was applied to the pial surface of preparations 30 min before and also during application of the APB. The results were evaluated with a two factor analysis of variance followed by Dunnett's test for the comparison of treatments with control.

Synaptic facilitation studies

Slices were prepared and perfused in the usual manner and stimulated at 0.1 Hz (supramaximal voltage) for 1 h to allow for the majority of the time-dependent increase in N-wave amplitude to take place (Pickles & Simmonds, 1976; 1978). Preparations were then presented with pairs of supramaximal stimuli (conditioning interval of 20 to 1700 ms) at 10 s intervals. The amplitudes of the N-waves evoked by the test stimuli were calculated in terms of their percentage differences from those evoked by the corresponding conditioning stimuli and the values plotted graphically *vs* the appropriate conditioning interval (Figure 3a). The interval at which maximum facilitation occurred was identified (usually 100 ms) and using this fixed interval, slices were stimulated with a range of voltages from threshold to supramaximal: this procedure was necessary as the magnitude of the facilitation was dependent both on the stimulus voltage (Figure 2) and on the amplitude of the conditioning response (Figure 3b). The experiments were then repeated in the presence of APB. Each experiment was carried out using a total of between 4 and 6 slices.

In other studies, the effects of drugs on the synaptic facilitation produced by 1 mM APB were assessed. All of the drugs tested altered the amplitude of the N-wave and in order to analyse their interactions with APB quantitatively, synaptic facilitation was monitored at a fixed conditioning interval of 100 ms and at stimulus voltages at which the amplitudes of the N-waves evoked by the conditioning stimuli were 0.5 and 1 and, where possible, 1.5 and 2 mV: the range of evoked amplitudes did not differ by more than 10% from these values. In each experiment, the facilitation and N-wave amplitude evoked by supramaximal stimuli were also recorded. The procedures were repeated, (i) in the presence of APB; (ii) following perfusion with drug-free solution for 1 h to achieve recovery; (iii) following application of the drug under investigation to the pial surface of the slice for 20 min and; (iv) in the

presence of the drug plus APB. The mean percentage facilitation was calculated and the values plotted against the amplitude of the N-wave evoked by the conditioning stimulus (Figure 6). The results were evaluated with a two factor analysis of variance followed by Dunnett's test for the comparison of treatments with control.

Release studies

The effect of APB on the K^+-evoked release of endogenous aspartate, glutamate and GABA from small cubes of olfactory cortex tissue perfused with solution containing tetrodotoxin (0.25 μM) was also measured. The experimental procedure employed in preparing and perfusing the tissue fragments is described elsewhere (Collins *et al.*, 1981). The amino acids were estimated by a double label microdansylation procedure (Clark & Collins, 1976).

Results

When the LOT of olfactory cortex slices was stimulated twice in rapid succession, the amplitude of the N-wave evoked by the second (test) stimulus was greater than that evoked by the first (conditioning) pulse (Figure 1a). In the example illustrated, facilitation was maximal at conditioning intervals of approximately 100 to 200 ms and was detectable over intervals of up to 1700 ms. Application of APB (1 mM) reduced the amplitudes of the responses to both conditioning and test stimuli but markedly increased synaptic facilitation (Figure 1b). The degree of enhancement of facilitation produced by APB was concentration-dependent over the range 0.01 to 5 mM (Figures 2 and 3a). Note that neither APB (Figure 3a) nor stimulus voltage (not shown) had any marked effect on the conditioning interval at which maximum facilitation was observed.

In the present experiments, either stimulus voltage or the amplitude of the conditioning response might have been an appropriate index of presynaptic conditioning. When facilitation was plotted against stimulus voltage (Figure 2), a biphasic relationship was observed: at supramaximal stimulation, facilitation was independent of the stimulus intensity whereas at clearly submaximal voltages, there was an inverse relationship between the stimulus and facilitation. Application of APB (0.1 and 1 mM) essentially increased facilitation by the same extent at all stimulus voltages so that the proportional increase at low voltages was less than at high. In contrast, when facilitation was plotted against the amplitude of the

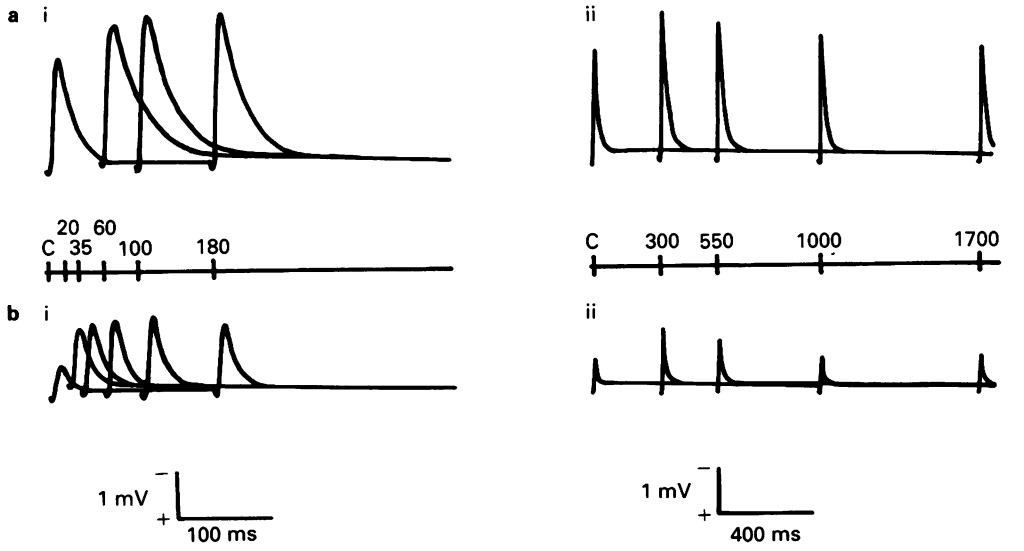

Figure 1 Synaptic facilitation evoked on supramaximal stimulation of the LOT of a single olfactory cortex slice using conditioning intervals between 20 and 1700 ms (see centre calibration). The traces (retouched) are of the N-waves evoked by the conditioning (c) and test stimuli and the recording timebase was changed between (i) and (ii) (see calibration bars) so that the potentials could be displayed conveniently. Each pair of stimuli was presented at 10 s intervals. In the absence of any drug (a) the maximum facilitation of the test response is approximately 15% whereas in the presence of 5 mM 2-amino-4-phosphonobutyrate, although the conditioning response is reduced in amplitude, facilitation of the test response is markedly increased (b).

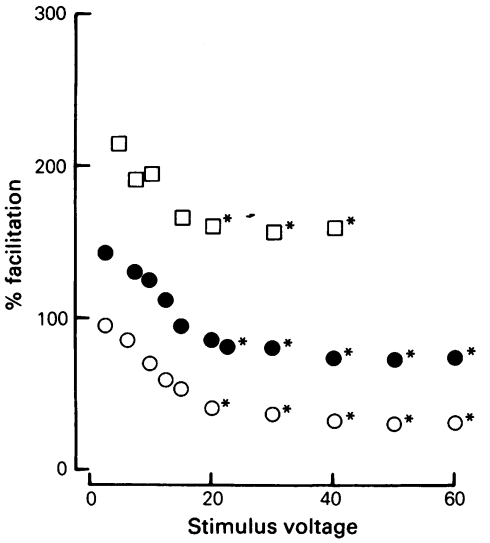

Figure 2 Effect of 2-amino-4-phosphonobutyrate (APB) on synaptic facilitation using stimulus voltage as the index of presynaptic conditioning. The results are from a single slice. Facilitation was measured at a conditioning interval of 100 ms over a range of stimulus voltages in the absence of drug (○) and during application of APB (0.1 mM, ●; 1.0 mM, □). An asterisk adjacent to a point indicates that the stimulus voltage was supramaximal. Similar results were seen in 3 other slices.

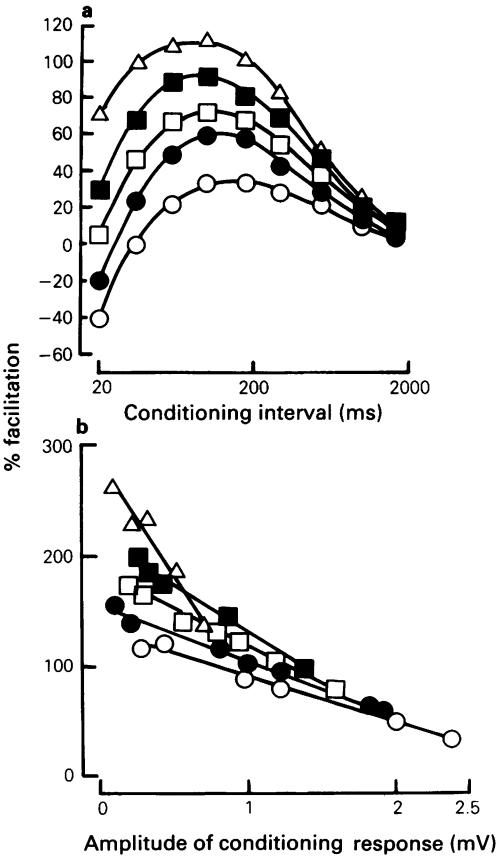

Figure 3 Concentration-dependency of the effects of 2-amino-4-phosphonobutyrate (APB) on synaptic facilitation. The results are from a single slice. Using supramaximal stimuli, facilitation was monitored over a range of conditioning intervals (a, ○). Then, at a fixed conditioning interval of 100 ms, facilitation was measured at stimulus voltages from threshold to supramaximal (b, ○). The procedures were repeated following application of APB (0.01 mM, ●; 0.1 mM, □; 1 mM, ■; 5 mM, △). Similar results were observed in 4 other slices. Note the progressive shift in the maximum limit of the conditioning response amplitude to lower values, reflecting the reduction in the amplitude of the N-wave by APB (see Figures 1 and 5).

conditioning response, a linear relationship with negative slope was observed (Figure 3b). Application of APB (0.01 to 5 mM) increased synaptic facilitation in a dose-dependent manner with an accompanying increase in the slope of the facilitation versus conditioning response relationship so that the proportional increase in facilitation was greater at low than at high amplitudes of the conditioning response. In spite of these differences, the amplitude of the conditioning response has been used as the index of presynaptic conditioning in all successive experiments (see Discussion).

Two other characteristics of the facilitation are shown in Figure 4. First in the absence of APB, facilitation was greater in preparations perfused with solution containing 1.25 mM (half-normal) calcium than when 5 mM was present (Figure 4a). In contrast, the increase in facilitation induced by 1 mM APB was less in slices perfused with 1.25 mM calcium than with the higher concentration; for example, in the experiment illustrated in Figure 4a, at a conditioning stimulus which evoked an N-wave of 0.5 mV in amplitude, APB in the presence of 1.25 mM calcium had no effect on facilitation whereas with 5 mM calcium, facilitation was marked. The second characteristic of the effect of APB on synaptic facilitation was that the L-(+)-isomer was markedly more potent than the D-(−)-isomer in increasing facilitation (Figure 4b).

As noted earlier, APB reduces the amplitudes of the N-waves evoked by both the conditioning and test stimuli (Figure 1). In experiments in which slices were stimulated using single stimuli at a rate of 0.1 Hz the reduction in N-wave amplitude by APB was concentration dependent over the range 0.01 to 5 mM (Figure 5) and was significantly greater in slices perfused with

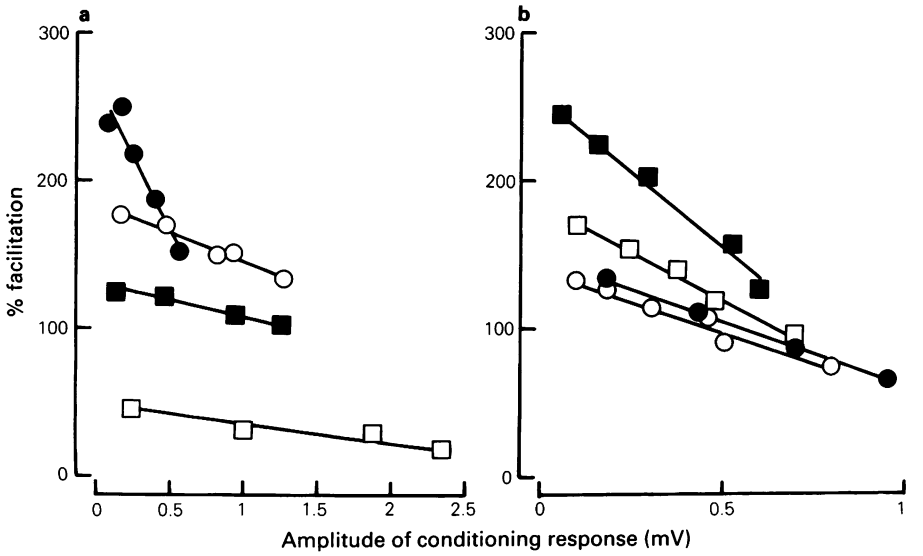

Figure 4 Some characteristics of the increase in synaptic facilitation produced by 2-amino-4-phosphonobutyrate (APB). The results shown in each panel are from different preparations. (a) Effects of calcium concentration on facilitation. The slice was preincubated and perfused with solution containing 1.25 mM calcium (half normal). Facilitation was measured at a fixed conditioning interval of 100 ms using a range of stimulus voltages both before (○) and during (●) application of 1 mM APB. Following perfusion with drug-free solution containing 5 mM calcium, facilitation was again monitored both before (□) and during(■) application of APB. Similar results were observed in 3 other slices. (b) Stereoselectivity of the increase in facilitation produced by APB (10 μM). In this experiment, following perfusion with solution containing normal calcium, facilitation was monitored at a fixed conditioning interval of 100 ms over a range of stimulus voltages both before (○) and during application of D-(−)-(●), DL-(±)-(□) and L-(+)-(■) APB. Similar results were observed in 3 other slices. Again, note the shift in the maximum limit of the conditioning response amplitude to lower values in the presence of APB (see legend to Figure 3).

solution containing 1.25 mM calcium than when 5 mM calcium was present (not shown).

If the site of action of APB in mediating an increase in synaptic facilitation and reduction in N-wave amplitude is presynaptic then its mechanism could involve either a reduction of calcium influx or, perhaps, potentiation of outward potassium currents. In order to test the latter possibility, the effects of the potassium channel blockers 4-aminopyridine, 3,4-diaminopyridine (Llinas *et al.*, 1976; Thompson, 1977; Kirsh & Narahashi, 1978; Bartschat & Blaustein, 1985), tetraethylammonium (Stanfield, 1983) and catechol (Ito & Maeno, 1986) on the actions of APB were investigated. Application of 4-aminopyridine (20 to 500 μM) or 3,4-diaminopyridine (5 to 500 μM) decreased whereas tetraethylammonium (5 to 20 mM) and catechol (0.05 to 2 mM; Collins & Dewhurst, 1986) both increased the amplitude of the N-wave in a concentration-dependent manner (not shown). The interactions of these drugs with APB were assessed at single concentrations (4-aminopyridine 0.25 mM, 3,4-diaminopyridine 0.1 mM, tetraethylammonium 10 mM and catechol 1 mM) which would have been at least partially effective in blocking potassium channels (see references above). All 4 drugs significantly attenuated synaptic facilitation but did not antagonize the increase in facilitation produced by APB either when stimulus voltage (not shown) or conditioning response amplitude (Figure 6) was used as the index of presynaptic conditioning. Protoveratrine (10 μM,), which opens tetrodotoxin-sensitive sodium channels but does not block potassium channels (Ulbricht, 1969), increased the amplitude of the N-wave but did not affect synaptic facilitation in either control or APB-treated slices (Figure 6). In single stimulus experiments, all 4 potassium channel blockers antagonized the reduction in N-wave amplitude produced by APB whereas protoveratrine was without effect (Figure 5).

In the final experiment, the effect of 1 mM APB on the release of endogenous amino acids from slices of olfactory cortex was monitored (Table 1). Alone, APB had no effect on release of aspartate, glutamate or GABA. However, it significantly reduced the K^+-evoked release of aspartate and glutamate but not of GABA.

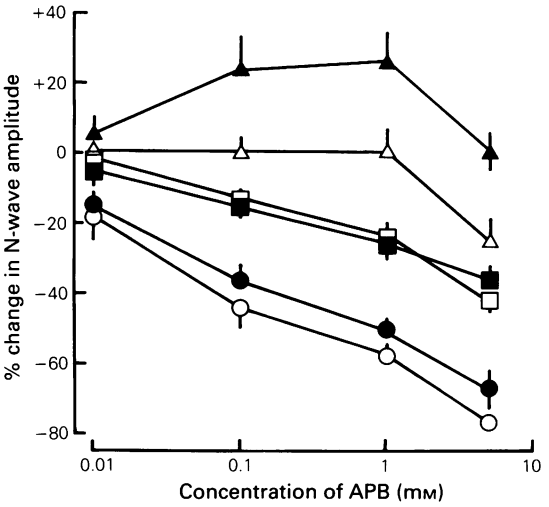

Figure 5 Drug effects on the reduction in N-wave amplitude produced by 2-amino-4-phosphonobutyrate (APB) alone (○) and in slices exposed to solutions of protoveratrine (5 μM, ●), catechol (1 mM, ■), tetraethylammonium (10 mM, □), 3,4-diaminopyridine (0.1 mM, △) or 4-aminopyridine (0.25 mM, ▲). Each point is a mean % change in amplitude (n between 4 and 6), the s.e.mean being indicated by the vertical bars. Apart from protoveratrine, all drugs significantly ($P<0.05$) antagonized the reduction in N-wave amplitude produced by APB (0.1 to 5 mM).

Discussion

The majority of the experiments described in this paper concerns the effect of APB on synaptic transmission and facilitation in the olfactory cortex slice. Although transmission is readily monitored, a problem arises in identifying whether stimulus voltage or the amplitude of the conditioning response is the better index of presynaptic conditioning during facilitation. In spite of the similarities between the relationships of facilitation and sub-maximal voltage stimuli (Figure 2) and amplitude of conditioning response (Figure 3b) the effects of APB are not identical, suggesting that the choice of conditioning parameter is of some significance. Despite the failure of the present results to resolve the problem, the amplitude of the conditioning response has been used generally as the indicator of presynaptic conditioning. First, the precise relationship between stimulus voltage and activation of the LOT fibres is obscure for there is no reason to assume that the fraction of the stimulus which depolarizes the fibres (as distinct from being shunted, for example, through the extracellular fluid) is constant at all stimulus voltages. Second, the LOT is composed of heterogeneous groups of fibres of varying diameters (Price & Sprich, 1975) which will differ in their stimulus thresholds. Third, the quantitative effects of APB on facilitation when applied alone or in the presence of potassium channel blockers remained statistically significant whichever index of presynaptic conditioning was employed. Nevertheless, identification of the appropriate estimate of presynaptic conditioning is important when considering the mechanism of action of APB (see later).

The results of the experiments described in this paper show that APB reduces transmission and increases facilitation at the LOT-superficial pyramidal cell synapse of the olfactory cortex. The L-(+)-isomer is markedly more potent than the D-(−)-form in causing both these effects (Collins, 1982; see Figure 4) suggesting that APB interacts with specific receptor sites (see also Koerner & Cotman, 1981; Davies & Watkins, 1982; Foster & Fagg, 1984). It is proposed that these sites are located on the LOT terminals. First, synaptic facilitation reflects a presynaptic phenomenon (for references see Introduction). The potentiation of facilitation by APB is best explained as a reduction in the amount of transmitter released by the conditioning stimulus so that more is available for release by the test pulse. Certainly, inhibition of transmitter release by lowering extracellular calcium levels (Figure 4a) or reducing stimulus intensity (Figures 2 and 3b) mimic the effects of APB on synaptic facilitation. Second, although there is controversy regarding the identity of the LOT transmitter(s) (ffrench-Mullen *et al.*, 1985; Collins, 1986), APB

Table 1 K^+-evoked release of endogenous amino acids from slices of rat olfactory cortex

Drugs	*Amino acid* *Aspartate*	*Glutamate*	*GABA*
K^+ 50 mM (control)	33.7 ± 5.2	136 ± 21	67.2 ± 8.1
K^+ 50 mM plus APB 1 mM	10.8 ± 3.7[a]	71.9 ± 8.3[b]	74.9 ± 11
APB 1 mM	0.9 ± 2.8	−1.2 ± 4.7	1.8 ± 7.3

APB: 2-amino-4-phosphonobutyrate; GABA: γ-aminobutyric acid. Each value is a mean release of amino acid (pmol mg^{-1} wet weight) ± s.e.mean of 5 observations. Significant difference (unpaired Student's t test) when compared with control [a]$P<0.01$; [b]$P<0.05$.

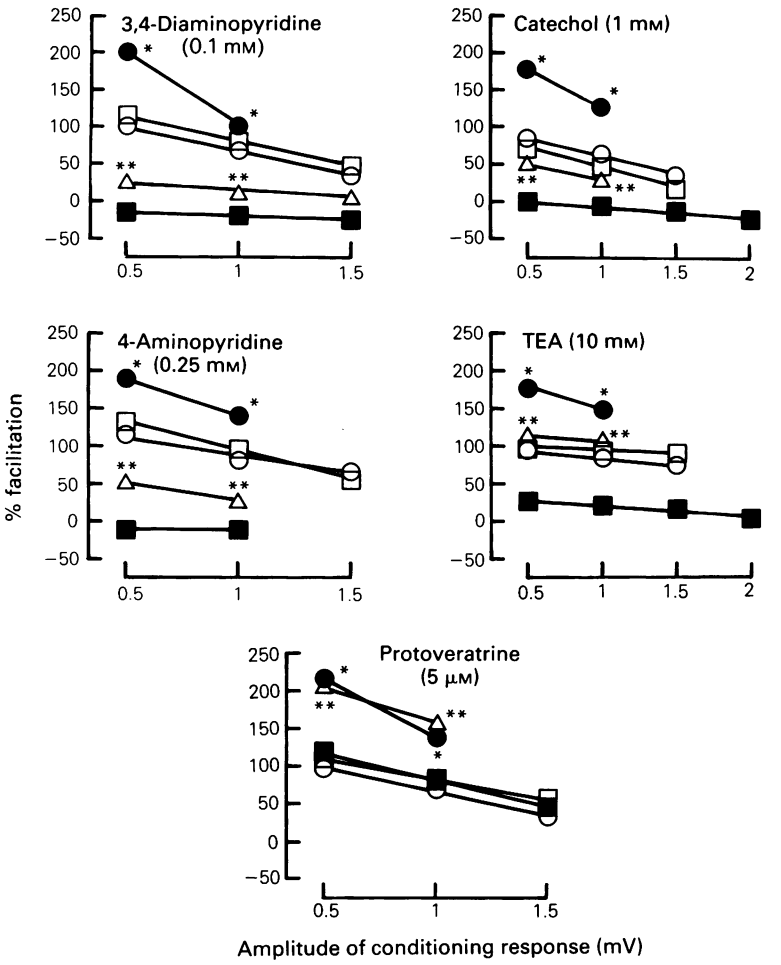

Figure 6 Drug effects on the increase in synaptic facilitation produced by 2-amino-4-phosphonobutyrate (APB) (1 mM). Each point is the mean % facilitation (n between 3 and 6) measured at fixed amplitudes of the conditioning response in slices stimulated at a constant conditioning interval of 100 ms. For clarity, the s.e.means are not shown. Facilitation was recorded in the sequence: control (○); plus APB (●); recovery (□); plus drug under investigation (■); drug plus APB (△). * Indicates a significant ($P < 0.05$) increase in facilitation by APB when compared with control. ** Indicates a significant ($P < 0.05$) increase in facilitation when compared with that occurring in the presence of the drug under test. Apart from protoveratrine, all drugs significantly ($P < 0.05$) reduced facilitation when applied alone.

inhibits the K^+-evoked release of L-aspartate and L-glutamate, two of the transmitter candidates of the LOT. Third, 5 mM APB is known to be ineffective as an antagonist of the postsynaptic actions of L-aspartate and L-glutamate in the olfactory cortex (Surtees & Collins, 1985; Collins & Brown, 1986). Even if the identities of the LOT transmitters are at fault, antagonism of postsynaptic receptors by APB would seem unlikely for it has been reported that at the perforant-path-dentate gyrus of the hippocampus, a site which also exhibits synaptic facilitation, postsynaptic receptor blockers reduce conditioning and test responses equally (Harris & Cotman, 1985).

There are numbers of mechanisms by which APB might inhibit transmitter release. For example, the compound might block an inhibitory conductance in the nerve terminals evoked by the conditioning stimulus. Such a possibility was suggested as a basis for the effects of adenosine on synaptic facilitation in the hippocampus (Dunwiddie & Haas, 1985) but rejected as the effects of a drug acting by such a mechanism would be greatest when the inhibitory

conductance was maximal (at high stimulus intensities and short conditioning intervals). The maximal increase in facilitation produced by adenosine (Dunwiddie & Haas, 1985) and APB (Figure 3) occur at low stimulus intensities and longer conditioning intervals. A second mechanism might involve a potentiation of outward potassium currents, thereby limiting calcium influx and hence transmitter release. The inability of a range of potassium channel blockers to antagonize the increase in synaptic facilitation produced by APB is not consistent with such a hypothesis. Presumably the ability of the blockers to reduce facilitation (Figure 6) and antagonize the reduction in N-wave amplitude by APB (Figure 5) is the result of an increased calcium influx and hence transmitter release. Perhaps the most attractive mechanism would be a direct inhibition of calcium into the nerve terminals by APB. However, if this were so, then calcium accumulation and transmitter release might be expected to be reduced in parallel so that the slope of the relationship between facilitation and conditioning response amplitude would remain constant (but see Figures 3b and 4a) as is the case when stimulus voltage is used as the index of presynaptic conditioning (Figure 2). An alternative explanation might be that APB interferes with transmitter release at a step subsequent to the influx of calcium.

In summary, the effects of APB on N-wave amplitude and synaptic facilitation are probably mediated by an inhibition of transmitter release from the LOT fibre terminals. This conclusion provides further support for the concept of a presynaptic site of action of APB.

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