J. Pharm. Pharmacol. 1991, 43: 417–420
Received September 10, 1990

Blockade by 4-Aminopyridine of the Muscarinic-receptor-mediated Responses of Guinea-pig Atria and Trachea

ROBERT A. URQUHART AND KENNETH J. BROADLEY

Department of Pharmacology, Welsh School of Pharmacy, University of Wales College of Cardiff, PO Box 13, Cardiff CF1 3XF, UK

Abstract—The potassium channel blocker 4-aminopyridine (4-AP) has been shown to antagonize the negative inotropic effects of muscarinic receptor agonists on atrial preparations. This is consistent with muscarinic agonists mediating their negative inotropy through the opening of potassium channels. In the present study, the possibility of a direct antagonism of the muscarinic receptor was examined by comparing the effects of 4-AP upon the responses to carbachol of isolated left atria (negative inotropy) and tracheal spirals (contraction) from reserpine pretreated guinea-pigs. The latter response is K^+ channel-independent. The concentration-response curve for carbachol on the paced left atria was displaced 520-fold to the right by 4-AP (10 mM). 4-AP alone caused dose-related contractions of the tracheal spirals. Carbachol-induced contractions were, however, superimposed upon the raised tone and there were substantial rightwards shifts of the concentration-response curves of 4·7- and 31·4-fold by 1 and 10 mM of 4-AP, respectively. Thus 4-AP appears to have muscarinic receptor antagonistic blocking properties. The blockade of the atrial responses was, however, substantially greater and could be attributed to an additional blockade of muscarinic receptor-linked potassium channels. The negative inotropic responses of the A_1- adenosine receptor agonist L-phenylisopropyladenosine (L-PIA) were also antagonized by 4-AP, but to a lesser extent than for carbachol. After allowing for the muscarinic receptor blocking activity of 4-AP, carbachol was still antagonized more effectively than L-PIA. This could be due to the presence of a K^+ channel-independent component in the response to L-PIA which is not susceptible to 4-AP.

4-Aminopyridine (4-AP) is a selective antagonist of potassium (K^+) channels in excitable membranes (Glover 1982). It has been shown to antagonize the negative inotropic effects of muscarinic agonists in guinea-pig (Freeman 1979; De Biasi et al 1989) and rabbit atria (Ray & MacLeod 1990). This finding supports the suggestion that muscarinic agonists mediate their negative inotropic effects through the opening of K^+ channels. The muscarinic receptor is thought to be directly linked to the K^+ channel by a pertussis toxin-sensitive guanine nucleotide binding protein (Pfaffinger et al 1985; Kurachi et al 1986; Yatini et al 1987). The increase in K^+ efflux arising from muscarinic receptor stimulation causes a reduction in action potential duration, decreases the time available for calcium influx and thus reduces the force of atrial contraction (Belardinelli & Isenberg 1983; Kurachi et al 1986; Kemmer et al 1989).

The negative inotropic effects of A_1-adenosine receptor agonists such as L-phenylisopropyladenosine (L-PIA) are also thought to be mediated in part at least via activation of K^+ efflux (Belardinelli & Isenberg 1983; Jochem & Nawrath 1983). However, De Biasi et al (1989) have recently found that 4-AP antagonized the muscarinic effects of carbachol in guinea-pig atria without affecting the negative inotropic response to L-PIA. They concluded that only the negative inotropy mediated via muscarinic receptors was dependent upon K^+ efflux. However, the possibility exists that 4-AP may additionally have muscarinic receptor blocking activity. Indeed, it has been shown to displace the binding of the muscarinic ligand [^{3}H]dexetimide from rat striatal muscarinic receptor binding sites (Drukarch et al 1989). The present study therefore compares the effects of 4-AP upon the responses of the atria to carbachol and L-PIA which are thought to be mediated via outward K^+ channels with a K^+ channel-independent response to carbachol, namely the contraction of the guinea-pig trachea.

Correspondence: K. J. Broadley, Department of Pharmacology, Welsh School of Pharmacy, University of Wales College of Cardiff, PO Box 13, Cardiff CF1 3XF, UK.

Materials and Methods

Drugs

4-Aminopyridine, carbamylcholine chloride (carbachol), L-phenylisopropyladenosine (L-PIA) and reserpine were obtained from Sigma. Reserpine was dissolved by adding benzyl alcohol (0·08 mL) to reserpine (10 mg) and citric acid (10 mg). To this was added Tween 80 (0·4 mL) and the concentration adjusted to 2·5 mg of reserpine per mL with distilled water. All other drugs were dissolved in distilled water.

Isolated tissues

Guinea-pigs (Dunkin-Hartley, 250–450 g, male) were pretreated with reserpine (5 mg kg^{-1} i.p.) 24 h before use. Animals were killed by a blow to the head and exsanguinated under running water. The rib cage was opened and the left atrium removed with cottons attached to the tip of the left atrial appendage and the atrioventricular junction. The latter cotton attached the atrium to the electrode tips of a Harvard bipolar platinum electrode and the first cotton was attached to an isometric transducer (Devices, UFI, 57 g sensitivity range).

The trachea was dissected out down to the bronchi and any fat or connective tissue removed. A spiral was cut (70 × 5 mm) and one end anchored to a tissue holder. A cotton secured to the other end was attached to an isometric transducer.

Both tissues were immersed in 50 mL organ baths containing Krebs-bicarbonate solution of composition (mM) in double distilled water: NaCl 118, KCl 4·69, $CaCl_2 \cdot 2H_2O$ 2·52, $MgSO_4 \cdot 7H_2O$ 1·18, $KH_2PO_4 \cdot 2H_2O$ 1·18, $NaHCO_3$ 25, glucose 11·7, which was maintained at 37·5±0·5°C and gassed with 5% CO_2 in oxygen. The transducers were adjusted to apply resting tensions of 0·5–0·75 g to the atria and 0·8–1·0 g to the trachea. Isometric tension was recorded on a Devices M19 polygraph (Lectromed, Welwyn Garden City).

The atria were paced at 2 Hz with square-wave pulses of 5 ms duration at threshold voltage (+50%) delivered by a Harvard Research 50–75 stimulator.

Construction of concentration-response curves

An equilibration period of approximately 60 min was allowed, during which time the tissue was washed every 15 min. In the atria, a single concentration-response curve to carbachol or L-PIA was obtained in each preparation by cumulative addition of log increments in concentration. The curve was obtained either in the absence or presence of 4-AP added 5–10 min before commencing the agonist curve.

In tracheal spirals, a concentration-response curve for carbachol was obtained by cumulative addition in half log increments in concentration to the maximum contraction. The tissue was then washed 5 times over 60 min to restore tension to the resting level, 4-AP added and a second curve for carbachol obtained in its presence. To allow for time-dependent changes in tracheal sensitivity, control experiments were performed in which two carbachol concentration-response curves were obtained but without addition of 4-AP between them.

Measurement of responses

Atrial developed tension was measured at the resting level and at the peak effect of each concentration of agonist. The concentration of agonist producing a 30% inhibition of resting developed tension (IC30) was determined and the geometric mean IC30 values calculated with their 95% confidence limits.

Tracheal tension increases in response to each concentration of carbachol were measured. From individual control experiments, the tension of the second curve was divided by the tension of the first curve at the corresponding concentration of carbachol. Mean ($n \geq 4$) correction factors were thus obtained for each concentration of carbachol. The tension changes on the pre-4-AP concentration-response curves of test experiments were then corrected by multiplying by the appropriate correction factor. The tension changes were then plotted as a percentage of the maximum response in two ways. Firstly, the increases in tension induced by carbachol alone in the first curve and by 4-AP plus carbachol in the second curve, above the initial resting level, were plotted as a percentage of the pre-4-AP corrected maximum response. Secondly, the increases in tension induced by carbachol alone were expressed as percentages of their own maximum response. The concentration of carbachol producing a 50% increase in tension by the latter method (EC50) was determined and geometric mean EC50 values with their 95% confidence limits were calculated.

Statistical comparisons were made by Student's unpaired (atria) or paired (trachea) *t*-tests applied to tension values or to IC30 or EC50 values. A significant difference was judged to occur when $P < 0.05$.

Results

Left atria

4-AP (10 mM) caused a large transient increase in left atrial tension; however, within 3–4 min the developed tension had returned to a level (0·94±0·09 g) not significantly different from that before 4-AP addition (0·88±0·08 g).

The concentration-response curve for the negative inotropic response to carbachol was displaced to the right by 4-AP (10 mM) (Fig. 1A). The IC30 increased significantly from 71(36–142) nM to 37(12–114) μM, a 520-fold shift.

In the presence of 4-AP (10 mM), the negative inotropic response to L-PIA was inhibited (Fig. 1B). The inhibition of atrial tension by the maximum concentration of L-PIA in the presence of 4-AP (56·5±6·1%) was significantly less than in its absence (86·3±3·4%). The IC30 was significantly increased from 54(27–107) to 198(36–577) nM.

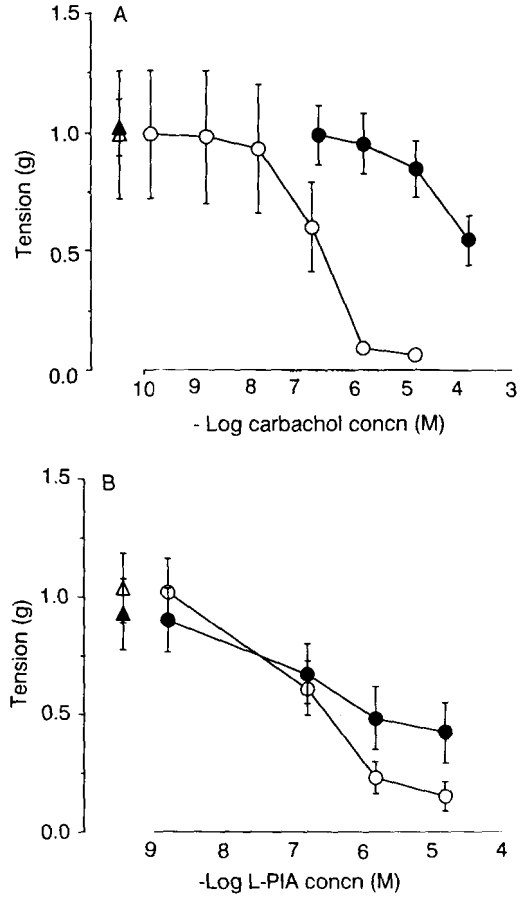

FIG. 1. Effects of 4-aminopyridine (10 mM) (4-AP) on the negative inotropic responses of guinea-pig paced left atria to A. Carbachol and B. L-PIA. A single cumulative concentration-response curve was obtained in each atrium either in the absence (○) or presence of 4-AP (●). Mean ($n \geq 4$) absolute developed tensions (g) are shown at the resting levels in the absence (△) and presence of 4-AP (▲) immediately before the first agonist addition, and at each concentration of agonist. Vertical bars represent the s.e.m.

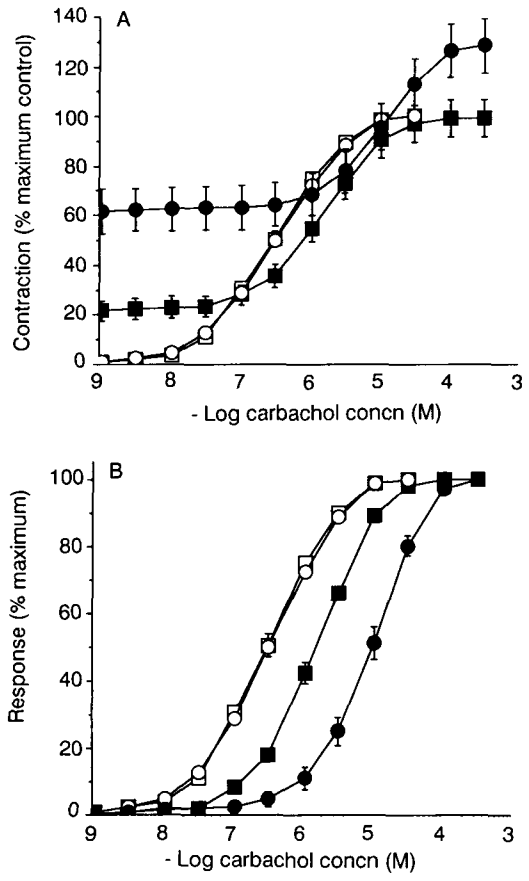

FIG. 2. Effects of 4-aminopyridine (4-AP) on the contractile responses of guinea-pig tracheal spirals to carbachol. Concentration-response curves were obtained before (open symbols) and in the presence of 4-AP, 1 mM (■) or 10 mM (●). A. Contractions measured from the initial baseline and expressed as a percentage of the maximum contraction obtained before 4-AP. B. Contractions measured from the baseline immediately preceding the curve and expressed as a percentage of their own maxima. Pre-4-AP curves were corrected for time-dependent changes in sensitivity from time-matched controls as described in the text. Each point is the mean of at least 4 tissues and vertical bars represent the s.e.m.

Tracheal spirals

In the absence of 4-AP, carbachol produced concentration-dependent contractions of the tracheal spiral with an EC50 value of 322(271–383) nM. 4-AP alone produced significant concentration-dependent contractions of the tracheal spirals. These were equivalent to 21·6±4·2 and 61·8±6·0% of the maximum carbachol-induced contractions obtained before adding the 4-AP, for the 1 mM and 10 mM concentrations, respectively (Fig. 2A). The maximum contraction produced by carbachol in the presence of 4-AP (1 mM) (0·97±0·13 g) was not significantly different from that of carbachol alone (0·99±0·12 g). However, the maximum contraction produced by carbachol in the presence of 4-AP (10 mM) (1·22±0·1 g) was significantly greater than that produced by carbachol alone in the same tissues (0·98±0·12 g). When the tracheal contractions induced by carbachol both in the absence and presence of 4-AP were plotted as percentages of their own maximum responses (Fig. 2B), the rightwards shifts of the curves in the presence of 4-AP were apparent. In the presence of 4-AP (1 mM), the carbachol EC50 value was significantly increased from 0·32 (0·20–0·52) to 1·5 (1·0–2·3) μM, a 47-fold increase. Similarly, in the presence of 4-AP (10 mM), the carbachol EC50 value was significantly increased from 0·32 (0·26–0·41) to 10·2 (6·4–16·2) μM, a 31·4-fold increase.

Discussion

The increase in left atrial tension induced by 4-AP confirms previous reports (Glover 1981; Furukawa et al 1985) and is consistent with an inhibitory effect upon K^+ channels, the reduced efflux of K^+ permitting prolongation of the action potential which promotes a rise in intracellular Ca^{2+} (Glover 1982). However, the effect did not persist. Part of the positive inotropy seen in rabbit papillary muscles has been attributed to the concomitant rise in extracellular pH with 4-AP (Shahid & Rodger 1989). However, the present response was largely unaffected by simultaneous neutralization with HCl (unpublished observation). 4-AP is also known to release catecholamines (Glover 1982); however, this cannot explain the positive inotropy observed here because the animals were routinely pretreated with reserpine to deplete endogenous catecholamines.

The negative inotropic responses to both the A_1-receptor and muscarinic receptor agonists L-PIA and carbachol were antagonized by 4-AP. At first sight this is consistent with the view that muscarinic and A_1-receptors are linked to K^+ channels and mediate, at least in part, the negative inotropic responses to carbachol and L-PIA (Belardinelli & Isenberg 1983; Kurachi et al 1986). The antagonism of L-PIA was substantially less and is thus compatible with the observation of De Biasi et al (1989) who found substantial antagonism of carbachol but not of L-PIA. They inferred that K^+ channels were not involved in the response to L-PIA. The present results do, however, suggest their involvement, although because of the minor degree of antagonism, other mechanisms may also be responsible for the negative inotropy. The greater degree of antagonism of the carbachol response, as suggested by De Biasi et al (1989), may indicate a more important role for K^+ channels in the response to muscarinic receptor stimulation. It is unlikely that the selectivity lies in a differential susceptibility of the K^+ channels to blockade by 4-AP since the channels activated by A_1- and muscarinic-receptors in the left atria are part of the same K^+ channel population (Belardinelli & Isenberg 1983; Kurachi et al 1986).

An alternative explanation for the differential effect upon L-PIA and carbachol responses, hitherto ignored, is that 4-AP may have muscarinic receptor antagonistic activity. This possibility was examined by determining the effect of 4-AP upon the K^+ channel-independent contractile response of guinea-pig trachea to carbachol. Interpretation of the effects of 4-AP upon the carbachol concentration-response curve was complicated by the fact the 4-AP itself caused a dose-dependent contraction of the tracheal spirals. Stimulation of acetylcholine release has been reported to account for the majority of 4-AP-induced contractions in smooth muscle preparations (Glover 1982). Indeed, the 4-AP-induced contraction of canine tracheal muscle is atropine sensitive (Kannan & Daniel 1979). This possible release of acetylcholine would further complicate the determination of any

atropine-like activity of 4-AP, since this would tend to be opposed by its ability to release acetylcholine. Thus any estimate of 4-AP activity as a muscarinic antagonist would be an underestimate of its true activity.

When the concentration-response curves in the presence of 4-AP were plotted as a percentage of their own maxima, clear parallel rightwards shifts of the curves were obtained; 4·7- and 31·4-fold shifts of the curves were obtained with 1 and 10 mM concentrations of 4-AP, respectively. Therefore, 4-AP is an antagonist of the muscarinic receptors mediating the carbachol-induced contraction of the guinea-pig trachea. This supports the finding that 4-AP displaces the binding of the muscarinic ligand [^{3}H]dexetimide from rat striatal muscarinic receptor binding sites (Drukarch et al 1989).

Therefore, the selective blockade by 4-AP of muscarinic receptor- compared with A_1-receptor-mediated negative inotropy in left atria may be partly explained by the muscarinic antagonistic activity of 4-AP. This possibility was not considered by De Biasi et al (1989). 4-AP (10 mM) induced a 31·4-fold increase in the carbachol EC50 value in tracheal spirals whereas in left atria, the same concentration produced a 520-fold increase in carbachol IC30. Therefore, the negative inotropic responses of left atria to carbachol are more sensitive to 4-AP than the contractile responses of tracheal spirals. This may be due to a difference in the affinity of 4-AP for different muscarinic receptor subtypes. The cardiac muscarinic receptors are M_{2A}- or M_2- receptors, whereas the muscarinic receptors found in smooth muscle are M_{2B}- or M_3-receptors (Eglen & Whiting 1986). If 4-AP has a higher affinity for the M_2-receptors, then it would block cardiac effects of muscarinic receptor agonists more effectively than the tracheal smooth muscle effects. However, such a simple molecule as 4-AP is unlikely to exhibit such a high degree of selectivity and therefore a difference in affinity for the muscarinic receptor subtypes is unlikely to explain the far greater blockade of atrial responses.

The most likely explanation for the selective antagonism of atrial responses is that whereas the antagonism of atrial responses to carbachol is due to a combination of muscarinic receptor antagonism and blockade of muscarinic receptor-linked K^+ channels, the antagonism of tracheal responses is due to muscarinic receptor blockade alone.

If the muscarinic antagonist activity of 4-AP is taken into account (31·4-fold shift) when examining the antagonism of the negative inotropic responses to carbachol and L-PIA, the responses to carbachol are still more sensitive to 4-AP than are those to L-PIA. Therefore, the greater sensitivity to 4-AP of the negative inotropic responses to the muscarinic agonist carbachol compared with the A_1-receptor agonist L-PIA, are only partially explained by the muscarinic receptor blocking properties of 4-AP. The resulting lesser blockade of L-PIA compared with carbachol may arise because the negative inotropic response to L-PIA has an additional component that is potassium efflux-independent and thus not susceptible to 4-AP.

Acknowledgements

This work was supported by an SERC CASE studentship in collaboration with SmithKline Beecham, Welwyn, Herts. We thank Dr Alan Rothaul of that Company for helpful discussions.

References

Belardinelli, L., Isenberg, G. (1983) Isolated atrial myocytes: adenosine and acetylcholine increase potassium conductance. Am. J. Physiol. 244: H734–H737

De Biasi, M., Froldi, G., Ragazzi, E., Pandolfo, L., Caparrotta, L., Fassini, G. (1989) Potassium channel blockers differentially affect carbachol and (−)-N^6-phenylisopropyladenosine on guinea-pig atria. Br. J. Pharmacol. 97: 866–872

Drukarch, B. N., Kits, K. S., Leysen, J. E., Schepens, E., Stoof, J. C. (1989) Restricted usefulness of tetraethylammonium and 4-aminopyridine for the characterization of receptor-operated K^+-channels. Ibid. 98: 113–118

Eglen, R. M., Whiting, R. L. (1986) Muscarinic receptor subtypes: a critique of the current classification and a proposal for a working nomenclature. J. Auton. Pharmacol. 6: 323–346

Freeman, S. E. (1979) Cholinergic mechanisms in heart: interactions with 4-aminopyridine. J. Pharmacol. Exp. Ther. 210: 7–14

Furukawa, Y., Saegusa, K., Chiba, S. (1985) The mode of action of 4-aminopyridine on the chronotropic and inotropic responses in the isolated, blood perfused dog heart. Eur. J. Pharmacol. 114: 317–323

Glover, W. E. (1981) Cholinergic effect of 4-aminopyridine and adrenergic effect of 4-methyl-2-aminopyridine in cardiac muscle. Ibid. 71: 21–31

Glover, W. E. (1982) The aminopyridines. Gen. Pharmacol. 13: 259–285

Jochem, G., Nawrath, H. (1983) Adenosine activates a potassium conductance in guinea-pig atrial heart muscle. Experientia 39: 1347–1349

Kannan, M. S., Daniel, E. E. (1979) Formation of gap junctions by treatment in vitro with potassium conductance blockers. J. Cell Biol. 78: 338–348

Kemmer, M., Jakob, H., Nawrath, H. (1989) Pronounced cholinergic but only moderate purinergic effects in isolated atrial and ventricular heart muscle from cats. Br. J. Pharmacol. 97: 1191–1198

Kurachi, Y., Nakajima, T., Sugimoto, T. (1986) On the mechanism of activation of muscarinic K^+ channels by adenosine in isolated atrial cells: involvement of GTP-binding proteins. Pflügers Arch. 407: 264–274

Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. M., Hille, B. (1985) GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature 317: 536–538

Ray, A., MacLeod, K. M. (1990) Adrenergic-cholinergic interactions in left atria: a study using K^+ channel agonists, antagonists and pertussis toxin. Br. J. Pharmacol. 99: 661–666

Shahid, M., Rodger, I. W. (1989) The inotropic effect of 4-aminopyridine and pH changes in rabbit papillary muscle. J. Pharm. Pharmacol. 41: 601–606

Yatini, A., Codina, J., Brown, A. M., Birnbaumer, L. (1987) Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein G_k. Science 235: 207–211