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Effects of phospholipase A_2 inhibitors on coupling of α_2-adrenoceptors to inwardly rectifying potassium currents in guinea-pig submucosal neurones

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1 Noradrenaline hyperpolarizes guinea-pig submucosal neurones by opening inwardly rectifying potassium channels. Intracellular recordings were made from submucosal neurones and the possible involvement of the phospholipase A_2 pathway in this response was examined.

2 The non-specific phospholipase A_2 inhibitors, quinacrine (10 μM) and 4-bromophenacyl bromide (4-BPB, 10 μM) inhibited nerve-evoked inhibitory synaptic potentials (i.p.s.ps) and hyperpolarizations to somatostatin and UK 14304. Quinacrine and 4-BPB also blocked the inward rectification present in current-voltage curves in the absence of somatostatin or UK 14304.

3 The more selective phospholipase A_2 inhibitor, cyclosporin A (10 μM) and the lipoxygenase and cyclo-oxygenase inhibitor, eicosatetraynoic acid (ETYA, 20 μM) and nordihydroguairetic acid (NDGA, 20 μM) did not alter i.p.s.ps or hyperpolarizations to UK 14304.

4 Exogenously applied arachidonic acid (1–300 μM) did not mimic the i.p.s.p. or the hyperpolarization to UK 14304.

5 We conclude that arachidonic acid or its eicosanoid metabolites produced by phospholipase A_2 stimulation are unlikely to be involved in the receptor G-protein coupled activation of potassium currents in submucosal neurones. The inhibition of the noradrenaline-induced hyperpolarization by quinacrine and 4-BPB is most likely due primarily to blockade of the basal inwardly rectifying potassium conductance present in these neurones.

Keywords: Enteric neurones; arachidonic acid; phospholipase A_2; electrophysiology; G-protein coupled receptors

Introduction

Arachidonic acid can be released following the activation of phospholipase A_2 by receptor-coupled G proteins (Axelrod *et al.*, 1988; Piomelli & Greengard, 1990). Arachidonic acid or its eicosanoid metabolites have been shown to act as second messengers in neuronal inhibition by G-protein-coupled receptors which act by increasing potassium conductances (Piomelli *et al.*, 1987; Kurachi *et al.*, 1989; Volterra & Siegelbaum, 1989; Miller *et al.*, 1992). This mechanism of action has been particularly well characterized for the FMRF-amide induced activation of the S-channel in *Aplysia* neurones (see review by Volterra & Siegelbaum, 1989). There is much less information regarding the involvement of this pathway in activation of G-protein coupled potassium conductances in mammalian neurones. In rat hippocampal pyramidal neurones the increase in neuronal M-current by somatostatin can be mimicked by the application of arachidonic acid or its eicosanoid metabolite, leukotriene C_4 (Schweitzer *et al.*, 1990) and arachidonic acid has been shown to open potassium channels in excised membrane patches obtained from these neurones (Premkumar *et al.*, 1990).

The opening of potassium channels following the activation of inhibitory receptors has been studied extensively in guinea-pig submucosal neurones (North, 1989). These neurones receive inhibitory synaptic inputs through the activation of α_2-adrenoceptors by noradrenaline released from sympathetic nerves and by somatostatin released from intrinsic enteric nerves (Mihara *et al.*, 1987a,b; Surprenant & North, 1988; Bornstein *et al.*, 1988). These hyperpolarizations are mediated through the activation of an inwardly rectifying potassium conductance. The transduction is blocked by pertussis toxin (Surprenant & North, 1988), and is guanosine 5′-triphosphate-sensitive (Shen *et al.*, 1992) demonstrating the activation is G-protein coupled. The involvement of protein kinases A and C as second messengers in this transduction pathway has been discounted (Surprenant & North, 1988). We have shown previously that the opening of potassium channels by noradrenaline can be recorded in excised patches of submucosal neurones (Shen *et al.*, 1992). These results suggest that either the potassium channel is modulated directly by the G-protein coupled receptor or the second messenger system is closely associated with the cell membrane. A possible candidate for such a second messenger system is the production of arachidonic acid and its metabolites from membrane phospholipid by the membrane-bound phospholipase, phospholipase A_2 (Kennedy, 1992). Recently, it has been suggested that α_2 adrenoceptor activation may be coupled to the stimulation of phospholipase A_2 and the production of arachidonic acid (Jones *et al.*, 1991; Gonzales *et al.*, 1991). The aim of the present study was to examine whether arachidonic acid or its metabolites may be involved in the hyperpolarizations to somatostatin and α_2-adrenoceptor activation in guinea-pig submucosal neurones.

Methods

Submucosal plexus preparations were obtained from the small intestine of guinea-pigs (150–250 g); methods of tissue preparation were as described previously (Surprenant, 1984). Tissues were superfused at 3–4 ml min^{-1} with a physiological solution of the following composition (mM): NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 5, $NaHCO_3$ 25 and glucose 11; gassed with 95% O_2 and 5% CO_2. The temperature was maintained at 34–36°C. Test agents were added to the superfusion medium to give the required final concentration.

Changes in membrane potential of submucosal neurones

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were recorded with glass microelectrodes, filled with 1 M KCl and 1% neurobiotin (Vector laboratories) with tip resistances of 70–180 MΩ. Signals were recorded with an Axoclamp 2A amplifier and displayed on a Gould 2400S chart recorder. When successful impalements were obtained with microelectrodes of approximately 70–90 MΩ, membrane currents were measured with a single-electrode voltage clamp amplifier (Axoclamp 2A) with a switching frequency of 1–3 kHz. Steady-state voltage/current relationships were obtained by measuring membrane current during slow (5 mV s^{-1}) ramps of membrane potential. Signals were also digitised at 5–10 kHz and data were acquired using pClamp 5.5 software.

A blunt glass microelectrode (tip diameter 20–50 μm) filled with physiological saline was used to evoke synaptic potentials from submucosal neurones following the stimulation of adjacent ganglia (for nicotinic excitatory postsynaptic potentials (e.p.s.ps) 0.1 Hz, pulse width 0.1 ms, 5–15 V, and noradrenergic inhibitory postsynaptic potentials (i.p.s.ps) 5 pulses 20 Hz, pulse width 0.2 ms, 50 V). I.p.s.ps and agonist-induced hyperpolarizations were recorded at − 60 mV; nicotinic e.p.s.ps were recorded at a holding potential of − 90 mV in order to suppress action potential initiation.

Acetylcholine (ACh) (1 mM) was applied by ionophoresis from fire polished patch clamp electrodes (resistance 3–5 MΩ). Duration of the ionophoretic pulses ranged from 10 to 50 ms (30–50 V). Ionophoretic pulses were delivered at a frequency of 0.1 Hz throughout the course of the experiment.

Drug effects are expressed as a percentage of the response before drug application; all values are mean ± s.e.mean. Tests of significance were by Student's t test; $P<0.05$ was considered statistically significant.

Drugs

The following drugs were used, acetylcholine, arachidonic acid (sodium salt), 4-bromophenacyl bromide (4-BPB), 5,8,11,14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), quinacrine, tetrodotoxin and somatostatin (Sigma); cyclosporin A (Sandoz), UK 14304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline, gift from Pfizer). Drugs were made up as concentrated stock solutions. 4-BPB was dissolved in dimethylsulphoxide (DMSO), arachidonic acid, ETYA and NDGA were made up in ethanol; these solvents had no effects on responses when applied in vehicle control experiments.

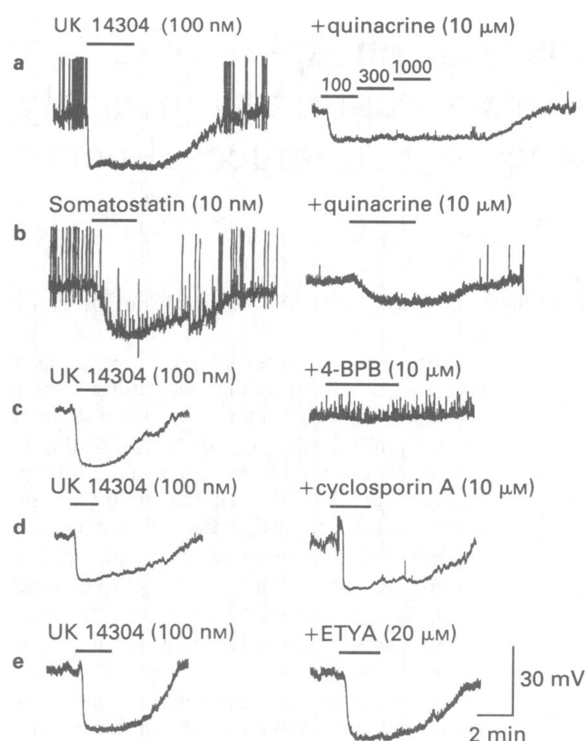

Figure 1 Effects of phospholipase A_2 inhibitors, quinacrine, 4-BPB, and cyclosporin A, and the cyclo-oxygenase and lipoxygenase inhibitor ETYA on somatostatin and α_2-adrenoceptor-mediated hyperpolarizations. Traces in (a)-(e) are recordings obtained before (lefthand records) and during the application of phospholipase A_2 inhibitors (righthand records); each set of recordings were obtained in separate experiments. (a,b) Quinacrine (10 μM) reduced the hyperpolarization produced by the α_2-adrenoceptor agonist, UK 14304 (UK, a) or somatostatin (b). (c) 4-BPB (10 μM) abolished the UK 14304-induced hyperpolarization; note the high frequency of spontaneous nicotinic e.p.s.ps in the presence of 4-BPB. (d,e) The hyperpolarization in response to UK 14304 was unaltered in the presence of cyclosporin A (10 μM) (d) or ETYA (20 μM) (e). For abbreviations, see text.

Results

Electrophysiological properties of submucosal neurones

Recordings were made from submucosal neurones that exhibited noradrenergic inhibitory postsynaptic potentials (i.p.s.ps) and/or were hyperpolarized by the α_2-adrenoceptor agonist, UK 14304 or somatostatin. Resting membrane potentials ranged from − 48 mV to − 63 mV (mean ± s.e.mean = − 54 ± 1.6 mV, n = 31). These neurones, which also show nicotinic excitatory postsynaptic potentials (e.p.s.ps), are classified as S or type 1 neurones (Nishi & North, 1973; Hirst *et al.*, 1974). Electrical stimulation (5 pulses at 20 Hz) evoked i.p.s.ps; the peak amplitude of these was 26.8 ± 1 mV (n = 20) (Figure 2). Superfusion with UK 14304 (100 nM) or somatostatin (10 nM) hyperpolarized these submucosal neurones by 24.8 ± 1.3 mV (n = 20) and 24.6 ± 3 mV (n = 4) respectively. Agonist-induced hyperpolarizations were reversed on washout (Figure 1).

Effects of phospholipase A_2 inhibitors on i.p.s.ps and hyperpolarizations to UK 14034 and somatostatin

The non-specific phospholipase A_2 inhibitors, quinacrine (mepacrine) and 4-BPB, and the relatively more specific inhibitor cyclosporin A (Wallach & Brown, 1981; Blackwell & Flower, 1983; Fan & Lewis, 1985; Niwa *et al.*, 1986; Schweitzer *et al.*, 1990; El-Etr *et al.*, 1992) were used to examine whether phospholipase A_2 may be coupled to the G-protein activation of inwardly rectifying potassium currents in submucosal neurones. Quinacrine (10 μM) reduced significantly the nerve evoked i.p.s.p. (Figure 2a), and the hyperpolarizations to UK 14304 (100 nM) and somatostatin (10 nM) by 92 ± 4% (n = 8), 58 ± 8% (n = 11) and 70 ± 5% (n = 4) respectively (Figure 1). Increasing the concentration of UK 14304 ten fold produced no further hyperpolarization (Figure 1a) indicating that quinacrine was not acting competitively. 4-BPB (10 μM) reduced the nerve-evoked i.p.s.p. by >95% (n = 5; Figure 2b) and reduced the hyperpolarization to UK 14304 by 94 ± 8% (n = 4; Figure 1c). An increase in the frequency of spontaneous e.p.s.ps was often recorded during the application of 4-BPB (e.g. Figure 1c). The inhibition of the UK 14304-induced hyperpolarization by quinacrine and 4-BPB was similar when experiments were carried out in the presence of tetrodotoxin (1 μM) to block indirect effects that might occur due to possible release of neurotransmitters by quinacrine or 4-BPB (n = 2). The effects of quinacrine and 4-BPB were not reversed after 20 min washout. Cyclosporin A (10 μM) had no effect on the i.p.s.p. amplitude (Figure 2c) or the hyperpolarization to UK 14304 (Figure 1d; 105.5 ± 2.5% (n = 8) and 108.7 ± 3.3% (n = 6) of control respectively).

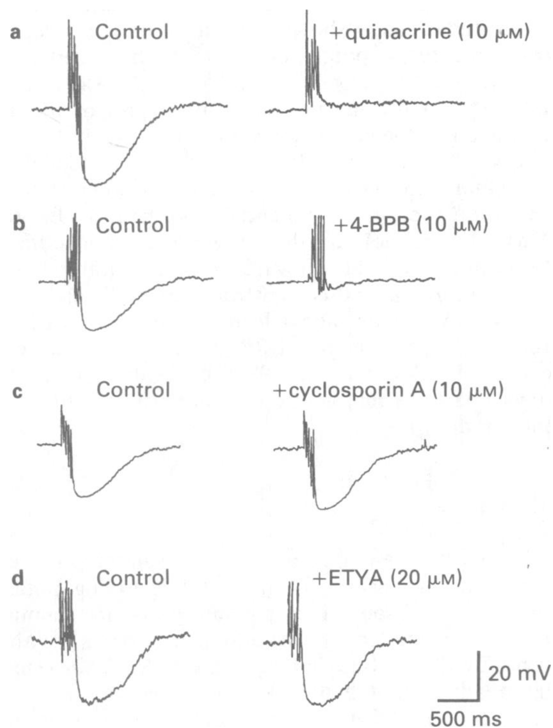

Figure 2 Effects of phospholipase A_2 inhibitors, quinacrine, 4-BPB, and cyclosporin A, and the cyclo-oxygenase and lipoxygenase inhibitor ETYA on nerve evoked i.p.s.ps. I.p.s.ps were evoked by electrical stimulation of adjacent ganglia with 5 pulses at 20 Hz. Traces in (a)-(d) are recordings obtained before (lefthand records) and during the application of drugs (righthand records); each set of recordings were obtained in separate experiments. (a,b) Quinacrine (10 μM) (a) and 4-BPB (10 μM) (b) reduced nerve evoked i.p.s.ps by >95%. (c,d) The amplitude of nerve evoked i.p.s.ps was unaffected by cyclosporin A (10 μM) (c) or ETYA (20 μM) (d). For abbreviations, see text.

Effects of cyclo-oxygenase and lipoxygenase inhibitors on hyperpolarizations to UK 14304

Arachidonic acid can be metabolised by two main routes: by cyclo-oxygenase to produce prostaglandins, and/or by lipoxygenase to produce hydroperoxyeicosatetraenoic acid derivatives. In this study neither the lipoxygenase and cyclo-oxygenase inhibitor ETYA (20 μM; Figure 1e) nor the lipoxygenase inhibitor NDGA (20 μM) inhibited the hyperpolarizations to UK 14304; UK 14304-induced hyperpolarizations were 89 ± 5% ($n = 5$) and 115 ± 15% ($n = 2$) of control respectively. Similarly, ETYA (20 μM) did not alter the i.p.s.p. amplitude (96 ± 4% of control i.p.s.p., $n = 3$; Figure 2d).

Effects of exogenously applied arachidonic acid

The exogenous application of arachidonic acid or its metabolites have been demonstrated to mimic G-protein activation in those cases where phospholipase A_2 stimulation has been well documented in signal transduction (Buttner *et al.*, 1989; Schweitzer *et al.*, 1990). In the present study superfusion with arachidonic acid (1–20 μM) produced no significant change in membrane potential ($n = 4$). Higher concentrations (100–300 μM) also did not alter the membrane potential ($n = 2$) or produced a 10–15 mV depolarization ($n = 3$). Arachidonic acid (100 μM) was without effect when it was applied in the presence of ETYA to prevent the enzymatic degradation of arachidonic acid ($n = 2$). Therefore, it appears that the depolarization to the higher concentrations of arachidonic acid may have been due to an action by one or more of its eicosanoid metabolites. Alternatively, these high concentrations of arachidonic acid may well have caused direct membrane damage which would be expected to result in a depolarization.

Effects of quinacrine on nicotinic responses and action potentials

In addition to its action as a phospholipase inhibitor, quinacrine has been shown to block ion channels and nicotinic ACh receptors (Adams & Feltz, 1980; Kehl, 1991; Mironov & Lux, 1992). We further examined the effects of quinacrine on nicotinic synaptic and ionophoretic potentials and on directly evoked action potentials. Quinacrine (10 μM) reduced nicotinic e.p.s.p.s by >95% ($n = 4$; Figure 3a). Ionophoretic application of ACh evoked nicotinic depolarizing potentials or inward currents; these were inhibited by 63 ± 9% by quinacrine (10 μM, $n = 8$; Figure 3b). Directly evoked action potentials were recorded in response to depolarizing current

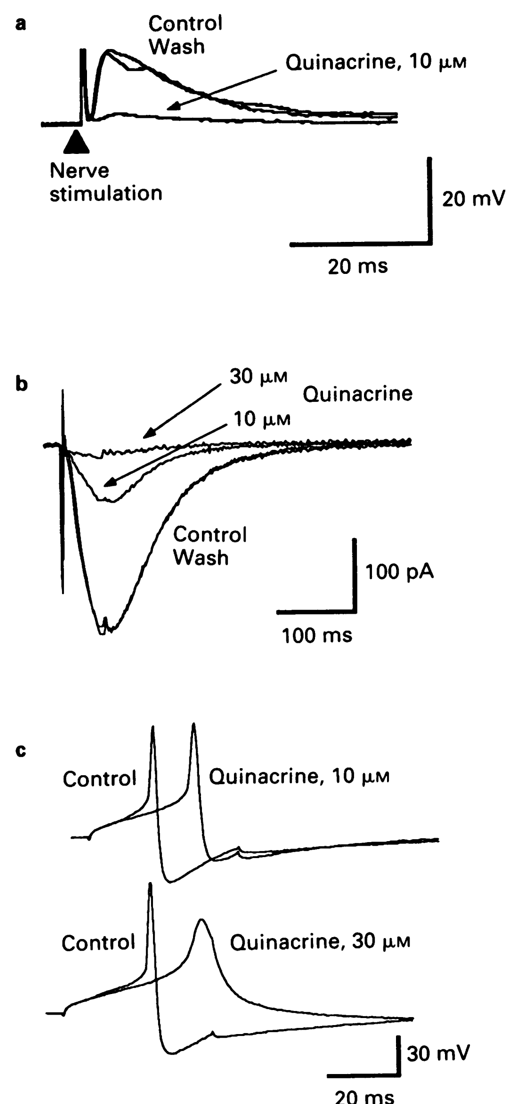

Figure 3 Effects of quinacrine on nicotinic responses and action potentials. (a) Records show averages of 10 nicotinic e.p.s.ps evoked at 0.1 Hz. Quinacrine (10 μM) reversibly reduced nicotinic e.p.s.ps by >95%. (b) Records show averages of 10 inward currents evoked by the ionophoretic application of acetylcholine (ACh, 1 mM) (50 ms pulse width, 30 V). Nicotinic inward currents were reduced reversibly by quinacrine (10–30 μM). (c) Injection of depolarizing current pulses (40 ms) evoked action potentials. The action potential threshold and duration was increased, and the peak amplitude and undershoot decreased by the application of quinacrine (10–30 μM).

pulses; quinacrine depressed the amplitude and increased the duration of these action potentials (Figure 3c).

Effects of quinacrine and 4-BPB on the voltage-current relationship

To determine if the inhibitory effects of quinacrine and 4-BPB may be independent of their action as phospholipase inhibitors, steady state voltage/current relations were examined between − 40 mV and − 140 mV. The control voltage/current curve shows marked inward rectification at potentials negative to E_K (Figure 4a; see Surprenant & North, 1988). Application of UK 14304 produced a hyperpolarization associated with an increase in membrane conductance (Figure 4). Previous studies have shown that it is the opening of an inwardly rectifying potassium current that mediates the hyperpolarization (Surprenant & North, 1988). Quinacrine (10 μM) reduced the inward rectification present in the absence of agonist; the conductance ratio measured at − 60 and − 115 mV was reduced from 1:3 ± 0.3 to 1:0.93 ± 0.1 ($n = 3$). Quinacrine produced no significant alteration in the current/voltage curve at potentials positive to E_K (about − 90 mV; Figure 4c). In the presence of quinacrine, UK 14304 produced a small outward current at potentials positive to E_K but no inward current was recorded negative to E_K (Figure 4c). A similar blockade of inward rectification was recorded in the presence of 4-BPB (30 μM, $n = 3$). Lower, and more selective (Okada *et al.*, 1989), concentrations of 4-BPB (3–10 μM) partially inhibited the basal inward rectification of the membrane ($n = 3$).

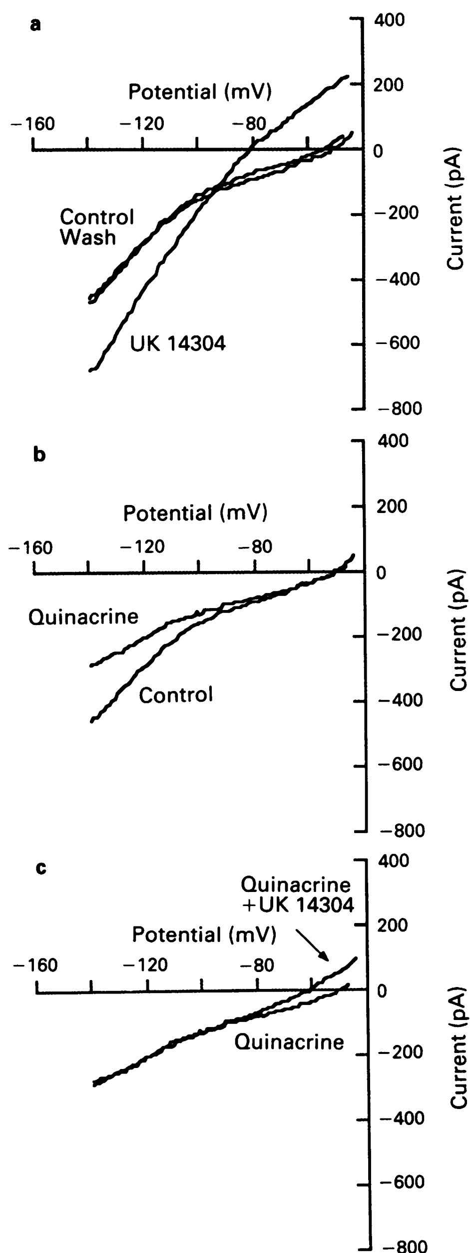

Figure 4 Effects of quinacrine on the voltage-current relationship. (a) The voltage-current curve shows marked inward rectification at potentials negative to E_K. UK 14304 (100 nM) produced a hyperpolarization associated with an increase in the membrane conductance. (b) In control physiological solution, quinacrine (10 μM) reduced the inward rectification at potentials negative of E_K with no effect on the voltage/current relationship at potentials positive of E_K. (c) In the presence of quinacrine the UK 14304-induced outward current recorded at potentials positive to E_K was greatly reduced and no inward current was recorded at potentials negative to E_K. All recordings obtained from the same neurone.

Discussion

The aim of the present study was to examine whether the receptor coupled opening of inwardly rectifying potassium current by activation of α_2-adrenoceptors in submucosal neurones was mediated by arachidonic acid or its metabolites generated by the action of phospholipase A_2. Quinacrine and 4-BPB greatly inhibited both the noradrenergic i.p.s.p. and the agonist-induced hyperpolarizations; these results initially suggested a possible involvement of the phospholipase A_2 pathway in signal transduction following α_2-adrenoceptor stimulation. However, we also found that quinacrine blocked a number of other ionic currents in these neurones, including the nicotinic current and currents underlying the directly evoked action potential. These actions of quinacrine probably account for the inhibition of spontaneous action potential firing that was observed in a number of cells when quinacrine was applied (e.g. Figure 1a,b). These results are in keeping with previous studies which have demonstrated that quinacrine blocks nicotinic currents (Adams & Feltz, 1980), fast transient outward potassium currents (Kehl, 1991), calcium currents (Mironov & Lux, 1992; Sargent *et al.*, 1992) and calcium and creep currents in isolated atrial myocytes (Bielfeld *et al.*, 1986; Yang & Vassalle, 1989). In the present study, quinacrine and 4-BPB also blocked the inward rectification present in the voltage/current curves recorded in control solution (Figure 4), in a manner analogous to the blockade of the inwardly rectifying potassium channels by rubidium or barium (Surprenant & North, 1988). Barium and rubidium inhibit somatostatin and α_2-adrenoceptor-activated potassium conductance increases in these neurones by this blockade of the inward rectification of the resting membrane (Surprenant & North, 1988). As has been described previously for barium (North & Surprenant, 1985), quinacrine and 4-BPB inhibited the outward current to UK 14304 recorded at potentials positive to E_K. It has not yet been established whether α_2-adrenoceptor activation shifts the current-voltage relation for the resting inward rectifier to more positive potentials or whether an additional conductance the pharmacology of which is identical to the inward rectifier present in these cells is activated (see Surprenant & North, 1988; Shen *et al.*, 1992). In any event, the most likely explanation for the actions of quinacrine and 4-BPB in inhibiting the somatostatin and UK 14304-induced hyperpolarization is a direct blockade of the inwardly rectifying potassium channels the activity of which is increased by these agonists. The inhibition by quinacrine and 4-BPB of the postsynaptic α_2-adrenoceptor response (the agonist-induced hyperpolarization as well as the adrenergic i.p.s.p.) were irreversible or only slowly reversible while the inhibition of the nicotinic e.p.s.p. was rapidly reversible. The inhibition of the e.p.s.p. is probably due to direct blockade of the nicotinic receptor-channel as has been described for the quinacrine-induced blockade of the endplate potential at frog neuromuscular junction

(Adams & Feltz, 1980). We have not examined possible mechanisms of action of quinacrine or 4-BPB in inhibiting the α_2-adrenoceptor response but because of its slow time course its action may involve more than direct blockade of the inward rectifying potassium channels. The present results, in addition to the previously mentioned studies, make it clear that neither quinacrine nor 4-BPB-mediated inhibition can be considered adequate criteria for involvement of the phospholipase A_2 pathway in a given cellular effect.

In the present study the more specific phospholipase A_2 inhibitor, cyclosporin A, or the inhibitors of arachidonic acid metabolism, ETYA and NDGA, had no effect on the response of submucosal neurones to α_2-adrenoceptor activation. In addition arachidonic acid itself did not mimic the UK 14304-induced membrane hyperpolarization of submucosal neurones. These results strongly indicate that in guinea-pig submucosal neurones the increase in the inwardly rectifying potassium current following the activation of α_2-adrenoceptors is not likely to be mediated by arachidonic acid or its eicosanoid metabolites produced through the stimulation of phospholipase A_2. Previous studies on submucosal neurones have failed to provide evidence for a role of protein kinase A, protein kinase C or nitric oxide in mediating the hyperpolarization to α_2-adrenoceptor or somatostatin receptor activation (Mihara *et al.*, 1987a; Surprenant & North, 1988; R.J.E. & A.S., unpublished observations). The present finding that the phospholipase A_2 pathway also is unlikely to play a role in transducing this response strengthens the conclusion that receptor-activated G-proteins modulate the activity of inwardly rectifying potassium channels directly.

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