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Investigation of the antimuscarinic and other actions of proadifen in-vitro

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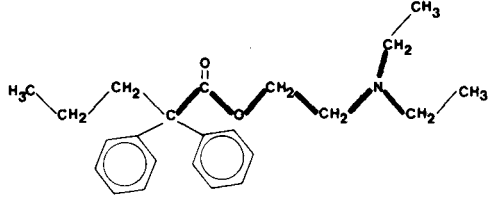
The possibility that proadifen (SKF 525A) antagonizes endothelium-dependent relaxations to acetylcholine (ACh) in isolated blood vessel preparations via a muscarinic receptor blocking action has been investigated. In phenylephrine-contracted rat isolated aortic ring preparations (with endothelium), proadifen (10–100 μM) shifts ACh relaxant curves to the right without affecting the maximal response, yet endothelium-dependent relaxations to ATP are unaffected. At lower concentrations, proadifen (1–10 μM) (i) antagonizes negative inotropic responses to ACh and ATP in guinea-pig left atria, (ii) antagonizes contractile responses to ACh and elevated [K^+] in guinea-pig ileal preparations, (iii) displaces (−)-[^{3}H]quinuclidinyl benzilate from muscarinic binding sites in membrane homogenates of guinea-pig ileal longitudinal muscle and (iv) reduces contractile responses to elevated [K^+] in rat aortic ring preparations. It is concluded that proadifen may possess (i) complex interactions with muscarinic receptors and (ii) Ca^{2+} entry blocking properties in concentrations 10–100 times lower than those reported to inhibit cytochrome P450-catalysed reactions.

The drug proadifen (SKF 525A; 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride, I) is well known as an inhibitor of the cytochrome P450 enzymes. In this context the drug has recently been used to investigate the role of cytochrome P450 enzymes in the production of the so-called endothelium-derived relaxing factor (EDRF) in rings of rabbit aorta (Singer et al 1984). In rabbit aorta, proadifen (10 μg ml^{-1}, 28 μM) depressed the maximal relaxant effect of the calcium ionophore calimycin (A23187), and produced a parallel shift to the right of methacholine concentration-effect curves without altering its maximal response. The effect of proadifen on methacholine responses are thus typical of those of a competitive antagonist and inspection of its chemical structure indicates similarities to acetylcholine.



I. Chemical structure of proadifen. Bonds shown as bold lines emphasize the structural similarity of proadifen and acetylcholine (ACh).

In the present study the effects of proadifen on acetylcholine-mediated (i) contractile effects in guinea-pig ileal preparations, (ii) negative inotropic effects in guinea-pig atrial preparations, and (iii) endothelium-dependent relaxant effects in rat aortic ring preparations, have been assessed.

To test the specificity of action, the ability of proadifen to alter responses to elevated [K^+] in ileal and aortic ring preparations, and to ATP in atrial and aortic ring preparations was investigated. In view of the results obtained in ileal preparations, studies on the ability of proadifen to displace (−)-[^{3}H]-quinuclidinyl benzilate ([^{3}H]QNB) from binding sites in homogenates of longitudinal muscle of guinea-pig ileum were also conducted.

METHODS

Isolated tissues were bathed in McEwen's solution (McEwen 1956) gassed with 5% CO_2 in O_2 and maintained at 37 °C. The experimental procedures used in driven left atrial (3 Hz, 2 ms, 2 × supramaximal voltage) and ileal preparations have been previously described (Choo & Mitchelson 1985). Reproducible responses of three to six concentrations of acetylcholine were recorded under a resting tension of 0·5 g in the absence and presence of proadifen. Additionally, responses to ATP were obtained in atria, and in ileal preparations contractions were obtained to 5, 10 and 20 mM K^+. Rat aortic ring preparations with endothelium were suspended under 2 g tension, contracted with phenylephrine (1 μM) and after steady-state contractions had been obtained, reproducible cumulative relaxant curves to acetylcholine or ATP established. In

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some preparations endothelium was removed by gently rubbing the intimal surface of the rings.

In aortic ring preparations without endothelium, contractions to 100 mM K^+ were obtained by exchanging the McEwen's solution for one in which part of NaCl was replaced by KCl.

In all tissues, dose-ratios for assessment of antagonism were calculated from half-maximal effects of the agonists or from 50% inhibition of force of contraction in left atrial preparations in the absence and presence of antagonists.

Binding experiments were performed on homogenates of guinea-pig ileal longitudinal muscle using [^{3}H]QNB, 40 pM as the ligand in 50 mM sodium phosphate buffer (pH 7·4) at 37 °C. Incubations were of 60 min duration and terminated by rapid filtration through HAWP 02500 filters (Millipore) positioned over a vacuum followed by 3 × 5 ml washes with ice-cold buffer. Radioactivity was determined after addition of the filter to a vial containing 10 ml of Filter Count (Packard) by liquid scintillation spectrometry as described by Choo et al (1985). Data were analysed using the non-linear curve fitting programme LIGAND (Munson & Rodbard 1980).

RESULTS

In the guinea-pig left atrium both acetylcholine (ACh) and ATP elicited negative inotropic effects. Proadifen (1–3 μM) did not alter basal tension but produced rightward shifts of ACh and ATP concentration-response curves, the geometric mean dose-ratios being 12·6 (2·86–55·9, 95% C.I., n = 4) and 33·6 (8·26–136, 95% C.I., n = 3) respectively. Incubation times with the antagonist of approximately 180 min were required before consistently reproducible responses could be obtained to the agonists.

Proadifen (1–10 μM), after a 60 min incubation, reproducibly inhibited responses to ACh in guinea-pig isolated ileal longitudinal muscle preparations producing rightward shifts in the curves, depression of the maximal response and a decrease in the slope of the concentration-response curves (Fig. 1). These effects of proadifen were not reversed by repeated washing.

Concentration-dependent contractions to 5, 10 and 20 mM K^+ were reduced after 60 min contact with proadifen (1–10 μM). With 10 μM proadifen, contractile tension to 20 mM K^+ was reduced from a mean (± s.e.m.) of 2·85 ± 0·20 g (n = 4) to 0·48 ± 0·13 g (n = 4), the reduction in the response being similar to that observed for 1 μM ACh (Fig. 1).

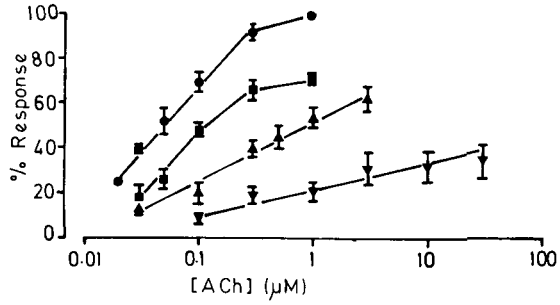

FIG. 1. Concentration-response curves to acetylcholine (ACh) in guinea-pig ileal longitudinal muscle in the absence (●) and presence of proadifen, 1 (■), 3 (▲) and 10 μM (▼). Each point is the mean of 3–5 experiments. All responses expressed as a percentage of the maximal response to ACh (1 μM).

Proadifen (0·05–10 μM) displaced specifically bound [^{3}H]QNB from membrane homogenates of the longitudinal muscle layer of guinea-pig ileum. The mean (± s.e.m.) slope factor (pseudo Hill coefficient) of the displacement curve was 0·98 ± 0·08 (n = 4) and the mean K_i (apparent dissociation constant) for proadifen was 0·39 ± 0·07 μM (n = 4). At concentrations greater than 10 μM, proadifen displaced [^{3}H]QNB below the level of specific binding as delineated by 10 μM atropine. With 100 μM proadifen [^{3}H]QNB binding was displaced 13·8 ± 2·9% (n = 4) below that determined by 10 μM atropine (Fig. 2).

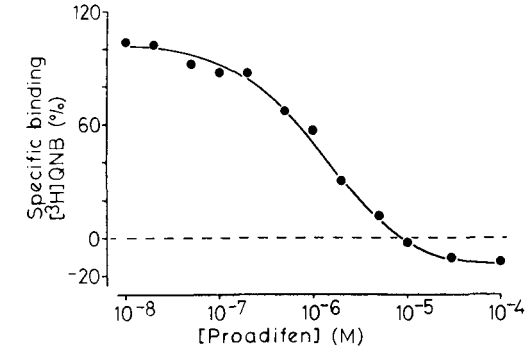

FIG. 2. Illustration of the effect of proadifen on the binding of (−)-[^{3}H]quinuclidinyl benzilate (40 pM) in an experiment with guinea-pig ileal longitudinal muscle. At high concentrations proadifen displaced the binding of quinuclidinyl benzilate to a greater extent than that observed in the presence of 10 μM atropine; used to determine non-specific binding and indicated by the zero point on the ordinate.

In the presence of endothelium, ACh elicited highly reproducible and concentration-dependent relaxations of phenylephrine-induced contractions of rat aorta. The mean (± s.e.m.) EC50 value for

ACh was 0·63 ± 0·12 μM (n = 14) and the maximal relaxation ranged from 60–100% of the phenylephrine contraction. Proadifen (10–30 μM) did not alter basal tone but depressed phenylephrine contractions (principally the tonic and not the phasic component) by approximately 30%. At 100 μM, proadifen alone produced a slowly-developing contraction which was approximately 20% of the response to 1 μM phenylephrine—subsequent contractions to phenylephrine were also depressed by proadifen by 30%. When only one concentration of proadifen was tested on each ring (10, 30 or 100 μM, 20 min incubation), the resultant curves to ACh were reproducibly displaced to the right in a parallel fashion without depression of the maximal effect. If the preparations were exposed to more than one concentration of the antagonist, the relaxant curves to ACh in the presence of the second and subsequent concentrations of antagonist were markedly shifted to the right and the maxima depressed in an apparently non-concentration-dependent manner. Using data from the experiments where each tissue was exposed to only one concentration of antagonist, analysis of ACh dose-ratios by the method of Arunlakshana & Schild (1959) gave a mean (± s.e.m.) value of slope of 0·83 ± 0·19 (n = 14 data points), suggesting the presence of competitive antagonism (Fig. 3). The mean (± s.e.m.) pK_B value for proadifen was 5·39 ± 0·08 (n = 14). By comparison, sequentially increasing concentrations of atropine (5–20 nM, 60 min) produced parallel shifts to the right in curves to ACh without altering the maximal effect. For atropine, the mean (± s.e.m.) pK_B value was 8·86 ± 0·07 (n = 9 data points) with a slope value of 1·01 ± 0·16 (n = 3 preparations).

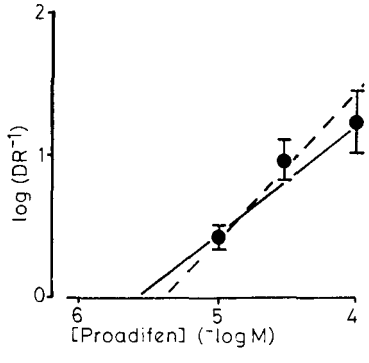

FIG. 3. Arunlakshana-Schild plot for the effect of proadifen on relaxant responses to acetylcholine in the rat isolated aorta. The dotted line indicates the computed regression with slope constrained to unity. Each point (± s.e.m.) represents the mean of 4–6 experiments.

ATP (1–100 μM) induced concentration-dependent relaxations of rat aorta with mean half-maximal effects occurring at 2·8 ± 0·4 μM (n = 6) in preparations with endothelium, while in preparations without endothelium threshold relaxant effects were evident only at concentrations >100 μM. The maximal relaxant effect of ATP (in preparations with endothelium) represented 60–90% relaxation of the phenylephrine contraction. Relaxations to ATP as well as those to ACh were unaltered by prior incubation of tissues with indomethacin (5–30 μM). Proadifen (10–100 μM, 20 min) had no effect on relaxations mediated by ATP, the mean EC50 values being 2·56 ± 0·50 μM (n = 4) before proadifen (100 μM) and 3·28 ± 0·83 μM (n = 4) after the inhibitor.

In aortic ring preparations devoid of endothelium, proadifen (1–30 μM) depressed contractions elicited by 100 mM K^+. After 20 min incubation with 10 and 30 μM of the inhibitor, steady-state contractions to K^+ were reduced to 20·5 ± 6·8% (n = 3) and 13·4 ± 1·9% (n = 3) of control responses (1·82 ± 0·24 g, n = 3), respectively.

DISCUSSION

Perhaps the most well-known action of proadifen is its ability to inhibit cytochrome P450 enzymes. The concentrations of proadifen used in previous studies to inhibit the in-vitro metabolism of drugs in slices of rat and rabbit liver range from 20–400 μM (Fouts & Brodie 1955; Brodie 1956; Gaudette & Brodie 1959; Gillette 1963: Henderson & Dewaide 1969).

An apparently competitive interaction between proadifen and ACh was observed in the present study in rat aorta and was similar to the effect found by Singer et al (1984) with methacholine in rabbit aorta. This effect of proadifen was specific for ACh since endothelium-dependent relaxations to ATP were unaffected. Analysis of the Schild plot obtained from the rightward shifts of ACh curves with various concentrations of proadifen (10, 30, 100 μM) yielded a slope value not significantly different from unity. Although these data are compatible with the presence of competitive antagonism, some caution should be exercised since, unlike atropine, it was not possible to obtain sequential rightward shifts with increasing concentrations of proadifen in the same experiment. It is important to note that the concentrations of proadifen used displaced non-specific binding determined by 10 μM atropine. Furthermore, other investigations performed in the present study indicate that lower concentrations of proadifen (1–10 μM) exert complex actions at muscarinic and

other receptor sites in other tissues. Nevertheless, the results of the proadifen/ACh and proadifen/ATP studies in rat aorta point to either a specific effect of the antagonist with muscarinic receptor function or the possibility that ACh and ATP release different EDRFs in rat aorta.

An inhibition of the action of ACh was evident with lower concentrations of proadifen (1–10 μM) in the guinea-pig isolated ileal and driven left atrial preparations. However, in the latter preparation the antagonism could not be considered to be specific since similar concentrations of proadifen also inhibited the negative inotropic effects of ATP, and the effect of proadifen required up to 180 min to equilibrate. Such a prolonged equilibration period for a relatively weak antagonist of muscarinic responses is suggestive of some action other than a simple competitive interaction at a muscarinic receptor. Although the actions of proadifen in the ileal preparation may also be considered to be non-specific since both ACh and K^+ contractions were equally inhibited, studies using [^{3}H]QNB in ileal muscle membrane homogenate indicate an interaction of proadifen with muscarinic receptor sites. As with the rat aorta results, the proadifen/[^{3}H]QNB interaction appeared to be competitive since the slope factor of the displacement curve was very close to unity but proadifen (>10 μM) also displaced [^{3}H]QNB below the level of specific binding. These results thus cast doubt on the presence of a solely competitive interaction between proadifen and muscarinic agonists. It is of interest that proadifen has been reported to displace skeletal neuromuscular blocking drugs from non-specific sites also (Bowman & Rand 1980) and binds to the ion channel associated with the nicotinic receptor (Krodel et al 1979).

Kalsner et al (1970) demonstrated that 26 μM proadifen possessed actions in rabbit aorta which, in current terminology, would be regarded as representing calcium-entry blocking properties. In the present experiments, proadifen (1–10 μM) antagonized K^+-induced contractions in guinea-pig ileal and rat aortic ring preparations devoid of endothelium. At 10 μM of the antagonist, responses to K^+ were reduced to approximately 20% of control. In the rat aorta the effect of proadifen was relatively specific for K^+ since phenylephrine contractions were reduced by only 30% with concentrations of proadifen up to 100 μM. Kalsner et al (1970) reported a similar specificity for proadifen in rabbit aorta.

In view of the relatively high concentrations of proadifen required to inhibit cytochrome P450 in-vitro, the results of the present study suggest that due care should be exercised in attributing the actions of this drug solely on the basis of inhibition of cytochrome P450 and it is possible that the drug interacts at other sites to interfere with agonists and antagonists acting at muscarinic receptors as well as drugs using voltage-dependent calcium channels in various tissues.

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