Desensitization and functional antagonism by β -adrenoceptor and muscarinic receptor agonists, respectively: a comparison with receptor alkylation for calculation of apparent agonist affinity

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1 Apparent affinity constants (K_D) for prenalterol, an agonist of low intrinsic efficacy at β_1 -adrenoceptors in rat left atria, have been determined by use of receptor desensitization and functional antagonism induced by isoprenaline and carbachol, respectively. The values obtained have been compared to those values estimated with the irreversible β -adrenoceptor antagonist, bromoacetylalprenololmenthane (BAAM).

2 The $-\log K_D$ values for prenalterol estimated by desensitization or irreversible antagonism ranged from 6.8–7.1 and 6.2–7.1, respectively.

3 Carbachol produced functional antagonism of the response to prenalterol even though it was removed before addition of prenalterol. This effect was mediated by M_2 -muscarinic receptors. Pretreatment of animals with pertussis toxin did not affect the functional antagonism elicited by carbachol. The apparent K_D value obtained after pre-exposure to carbachol (6.8) was similar to those estimated by use of either alkylation with BAAM or desensitization with isoprenaline (see above).

4 It is concluded that acute desensitization or functional antagonism of responses to agonists of low intrinsic efficacy provides a means to estimate apparent K_D constants. This approach could be useful to characterize receptors for which an irreversible antagonist may not be available.

Keywords: β -adrenoceptors; muscarinic receptors; prenalterol; agonist dissociation constants; BAAM (bromoacetylalprenololmenthane); functional antagonism; desensitization

Introduction

Traditionally, receptors are classified on the basis of differential antagonist affinities. However, concern has been expressed as to the correct manner to classify receptor subtypes (see Black, 1987; Green, 1990 for reviews) since the receptor may exhibit a more stringent structure-activity requirement for the endogenous ligand than for a synthetic ligand, such as antagonists (Kenakin, 1987). The multiplicity of 'pharmacological' sites may reflect the binding of antagonists to accessory membrane sites as well as to the 'receptive' site (Leff & Martin, 1988; Bevan *et al.*, 1989). The unambiguous determination of agonist apparent affinities ($-\log K_D$), therefore, would be useful in receptor characterization. The measurement of affinities for full agonists (Furchgott, 1966), however, requires irreversible antagonists for the receptor in question, which are not available for most receptors or receptor types (Kenakin, 1987). Furthermore, the potential of irreversible antagonists to bind to extra receptor sites in the membrane may influence apparent agonist affinity and consequently selectivity.

An alternative approach to the use of irreversible antagonists for the determination of apparent K_D values for agonists may be to use desensitization. In the sustained presence of high agonist concentrations, many receptor types undergo desensitization. The effects are qualitatively similar to those of receptor alkylation, i.e., the concentration-response curves are dextrally shifted and the maxima, upon depletion of the spare receptors, are depressed (Eglen *et al.*, 1987; Herepath & Broadley, 1990). However, unless the desensitization process is homologous, i.e. tissue responses to a single receptor type are desensitized, then null methods, which assume equal responses with equal degrees of receptor occupancy (Furchgott, 1966), may not apply since the receptor coupling system, in addition to the active receptor population, could be affected (Kenakin, 1987; Eglen *et al.*, 1987).

Application of such null methods to heterologous desensitization of the ileal M_3 -muscarinic receptor results in apparent K_D values for several full agonists but not partial agonists, that were incorrect in comparison to the apparent K_D values estimated by alkylation (Eglen *et al.*, 1987). In cultured AH31 cells, the effect of homologous β_2 -adrenoceptor desensitization was, however, analogous to that seen after receptor alkylation with N^8 (bromoacetyl)- N -[3-(4-indolyloxy)-2-hydroxy-propyl]-(2)-1,8-diaminomenthane (BIM; Lohse, 1990). Moreover, apparent agonist K_D values estimated by the two methods were in good agreement.

The use of homologous desensitization has been examined in the present study. Positive inotropic responses to β -adrenoceptor agonists in rat isolated left atria were used as a model to test this approach for several reasons. Firstly, rat atria have been well characterized in terms of the receptor populations by both radioligand and functional binding techniques (Juberg *et al.*, 1985; Montgomery *et al.*, 1988a; Herepath & Broadley, 1990). Secondly, short term β -adrenoceptor desensitization in cardiac tissue is generally homologous (see Harden, 1983 for review). Thirdly, an irreversible β -adrenoceptor antagonist, bromoacetylalprenololmenthane (BAAM), was available with which to compare directly apparent K_D values calculated by desensitization. Prenalterol, an agonist of low relative efficacy, was employed because (a) its affinity at β -adrenoceptors has been measured by both functional and radioligand binding methods (Kenakin & Beek, 1982; 1984; Kenakin, 1987; Gurden *et al.*, 1989), and (b) errors involved in the estimation of the apparent K_D by the method of Furchgott (1966) for partial agonists are less than those associated with full agonists (Eglen *et al.*, 1987; Leff *et al.*, 1990a,b).

A second aspect of this paper concerns functional antagonism. Stimulation of muscarinic receptors has been shown to produce functional antagonism of β -adrenoceptor responses (O'Donnell & Wanstall, 1977; Broadley & Nicholson, 1979; Buckner & Saini, 1975; Leff *et al.*, 1985; Molenaar & Malta,

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1986), also resulting in effects qualitatively similar to those seen after receptor alkylation described above. There are, however, theoretical limitations to the use of functional antagonism to estimate apparent K_D values, particularly in cardiac tissue (MacKay, 1981; Leff *et al.*, 1985; Molenaar & Malta, 1986). Notably, the baseline tension developed by isolated atrial tissue in the presence of carbachol is reduced, thereby changing the state of the tissue and invalidating one of the assumptions of the null hypothesis developed by Furchgott (1966). In the present study, a procedure modified from that described by Broadley & Nicholson (1979) has been used, i.e., carbachol was removed from the organic bath prior to construction of the second concentration-response curve, allowing the baseline tension to re-attain control values. The apparent K_D values estimated with these data have been compared with those estimated by use of desensitization and receptor alkylation.

Some of these data have been presented in a preliminary form to the British Pharmacological Society (Montgomery *et al.*, 1988a) and to the American Society for Pharmacology and Experimental Therapeutics (Montgomery *et al.*, 1988b).

Methods

Tissue preparation

Rats (male, Sprague-Dawley, 200–250 g) were pretreated with reserpine (2.5 mg kg^{-1} , i.p., twice daily, for two days; the last dose was administered 18 h before tissue removal). Preliminary experiments showed that 0.1 mM tyramine did not elicit a positive inotropic response in isolated atria from reserpine-treated animals, suggesting a substantial reduction in endogenous catecholamine concentrations. Rats were lightly anaesthetized with CO_2 and decapitated. Isolated atria were then placed in warm (34°C) oxygenated (95% O_2 :5% CO_2) Krebs-Henseleit physiological salt solution (composition mM: NaCl 118, KCl 4.7, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 2.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.6, NaHCO_3 24.9, KH_2PO_4 1.2, glucose 5.6, EDTA 0.05) and cleaned of adhering connective tissue. The atria were then placed under 0.5 g tension in 10 ml organ baths containing Krebs solution (37°C) and placed between parallel platinum electrodes (Eglen *et al.*, 1988). The atria were electrically paced during the experiments (2 Hz, 5 ms duration, threshold voltage +12%) with electrical stimulation provided by a Grass S88 stimulator via a Buxco signal splitter. Tissues were allowed 60 min to equilibrate with overflow washing at 10 min intervals.

Receptor characterization

To characterize the β -adrenoceptor population present in the rat left atria, affinities i.e., $-\log K_B$ values, for the antagonists propranolol (nonselective), atenolol (β_1 -selective), and ICI 118,551 (β_2 -selective) were determined. Concentration-response curves to isoprenaline were established in the presence and absence of these antagonists after 60 min of equilibration (with regular washing at 15 min intervals). The muscarinic receptor subtype mediating negative inotropic responses to carbachol was also characterized (see Eglen & Whiting, 1986, for review) by determining $-\log K_B$ values for atropine (nonselective), pirenzepine (M_1 -muscarinic receptor-selective), methoctramine (M_2 -muscarinic receptor-selective), and para-fluoro-hexahydrosiladiphenidol (p -F-HHSiD; M_3 -muscarinic receptor-selective).

Receptor inactivation studies

After the equilibration period, a single dose of 100 nM isoprenaline was added for 5 min to obtain an estimate of the maximum response of the tissue. The tissues were then washed at 10 min intervals over the following 60 min and allowed to

re-attain baseline tension. A cumulative concentration-response curve to prenalterol was then established in the cumulative fashion (Van Rossum, 1963) with incremental increases of concentration spaced at 0.5 log intervals. After the maximum response was attained the tissues were washed by overflow at 10 min intervals over the succeeding 30 min and allowed to re-attain baseline tension.

The irreversible β -adrenoceptor antagonist BAAM (Posner *et al.*, 1984) was added to the organ baths for a period of 60 min (32–3200 nM) with washing at 10 min intervals. Each preparation was exposed to only one concentration of BAAM. The tissues were washed at 10 min intervals over the succeeding 30 min and a second cumulative concentration-response curve to prenalterol was then established. The apparent K_D value was then estimated by the method of Furchgott (1966) as described below.

The apparent K_D value was also estimated by the method of Furchgott & Bursztyn (1967). This latter method reduces the efficacy of a partial agonist thereby approximating it to a competitive antagonist. In separate experiments to those described above, tissues were initially exposed to either 32 nM or $3.2 \mu\text{M}$ BAAM. After a 30 min washout period, concentration-response curves to isoprenaline were then constructed in the presence or absence of $10 \mu\text{M}$ prenalterol (with a 60 min equilibration period). The dextral shift in the concentration-response curves to isoprenaline was then used to calculate the apparent K_D value for prenalterol (see below). It should be noted that the constants calculated are only apparent, since drug and receptor $\rightleftharpoons$ drug/receptor complex is not a realistic description of agonist action at G-protein-coupled receptors.

Receptor desensitization studies

The procedure employed was similar to that described above except that isoprenaline (3.2–560 nM) was substituted for BAAM, and left in contact with the preparations for 120 min. This period was chosen since Herepath & Broadley (1990) have shown that exposure greater than 4 h resulted in irreversible desensitization (i.e. down regulation). After 120 min exposure, washing at 10 min intervals was continued for the next 60 min or until baseline tension was restored. A second concentration-response curve to prenalterol was then established. In separate experiments, apparent K_D values were also estimated by the method of Furchgott & Bursztyn (1967). The tissues were initially exposed to isoprenaline (3200 nM), and, after washout, a concentration-response curve to isoprenaline was constructed in the presence of $10 \mu\text{M}$ prenalterol.

Functional antagonism

The procedure followed was similar to that described above for isoprenaline, except that the tissues were exposed to carbachol ($0.1 \mu\text{M}$ – 0.1 mM) for 120 min. After exposure, the tissues were washed at 10 min intervals over the next 60 min. In all experiments, the baseline tension was returned to pre-carbachol values prior to the construction of a second curve to prenalterol. In a separate series of experiments, the muscarinic receptor subtype was characterized. The preparations were exposed simultaneously to atropine (320 nM) and carbachol ($10 \mu\text{M}$) for 120 min, prior to the construction of the second concentration-response curve to prenalterol. Similar experiments were performed with pirenzepine at a concentration (100 nM) that has been shown to antagonize selectively M_1 -muscarinic receptors (Eglen & Whiting, 1986).

Pretreatment with pertussis toxin

Pertussis toxin ($10 \mu\text{g kg}^{-1}$) or vehicle was injected via the tail vein 48 h prior to the removal of tissues (Eglen *et al.*, 1988). This regimen was shown to abolish the negative inotropic responses to muscarinic agonists due to ADP-ribosylation of the G_i protein (Tajima *et al.*, 1987; Eglen *et al.*, 1988).

Effect on responses to forskolin

To estimate the specificity of the inactivating effects of BAAM, isoprenaline-induced desensitization, or carbachol-induced functional antagonism, concentration-response curves to the adenylate cyclase activator, forskolin (1 nM–10 μ M, 5 min exposure at each concentration) were established before and after exposure to each of these agents. The method and conditions were similar to those described above.

Measurement of responses

All tissue responses were determined as changes in isometric tension (mg). Responses were measured with a Grass FT03 isometric force transducer and displayed on a Grass 8 channel recorder. These values were determined at each agonist concentration. If necessary, values on the post-exposure curves were corrected for any time-dependent changes (2–5 fold) in sensitivity derived from corresponding control studies. The increases in developed tension were then calculated as a percentage of the response to 100 nM isoprenaline.

Analysis of responses

The potencies and maximal responses to prenalterol from the pre- and post-exposure curves were defined by the EC_{50} and percentage of the maximum of the pre-exposure curve. This latter parameter was derived by non-linear iterative curve fitting procedures (Parker & Waud, 1971) using RS1 software (BBN Corporation, MA, U.S.A.). Antagonist equilibrium dissociation constants ($-\log K_B$) were calculated by the method of Gaddum (1937):

$$K_B = \frac{[\text{antagonist concentration}]}{(\text{concentration-ratio} - 1)}$$

Apparent equilibrium dissociation constants (K_D) for prenalterol were calculated in two ways. All pre- and post-exposure curves to BAAM, isoprenaline or carbachol, were employed to calculate apparent K_D values by the method of Furchgott (1966):

$$\frac{1}{[A]} = \frac{1}{[A^1]} \frac{1}{q} + \frac{1}{K_D} \frac{(1-q)}{q}$$

where q is the fraction of receptors remaining after desensitization, K_D is the apparent equilibrium dissociation constant, and $[A]$ and $[A^1]$ are equally effective concentrations of prenalterol before and after exposure. These equieffective concentrations were determined at 10% increments of response. These values were then plotted in a double reciprocal fashion, and the apparent K_D value was derived after exposure. These equieffective concentrations were determined at 10% increments of response and the values were then plotted in a double reciprocal fashion, and the apparent K_D value derived from the resulting straight line as (slope $- 1$)/intercept.

In separate experiments following receptor inactivation or desensitization, values were derived by comparing curves constructed to isoprenaline in the presence or absence of prenalterol (10–100 μ M); the latter acting as an antagonist. In this instance, the following relationship was employed

$$[A] = \frac{[A^1]}{1 + ([P]/K_D)}$$

where $[A]$ and $[A^1]$ are concentrations of the full agonist (isoprenaline) in the absence and presence of the partial agonist P (prenalterol). The apparent equilibrium dissociation constant for prenalterol (K_D) was derived from the relationship $[P] \times \text{slope}/(1 - \text{slope})$. This relationship is a modification of the equation of Gaddum (1937) described above.

Statistical analysis

All values quoted are means $\pm$ s.e.mean, $n = 4-6$. Statistical differences were assessed by use of the Student's unpaired t test, with $P < 0.05$ considered to be significant.

Drugs used

The following compounds were employed: atenolol, ICI 118,551 (erythro-($\pm$)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ICI plc, Alderley Edge, U.K.), BAAM, forskolin, methoctramine (Research Biochemicals Inc., Natick, MA, U.S.A.), pertussis toxin (Peninsula Peptides, San Diego, CA, U.S.A.), pirenzepine (Boots plc, Nottingham, U.K.), and prenalterol (A.B. Hassle, Molndal, Sweden). Racemic p -F-HHSiD (*para*-fluoro-hexahydro-siladiphenidol) was synthesized by the Institute of Organic Chemistry, Syntex, Palo Alto, CA, U.S.A. The remaining compounds were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.).

Results

Isoprenaline and prenalterol elicited concentration-dependent positive inotropic responses. The maximal responses to prenalterol (relative to the maximal response of isoprenaline) varied between preparations, ranging from 55 to 112%. As a result, initial concentration-response curves to prenalterol were always established in each preparation prior to exposure of the tissues to BAAM, isoprenaline, or carbachol, thereby providing an internal control curve for each preparation.

β -Adrenoceptor and muscarinic receptor characterization

Antagonist affinities ($-\log K_B$) were derived to characterize the populations of β - and muscarinic receptors present (Table 1). Propranolol and atenolol (100 nM) shifted the concentration-response curve to isoprenaline to the right in a parallel fashion, whereas no effect was observed with ICI 118,551 (100 nM). Concentration-response curves to carbachol were shifted to the right in a parallel fashion by atropine (100 nM), methoctramine (100 nM) and pirenzepine (3000 nM) but not by p -F-HHSiD (100 nM). In all of these studies, no diminution in the maximal response to either isoprenaline or carbachol was observed.

β -Adrenoceptor inactivation

BAAM, (32 and 100 nM) reduced both the potency and maxima of the concentration-response curve to prenalterol (Figure 1, Table 2). After exposure to 320 and 1000 nM BAAM, no further significant decreases in maxima were observed. Exposure to 3200 nM, however, resulted in a further decrease in maxima, which was accompanied by a larger dextral shift. The apparent affinities ($-\log K_D$) for prenalterol were similar

Table 1 Apparent antagonist affinities ($-\log K_B$) in rat left atria

Response	Agonist	Antagonist	$-\log K_B$
Positive inotropic	Isoprenaline	Propranolol	8.6 ± 0.1
	Isoprenaline	Atenolol	8.0 ± 0.1
	Isoprenaline	ICI 118,551	< 7.0
Negative inotropic	Carbachol	Atropine	8.6 ± 0.1
	Carbachol	Methoctramine	7.9 ± 0.1
	Carbachol	p -F-HHSiD	< 7.0
	Carbachol	Pirenzepine	6.9 ± 0.1

Values are mean $\pm$ s.e.mean, $n = 6$. All antagonists were studied at a concentration of 0.1 μ M with the exception of pirenzepine (0.3 μ M). p -F-HHSiD-*para*fluorohexahydrosiladiphenidol.

Figure 1 Responses to prenalterol in rat left atria in the absence and after exposure to bromoacetylalprenolol menthane (BAAM). The concentrations of BAAM were 32 nM (Δ), 0.1 μ M ($\square$), 0.32 μ M ($\bullet$), 1 μ M ($\circ$) and 3.2 μ M ($\blacksquare$). Points show mean values with vertical bars indicating s.e.mean.

after inactivation by 32 and 100 nM BAAM, but these values progressively decreased after exposure to higher concentrations (Table 2).

The apparent K_D values were also estimated by the method of Furchgott & Bursztyn (1967) following pretreatment of the tissues with BAAM (32 and 3200 nM). The apparent $-\log K_D$ values were significantly ($P < 0.05$) different (7.07 ± 0.05 and 6.02 ± 0.08 , respectively) in tissues exposed to 32 or 3200 nM BAAM. These apparent $-\log K_D$ values were similar, however, to those determined by the method of Furchgott (1966) using these concentrations of BAAM (6.7 and 6.2, respectively; Table 2).

Isoprenaline-induced desensitization

After exposure to isoprenaline (3.2–560 nM) for 120 min, there was a concentration-dependent diminution of the maxima of the concentration-response curve to prenalterol (Table 3). The

apparent affinities ($-\log K_D$), in contrast to those estimated with BAAM, were similar after exposure to each concentration of isoprenaline. The apparent K_D value estimated by the method of Furchgott & Bursztyn (1967) following exposure of the tissues to 560 nM isoprenaline was 7.14 ± 0.03 .

Functional antagonism

Exposure of the tissues to 0.32 and 1 μ M carbachol, followed by a 30 min washout period, did not significantly inhibit the responses to prenalterol (Table 4). However, after exposure to 10 μ M carbachol and a similar washout period, the potency and the maximum of the prenalterol concentration-response curve were significantly reduced (Table 4). The affinity estimated after exposure to 10 μ M carbachol was similar to that obtained with both BAAM (32–100 nM) and isoprenaline-induced desensitization (Table 4).

In some experiments, tissues were exposed to both atropine (320 nM) and carbachol (10 μ M) for 3 h, prior to construction of the second concentration-response curve to prenalterol. Under these conditions, the desensitizing effects of carbachol were reversed (Table 5). Pirenzepine (100 nM), in contrast, did not reverse the carbachol-induced desensitization of responses to prenalterol (Table 5). Pretreatment of the animals with pertussis toxin (10 mg kg⁻¹, i.v.) abolished negative inotropic responses to carbachol, but revealed a positive inotropic response at carbachol concentrations $>10^{-5}$ M (Figure 2). Moreover, pertussis toxin pretreatment did not alter the functional antagonism by carbachol of the responses to prenalterol (Table 5, Figure 3). The positive inotropic responses to prenalterol or isoprenaline alone were unaffected by pertussis toxin pretreatment (data not shown).

In order to obtain some measure of the specificity of the effects of BAAM or isoprenaline on functional antagonism, concentration-response curves to forskolin were constructed following pretreatment with the agents. It was observed that pre-exposure to BAAM (3200 nM; 60 min), isoprenaline (560 nM; 120 min), or carbachol (10 μ M; 180 min) did significantly ($P < 0.05$), but not meaningfully, affect either the potency or maximal responses observed with forskolin (Table 6).

Table 2 Potencies ($-\log EC_{50}$), maximal responses, and apparent affinities ($-\log K_D$) of prenalterol after receptor inactivation by bromoacetylalprenolol menthane (BAAM)

BAAM concentration (nM)	$-\log EC_{50}$	% max. isoprenaline response	Max. isoprenaline response (mg)	$-\log K_D$	q
Control	7.8 ± 0.1	112 ± 8	670 ± 40	—	—
32	7.4 ± 0.1	84 ± 5	693 ± 50	6.7 ± 0.1	0.37
100	6.9 ± 0.1	62 ± 8	730 ± 90	7.0 ± 0.1	0.15
320	6.5 ± 0.1	57 ± 5	644 ± 60	6.5 ± 0.1	0.05
1000	6.3 ± 0.1	59 ± 6	679 ± 73	6.2 ± 0.1	0.03
3200	5.6 ± 0.1	20 ± 3	632 ± 45	6.2 ± 0.1	0.004

Values are mean $\pm$ s.e.mean, $n = 4-8$. Control values are those determined prior to exposure to BAAM. Equilibrium dissociation constants (K_D) and proportion of receptors remaining after inactivation (q) were derived after the method of Furchgott (1966).

Table 3 Potencies ($-\log EC_{50}$), maximal responses, and apparent affinities ($-\log K_D$) of prenalterol after desensitization by isoprenaline

Isoprenaline concentration (nM)	$-\log EC_{50}$	% Max isoprenaline response	Max isoprenaline response (mg)	$-\log K_D$	q
Control	7.6 ± 0.1	80 ± 5	749 ± 68	—	—
3.2	7.0 ± 0.1	52 ± 8	639 ± 94	7.1 ± 0.1	0.52
32	6.8 ± 0.1	36 ± 5	791 ± 83	6.9 ± 0.1	0.34
320	6.7 ± 0.1	30 ± 7	753 ± 53	6.9 ± 0.1	0.15
560	6.5 ± 0.1	21 ± 4	721 ± 46	6.8 ± 0.1	0.08

Values are mean $\pm$ s.e.mean, $n = 4-8$. Control values are those determined prior to exposure to isoprenaline. See legend to Table 2 for explanation of K_D and q values. Apparent $-\log K_D$ values estimated by method of Furchgott (1966).

Table 4 Potencies ($-\log EC_{50}$) and apparent affinities ($-\log K_D$) of prenalterol maximal responses after functional antagonism by carbachol

Carbachol concentration (μM)	$-\log EC_{50}$	% Max isoprenaline response	Max isoprenaline response (mg)	$-\log K_D$	q
Control	7.20 ± 0.1	58 ± 5	695 ± 35	—	—
0.32	7.16 ± 0.1	52 ± 6	778 ± 67	NC	
1	6.88 ± 0.1	43 ± 4	650 ± 97	NC	
10	6.74 ± 0.1	18 ± 3	800 ± 38	6.8 ± 0.1	0.17

Values are mean $\pm$ s.e.mean, $n = 4-6$. NC = not calculated. Control values are those determined prior to exposure to carbachol. Carbachol ($10 \mu M$) was applied to the atria for 120 min and then washed out over a period of 60 min. See legend of Table 2 for explanation of K_D and q values.

Table 5 Effect of pre-exposure to carbachol after various conditions on responses to prenalterol

Pre-exposure conditions	$-\log EC_{50}$	Max % isoprenaline response	Max isoprenaline response (mg)
Pre carbachol	7.20 ± 0.08	58 ± 5	695 ± 35
Post carbachol	6.74 ± 0.08	18 ± 3	800 ± 38
Post carbachol (+0.32 μM atropine)	6.79 ± 0.05	46 ± 5	825 ± 56
Post carbachol (+0.1 μM pirenzepine)	6.95 ± 0.11	19 ± 8	588 ± 80
Post carbachol (+ vehicle) ^a	6.93 ± 0.05	23 ± 3	506 ± 99
Post carbachol (+ PTx pretreatment)	7.04 ± 0.03	24 ± 8	492 ± 99

Values are mean $\pm$ s.e.mean, $n = 4-6$. The maximal isoprenaline concentration employed was $0.1 \mu M$. The carbachol ($10 \mu M$) exposure period was 180 min. PTx: pertussis toxin.

^a Vehicle-treated animals were pretreated with 0.3 ml H_2O injected 3 h prior to tissue removal.

Table 6 Effect of pre-exposure to bromoacetylalprenolol menthane (BAAM), isoprenaline, or carbachol on responses to forskolin

Pre-exposure conditions	$-\log EC_{50}$	Max. % isoprenaline response	Max. isoprenaline response (mg)
Control	6.26 ± 0.09	96 ± 7	748 ± 63
BAAM (3200 nM; 60 min)	6.55 ± 0.13	93 ± 4	734 ± 112
Isoprenaline (560 nM; 120 min)	6.34 ± 0.11	98 ± 7	842 ± 98
Carbachol (10,000 nM; 180 min)	$5.89 \pm 0.04^*$	104 ± 8	970 ± 49

Values are mean $\pm$ s.e.mean, $n = 4-8$. Atria were pretreated as indicated prior to construction of forskolin concentration-response curves.

* Significantly different ($P < 0.05$) from control value.

Figure 2 Effects of pretreatment of animals with pertussis toxin (●) or vehicle (○) on inotropic responses to carbachol in rat left atria. Points show mean values with vertical bars indicating s.e.mean. **Figure 3** Effects of pre-exposure of rat left atria to carbachol on responses to prenalterol in vehicle-treated (●) or pertussis toxin-treated (▲) animals. Effects in control tissues (no carbachol treatment) are also shown in pertussin toxin-treated (○) or vehicle-treated (Δ) animals. Points show mean values with vertical bars indicating s.e.mean.

Discussion

Affinity and efficacy are fundamental parameters in the analysis of agonist/receptor interactions and receptor alkylation is extensively employed to measure these parameters. Many receptor systems lack such selective alkylating agents, thus precluding the use of this approach. The classification of the many receptors, therefore, is based on the differential affinities of competitive reversible antagonists. In the present study desensitization has been used as an alternative method to determine agonist affinity since many receptors undergo desensitization and the consequences are similar, at least temporarily, to those observed after alkylation. If, however, desensitization influences post-receptor events, then incorrect estimates of agonist affinities may result (MacKay, 1981; Leff *et al.*, 1985; Eglen *et al.*, 1987; Lohse, 1990).

In order to characterize the adrenoceptor and muscarinic receptor populations present, antagonist studies were undertaken. The affinities ($-\log K_D$) of propranolol (non-selective), atenolol (β_1 -adrenoceptor selective), and ICI 118,551 (β_2 -adrenoceptor selective) suggest that the positive inotropic response was mediated through β_1 -adrenoceptors while the data obtained with atropine (nonselective), pirenzepine (M_1 -muscarinic receptor-selective), methoctramine (M_2 -muscarinic receptor-selective), and *p*-F-HHSiD (M_3 -muscarinic receptor-selective) suggest that the negative inotropic responses to carbachol were mediated through an M_2 -muscarinic receptor (see also Juberg *et al.*, 1985; Eglen & Whiting, 1986, for pA_2 values and further references).

The effects of BAAM on the responses to prenalterol were different from the effects of isoprenaline-induced desensitization. The apparent K_D values were independent of the level of inactivation only for isoprenaline-induced desensitization but not for alkylation with BAAM. The apparent K_D values (6.8–7.1) measured by the methods of Furchgott (1966) or Furchgott & Bursztyn (1967) agreed with the apparent affinity ($pA_2 = 6.8$) reported by Kenakin & Beek (1984), who used prenalterol as a competitive antagonist. These values were also similar to those obtained in the rat atria with radioligand binding techniques ($-\log K_1 = 6.81$, Juberg *et al.*, 1985). In guinea-pig left atria, prenalterol acted as a competitive antagonist with a pA_2 value of 7.2 (Molenaar *et al.*, 1985). Taken together, these data suggest that reproducible apparent K_D values for prenalterol can be estimated by the methods described by Furchgott (1966) or Furchgott & Bursztyn (1967). The theoretical limitations recently discussed in the literature (see Mackay, 1988; 1990; Kenakin, 1989 for reviews) may not apply. Similar findings have been reported by Leff *et al.* (1990a,b) and Eglen *et al.* (1987) for the affinity of pilocarpine at the cardiac M_2 or ileal M_3 muscarinic receptor, respectively.

BAAM at higher concentrations proved an unreliable tool, since the apparent K_D values varied. BAAM (10–30 μM) has been shown to act as a competitive antagonist of responses in the guinea-pig atria to isoprenaline and the partial agonist oxymethylene-isoprenaline (Ng & Malta, 1989). Black *et al.* (1985) found that, at concentrations greater than 10 μM , BAAM depressed the basal rate of the beating of rat atria. These observations, together with the data from the present study, discourage the use of BAAM at concentrations greater than 320 nM. This is in contrast to data reported by Doggrell (1989), who has employed 3 μM BAAM to estimate an apparent K_D value for isoprenaline at β_1 -adrenoceptors in the right ventricle of the rat.

Acute desensitization by isoprenaline also permitted a reliable estimation of the apparent K_D value for prenalterol (this study). The apparent K_D values obtained by this method were similar to those reported in the literature (see above) and to those estimated with low concentrations of BAAM. Exposure

to isoprenaline for 180 min did not affect either the potency or the maximal response to forskolin. This indicated that the isoprenaline-induced β -adrenoceptor desensitization did not directly affect the catalytic subunit of adenylate cyclase, though it should be noted that this does not exclude the possibility of changes in the G_s guanine nucleotide binding protein.

In the experiments on functional antagonism by carbachol, the procedure described by Broadley & Nicholson (1979) was modified by removing carbachol from the tissue by extensive washing before exposure to prenalterol. The inotropic responses to prenalterol were still inhibited, and analysis yielded an apparent K_D value similar to that reported in the literature and that from BAAM inactivation or isoprenaline-induced desensitization (this study). There was a significant but not meaningful inhibition of forskolin activity after carbachol exposure, implying that the functional antagonism was unrelated to effects on the catalytic unit of adenylate cyclase. These data suggest that under the correct experimental conditions, functional antagonism may accurately measure agonist affinity, at least for partial agonists such as prenalterol.

Other workers using carbachol in atria (Broadley & Nicholson, 1979) or trachea (Buckner & Saini, 1975; O'Donnell & Wanstall, 1977) have utilized carbachol, present throughout the experiment, to produce functional antagonism responses to β -adrenoceptor agonists. However, the apparent K_D values for isoprenaline estimated in this fashion differed from those estimated in radioligand binding studies (see Leff *et al.*, 1985 for references). Molenaar & Malta (1986) have argued that these disparities undermine the validity of functional antagonism for determination of β -adrenoceptor agonist affinities. Kenakin (1987), in contrast, has shown that the latter method may be applicable when the efficacy of a partial agonist is depressed to such an extent that it acts as a competitive antagonist.

Pretreatment of animals with pertussis toxin, at a dose which has been shown (Eglen *et al.*, 1988) to abolish the negative inotropic response to carbachol, failed to influence the carbachol-induced desensitization. This suggested that the M_2 -muscarinic receptor responsible for carbachol-induced β_1 -adrenoceptor desensitization was not coupled via the guanine nucleotide binding protein, G_i . Moreover, sustained inhibition of adenylate cyclase was not a prerequisite for the desensitization, since this was observed even after removal of the carbachol (where baseline developed tension had return to control levels). The insensitivity to pertussis toxin of both functional antagonism by carbachol (this study) and the inotropic response to muscarinic agonists (Eglen *et al.*, 1988), suggests a common mechanism. Furthermore, the concentration range over which carbachol elicits these effects is also similar (desensitization: 1–100 μM ; positive inotropy: 1–1000 μM). Taken together, these data suggest that the cardiac M_2 -muscarinic receptor elicits multiple responses (negative, positive inotropy, β -adrenoceptor desensitization) dependent upon the G proteins to which it is coupled. This provides further evidence for promiscuity of G protein coupling by the M_2 -muscarinic receptor, as recently suggested by Kenakin & Boselli (1990).

In conclusion, the present study has demonstrated that desensitization with β_1 -adrenoceptor agonists or functional antagonism by M_2 -muscarinic receptor agonists can be used to estimate reproducible, apparent K_D values for prenalterol. The use of these methods to derive apparent K_D values may have a more general utility, specifically in instances where the agonist exhibits low intrinsic efficacy.

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