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Pharmacological analysis of agonist-antagonist interactions at acetylcholine muscarinic receptors in a new urinary bladder assay

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1 Agonist-antagonist interactions at acetylcholine (ACh) muscarinic receptors have been analysed by use of an improved urinary bladder assay, isolated and intact, from the mouse. With 5-methylfurmethide as agonist, validated cumulative concentration-effect curves were obtained in less than 7 min by re-dosing before the response plateaux began to fade.

2 The pK_B value estimated for pirenzepine was 6.76. The pK_B values estimated for atropine and N-methylatropine from data obtained at concentrations which produced dose-ratios greater than 20 and 60 were 8.90 and 9.58, respectively.

3 The deviation from simple competitive behaviour at low dose-ratios with atropine and N-methylatropine was consistent with the operation of saturable antagonist removal processes. The deviation observed with atropine was corrected by pre-incubation with methylbutyrate, an alternative substrate for 'atropine esterase'.

4 The simple competitive behaviour of N-methylatropine was restored following pre-incubation with the neuronal choline uptake blocker hemicholinium-3 (HC-3). However, the pK_B estimated for N-methylatropine under these conditions was low. This latter result could be accounted for by the observed behaviour of HC-3 as a competitive antagonist of ACh muscarinic receptors ($pK_B = 4.01$).

5 We conclude that the modified mouse urinary bladder assay is suitable for the quantitative analysis of muscarinic receptor interactions. In addition, we postulate the existence of a previously undescribed uptake mechanism for quaternary muscarinic receptor antagonists.

Keywords: Receptor, muscarinic; parasympatholytics; parasympathomimetics; muscle, smooth, bladder; ammonium compounds; urinary tract, bladder; hemicholinium-3; esterase.

Introduction

The need for the development of new selective drugs to control the function of the urinary bladder is widely recognized (see McGuire, 1986). One approach is to use understood pharmacological principles and methods to define receptor targets (Black, 1989). Tactically, pharmacological models of agonist and antagonist action are applied in an attempt to relate experimental data to the model-defined chemical parameters which are imagined to govern ligand-receptor interactions. The challenge is to develop bioassays which can provide data of the necessary quality both to test the applicability of a model and to determine the confidence held in particular parameter estimates.

In vitro assays currently used for the pharmacological investigation of the urinary bladder are hindered by the intrinsic properties of the smooth muscle. The characteristic basal spontaneous activity which often increases during agonist-induced contraction and the transient nature of agonism, both make response level determination difficult (Edvardsen & Setekleiv, 1968; Sibley, 1984). As a result, experimental designs have evolved in which the preparations are superfused or washed between repeated applications of either single agonist doses or pulses of electrical stimulation (Holt *et al.*, 1985; 1986; Santicioli *et al.*, 1986). These experimental designs are, in our hands at least, inherently uneconomical and the data obtained highly variable. In addition, those assays using strips of bladder muscle are potentially further complicated because of the known regional variation in the hormone receptor (Levin *et al.*, 1980) and innervation density (Taira, 1972).

We describe the development of an *in vitro*, intact, mouse urinary bladder preparation for pharmacological assay. The assay has been validated by performing standard competitive analyses on the interaction between 5-methylfurmethide, an acetylcholine (ACh) muscarinic receptor agonist and three competitive antagonists, atropine, N-methylatropine and pirenzepine, previously well-characterized on other biosystems (see Black & Shankley, 1985). These validation procedures disclosed a new antagonist removal process and some of its properties.

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Methods

Urinary bladder assay

Bladders removed from adult male mice (18–28 g of Charles River CD-1 albino strain) were placed in Krebs-Henseleit solution of the following composition (in mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HPO_4^{2-} 1.0, SO_4^{2-} 1.2, D-glucose 10 and HCO_3^- 25. The whole bladder was drained, trimmed of extraneous tissue, ligated between two stainless steel wires and placed in an organ bath containing 20 ml of the above solution at 37°C, gassed with 95% O_2 and 5% CO_2. Tension was continuously recorded with an isometric transducer following the application of a 2 g load.

Guinea-pig trachea assay

Tissues were prepared as tracheal strips essentially as described by Emmerson & Mackay (1979). Briefly, the whole trachea from male guinea-pigs (Dunkin Hartley 400–600 g) was removed and cleared of extraneous tissue. The trachea was examined to locate the smooth muscle layer (posterior aspect). The anterior cartilage was cut longitudinally. The trachea was then pinned open on a board and kept moist with Krebs solution. The connective tissue between two rings was

cut on one side; then, the muscle layer was cut, leaving two adjacent rings separated from the rest of the trachea. Threads were tied to the open ends of the rings. Each preparation was suspended in an organ bath under identical recording conditions to those used for the bladder assay with the exception of the application of a 1 g load.

Experimental protocols

After the cumulative agonist dosing regime was established (see Results), the following protocol was used for the analysis of competitive antagonism. Twelve preparations were used simultaneously and after an initial 60 min stabilization period (solution replaced every 20 min), tissues were incubated for a further 60 min in the absence or presence of antagonist before a single cumulative concentration-effect curve was obtained using 5-methylfurmethide (5-MeF). Indomethacin (3 μM) was also included during this period in the tracheal assay to minimize interference from cyclo-oxygenase products. In additional experiments, tissues were also preincubated with hexamethonium (100 μM), methylbutyrate (100 μM), taurocholate (100 μM), choline (100 μM) or hemicholinium-3 (HC-3, 500 μM) for 60 min. Antagonist and other drug treatments were allocated on a block design so that each organ bath received a different treatment during the course of an experiment. Between 4 and 8 replicates were obtained for each treatment group.

Data analysis

Responses were expressed as changes in tension measured from the baseline immediately prior to starting the cumulative agonist concentration-effect curve or electrical stimulation. The concentration-effect curve data from individual preparations were fitted to a logistic function which provided estimates of the midpoint location ($\log[A_{50}]$), maximal asymptote (α) and midpoint slope (p) parameters, as described previously (Black & Shankley, 1985). These parameters were expressed as mean $\pm$ s.e.mean and used for both the analysis of the data and for display purposes by using them to simulate logistic curves which are shown superimposed upon the mean experimental data.

Competitive antagonism experiments were analysed according to methods described previously (Black *et al.*, 1985). In brief, if no significant differences were found in slopes and asymptotes of the curves in the absence and presence of the antagonist as tested by one-way ANOVA, then the $\log[A_{50}]$ values in the absence ($[A_{50}]$) and presence ($[A_{50}]^B$) of antagonist (B) were directly fitted to the following derivative of the Schild equation:

$$\log[A_{50}]^B = \log[A_{50}] + \log\left(1 + \frac{[B]^b}{10^{\log K_B}}\right)$$

If b, which is equivalent to the Schild slope parameter, was found to be not significantly different from unity, a second fit was performed with b constrained to unity, allowing the antagonist equilibrium dissociation constant (K_B) to be estimated as $\log K_B \pm$ s.e. For display purposes, mean $\log[A_{50}]$ data were expressed as dose-ratios (r) and plotted in Schild plot space.

A combined dose-ratio analysis was performed as described previously (Shankley *et al.*, 1988). In brief, data from the interaction between HC-3 (300 μM) and atropine (10 nM) in the presence of methylbutyrate (100 μM) were analysed to test compliance with the additive and multiplicative models.

A model describing the saturable uptake of a competitive antagonist (see Shankley, 1985; Kenakin & Beek, 1987) was used to simulate the data from the interaction between both N-methylatropine and atropine with 5-MeF. The following relation was used, $\log(r - 1) = \log[B_a] + pK_B$, where $[B_a]$, [B] in the receptor compartment, is given by the following equations,

$$[B_a] = \frac{-b + \sqrt{b^2 + 4ac}}{2a}$$

where $a = 1$, $b = (K_{BU} + U_M/k - [B])$ and $c = K_{BU}[B]$.

U_M is the maximum rate of uptake, k the diffusion rate constant for entry into the receptor compartment and K_{BU} the Michaelis-Menten constant.

Similarly, the combined competitive antagonism of muscarinic receptors by HC-3 (C) and N-methylatropine (B) was simulated using the relationship (Black *et al.*, 1986), $\log(r - 1) = \log([B]/K_B(1 + [C]/K_C))$ where K_B and K_C are the equilibrium dissociation constants for the two antagonists. The simultaneous expression of combined antagonism and antagonist uptake was simulated as follows,

$$\log(r - 1) = \log\left(\frac{[B_a]}{K_B(1 + [C]/K_C)}\right)$$

where $[B_a]$ is obtained from the equation describing the uptake system which is given above.

Drugs

Where possible, all drugs were freshly prepared in distilled water; methylbutyrate and indomethacin had to be prepared initially in absolute ethanol. The total volume of solvent added to the 20 ml organ bath did not exceed 350 μl. Drugs and their sources were as follows: atropine sulphate, N-methylatropine nitrate, hemicholinium-3 bromide, choline chloride, hexamethonium bromide, indomethacin, taurocholic acid (Sigma Chemical Co. Ltd.), methylbutyrate (Aldrich Chemical Co. Ltd.), 5-methylfurmethide iodide (Wellcome Research Laboratories Ltd.), pirenzepine dihydrochloride (A.B. Hassle Ltd.).

Results

Characterization of 5-methylfurmethide concentration-effect relations

Mouse urinary bladders, exposed to 5-MeF at concentrations between 0.1 μM and 10 μM, responded by a rapid increase in tone. After reaching a brief plateau, the tone faded with a half-life of a few minutes. Soon after the fade began, the muscle developed irregular, high frequency, twitch contractions (Figure 1a). This combination of fade and irregular twitchings made the identification and measurement of a 'steady-state' level nearly impossible. Apparently, 5-MeF concentration-effect relations would have to be measured from randomised sequences of individual doses, a difficult experimental design for studying agonist-antagonist interactions comprehensively.

Attempts to suppress the spontaneous activity of the bladder by pharmacological means were unsuccessful. Experiments were performed in the absence of calcium and in the presence of 1%, 3% and 10% of normal calcium concentration (2.5 mM) at organ bath temperatures of either 24°C and 37°C. Similarly, increasing the magnesium concentration (1.2 mM) up to 10 fold, in either a normal or low calcium solution (1%) did not affect spontaneous activity although the magnitude of the responses to 5-MeF was reduced.

However, one of us (P.A.C.D.) discovered that when a higher concentration of 5-MeF was added at the initial, twitch-free plateau of a previous dose-response, the superimposed contraction suppressed the onset of spontaneous twitching which was expected to develop after the antecedent dose. Thus it was possible, in a mere 7 min to obtain a complete agonist concentration-effect curve by cumulative dosing before the onset of fade at each level (Figure 1b). No significant differences were found between peak responses during

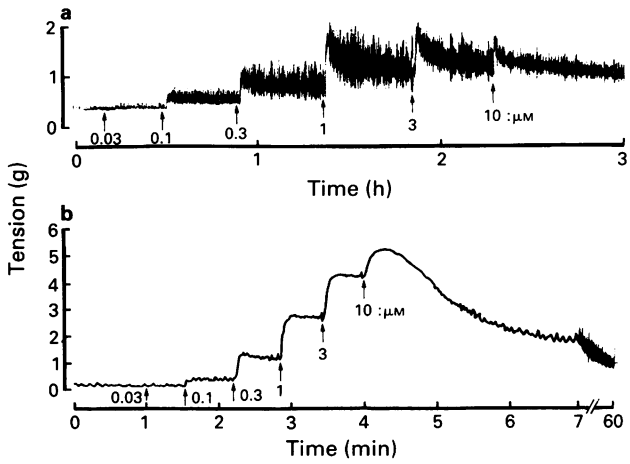

Figure 1 Experimental traces showing cumulative 5-methylfurmethide concentration-effect curves obtained on the intact mouse urinary bladder assay. Traces show (a) the development of fade and spontaneous activity following several minutes of exposure to the agonist and (b) the well-defined curve following adoption of the fast-dosing regime.

sequential cumulation of doses and those obtained from single doses given on a randomized schedule (Table 1). This table also shows that the experimental price to be paid for the ease and speed of cumulative dosing is serial correlation which shows up as progressively increasing variances.

Interaction between pirenzepine and 5-methylfurmethide

Pirenzepine, a polar derivative of benzodiazepine, is classified as a selective muscarinic receptor antagonist (Hulme *et al.*, 1990). It is useful for distinguishing M_1-receptors ($pK_B \sim 8.0$) from M_2- and M_3-receptors ($pK_B \sim 6.8$).

Pirenzepine, equilibrated with bladder muscle at concentrations between 0.3 μM–30 μM regularly moved the location of 5-MeF concentration-effect curves according to the Gaddum-Schild equation (Figure 2). The other parameters of the curves, slope and maximum, were not significantly changed. The simple competitive behaviour of pirenzepine was characterized by a pK_B estimate of 6.76 ± 0.09 (d.f. = 33). This value corresponds to the affinity of pirenzepine for M_2- and M_3-receptors.

Interaction between atropine and 5-methylfurmethide

Atropine, a basic organic ester, is classified as a non-selective antagonist of muscarinic receptors with a $pK_B \sim 9.0$.

On urinary bladder muscle, atropine produced concentration-dependent (3 nM–300 nM), parallel displacement of 5-MeF concentration-effect curves. Their slope parameters and maximal asymptotes were not altered. However, unlike pirenzepine, the displacements produced by atropine did not conform to the rectilinear simplicity of the Gaddum-Schild equation for competitive antagonism (Figure 2). The Schild plot, concave to the log[antagonist] axis, approached an asymptote of unit slope which gave an apparent pK_B intercept

Table 1 Comparison of the initial responses (tension, g ± s.e.mean, $n = 6$) to 5-methylfurmethide (5-MeF) obtained following single and cumulative dosing on the mouse urinary bladder assay

[5-MeF]: μM	Single dosing	Cumulative dosing
0.1	0.98 ± 0.19	1.20 ± 0.20
1	3.38 ± 0.20	3.60 ± 0.45
10	5.50 ± 0.29	5.33 ± 0.82

No significant differences were found (unpaired t test, $P > 0.05$).

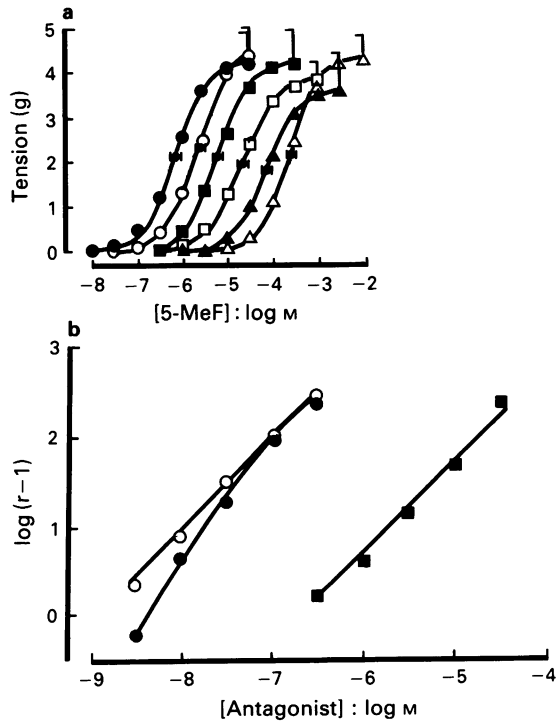

Figure 2 Atropine and pirenzepine pK_B determination on the mouse urinary bladder assay. (a) Methylfurmethide (5-MeF) concentration-effect curves obtained in the absence (●) and presence of atropine (nM), 3 (○), 10 (■), 30 (□), 100 (▲) and 300 (△) and in the presence of methylbutyrate (100 μM). (b) Schild plots for the 5-MeF/atropine (●), 5-MeF/atropine in the presence of methylbutyrate (100 μM) (○) and 5-MeF/pirenzepine (■) interactions on the mouse urinary bladder assay. The curve drawn through the data obtained from the 5-MeF/atropine interaction in the absence of methylbutyrate was simulated using a model describing the saturable removal of atropine (see legend to Figure 4 for details). pK_B estimates were as follows: atropine (+methylbutyrate) $pK_B = 8.99 \pm 0.07$, $b = 1.07 \pm 0.05$, pirenzepine $pK_B = 6.76 \pm 0.09$, $b = 1.11 \pm 0.07$.

of 8.90 ± 0.06 (d.f. = 25). Underreading of the effectiveness of atropine at low dose-ratios could occur if the tissue had a saturable removal process for atropine which operated in the vicinity of the cell surface receptors (Black & Shankley, 1985). In fact, Kenakin & Beek (1987), using rabbit ileum, found a similar saturable process which they attributed to the operation of an 'atropine esterase'. They showed that the enzyme could be inhibited by incubation with an excess (100 μM) of methylbutyrate, a substrate for the enzyme. Urinary bladders similarly incubated with methylbutyrate had the simple competitive behaviour of atropine fully restored, characterized by $pK_B = 8.99 \pm 0.07$ (d.f. = 28).

Interaction between N-methylatropine and 5-methylfurmethide

N-methylatropine, the highly polar quaternary ammonium derivative of atropine, is classified, like atropine itself, as a non-selective muscarinic receptor antagonist but with a $pK_B \sim 9.6$.

N-methylatropine produced concentration-dependent (3 nM–300 nM) parallel displacement of 5-MeF concentration-effect curves without alteration of slope parameters or upper asymptotes. Like atropine, the Schild plot was concave to the log[antagonist] axis but, at the higher dose-ratios, approached an asymptote of unit slope (Figure 3). Data from this region gave $pK_B = 9.58 \pm 0.07$ (d.f. = 20). Once again, it was possible to simulate these data with the model which describes the saturable uptake of a competitive antagonist (Figures 3 and 4). However, in this case, preincubation with methylbutyrate (100 μM) failed to disclose simple competitive

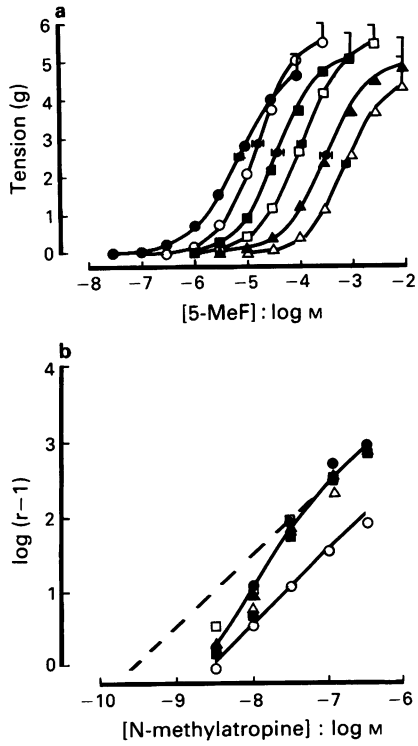

Figure 3 N-methylatropine pK_B determination on the mouse urinary bladder assay. (a) 5-Methylfurmethide (5-MeF) concentration-effect curves obtained in the presence of hemicholinium-3 (HC-3, 500 μM) and in the absence (●) and presence of N-methylatropine (nM), 3 (○), 10 (■), 30 (□), 100 (▲) and 300 (△). (b) Schild plots for the 5-MeF + N-methylatropine interaction in the absence (●) and presence of (○) HC-3 (500 μM), (■) methylbutyrate (100 μM), (□) choline (100 μM), (▲) hexamethonium (100 μM) and (△) taurocholate (100 μM). The curve drawn through the Schild plot data was simulated using a model describing the saturable removal of N-methylatropine. The pK_B value, in the absence of HC-3, (9.58 ± 0.07, b = 0.97 ± 0.09) was estimated using concentrations of N-methylatropine above 10 nM. A pK_B value of 8.61 ± 0.07, b = 0.95 ± 0.05 was estimated in the presence of HC-3.

behaviour suggesting that the N-methylatropine is not a substrate for the 'atropine esterase'.

Other antagonist removal processes were investigated. Taurocholate (100 μM), a recognized inhibitor of quaternary ammonium ion uptake in biochemical systems (Ruifrok, 1982) and hexamethonium (100 μM), a potential alternative substrate, had no effect on the Schild plot (Figure 3b). Similarly, choline (100 μM), a quaternary base with its own specific uptake process, did not affect the profile of antagonism (Figure 3b). However, preincubation with HC-3 (500 μM but not 30 μM), a specific inhibitor of the transport system which takes choline into cholinergic nerve-endings (see Ivy & Townsel, 1987), corrected the deviation from simple competitive behaviour in the Schild plot. However, the corresponding pK_B estimate of 8.61 ± 0.07 (d.f. = 21) was significantly lower than that obtained from the asymptote at high dose-ratios in the absence of HC-3 (Figure 3b).

The model simulations (Figure 4) indicate that the data obtained in the presence of HC-3 are consistent with the model describing the concomitant blockade of the N-methylatropine saturable uptake and muscarinic receptor competitive antagonism. This hypothesis was tested by application of the pharmacological resultant analysis (Figure 5). HC-3 produced concentration-dependent, parallel displacement of 5-MeF concentration-effect curves on both mouse bladder and guinea-pig trachea assays. This behaviour was compatible with HC-3 being a simple competitive antagonist of M-receptors in tracheal muscle with a $pK_B = 4.12 \pm 0.07$ (d.f. = 22). On the other hand, the Schild plot from the bladder assay was flat (b = 0.71 ± 0.04) and possibly nonlinear as well, data incompatable with simple competitive behav-

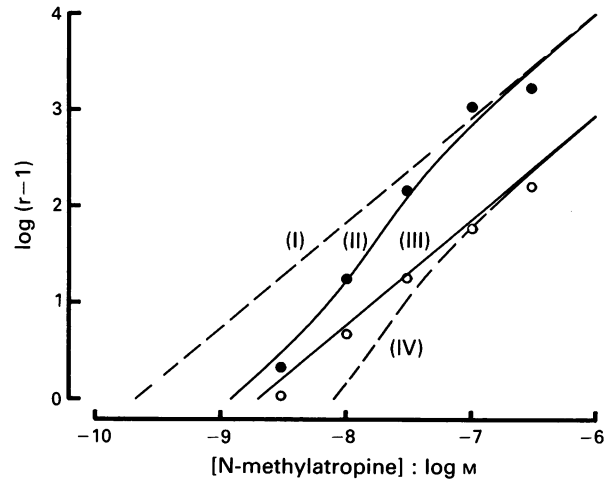

Figure 4 The effect of hemicholinium-3 (HC-3) on the N-methylatropine/5-methylfurmethide (5-MeF) interaction. Schild plots for the 5-MeF/N-methylatropine interaction in the absence (●) and presence (○) of 500 μM HC-3 on the mouse urinary bladder assay. The model-derived lines drawn superimposed on the data were obtained as described in the Methods section. Parameters as follows: (I) Simple competitive antagonism: simulated assuming a K_B value of 0.21 nM for N-methylatropine in the absence of HC-3 using the relationship $\log(r - 1) = \log[B] + pK_B$. (II) Saturable antagonist uptake in the absence of HC-3. $K_B = 0.21$ nM, $U_M = 15$ nmol s^{-1}, $k = 1$ s^{-1} and $K_{BU} = 3$ nM. (III) Antagonist uptake blocked and combined competitive antagonism of muscarinic receptors by HC-3 (C) and N-methylatropine (B). $K_B = 0.21$ nM, $K_C = 200$ μM and [C] = 500 μM. (IV) Combined competitive antagonism of muscarinic receptors by HC-3 (C) and N-methylatropine (B) with the uptake process still active. $K_B = 0.21$ nM, $K_C = 200$ μM, [C] = 500 μM and $[B_a]$ is given by the uptake model as above (II).

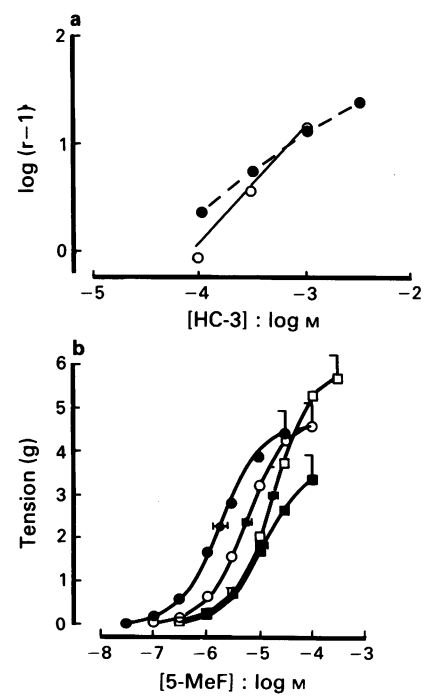

Figure 5 The effect of hemicholinium-3 (HC-3) on the N-methylatropine/5-methylfurmethide (5-MeF) interaction. (a) Schild plots for the 5-MeF/HC-3 interaction on the mouse urinary bladder (●, b = 0.71 ± 0.04) and guinea-pig trachea (○, $pK_B = 4.12 \pm 0.07$, b = 1.14 ± 0.12) assays. (b) Combined dose-ratio analysis. 5-MeF concentration-effect curves in the absence (●) and presence of (○) HC-3 (300 μM), (■) atropine (10 nM) plus methylbutyrate (100 μM) and (□) a combination of both antagonist treatments. The test statistic for the multiplicative model ($S_M = -0.32 \pm 0.18$, d.f. = 24), but not the additive model ($S_A = 0.12 \pm 0.26$, d.f. = 24), was significantly different from zero. Therefore the results are consistent with the additive model (for details of the analysis see Shankley et al., 1988).

iour. However, a combined dose-ratio analysis with atropine used as the reference antagonist showed, by the additivity of antagonism, that HC-3 was apparently acting syntopically with atropine (Figure 5). Importantly, it was not possible to simulate the effect of HC-3 on the N-methylatropine Schild plot by assuming that its action was solely due to muscarinic receptor blockade (Figure 4).

Discussion

Confidence in the application of models of drug action to bioassay data increases with the number of model-defined experimental criteria that are met. Models of agonism and antagonism, based on the applicability of the Law of Mass Action to the interactions among agonist, antagonist and receptor, require for simplicity that measurements of effect are made under equilibrium conditions. Ideally, measurements of effect are made when the agonist response achieves a clearly-defined, sustained plateau. This steady-state condition is usually assumed to indicate an underlying equilibrium condition at the receptors. The increase in tone of the urinary bladder muscle produced by 5-MeF, a muscarinic receptor agonist which is not a substrate for cholinesterases, faded rapidly after a brief plateau. We have presumed that this is due to the bladder wall plasticity which allows this organ to accommodate increases in volume without change in intravesical pressure. In spite of this, the adoption of the fast, cumulative dosing regime and increasing the chart speed did allow the reliable definition of the concentration-effect curve for 5-MeF. This was possible because each pre-fade contraction apparently suppressed not only fade but also the development of spontaneous, irregular twitch contractions.

However, the problem remained as to whether the brief period following application of the agonist to the organ bath was sufficient for equilibrium to be achieved. In reality, this problem is no different from that faced with all assays in which the receptor events are deduced rather than measured directly (perhaps as in ligand binding studies), whether or not sustained responses are achieved. Ideally, during preincubation, the antagonist diffuses into the tissue and ultimately it comes into concentration equilibrium with the receptor. According to the Law of Mass Action, the antagonist, B, will occupy a specified fraction of the available receptors. When the agonist, A, is applied, it diffuses in and competes with B to occupy receptors. This means that the occupancy of B previously established during the pre-incubation period must decrease as occupancy by A increases. For the fast dosing regime to be considered useful, this new equilibrium must be attained rapidly. A necessary but not sufficient test for rapid equilibrium was performed by analysing the interaction between 5-MeF and the three previously well-characterized muscarinic receptor antagonists. If the system was at equilibrium then the pK_B estimates would agree with those previously found (Black & Shankley, 1985). The results with pirenzepine show not only that this method of characterizing agonist concentration-effect curves exposes its expected simple competitive behaviour but, also gives a satisfactory estimate of its pK_B at M_2- or M_3-receptors (6.76 ± 0.09).

The competitive analysis with 5-MeF and atropine was initially confounded by significant deviation from competitive behaviour at low atropine concentrations. However, following pretreatment with methylbutyrate, the alternative substrate for 'atropine esterase', atropine behaved as a simple competitive antagonist and the pK_B value estimated (8.99 ± 0.07) was similar to values previously obtained on other muscarinic receptor assays (Hulme *et al.*, 1990). Interestingly, in contrast to the results of the study by Kenakin & Beek (1987), in which 'atropine esterase' activity was observed in only 25% of rabbit ileum preparations, the deviation from simple competitive behaviour was seen in all preparations in the present study. However, wide variation in the distribution and activity of the enzyme has been previously recognized (see Kenakin & Beek, 1987).

The deviation from simple competitive behaviour with N-methylatropine, in contrast to atropine, was not corrected by incubation with an excess concentration of methylbutyrate suggesting that N-methylatropine is not a substrate for 'atropine esterase'. We have previously suggested that N-methylatropine might be a substrate for a quaternary ammonium uptake system (Black & Shankley, 1985). However, as in our previous study, using the lumen-perfused mouse stomach assay, the biochemically-defined uptake inhibitor (Ruifrok, 1982), taurocholate, and the potential alternative substrates, hexamethonium and choline, were ineffective. However, the ability to estimate $\log[A_{50}]$ values in the presence of high concentrations of N-methylatropine, seemingly when the uptake process was saturated, did allow a pK_B value (9.58 ± 0.07) to be estimated which agreed with values previously found (Black & Shankley, 1985).

Considered together, the results obtained from the three competitive analyses indicate that equilibrium conditions are achieved rapidly, that is, within the period between agonist dosing and the corresponding effect level measurement.

The finding that the deviation from competitive behaviour of N-methylatropine was corrected by pre-incubation with HC-3 was initially confused by the significantly lower pK_B indicated for N-methylatropine (Figure 3). However, this result could be accounted for by assuming that the effects of HC-3 were due to concomitant uptake blockade and competitive antagonism at muscarinic receptors. The data could be simulated by assuming a pK_B value of 4.20 for HC-3 (Figure 4). The independent experiments on both the guinea-pig trachea and mouse urinary bladder assays provided estimates, 4.12 ± 0.07 and $4.01 \pm \pm 0.07$, respectively, which were very close to this model prediction. The choline uptake blocking property of HC-3 has been claimed to be unique to neuronal sites (see Ivy & Townsel; 1987) suggesting the possibility that N-methylatropine, but not atropine, is taken up by cholinergic neurones. The pharmacological significance of this finding merits further investigation. Is the N-methylatropine captured by the nerve-endings also taken up by the terminal vesicles? Is N-methylatropine released along with ACh during nerve stimulation?

In conclusion, the mouse urinary bladder assay described is suitable for the quantitative analysis of muscarinic receptor interactions. In addition we postulate the existence of a previously undescribed uptake mechanism for quaternary muscarinic receptor antagonists.

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References

BLACK, J.W. (1989). Drugs from emasculated hormones: The principle of syntopic antagonism. *Science*, **245**, 486–493.

BLACK, J.W., LEFF, P. & SHANKLEY, N.P. (1985). Further analysis of anomalous pK_B values for histamine H2-receptor antagonists on the isolated mouse stomach assay. *Br. J. Pharmacol.*, **86**, 581–587.

BLACK, J.W. & SHANKLEY, N.P. (1985). Pharmacological analysis of muscarinic receptors coupled to oxyntic cell secretion in the mouse stomach. *Br. J. Pharmacol.*, **86**, 601–607.

BLACK, J.W., GERSKOWITCH, V.P., LEFF, P. & SHANKLEY, N.P. (1986). Analysis of competitive antagonism when this property occurs as part of a pharmacological resultant. *Br. J. Pharmacol.*, **89**, 547–555.

EDVARDSEN, P. & SETEKLEIV, J. (1968). Distribution of adrenergic receptors in the urinary bladder of cats, rabbits and guinea-pigs. *Acta Pharmacol. Toxicol.*, **26**, 437–445.

EMMERSON, J. & MACKAY, D. (1979). The zig-zag tracheal strip. *J.*

Pharm. Pharmacol., **31**, 798.

HOLT, S.E., COOPER, M. & WYLLIE, J.H. (1985). Evidence for purinergic transmission in mouse urinary bladder and for modulation of responses to electrical stimulation by 5-hydroxytryptamine. *Eur. J. Pharmacol.*, **116**, 105–111.

HOLT, S.E., COOPER, M. & WYLLIE, J.H. (1986). On the nature of the receptor mediating the action of 5-hydroxytryptamine in potentiating responses of the mouse urinary bladder strip to electrical stimulation. *Naunyn Schmiedebergs Arch. Pharmacol.*, **334**, 333–340.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

IVY, M.T. & TOWNSEL, J.G. (1987). A comparative study of high affinity choline uptake and choline utilization in cholinergic and non-cholinergic tissues. *Comp. Biochem. Physiol.*, **86**, 111–120.

KENAKIN, T.P. & BEEK, D. (1987). The effects on Schild regressions of antagonist removal from the receptor compartment by a saturable process. *Naunyn Schmiedebergs Arch. Pharmacol.*, **335**, 103–108.

LEVIN, R.M., SHOFER, F.S. & WEIN A.J. (1980). Cholinergic, adrenergic and purinergic responses of sequential strips of rabbit urinary bladder. *J. Pharmacol. Exp. Ther.*, **212**, 536–540.

McGUIRE, E.J. (1986). Neuromuscular dysfunction of the lower urinary tract. Chapter 11 (pp. 616–638). In *Campbell's Urology*, Fifth edition. London: W.B. Saunders & Co.

RUIFROK, P.G. (1982). Uptake of quaternary ammonium compounds into rat liver plasma membrane vesicles. *Biochem. Pharmacol.*, **31**, 1431–1435.

SANTICIOLI, P., MAGGI, C.A. & MELI, A. (1986). The postganglionic excitatory innervation of the mouse urinary bladder and its modulation by prejunctional $GABA_B$-receptors. *J. Auton. Pharmacol.*, **6**, 53–66.

SHANKLEY, N.P., BLACK, J.W., GANELLIN, C.R. & MITCHELL, R.C. (1988). Correlation between log P_{OCT/H_2O} and pK_B estimates for a series of muscarinic and histamine H_2-receptor antagonists. *Br. J. Pharmacol.*, **94**, 264–274.

SHANKLEY, N.P. (1985). Pharmacological analysis of the regulators of gastric acid secretion. *Ph.D. thesis. University of London.*

SIBLEY, G.N.A. (1984). A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. *J. Physiol.*, **354**, 431–443.

TAIRA, N. (1972). The autonomic pharmacology of the bladder. *Annu. Rev. Pharmacol.*, **12**, 197–208.

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