DARIFENACIN REVEALS A FUNCTIONAL ROLE FOR M_4 MUSCARINIC ACETYLCHOLINE RECEPTORS IN GUINEA PIG GALLBLADDER

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ABSTRACT

Objective: Previous studies have revealed the presence of M_1 to M_4 muscarinic receptors in guinea pig gallbladder, with M_3 and M_4 receptors being claimed to mediate contractions.

Methods: In the present study, cumulative concentration-response curves to carbachol were constructed in the absence and presence of darifenacin, which has greater selectivity for M_3 over M_4 receptors, in guinea-pig gallbladder.

Results: Darifenacin caused concentration-dependent dextral shifts of the carbachol curve, however, at 1 μM, it also caused a degree of insurmountable antagonism. Using a novel analytical approach, we obtained a pK_B estimate for darifenacin (7.51 ± 0.14) that is in excellent agreement with its affinity for M_2/M_4, but not M_3 receptors.

Conclusion: Given our previous demonstration that M_2 receptors are unlikely to contribute to muscarinic contractions in this tissue, our current findings provide pharmacological evidence for a predominant role of M_4 muscarinic receptors in guinea pig gallbladder contractions.

Key Words: Muscarinic receptor, Guinea-pig, Gallbladder, Contraction, Darifenacin.

INTRODUCTION

Muscarinic acetylcholine receptors play an important role in regulating smooth muscle contractility (1). Although abundant evidence exists to suggest that the muscarinic M_3 receptor is the predominant subtype causing a contraction in most smooth muscle preparations (2), there are exceptions to this general observation. One notable example is the guinea pig gallbladder, where muscarinic receptor-mediated smooth muscle contraction helps to control bile outflow in response to feeding; despite previous claims of functional M_3 receptors being present in this tissue (3-6), a combination of biochemical and organ bath experiments have revealed the

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presence of M_1, M_2, M_3 and M_4 muscarinic receptor subtypes (7-11).

A major difficulty in ascribing functional roles to subtypes of muscarinic receptors has long been the lack of selective antagonists that can differentiate between receptors mediating the same response (1). Nevertheless, a comparison of the potencies of a group of antagonists may still be used to determine the possible contribution of different subtypes to a particular response. Using this strategy, we provided evidence for a functional role of both M_3 and M_4 receptors in mediating guinea-pig gall bladder contractions (11). However, that study was unable to determine which of the two subtypes played the predominant functional role. In the present study, we have utilized the antagonist, darifenacin, which is at least greater than 50 times more selective for the M_3 (pK_B approx. 9) over the M_4 receptor (pK_B approx. 7.5) (12,13), and investigated its effects on carbachol-mediated contractions in guinea-pig gallbladder.

MATERIAL AND METHODS

Guinea-pigs of either sex (300-350 g) were killed by CO_2 asphyxiation. Gallbladders were then removed and longitudinal strips were prepared and mounted in an organ bath containing Krebs solution (composition in mmol/l; NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; glucose, 11.1) at 37°C bubbled with a mixture of 95 % O_2 and 5 % CO_2. Tissues were allowed to equilibrate for 2 h under a resting tension of 0.5 g., and isometric contractions were recorded on a polygraph (Grass Model 7) via a force-displacement transducer (Grass FT03).

Control cumulative concentration-response curves were constructed to carbachol (Sigma, St. Louis, USA). An interval of 45 min was then allowed during which the tissues were washed with Krebs solution. A second concentration-response curve to carbachol was then constructed in the absence or presence of darifenacin. One or two concentrations of the antagonist were used for each strip.

Normalized concentration-response data were fitted to a three-parameter Hill equation using a pre-release version of Prism 4.0 (GraphPad Software, San Diego, CA). Because the highest concentration of darifenacin (1 μM) caused a significant reduction in the maximal response to carbachol (see Results), we determined equieffective agonist concentrations, in the absence or presence of antagonist, at a response level close to the tissue minimum (15%) in order to derive antagonist potency estimates. The validity of this analytical approach has been demonstrated previously (14-16). The agonist $pEC_{15\%}$ values were fitted to the following equation:

$$pEC_{15\%} = -\log([B]^s + 10^{-pK}) - \log c \quad (1)$$

where [B] denotes antagonist concentration, pK and logc are fitting constants, and s is equivalent to the Schild slope factor; when s=1, $pK=pK_B$ (15). For presentation purposes, the relationship between the estimated pK_B and the shift of the agonist concentration-response curves was displayed as a Clark plot (17).

Data are shown as mean ± s.e.m. Estimates of agonist concentration-response curve maxima were compared by one-way ANOVA. $P < 0.05$ was taken as significant.

RESULTS

Carbachol caused concentration-dependent contractions in guinea-pig gallbladder longitudinal muscle strips, with a pEC50 value of 5.63 0.18 (n = 7). The addition of darifenacin caused dextral shifts of the carbachol curve (Fig. 1A). However, the highest concentration of darifenacin also caused a significant reduction in the maximal response to carbachol, an observation not consistent with the expectations of simple competitive antagonism.

Because the insurmountable effect of 1 μM darifenacin meant that the carbachol pEC_{50} values do not all represent equieffective agonist concentrations, we fitted the data to equation (1) utilizing equieffective agonist concentrations that were determined at the $pEC_{15\%}$ response level (Fig. 1A). The analysis using this method yielded a pK_B value of 7.51 0.14 (n = 25). In this analysis, the Schild slope parameter was not significantly different from unity and was constrained as such for the estimation of the antagonist pK_B value.

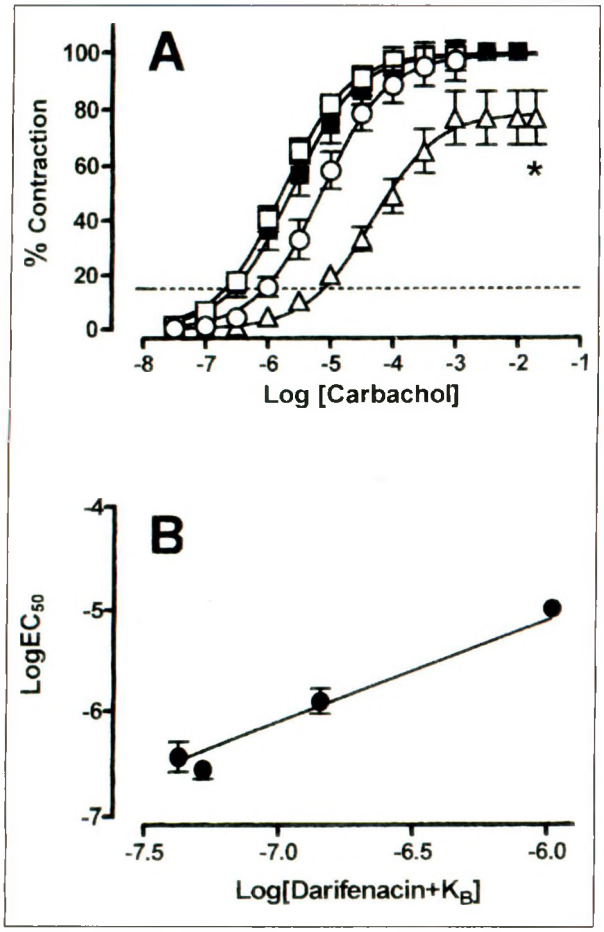

Fig. 1: (A) Concentration-response curves of carbachol in the absence (□) or presence of darifenacin 0.01 (■), 0.1 (○) or 1 μM (Δ) in guinea-pig gallbladder. Also indicated on the Figure is the 15% response level (dashed line) that was used for the derivation of equieffective agonist concentrations for subsequent determination of the antagonist pK_B from Equation (1) of the Materials and Methods. Data points represent the means of 4-8 experiments. * Significantly different ($P < 0.05$) than the control maximal response to carbachol. (B) Clark plots of the interaction between increasing concentrations of darifenacin with carbachol. The antagonist pK_B estimate was first derived by nonlinear regression analysis according to equation (1) in the Materials and Methods, and was subsequently used in the construction of the Clark plot.

DISCUSSION

Studies on most smooth muscle preparations have demonstrated a major role for functional muscarinic M_3 receptors in mediating contractions, even though these tissues invariably contain a mixture of muscarinic receptor subtypes (2). In a few smooth muscle preparations, however, contractile responses may be mediated by muscarinic receptor subtypes other than the M_3 receptor (18-22).

In the guinea-pig gallbladder, Von Schrenck et al. (6) reported that carbachol-induced inhibition in adenylate cyclase, but not stimulation of phosphoinositides, was pertussis toxin sensitive, indicating the presence of more than one subtype of muscarinic receptors. Subsequently, biochemical and functional studies have revealed the presence of M_1, M_2, M_3 and M_4 muscarinic receptor subtypes in this tissue (23,24,8,9). Of these subtypes, a predominant functional role in guinea pig gallbladder smooth muscle was suggested for the M_3 receptors (3-6), but we recently provided evidence for the coexistence of both functional muscarinic M_3 and M_4 receptor subtypes mediating Ca^{2+} mobilization in this tissue (11).

The present study extended our previous work by utilizing darifenacin to differentiate the muscarinic receptor subtype involved in carbachol-mediated contractions of the guinea pig gallbladder. Darifenacin is classed as an M_3-selective muscarinic receptor antagonist, showing 100-fold selectivity for M_3 receptors over M_2 receptors in atria and 30 fold over M_1 receptors in rabbit vas deferens (12). The pK_i values against human cloned muscarinic receptors have been reported as: M_1, 8.15; M_2, 7.35; M_3, 9.12; M_4, 7.34; M_5, 8.03 (13).

Although darifenacin was able to inhibit carbachol-mediated contractions in our preparation, the highest concentration used here resulted in a significant degree of insurmountable antagonism (Fig. 1A). This property of darifenacin has been reported previously at pig, rat and dog bladder smooth muscle, but not in rabbit and mouse bladder (25-29). Although the reason for the apparent insurmountability of darifenacin antagonism is not known, it is possibly related to slow binding kinetics at the muscarinic receptors, relative to the time scale of the measured responses, as previously noted with other muscarinic receptor antagonists (14). Irrespective of mechanism, apparent insurmountable antagonism invalidates the classic approaches for quantifying competitive antagonism that rely on the comparison of agonist EC_{50} values, because these values no longer represent equieffective agonist

concentrations and hence the analysis is no longer response-null. As a consequence, the use of EC_{50} values in the determination of antagonist potency under such conditions leads to an underestimation of the true antagonist potency (14-16). However, we have previously demonstrated in different cell and tissue preparations how the impact of this underestimation can be minimized by choosing truly equieffective agonist concentrations at an appropriate response level (14-16). In the present study, we utilized the 15% response level in equation (1).

Using our analytical method, we derived a pK_B estimate for darifenacin of 7.51 that is more than an order of magnitude lower than any previous estimates of the affinity of darifenacin for the M_3 receptor, but in excellent agreement with its affinity for either the M_2 receptor or the M_4 receptor (13). Because we have previously used a series of selective antagonists to rule out a functional role for muscarinic M_2 receptors in guinea pig gallbladder (11), our current findings with darifenacin lead us to conclude that the major functional muscarinic receptor subtype in this preparation is the M_4 receptor.

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