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Further evidence for the heterogeneity of functional muscarinic receptors in guinea pig gallbladder

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Abstract

Previous studies have suggested the presence of multiple muscarinic receptor subtypes in guinea pig gallbladder smooth muscle, although the relative abundance and functional role of these subtypes remains an area of significant research efforts. The present study utilized both radioligand kinetic and functional experiments to further probe the nature of the muscarinic receptors in gallbladder smooth muscle and their mode of coupling to intra- and extra-cellular Ca^{2+} sources. Dissociation kinetic studies using [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) indicated that the binding profile in guinea pig gallbladder smooth muscle could not be reconciled with that expected for a single muscarinic receptor subtype, the latter determined in parallel experiments conducted on the cloned muscarinic M_1–M_5 subtypes in Chinese hamster ovary (CHO) cells. Furthermore, comparison of the gallbladder data with the dissociation characteristics of [^{3}H]NMS in guinea pig urinary bladder revealed a significantly different kinetic profile, with the urinary bladder, but not the gallbladder, demonstrating biphasic radioligand dissociation kinetics. In functional experiments, carbachol caused a concentration-dependent contraction of guinea pig gallbladder smooth muscle strips in Ca^{2+}-free or 5 mM Sr^{2+}-substituted physiological salt solutions (PSS) with amplitudes of the maximal contractions corresponding to 45.8 ± 8.0% and 33.2 ± 6.6% of control responses in normal PSS, respectively. Furthermore, the stimulus–response characteristics of carbachol-mediated contraction appeared significantly altered in Ca^{2+}-free PSS relative to normal or Sr^{2+}-substituted PSS. The antagonist, methoctramine (1×10^{-7}–3×10^{-5} M), exerted only a slight inhibition of carbachol (10^{-5} M)-induced contractions in 5 mM Sr^{2+}-substituted medium, whereas it was significantly more potent in antagonizing gallbladder contractions in response to 10^{-5} M carbachol in the absence of extracellular Ca^{2+}. Both atropine and tripitramine were equipotent in antagonizing carbachol-induced contractions in Ca^{2+}-free (pIC_{50}: 6.85 ± 0.11 for atropine and 5.75 ± 0.32 for tripitramine) and Sr^{2+}-substituted media (pIC_{50}: 6.88 ± 0.25 for atropine and 5.70 ± 0.16 for tripitramine), and pirenzepine was only slightly more potent in Ca^{2+}-free PSS (pIC_{50}: 5.66 ± 0.23) than in Sr^{2+}-substituted PSS (pIC_{50}: 5.33 ± 0.21). Taken together, our data indicate that carbachol contracts guinea pig gallbladder by stimulating two distinct muscarinic receptor subtypes linked to extracellular Ca^{2+} influx and intracellular Ca^{2+} release. These two subtypes may represent the muscarinic M_3 and M_4 receptors, although the presence of the muscarinic M_2 receptor subtype is also suggested from the binding data.

Keywords: Muscarinic receptor; (Guinea pig); Gallbladder; Receptor heterogeneity; Radioligand binding; Kinetics; Ca^{2+}, Intracellular; Ca^{2+}, Extracellular; Sr^{2+}

1. Introduction

Five different muscarinic receptor subtypes have been identified based on studies of molecular structure, in vitro binding and function (Buckley et al., 1989; Hulme et al., 1990; Dörje et al., 1991). Muscarinic receptors mediate smooth muscle contraction, and in most preparations the predominant functional muscarinic receptor subtype has been identified as the muscarinic M_3 receptor (Eglen et al., 1994). However, contractile responses have also been found to be mediated by muscarinic M_2 receptors in a few smooth muscle preparations, such as porcine basilar artery (Van Charldorp and Van Zwieten, 1989) and guinea pig uterus (Eglen et al., 1989, 1991; Doods et al., 1993), and by muscarinic M_1 receptors in canine saphenous and

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femoral veins (Eglen and Whiting, 1990). Recently, we have provided evidence indicating that the contractile responses of guinea pig gallbladder smooth muscle cells may be mediated via muscarinic M_4 receptors (Özkutlu et al., 1993; Oktay et al., 1998; Karaalp et al., 1999), whereas other groups have claimed the presence of functional muscarinic M_3 receptors in this tissue (Von Schrenck et al., 1993, 1994; Takahashi et al., 1994; Eltze et al., 1997).

Interestingly, radioligand binding studies and Northern blot analysis have revealed the existence of a mixed population of muscarinic binding sites in the majority of smooth muscles from many species (Giraldo et al., 1987; Maeda et al., 1988; Michel and Whiting, 1988; Entzeroth and Mayer, 1991; Ford et al., 1991). In agreement with these findings, Takahashi et al. (1994) have reported that carbachol-induced inhibition of adenylate cyclase activity, but not phosphoinositide hydrolysis, is pertussis toxin sensitive in guinea pig gallbladder, suggesting the presence of more than one subtype of muscarinic receptor subserving a functional response in this tissue. Although the latter finding may also be indicative of promiscuous G protein coupling by a single receptor subtype, the presence of both muscarinic M_2 and muscarinic M_4 receptors in this tissue has also been demonstrated via radioligand binding and Western blot analysis (Oktay et al., 1998). Furthermore, Parkman et al. (1999) have claimed that presynaptic inhibitory M_1, excitatory M_2 and postsynaptic M_3 receptors all contribute to contractions in guinea pig gallbladder.

In light of the mixed reports in the literature regarding the subtypes and functional roles of the muscarinic receptors found in the gallbladder, a number of complementary approaches need to be employed in tandem in order to resolve this issue. One such approach may involve monitoring the kinetic binding profile of muscarinic receptor radioligands, as such studies have previously been shown to be quite sensitive indicators of receptor heterogeneity when compared to equilibrium binding studies utilizing the same radioligand (Flynn et al., 1997). A second approach has also been outlined in a few functional studies, whereby the experimental assay conditions have been modified in order to delineate muscarinic receptor subtypes. For example, Chen et al. (1995) have reported that muscarinic M_2 and M_3 receptor subtypes may be involved in the contractile responses of cat gallbladder. Specifically, by modifying the availability of extracellular and intracellular Ca^{2+} sources, these investigators concluded that muscarinic M_2 receptors contribute to smooth muscle contraction predominantly by stimulating extracellular Ca^{2+} influx, whereas muscarinic M_3 receptors account for intracellular Ca^{2+} mobilization via the phosphoinositide pathway. Similarly, in the guinea pig gallbladder, acetylcholine-induced contractions have been shown to involve both extracellular and intracellular Ca^{2+} pools (Renzetti et al., 1990). It is therefore possible that the contribution of both Ca^{2+} pools to the contractile responses in the guinea pig gallbladder may also be mediated by distinct muscarinic receptor subtypes, as found in the cat gallbladder (Chen et al., 1995).

Thus, the aims of the present study were two-fold. First, we chose to determine the binding kinetics of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) in homogenates of guinea pig gallbladder smooth muscle and to compare the observed binding profile to that obtained at the individual human M_1–M_5 muscarinic receptor subtypes expressed in Chinese hamster ovary (CHO) cells, as well as to that observed in guinea pig urinary bladder. The latter tissue was chosen because it has been well characterized as possessing a mixture of muscarinic M_2 and M_3 receptors, with the muscarinic M_2 receptor being most predominant (Wang et al., 1995). Second, we investigated whether the utilization of different Ca^{2+} sources in the contractile response of the guinea pig gallbladder smooth muscle is mediated by different muscarinic receptor subtypes by utilizing a number of subtype-selective and nonselective antagonists.

2. Materials and methods

2.1. Binding assays

2.1.1. Membrane preparation

CHO cells, stably expressing the human muscarinic M_1–M_5 acetylcholine receptors were kindly provided by Dr. Mark Brann (University of Vermont, Burlington, USA) and were grown for 4 days at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and 50 μg/ml geneticin in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were used 4 days after subculturing and were harvested by trypsinization followed by centrifugation ($300 \times g$, 3 min) and resuspension in sodium–potassium–phosphate buffer (SPPB; 30 mM K_2HPO_4, 20 mM NaH_2PO_4, 1 mM $MgCl_2$). Cells were then homogenized with an Ultra-Turrax (2×10 s bursts, with a 30-s period of cooling on ice between homogenizations) and centrifuged at $1000 \times g$ for 10 min. The resulting supernatant was collected and centrifuged at $30{,}000 \times g$ for 30 min, after which the pellet was re-suspended and immediately utilized in the binding assays.

Guinea pigs (weighing about 500–600 g) of both sexes were killed and gallbladders and urinary bladders were quickly removed and put in SPPB. The tissues were then weighed and cut into small pieces, before being homogenized in a volume of 20 ml buffer with an Ultra-Turrax homogenizer at a maximum speed for 30 s. Following centrifugation at 4°C for 10 min at $1000 \times g$, the supernatants were collected and immediately used in the binding assays.

2.1.2. Association and dissociation kinetic experiments

CHO, guinea pig urinary bladder or guinea pig gallbladder smooth muscle cell membranes (ca. 100 μg pro-

tein/ml) were pre-incubated in SPPB for 15 min at 25°C. Subsequently, 0.5-nM [^{3}H]NMS was added to the tubes at various times and the reaction was then terminated by rapid filtration. The amount of radioactivity thus determined at the various time intervals was utilized to obtain a quantitative estimate of the [^{3}H]NMS association rate, as described below. In a parallel series of experiments designed to asses radioligand dissociation rate, membranes were incubated with 0.5-nM [^{3}H]NMS for 60 min to ensure equilibrium, after which 1 μM atropine was added to the tubes at various time points to ensure maximal radioligand dissociation. Non-specific binding was defined using 10-μM atropine. Protein determinations were performed using the method of Bradford (1976) with bovine serum albumin as the standard.

For all binding experiments, incubation was terminated by filtration through Whatman GF/B or Skatron 11731 filters, positioned on a Brandell (Gaithersburg, MD, USA) or Skatron (Sterling, VA, USA) Cell Harvester, respectively. Filters were washed three times with 4-ml aliquots of ice-cold buffer and dried before radioactivity was measured using liquid scintillation counting.

2.2. Functional experiments

2.2.1. Isolated tissue preparation

Longitudinal strips of guinea pig gallbladders (500–600 g) of both sexes were prepared and mounted in an organ bath containing physiological salt solution (PSS) at 37°C bubbled with a mixture of 95% O_2 and 5% CO_2 under a resting tension of 1.0 g and were allowed to equilibrate for 3 h. Isometric contractions were recorded on a polygraph (Model 7, Grass Instruments, Quincy, MA, USA) via a force-displacement transducer (Model FT03, Grass Instruments).

Three different buffers were utilized. Normal PSS contained the following: 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 mmol/l. Ca^{2+}-free PSS was prepared by eliminating $CaCl_2$ from the buffer medium and substituting for it an equivalent amount of NaCl, and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added to a final concentration of 0.1 mM. For the third buffer, strontium (Sr^{2+}) chloride (5.0 mM) was added instead of $CaCl_2$ in the normal PSS to prepare Sr^{2+}-substituted PSS. Sr^{2+} replacement for Ca^{2+} in the medium blocks the processes that require Ca^{2+} release from intracellular storage sites. All solutions were titrated to pH 7.4 and gassed with a mixture of 95% O_2 and 5% CO_2.

2.2.2. Experimental protocol

Two functional experimental paradigms were utilized. For the first, cumulative concentration–response curves were constructed to the agonist, carbachol (3×10^{-8}–1×10^{-3} M), initially in normal PSS, using incremental increases in concentration spaced at 0.5 log intervals. Concentrations were added once a sustained response to the previous concentration was reached. An interval of 45 min was then allowed during which the tissues were washed with normal PSS. Following 20-min incubation in either Ca^{2+}-free or Sr^{2+}-substituted PSS, a second cumulative concentration–response curve to carbachol was constructed. Additionally, the onset of contractile responses to a single (10^{-5} M) concentration of carbachol in the different buffers was also tested.

The second functional paradigm involved the construction of antagonist inhibition curves. Initially, two consecutive tissue contractions were elicited by 10^{-5} M carbachol in normal PSS. Then, the buffer was replaced either with Ca^{2+}-free or Sr^{2+}-substituted PSS and the contractile responses were repeated. The strips were then incubated with a muscarinic receptor antagonist, atropine (1×10^{-8}–3×10^{-7} M), pirenzepine (1×10^{-7}–1×10^{-5} M), methoctramine (1×10^{-7}–3×10^{-5} M) or tripitramine (1×10^{-7}–1×10^{-5} M), for 15 min and contraction was re-induced with 10^{-5} M carbachol in the presence of the antagonist. This procedure was repeated with increasing concentrations of antagonist, and only one antagonist was used for each strip.

2.3. Data analysis

Radioligand kinetic parameters were determined via nonlinear regression analysis using the programs ORIGIN 5.0 (Microcal Software, Northampton, MA, USA) or PRISM 2.0 (GraphPad Software, San Diego, CA, USA) as follows.

Association kinetic data were fitted to the following equation describing a monoexponential association:

$$Y_t = Y_{eq}(1 - e^{k_{obs}t})$$

where

$$k_{obs} = k_{on} \times [\text{radioligand}] + k_{off}.$$

In the above equations, Y_t denotes the specific binding of the radioligand at time t, and k_{on} and k_{off} denote the association and dissociation rate constants, respectively. Dissociation kinetic data were fitted to the following equation describing monoexponential decay:

$$Y_t = Y_{eq}e^{k_{off}t}$$

where the parameters are as defined above. In practice, datasets for association and dissociation experiments that were obtained on the same receptor preparation on the same day were simultaneously analyzed using the program ORIGIN 5.0 (Microcal Software) according to the above equations in order to obtain direct estimates of k_{on} and k_{off}. In some instances where only a single association kinetic curve was obtained in one preparation, k_{on} was estimated from the relationship:

$$k_{on} = (k_{obs} - k_{off})/[\text{radioligand}]$$

Table 1
Kinetic rate constants for [^{3}H]NMS at the individual muscarinic receptor subtypes expressed in CHO cells, as well as in the native receptors of the guinea pig urinary bladder and gallbladder

Receptor	k_{off}^{a} (min^{-1})	$k_{off\ fast}^{b}$ (min^{-1})	$k_{off\ slow}^{b}$ (min^{-1})	n^{c}	k_{on}^{d} ($M^{-1}\ min^{-1}$)	n^{c}
CHO M_1	0.14 ± 0.02			3	$1.94 \pm 0.33 \times 10^9$	4
CHO M_2	0.46 ± 0.01			3	$1.70 \pm 0.32 \times 10^9$	4
CHO M_3	0.06 ± 0.02			4	$8.11 \pm 1.53 \times 10^8$	4
CHO M_4	0.09 ± 0.01			4	$1.03 \pm 0.30 \times 10^9$	4
CHO M_5	0.04 (0.05, 0.02)			2	$9.09 \pm 4.79 \times 10^7$	5
Guinea pig urinary bladder		1.04 ± 0.57	0.09 ± 0.03	5	$1.08 \pm 0.28 \times 10^9$	5
Guinea pig gallbladder	0.23 ± 0.02			4	$1.22 \pm 0.06 \times 10^9$	3

[a] Dissociation rate constant of [^{3}H]NMS, determined by nonlinear regression analysis according to a monoexponential decay equation. Data represent the means ± S.E.M., except for the value of the CHO M_5 receptor, where the individual determinations are shown in the parentheses next to the mean value.

[b] Dissociation rate constants of [^{3}H]NMS, determined by nonlinear regression analysis according to a biexponential decay equation, with $k_{off\ fast}$ denoting the fast dissociation rate constant, and $k_{off\ slow}$ denoting the slower rate constant. Data represent the means ± S.E.M.

[c] Number of experiments.

[d] Association rate constant for [^{3}H]NMS, determined by nonlinear regression analysis. Data represent the means ± S.E.M.

with the value of k_{off} taken from the mean value of all individual dissociation experiments for that particular receptor subtype. In addition, kinetic data were also fitted to biexponential association and dissociation kinetic equations, and the better fit was determined using an extra sum of squares test.

In the functional experiments, concentration–response curves were normalized to the maximal carbachol response obtained in Normal PSS, and fitted via nonlinear regression to the Hill equation:

$$Y = \frac{E_{max}[A]^{n_H}}{[A]^{n_H} + EC_{50}^{n_H}}$$

where Y represents percent response, $[A]$ represents agonist concentration, E_{max} represents asymptotic maximal response, EC_{50} represents the concentration of antagonist producing half E_{max} and n_H represents the Hill slope.

For the antagonist inhibition curve experiments, the amplitude of tissue contractions in the presence of the antagonist was expressed as percentage of the corresponding control response in the absence of the antagonist. IC_{50} values (concentration of antagonist yielding half-maximal inhibition of control response) were calculated by nonlinear regression analysis according to the Hill equation, the latter parameter being formally identical to the EC_{50}. In all instances, EC_{50} and IC_{50} values were converted to negative logarithms (pEC_{50} and pIC_{50}) prior to the application of statistical testing. Differences between means were assessed by Student's *t*-test or one-way analysis of variance (ANOVA), as appropriate, and the level of significance was considered to be $P < 0.05$. Data are expressed as mean ± S.E.M.

2.4. Chemicals

[^{3}H]NMS (specific activity: 83 Ci/mmol) was purchased from Amersham (Amersham, UK). Methoctramine and tripitramine were generously provided by Prof. C. Melchiorre (Italy). DMEM was purchased from Gibco BRL (Gaithersburg, MD, USA), bovine calf serum from Hyclone (Logan, UT, USA), geneticin from Calbiochem (La Jolla, CA, USA) and all other drugs and reagents from Sigma (St. Louis, MO, USA).

3. Results

3.1. Radioligand kinetic experiments

In CHO cell membranes expressing the individual human muscarinic receptor subtypes, [^{3}H]NMS (0.5 nM) displayed reversible and saturable binding, allowing for the determination of the kinetic rate constants for the radioligand at each receptor subtype. As shown in Table 1, the radioligand exhibited approximately a 20-fold difference in the range of association rates (k_{on} values) at the various subtypes, the rank order being $M_1 \geq M_2 > M_4 > M_3 > M_5$. The dissociation rate constant (k_{off} value) for [^{3}H]NMS also showed a greater than 10-fold difference in range

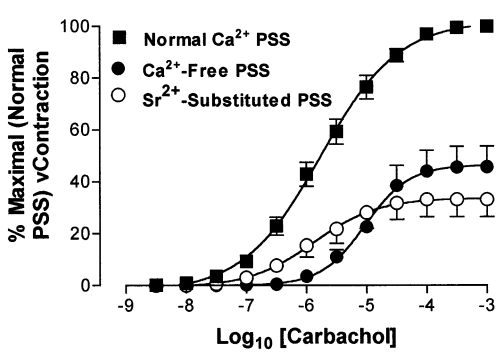

Fig. 1. Effect of calcium on carbachol-induced contraction of guinea pig gallbladder smooth muscle strips. The strips were incubated in normal calcium (2.5 mM), zero calcium or 5 mM strontium. Values are means ± S.E.M. of four to eleven experiments.

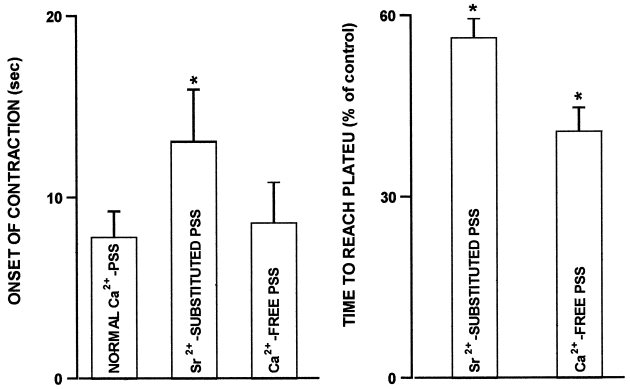

Fig. 2. The contractile effect of carbachol on guinea pig gallbladder in normal (2.5 mM) calcium containing, calcium-free and strontium-substituted saline solutions; the onset of contraction and time to reach plateau. Values are means ± S.E.M. of four to six experiments.

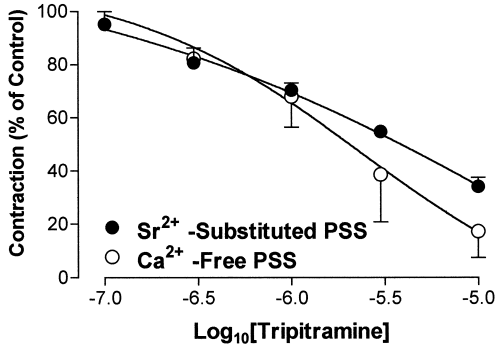

Fig. 4. Effect of increasing concentrations of tripitramine on 10^{-5} M carbachol-induced contraction of guinea pig gallbladder in Ca^{2+}-free and Sr^{2+}-substituted physiological salt solution. Values are means ± S.E.M. of four experiments.

across the subtypes, with a rank order of $M_2 > M_1 > M_4 \geq M_3 \geq M_5$.

Because kinetic experiments with [^{3}H]NMS potentially offer a greater sensitivity for the detection of subtype differences than equilibrium experiments, similar studies were undertaken in the guinea pig urinary bladder and gallbladder (Table 1). In the both tissues, the association kinetic data could not be statistically resolved into more than one phase. In contrast, [^{3}H]NMS displayed biphasic dissociation kinetics in the urinary bladder, but not the gallbladder. The values for all the rate constants determined from the curve fits to the data are shown in Table 1. When compared to the data obtained in the individual CHO cells, the kinetic profiles in either the urinary or gallbladders appear equivocal with regard to which muscarinic receptor subtype(s) involved, although it is possible that the slow rate of dissociation observed in the urinary bladder may be representative of an M_3 and/or M_4 profile. Irrespective, it is quite evident that the two bladder preparations display markedly different kinetic profiles, indicative of different muscarinic receptor subtypes.

3.2. Functional experiments

Carbachol contracted gallbladder smooth muscle strips in a concentration-dependent manner in either 2.5 mM Ca^{2+} containing PSS (Normal PSS), Ca^{2+}-free PSS or 5 mM Sr^{2+}-substituted PSS, indicating that both intra- and extra-cellular Ca^{2+} sources could be utilized for muscarinic receptor-mediated contractions (Fig. 1). Analysis of the data according to the Hill equation yielded the following parameter estimates: Normal PSS ($n = 11$), $pEC_{50} = 5.72 \pm 0.12$; $n_H = 0.79 \pm 0.04$; Ca^{2+}-free PSS ($n = 4$), $pEC_{50} = 5.05 \pm 0.08$; $n_H = 1.39 \pm 0.29$; 5 mM; Sr^{2+}-substituted PSS ($n = 6$), $pEC_{50} = 5.81 \pm 0.13$; $n_H = 0.89 \pm 0.07$. A one way ANOVA found that the curve obtained in the Ca^{2+}-free buffer demonstrated a significant difference ($P < 0.05$) in location and slope compared to the other two assay conditions. In addition, the amplitudes of the maximal contraction in both Ca^{2+}-free and 5 mM Sr^{2+}-substituted PSS were significantly lower ($P < 0.05$) than that observed using Normal PSS, being 45.8 ± 8.0% and 33.2 ± 6.6% of Normal PSS E_{max}, respectively (Fig. 1).

When carbachol was added to the medium at a single submaximal concentration (10^{-5} M), it caused a rapid contraction (onset of contraction = 7.8 ± 1.4 s), which

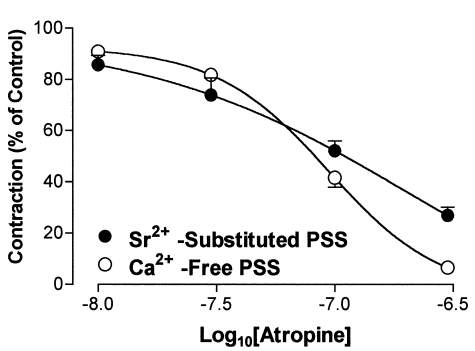

Fig. 3. Effect of increasing concentrations of atropine on 10^{-5} M carbachol-induced contraction of guinea pig gallbladder in Ca^{2+}-free and Sr^{2+}-substituted physiological salt solution. Values are means ± S.E.M. of eight experiments.

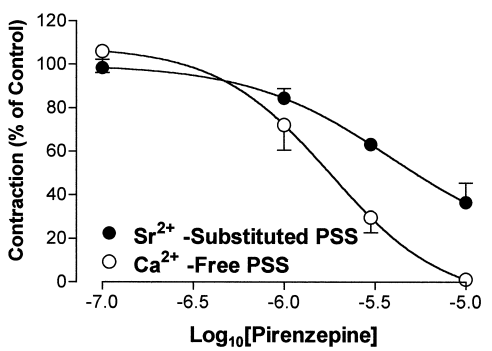

Fig. 5. Effect of increasing concentrations of pirenzepine on 10^{-5} M carbachol-induced contraction of guinea pig gallbladder in Ca^{2+}-free and Sr^{2+}-substituted physiological salt solution. Values are means ± S.E.M. of eight experiments.

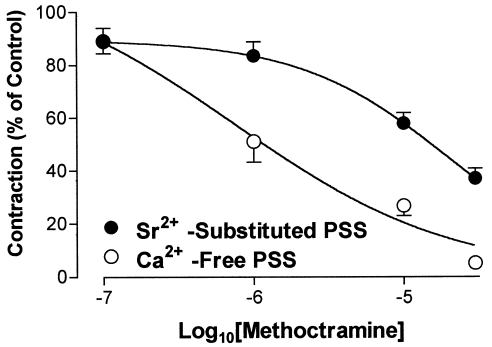

Fig. 6. Effect of increasing concentrations of methoctramine on 10^{-5} M carbachol-induced contraction of guinea pig gallbladder in Ca^{2+}-free and Sr^{2+}-substituted physiological salt solution. Values are means ± S.E.M. of six experiments.

reached its maximum in amplitude at a mean of 6.8 ± 0.4 min in Normal PSS. However, the contraction pattern changed when either intra- or extra-cellular Ca^{2+} was removed. The onset of contractile responses after the application of carbachol into the organ bath was significantly delayed in Sr^{2+}-substituted PSS (Fig. 2). Time to reach the plateau was significantly shorter in both Ca^{2+}-free and Sr^{2+}-substituted media (40.8 ± 3.9% and 56.3 ± 3.1% of Normal PSS, respectively).

In the antagonist experiments, atropine inhibited carbachol (10^{-5} M)-induced contractions in a concentration-dependent manner (Fig. 3). Antagonist pIC_{50} values in 5 mM Sr^{2+}-substituted PSS (where intracellular Ca^{2+} concentration was considered to be zero) and in Ca^{2+}-free PSS (where extracellular Ca^{2+} concentration was zero) were not significantly different from one another, being 6.88 ± 0.25 and 6.85 ± 0.11, respectively. Similarly, the antagonistic potency of tripitramine in the absence of intracellular or extracellular Ca^{2+} was not significantly different in either instance, with pIC_{50} values of 5.75 ± 0.32 and 5.70 ± 0.16, respectively (Fig. 4).

When pirenzepine was utilized as the antagonist, it displayed a slightly higher potency in antagonizing carbachol-induced contractions in Ca^{2+}-free PSS (pIC_{50} = 5.66 ± 0.23) than in Sr^{2+}-substituted PSS (pIC_{50} = 5.33 ± 0.21) (Fig. 5), yet this difference did not reach statistical significance. In contrast, methoctramine exerted only a slight inhibition on carbachol-induced contraction in Sr^{2+}-substituted PSS (pIC_{50} = 4.83 ± 0.30) whereas it was significantly more potent in Ca^{2+}-free PSS (pIC_{50} = 5.99 ± 0.19) (Fig. 6).

4. Discussion

The present study was designed to gain further insight into the muscarinic receptor profile of the guinea pig gallbladder smooth muscle, as previous studies have suggested a different mix of receptors in this preparation relative to other smooth muscle preparations. Radioligand kinetic studies with [^{3}H]NMS indicated that guinea pig gallbladder smooth muscle displays a distinctly different kinetic profile to urinary bladder smooth muscle from the same species. In particular, dissociation kinetic experiments identified both slow and fast components of binding in the latter preparation, but not the former (Table 1). Experiments conducted on the individual cloned muscarinic receptors in CHO cells indicated that fast [^{3}H]NMS dissociation may be expected from the muscarinic M_1 and, particularly, the muscarinic M_2 receptor, whereas slow dissociation would be expected from the muscarinic M_3, M_4 and M_5 receptors. This is in agreement with previous studies (Michel et al., 1989; Flynn and Mash, 1993; Flynn et al., 1997). Thus, the biphasic radioligand kinetics observed in the urinary bladder may be reconciled with previous observations indicating a mix of muscarinic M_2 and M_3 receptors in this tissue (Wang et al., 1995). In contrast, the monophasic dissociation kinetics in the gallbladder are at odds with an expectation of mixtures of receptors displaying both fast and slow kinetics. However, it should be noted that CHO muscarinic M_3 and M_4 receptors demonstrated markedly similar [^{3}H]NMS dissociation characteristics, and it would be expected that a tissue containing a heterogeneous mix of these two subtypes would still display an apparent monophasic dissociation. Based on theoretical simulations using the CHO cell data (not shown), the dissociation kinetic data in the gallbladder can be adequately explained assuming the presence of either (i) a single receptor subtype, (ii) a mixture of subtypes displaying similar kinetic profiles or (iii) a mixture of subtypes displaying different kinetic profiles, but with one subtype being the predominant form (approximately 80% abundance). The first possibility may be readily excluded given the ample evidence based on binding, molecular biological and functional experiments indicating a mix of receptor subtypes in guinea pig gallbladder (Von Schrenck et al., 1994; Oktay et al., 1998; Parkman et al., 1999). The discrimination between the latter two possibilities is, unfortunately, more complicated. If a mixture of subtypes displaying similar [^{3}H]NMS kinetic characteristics were involved, then the dissociation profile would have been expected to be quite fast if the mix was M_1 and M_2, and quite slow if the mix was M_3 and M_4. The observed dissociation rate constant was intermediate between that expected for this possibility, but is compatible with a mix of receptors displaying both fast and slow kinetic characteristics, with the former predominating such that the computerized nonlinear regression algorithm could not differentiate between more than one phase of dissociation. Interestingly, in no instance did the association kinetic profile detect more than one phase of binding (Table 1), but this was also in accordance with our simulations that assumed a predominant abundance of one subtype over all others (not shown). A point of disparity between the smooth muscle and CHO cell dissociation data is the actual difference in magnitude between the observed rate

constants. One possible explanation may be due to the species differences between guinea pig and human receptors. Another may be due to differences imposed upon the receptor protein by the membrane environment. In any case, based on our present data it may be concluded that the guinea pig gallbladder smooth muscle contains a different composition of muscarinic receptors to that found in the urinary bladder.

In functional studies, cell transfection experiments have demonstrated that individual muscarinic receptor subtypes can regulate multiple effector pathways (for recent reviews, see Felder, 1995; Eglen et al., 1996), and that it is not necessary to invoke distinct receptor subtypes mediating response. For example, Ashkenazi et al., (1987) demonstrated that treatment of muscarinic M_2 receptors transfected CHO cells with carbachol induced the formation of inositol phosphates in addition to inhibition of cAMP accumulation. The cloned rat muscarinic M_1 receptor gene expressed in the rat embryonic fibroblast (RAT-1) cell line was shown to be coupled to the stimulation of phosphoinositide turnover as well as to the inhibition of adenylate cyclase (Stein et al., 1988). Peralta et al. (1988) suggested that muscarinic M_2 and M_4 receptors, which inhibit adenylate cyclase activity efficiently, could also lead to phosphoinositide hydrolysis, albeit poorly. Even in native tissues, such as the rat parotid gland, both second messenger systems have been shown to be linked to a single (M_3) receptor subtype (Dai et al., 1991). However, differential regulation of phosphoinositide hydrolysis and adenylate cyclase activity by separate muscarinic receptors has also been demonstrated in tissues such as the rat brain (Gil and Wolfe, 1985), and embryonic chick heart cells (Brown and Brown, 1984). Further, in the canine colonic smooth muscle (Zhang and Buxton, 1991) and guinea pig myometrium (Leiber et al., 1990), phosphoinositide hydrolysis is mediated by muscarinic M_3 receptors while cAMP inhibition is linked to muscarinic M_2 receptors. Thus, ample evidence also exists for different subtypes mediating different signal transduction pathways in the same tissue.

Previous functional studies have indicated that the modification of either intracellular or extracellular Ca^{2+} could also be utilized to differentiate the contribution of distinct muscarinic receptor subtypes to the contractile process (Chen et al., 1995). The use of Ca^{2+}-free PSS eliminates the contribution of any influx of extracellular Ca^{2+}. Sr^{2+}-substituted PSS was used because Sr^{2+} can replace the role of intracellular Ca^{2+} (Baba et al., 1985; Lee et al., 1989), by being incorporated into the endoplasmic reticulum (Somlyo and Somlyo, 1971), displacing Ca^{2+} from high-affinity binding sites (Yasuda and Sakai, 1984), but cannot be easily released (Brown and Birnbaumer, 1988). In the present study, when either of the Ca^{2+} stores was removed, the amplitude of the contractile response to carbachol was significantly reduced (Fig. 1), and the time to reach a steady-state plateau response was significantly shortened (Fig. 2). Moreover, a marked delay was observed in the onset of contraction after the application of carbachol into the Sr^{2+}-substituted medium. These findings, in accordance with previous reports (Renzetti et al., 1990; Chen et al., 1995), clearly indicate that muscarinic receptor activation can mobilize either intracellular or extracellular Ca^{2+} stores to yield a contraction. However, the reduction of the maximal contractile response to carbachol in either Ca^{2+}-free or Sr^{2+}-substituted PSS indicates that both Ca^{2+} sources are needed for guinea pig gallbladder to give an appropriate contractile response to an acetylcholine receptor agonist. This latter finding differs from that observed in cat gallbladder by Chen et al., (1995), whereby the potency and intrinsic activity of acetylcholine was unaltered under any of the experimental assay conditions, and may be indicative of species differences between the two tissues.

A further examination of the concentration–response relationship to carbachol in the different buffers also suggests distinct modes of coupling to Ca^{2+} sources that mediate the final observed contraction. Although the reduced maximal contraction to the agonist in either Ca^{2+}-free or Sr^{2+}-substituted PSS may simply be due to limited Ca^{2+} availability, this would not explain the significant change in agonist curve shape and location observed for carbachol in the Ca^{2+}-free PSS (Fig. 1). Specifically, agonist potency was reduced whereas the sensitivity of the system to concentration increments (as measured by the Hill slope) was significantly increased. Changes in curve location and slope obtained in the same tissue to the same agonist under different conditions are indicative of a different mode of stimulus–response coupling. Thus, the removal of extracellular Ca^{2+} modifies the ability of carbachol to mediate a contractile response in a manner that goes beyond simply limiting the amount of Ca^{2+} available. The observed changes in stimulus–response coupling may therefore be indicative of a differential contribution of multiple receptor subtypes to the same, final response, and may be presumptive evidence of such a mechanism operating in guinea pig gallbladder.

In accord with the suggestion of multiple muscarinic receptor subtypes mediating the same response in this tissue, previous studies have identified M_3 (Von Schrenck et al., 1993, 1994; Takahashi et al., 1994; Eltze et al., 1997) and M_4 receptors (Özkutlu et al., 1993; Oktay et al., 1998; Karaalp et al., 1999) as playing a role in the contraction of guinea pig gallbladder smooth muscle cells. Barocelli et al. (1994) have also concluded that gallbladder muscarinic receptors may be distinct from ileal smooth muscle receptors. Recently, Parkman et al. (1999) have claimed that prejunctional facilitatory M_1, prejunctional inhibitory M_2 and postjunctional M_3 receptors mediate contractions in this tissue. It has also been reported that carbachol-induced inhibition in adenylate cyclase activity, but not phosphoinositide hydrolysis, was pertussis toxin sensitive in guinea pig gallbladder (Takahashi et al., 1994), indicating the presence of more than one subtype of mus-

carinic receptor. The presence of both muscarinic M_2 and M_4 receptors, the former being predominant (approximately 80%), has also been demonstrated via Western blot analysis (Oktay et al., 1998).

As a consequence of the above findings, further functional studies were undertaken in the present study by exploiting the availability of relatively subtype-selective muscarinic receptor antagonists. Unfortunately, no antagonist is currently available that can effectively discriminate a single muscarinic receptor subtype to the exclusion of all others (Caufield and Birdsall, 1998), but a comparison of the potencies of a group of antagonists may be used to determine the possible contributions of different subtypes. Given that a contractile response is observed in this tissue when either intracellular or extracellular Ca^{2+} stores are depleted, and given the aforementioned evidence indicating a mixture of receptor subtypes in gallbladder smooth muscle, it is reasonable to assume that the different functional subtypes present in this tissue should be preferentially linked to either one or the other sources of Ca^{2+}. Chen et al. (1995) have indeed shown this to be the case for muscarinic M_2 and M_3 receptors in cat gallbladder. However, it cannot be assumed that this is the same receptor profile responsible for the response in guinea pig gallbladder, and the data obtained with the antagonists used in our study support this conclusion.

As expected, the nonselective muscarinic antagonist atropine was equipotent in inhibiting carbachol (10^{-5} M)-induced contractions in 5 mM Sr^{2+}-substituted PSS, where intracellular Ca^{2+} concentration was considered to be zero, and in Ca^{2+}-free PSS where extracellular Ca^{2+} concentration was zero (Fig. 3). Thus, beyond demonstrating the muscarinic specificity of the contractile response, no further subtype discrimination was possible with this antagonist. However, the remaining antagonists exhibit different selectivity profiles (Caufield and Birdsall, 1998). For instance, pirenzepine has a similar, high affinity for muscarinic M_1 and M_4 receptors, but an approximately 10-fold lower affinity for muscarinic M_2 and M_3 receptors. Methoctramine displays a greater than 10-fold higher affinity for muscarinic M_2 and M_4 receptors over muscarinic M_3 receptors, whereas tripitramine displays a 100-fold selectivity for M_2 over M_3, 30-fold selectivity for M_4 over M_2, but less than 10-fold selectivity for M_4 over M_3 (Caufield and Birdsall, 1998). Given these affinity differences, and assuming that the muscarinic receptor subtypes in the gallbladder are preferentially linked to different sources of Ca^{2+}, the following conclusions may be drawn: (i) If the functional receptor mix was predominantly M_2 and M_3, then both methoctramine and tripitramine would have been expected to have demonstrated significant potency differences in their ability to inhibit carbachol-mediated contraction between the two buffers. This was not the case, however, as only the former antagonist displayed a significantly higher potency in Ca^{2+}-free PSS relative to Sr^{2+}-substituted PSS (Fig. 6). (ii) If the mix were predominantly M_2 and M_4, then tripitramine would have been expected to differentiate between the two under the different assay conditions. Again, this was not observed (Fig. 4). (iii) If the functional mix were predominantly M_3 and M_4, then methoctramine, but not tripitramine, would be expected to differentiate between the two subtypes. This was, indeed, observed in our study (Figs. 4 and 6). It may be argued that pirenzepine should also be able to distinguish between muscarinic M_3 and M_4 receptors, given its 10-fold higher affinity for the latter, relative to the former. However, this difference may be insufficient to ensure a statistically relevant discrimination amongst subtypes, although a slightly higher potency for this antagonist was, in fact, noted in the Ca^{2+}-free buffer (Fig. 5).

Taken together, our results therefore provide functional evidence for the coexistence of functional muscarinic M_3 and M_4 receptor subtypes mediating Ca^{2+} mobilization in guinea pig gallbladder smooth muscle, as well as the presence of the muscarinic M_2 receptor subtype. It remains to be seen whether muscarinic M_3 and M_4 receptors are also responsible for coincident activation of other signal transduction cascades, such as the phosphoinositide or cAMP response, and whether both effectors play a role in the observed gallbladder muscle contraction.

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