Muscarinic Receptors Controlling the Carbachol-Activated Nonselective Cationic Current in Guinea Pig Gastric Smooth Muscle Cells

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Received August 17, 1999 Accepted January 11, 2000

ABSTRACT—Muscarinic receptor subtypes controlling the nonselective cationic current in response to carbachol (ICCH) were studied in circular smooth muscle cells of the guinea pig gastric antrum using putative muscarinic agonists and antagonists. Both oxotremorine-M (an M₂-selective agonist) and CCh dose-dependently activated the cationic current with EC₅₀ values of 0.21 ± 0.01 µM and 0.97 ± 0.06 µM, respectively. In contrast, pilocarpine and McN-A 343 (an M₁-selective and a putative M₄ agonist) were weak partial agonists. In response to 10 µM CCh, 4-DAMP, methoctramine and pirenzepine dose-dependently inhibited ICCH and had IC₅₀ values of 1.91 ± 0.2 Nm, 0.46 ± 0.07 µM and 8.33 ± 0.4 µM, respectively. 4-DAMP, methoctramine and pirenzepine shifted the concentration-response curves of ICCH to the right without significantly reducing the maximal current. Values of the apparent dissociation constant pA₂ obtained from Schild plot analysis were 9.24, 7.72 and 6.62 for 4-DAMP, methoctramine and pirenzepine, respectively. Also, pertussis toxin completely blocked ICCH generation. These results suggest that the M₂-subtype plays a crucial role in the activation of the ICCH, and a block of the M₂-subtype reduces the sensitivity of the M₂-mediated response with no significant reduction of maximum response.

Keywords: Muscarinic receptor subtype, Nonselective cationic current, Carbachol, Smooth muscle

In the gastrointestinal (GI) tract, acetylcholine is the major excitatory neurotransmitter mediating smooth muscle contraction. Radioligand binding studies have shown that the M₂ subtype is the predominant population of muscarinic receptors (approx. 80%) expressed in GI smooth muscle cells (SMCs) (1, 2). Although functional studies have shown that the contractile response is mainly mediated by the minor population of M₁ subtype (approx. 20%), the role of the major M₂ subtype in excitation-contraction coupling in GI smooth muscle is generally believed to be indirect (3). It has been well described that the stimulation of muscarinic receptors depolarizes membrane potential via opening of nonselective cation channels (4, 5), and recent pharmacological experiments demonstrated that the opening of cation channels is mainly mediated by the M₂ subtype in tracheal (6) and intestinal SMCs (7, 8).

As in intestinal smooth muscle (4, 5), stimulation of the muscarinic receptors by carbachol (CCh) activates nonselective cationic current (ICCH) in guinea pig gastric SMCs (9). Although biophysical and signal transducing properties of the ICCH in guinea pig gastric SMCs have been described (9–12), the specific muscarinic receptor subtypes mediating ICCH are still not determined. Therefore, in the present study, we aim to determine the muscarinic subtypes mediating ICCH in circular SMCs of guinea pig gastric antrum by evaluating pharmacological affinity profiles of both agonists and antagonists for the muscarinic receptors. Parts of our results were previously reported (13).

MATERIALS AND METHODS

Single cell isolation and whole-cell patch clamp

Fresh, single SMCs were isolated enzymatically from the antral circular layer of guinea pig stomach as previously described (11). Isolated SMCs were stored at 4°C until use, and all experiments were performed within 10 h after cell dispersion. An aliquot of single smooth muscle cells in suspension was added to the recording chamber (0.3 ml) mounted on an inverted microscope (Diaphot 300; Nikon, Tokyo). Solutions were perfused through the chamber by
gravity at a rate of approx. 2 ml/min and the experiments were performed at room temperature (approx. 25°C). Whole-cell currents were measured using the standard patch clamp technique. Patch pipettes (free-tip resistance of 2–4 MΩ) were connected to the head stage of a patch-clamp amplifier (Axopatch-1D; Axon Instruments, USA). Before starting each experiment, liquid junction potentials were corrected with an offset circuit. Whole-cell currents were filtered at 10 kHz (−3 dB frequency), and the data were displayed on a digital oscilloscope (PM 3350A; Philips, Netherlands), a computer monitor, and a pen recorder (RS 3200; Gould, USA). For later analysis, data were saved in a digital tape recorder (DTR-1204; BioLogic, France). All data analysis was performed with pCLAMP software 6.0.2 (Axon Instruments) and Origin 5.0 (Microcal Software Inc., USA). The cells had an average capacitance of 50 pF as reported previously (14). The series resistance (10–15 MΩ) compensation was not introduced.

Nonselective cationic currents in whole-cell voltage-clamp mode were measured in CsCl-rich external solution containing 135 mM CsCl, 10 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.35 with CsOH). The internal pipette solution contained: 135 mM CsCl, 3 mM Mg-ATP, 5 mM di-tris-creatine phosphate, 0.5 mM tris-GTP, 2 mM EGTA and 10 mM HEPES (pH 7.2 with CsOH). The use of Cs⁺-rich external and bating solutions result in a larger than normal cationic current that improves the signal to noise ratio of the recording (11). All cells presented in this paper were voltage-clamped at −60 mV, unless otherwise stated.

Analysis and statistics

Although the inclusion of 2 mM EGTA in the pipet solution markedly prevents desensitization of I_{CCS} (10), it is still insufficient to give full agonist concentration-response curves, especially in the presence of several antagonist concentrations. Thus, the consecutive concentration-response curves of I_{CCS} were selected only from the cells that are slightly desensitized during the full-sessions of experiments. Log EC₅₀ values were determined by fitting lines of the following logistic sigmoid function of the log concentration-response curves: I / I_{max} = [1 + ([EC₅₀] / [A])⁵⁻¹, where I is the cationic current activated at a given CCh concentration and I_{max} is the cationic current at a maximal CCh concentration (50 µM). EC₅₀ is the agonist concentration ([A]) when I was 50% of I_{max} and h is the slope factor of the agonist curve. Schild analysis (15) was performed by calculating dose ratios (DR) at equieffective concentrations of CCh (EC₅₀ concentration) for each concentration of antagonist and plotting log (DR − 1) vs − log [B], where DR = [A₂] / [A₁], [A₁] = EC₅₀ concentration of CCh in the absence of antagonist, [A₂] = EC₅₀ concentration of CCh in the presence of antagonist, and [B] = concentration of antagonist. The DR for each antagonist concentration was derived from the concentration-response curves. Linear regression analysis of the Schild plots was performed to estimate the slopes and intercepts. The pA₂ value, defined as the negative logarithm of the antagonist concentration that produces a two-fold rightward shift in the concentration-response curve, was extrapolated from the regression equation as the x-intercept.

The data are presented as the mean ± S.E.M. with n, the sample size. Significant differences were detected by Student’s unpaired t-test (P<0.05).

Chemicals

The following compounds were used: 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), pirenzepine hydrochloride, methoctramine hydrochloride, 4-(3-chlorophenylcarbamoyloxy)2-butylnitrimethylammonium chloride (MnCNA 343) and pilocarpine hydrochloride, all purchased from Research Biochemical International (Natick, MA, USA). Oxotremorine-M was purchased from Tocris (Bristol, UK). Carbachylcholine chloride (CCh), pertussis toxin (PTX) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

I_{CCS} were recorded in the cells voltage-clamped at −60 mV. The maximum currents were obtained with approx. 50 µM CCh, and the EC₅₀ was 0.97 ± 0.06 µM (n = 15). At a holding potential of −60 mV, concentration-response curves of each muscarinic agonist were compared to that of I_{CCS} (Fig. 1). Oxotremorine-M (an M₂-selective agonist) dose-dependently activated the cationic current and had an EC₅₀ of 0.21 ± 0.01 µM (n = 6). In contrast, pilocarpine (an M₁ agonist) and MnCNA 343 (an M₁-selective and a putative M₄ agonist) (16) showed weak partial agonistic effects. After reaching their maximal responses, additional CCh (50 µM) further elicited cationic current (Fig. 1A). The maximum currents in response to pilocarpine (500 µM) and MnCNA 343 (800 µM) were 18.7 ± 1.5% (n = 5, P<0.01) and 4.0 ± 0.6% (n = 4, P<0.01) of the I_{CCS} at 50 µM, respectively.

The voltage dependence of the cationic current varies with CCh concentration (17) and could affect the potency of antagonists for inhibition of I_{CCS} (8). Therefore, we compared inhibitory effects of antagonists on I_{CCS} in response to 10 µM CCh. The mean current amplitude in response to 10 µM CCh was −620 ± 30 pA at −60 mV (n = 52). 4-DAMP, methoctramine and pirenzepine each inhibited the I_{CCS} in a dose-dependent manner. IC₅₀ values obtained from the dose-response curves were 1.91 ± 0.2 nM, 0.46 ± 0.07 µM and 8.33 ± 0.4 µM for 4-DAMP (n = 6), methoctramine (n = 5) and pirenzepine (n = 4),
results respectively (Fig. 2). The slope factors \((h)\) of the curves were not different among groups \((P>0.05)\), and had \(2.2 \pm 0.16, 2.1 \pm 0.4\) and \(2.4 \pm 0.12\) for 4-DAMP, methoctramine and pirenzepine, respectively.

Concentration-response curve of \(I_{\text{CCb}}\) was obtained by the application of the ascending concentrations of CCh, and the antagonism by 4-DAMP, methoctramine and pirenzepine were observed. Figure 3 shows examples of the effects of antagonists on \(I_{\text{CCb}}\). All the antagonists shifted the concentration-response curve of \(I_{\text{CCb}}\) to the right without significant reduction in the maximal responses. The \(EC_{50}\) changed from \(1.2 \pm 0.1 \mu M\) to \(4.0 \pm 0.2, 27.2 \pm 1.0\) and \(84.9 \pm 2.8 \mu M\), in the presence of 1, 4 and 10 nM 4-DAMP, respectively (Fig. 4A). In the case of methoctramine, the \(EC_{50}\) increased from \(1.0 \pm 0.1 \mu M\) to \(6.2 \pm 0.2, 18.6 \pm 1.3\) and \(45.7 \pm 4.4 \mu M\) with 0.1, 0.5 and 1 \(\mu M\), respectively (Fig. 4B). \(EC_{50}\) shifted from \(1.0 \pm 0.1 \mu M\) to \(4.9 \pm 0.2, 15.5 \pm 1.4\) and \(47.9 \pm 2.3 \mu M\) by 1, 5 and 10 \(\mu M\) pirenzepine, respectively (Fig. 4C). When a Schild plot was constructed, the slope was not significantly different from unity and when constrained to unity gave \(pA_2\) values of \(9.24, 7.72\) and \(6.62\) for 4-DAMP, methoctramine and pirenzepine, respectively (Fig. 5). \(pA_2\) values obtained from each antagonist were summarized and compared with those described in other preparations (2, 7) (Table 1).

In SMCs incubated with PTX (5 \(\mu g/ml\), 3 h at 37°C), no discernible cationic current was activated by CCh. \(I_{\text{CCb}}\) values in response to 10 \(\mu M\) CCh at a holding potential of \(-60\) mV were \(-330 \pm 32\) pA \((n=4)\) and \(-15 \pm 3.0\) pA \((n=6)\) in the control and PTX-treated cells, respectively \((P<0.01)\) (data not shown).
DISCUSSION

We used a number of putative agonists and antagonists for muscarinic receptors to determine the subtype mediating $I_{CCh}$ in guinea pig gastric antral SMCs. From the affinity profiles of the receptors and PTX sensitivity, mediation of $I_{CCh}$ by the M$_2$-subtype was suggested. Furthermore, it was deduced that the block of M$_2$-subtype reduces the sensitivity of the M$_2$-mediated channel opening with no significant reduction of maximum response.

For the comparison of potency of muscarinic agonists, we constructed the concentration-response curves of each agonist. The potency of oxotremorine-M (an M$_2$-selective agonist; EC$_{50}=0.21$ $\mu$M) in the activation of the $I_{CCh}$ was about five times higher than that of CCh (a non-specific agonist; EC$_{50}=0.97$ $\mu$M). On the other hand, putative M$_2$- and M$_1$-selective agonists (McN-A 343 and pilocarpine) were much less potent than CCh and act as weak partial agonists. Therefore, these results may suggest the M$_2$-subtype mediation of the cation channel opening.

We compared the inhibitory effects of antagonists on $I_{CCh}$ in response to 10 $\mu$M CCh. The order of potency was 4-DAMP (IC$_{50}=1.91$ nM) $>$ methoctramine (IC$_{50}=0.46$ $\mu$M) $>$pirenzepine (IC$_{50}=8.33$ $\mu$M). The obtained pA$_2$ value of methoctramine (7.72), a putative M$_2$-selective antagonist, is similar not only to the M$_2$-mediated $I_{CCh}$ in guinea pig ileum (pA$_2=8.11$) (7) but also to the M$_2$ in other tissues (pA$_2=7.8-8.3$) (2) (see Table 1). The obtained pA$_2$ value of pirenzepine (6.62), a putative M$_1$-selective antagonist, is within the range of those found in the M$_2$ (pA$_2=6.7$) and the M$_3$ (pA$_2=6.7-7.1$) subtypes, and it is far different from those of the M$_1$ (pA$_2=8.1-8.5$) and the M$_4$ (pA$_2=7.7-8.1$) subtypes (2). Thus, all these pharmacological profiles suggest that the M$_2$ subtype, rather than M$_1$ and M$_4$, mediates $I_{CCh}$ in guinea pig gastric SMCs.

As in the previous reports on guinea pig small intestine (5, 18), $I_{CCh}$ was completely blocked by PTX in our preparations. In guinea pig ileum, PTX blocks M$_2$-mediated muscle contraction and cAMP generation with no interference with M$_3$-mediated contraction and phosphoinositide
Fig. 3. Current traces depicting antagonism of $I_{\text{CC}}$ by the muscarinic antagonists. A series of ascending concentrations of CCh was applied onto the cells after they were held at $-60\,\text{mV}$. 4-DAMP (A), methoctramine (B) and pirenzepine (C) competitively inhibited the CCh responses without significantly reducing the maximal response. In the case of 4-DAMP, five series of current traces are drawn. Vertical deflections represent ramp applied to determine current-voltage relationship.

Fig. 4. Effects of the muscarinic antagonists on the concentration-response curves for CCh. A: 4-DAMP antagonisms on the $I_{\text{CC}}$: control (○) and 1 (△), 4 (□) and 10 nM (●) 4-DAMP (n = 4). B: Methoctramine antagonisms on the $I_{\text{CC}}$: control (○) and 0.1 (△), 0.5 (□) and 1.0 μM (●) methoctramine (n = 4). C: Pirenzepine antagonisms on the $I_{\text{CC}}$: control (○) and 1 (△), 5 (△) and 10 μM (●) pirenzepine (n = 4). $I_{\text{CC}}$ in response to 50 μM CCh was obtained before the start of the experiments and was taken as 100% response.
hydrolysis (19). Wang et al. (6) proved that the activation of M_2 receptors leads to the opening of nonselective cation channels through G_4/G_5 proteins in tracheal SMCs. In guinea pig gastric antral SMCs (20), antibodies against the alpha-subunit of G_4 protein suppress I_{CCH} generation, suggesting that the I_{CCH} is mediated via a M_2 subtype—PTX-sensitive G proteins cascade.

In our experiments, although the pharmacological profiles of oxtremorine-M, methoctramine, pirenzepine and PTX-sensitivity strongly prove M_2-subtype mediation of the I_{CCH}, the involvement of M_3-subtype should be taken into account. 4-DAMP showed a high affinity (pA_2 = 9.24) that was approximately ten times higher than that of the M_2 subtype (pA_2 = 8.0–8.4) but similar to that of the M_3 subtype (pA_2 = 8.9–9.3) (2). Because 4-DAMP shifted dose-response curves of the I_{CCH} to the right with no great reduction of maximal response, this may indicate that M_2-mediated function may become less sensitive to CCh when M_3 receptors are blocked. If M_3 receptor generates a potentiating signal that affects the M_2 signaling, selective blockade of M_3 receptor by 4-DAMP might blunt the channel opening processes. This effect may result in the right shift of the dose-response curves without reduction of maximal response as we observed (Fig. 4A). Therefore, these findings support the idea that M_2 receptors are functionally associated with the M_3 receptors in SMCs as previously suggested (6, 7).

In tracheal SMCs, Wang et al. (6) suggested that the I_{CCH} be mediated by both M_2 and M_3 receptors. They showed that the stimulation of M_2 receptors alone is not sufficient to open nonselective cation channels, and a rise in [Ca^{2+}]_i associated with the stimulation of M_3 receptors is necessary for the activation of I_{CCH}. In guinea pig ileal myocytes (7), M_3 antagonists (himbacine, triptiaramine and methoctramine) competitively inhibited I_{CCH} with affinities indicating typical antagonism at the M_3 receptor. On the other hand, a selective block of the M_3 receptors by zamifenacain, 4-DAMP and p-F-HHSD strongly reduced the maximum cationic current with no change in the EC_{50}, thus suggesting that M_3 receptor activation exerts a potent modulatory effect on channel opening in an entirely unexpected way. This depression of cationic current by M_3-receptor antagonists is brought about by an action at an earlier point in the transduction process but not by direct channel block, since the GTPγS-induced current was not depressed by M_3 antagonists (7). As in ileal SMCs, a high concentration of 4-DAMP (>50 nM) did not suppress the GTPγS-induced current in our preparation (data not shown). In contrast to the ileal SMCs (7), our result shows that a low concentration of 4-DAMP (1–10 nM) increases the EC_{50}, while it does not reduce the maximal current. These differences might be due to the use of a different concentration of 4-DAMP (1–10 nM vs 50 nM). In spite of this possibility, a more plausible explanation is the involvement of cytoplasmic Ca^{2+}, which is well known to potentiate I_{CCH} (11, 21). We used a pipette solution containing 2 mM EGTA with no Ca^{2+} added, while Zholos and Bolton (7) used a pipette solution in which [Ca^{2+}]_i was clamped to approx. 100 nM by 10 mM BAPTA. Furthermore, we recorded I_{CCH} in 2 mM Ca^{2+}-containing Cs^{+}-rich bathing solution, while they used a Ca^{2+}-free bath solution. We have previously shown that CCh produces the sustained [Ca^{2+}]_i increase under our

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<td>Pirenzepine</td>
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<td>Methoctramine</td>
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The pA_2 values obtained from the present study were compared with those values reported by Eglen et al. (2).
experimental conditions (10). Therefore, it can be postulated that depending on the [Ca$^{2+}$]$_i$ level, the selective block of M$_1$ receptor affects the M$_2$-activated $I_{Ca,ao}$ in a different way. The precise functional relationships between M$_2$ and M$_1$ receptors remain to be elucidated, and the species and/or tissue-specific differences should be tested in the further studies.

Acknowledgements

This study was supported by grants from the Samsung Biomedical Research Institute (C-95-002-3 and C-95-005), a grant from the Seoul National University Hospital Research Fund (no. 02-1996-349-0) and a grant awarded to TM Kang from Sungkyunkwan University School of Medicine, Korea.

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