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Different sensitivities to agonist of muscarinic acetylcholine receptor subtypes

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Muscarinic acetylcholine receptor (mAChR) III expressed in *Xenopus* oocytes, like mAChR I, mediates activation of a Ca^{2+}-dependent Cl^- current, whereas mAChR IV, like mAChR II, principally induces activation of Na^+ and K^+ currents in a Ca^{2+}-independent manner. mAChR III has a sensitivity to agonist of about one order of magnitude higher than that of mAChR I in mediating the Ca^{2+}-dependent current response in *Xenopus* oocytes and in stimulating phosphoinositide hydrolysis in NG108-15 neuroblastoma-glioma hybrid cells. The agonist-binding affinity of mAChR III is also about one order of magnitude higher than that of mAChR I.

Muscarinic acetylcholine receptor subtype; Ca^{2+}-dependent Cl^- current; Na^+ current; K^+ current; Phosphoinositide hydrolysis; Sensitivity to agonist; Agonist-binding affinity

1. INTRODUCTION

An effective approach to studying the functional heterogeneity of the muscarinic acetylcholine receptor (mAChR) has been provided by expression of cloned DNAs encoding molecularly distinct mAChR subtypes. The antagonist-binding properties of four individual mAChR subtypes expressed in *Xenopus* oocytes [1–3] indicate that mAChR I, mAChR II and mAChR III correspond most closely to the pharmacologically defined M_1 (I), M_2 cardiac (II) and M_2 glandular (III) subtypes [4–6], respectively. Hybridization analysis has shown that the mRNAs encoding the four mAChR subtypes are differentially distributed in tissues, that is, the mAChR II mRNA in heart [7–9], both mAChR II and mAChR III mRNAs in smooth muscles [9], both mAChR I and mAChR III mRNAs in exocrine glands [9] and all four mAChR mRNAs in cerebrum [1,7–10]. Thus, the mAChR heterogeneity in tissues with respect to antagonist binding can be accounted for by the presence of individual molecularly distinct mAChR subtypes or various combinations of them. Furthermore, DNA expression studies with *Xenopus* oocytes and mammalian cells have revealed that mAChR subtypes are selectively coupled with different effector systems, albeit not exclusively. Initially, evidence has been obtained to indicate that mAChR I expressed in *Xenopus* oocytes mediates activation of a Ca^{2+}-dependent Cl^- current, whereas mAChR II principally induces activation of Na^+ and K^+ currents in a Ca^{2+}-independent manner [1,2]. Expression studies in mammalian cells have further shown that mAChR I and mAChR III are coupled efficiently with phosphoinositide hydrolysis [11–13], intracellular Ca^{2+} release [14], activation of Ca^{2+}-dependent K^+ currents [11,14,15] and inhibition of the M current [11], whereas mAChR II and mAChR IV are linked preferentially with adenylate cyclase inhibition [12,16].

In the present investigation, we have examined

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Abbreviations: mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate

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the functional properties of mAChR III and mAChR IV expressed in *Xenopus* oocytes. The results obtained show that mAChR III and mAChR IV mediate current responses similar to those induced by mAChR I and mAChR II, respectively. Furthermore, expression studies in *Xenopus* oocytes and in NG108-15 neuroblastoma-glioma hybrid cells reveal that the sensitivity to and the binding affinity for agonist of mAChR III are about one order of magnitude higher than those of mAChR I.

2. MATERIALS AND METHODS

mRNAs specific for porcine mAChR I, porcine mAChR III, rat mAChR III and rat mAChR IV were synthesized by transcription in vitro of the respective cloned DNAs as in [2,3]. Each of the mAChR subtype-specific mRNAs was injected into *Xenopus laevis* oocytes; the mRNA concentration used was 1 μg/μl for the mAChR I- and mAChR IV-specific mRNAs or 1 ng/μl for the mAChR III-specific mRNAs, unless otherwise indicated, and the average volume injected per oocyte was ~40 nl. The injected oocytes were incubated at 19°C for 2 days as in [1].

Electrophysiological measurements on *Xenopus* oocytes were carried out at 20–24°C in Ringer's solution under the conditions described previously [1], unless otherwise specified. The Na^+-free solution was composed of 120 mM KCl, 1.8 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.2), and the K^+-free solution of 120 mM NaCl, 1.8 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.2). Our detectable limit for acetylcholine (ACh)-activated currents was ~10 nA. EGTA was injected ionophoretically into oocytes as in [2], except that the duration was 10 min. (−)-[^{3}H]*N*-methylscopolamine (NMS)-binding activity on the oocyte surface was estimated as in [2]. Displacement of (−)-[^{3}H]quinuclidinyl benzilate (QNB) binding by unlabelled carbamylcholine was measured using oocyte extracts as in [3].

For the assay of inositol phosphate formation, NG108-15 cells transformed to express porcine mAChR I, porcine mAChR III or rat mAChR III [11] were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6 μCi/ml *myo*-[^{3}H]inositol (spec. act. 20.0 Ci/mmol) at 37°C for 2.5 h and then in DMEM without [^{3}H]inositol for 1 h. After preincubation in DMEM containing 10 mM LiCl at 37°C for 5 min, the labelled cells were stimulated by various concentrations (10 nM to 1 mM) of carbamylcholine at 37°C for 10 min. Control cells were incubated without carbamylcholine. Total inositol phosphates were extracted [17], isolated [18] and counted for radioactivity.

3. RESULTS AND DISCUSSION

Porcine and rat mAChR III and rat mAChR IV were expressed in *Xenopus* oocytes by microinjection of the respective subtype-specific mRNAs derived from the cloned DNAs. The (−)-[^{3}H]NMS-binding activity on the cell surface per oocyte was 1.2 ± 0.2 fmol (mean ± SD, n = 3) for porcine mAChR III, 1.5 ± 1.4 fmol (n = 3) for rat mAChR III and 65 ± 13 fmol (n = 3) for rat mAChR IV; note that the concentrations of the mAChR III- and mAChR IV-specific mRNAs injected were 1 ng/μl and 1 μg/μl, respectively. All the injected oocytes showed a current response to ACh. At −70 mV membrane potential, the peak inward current elicited by 10 nM ACh was 1.53 ± 0.47 μA (n = 20) for porcine mAChR III and 1.41 ± 0.35 μA (n = 20) for rat mAChR III (see also fig.3). These currents were much larger than the peak inward current activated by 1 μM ACh in oocytes implanted with rat mAChR IV (166 ± 72 nA, n = 30). No detectable response to ACh (1 μM) was observed in 20 noninjected oocytes. The ACh response observed in oocytes implanted with either mAChR species was abolished by atropine or (−)-scopolamine (0.1 μM each) in a reversible manner, whereas (+)-tubocurarine (2.5 μM) and α-bungarotoxin (0.1 μM) exerted no effect.

The ACh-activated inward current observed in mAChR III-implanted oocytes was oscillatory in nature (fig.1A, a), whereas mAChR IV-implanted oocytes typically showed a response comprising an initial smooth inward current followed by an oscillatory component (fig.1B, a). Application of hyperpolarizing test pulses indicated that these inward currents were accompanied by increased membrane conductance. The oscillatory current evoked by mAChR IV varied in amplitude among the oocytes tested and was undetected in some of them. The latency of the ACh response in mAChR IV-implanted oocytes was shorter than that in mAChR III-implanted oocytes, the former being mostly attributable to the dead-space time in the perfusion system (~7 s). The current response in mAChR III-implanted oocytes occurred after an additional delay (2 ± 1 s, n = 10). Replacement of extracellular Ca^{2+} by Mg^{2+} did not significantly affect the ACh response in oocytes implanted with either mAChR III or mAChR IV. However, intracellular injection of the calcium-chelating agent EGTA almost completely abolished the ACh-activated current in mAChR III-implanted oocytes (fig.1A, b), leaving only a small long-lasting inward current (32 ± 10 nA in amplitude and 8 ± 2 min in duration, n = 8, no detectable current be-

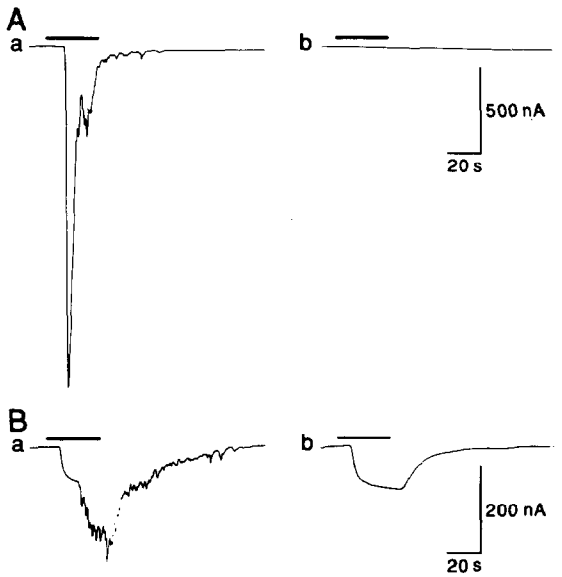

Fig.1. Effect of EGTA on ACh-activated currents in *Xenopus* oocytes injected with the porcine mAChR III-specific mRNA (A) or the rat mAChR IV-specific mRNA (B). Whole-cell currents activated by bath application of 10 nM (A) or 1 μM ACh (B) were recorded under voltage clamp at -70 mV membrane potential before (a) and after (b) intracellular injection of EGTA. Inward current is downward. The duration of ACh application is indicated by bars without taking into account the dead-space time in the perfusion system (~7 s). The ACh response in oocytes injected with the rat mAChR III-specific mRNA was similar to that in oocytes injected with the porcine mAChR III-specific mRNA.

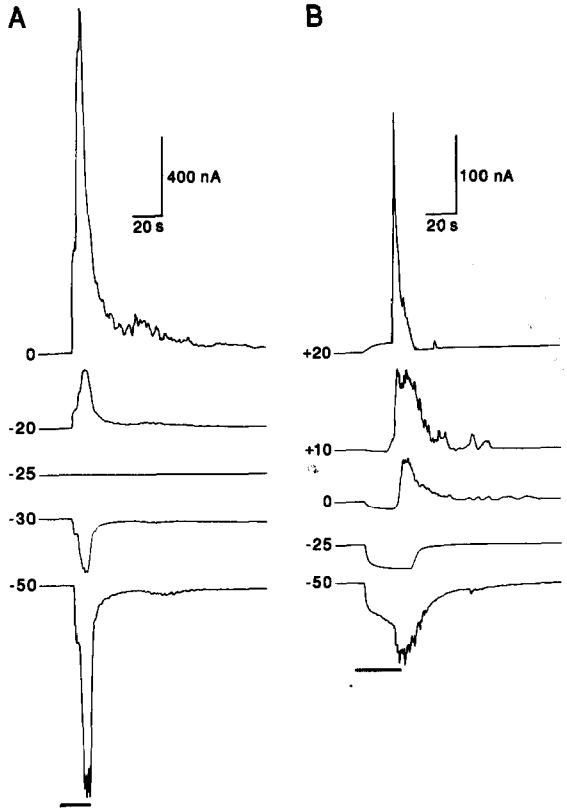

Fig.2. Membrane-potential dependence of ACh-activated currents in *Xenopus* oocytes injected with the porcine mAChR III-specific mRNA (A) or the rat mAChR IV-specific mRNA (B). Whole-cell currents activated by bath application of 10 nM (A) or 1 μM ACh (B) were recorded under voltage clamp at different membrane potentials (mV) as indicated on the left-hand side of each trace. Inward current is downward. The duration of ACh application is indicated as in fig.1. The membrane-potential dependence of ACh-activated currents in oocytes injected with the rat mAChR III-specific mRNA was similar to that in oocytes injected with the porcine mAChR III-specific mRNA.

ing observed in 2 of the 10 oocytes tested). The oscillatory current in mAChR IV-implanted oocytes similarly disappeared after this treatment, whereas the smooth current was virtually unaffected (fig.1B, b).

Fig.2 shows the ACh-activated currents recorded from an oocyte implanted with mAChR III (A) or mAChR IV (B) at various membrane potentials. The reversal potential of the oscillatory current elicited by mAChR III in Ringer's solution was -26 ± 2 mV ($n = 13$), which is close to the equilibrium potential of chloride ions in *Xenopus* oocytes [19]. In fact, the reversal potential shifted by 16 ± 1 mV ($n = 3$) to a more positive value when the external Cl^- concentration was halved by substitution with SO_4^{2-}. The oscillatory current in mAChR IV-implanted oocytes was reversed in polarity at a potential around -25 mV, whereas the reversal potential of the smooth current was around 10 mV. In mAChR IV-implanted oocytes loaded with EGTA, the reversal potential of the smooth current in Ringer's solution was 11 ± 2 mV ($n = 13$) and was virtually unchanged even when external Cl^- was completely replaced by SO_4^{2-}. In a K^+-free solution (120 mM Na^+), the reversal potential of the smooth current was 11 ± 2 mV ($n = 3$) and shifted to -1 ± 2 mV ($n = 3$) when the Na^+ concentration was reduced to one half by substitution with Tris. The reversal potential in a Na^+-free solution (120 mM K^+) was -2 ± 2 mV ($n = 3$) and changed to -16 ± 2 mV ($n = 3$) when

the K^+ concentration was halved by substitution with Tris. These results indicate that mAChR III, like mAChR I, mediates activation of a Ca^{2+}-dependent Cl^- current, whereas mAChR IV, like mAChR II, principally induces activation of Na^+ and K^+ currents in a Ca^{2+}-independent manner.

Fig.3 shows dose-response relationships for ACh-activated peak inward currents obtained from oocytes implanted with mAChR I (circles) or mAChR III (squares). It is to be noted that the concentrations of the mAChR I- and mAChR III-specific mRNAs injected were 1 μg/μl and 1 ng/μl, respectively. Under these conditions, the receptor density on the cell surface, as estimated by (−)-[^{3}H]NMS binding, was comparable for mAChR I-implanted oocytes (1.9 ± 0.5 fmol/oocyte, n = 3) and mAChR III-implanted oocytes (see above). Nevertheless, the dose-response curves for mAChR I and mAChR III were different. mAChR III-implanted oocytes responded clearly to 1 nM ACh (59 ± 35 nA, n = 10, for porcine and 51 ± 26 nA, n = 10, for rat), whereas mAChR I-implanted oocytes showed no detectable response to 10 nM ACh. To elicit a current of similar amplitude, mAChR I required a ~30-fold higher concentration of ACh than did mAChR III. When oocytes were injected with increasing concentrations of the porcine mAChR III-specific mRNA (1 ng/μl to 1 μg/μl), the (−)-[^{3}H]NMS-binding activity on the cell surface increased from 1.2 to 90 fmol/oocyte, but the current response to ACh (1 nM and 10 nM) was not appreciably augmented.

We further examined whether mAChR I and mAChR III expressed in mammalian cells also differed in sensitivity to agonist. Our previous study has revealed that mAChR I and mAChR III, but not mAChR II and mAChR IV, efficiently mediate phosphoinositide hydrolysis in NG108-15 cells [11]. Fig.4 shows the effects of increasing concentrations of carbamylcholine on the formation of total [^{3}H]inositol phosphates in NG108-15 cells expressing mAChR I (circles) or mAChR III (squares). The EC_{50} value for mAChR III-transformed cells (2.9–3.8 μM) was 7–9-fold smaller than that for mAChR I-transformed cells (27 μM), although the (−)-[^{3}H]QNB-binding activities of the two types of cells were comparable [11].

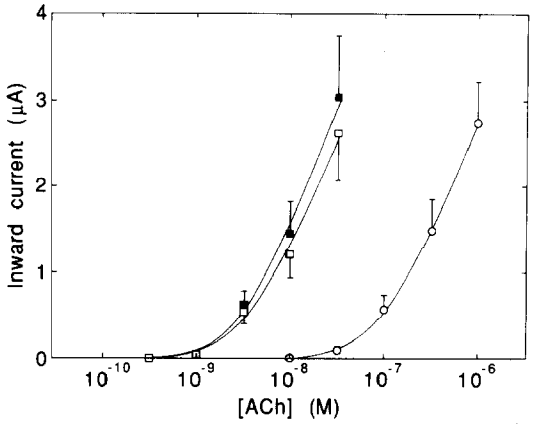

Fig.3. Dose-response curves for ACh-activated peak inward currents in *Xenopus* oocytes injected with the mRNA specific for porcine mAChR I (○), porcine mAChR III (□) or rat mAChR III (■). Each point represents the mean ± SD of measurements on 10 oocytes at −70 mV membrane potential.

The agonist binding affinities of mAChR I and mAChR III were examined using oocyte extracts (fig.5). The apparent dissociation constant (K_d) for carbamylcholine, obtained by measuring displacement of (−)-[^{3}H]QNB binding, was 7–10-fold lower for mAChR III (61–78 μM) than for mAChR I (580 μM); mAChR I and mAChR III have similar K_d values for (−)-[^{3}H]QNB [3]. The presence of 100 μM guanosine 5′-(β,γ-imido)triphosphate (GppNHp) did not appreciably affect the extents of displacement by carbamylcholine of (−)-[^{3}H]QNB binding to mAChR I and mAChR III. This may indicate that the proportion of high-affinity sites is low and that the K_d values obtained represent those of low-affinity sites [21].

The present investigation reveals that mAChR III has a sensitivity to agonist of about one order of magnitude higher than that of mAChR I in mediating activation of a Ca^{2+}-dependent Cl^- current in *Xenopus* oocytes and in stimulating phosphoinositide hydrolysis in NG108-15 cells. In accord with this finding is the observation that mAChR III, as compared with mAChR I, has a higher sensitivity to ACh in mediating an increase in the concentration of free intracellular Ca^{2+} in NG108-15 cells [14]. The present study also shows that the agonist-binding affinity of mAChR III is about one order of magnitude higher than that of mAChR I. The higher sensitivity to agonist of mAChR III is probably attributable, at least part-

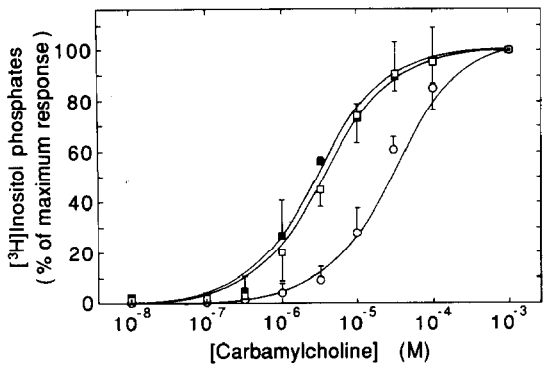

Fig.4. Dose-response curves for the formation of total [^{3}H]inositol phosphates in transformed NG108-15 cells with porcine mAChR I (clone NGPM1-8; ○), porcine mAChR III (clone NGPM3-332; □) or rat mAChR III (clone NGRM3-309; ■). Data are expressed as percentage of the maximum response obtained with 1 mM carbamylcholine. Each point represents the mean ± SD of 3–4 experiments. The formation of total [^{3}H]inositol phosphates in cells stimulated by incubation with 1 mM carbamylcholine, expressed as ratio to that in control cells incubated without carbamylcholine, was 3.7 ± 0.5 (n = 4), 4.5 ± 0.5 (n = 4) and 4.7 ± 0.2 (n = 3) for porcine mAChR I-, porcine mAChR III- and rat mAChR III-transformed cells, respectively. The theoretical curves have been drawn by nonlinear least-square analysis according to the equation $y = x/(x + EC_{50})$, where x represents the carbamylcholine concentration.

ly, to its higher agonist-binding affinity. It has been reported that muscarinic agonists are more potent in stimulating phosphoinositide hydrolysis in parotid gland than in cerebrum [22,23]. The presence of both mAChR I and mAChR III in exocrine glands and in cerebrum is suggested by the results of hybridization analysis which show that the corresponding mRNA species coexist in these tissues [1,8–10]. The EC_{50} value of carbamylcholine in stimulating phosphoinositide hydrolysis in the parotid gland (2.6–10.5 μM [22,23]) is comparable to that in mAChR III-transformed NG108-15 cells (2.9–3.8 μM), whereas a similar correlation is found between the EC_{50} values for cerebrum (43–68 μM [22,23]) and mAChR I-transformed NG108-15 cells (27 μM). Thus, it seems that mAChR III predominantly contributes to the response in the parotid gland, whereas mAChR I is mainly involved in the response in cerebrum. The mechanism underlying the preferential involvement of the individual mAChR subtypes in the response in these tissues remains to be elucidated.

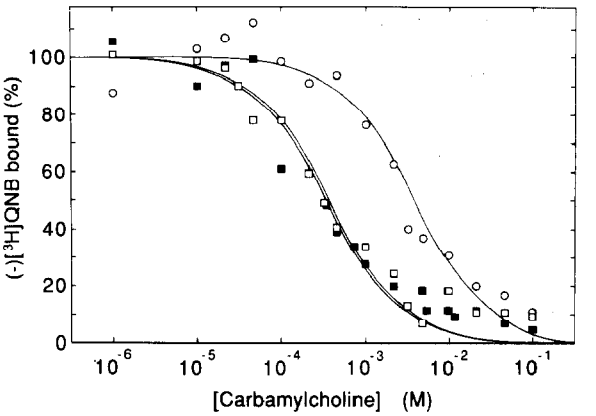

Fig.5. Effect of carbamylcholine on (−)-[^{3}H]QNB binding in extracts from *Xenopus* oocytes injected with the mRNA specific for porcine mAChR I (○), porcine mAChR III (□) or rat mAChR III (■). The concentration of the porcine and the rat mAChR III-specific mRNA injected was 32 ng/μl. Data are from 3 experiments. Values for 100% and 0% binding were determined by measurements in the absence of carbamylcholine and in the presence of 10 μM atropine, respectively. The 0% values [43–250 dpm (○), 39–279 dpm (□) or 55–217 dpm (■)] were 3–17% (○), 1–21% (□) or 3–18% (■) of the 100% values. The theoretical curves have been drawn by nonlinear least-square analysis as in [1]. The IC_{50} value of carbamylcholine was 3.67 mM for porcine mAChR I, 371 μM for porcine mAChR III and 336 μM for rat mAChR III. The K_d values for carbamylcholine were calculated from the IC_{50} values [20], using the K_d values for (−)-[^{3}H]QNB taken from [3].

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