0026-895X/88/040421-06$02.00/0

MOLECULAR PHARMACOLOGY, 34:421-426

ACCELERATED COMMUNICATION

Cloned Muscarinic Receptor Subtypes Expressed in A9 L Cells Differ in Their Coupling to Electrical Responses

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Received April 22, 1988; Accepted July 11, 1988

SUMMARY

The electrophysiological responses to cholinergic stimulation of four cloned muscarinic receptor subtypes (m1–m4) were studied in A9 L cells transfected with the expression plasmids of each of the different subtypes, using the tight-seal whole-cell recording technique. Cells transfected with m1 and m3 muscarinic receptor subtypes were hyperpolarized by acetylcholine (ACh), whereas m2- and m4-transfected cells did not respond to ACh concentrations of up to 1 mM. Stimulation of both m1 and m3 muscarinic receptor subtypes evoked outward currents in cells voltage-clamped at −50 mV, associated with an increase in membrane conductance. These outward currents were blocked by atropine but not by tubocurarine. The ACh-induced currents of m1- and m3-transfected cells primarily involved potassium ions, although chloride ions also contributed to a minor extent. The potassium and chloride conductances were blocked by barium or cobalt and by buffering the intracellular calcium to low levels with 5 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid, showing a dependence of these conductances on calcium. Thus, m1- and m3-transfected cells respond to ACh in a manner that is qualitatively similar, evoking calcium-dependent potassium and chloride conductances, whereas m2- and m4-transfected cells are not coupled to electrically detectable responses in A9 L cells.

Stimulation of muscarinic receptors by the neurotransmitter ACh or by other agonists modulates several species of ion channel (1, 2). Many of the changes in ion channel activity induced by stimulation of muscarinic receptors are mediated directly or indirectly by G proteins (3). Muscarinic receptors directly activate potassium channels in heart (4) and clonal pituitary cells via a G protein called G_K (5), which is inhibited by pertussis toxin. Other muscarinic effects on ion channel activity are mediated indirectly through G proteins by a variety of second messengers (3, 6). Muscarinic receptors are coupled via a G protein to phospholipase C, which, when activated by ACh, hydrolyses phosphatidylinositol to IP_3 and diacylglycerol. IP_3 mobilizes calcium from intracellular stores and activation of calcium-dependent potassium and chloride conductances by muscarinic receptors can be mimicked by intracellular injection of IP_3 in lacrimal glands (7) and *Xenopus* oocytes (8, 9). Adenosine-3′,5′-cyclic monophosphate and guanosine-3′,5′-cyclic monophosphate have also been implicated in mediating the actions of muscarinic receptors on voltage-gated calcium channels (10, 11). Stimulation of protein kinase C by phorbol esters (analogs of diacylglycerol) mimics muscarinic depression of a calcium-dependent potassium conductance in hippocampus (12) and of a potassium channel in *Xenopus* oocytes (13).

Distinguishing among electrical responses to muscarinic receptor stimulation was aided by the discovery of the selective antagonist pirenzepine. Pirenzepine defines two muscarinic receptor classes of either high (M1) or low (M2) affinity (14). Based on this subtype classification, presynaptic (M2) and postsynaptic (M1) receptors have been proposed in peripheral ganglia, using electrophysiological techniques (15). M1 activation mediates the resting depolarization in hippocampal and cortical neurons (16, 17) thought to be due to inhibition of a non-voltage-dependent potassium conductance (18). M2 activation has been associated with a negative chronotropic effect in heart (19) thought to be due to an increase in a potassium conductance; with inhibition of a calcium-dependent potassium conductance thought to underlie the after-hyperpolarization (20) in cortical neurons (16, 17), and other actions in various brain regions, including increases in potassium conductances (21, 22) and suppression of the m-current (23).

ABBREVIATIONS: ACh, acetylcholine; G protein, GTP-binding protein; IP_3, inositol-1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NMGMeSO_3$, *N*-methyl-D-glucamine methylsulfonate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; QNB, quinuclidinyl benzylate.

Using molecular cloning techniques four muscarinic receptor subtypes (m1-m4) have now been isolated (24–27). To investigate the possibility that these muscarinic receptors may mediate distinct electrophysiological responses, each muscarinic receptor subtype was expressed in isolation in A9 L cells, using DNA transfection techniques (28). Previous studies initially suggested that m1 muscarinic receptors activate a calcium-dependent chloride conductance (28); however, ion substitution experiments showed that both calcium-dependent potassium and chloride conductances are activated, when expressed in A9 L cells (29). In the present study, we have investigated the actions of m2–m4 muscarinic receptors when expressed in A9 L cells using electrophysiological techniques and compared them with the responses obtained in m1-transfected cells.

Materials and Methods

The m1 and m3 expression plasmids used in this study were cDNAs from rat brain and the m2 and m4 expression plasmids were derived from the human genome (26). A9 L cells were co-transfected with each of the muscarinic receptor plasmids and a plasmid containing the neomycin-resistance gene. After selection for 2 weeks with the neomycin analogue G418 resistant colonies were subcloned at limiting dilution. Monoclonal cell lines were isolated and levels of [^{3}H]QNB binding sites were determined as previously described (28). The clones isolated for the present study have comparable levels of [^{3}H]QNB binding (except for m2, which was somewhat lower), being 509, 84, 401, and 202 fmol/mg of protein for the m1–m4 respectively. A detailed account of the preparation of these cells is presented in Brann *et al.*[1] These clones were selected because their levels of receptor expression were comparable to the levels of receptor expression observed endogenously in various frequently studied tissues and cell lines (30).

Cells were incubated at 37° in 95% air and 5% CO_2 in Dulbecco's modified Eagles medium with 10% fetal calf serum for at least 24 hr before recordings. Cells were studied at room temperature (20–24°) on the inverted stage of a phase contrast microscope at a magnification of ×320. The tight-seal whole-cell recording technique was used to record membrane currents and potentials, using a LIST EPC-7 amplifier (31). The patch electrodes were made from thin-wall capilliary borosilicate glass (WPI, New Haven, CT) and pulled on a two stage horizontal electrode puller (Mecanex, Basel, Switzerland). Pipette resistances were 4–10 MΩ when placed in the bath and varied with pipette solution. Pipette seals of between 5 and 20 GΩ were obtained before disruption of the membrane patch. Once access to the cell interior was established, cell input resistances of 1–3 GΩ were obtained at resting potential (approximately −50 mV). Series resistance compensation was applied in voltage-clamp.

The ionic composition of the extracellular medium was (in mM): 140 NaCl, 5 KCl, 5 HEPES, 5.6 D-glucose, 2 $CaCl_2$, and 1 $MgCl_2$, pH adjusted to 7.4 with NaOH. The osmolarity was adjusted to 330–335 mOsmolar with sucrose. Other extracellular solutions used include a high K^+ medium containing (in mM): 140 NaCl, 50 KCl, 5 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, and 5.6 D-glucose. In most experiments the intracellular patch pipette solution contained (in mM): 150 K-gluconate, 2 $MgCl_2$, 5 HEPES, 1.1 EGTA, 0.1 $CaCl_2$, 5 Mg-ATP, and 0.1 Li-GTP, pH adjusted to 7.2 with KOH. The osmolarity was adjusted to 320–325 mOsmolar with sucrose. The free Ca^{2+} concentration was estimated to be about 20 nM (32). Another intracellular pipette solution used contained 150 mM $NMGMeSO_3$ instead of 150 mM K-gluconate. An aqueous lysate of the A9 L cells was made by replacing the solution in a Petri dish containing almost confluent cells with 1 ml of distilled water for 10–20 min (33). This caused visible disruption of the cells. The solution was decanted and filtered through a 0.22-μm Millipore filter and then used to formulate the intracellular pipette solution as a diluent in place of distilled water. Liquid-junction potentials between the bath solution and intracellular electrode solutions were eliminated by filling a side bath containing the reference electrode with the intracellular solution. Connections between the two baths were made via a low resistance bridge.

Current-clamp recordings were made at resting potential. Membrane currents were usually recorded from cells voltage clamped at −50 mV. Either resting current measurements were taken or the currents produced by a series of 10-mV, 100-msec, hyperpolarizing and depolarizing voltage steps applied at a frequency of 1 Hz were recorded. ACh (1–50 μM) was applied by low (1–2 lb sq in^{-1}) pressure from a micropipette placed 5–10 μm from the cell. ACh pulses were usually applied at 3–4-min intervals, as this was sufficient to evoke constant amplitude current responses. The current and voltage responses were displayed on a Gould pen recorder (filtered at 50 Hz) and also sampled and digitized on-line by a PDP 11/23 mini-computer with an analogue-to-digital Data Translation converter (12 bit, ±5 V range), at a digitization rate of 2.5 KHz. Signals were filtered at 1 KHz. Antagonists were applied directly to the bath and solution changes were obtained by perfusion of the 1.5-ml bath with fresh medium at a flow rate of 2 ml min^{-1}. All results are expressed as mean ± standard error.

Results

Comparison of the electrical responses to ACh of A9 L cells transfected with m1–m4 muscarinic receptors. The A9 L cells transfected with m1, m2, m3, or m4 muscarinic receptors were morphologically indistinguishable when examined with phase contrast optics at a magnification of ×320. They had resting membrane potentials of −47 ± 3 (n = 19), −47 ± 7 (n = 13), −49 ± 2 (n = 42), and −46 ± 3 mV (n = 21), respectively, when whole-cell recording was first obtained. Application of 1–50 μM ACh from a pressure ejection pipette to A9 L cells transfected with m1 (n = 5) and m3 (n = 5) muscarinic receptors hyperpolarized the cells from resting to around −67 mV but had no detectable effect on m2- and m4-transfected cells (not shown). In voltage clamp, ACh induced an outward current response at −50 mV in all cells transfected with m1 (Fig. 1A) and most cells transfected with m3 (Fig. 1C) but produced no response in cells transfected with muscarinic receptor subtypes m2 (n = 13) (Fig. 1B) and m4 (n = 25) (Fig. 1D) even at ACh concentrations of 1 mM. Inclusion of an aqueous lysate of the cells in the intracellular pipette solution did not alter ACh-induced responses in cells transfected with m1 (n = 3) and m3 (n = 5), nor could responses to ACh in cells transfected with m2 (n = 4) and m4 (n = 4) be elicited (not shown).

Comparison of the currents activated by stimulation of m1 and m3 muscarinic receptors. The onset of the responses of m1- and m3-transfected cells to ACh applications from pipettes positioned within 10 μm from the cell surface were both characteristically delayed. Responses of m1-transfected cells were delayed by 2.7 ± 0.3 sec (n = 27) and those of m3-transfected cells by 2.0 ± 0.4 sec (n = 18). The delay was not due to the time required for diffusion of ACh from the pipette to the cell surface because application of 150 mM KCl depolarized the cells within 50 msec. As with the m1 muscarinic responses (29), ATP and GTP were required in the intracellular pipette solution to maintain m3 muscarinic receptor responses.

A comparison of the contribution of K^+ to the current response in cells transfected with the m1 and m3 muscarinic receptors is shown in Fig. 2. Current-voltage (I-V) relationships were determined by 100-msec voltage steps from −50 mV to a

[1] Brann, M. R., B. Conklin, N. M. Dean, R. M. Collins, T. I. Bonner, and N. J. Buckley. Cloned muscarinic receptors couple to different G-proteins and second messengers. Submitted for publication.

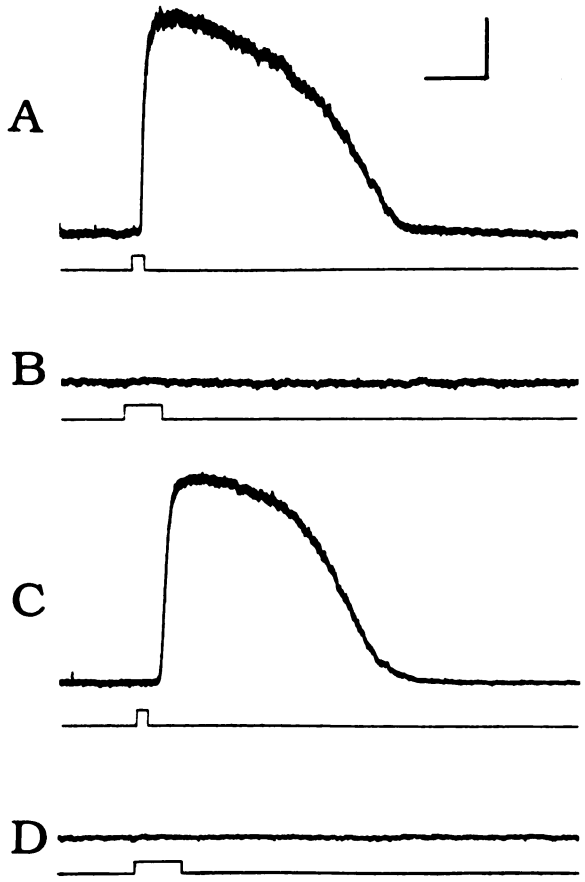

Fig. 1. Only m1- and m3-transfected cells respond to ACh. Current responses (*top traces*) to applications of 50 μM ACh (*lower traces*) in A9 L cells voltage-clamped at −50 mV. A, m1 muscarinic receptor activation leads to a delayed and sustained outward current; B, A9 L cells transfected with m2 do not respond to application of ACH; C, ACh triggers a delayed outward current response in m3-transfected cells greater in amplitude than responses in m1-transfected cells; D, ACh elicits no response in cells transfected with m4 muscarinic receptors. Upward deflections denote outward current. Horizontal calibration, 10 sec; vertical calibration: 25 pA (A and B) and 50 pA (C and D).

range of potentials in extracellular solutions containing either 5 or 50 mM KCl. The currents were measured at the end of the 100-msec steps. In both solutions I-V curves of m1- and m3-transfected cells were similar. The I-V relationship was linear over the range −100 to +60 mV with no evidence of voltage-activated conductances. ACh (50 μM) increased the slope conductance (calculated from the slope of the I-V curve) about 10-fold from 0.6 ± 0.1 nS in control to 6.1 ± 1.4 nS (n = 5) in m1-transfected cells. In m3-transfected cells 50 μM ACh produced a larger response, increasing the slope conductance 15-fold from 0.5 ± 0.1 to 7.7 ± 1.2 nS (n = 12). The reversal potential for the m1 response was shifted 49 mV from −67 ± 3 mV (n = 5) to −18 ± 3 mV (n = 2) and from −67 ± 1 mV (n = 9) to −18 ± 1 mV (n = 5) for m3-transfected cells when the KCl concentration was increased from 5 to 50 mM.

Using the Nernst equation:

$$E_{ion} = \frac{RT}{ZF} \log_e [ion]_{out}/[ion]_{in}$$

where E_{ion} is the equilibrium potential for that ion; Z is the valency of the ion; R is the gas constant; F is the Faraday constant, and T is temperature in degrees Kelvin, the equilibrium potential (i.e., the potential at which there is no net current flow) calculated for recordings in 5 mM KCl is −86 mV for potassium (E_K) and −92 mV for chloride (E_{Cl}). Thus, when the extracellular solution was changed to one containing 50 mM KCl, E_K theoretically changed to −28 mV and E_{Cl} to −99 mV.

These results suggest that, as with A9 L cells transfected with m1 muscarinic receptor, K^+ ions are primarily involved in the response rather than chloride ions. However, in K^+-free solutions ($NMGMeSO_3$ as the K^+ substitute in the intracellular electrolyte) responses to ACh could still be obtained. The I-V relationship produced under these conditions is shown in Fig. 2C. The reversal potential of the response was −98 ± 6 mV (n = 4), which is close to E_{Cl} (−93 mV) in these solutions. The slope conductance of the m3 response in the K^+-free solution was increased 5-fold from 0.13 ± 0.04 nS in control to 0.68 ± 0.19 nS (n = 4) in the presence of ACh. This is slightly greater than the 2-fold increase in conductance induced by ACh in m1-transfected cells bathed in K^+-free solutions (see Ref. 29).

Pharmacology of the ACh-induced current response in A9 L cells transfected with the m3 muscarinic receptor. The ACh-induced responses to m3 muscarinic receptor stimulation were inhibited by 1 μM atropine, a muscarinic receptor antagonist, but were not inhibited by the nicotonic receptor antagonist tubocurarine (50 μM) (Fig. 3), suggesting the response is muscarinic in nature. Similarly to the ACh-induced responses in cells transfected with m1 muscarinic receptors, the ACh-induced current responses in cells transfected with the m3 muscarinic receptor were blocked by barium and cobalt (Fig. 3). Neither 5 mM barium nor 5 mM cobalt affected the binding of [^{3}H]QNB to the ACh receptor (29). Rapidly buffering intracellular Ca^{2+} to low levels, by addition of 5 mM BAPTA to the patch pipette solution (34), eliminated the ACh-induced response in cells transfected with the m3 muscarinic receptor (n = 10), suggesting that like the m1, stimulation of the m3 muscarinic receptor activates conductances dependent on increases in intracellular Ca^{2+}. Thus, stimulation of both m1 and m3 muscarinic receptors activates Ca^{2+}-dependent K^+ and to a lesser extent Ca^{2+}-dependent Cl^- conductances, showing that in A9 L cells their actions are qualitatively similar.

Discussion

The four muscarinic receptor subtypes were expressed in A9 L cells to study their electrophysiological functions in isolation. Stimulation by ACh of A9 L cells transfected with m1 or m3 muscarinic receptors hyperpolarized cells primarily by activation of a calcium-dependent potassium conductance and to a lesser extent activation of a calcium-dependent chloride conductance. A9 L cells transfected with muscarinic receptor subtypes m2 and m4 were devoid of any electrically detectable responses under the conditions used here.

Muscarinic activation of calcium-dependent potassium and chloride conductances has been reported in lacrimal (7, 35) and salivary glands (36), which appear to be similar to the m1 and m3 muscarinic responses recorded in transfected A9 L cells. The mRNAs for both m1 and m3 are found in the salivary glands,[2] therefore, this suggests a further correlation of the function of m1 and m3 with responses in these tissues. As

[2] Unpublished observations.

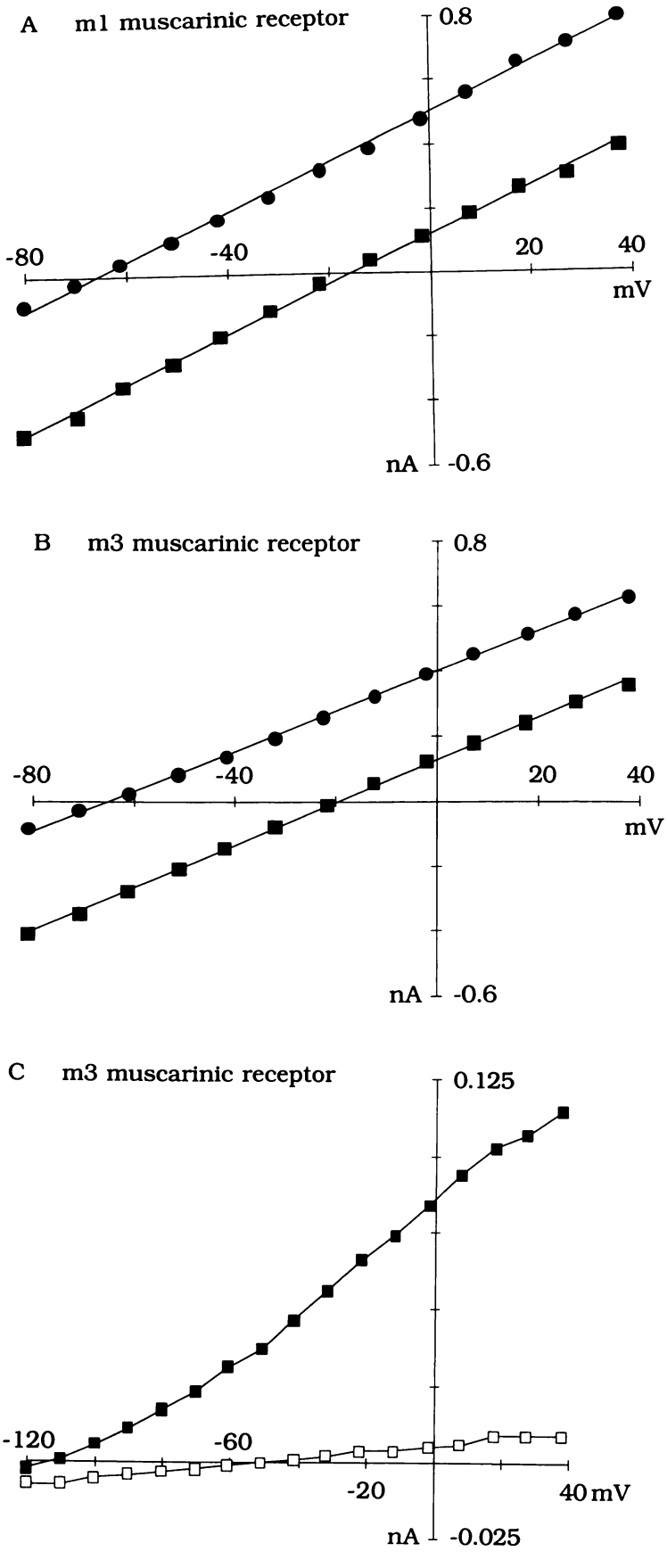

Fig. 2. Reversal potentials of ACh-induced responses shift with changes in K^+ and Cl^- gradients. Current-voltage relationships of m1-transfected (A) and m3-transfected (B) A9 L cells recorded with K-gluconate containing intracellular pipette solutions, showing the reversal potentials (E_{ACh}) of the ACh-induced responses. Increasing extracellular K^+ from 5 (●) to 50 mM (■) alters E_{ACh} from approximately −67 mV to approximately −18 mV in m1- and m3-transfected cells. C, ACh-induced currents can be elicited in K^+-free solution with 150 mM *N*-methyl-D-glucamine chloride in the extracellular solution and 150 mM $NMGMeSO_3$ in the intracellular pipette solution. E_{ACh} under these conditions is approximately −110 mV, close to the calculated value for E_{Cl}. □, Control; ■, 50 μM ACh.

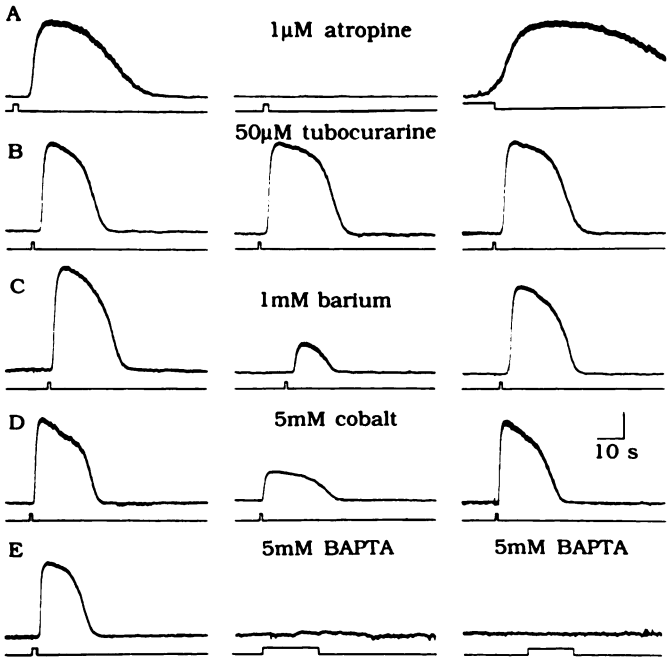

Fig. 3. Pharmacology of muscarinic receptor current response in m3-transfected A9 L cells. The cells were voltage-clamped at −50 mV and ACh was applied for several seconds. The *first column* shows ACh-induced responses under control conditions, the *second* shows the responses about 10 min after the addition of indicated drug to the bathing medium. The *third column* shows the recovery of the responses after drug washout. *Upper panels* show ACh-induced currents, *lower panels* show application periods of ACh. A, Atropine (1 μM) abolishes responses to 1 μM ACh. B, Tubocurarine (50 μM) has no effect on current responses induced by 10 μM ACh. Barium (1 mM) (C) and 5 mM cobalt (D) attenuate the responses to 10 μM ACh application. In E, the responses to 50 μM ACh were recorded in two different cells with patch pipette solutions containing either 1.1 mM EGTA (used in all other experiments) or 5 mM BAPTA to buffer intracellular Ca^{2+}. The currents recorded with BAPTA are from the same cell, showing no response to ACh using the same pressure ejection pipette as used for responses elicited with EGTA-containing intracellular pipette solutions. Upward deflections denote outward current. Vertical calibration, 100 pA in A–C, 50 pA in D and *first column* of E; *second* and *third column* of E, vertical calibration is 25 pA.

mentioned previously, intracellular injection of IP_3 in lacrimal glands mimicked the muscarinic response. The m1 and m3 muscarinic receptors stimulate inositol metabolism in A9 L cells[1] and thus IP_3 may play a role in the increase in intracellular calcium induced by ACh, although the entry of calcium from the extracellular solution cannot be ruled out at present.

Although both m1 and m3 muscarinic receptor mRNAs are abundant in central nervous system tissues (37, 38), muscarinic activation of either calcium-dependent potassium or chloride conductances has not as yet been reported in the central nervous system. Thus, the phenotype of the cell in which the receptor is endogenously expressed may be critical in determining the physiological responses coupled to muscarinic receptor stimulation. Expressing m1 and m3 muscarinic receptors in other cell types, such as neurons, may reveal different physiological responses.

The m2 and m4 muscarinic receptor subtypes expressed in A9 L cells did not respond electrically to ACh under the same experimental conditions used to reveal the ACh-induced responses of the m1 and m3 receptors. It has been shown that stimulation of either the m2 or the m4 muscarinic receptor has no effect on inositol phosphate metabolism but decreases levels of cAMP when expressed in A9 L cells.[1] The m2 and m4 muscarinic receptors couple to a G protein in A9 L cells as demonstrated by a pertussis toxin-sensitive effect of guanine nucleotides on agonist binding.[1] It is clear that neither m2 nor m4 receptors respond via the same mechanism as the m1 and m3 receptors in A9 L cells. This is not due to their slightly lower levels of expression, inasmuch as concentrations of ACh that occupy less than 1% of the receptors (20 nM) can still elicit maximum electrical responses in m1- and m3-transfected cells. It is, therefore, reasonable to assume that the m2 and m4 receptors couple to a response that is lacking an essential component in A9 L cells. One possibility is that a factor or second messenger of some sort is required for activation of other conductances and is being dialyzed out of the cell by the tight-seal whole-cell recording technique. However, inclusion of a dilute aqueous lysate of the cells, which presumably contains soluble cytoplasmic components, in the intracellular electrolyte solution did not support any muscarinic receptor-coupled electrical responses.

The m2 muscarinic receptor subtype is thought to activate an inwardly rectifying potassium conductance in heart cells. This conductance is not expressed in A9 L cells; thus, the inward rectifying potassium channel may be critical to expression of m2 receptor function. This contrasts with responses to m2 muscarinic receptor activation in *Xenopus* oocytes, which include both a calcium-dependent chloride conductance and a nonspecific cation conductance (39). The activation of a calcium-dependent chloride conductance has been previously associated with m1 activation in *Xenopus* oocytes (24). The activation of this conductance by the m2 muscarinic receptor produces a much smaller chloride current and may represent a nonspecific interaction of the m2 receptor, inasmuch as densities of the m2 receptor expressed in the oocytes were over 30 times greater than those of the m1 (39). The lack of activation of a cation conductance by m2 receptors in A9 L cells may reflect the absence of such a conductance within the A9 L cell membrane.

The results of this study show that muscarinic receptor subtypes m1 and m3 are functionally similar and differ from m2 and m4 when expressed in A9 L cells. Interestingly, this correlates with the observation that the primary structure of the m1 and m3 receptors are more closely related to each other than they are to either m2 or m4 receptors.

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