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MUSCARINIC REGULATION OF MEMBRANE ION CHANNELS IN AIRWAY SMOOTH MUSCLE CELLS

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Abstract—We have demonstrated that stimulation of airway smooth muscle by muscarinic agonists results in a coordinated modulation of two membrane ion channel proteins. Both channels are modulated in a similar way, although their effects on open-channel probability are opposite. The voltage-dependence of channel activity is shifted to more positive potentials in the case of K_{Ca}, and to more negative potentials in the case of the voltage-dependent calcium channels. Similarly, K_{Ca} channel dwell-time kinetics are shifted to short open lifetimes, whereas the long open state is favored for the large-amplitude voltage-dependent calcium channel. Although little is known about the molecular coupling of calcium channels, muscarinic inhibition of K_{Ca} channels is mediated through a pertussis toxin-sensitive guanine nucleotide binding protein.

Muscarinic regulation of smooth muscle is the principle determinant of smooth muscle tone in a variety of organ systems including the lung. In airway smooth muscle cells, exposure to a muscarinic agonist results in a graded depolarization [1, 2], and a transient and sustained rise in intracellular calcium [3]. Although the role of membrane ion channels in these processes is not yet well understood, specific information about the linkage between muscarinic receptor activation and membrane ion channel proteins is emerging. This review will summarize data from our laboratory on the coupling between muscarinic stimulation, and potassium and calcium channel function.

Muscarinic inhibition of calcium-activated potassium channels

Calcium-activated potassium channels are a ubiquitous component of single-channel currents in membrane patches of airway smooth muscle cells [4–7]. At physiologic E_m, cytosolic calcium and pH, however, open channel probability (P_o) is quite low. It is therefore uncertain whether these channels contribute substantially to resting membrane potassium conductance. Since single-channel conductance and channel density are quite high, it is possible that even at very low channel P_o, K_{Ca} channels contribute substantially to total membrane conductance. During cellular excitation by contractile agonists, however, two events occur that would occasion a steep increase in P_o. Firstly, airway smooth muscle cells depolarize between 15 and 20 mV in a graded and sustained manner [1, 2]. Secondly, intracellular calcium rises from resting levels of approximately 100 nM to levels that transiently approach 1 μM and are then sustained at 150–200 nM [8]. Membrane depolarization and rises in cytosolic calcium combine to augment markedly the open-probability of this voltage- and calcium-sensitive channel. If this occurred during excitation, however, it is difficult to imagine how a sustained depolarization would be maintained. It is more likely that membrane depolarization requires an inhibition of voltage-dependent potassium channels, allowing a net movement away from the potassium equilibrium potential. This is likely to be particularly true of the calcium-activated potassium channel, since this channel is also influenced markedly by associated rises in cytosolic calcium. Recent patch-clamp experiments in our laboratory have examined the coupling between potassium channel activity and muscarinic receptor occupancy, and have demonstrated that muscarinic activation results in a marked inhibition of K_{Ca} channel activity [9].

Figure 1a shows that following exposure of the extracellular membrane surface to methacholine (50 μM), channel activity was inhibited markedly. Muscarinic inhibition of channel activity was observed in the absence of exogenous guanine nucleotides in the pipette solution (cytosolic surface) but was augmented by inclusion of guanosine triphosphate (GTP†) or GTPγS (100 μM). Inhibition resulted in a marked shift in the relationship between open-state probability and potential (Fig. 1b). These results are similar to those of Toro *et al.* [10], who observed that angiotensin II inhibited K_{Ca} channels from coronary artery membranes that had been reconstituted in lipid bilayers. The augmentation of channel inhibition observed when guanine nucleotides were added to the pipette solution suggested the involvement of a regulatory G protein. We have examined the guanine nucleotide dependence of this inhibition and have determined some of the critical coupling processes that interpose between receptor occupancy and channel inhibition.

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† Abbreviations: G protein, guanine nucleotide binding protein; GTP, guanosine 5′ triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate).

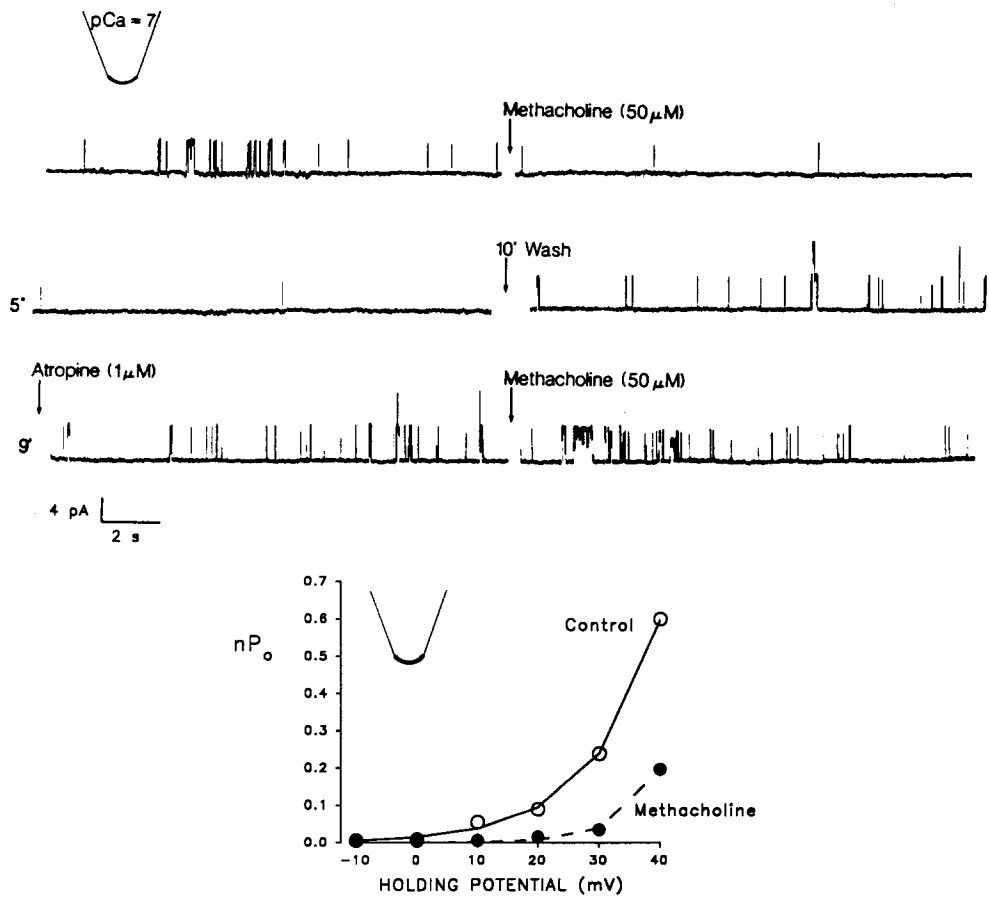

Fig. 1. Methacholine Inhibits K_{Ca} channels in outside-out patches. Addition of methacholine (50 μM) results in a marked decrease in channel opening. Channel activity slowly returned to baseline and remained constant following washout. Addition of methacholine in the presence of atropine did not result in channel inhibition. Holding potential was 0 mV. (B) Open-probability vs voltage for a similar outside-out experiment, in which holding potentials were varied from −10 to 40 mV. The average nP_o was determined over a 30 sec period at each potential before (○) and during (●) methacholine (50 μM) exposure. A prominent shift toward positive potentials can be seen in the presence of methacholine. The lines show the best-fit of a Boltzman equation to the relationship. The V_{50} for the fits were −37.4 and −47.7 mV for control and methacholine, respectively, indicating a shift of approximately 10 mV.

Figure 2 shows that when BDPβS was included in the patch pipette in outside-out experiments, no muscarinic inhibition of channel activity was observed. The role of a coupling G protein was established more directly using inside-out patches. In these experiments, methacholine (50 μM) was included in the patch thereby activating muscarinic receptors at the extracellular membrane surface. Following patch excision, the cytosolic membrane surface was exposed to GTP resulting in a prompt inhibition of channel activity (Fig. 3). When GTP was removed, channel activity slowly returned to baseline, consistent with the slow hydrolysis of GTP observed *in vitro* for many GTPase proteins [11]. Addition of GDPβS further increased channel activity, presumably by promoting the stability of the heterotrimeric $\alpha\beta\gamma$ complex. We have also been successful in establishing the nature of the coupling G protein in this system. Incubation of dissociated cells with native pertussis toxin (0.1 μg/mL) resulted in a loss of the coupling between recep-

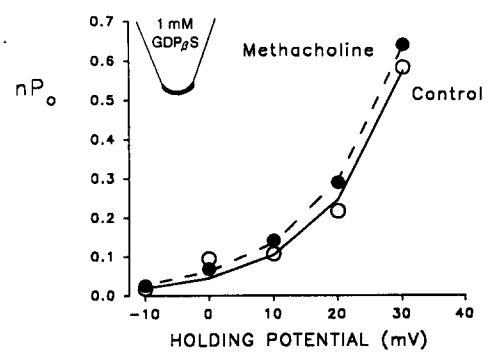

Fig. 2. Muscarinic inhibition of K_{Ca} is guanine nucleotide-dependent. Open-probability vs holding potential for an outside-out patch in which GDPβS (1 mM) was included in the pipette. Methacholine (50 μM) addition did not inhibit channel activity in the presence of GDPβS. Lines shown are Boltzman fits.

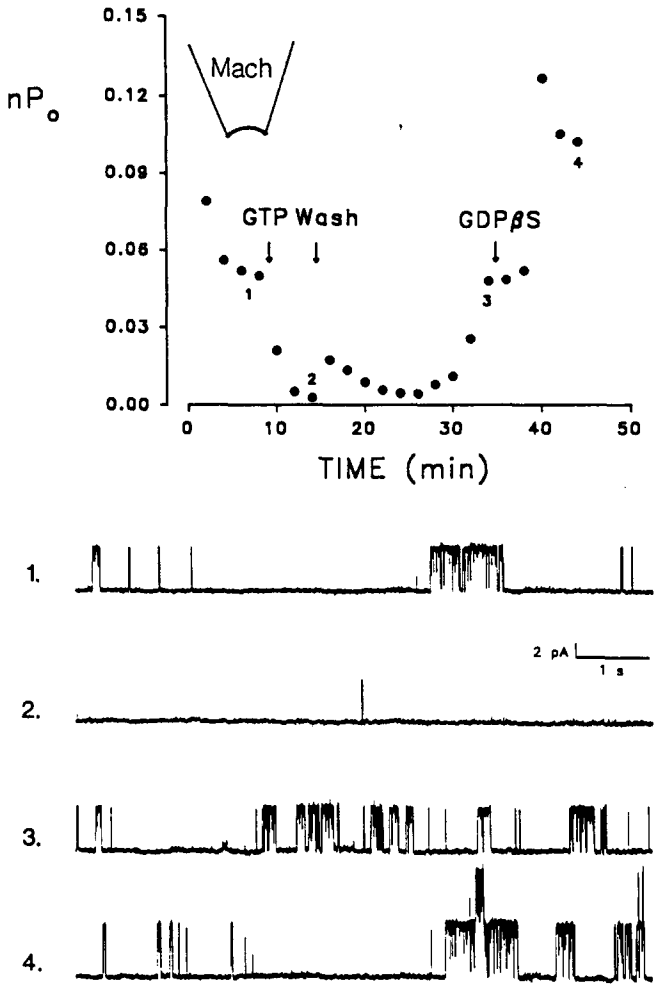

Fig. 3. GTP inhibition of K_{Ca} in inside-out patches. An inside-out experiment in which methacholine was included in the patch pipette and GTP (100 μM) was added to the bath (arrow). Data are averaged open-probability over 2 min. Following addition of GTP, channel open-probability decreased and channel openings were very brief. Following washout, channel activity slowly returned to baseline, then increased following addition of GDPβS. Traces below show 8 sec of data from each experimental condition at the points indicated.

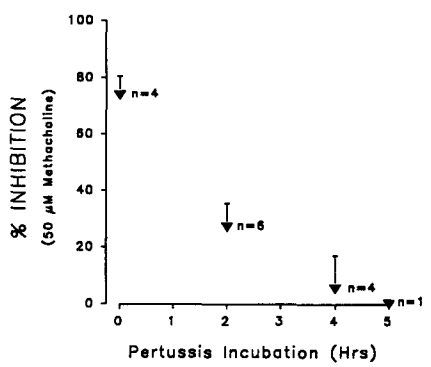

Fig. 4. Pertussis toxin blocks muscarinic inhibition of K_{Ca}. Summary of the relationship between time of incubation with pertussis toxin (0.1 μg/mL) and % inhibition of channel activity by methacholine (50 μM). The number of patches and SE are shown for each data point.

tor and channel. As shown in Fig. 4, channel inhibition was blocked completely following 4 hr of pertussis toxin incubation and intermediate incubation times resulted in a less than complete block.

Taken together, these data suggest that during muscarinic excitation, K_{Ca} channels are potently inhibited by a process that has features similar to those of the Gk activation of cardiac potassium channels. We believe that this channel inhibition is part of a coordinated membrane response to contractile agonists, which lowers potassium conductance allowing membrane potential to move to a more negative equilibrium potential and an attendant triggering of other voltage-dependent conductances. Experiments to identify the precise G protein subunits responsible for mediating channel inhibition are currently being performed.

Muscarinic activation of voltage-dependent calcium channels

The role of dihydropyridine-sensitive, voltage-

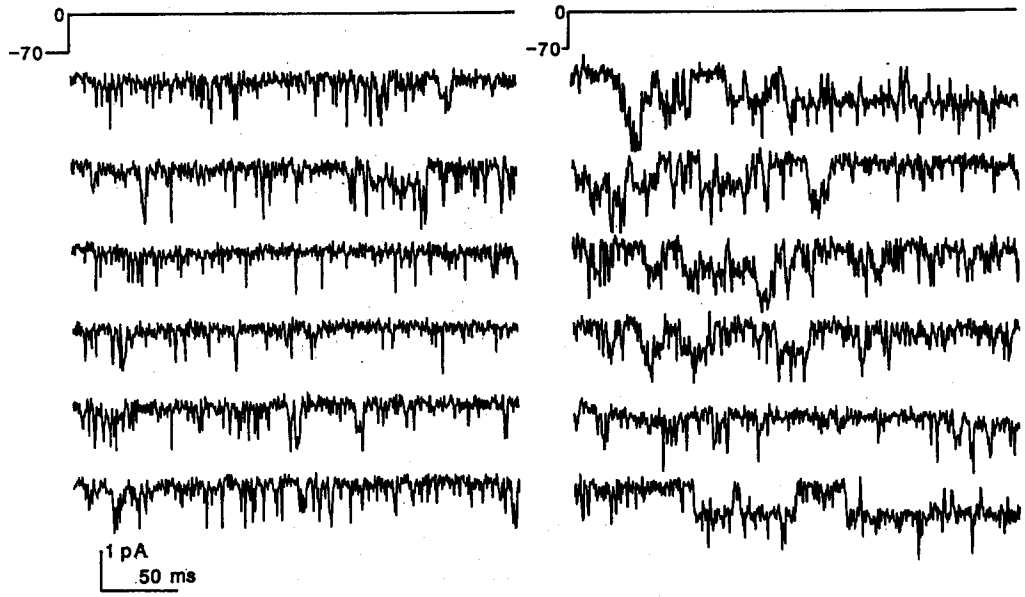

Fig. 5. Methacholine activates large-conductance, voltage-dependent calcium channel in airway smooth muscle cells. Representative 300 msec current traces from on-cell membrane patches showing baseline activity of channel (left) and activity following bath addition of methacholine (50 μM) (right).

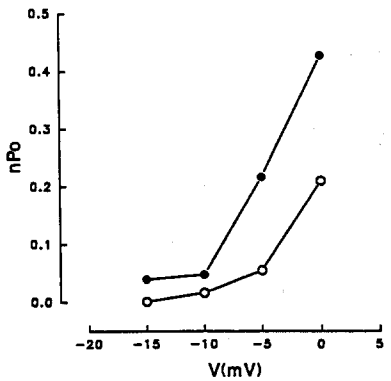

Fig. 6. Methacholine shifts the voltage-dependence of channel activity. Plot shows nP_{open} vs step potential for control (○) and post agonist exposure (●). Agonist exposure resulted in leftward shift of the open-probability/voltage relationship.

dependent calcium channels in airway smooth muscle excitation–contraction coupling has been controversial. Although these channels play a pivotal role in the production of tone in some smooth muscle tissues [12], dihydropyridine calcium channel antagonists are only modestly effective in inhibiting cholinergic contraction of airway smooth muscle [13]. Moreover, calcium influx pathways that are insensitive to dihydropyridines have been shown to exist in human airway smooth muscle cells [8].

Voltage-dependent calcium currents have been reported at the whole cell [14, 15] and single-channel [16] level in airway smooth muscle. We reasoned that if these channels were important functionally in excitation–contraction coupling, a necessary condition would be the existence of coupling pathways between receptor stimulation and channel modulation. Since studies in other smooth muscle cell types have demonstrated agonist-induced augmentation of calcium channel activity [12, 17, 18], we set out to determine whether such augmentation is present in airway smooth muscle [19], a tissue in which voltage-dependent calcium channel antagonists are only modestly effective at inhibiting agonist-induced contraction [13, 20, 21].

Acutely dissociated canine smooth muscle cells were studied using on-cell, patch-clamp techniques. Currents were recorded using 80 mM Ba^{2+} as the charge carrier at step potentials ranging from −10 to 10 mV. In this preparation, two distinct channel conductances could be observed, although both were inhibited by dihydropyridines. As shown in Fig. 5, application of 50 μM methacholine to the extracellular bath resulted in a marked increase in channel activity recorded in a patch of membrane that was not exposed directly to the agonist. The activity of both channel amplitudes was increased, although the large-conductance channel was observed most commonly. The average single-channel amplitudes at a given potential were not significantly different before and after activation, indicating that methacholine did not increase channel open probability by simply depolarizing the cell. Rather, as shown in Fig. 6, a shift in the relationship between open-probability and voltage resulted following exposure to the agonist. This shift in channel sensitivity to voltage has also been described in vascular smooth muscle cells [12]. Additionally, shifts in open-time kinetics could be observed for

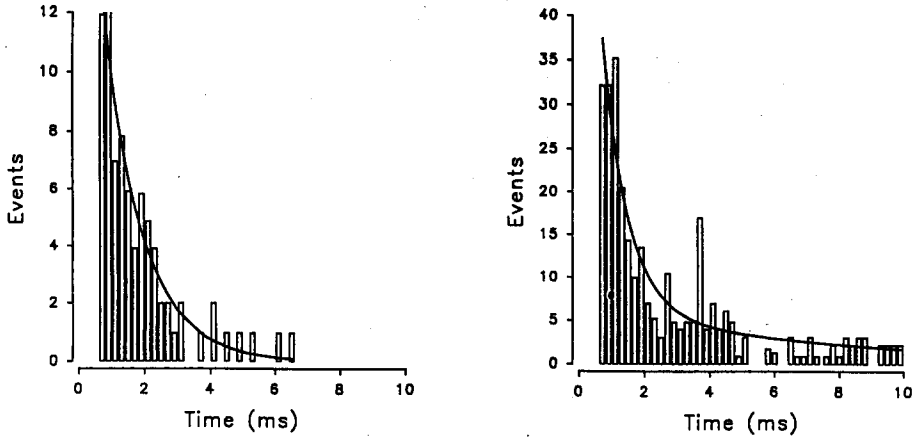

Fig. 7. Effect of methacholine on open-time distribution of calcium channels. Left, control open-time histogram of large-conductance calcium channel in CASM obtained from 20 current traces at 0 mV step potential. A single exponential decay curve is fit to the data suggesting a single open-state; τ is 1.2 msec. Right, histogram from same cell following exposure to 50 μM methacholine (20 traces, note difference in scaling). Following agonist activation, the open-times are fit with a bi-exponential decay curve suggesting two open states: $\tau_{fast} = 0.8$ msec; $\tau_{slow} = 7.6$ msec.

the large-conductance calcium channel. Activation resulted in an increase in the probability that a single opening would be of relatively long mean duration, as shown in Fig. 7. This effect is reminiscent of the action of BAY K8644 on the channel [22].

The demonstration of cholinergic augmentation of channel open-state probability at a fixed voltage demonstrates that the molecular pathways linking receptor occupancy and calcium channel activity exist in airway smooth muscle. However, the role of this channel activation in excitation–contraction coupling processes has not been established. This fact bears emphasis, since agents that are capable of completely inhibiting calcium channel currents [14, 16, 23] do not completely inhibit muscarinic contractions [13], and since dihydropyridine-insensitive mechanisms of calcium influx have been demonstrated in these cells [8]. On the other hand, it seems likely that these channels are activated during muscarinic contraction and play some role in agonist-induced contraction. Although at very high doses of cholinergic agonists calcium channel antagonists are ineffective, they can effectively inhibit low-dose contractions [13].

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