rapid communications

Trypsin activation of atrial muscarinic K^+ channels

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KIRSCH, G. E., AND A. M. BROWN. *Trypsin activation of atrial muscarinic K^+ channels.* Am. J. Physiol. 257 (Heart Circ. Physiol. 26): H334–H338, 1989.—The atrial muscarinic K^+ channel normally is opened by the activated G protein, G_k. Based on the assumption that an inactivating particle keeps the channel closed, several protein-modifying agents including trypsin, papain, glyoxal, and phenylglyoxal that remove Na^+-channel inactivation were tested. K^+ channels were studied in inside-out excised membrane patches from primary cultures of neonatal rat atrial myocytes. Of the agents tested, only trypsin activated muscarinic K^+ channels, and it did so irreversibly. Trypsin was effective in the absence of muscarinic agonist or intracellular Mg^{2+} and guanosine 5′-triphosphate. Heat-denatured trypsin was ineffective, and trypsin inhibitor blocked the effect. Because trypsin is known to inactivate G proteins, the effect was probably on the K^+ channel or a structure closely associated with it. Trypsin activation produced single-channel currents in which inward rectification, single-channel conductance, mean open time, and burst duration were indistinguishable from muscarinic activation. Trypsin cleaves proteins at lysine or arginine residues, and the arginine-specific reagents, glyoxal and phenylgloxal, did not activate K^+ channels. We conclude that trypsin disrupts an inhibitory gating mechanism that normally holds the channel closed in the absence of activated G_k. The inhibitory gate is physically distinct from the gate that mediates bursting and must contain at least one trypsin cleavage point located at a lysine residue accessible from the cytoplasmic surface of the cell membrane.

guanosine 5′-triphosphate-binding protein; muscarinic receptor; potassium; heart; myocyte; ion channel

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ACETYLCHOLINE (ACh) activates atrial K^+ channels (5, 21) that are directly coupled to muscarinic receptors through the guanosine 5′-triphosphate (GTP)-binding protein, G_k (2, 16, 20, 24). In the presence of muscarinic agonist, atrial K^+ channels (K^+[ACh]) can be opened by intracellular application of GTP (16) or in the absence of agonist by GTPγS because of activation of endogenous G_k (12). Furthermore, K^+[ACh] channels are activated directly by the application of exogenous G_k or its α-subunit after preactivation with GTPγS (3, 9, 24). Unlike voltage-gated inwardly rectifying K^+ channels (19), which are open at the resting potential, K^+[ACh] channels are normally closed in the absence of agonist. One explanation is that an inhibitory or inactivating particle keeps the channel closed and one approach would be to use protein-specific reagents to modify channel function (1, 4, 17). In the present paper, we show that single K^+[ACh] channels are activated irreversibly by mild tryptic digestion, a treatment that removes inactivation in Na^+ channels (1, 17) and causes inactivation of G proteins (23). Trypsin-activated and agonist-activated K^+[ACh] channels have similar single-channel conductance, bursting, and open times.

We propose that K^+[ACh] channels in unstimulated membranes are inhibited from opening by a gating subunit located near the intracellular surface and containing at least one tryptic cleavage site. This inhibitory component normally holds the channel closed in the absence of G protein stimulation.

METHODS

Cell culture. Atrial cell cultures were prepared from hearts of 1- to 3-day-old neonatal rats by trypsin digestion as described previously (9).

Electrical recording. Single-channel currents were measured using the gigaseal patch-clamp method (6) and were analyzed as described previously (9). All experiments were performed at room temperature (20–22°C). Where appropriate, data are expressed as means ± SE.

Solutions. Solutions in the pipette and bath contained (in mM) 140 KCl, 5 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 2 $MgCl_2$, and 10 *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES). pH was adjusted to 7.3 with tris-(hydroxymethyl)aminomethane (Tris) base. Mg-free solutions were prepared by replacement of EGTA with 11 mM EDTA. High pH solution for use with glyoxal was prepared by replacement of HEPES with 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) and titration to pH 9. Trypsin, trypsin inhibitor, papain, glyoxal, and phenylglyoxal were obtained from Sigma Chemical (St. Louis, MO) and were added directly to the bath in 0.01-ml aliquots at 100 times the final concentration and were mixed using the pipette. Washout was achieved by continuous flow of solution through the chamber at a rate of 3.5 ml/min.

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RESULTS

In cell attached patches (Fig. 1*A*), Carbachol at 1 μM in the pipette activated single-channel K^+[ACh] currents (Fig. 1*A*, trace 1). Excision of the patch into an inside-out configuration (intracellular surface exposed to the bathing solution, Fig. 1*A*, trace 2) resulted in both a decrease of single-channel K^+[ACh] currents because of the washout of GTP from the intracellular surface of the patch and, rarely, a stimulation of single-channel ATP-dependent K^+ currents (K^+_{ATP}) because of the washout of ATP (15). K^+_{ATP} currents could be distinguished by their larger conductance (80 vs. 40 pS), bursting pattern, and sensitivity to inhibition by intracellular ATP. K^+_{ATP} currents were most active immediately after excision and spontaneously declined thereafter (22). After washout of GTP from the excised patch and spontaneous run-down of the K^+_{ATP} currents, single-channel K^+[ACh] currents could be restored by addition of GTP at 0.1 mM (Fig. 1*A*, trace 3). Single-channel currents ceased when GTP was washed out of the bath (Fig. 1*A*, trace 4). At this point (Fig. 1*A*, trace 5), the addition of trypsin to the bathing solution at 0.25 mg/ml activated K^+[ACh] currents. Trypsin activation was observed in 15 of 15 patches.

As shown previously (9), patch excision often resulted in the formation of a vesicle rather than a membrane patch. Because the intracellular surface of the vesicle was not accessible to the bathing solution, the characteristic decline due to nucleotide washout of single-channel K^+[ACh] (e.g., Fig. 2*A*, trace 1 and *B*, trace 1) and activation of K^+_{ATP} currents did not occur. Vesicles could sometimes be converted to patches by exposure to air (6), and a successful conversion was marked by the reappearance of single-channel currents, which responded to wash in or wash out of nucleotides. We were careful to exclude the possibility that the trypsin effect (Fig. 1*A*) was an artifact arising from trypsin-induced vesicle conversion by demonstrating complete access to the intracellular surface of the patch from the bathing solution before trypsin application. The signs of free access were *1*) activation of the K^+_{ATP} currents following excision into ATP-free solution (Fig. 1*A*, trace 2) and *2*) reversible GTP-dependent activation of K^+[ACh] currents (Fig. 1*A*, traces 3 and 4). Furthermore, unlike the

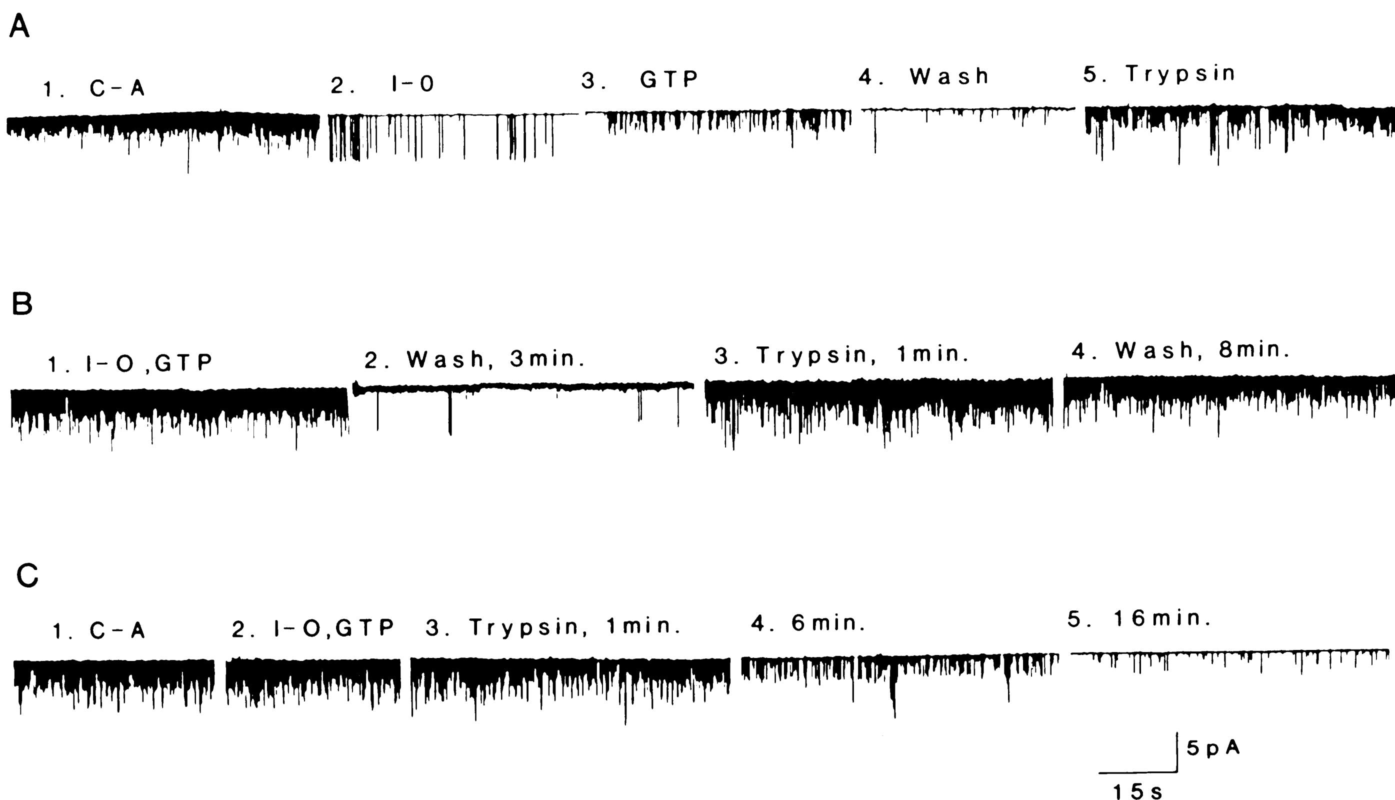

FIG. 1. Effects of guanosine 5′-triphosphate (GTP) and trypsin on single-channel muscarinic atrial K^+-channel currents in inside-out patches excised from neonatal rat atrial myocytes. *A*: currents were recorded as *trace 1*) in cell-attached (C-A) mode with carbachol at 1 μM in pipette; *trace 2*) in inside-out (I-O) mode 40 s after excision of patch into nucleotide-free bathing solution; *trace 3*) 15 s after addition of GTP at 0.1 mM to bathing solution; *trace 4*) 4 min after beginning washout with GTP-free solution; and *trace 5*) 50 s after addition of trypsin at 0.25 mg/ml to bathing solution. Large amplitude single-channel currents in I-O mode arose from ATP-sensitive K^+ channels as described in text. *B*: currents were recorded as *trace 1*) in I-O mode with GTP at 0.1 mM in bathing solution; *trace 2*) 3 min after beginning washout with GTP-free solution; *trace 3*) 1 min after addition of trypsin at 0.19 mg/ml to bath; and *trace 4*) 8 min after beginning washout in trypsin-free solution. *C*: currents were recorded as *trace 1*) in C-A mode with carbachol at 1 μM in pipette; *trace 2*) in I-O mode with GTP at 0.1 mM in the bath; *trace 3*) 1 min after exposure to trypsin at 0.1 mg/ml; *trace 4*) 6 min after exposure to trypsin; and *trace 5*) 16 min after exposure to trypsin. Holding potential was −80 mV. Brief single-channel events were attenuated by limited frequency response of pen recorder (−3 dB at 125 Hz).

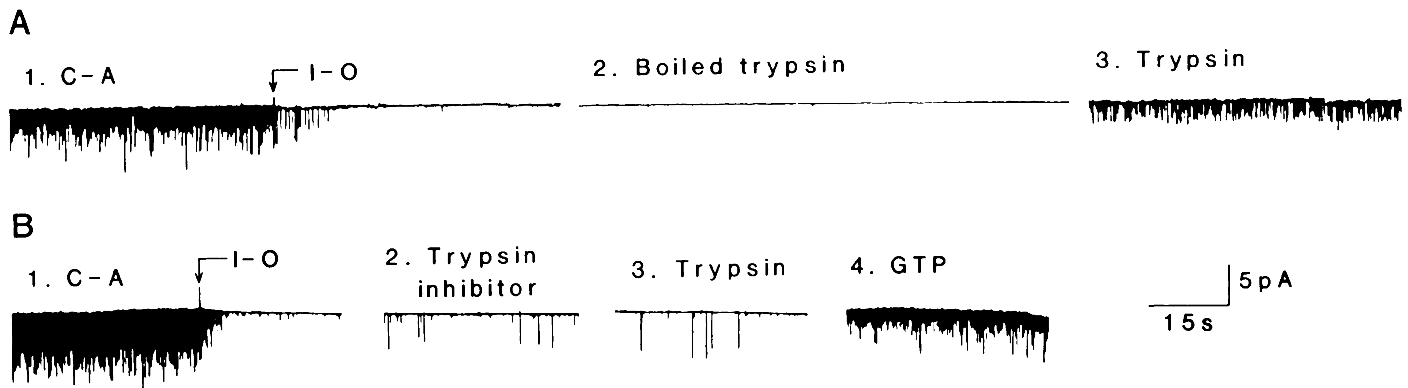

FIG. 2. Specificity of effect of trypsin on single-channel atrial K^+[ACh] currents. *A*: currents were recorded as *trace 1*) in cell-attached mode (C-A) with carbachol at 1 μM in pipette [at arrow, excision, to inside-out (I-O) configuration in guanosine 5′-triphosphate (GTP)-free bathing solution was performed]; *trace 2*) after 1 min exposure to heat-denatured trypsin at 0.15 mg/ml; and *trace 3*) after 1 min exposure to normal trypsin at 0.15 mg/ml. *B*: currents were recorded as *trace 1*) same as in *A*; C-A mode, *trace 2*) after 90 s exposure to trypsin inhibitor at 0.25 mg/ml; *trace 3*) after 2 min exposure to trypsin at 0.15 mg/ml in presence of inhibitor; and *trace 4*) 60 s after addition of GTP at 0.1 mM to bath. Other recording conditions as in Fig. 1.

GTP-dependent effect, trypsin-activation was not washed out (Fig. 1*B*) and appeared to be an irreversible modification. Similar results were obtained in two additional patches.

Prolonged exposure of the patch to trypsin resulted in a gradual diminution of channel activity (Fig. 1*C*) in the presence of agonist plus GTP. A similar decrease occurred in the presence of trypsin alone or after addition of trypsin to a patch preactivated by GTPγS. Trypsin activation of single-channel K^+ currents could be studied for extended periods if exposure to trypsin was brief (1–2 min). As shown in Fig. 1*B*, prolonged washout of patches treated in this way showed sustained channel activity.

To eliminate the possibility that the trypsin effect was caused by a contaminant in the trypsin stock solution, we performed two types of control experiments. First, the trypsin solution was heat-denatured by boiling. As shown in Fig. 2*A*, the application of trypsin (Fig. 2*A*, trace 3) but not denatured trypsin (Fig. 2*A*, trace 2) evoked K^+[ACh] currents in the absence of nucleotides. Second (Fig. 2*B*), we added a saturating concentration (0.25 mg/ml) of trypsin inhibitor (Fig. 2*B*, trace 2), which by itself had no effect on channel activity, and found that subsequent application of trypsin at 0.15 mg/ml (Fig. 2*B*, trace 3) was ineffective even though the K^+[ACh] currents were responsive to GTP (Fig. 2*B*, trace 4). Similar results were obtained in four additional experiments. The activation of K^+ currents by trypsin, therefore, was due to its enzymatic activity.

Single-channel currents are illustrated at a faster time base in Fig. 3, *A* and *B*, which compares typical records from agonist- and trypsin-activated channels in the same patch, the agonist-activated current having been recorded first. The pattern of short bursts separated by long closed intervals was characteristic of single-channel K^+[ACh] currents (18). Analysis of single-channel bursts in the experiment illustrated (Fig. 3, *A* and *B*) revealed that the closed intervals within bursts and burst length averaged 0.5 and 1.6 ms, respectively, for the carbachol-

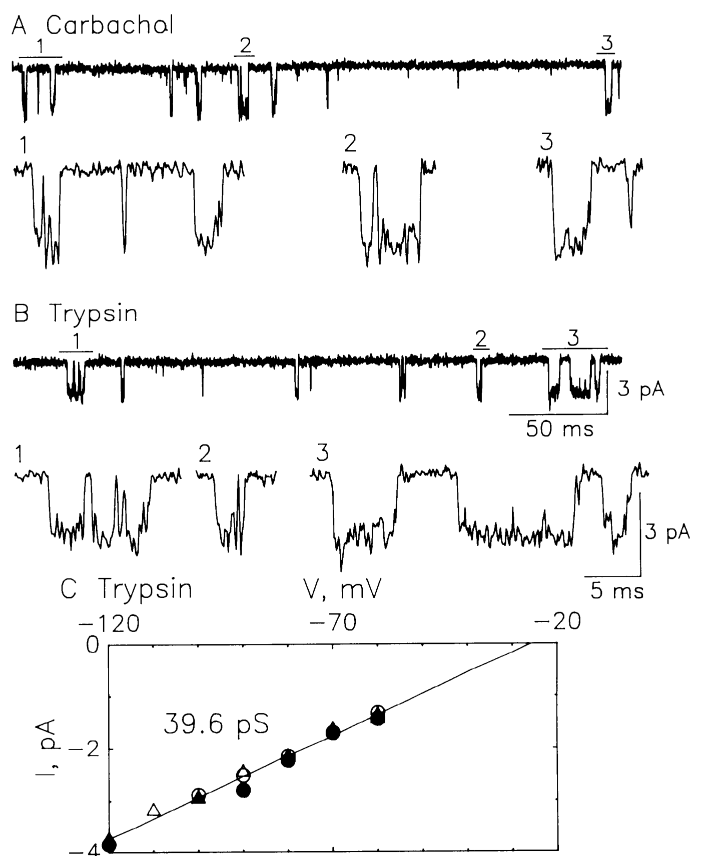

FIG. 3. Typical single-channel atrial K^+[ACh] currents activated by carbachol (*A*) and trypsin (*B*). *C*: current-voltage (*I*–*V*) relationship of trypsin-activated channels. *A*: top trace is a continuous record from a cell-attached patch with carbachol at 1 μM in the pipette. Bottom row of traces show *regions 1–3* of the continuous record at higher resolution. Same calibrations as in *B*. *B*: continuous record of trypsin-induced currents in same patch after excision into guanosine 5′-triphosphate-free solution and 2 min exposure to trypsin at 0.15 mg/ml. Holding potential −80 mV, low-pass filter 3 kHz (−3 dB). *C*: single-channel *I*–*V* relationship for trypsin-activated single-channel currents recorded in 4 patches. Slope conductance calculated by linear regression.

activated currents (Fig. 3*A*), and 0.6 and 1.5 ms, respectively, for the trypsin-activated currents (Fig. 3*B*). Mean open times in carbachol- and trypsin-activated channels were 0.6 ± 0.1 and 0.8 ± 0.4 ms ($n = 8$ patches), respectively. The slope conductance (39.6 pS, Fig. 3*C*) of the trypsin-activated channels was nearly the same as that of the agonist-activated channel (43 pS, Ref. 9). The trypsin-activated currents showed inward rectification; in isotonic KCl, in the presence of intracellular Mg^{2+}, single-channel currents disappeared at holding potentials more positive than −20 mV. The strong similarity between trypsin-activated and muscarinic agonist-activated single-channel K^+ currents suggests that short-term exposure to trypsin did not alter the gating properties of the activated channel.

Trypsin activated K^+[ACh] currents in the absence of carbachol (4 patches) or intracellular Mg^{2+} (3 patches). Hence, neither receptor occupancy by agonist nor endogenous G protein activation was necessary. This suggests that the enzyme operated directly on the K^+[ACh] channel rather than on the muscarinic receptor or G_k. To more narrowly define the trypsin-sensitive site, we tested the effects of several other group-specific protein reagents that mimicked the effect of trypsin on Na^+ channels (4). In K^+[ACh] channels, however, these other agents [papain (0.5 mg/ml, $n = 4$), glyoxal (5 mM, $n = 3$, applied at pH 9 optimal for arginine-specific reaction), and phenylglyoxal (1 mM, $n = 3$, applied at pH 7)] were found to be ineffective. Glyoxal and phenylglyoxal share with trypsin a specificity for arginine residues, suggesting that the trypsin effect on K^+[ACh] channels, unlike the effect on Na^+ channels, was achieved via cleavage at lysine residues.

DISCUSSION

Our main finding is that intracellular trypsin treatment converts K^+[ACh] channels from a resting, closed state to an activated, bursting state indistinguishable from that achieved by channel activation via the muscarinic receptor and G_k. The effects of trypsin were easily distinguished from the nonspecific noise currents often observed in partially disrupted membrane patches. Such noise does not have the well-defined unitary conductance, inward rectification, and bursting kinetics of single-channel K^+[ACh] currents.

The most plausible mechanism of trypsin's action is that it acts directly on the K^+[ACh] channel to cleave an inhibitory component at an intracellularly accessible site. Our main evidence is that trypsin-induced activation occurred in the absence of any of the requirements of the normal activation pathway, including ligand, nucleotides, and Mg^{2+}. A similar mechanism has been proposed for the action of proteolytic enzymes to remove inactivation from voltage-dependent Na^+ (1) and K^+ (13) channels in neurons and to cause irreversible, Ca^{2+}-insensitive activation of Ca^{2+}-dependent K^+ channels in paramecia (10). Alternatively, trypsin could act indirectly by modifying regulatory proteins that are separate from the channel itself, such as G_k or phospholipase A_2. G proteins are unlikely sites of action because trypsin has been shown to inactivate G proteins purified from brain (23), and the trypsin effect on K^+[ACh] channels occurred in the absence of Mg^{2+} or nucleotides, both of which are needed to activate G proteins under normal conditions. Phospholipase A_2, which stimulates K^+[ACh] channels via the arachidonic acid pathway (8, 11), also is unlikely, since this pathway requires GTP (11). Other as yet unexplored possibilities might include dephosphosphorylation of the channel through a trypsin-catalyzed activation of a protein phosphatase. However, in the absence of evidence for such a mechanism, we favor the idea that trypsin acts directly on the channel.

The interaction of trypsin with the K^+ channel appears to be a covalent modification at one or more intracellularly accessible lysine residues, since activation is irreversible and is blocked by enzyme denaturation or pretreatment with trypsin-specific inhibitor. Lysine rather than arginine residues may be involved, since arginine-specific reagents were found to be ineffective. However, it is possible that channel activation requires cleavage at a number of sites, and trypsin is effective simply because it is less selective. It is very unlikely that extracellular trypsin cleavage sites would have a similar effect, since our tissue-cultured cells were dissociated by trypsinization and muscarinic K^+ channel currents were not present in cell-attached patches in the absence of agonist.

We conclude, therefore, that muscarinic K^+ channels in unstimulated membranes are inhibited from opening by a gating component that contains at least one trypsin cleavage point located near the intracellular surface. A physically distinct, trypsin-resistant, G_k-independent gating mechanism governs the characteristic single-channel open time and burst duration of muscarinic K^+ channels because such bursting persists in the presence of trypsin without G protein activation. Inhibitory regulation of G protein targets is not without precedent. Guanosine 3′,5′-cyclic monophosphate phosphodiesterase, the effector for transducin, is normally inactivated by its γ-subunit. Trypsin cleaves the γ-subunit activating the phosphodiesterase (7, 14). Hence, it is possible that effectors for which G protein activation is obligatory are normally regulated by inhibitory subunits.

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