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A9 Fibroblasts Transfected with the m3 Muscarinic Receptor Clone Express a Ca^{2+} Channel Activated by Carbachol, GTP and GDP

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Abstract. Muscarinic m3 receptor-mediated changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) occur by activation of Ca^{2+} release channels present in the endoplasmic reticulum membrane and Ca^{2+} entry pathways across the plasma membrane. In this report we demonstrate the coupling of m3 muscarinic receptors to the activation of a voltage-insensitive, cation-selective channel of low conductance (3.2 ± 0.6 pS; 25 mM Ca^{2+} as charge carrier) in a fibroblast cell line expressing m3 muscarinic receptor clone (A9m3 cells). Carbachol (CCh)-induced activation of the cation-selective channel occurred both in whole cell and excised membrane patches (CCh on the external side), suggesting that the underlying mechanism involves receptor-channel coupling independent of intracellular messengers. In excised inside-out membrane patches from nonstimulated A9m3 cells GTP (10 μM) and GDP (10 μM) activated cation-selective channels with conductances of approximately 4.3 and 3.3 pS, (25 mM Ca^{2+} as charge carrier) respectively. In contrast, ATP (10 μM), UTP (10 μM) or CTP (10 μM) failed to activate the channel. Taken together, these results suggest that carbachol and guanine nucleotides regulate the activation of a cation channel that conducts calcium.

Key words: Ca^{2+} channel — Receptor — Carbachol — Acetylcholine — Voltage-independent Ca^{2+} channel — Cation channel

Introduction

Changes in cytosolic free-calcium concentration, $[Ca^{2+}]_i$, are regulated, in part, through the opening and closing of calcium channels located in the plasma membrane (Tsunoda, 1993; Felder, Singer-Lahat & Mathes, 1994; Clapham, 1995). Ca^{2+} influx through voltage-insensitive Ca^{2+} channels has been shown to be present in different cell types such as human platelets (Sage & Rink, 1987; Merritt et al., 1990; Mahaut-Smith, Sage & Rink, 1990; Rink & Sage, 1990), rat smooth muscle (Sage & Rink, 1987; Benham & Tsien, 1987), human endothelial cells, human neutrophils (Merritt, Jacob & Hallam, 1989), rat parotid (McMillian et al., 1988), murine thymocytes (El-Motassim et al., 1989), rat mast cells (Hoth & Penner, 1992), rat lymphocytes (Mason, Mahaut-Smith & Grinstein, 1991), mouse neuronal cells (Mathes & Thompson, 1994), rat neonatal cardiomyocytes (Merie, Feige & Verdetti, 1995) and human T cells (Zweifach & Lewis, 1993), suggesting the existence of a new class of voltage-insensitive Ca^{2+} channel.

Depending on the mechanism of activation, voltage-insensitive Ca^{2+} channels can be grouped in the following types (Tsunoda, 1993; Felder et al., 19940: (i) Channels activated by second messengers following depletion of intracellular Ca^{2+} stores. (ii) Calcium channels which are activated by second messengers following receptor stimulation. (iii) Calcium channels which are directly operated by a receptor. Due to their extremely low conductance, voltage-insensitive Ca^{2+} channels have evaded direct measurement and only recently has the electrophysiological characterization of these currents been possible. Most of the voltage-insensitive Ca^{2+} channels characterized to date belong either to the depletion-operated Ca^{2+} channel group or to the second messenger-operated Ca^{2+} channel group, but very little is currently known about the receptor-operated Ca^{2+} channel group. The first direct measurement of a receptor operated-Ca^{2+} channel was performed in smooth muscle cells, where

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ATP stimulated the opening of a low conductance calcium-selective channel that was both second messenger- and voltage-insensitive (Benham & Tsien, 1987). Little is known, however, about the mechanism of coupling between the receptor and channel proteins and if this type of channel is present in nonexcitable cells. We have described a marked elevation of Ca^{2+} influx following muscarinic m3 receptor activation in murine fibroblasts that is also independent of second messenger release and changes in intracellular calcium, and appeared to be of the receptor-operated Ca^{2+} channel type (Felder, Poulter & Weiss, 1992). The muscarinic receptor-activated calcium influx was shown to play an important role in the transduction of signaling mediated by phospholipase A_2, phospholipase Cγ, tyrosine kinase and phospholipase D (Felder, 1995). Furthermore, recent studies have linked muscarinic receptor-stimulated calcium influx to the reversal of CHO cells from a tumorigenic to a nontumorigenic phenotype (Felder et al., 1993; Singer-Lahat, Ma & Felder, 1996).

To further characterize Ca^{2+} influx stimulated by muscarinic receptors, whole-cell and single-channel currents were recorded. We report here that external application of the muscarinic agonist, carbachol, can activate a cation-selective channel in excised outside-out membrane patches. In addition, GTP or GDP can also activate cation-selective channels in inside-out membrane patches. Furthermore, GTP- or GDP-activated cation channels exhibited characteristics (conductance and kinetics) which were remarkably similar to those of the carbachol-activated cation channels. Taken together, these results suggest that CCh, GTP and GDP regulate a common cation channel which conducts calcium. The negative results with other nucleotides further suggest the involvement of a guanine nucleotide binding domain that modulates channel activity.

Materials and Methods

Materials and Electrophysiological Solutions

Whole-cell membrane currents and single-channel currents were recorded from outside-out excised membrane patches as described elsewhere (Kukuljan et al., 1992). The extracellular medium was (in mM): 111 NaCl or 131 TEACl 5.4 KCl, 25 $CaCl_2$, 0.8 $MgSO_4$, 5 glucose, 10 NaHepes at pH 7.4. The inner aspect of the membrane was always exposed to a solution designed to minimize ion flow through K^+ channels (in mM: 18 CsCl, 70 $CsSO_4$, 5 $MgCl_2$, 10 NaHepes, pH 7.2).

Carbachol was purchased from Sigma. GTP, GTPγS, GDP and GDPβS were purchased from Boehringer Mannheim (Indianapolis, IN).

Cell Culture

A9 fibroblasts were obtained from ATCC (American Type Culture Collection, MD) and transfected with the muscarinic cholinergic receptor m3 clone as described elsewhere (Bonnel et al., 1988). A day before the experiment, cells were plated on 35 mm dishes (Corning Glass Works, NY) at a density of 70,000 cells/ml (3 ml/dish) and cultured at 37°C in an atmosphere of 90% air and 10% CO_2.

Electrophysiology

Patch-clamp experiments were carried out at room temperature (20–25°C). Measurements of membrane potential (V_m), under current-clamp conditions or membrane current (I_m), under voltage-clamp conditions were made using an EPC-7 patch-clamp amplifier (List-Electronics, Darmstadt-Eberstadt, Germany). Patch pipettes were pulled from soda glass capillary tubes using a BB-CH-PC puller (Mecanex, Switzerland). For single-channel current recordings, the pipettes were coated to the tip with slygard (Corning, NY). Pipettes filled with solutions had tip resistances from 4 to 6 MΩ. V_m and I_m signals at the outputs of the EPC-7 amplifier were made using a 4 channel analog magnetic tape recorder (frequency response 0-25 kHz). The corresponding records were digitized using the TL-1, 12-bit analog converter board interface controlled by a Labmaster DMA board (Axon Instruments, Saratoga, CA) and data analyzed with Axotape 2.2 and pClamp 5.51 software (Axon Instruments, Saratoga, CA). A low pass 8-pole Bessel filter set at 200 Hz was used to filter the current output of the feedback amplifier. Single-channel analysis was performed using TRANSIT (A.M.J. VanDongen, Duke University, Chapel Hill, NC; Ottolia & Toro, 1994).

Results

To insure extensive dialysis of cytoplasmic components which might be second messengers, cells were dialyzed for at least 15 min prior to measurement of whole-cell ion currents.

Inward Current Activated by CCh in Fibroblasts Expressing m3 Muscarinic Acetylcholine Receptor

Under voltage-clamp conditions, with the membrane potential held at the reversal potential for $Cl^-/[Cl]_o$ = 166.4 mM; $[Cl]_i$ = 16 mM; V_{rev} = −60 mV), the holding current remained unchanged in the presence of Cs^+ solution. After an equilibration period, CCh-induced changes in holding current were investigated. As shown in Fig. 1*A*, upon application of the agonist, a transient inward current was detected. Since the membrane potential was held at the V_{rev} for Cl^-, the inward current elicited by CCh must be carried by either Na^+ or Ca^{2+}, or both. The average peak value of the transient current was −30.7 ± 3.5 pA (n = 3).

Plasma membrane resident voltage-insensitive channel gating depends on receptor occupancy (Tsunoda, 1993). Figure 1*B* depicts a family of superimposed current records in response to single pulses taking the membrane potential to different levels (from −55 to −95 mV in 5-mV increments). Since each voltage step was 85 msec in duration, we observed only transient currents (the first peak of the ion response in Fig. 1*A*). It is im-

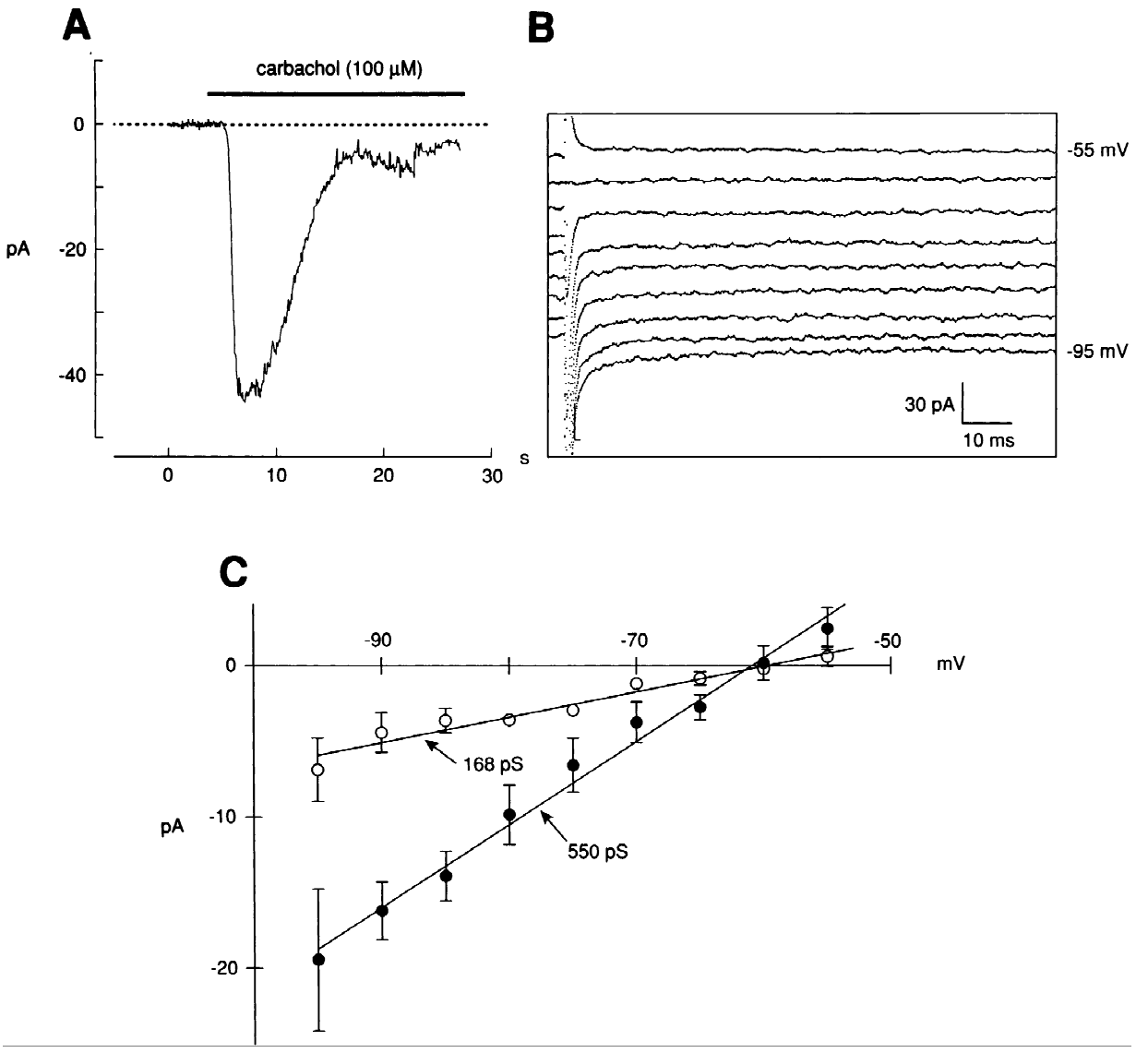

Fig. 1. CCh-activated inward current in the whole cell configuration. Membrane potential was held at −60 mV corresponding to the reversal potential for Cl^-. (*A*) Temporal course of inward current elicited by external application of CCh (100 μM). Data shown are a representative record selected from at least 3 different cells. (*B*) Superimposed whole cell current records in response to single pulses (107 msec duration) taking the membrane potential to different levels in 5- mV increments. The absolute membrane potential during the pulses is indicated in mV next to the corresponding trace. A low pass 8-pole Bessel filter set at 10 Hz was used to filter the current output of the feedback amplifier. Data shown are representative recordings from at least 3 different cells. (*C*) *I-V* curves in the absence (○) and in the presence of CCh (●). The vertical axis represents the mean ($n = 3$) value of the current at 100 msec after the onset of the pulses has been corrected to take into account the holding current.

mediately apparent that the current flowing through open channels is rather insensitive to the transmembrane potential during the pulses (Fig. 1*C*). As shown in Fig. 1*B*, the time course of the current at different potentials remained unchanged. *I-V* curves from nonstimulated (○) and CCh-stimulated (●) cells were linear with a slope conductance of 168 and 550 pS, respectively (Fig. 1*C*). Thus, CCh stimulation of A9m3 cells induced a conductance increase of approximately 382 pS. The records from CCh-stimulated cells were acquired when the transient current depicted in Fig. 1*A* reached its maximum level. The experiments described in the following section were designed to determine the characteristics of the single channels involved in CCh-induced currents.

CCh-INDUCED Ca^{2+} CHANNEL ACTIVITY IN A9m3 CELL MEMBRANE PATCHES

The microscopic characteristics of the CCh-induced current were examined in outside-out excised membrane

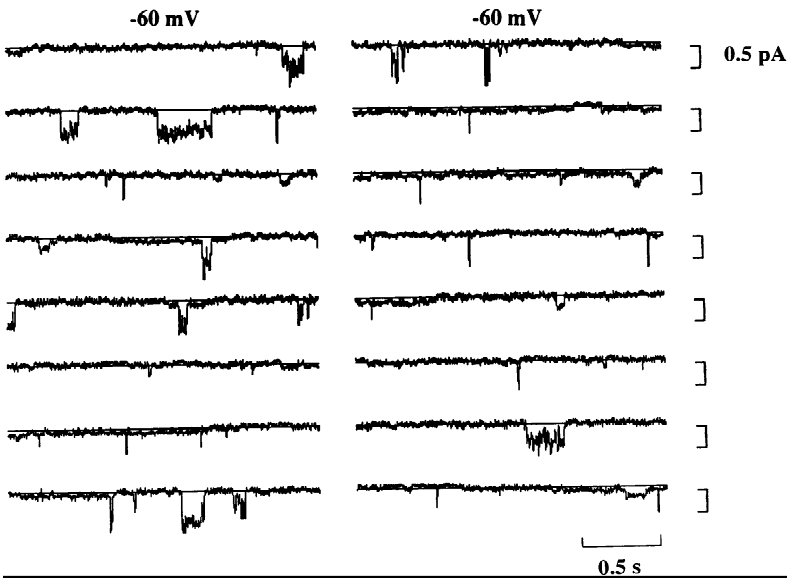

Fig. 2. CCh-activated channel activity. Outside-out excised membrane patch from a resting A9m3 cell. These are representative steady sequential traces at a membrane holding potential of −60 mV. The records were filtered at 200 Hz. Data are representative of at least 5 cells.

patches. Nonstimulated A9m3 cells were internally dialyzed for approximately 10 min. Outside-out membrane patches were then excised and single-channel activity was recorded during a 9-min control period. Prior to the application of CCh, channel events were infrequent. Figure 2 demonstrates ion channel activity in the presence of carbachol while the membrane potential was held at −60 mV. After a brief delay, CCh stimulated channel activity (Fig. 2; left panel, top record). Furthermore, the channel remained active for many minutes after a complete washout with CCh-free solution, and after the application of atropine (100 μM), a muscarinic receptor antagonist (*data not shown*).

Amplitude histograms (Fig. 3*A*) calculated from the data in Fig. 2 revealed 3 components with mean amplitudes of −0.2, −1.6, and −1.9 pA. Single-channel conductance (γ_{Ca}) estimated from the corresponding *I-V* curve was 3.2 ± 0.6 pS 25 mM Ca^{2+} (*data not shown*). Single-channel conductance was insensitive to the addition of Na^+ as charge carrier (in mM: 121 Na^+, 25 Ca^{2+}). Under these conditions the average single-channel conductance was 3.5 ± 0.3 pS (*data not shown*). Open and close time distributions are shown in Fig. 3*B* and *C*, respectively. Two time constants were required to fit the open time histogram in Fig. 3*B*, 8.3 and 59.7 msec. The close time histogram in Fig. 3*C* also required two time constants, 2.8 and 838 msec.

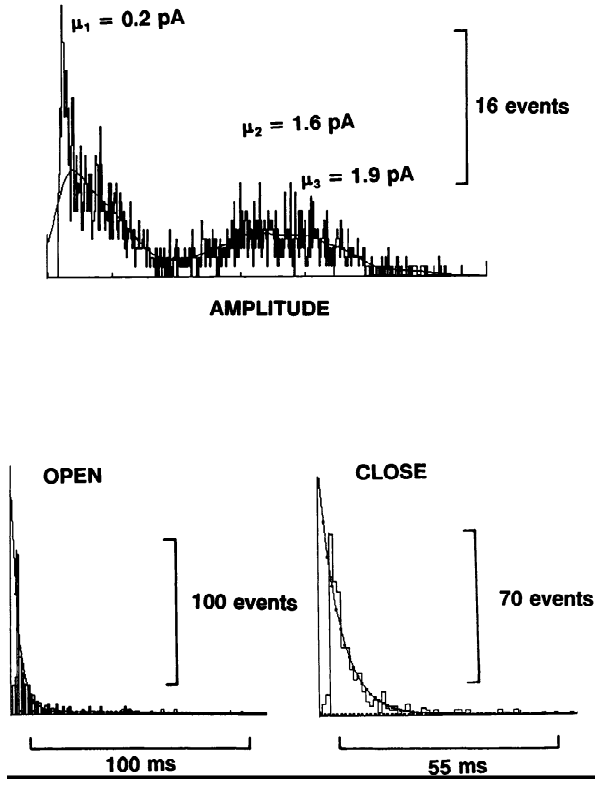

Fig. 3. Amplitude, open-time and close-time characteristics of the channel activated by CCh. Data were obtained from the a trace of current that part of it is shown in Fig. 2. (*A*) Amplitude histogram. The smooth curve was calculated to fit the channel amplitude data and revealed 3 levels (μ). (*B*) Open-time distribution. Two time constants were required to fit the histogram (ι_1 = 8.4 and ι_2 = 59.7 msec). (*C*) Close-time distribution. Two time constants were required to fit the histogram. (ι_1 = 2.8 and ι_2 = 838 msec).

Effects of Guanine Nucleotides on the Activity of the Channel

To test the involvement of G-proteins in activation of the ion channel, the internal aspect of the cell membrane was exposed to GTP or GTPγS (10 μM) and channel events

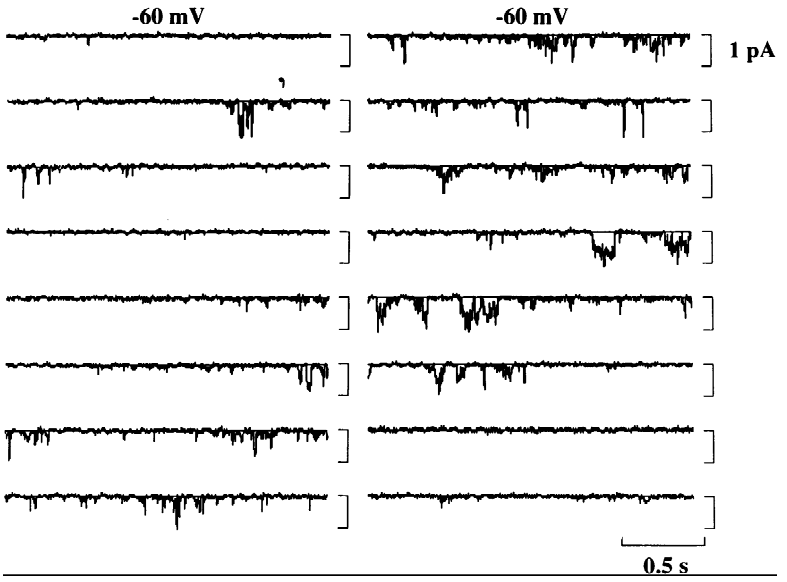

Fig. 4. GTP-evoked channel activity. The channel activity was initiated by GTP (10 μM) recorded from an inside-out membrane patch. These are representative steady sequential traces at a membrane holding potential of −60 mV (cytosolic side positive). The patch was exposed to the same solutions (internal and external) as in Fig. 2. Records filtered at 200 Hz. Data are representative of at least 4 cells.

were recorded. In these recordings the same solutions were used as for the carbachol-activated currents in outside-out excised membrane patches (Fig. 2). Prior to the application of guanine nucleotides only infrequent channel openings were observed when the membrane potential was held at the reversal potential for Cl (−60 mV). This type of infrequent activity remained unaltered by shifting the holding potential to +60 mV (intracellular side positive). In contrast, application of GTP (10 μM) to the intracellular side of the membrane patch evoked a profound stimulation of the channel activity. Figure 4 shows ion channel events in the presence of GTP. Holding the membrane potential at +60 mV, 120 mV more positive than the reversal potential for Cl^-, the amplitude of the single-channel event was 0.2 ± 0.03 ($n = 6$; *data not shown*). Single-channel conductance (γ_{Ca}) with 25 mM Ca^{2+} as a charge carrier estimated from the *I-V* curve was 4.3 ± 0.8 pS. Two time constants were required to fit the open-time histogram (3.1 and 23.7 msec) and the close time histogram (19.2 and 470 msec) revealed from the channel events that are shown in Fig. 4.

We tested the ability of GDP and the nonhydrolyzable analogue GDPβS, to block the coupling of m3 receptor to channel activation. However, instead of blocking, GDP and GDPβS (10 μM) applied to the cytosolic side of the membrane (Fig. 5) activated a cation-selective channel. Shown in Fig. 5 are 12 segments of a continuous record of the channel activity recorded immediately after the application of GDP (10 μM). Single-channel conductance (γ_{Ca} estimated from the corresponding *I-V* curve was found to be 3.2 ± 0.8 pS (25 mM Ca^{2+} as charge carrier). Thus, the single-channel conductance activated by GTP, GDP or GDPβS ranges from 3.2 to 4.3 pS. Two time constants were required to fit the open-time histogram (2.2 and 30.6 msec) and the close time histogram (6.4 and 503 msec) revealed from the channel events that are shown Fig. 5.

Discussion

We report here compelling evidence suggesting that the m3 receptor might be coupled to a cation-selective channel which is insensitive to transmembrane potential. The underlying mechanism might involve an as yet unidentified GTP binding protein.

Application of CCh from the extracellular side of the A9 fibroblast membrane, both in whole-cell and excised outside-out patches, activated a cation-selective channel of low conductance (*ca.* 3.2 pS with 25 mM Ca^{2+} as charge carrier). It was previously shown in chromaffin cells (Pusch & Neher, 1988), that the time constant of exchange between the cell and pipettes with resistance of 10 mΩ was 1 min for substance of molecular weight around 1,000. Since the cells were dialyzed for at least 15 minutes prior to measurement of whole-cell ion currents, the concentration of diffusible messengers on the cytosolic side of the membrane was probably diluted to a minimal level. Thus, the CCh-activated ion channel activity is most likely a membrane delimited process.

Transitory Early and Delayed Sustained Inward Cation Current Evoked by CCh

CCh can activate an early, transient inward current (peak conductance 382 pS) which is followed by a sustained smaller inward current (external solution in mM: 25 Ca^{2+} and 121 Na^+). Since the membrane potential was held at the calculated reversal potential Cl^-, the inward current

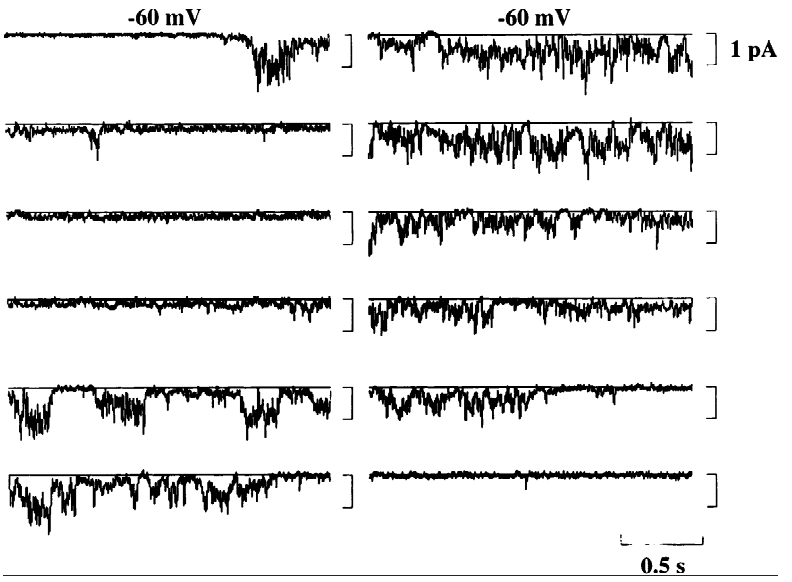

Fig. 5. GDP (10 μM)-evoked channel activity. Experimental conditions as in experiment described in Fig. 4. These are representative steady sequential traces at a membrane holding potential of −60 mV Records filtered at 200 Hz. Data are representative of at least 5 cells.

is most likely to be carried out by either Ca^{2+}, Na^+ or both.

Assuming that CCh activates only one type of cation channel, the early transitory component of the inward current must result from the activity of a large number of such channels. The transitory nature of the inward currents suggests that in the maintained presence of the nonhydrolyzable agonist, CCh, the coupling mechanism partially inactivates. This kind of inactivation was not observed when CCh was added to the extracellular solution and activated the cation channels. Moreover, activation of the cation channel could not be blocked by atropine or by washout of CCh. Therefore, it is possible that in excised patches an intracellular component involved in cation modulation is missing.

GTP-binding Proteins Might be Involved in the Activation of the Cation Channels

As shown in the Table, application of GTP, GDP and GDPβS to the internal aspect of the cell membrane resulted in the rapid activation of the channel. It should be noted that the single channel parameters, i.e., conductance, mean open time and mean closed time, for the channel activity evoked by external application of CCh, internal application of GTP and GDP (or GDPβS) are very similar (Table). CCh-activated channel conductance of 3.2 pS is identical to that obtained with GDP (or GDPβS). In addition, channel kinetics are similar in the three situations (open time and close time; *see* Table). Activation of the cation channel by CCh, GTP, or GDP revealed bursts of channel events with a long opening time (Figs. 2, 4, and 5) and long close time constants (Table) which might be characteristic of this channel. Another possibility is that the low frequency of the filter used during the experiment masked short openings of the channel. Since we are dealing with a cation channel with a very small conductance, we were forced to use this filter to overcome excessive noise. Low concentrations of guanine nucleotides were required to open the cation channels. This explains the ability of CCh to activate the cation channel in excised membrane patch configuration. Application of GTP or GDP to the bath in the inside-out configuration patch, after activation of the cation channel with CCh (in the presence of CCh in the pipette), did not attenuate activation of the channel (*data not shown*) which suggests that the same type of channel is activated by the three compounds. The observation that ATP, CTP or UTP were without effect is further evidence that the domain on the channel or proteins that control the opening of the cation channel specifically requires guanine nucleotide in order to activate the channel.

Activation of the channel by GDP and the nonhydrolyzable analogue GDPβS is not consistent with the G-protein mechanism. Indeed, it is well established that binding of GDP (or GDPβS) to the α-subunit of heterotrimeric G-proteins renders the α-subunit inactive and prevents G-protein mediated receptor-channel coupling. However, it was shown previously in cardiac sarcolemmal membranes that application of GDP and GDPβS inhibits adenylate cyclase in a similar way as application of CCh or GTP (Quist, Powell & Vasan, 1991). Our data further support the suggestion that the muscarinic receptor-effector coupling mechanism may be unique in some tissues (Quist et al., 1991). Another novel interaction between a G-protein-coupled receptor and Ca^{2+} channel was shown in Dictyostelium in which G-proteins were not involved in the interaction between the receptor and the Ca^{2+} channel (Milne et al., 1995).

Table Comparison of the kinetics characters of the cation channels that are activated by carbachol (100 μM), GTP (10 μM) or GDP (10 μM)

Property	Carbachol	GTP	GDP/GDPβS
Number of cells (*n*)	5	4	5
Single-channel current (pA; membrane potential at −60 mV)	−0.15 ± 0.04	−0.2 ± 0.03	−0.14 ± 0.003
Conductance (pS)	3.2 ± 0.6	4.3 ± 0.8	3.2 ± 0.8
Open-time constants (msec)	3.2 ± 1	3.9 ± 1	4.5 ± 2
	42.4 ± 10	24.6 ± 12	46 ± 20
Close-time constants (msec)	6.8 ± 1	14 ± 4	5.4 ± 2
	748 ± 249	684 ± 200	443 ± 98

Values are mean ± SEM. Two time constants were required to fit the open-time and close-time histograms.

Potential Physiological Roles of Muscarinic Receptor-operated Ca^{2+} Channels

Receptor-operated Ca^{2+} channel may provide the predominant source of calcium required for the stimulation of extracellular calcium-dependent effector enzymes such as tryosine kinase and phospholipases A2, D, and Cγ (Felder, 1995). It is unlikely that the carbachol-stimulated channel activity observed in excised patches plays a significant role in refilling of cytoplasmic calcium pools, as this process is thought to be under the regulation of diffusible messengers (Brown & Birnbaumer, 1990; Felder et al., 1994). Recent studies (Felder et al., 1993; Singer-Lahat et al., 1996) linked carbachol-activated Ca^{2+} channels to the reversal of CHO cells from a tumorigenic to a nontumorigenic phenotype, suggesting their role in a variety of physiological processes.

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