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Regulation of a Potassium-Selective Current in Rabbit Corneal Epithelium by Cyclic GMP, Carbachol and Diltiazem

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Summary. The effects of cyclic GMP (cGMP), carbachol and diltiazem on a potassium-selective, delayed-rectifier current in freshly dissociated rabbit corneal epithelial cells were studied using a modified perforated-patch-clamp technique. The current was stimulated by both 500 μM cGMP (2.3–4.5-fold, mean = 2.9) and 250 nM carbachol, a muscarinic agonist (1.12–7.04-fold, mean = 3.8), and the stimulated current was totally blocked by diltiazem (10 μM). The effects of cGMP appeared to be, at least in part, different from those of carbachol as they required the presence of external calcium. Single-channel data suggest that cGMP and carbachol activate the potassium current by increasing the open probability of the channel via a second-messenger system and that the action of diltiazem is probably through a direct blocking effect on the open channel.

Key Words rabbit corneal epithelium · potassium current · cyclic GMP · carbachol · diltiazem · patch clamp

Introduction

The apical cell layers of the rabbit corneal epithelium largely exhibit chloride conductance (Klyce, 1975), while the basolateral cell layers are the major site of potassium conductance (Nagel & Reinach, 1980). The basolateral cells can be reliably identified in a dissociated-cell preparation by their smaller size and have been shown to contain both a nonselective-cation channel, found only in excised patches, and a large-conductance (167 pS in 150 mM K), stretch-activated potassium channel (Rae et al., 1990; Rae, Dewey & Rae, 1992). Recordings from these cells, patch clamped in a whole-cell configuration, have demonstrated a potassium-selective, delayed outward-rectifying current. This current appears to be made up of at least three components: a large K channel identical to the one seen in single-channel recordings, a time-dependent smaller channel and a component due to "leak." This whole-cell current is blocked by barium and quinidine and is found in practically all cells patched (Rae & Farrugia, 1992). Furthermore, Reinach reported that in whole bullfrog corneas bathed in NaCl Ringer, the basolateral potassium-ionic conductance is inhibited by diltiazem and that this inhibition is greater than that of barium (Reinach, 1985).

Corneal epithelium in tissue preparations has been shown to contain acetylcholine concentrations that are among the highest in the body (Williams & Cooper, 1965), and recently muscarinic cholinergic receptors have been shown to be present in human corneal epithelium (Walkenbach & Ye, 1991). Stimulation of these receptors has been shown to result in up to threefold stimulation of intracellular cGMP levels. The mechanism of action of muscarinic receptors in rabbit corneal epithelium is unknown. In other tissues, mechanisms include inhibition of adenylate cyclase (Gilman, 1984; Katada et al., 1984), activation of inositol phosphates (Berridge, 1984), and a direct action on potassium (Loffelholz & Pappano, 1985) and calcium channels (Trautwein, McDonald & Tripathi, 1975).

We report here our results on the activation of the whole-cell potassium current by cGMP and carbachol, as well as its blockade by diltiazem, using the patch-clamp technique.

Materials and Methods

All measurements were made from adult New Zealand White rabbits with an average weight of 2.5 kg. The rabbits were euthanized with an overdose of a barbiturate (Sleepaway, Fort Dodge Laboratories) injected into a marginal ear vein. All animals were handled according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research. Single-cell preparation was identical to that described in the previous paper (Rae & Farrugia, 1992). Briefly, the corneal epithelium was placed in 2.4 μg/ml dispase and incubated for 70

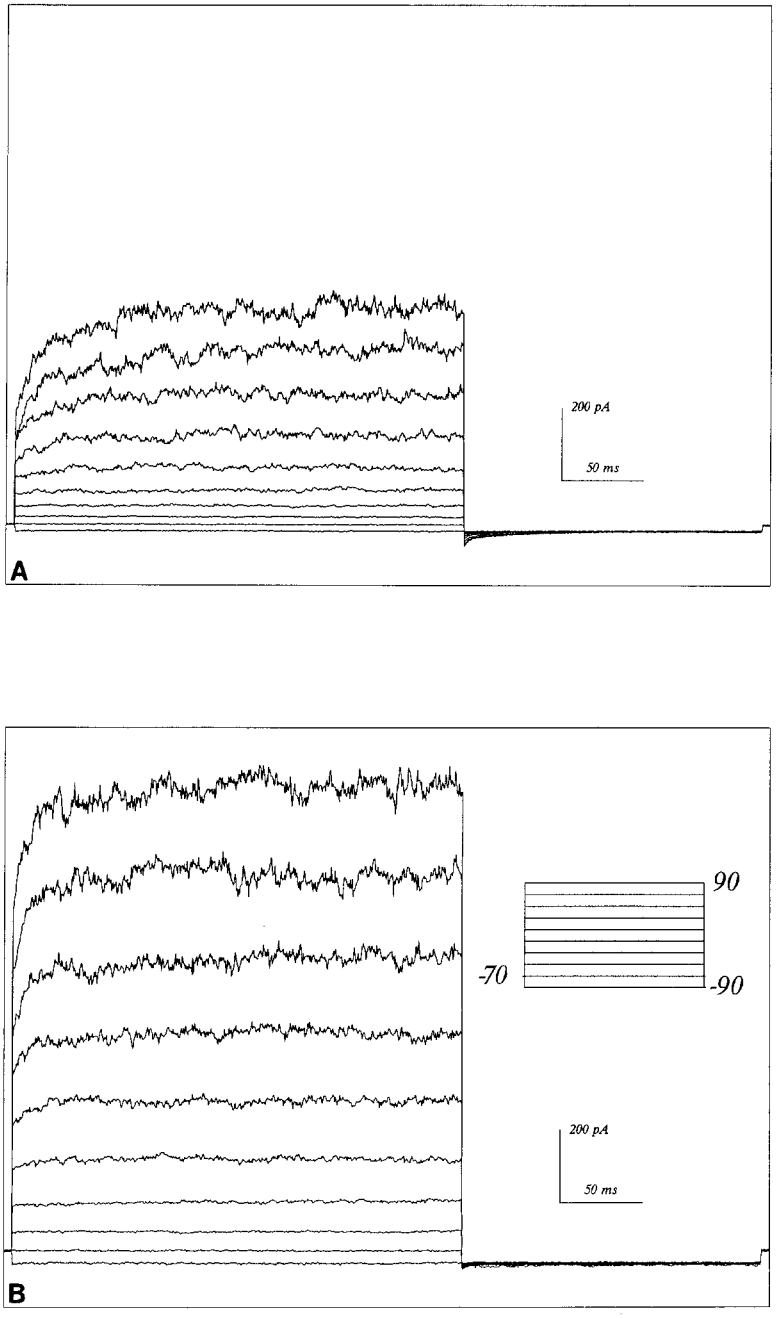


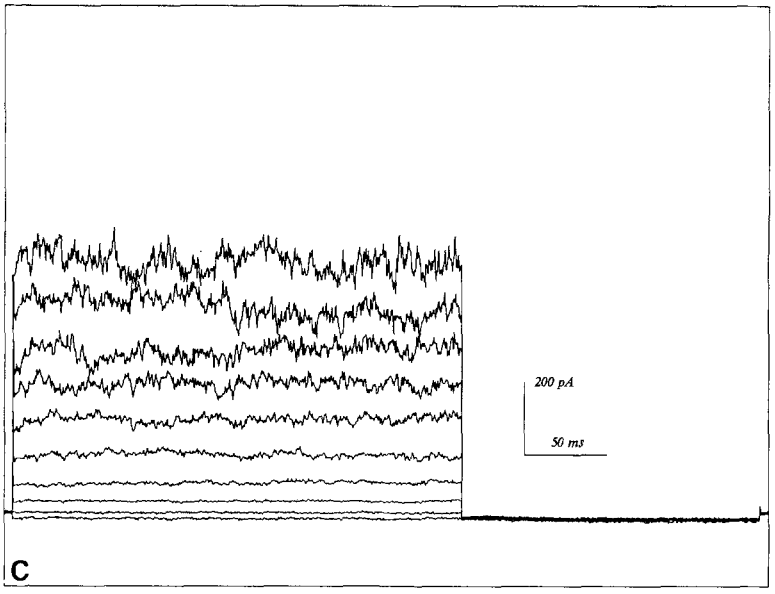
Fig. 1. Activation of the K current with 500 μM cGMP. (*A*) Unactivated current. (*B*) Current 10 min after application of cGMP showing a 2.3-fold increase in the current.

min. The preparation was then triturated in 0.1% trypsin to obtain single cells, which were resuspended in NaCl Ringer.

All cells were used within 4 hr of preparation. The cells were patch-clamped in a custom-built 300-μl chamber, after they adhered to a 4- by 8-mm piece of coverslip glass placed at the bottom of the chamber.

The patch electrodes were made from Kimble KG-12 glass (Garner Glass, Claremont, CA) and pulled on a Sutter puller P.80/PC (Sutter Instruments, Novata, CA). The electrodes were Sylgard® #184 (Dow Corning, Midland, MI) coated and, after fire polishing, had resistances of approximately 3 MΩ. Recordings were made using an Axopatch 1B patch-clamp and the data recorded on a modified IBM AT computer via a TL-1 A/D-D/A interface (Axon Instruments, Foster City, CA). The current records were filtered at 1 kHz through a 4-pole Bessel filter and were sampled at 500 μsec per point.

Whole-cell recordings were made using a modified perforated-patch-clamp technique (Rae et al., 1991). The electrode tips were filled with an amphotericin-free intracellular solution to a distance of about 300 μm and then backfilled with the same solution containing amphotericin B at a concentration of 240 μg/ml. This allowed seal formation and subsequent amphotericin B-induced pore formation. Partition times varied from 5–15 min, and the final access resistances averaged 10 to 15 MΩ.



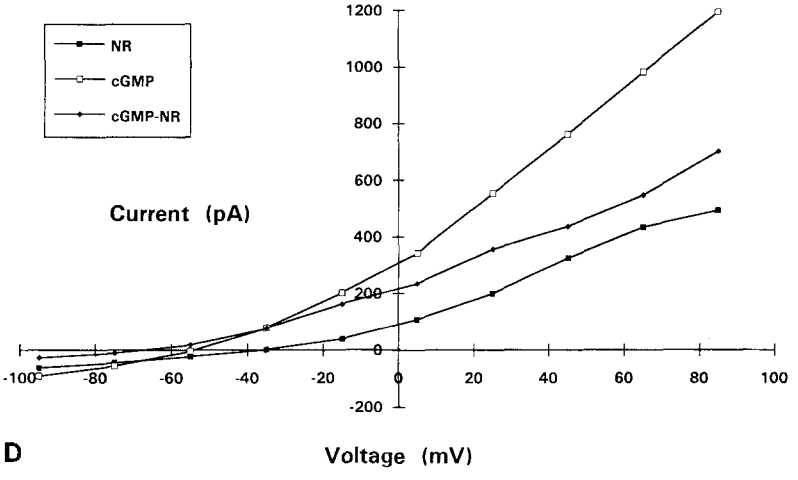


Fig. 1.—Continued. (*C*) Current obtained after subtracting the initial current from the activated one (the difference current), and (*D*) steady-state *I-V* relationships for these currents. Note the shift in the resting membrane potential from −38 to −55 mV with the difference current (*cGMP-NR*) reversing at −68 mV.

Solutions

Ringer's solution contained (in mM): 4.74 KCl, 149.2 NaCl and 2.54 $CaCl_2$, with an osmolarity of 293 mosM/kg, while the intracellular solution contained (in mM): 125 $KMeSO_3$, 25 KCl and 2 EGTA, with an osmolarity of 281 mosM/kg. All pharmacologic agents were made up in Ringer solution. All solutions were buffered with 5 mM HEPES. The external solutions were buffered to a pH of 7.35, and the internal solution was buffered to 7.00.

Drugs

The drugs used in this study were cyclic 8-brom-G-3 : 5-MP, cyclic 8-brom-A-3 : 5-MP from Boehringer Mannheim (Indianapolis, IN) and atropine, carbachol and diltiazem from Sigma (St. Louis, MO). To ensure that the correct drug concentration was applied to the cell each solution change was carried out at least four times using 1.2 ml each time. This resulted in a 16× bath volume change on each application. Drugs were applied at least for 30 min before ineffectiveness was assumed.

Results

Ninety-two cells were voltage clamped. The mean cell capacitance and resting voltage (±SD) were 5.6 ± 2.25 pF and −38 ± 25 mV, respectively. The whole-cell pulse protocol used spanned a voltage range from −90 or −110 to +90 mV, in 10–11 increments of 20 mV, with a pulse length of 200 msec (Fig. 1*B inset*). Five runs were averaged to produce the recorded data in each experiment, and the cells were held at −70 mV during the interpulse interval.

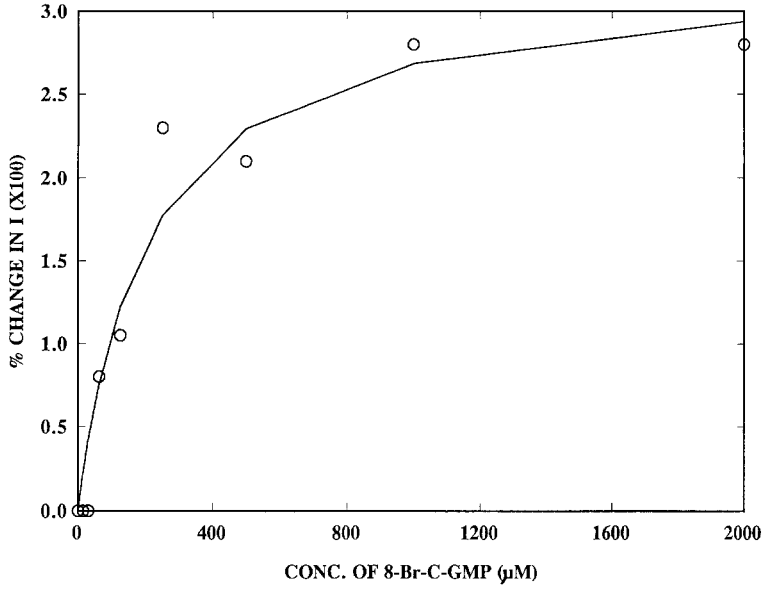


Fig. 2. Dose-response curve of the K current to cGMP. The data, taken from 10 cells, is fitted with the Michaelis-Menton equation giving an effective Km (half-maximal stimulation) of 206 μM and a peak activation of 2.9 times baseline.

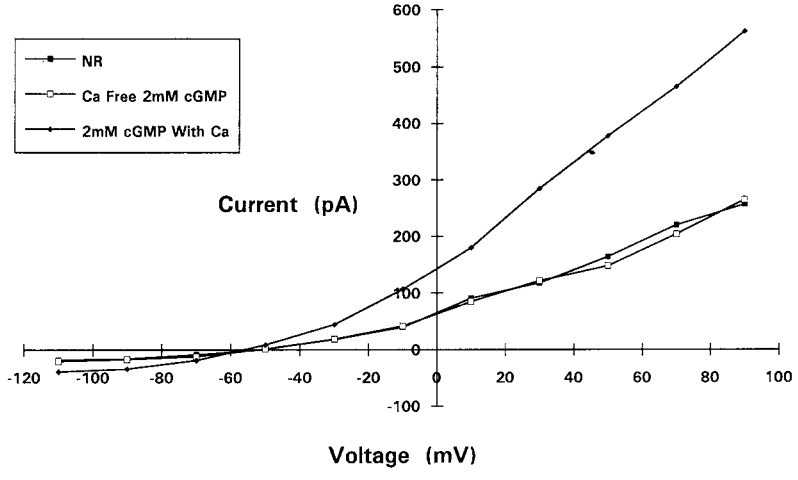


Fig. 3. Steady-state *I-V* relationships showing the lack of activation by cGMP in the absence of external calcium, with full activation after addition of calcium to the bath solution.

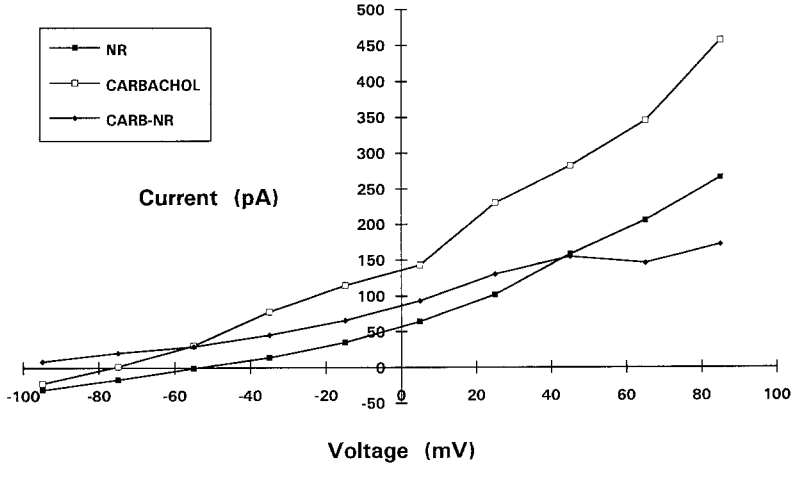


Fig. 4. Steady-state *I-V* relationships showing activation of the K current with 250 nM carbachol. The unactivated current (*NR*) has a reversal potential of -55 mV, the activated current a reversal potential of -78 mV, while the reversal potential of the difference current (*CARB-NR*) was undistinguishable from E_K.

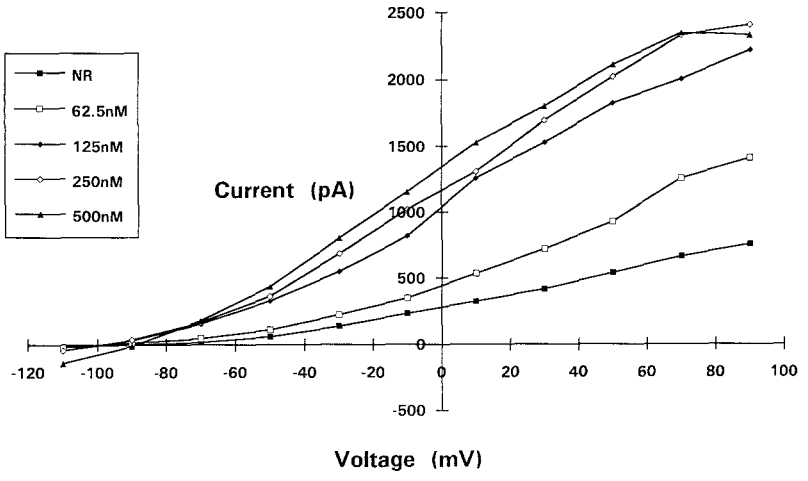


Fig. 5. Typical dose response for carbachol, showing initial activation at 62.5 nM and full activation at 250 nM, resulting in a fivefold increase in the current.

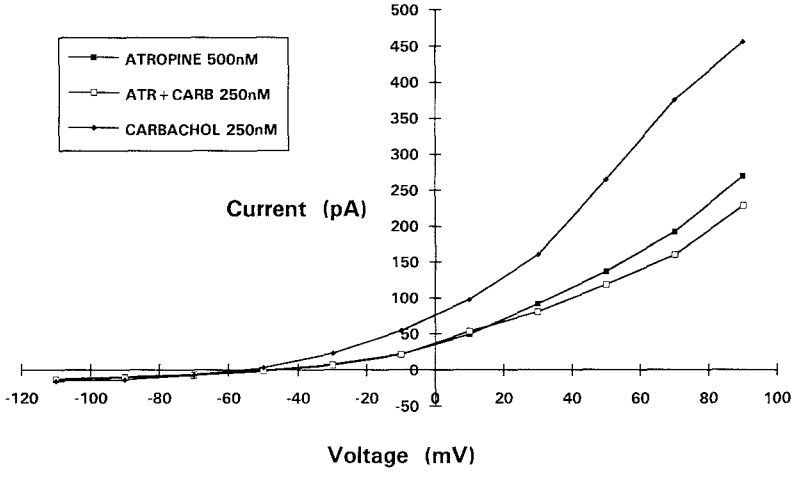


Fig. 6. Steady-state *I-V* relationships showing total blockade of carbachol-stimulated activation with 500 nM atropine and subsequent full activation on removal of the atropine from the bath.

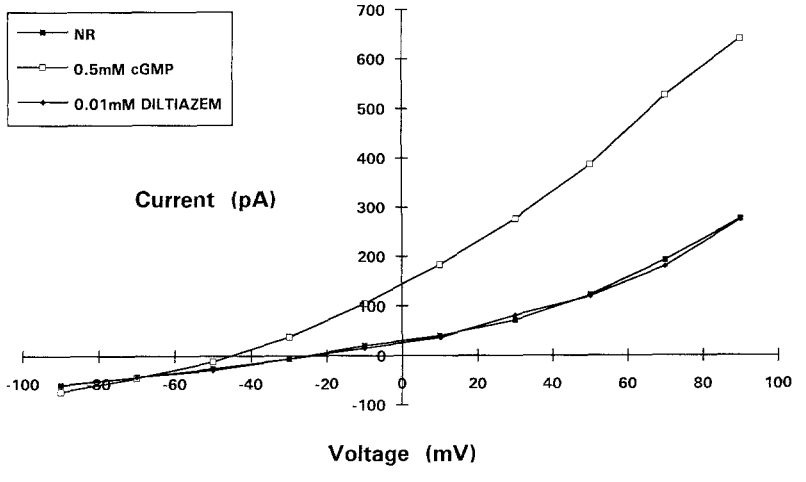


Fig. 7. Effect of 10 μM diltiazem on the cGMP-activated current (500 μM), showing complete blockade of activation with diltiazem.

Cyclic GMP

Figure 1 demonstrates the effect of 500 μM 8-bromo-cGMP on the K current. Figure 1*A* shows the unstimulated current, Fig. 1*B* shows the current 10 min after the application of the cGMP, the current increasing threefold, and Fig. 1*C* the difference current obtained by subtracting the stimulated current from the baseline current. Figure 1*D* shows the steady-state *I-V* relationships showing a shift in the reversal potential from -38 to -55 mV, while the difference current reversed at -68 mV. This effect

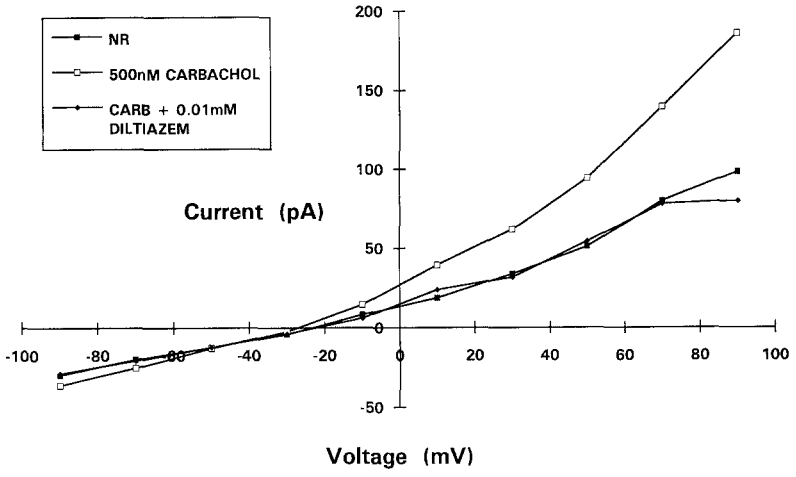


Fig. 8. Effect of 10 μM diltiazem on the carbachol-activated current (250 nM), showing complete blockade of carbachol activation.

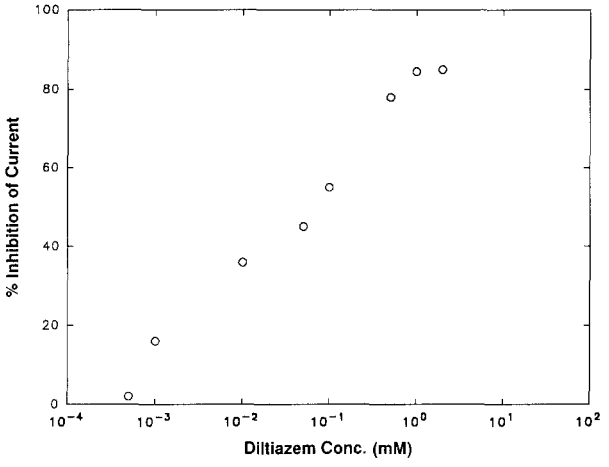


Fig. 9. Typical dose-response curve for diltiazem inhibition of the K current plotted on the semilogarithmic scale. Initial blockade of the current was seen at 1 μM and 85% blockade at 1 mM.

was seen in 12 of 14 cells and had a delay in onset of an average of 7 min (range 3–17). Maximal drug effect on current amplitude varied from 2.3–4.5 times the original current with a mean of 2.9 (at 100 mV). The drug effect could be reversed by replacing the external solution with Ringer solution lacking cGMP. The mean reversal time was about 17 min (13–24). Figure 2 shows the dose-response curve for 8-bromo cGMP. The data points are fitted with the Michaelis-Menton equation

$$FI = I_{max}/(1 + K_m/G)$$

where FI is the calculated current, I_{max} the maximal current, K_m the cGMP concentration required for half-maximal stimulation (the effective K_m), and G the cGMP concentration. The effective K_m for the drug was 206 μM, while a maximal effect was seen with 1 mM. Cyclic GMP also increased the "leak" current. The mean increase was 3.6 times (1.6–5.7).

Changing the normal Ringer external solution to a calcium-free solution containing 2 mM EGTA and 2 mM cGMP failed to stimulate the current in 4 of 4 cells. Subsequent addition of calcium to the external solution containing cGMP resulted in full stimulation of the current (Fig. 3). Both barium and quinidine were effective blockers of the stimulated current (*data not shown*).

Carbachol

Figure 4 shows the effect of 250 nM carbachol on the current. In this example the unstimulated current had a reversal potential of −55 mV. After stimulation with carbachol this shifted to −78 mV and the reversal potential of the difference current was undistinguishable from E_K. This effect was seen in 13 of 14 cells, and maximal effects were noted within 1 min. Carbachol stimulation was variable with a mean maximal effect of a 3.8-fold increase in the current (1.12–7.04) at +100 mV. An effect was first noted with 31.25 nM; the effective K_m was 125 nM, while a maximal effect was seen with 250 nM carbachol. Doses above 500 nM resulted in reduced maximal currents, as did repeated application of the drug. A typical dose response is shown in Fig. 5. The stimulatory effect was not reversible on changing external solution to Ringer (observed for 45 min), and its effect could be totally blocked by adding 500 nM atropine to the bath prior to carbachol (Fig. 6). Addition of atropine after stimulation with carbachol did not reverse the stimulation in 2 of 2 cells. The effect of carbachol was not dependent on the presence of external calcium, and again barium and quinidine blocked the current.

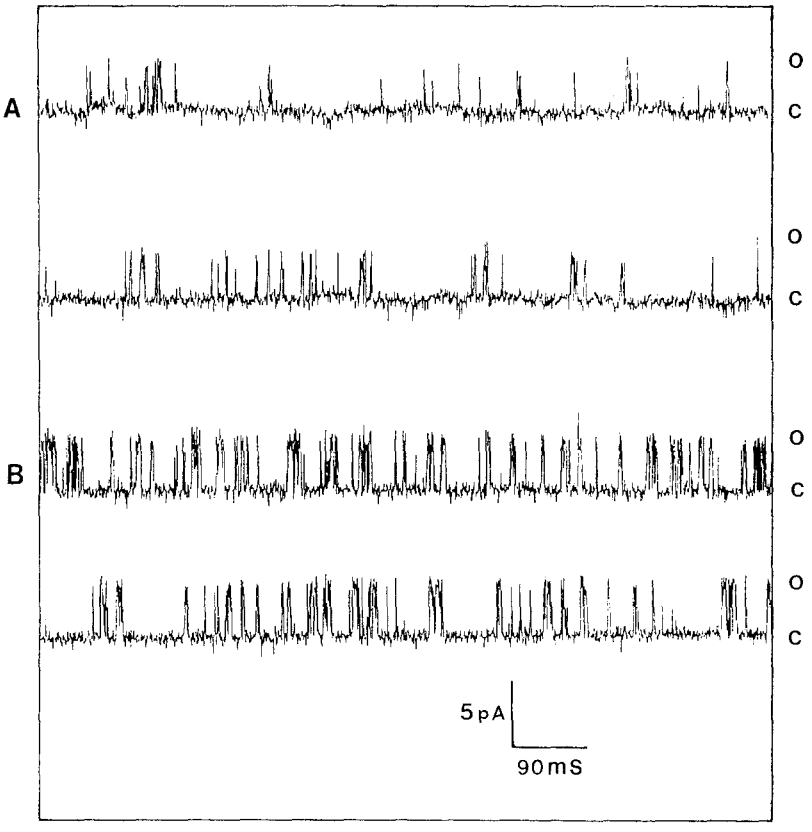


Fig. 10. Single-channel data from an on-cell patch, showing the 167-pS (in 150 mM KCl) channel before (*A*) and after (*B*) application of 2 mM cGMP to the bath with marked activation of the channel post application (10 min).

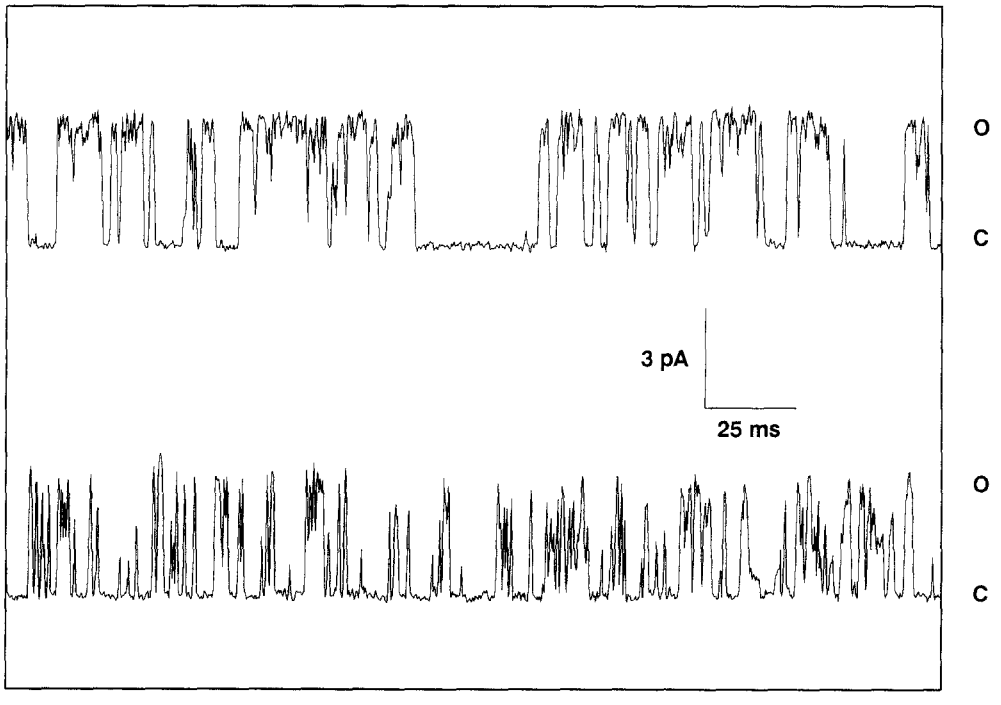


Fig. 11. Effect of 50 μM diltiazem, in the pipette, on the large K channel, showing the 'flickery' blockade.

Diltiazem

Diltiazem at a dose of 10 μM completely blocked the stimulatory effects of both cGMP and carbachol (Figs. 7 and 8). Blockade was rapid (in seconds) as was reversal of the blockade on changing the bath back to normal Ringer. This blockade also resulted in a decrease in the resting membrane potential, suggesting that its effect is through blockade of a K channel. This effect is further examined in our single-channel experiments. Diltiazem also blocked the unstimulated current, but its effect was less marked and required higher doses. Initial effects were seen at 1 μM with maximal blockade occurring at 1 mM. A typical dose-response curve is shown in Fig. 9. Maximal doses did not result in total blockade of the current, and the effect of diltiazem appeared to depend on the size of the initial current, with greater blockade occurring with larger initial currents. As with its effects on cGMP and carbachol, both blockade and recovery were rapid (in seconds).

Single-Channel Data

The whole-cell current is contributed to by at least three channels. To show that the drug effects were specifically on the large K channel, the major contributor to the unstimulated current, various configurations in which single-channel currents could be studied were used. In one configuration the drugs were applied to the exterior of the cell while recording from an on-cell patch. An increase in the single-channel activity here would suggest a second-messenger effect. The drugs were also applied to the bath of an inside-out patch to assess their action from the interior aspect of the channel. Lastly, the drugs were backfilled into the pipette after the tip was filled with Ringer solution lacking the drug. This enabled us to obtain control records and then assess the direct effect of the drugs after the drug diffused to the electrode tip. These results suggest that the action of these drugs is on the large K channel, but full single-channel kinetic analysis will be required to further quantify their effects.

Cyclic GMP and carbachol activated the large K channel when applied to the outside of the cell in a cell-attached recording (Fig. 10), while they had no effect when applied to inside-out patches or directly in the pipette, suggesting that second messengers are necessary for their actions. Diltiazem was extremely effective in blocking the large K channel, when applied directly to the channel via the pipette, and was ineffective, when applied to an inside-out patch or to bath in a cell-attached recording. Figure 11 shows the effect of 50 μM diltiazem applied to the channel via the pipette in an on-cell patch, showing the characteristic "flickery" effect. Present data suggests that diltiazem is an open-channel blocker in these cells, but more detailed single-channel studies are required.

Discussion

We characterized the effects of cGMP, carbachol and diltiazem on a potassium-selective, delayed-rectifier current. The role of cGMP in cellular function is poorly defined at present. Cyclic GMP, and the enzymes that synthesize and degrade it, are widely distributed in many cell types, and there are several receptors that appear to modulate its formation. Among these are the muscarinic receptor in nervous tissue (Ferrendelli et al., 1970), smooth muscle (Lee, Kuo & Greengard, 1972) and heart (George et al., 1970). In these tissues the muscarinic receptor is thought to activate the soluble form of guanylate cyclase, resulting in an increase in intracellular cGMP. The activation of guanylate cyclase is thought to be dependent on a second messenger, with calcium appearing as a likely candidate (McKinney & Richelson, 1990).

It is not clear from our studies if the effects of carbachol and cGMP stimulation were via the same intracellular mechanism. The stimulated currents observed appear to be different, with cGMP decreasing the activation time more than carbachol (*data not shown*). Cyclic GMP stimulated both the K current and the "leak" current, while carbachol appeared to stimulate only the K current, as the reversal potential of the difference current was undistinguishable from E_K. Furthermore, the ability of carbachol to stimulate the current in the absence of external calcium, unlike cGMP, suggests that, at least in part, its mechanism of action in these cells is independent of cGMP. It is well established that external calcium is necessary for the action of guanylate cyclase (Schultz et al., 1973). Our data seem to suggest that this is also necessary for the action of cGMP on this channel.

The effects of diltiazem were somewhat surprising. The rapidity of onset of action, as well as its reversal, together with the single-channel data suggest that the drug acts directly on the channel as an open-channel blocker. Diltiazem is traditionally thought of as a selective calcium-channel blocker. Since this K channel is an important determinant of the resting membrane potential and may play a role in volume regulation, this blockade may have clinical importance given the widespread use of this drug in cardiovascular disease.

The K channel responsible for a large part of the whole-cell current described here has previously

been shown to be stretch activated in on-cell configurations (Rae et al., 1990). There has recently been considerable controversy over the presence and possible role of stretch-activated channels in the intact cell (Morris & Horn, 1991; Sachs et al., 1991). Others have considered stretch channels to be artifacts (Milton & Caldwell, 1990). We show here that this stretch-activated K channel is modulated by cGMP, carbachol and diltiazem both when recording from on-cell and whole-cell configurations. It is unlikely that these drugs are activating the channel by stretch, as we monitored cell size through an eyepiece micrometer and this did not change. It is presently not known if stretch activation has a physiological role in cellular function in these cells although we have preliminary evidence that osmotic swelling increases the potassium current in the cells studied here.

Finally, the role of muscarinic receptors and the effects of cGMP on corneal epithelium are at present unknown. A possible role has been suggested by Cavanagh and Colley (1982), who noted an increased incorporation of thymidine and leucine in the presence of cGMP and decreased uptake in the presence of cAMP. They hypothesized that growth and proliferation of corneal epithelium during healing is regulated by a balance between cAMP-enhancing substances, such as catecholamines, which inhibit regrowth, and cGMP-mediated cholinergic effects that enhance it. Our studies suggest a role in volume regulation and ion transport as well.

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