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Mechanism of Activation of K^+ Channels by Minoxidil-Sulfate in Madin-Darby Canine Kidney Cells

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Summary. We studied the mechanism of K^+ channel activation by minoxidil-sulfate ($MxSO_4$) in fused Madin-Darby canine kidney (MDCK) cells. Patch-clamp techniques were used to assess single channel activity, and fluorescent dye techniques to monitor cell calcium. A Ca^{2+}-dependent inward-rectifying K^+ channel with slope conductances of 53 ± 3 (negative potential range) and 20 ± 3 pS (positive potential range) was identified. Channel activity is minimal in cell-attached patches. $MxSO_4$ initiated both transient channel activation and an increase of intracellular Ca^{2+} (from 94.2 ± 9.1 to 475 ± 12.6 nmol/liter). The observation that K^+ channel activity of excised inside-out patches was detected only at Ca^{2+} concentrations in excess of 10 μmol/liter suggests the involvement of additional mechanisms during channel activation by $MxSO_4$.

Transient K^+ channel activity was also induced in cell-attached patches by 10 μmol/liter of the protein kinase C activator 1-oleoyl-2-acetyl-glycerol (OAG). OAG (10 μmol/liter in the presence of 1.6 mmol/liter ATP) increased the Ca^{2+} sensitivity of the K^+ channel in inside-out patches significantly by lowering the K_m for Ca^{2+} from 100 μmol/liter to 100 nmol/liter. The channel activation by OAG was reversed by the protein kinase inhibitor H8. Staurosporine, a PKC inhibitor, blocked the effect of $MxSO_4$ on K^+ channel activation. We conclude that $MxSO_4$-induced K^+ channel activity is mediated by the synergistic effects of an increase in intracellular Ca^{2+} and a PKC-mediated enhancement of the K^+ channel's sensitivity to Ca^{2+}.

Key Words fused MDCK cells · K^+ channel · minoxidil-sulfate · Ca^{2+} · protein kinase C

Introduction

The initial and cortical collecting ducts play an important role in renal K^+ homeostasis. A large body of evidence supports the view that a small-conductance K^+ channel of the apical membrane of principal cells mediates K^+ secretion in distal nephron segments [8]. The properties of this K^+ channel with respect to its regulation by pH, ATP, protein kinases A and C have been studied extensively [33, 34, 35].

Recently, a group of K^+ channel openers such as pinacidil, cromakalim or minoxidil-sulfate ($MxSO_4$) evolved as a new class of drugs that act on ATP-regulated K^+ channels (K^+_{ATP} channel [3, 4, 36, 38]. $MxSO_4$, the active metabolite of the antihypertensive drug minoxidil, also stimulates $^{42}K^+$ efflux from the smooth muscle of rabbit mesenteric artery [23] and induces vasodilation [15] which can be specifically blocked by glyburide [39], an inhibitor of K^+_{ATP} channels in insulin-secreting cells. Whole-cell K^+ currents are also stimulated by $MxSO_4$ [19].

In the present study we explore the effects of $MxSO_4$ on renal ion channels to further elucidate their regulation. Ion channel modulation by protein kinase C (PKC) had been suggested in studies on single MDCK cells in which K^+ channel activation by bradykinin [18] or ATP [25] is paralleled by increased production of 1,4,5-inositol-triphosphate (IP3).

In our studies we chose Madin-Darby canine kidney (MDCK) cells, a stable line of epithelial cells derived from the dog kidney [21]. These cells share important transport properties with renal collecting duct cells [24, 32]. We fused single MDCK cells to "giant" cells because macroscopic current measurements in fused MDCK cells show a low K^+ conductance of the cell membrane under resting conditions, yet channel activity can be dramatically increased after a hypotonic shock [17] and following addition of channel activators [6, 7, 18]. Accordingly, the stimulating effects of agents such as $MxSO_4$ on quiescent channels could be easily detected. In this paper we demonstrate the stimulation of Ca^{2+}-dependent K^+ channels in fused MDCK cells by $MxSO_4$ and their modulation by intracellular Ca^{2+} and PKC.

Materials and Methods

Cell Culture

MDCK cells were obtained from American Type Culture Collection (Rockville, MD; no. CCL 34), and grown in a humidified atmosphere of 95% O_2 and 5% CO_2 in Eagle's Minimum Essential Medium (MEM; Sigma Chemical, St. Louis, MO), supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 24 mmol/liter HCO_3^-, 100,000 U/liter penicillin and 100 μmol/liter streptomycin (GIBCO, Grand Island, NY). When grown to subconfluence, cells were in part passaged and in part fused to "giant MDCK cells" according to the protocol described below. We used cells from passages 53–65.

Cell Fusion

We applied the previously described polyethylene glycol (PEG) fusion technique for MDCK cells [16]. In short, cells are grown to subconfluence in plastic culture flasks (Falcon, Lincoln Park, NJ) and washed in Ca^{2+}-free EDTA-containing phosphate-buffered saline (EDTA-PBS) before 0.025% trypsin (GIBCO) is added. When cells detach from the bottom of the culture flask, trypsinization is stopped by adding culture medium. A small part of this cell suspension is used for further passaging the cells; the larger part, however, undergoes cell fusion. After centrifugation, we discarded the supernatant and then slowly suspended the cells in 2 ml of fusion medium (50% wt/vol PEG, Mr 4,000 (Fluka Chemie AG, Buchs, Switzerland) in MEM culture medium without FCS, pH 7.8). Cells were centrifuged again after 4 min and resuspended in culture medium with FCS. For experiments we plated the fused cells on small coverslips coated with a 0.01% poly-L-lysine solution (Sigma). We performed patch-clamp experiments and measurements of intracellular Ca^{2+} on day 1 and 2 after cell fusion.

Experimental Solutions

Cells were bathed during all experiments in Ringer solution having the following composition (in mmol/liter): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES, pH 7.4 (control Ringer). For low Ca^{2+} Ringer solutions (5 nmol/liter – 100 μmol/liter free Ca^{2+}) we used 1.3 mmol/liter EGTA as Ca^{2+} buffer and calculated the amount of Ca^{2+} needed for the desired free Ca^{2+} activities. Adenosine triphosphate (ATP; Sigma) and N-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (H8; Seikagaku America, St. Petersburg, FL) were dissolved each day in Ringer solutions with the appropriate Ca^{2+} activity at a concentration of 20 mmol/liter and 10 mmol/liter, respectively. Minoxidil-sulfate ($MxSO_4$; Upjohn, Kalamazoo, MI) and 1-oleoyl-2-acetyl-glycerol (OAG; Sigma) were stored as DMSO stock solutions. Final DMSO concentration never exceeded 0.03%. Ionomycin (Calbiochem, San Diego, CA) was dissolved in ethanol as 1 mmol/liter stock solution. To yield the desired final concentration, the appropriate amount of diluted stock solution was added directly to the bath. The pipette solution in patch-clamp experiments contained, in mmol/liter: 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4. All experiments were carried out at 37°C.

Patch-Clamp Technique

Patch-clamp experiments were performed according to the methods of Hamill et al. [10]. We pulled patch pipettes from prism™ hard-glass capillaries (Dagan, Minneapolis, MN). They had a resistance of 5–8 MΩ when filled with 140 mmol/liter KCl. Recordings were made using a List L/M-EPC7 patch-clamp amplifier (List Medical, Darmstadt, FRG). Single channel currents were filtered at 1.5 kHz by an eight-pole low-pass Bessel filter (902 LPF; Frequency Devices, Haverhill, MA). Data were stored on a video cassette recorder (VCR; Sony SL-HF 350) via a digital data recorder (VR-10; Instrutech, Mineola, NY). Data analysis was performed with an Atari Computer (SH 204) using Atari Data Acquisition and Analysis Software (Instrutech).

Open probability P_o was calculated according to the equation

$$P_o = \sum_{n=1}^{N} (t_n \times n)/(N \times T) \quad (1)$$

where t_n is the dwell time spent at current levels corresponding to $n = 1, 2, \ldots N$ channels in the open state, n is the number of open channels, N the number of active channels in the patch and T the total time of the analyzed record. Current amplitudes were determined by fitting a Gaussian distribution curve to an amplitude histogram.

For kinetic analysis, histograms for open and closed dwell times were used, and time constants determined by performing maximal likelihood fitting with one or more exponentials for the open and closed time histograms, respectively.

Ca^{2+} Measurements

Ca^{2+} measurements were carried out on MDCK cells which had undergone cell fusion 1 or 2 days prior to the experiments. Cells, grown on small coverslips, were incubated for 60 min in serum-free culture medium (MEM) containing 7 μmol/liter of the fluorescent dye Fura-2 am (Molecular Probes, Eugene, OR; dissolved in DMSO; final DMSO concentration 0.6%) in a 95% O_2/5% CO_2 atmosphere at 37°C. Cells were rinsed with control Ringer solution before transfer to the experimental chamber on the microscope stage.

For excitation of dye-loaded cells, a 150-W Xenon lamp was used. In combination with a monochromator, this device generated light of a single wave length of 340 nm which was passed via fiber optic to the microscope (Nikon Diaphot). The emitted light signal, measured at 510 nm, was amplified and recorded by an intensified videocamera (Videoscope/Dage CCD) and then transmitted to the imaging processing board (Analytical Imaging, GA). Areas corresponding to individual cells were defined on the monitor of the imaging system and fluorescence was determined as average value for these areas. We measured Ca_i^{2+} under control conditions and its peak value after application of $MxSO_4$.

Intracellular Ca^{2+} activity was calculated according to the following equation [9]:

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F). \quad (2)$$

K_d is the dissociation constant for Fura-2 (225 nmol/liter) and F the measured cell fluorescence. Values for F_{max} and F_{min} were

determined during calibration after each experiment. F_{max} was measured after permeabilizing the cell membrane for Ca^{2+} with the Ca^{2+} ionophores Br-A 23187 (4 μmol/liter; Molecular Probes) or ionomycin (10 μmol/liter; Sigma). F_{max} represents maximal cell fluorescence after saturating the intracellular dye with Ca^{2+}. After the cell membrane was made permeable for Ca^{2+}, F_{min} was obtained by adding 3 mmol/liter $MnCl_2$ to the bath. $MnCl_2$ enters the cell and effectively quenches the Fura-2-related fluorescence signal. Measured fluorescence intensities were always corrected for background fluorescence. Ca^{2+} activity measured for individual cells represents average values.

Results

Characterization of Spontaneous K^+ Channel Activity

We confirmed previous results [2, 6, 7] by observing two types of K^+ channels in fused MDCK cells: a maxi-K^+ channel with a conductance of 150 pS, and another Ca^{2+}-sensitive K^+ channel with a slope conductance of 53 ± 3 pS for inward current and of 20 ± 3 pS for outward current (n = 10). The incidence of the K^+ channel with the lower conductance was much higher (present in 70% of successful patches) than that of the high conductance K^+ channel (present in 10% of successful patches). In this paper we shall consider only the K^+ channel with the lower conductance.

In a first series of experiments we characterized the basic properties of the K^+ channel in fused MDCK cells. Figure 1 shows a representative recording of the current-voltage relationship of the 53 pS K^+ channel. The cell is bathed in control Ringer solution and the patch pipette contains 140 mmol/liter KCl. Inspection of Fig. 1 indicates an asymmetry of current flow suggesting inward rectification. This was confirmed in experiments in which channel currents were studied in inside-out excised patches with symmetrical pipette and bath solutions (140 mmol/liter KCl). Under these conditions, inward and outward conductances were 55 ± 4 pS and 20 ± 3 pS, respectively (n = 4).

In inside-out patches with control Ringer solution in the bath, we observed no significant voltage dependence of the channel's open probability over a voltage range from −40 to −80 mV (cell interior negative). Open probability P_o is 0.41 ± 0.03 (n = 11) under these conditions. For kinetic analyses, the data obtained at these different holding potentials were pooled.

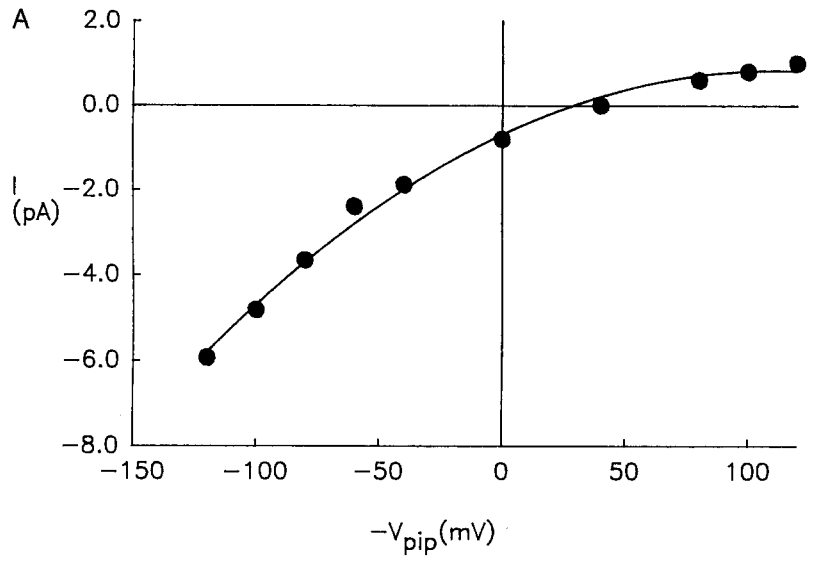
Figure 1*B* summarizes results from a kinetic analysis of open and closed times of the channel whose current-voltage relationship is displayed in Fig. 1A. At a calcium concentration of 1 mmol/liter in the bath, channel kinetics are characterized by brief openings and closures, occasionally interrupted by long channel closures. The frequency distribution of the closed time histogram shows that short events predominate and that few channel closures last longer than 100 msec. Kinetic analysis of the channel is consistent with one open state and several closed states. The following time constants were obtained (relative frequencies of closed states given in parentheses): t_{open} = 5.6 ± 0.5 msec, t_{c1} = 1.5 ± 0.1 msec (77 ± 2%), t_{c2} = 9.3 ± 0.6 msec (20 ± 2%), and t_{c3} = 128 ± 17 msec (2.4 ± 0.6%).

The channel was selective for K^+ over Na^+. The relative channel selectivity was calculated from the shift of the reversal potential following a change of the bath solution from control Ringer solution (140 mmol/liter NaCl) to one containing 140 mmol/liter KCl. The selectivity ratio K^+/Na^+ was 5:1 (n = 4). Previously, the selectivity ratio K^+/Na^+ of this channel was reported to be 12:1 [37]. Possibly this discrepancy is related to methodical differences. We determined channel selectivity in the inside-out patch configuration in which virtually no outward current is visible with control Ringer solution (140 mmol/liter NaCl) in the bath. Hence, an underestimation of the reversal potential and, thereby, of the selectivity ratio is likely. Weiss et al. [37] circumvented this problem by determining channel selectivity in the cell-attached patch configuration.

Even though selectivity and Ca^{2+} sensitivity (*see below*) of the presently studied K^+ channels from fused MDCK cells are not the same as reported for single MDCK cells [6, 7], we think that the K^+ channels from both preparations are identical. This conclusion is supported by the great resemblance of all other parameters tested. Both channels are selective for K^+, have similar slope conductances (60 pS in single subconfluent MDCK-cells *vs.* 53 pS in fused MDCK cells), and are inward-rectifying. Furthermore, their open probability shows no significant voltage dependence at membrane potentials between −40 and −80 mV and both channels are Ca^{2+} dependent.

Effects of $MxSO_4$ on Channel Activity and Intracellular Ca^{2+}

Figure 2 shows the results of an experiment in which addition of $MxSO_4$ led to multiple channel activation in a cell-attached patch. Following addition of 5 μmol/liter $MxSO_4$ to the bath, several K^+ channels are transiently activated before channel activity subsides. On the average, channel activity



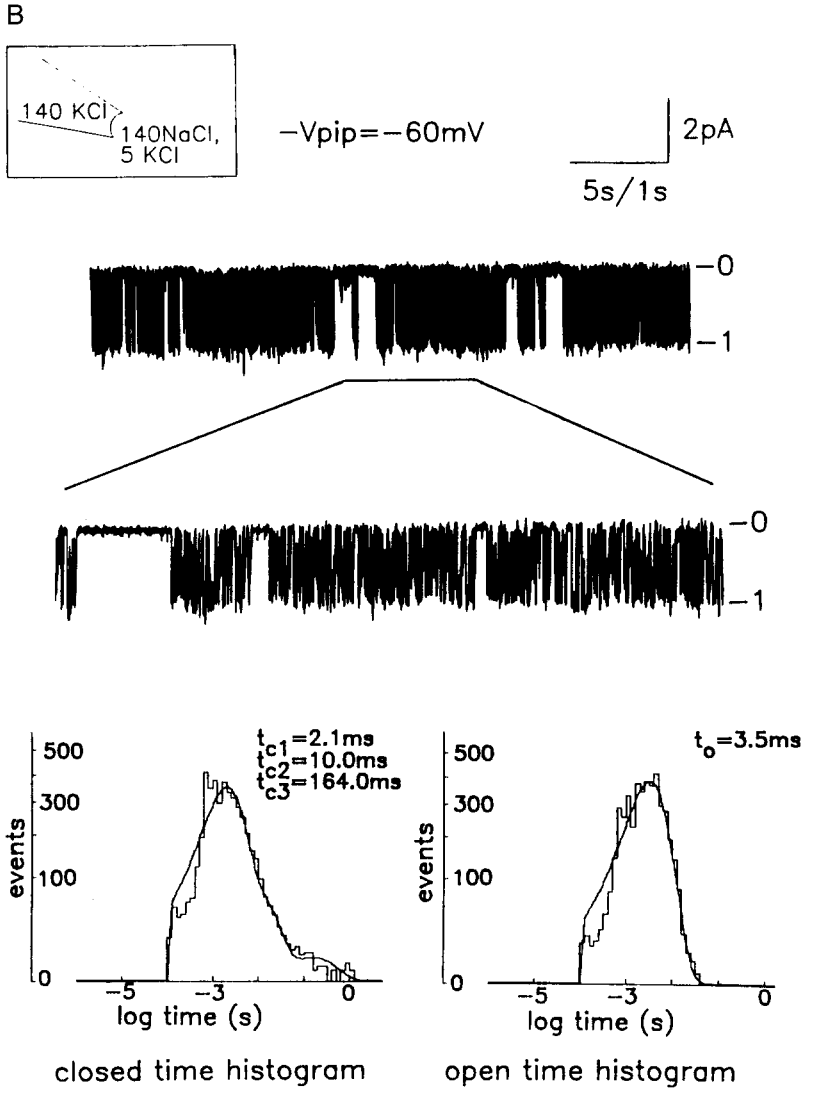


Fig. 1. (*A*) Analysis of a single channel recording in the cell-attached patch configuration of a fused MDCK cell. Single channel currents plotted as a function of holding potentials between bath and pipette. The inward slope conductance is 50 pS and the outward slope conductance is 10 pS. Bath (in mmol/liter): 140 NaCl, 5 KCl; pipette: 140 KCl. (*B*) Kinetic analysis of the same channel now in the inside-out mode (bath Ca^{2+} = 1 mmol/liter). The channel recording is shown in a low and a high time resolution. The abcissas (dwell times) of open and closed time histograms are in logarithmic scale. Maximal likelihood fitting of the histograms reveals three closed time constants and one open time constant. Bath: control Ringer solution; pipette: 140 mmol/liter KCl; Bessel-filtered at 1.5 kHz.

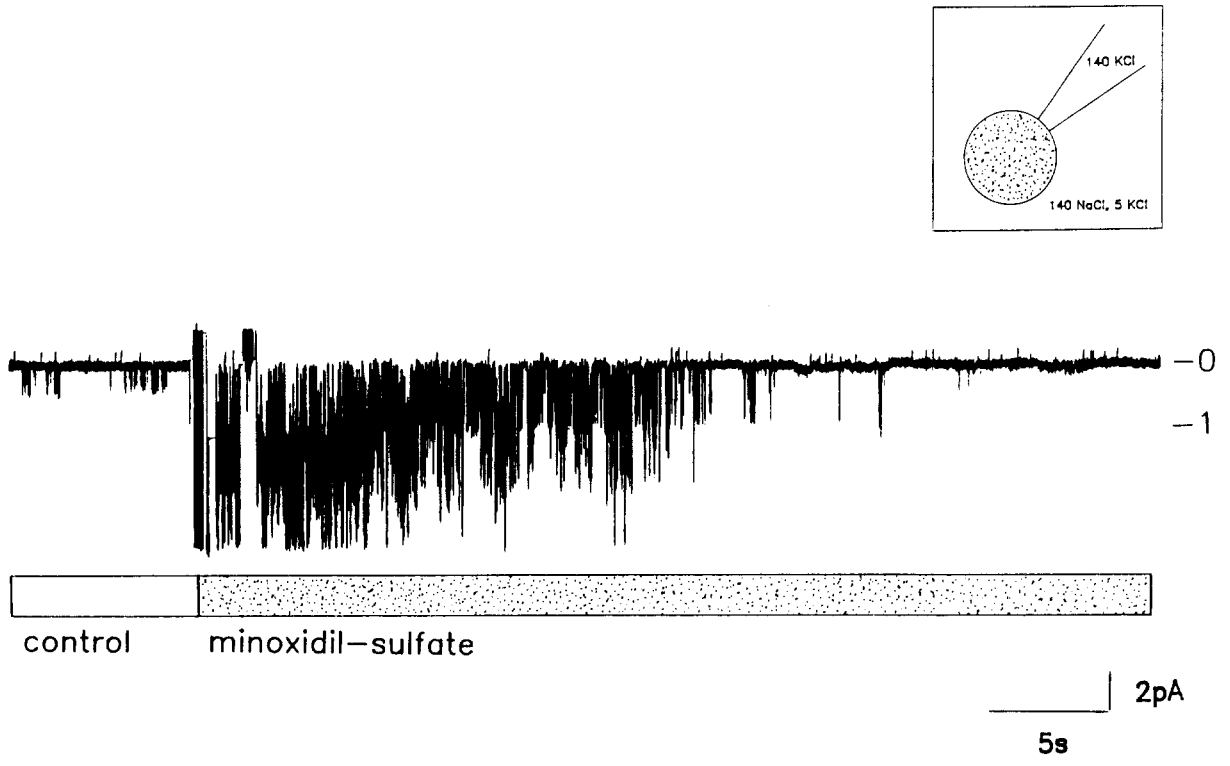


Fig. 2. Activation of K^+ channels by $MxSO_4$ in the cell-attached patch configuration. After addition of $MxSO_4$ (5 μmol/liter) to the bath up to three K^+ channels are transiently activated. Holding potential V_{pip} is 0 mV under control and −20 mV under experimental conditions. However, the increase in channel amplitude is also due to hyperpolarization of the cell membrane potential.

returned to the baseline level after 44 ± 11 sec (n = 5). We also observed an increase of single channel amplitude which is consistent with cell hyperpolarization by the activation of K^+-selective channels. In contrast to the marked stimulatory effect of $MxSO_4$ in the cell-attached patch configuration, $MxSO_4$ had no effect on channel activity in inside-out patches when similar concentrations of $MxSO_4$ were added to the bath solution (n = 12). We conclude from these observations that additional cellular mechanisms are involved in the stimulation of K^+ channels by $MxSO_4$.

In view of several observations that maneuvers activating K^+ channels in MDCK cells change cell Ca^{2+} [18, 25], we tested whether $MxSO_4$ affects intracellular Ca^{2+}. Figure 3 shows the time course and magnitude of intracellular Ca^{2+} changes following addition of 5 μmol/liter $MxSO_4$. Common to all observations was a rapid and significant initial increase of cell Ca^{2+} and a slow return to baseline levels over a time period of 70–200 sec. Figure 4 summarizes control and peak values of intracellular Ca^{2+} after addition of 5 μmol/liter $MxSO_4$. Ca^{2+} levels rose from a mean control value of 94.2 ± 9.1 nmol/liter to a peak concentration of 475 ± 12.6 nmol/liter (n = 7).

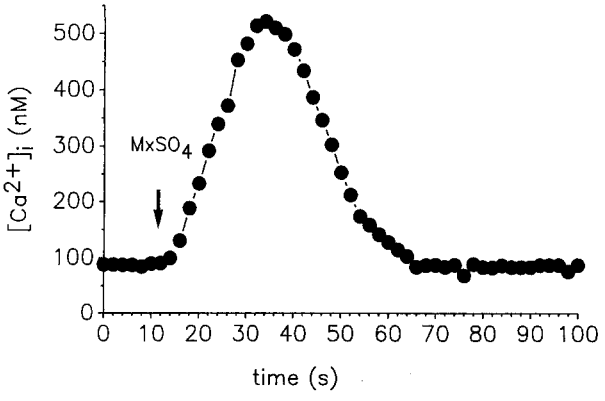


Fig. 3. Time course of Ca^{2+} concentration change in MDCK cell following addition of 5 μmol/liter $MxSO_4$.

Effects of Calcium on K^+ Channel Activity

Experiments in Cell-Attached Patches

The activation of K^+ channels and the $MxSO_4$-induced sharp increase of cell Ca^{2+} suggests that cell calcium changes may be linked to the stimulation of potassium channel activity. To demonstrate

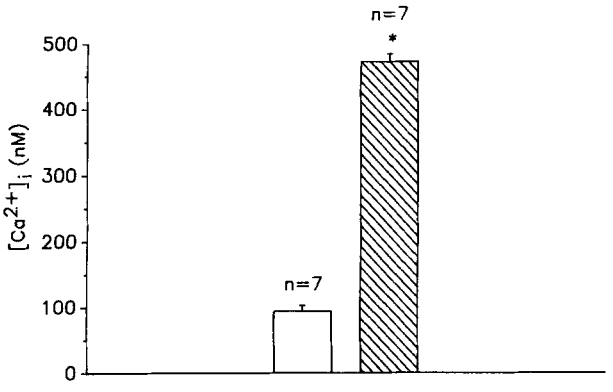


Fig. 4. Mean intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ in the control state (□) and peak values after addition of 5 μmol/liter $MxSO_4$ (▨).

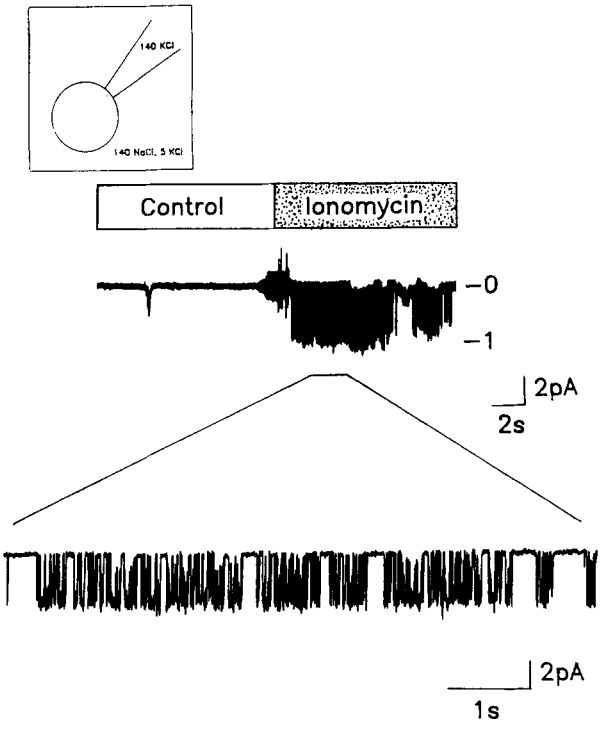


Fig. 5. K^+ channel activation by ionomycin (2 μmol/liter) in fused MDCK cell. This experiment demonstrates that an increase in intracellular Ca^{2+} concentration can activate the K^+ channel in a cell-attached patch. Open probability and kinetic properties of this channel are almost identical to inside-out patches in control Ringer solution: P_o = 0.39; t_{c1} = 1.75 msec (77%), t_{c2} = 12.5 msec (21%), t_{c3} = 142 msec (2.6%). Bath: control Ringer solution, pipette: 140 mmol/liter KCl; Bessel-filtered at 1.5 kHz; $-V_{pip}$ = −20 mV.

that intracellular Ca^{2+} changes can modulate K^+ channels, we carried out experiments in which the intracellular Ca^{2+} concentration was changed by addition of the Ca^{2+} ionophore ionomycin to the bath solution. Figure 5 summarizes results obtained in

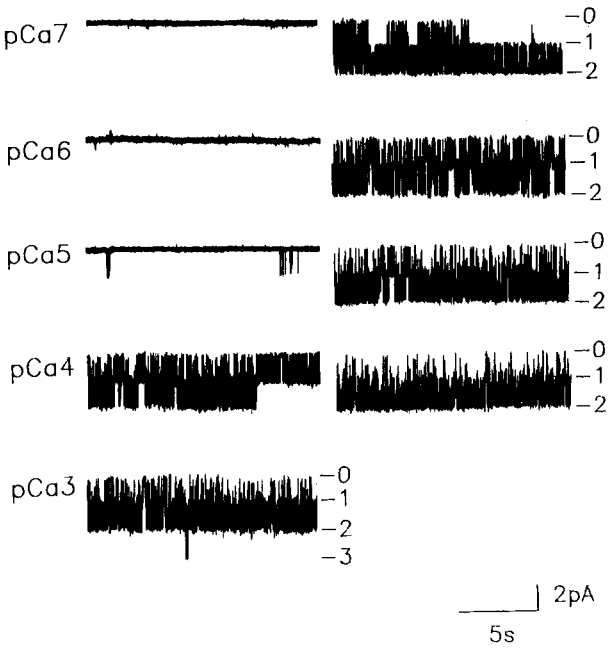


Fig. 6. Comparison of K^+ channel activity in an inside-out patch at different Ca^{2+} concentrations in the absence and presence of PKC activators. Activation of PKC with 1-oleoyl-2-acetyl-glycerol (OAG (10^{-5} μM); plus ATP (1.6 mM) as substrate) increases the Ca^{2+} sensitivity of the channel by three orders of magnitude. Bath: Ringer with varying Ca^{2+} concentrations; pipette: 140 mmol/liter KCl; $-V_{pip}$ = −60 mV.

one out of five cell-attached patches. It is apparent that addition of 2 μmol/liter ionomycin, with 1 mmol/liter Ca^{2+} in the bath, leads to the very rapid and sustained activation of channel activity. Under these conditions we also measured a sustained elevation of intracellular Ca^{2+} to 895 ± 26 nmol/liter (n = 26). Accordingly, open probability and kinetic behavior of the ionomycin-activated channels closely mimic the channel kinetic observed in inside-out patches with 1 mmol/liter Ca^{2+} in the bath. Lowering bath Ca^{2+} to 20 nmol/liter returned the channel activity control values. These experiments indicate that K^+ channel activity in MDCK cells depends on the intracellular Ca^{2+} concentration.

Experiments in Excised Inside-Out Patches

To test whether the $MxSO_4$-induced rise in cell Ca^{2+} is adequate to account for the K^+ channel activation after $MxSO_4$, we carried out experiments on inside-out patches from MDCK cells during changes of bath Ca^{2+} concentration. These experiments are summarized in the left panel in Fig. 6, in Figure 8 (open circles) and in Table 1. K^+ channel activity was monitored over a range of Ca^{2+} concentrations from 5 nmol/liter to 2 mmol/liter. Our results show that channel activity is present only at calcium con-

Table 1. Comparison: Open probability of nonstimulated and stimulated K^+ channels in dependence of Ca^{2+}

Ca^{2+} concentration	P_o nonstimulated (n)	P_o stimulated (n)
2 mM	0.42 ± 0.07 (6)	
1 mM	0.41 ± 0.03 (11)	
100 μM	0.21 ± 0.06 (7)	0.38 ± 0.03 (2)
10 μM	0.01 ± 0.003 (9)	0.36 ± 0.07 (4)
1 μM	0.0 (6)	0.28 ± 0.03 (6)
100 nM	0.0 (10)	0.26 ± 0.02 (4)
20 nM	0.0 (3)	0.15 ± 0.11 (3)
10 nM	0.0 (7)	0.10 ± 0.03 (7)
5 nM	0.0 (8)	0.01 ± 0.01 (4)

Open probability of K^+ channels in the inside-out patch configuration is shown as a function of bath Ca^{2+}. For nonstimulated channels the bath contained Ringer solution with the appropriate Ca^{2+} concentration, whereas for stimulation of channels, 10 μmol/liter OAG and 1.6 mmol/liter ATP were added to the bath.

centrations exceeding 10 μmol/liter. Channel activity is absent at bath Ca^{2+} concentrations of 1 μmol/liter.

These results are in contrast to previous experiments [6] in which the K^+ channel was found to be 100 times more Ca^{2+} sensitive. We have no explanation for this difference. Interestingly, in transformed MDCK cells which also express this K^+ channel its Ca^{2+} sensitivity is in between that from single and from fused MDCK cells [29]. We do not believe that trypsinizing cells before the experiments caused the difference of Ca^{2+} sensitivity [27] since trypsin did not have access to intracellular Ca^{2+} binding sites of the channel. However, we must emphasize that K^+ channel activity responded very reproducibly to changes of Ca^{2+} concentration on the cytoplasmic side of excised inside-out patches.

Our observations that $MxSO_4$ induces K^+ channel activation at cell calcium concentrations of 470 nmol/liter, yet the concentration of Ca^{2+} necessary for channel activation in inside-out patches is much higher (10 μmol/liter), suggest strongly that changes in cell Ca^{2+} are not the only mediator of stimulation of K^+ channels by $MxSO_4$.

Modulation of Channel Activity by Protein Kinase C

A possible role of protein kinase C (PKC) in the activation of inward-rectifying K^+ channels in MDCK cells was suggested by observations showing that bradykinin [18] and ATP [25] increase production of 1,4,5-inositol-triphosphate (IP3). Accordingly, we investigated the possibility that enhanced PKC activity could explain our observations that $MxSO_4$ induces channel activation in cell-attached patches at Ca^{2+} concentrations that are at least 20 times lower that those necessary to activate the K^+ channel in inside-out patches.

Experiments in Cell-Attached Patches

Inspection of Fig. 7 shows that application of 10 μmol/liter of the PKC activator OAG (1-oleoyl-2-acetyl-glycerol) [1] to the bath initiates, after a short lag period, several intermittent activation periods of channel activity. Channel activity usually lasts for about 1 min and is followed by a period of quiescence before channel activity reappears. We also observed that the number of channels activated with each subsequent oscillatory stimulation decreases progressively. Although we have no adequate explanation for the oscillatory pattern of channel openings, our experiments demonstrate that PKC is a potent activator of K^+ channel activity in MDCK cells.

The involvement of PKC in mediating the effects of $MxSO_4$ on K^+ channel activity was further investigated in a series of experiments in which the permeant PKC inhibitor staurosporine was used. When staurosporine was added to the bath solution (500 nmol/liter for periods of 15–30 min), addition of $MxSO_4$ (5 μmol/liter) failed to induce K^+ channel activity in seven of eight experiments.

Experiments in Excised Inside-Out Patches

To obtain further information about the mode of action of PKC on K^+ channels in MDCK cells, we carried out experiments in excised membrane patches at a constant concentration of OAG at varying Ca^{2+} concentrations. This allowed us, first, to investigate whether PKC affects the Ca^{2+} sensitivity of the K^+ channel and second, whether the inactive PKC molecule is present in a membrane-bound or cytosolic form.

Figure 8 and Table 1 summarize the results obtained in experiments in which addition of 10 μmol/liter of OAG and 1.6 mmol/liter ATP in the bath was used for stimulation of PKC in inside-out patches over a range of Ca^{2+} concentrations from 5 nmol/liter to 100 μmol/liter. K^+ channel activity was compared at different Ca^{2+} concentrations before (Fig. 6, left panel) and after stimulation of PKC by OAG (Fig. 6, right panel). There was a dramatic increase of K^+ channel sensitivity to Ca^{2+} ions in the presence of enhanced PKC activity. Following stimulation of PKC, K_m for Ca^{2+} was lowered by three orders of magnitude, from 100 μmol/liter to 100 nmol/liter. We conclude that PKC stimulation is an effective mediator between cell Ca^{2+} and K^+

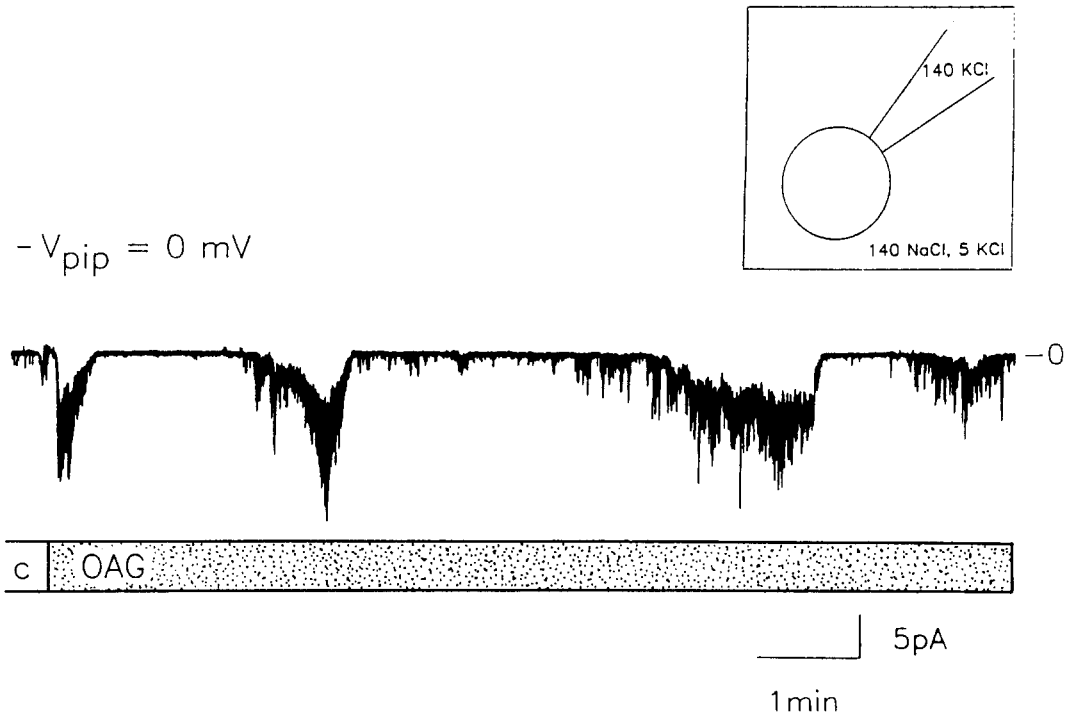


Fig. 7. Activation of K^+ channels in the cell-attached patch configuration by 10 μmol/liter of PKC activator 1-oleoyl-2-acetyl-glycerol (OAG). Only minimal channel activity is present under control (*c*) conditions. This figure depicts the first four episodes of oscillatory channel activity in which at least four channels are activated. Bath: control Ringer solution, pipette: 140 mmol/liter KCl, $-V_{pip}$ = 0 mV.

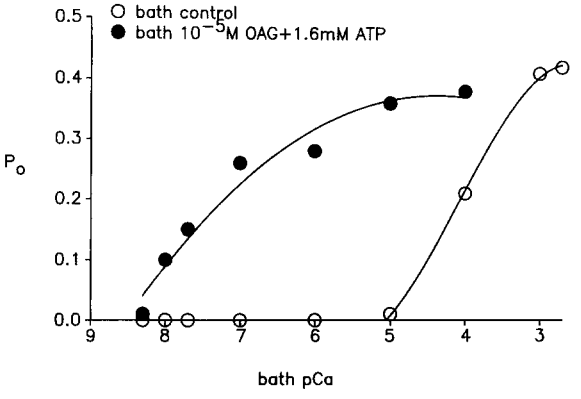


Fig. 8. Ca^{2+} dose-response curves summarizing experiments from Fig. 6. In the presence of activated protein kinase C, Ca^{2+} sensitivity of the K^+ channel in the inside-out patch rises dramatically. Half-maximal channel activation requires 100 nmol/liter in the stimulated patch, whereas 100 μmol/liter Ca^{2+} are needed in the nonstimulated patch. Bath (in mmol/liter): 140 NaCl, 5 KCl; pipette: 140 KCl.

channel activity by significant modulation of the potassium channel's sensitivity to calcium ions. The stimulation of channel activity by PKC in excised patches strongly suggests that inactive PKC is present in a membrane-bound form.

The involvement of PKC in the observed channel stimulation was tested in additional experiments by application of the protein kinase inhibitor H8 (200 μmol/liter; n = 5). Channel activity was monitored in inside-out patches at bath Ca^{2+} concentrations of 10 nmol/liter and 10 μmol/liter. Whereas the channel activity was significantly stimulated under these conditions by OAG (*see* Fig. 7), addition of H8 to the bath sharply reduced the channel's open probability to 0.03 (10 μmol/liter Ca^{2+}) and 0.01 (10 nmol/liter Ca^{2+}) (*data not shown*).

Discussion

Effects of $MxSO_4$ on K^+ Channel Activity

Agents activating K^+ channels are a new class of drugs that generally act on ATP-sensitive K^+ channels [3, 4, 14, 30, 38]. In vascular smooth muscle cells they induce vasodilation and are potentially useful as antihypertensive agents [38]. $MxSO_4$ is thought to activate ATP-sensitive K^+ channels in vascular smooth muscle because $MxSO_4$-induced vasodilation is specifically antagonized by the K^+_{ATP} channel blocker glyburide [39].

The present study indicates that the mechanism of action of $MxSO_4$ in renal MDCK cells differs from that in smooth muscle. First, it appears that $MxSO_4$ does not act directly on K^+ channel activity

in MDCK cells. This contrasts with the behavior in ventricular myocytes in which potassium channel openers such as chromakalin and pinacidil directly restore K^+_{ATP} channel activity (i.e., channel "run down") after excision of membrane patches [4, 5]. Moreover, competition between ATP and the K^+ channel openers pinacidil and RP 49356 has been suggested for K^+_{ATP} channels in cardiac cells [4, 31]. Second, it appears that the K^+ channel in MDCK cells is not a member of the family of ATP-sensitive K^+ channels that have been identified in renal tubule cells. For instance, ATP-sensitive K^+ channels in principal cells of the renal collecting duct are Ca^{2+}-insensitive, completely blocked by 1 mmol/liter ATP, stimulated by PKA and inhibited by PKC [33, 34]. In sharp contrast, the K^+ channel in the present study of MDCK cells is highly Ca^{2+} sensitive, still active at ATP concentrations as high as 2.4 mmol/liter (inside-out patch: open probability with 100 nmol/liter Ca^{2+} and 10 μmol/liter OAG in the bath: $P_o = 0.29 \pm 0.1$; $n = 3$), and stimulated by PKC; PKA appears to have no significant effect on channel activity. These considerations lead us to conclude that the mechanism of action of $MxSO_4$ as a potent K^+ channel opener differs in epithelial and excitable tissues.

When K^+ channel openers such as $MxSO_4$ are used clinically, renal sodium and fluid retention are not uncommon [20]. In addition to α-adrenergic stimulation, our experiments on renal epithelial cells may provide an additional explanation for this phenomenon. Activation of K^+ channels in cells of the distal nephron would inevitably lead to hyperpolarization, an event that increases the electrochemical driving force for sodium entry from lumen to cytoplasm. Such enhanced sodium entry would then stimulate basolateral Na^+/K^+-ATPase activity and accelerate net sodium absorption.

Role of Intracellular Ca^{2+}

Two observations of the present study suggest that changes in cell Ca^{2+} play a key role in mediating the increase of K^+ channel activity in MDCK cells after $MxSO_4$ application. First, $MxSO_4$ induced a significant transient elevation of Ca^{2+}. Second, the mechanism by which $MxSO_4$ activates K^+ channels involves an interaction between Ca^{2+} and PKC.

The fact that $MxSO_4$ induces a rise in cell Ca^{2+} differs from observations made in some other tissues. Whereas, as shown in the present study, intracellular Ca^{2+} increases in MDCK cells after $MxSO_4$, inhibition of L-type calcium channels and a decline of cell Ca^{2+} is observed in vascular smooth muscle cells [19]. Although intracellular Ca^{2+} increases in MDCK cells, we could not resolve the source of Ca^{2+} responsible for the rise in intracellular Ca^{2+}. Preliminary evidence is consistent with the notion that activation of Ca^{2+} release from cell stores is involved.[1]

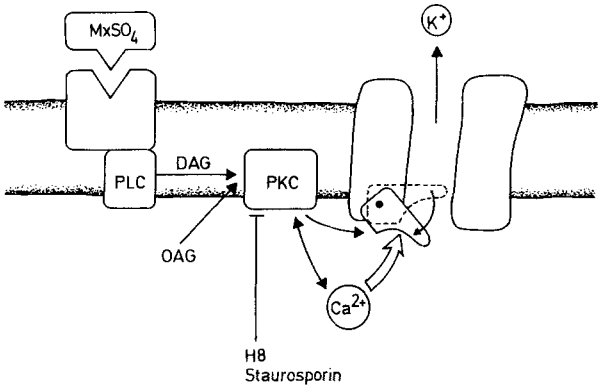


Fig. 9. Cell schematic of possible mechanism of activation of K^+ channels in MDCK cells. $MxSO_4$ binds from the extracellular side to a hypothetical receptor and subsequently generates diacetyl-glycerol (DAG). DAG and Ca^{2+} ions interact with membrane-bound PKC to phosphorylate a critical site of the K^+ channel. This stimulates channel activity by dramatically increasing its Ca^{2+} sensitivity.

Our study supports the view that interaction between cell Ca^{2+} and PKC plays a key role in the stimulation of K^+ channels after $MxSO_4$. Not only did PKC increase sharply the sensitivity of the K^+ channel to Ca^{2+} ions but K^+ channel activation was prevented by inhibiting the action of PKC. It has been demonstrated that PKC binding to cell membranes is significantly enhanced in the presence of phorbol esters when Ca^{2+} ions are elevated from 100 to 500 nmol/liter [22, 40], a concentration range similar to that observed in MDCK cells treated with $MxSO_4$. We conclude that the effects of $MxSO_4$ on K^+ channel activity are indirect and most likely mediated by its interaction with PKC and its physiological activators such as diacetylglycerol (DAG).

Role of Protein Kinase C

Figure 9 provides a cell schematic suggesting a possible mechanism of action of $MxSO_4$ on K^+ channels in MDCK cells. Important aspects include a

[1] Two lines of evidence strongly suggest that release of Ca^{2+} from intracellular stores is involved. We observed in three out of six experiments that $MxSO_4$-induced channel activation persisted when MDCK cells were bathed in Ringer solution with Ca^{2+} as low as 10 nmol/liter. The role of intracellular Ca^{2+} stores is further underscored by the finding that pretreatment with 100 μmol/liter TMB-8 (3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester) blocked the stimulating effect of $MxSO_4$ on K^+ channel activity. TMB-8 is an agent that interferes with Ca^{2+} release from cell stores. It is a useful tool to distinguish between Ca^{2+} entering the cell through Ca^{2+} channels and Ca^{2+} released from cell stores [26].

hypothetical $MxSO_4$ receptor and the generation of DAG. The sources of elevated cell Ca^{2+}—i.e., entry of Ca^{2+} through channels in the cell membrane and/or Ca^{2+} release from internal stores—are not incorporated into the model. Ca^{2+} ions and DAG interact with membrane-bound PKC to phosphorylate a critical site of the K^+ channel, a process that stimulates channel activity by dramatically increasing its Ca^{2+} sensitivity. Ca^{2+} ions also stimulate K^+ channel activity directly but the magnitude of this effect is insufficient to explain the effects of $MxSO_4$. Activation of inwardly rectifying K^+ channels in single MDCK cells by bradykinin and ATP is associated with increased production of IP3 [18, 25], confirming the possible involvement of PKC in K^+ channel activation. Although the proposed cell schematic contains several assumptions, it serves as a starting point to test and expand our proposal for a mechanism of action of $MxSO_4$ in renal tubule cells.

Kinetic studies of the effects of PKC activators on the interaction of PKC with its substrates demonstrate the existence of three distinct forms of PKC: a soluble, cytoplasmic form which is inactive, a membrane-associated, "primed" form which is also inactive, and an active, membrane-associated form which results from interaction with DAG or phorbol esters [11]. Our experiments demonstrate that PKC-mediated channel stimulation can be elicited in cell-free inside-out patches. Accordingly, we conclude that inactive PKC exists in MDCK cells in a membrane-associated "primed" form. If the inactive molecule were released into the cytosol, it would be expected to be absent in excised patches and reactivation of PKC would be impossible.

The activation of PKC shifts the Ca^{2+} dose-response curve of K^+ channel activation by three orders of magnitude to the left so that half-maximal channel stimulation is achieved at 100 nmol/liter Ca^{2+}. In the range of Ca^{2+} concentrations measured in the present experiments, between 100 nmol and 1 μmol/liter, open probability rises only minimally in excised patches in which PKC was activated. Hence, the $MxSO_4$-induced initial rise in intracellular Ca^{2+} does not induce activation of K^+ channels directly but triggers events that stimulate PKC.

Two types of protein kinase inhibitors were used in our study to evaluate the role of PKC in mediating the response of K^+ channels to $MxSO_4$. The protein kinase inhibitor H8 blocked the stimulating effect of OAG on K^+ channels. Some potential problems of specificity arise because H8 also blocks cAMP-dependent protein kinase A (PKA) [13]. However, we observed no effects of exogenous PKA on K^+ channel activity. Accordingly, the effects of H8 are related to its inhibitory effects upon PKC.

Different Effects of PKC Activation on Renal K^+ Channel Activity

The effects of PKC on the potassium channel in the present study differs from those observed in principal cells of the cortical collecting duct. A low-conductance K^+ channel in the apical membrane of these cells has been identified and strongly implicated in regulating potassium secretion in the distal nephron [35]. Its properties include inwardly directed rectification, inhibition by high concentrations of ATP and by acidification, and, importantly, downregulation by PKC. Accordingly, K^+ transport in rabbit cortical collecting duct is inhibited by PKC [12]. The modulatory effect of PKC upon K^+ channel activity also varies in other tissues. Thus, PKC activates K^+ currents in guinea pig cardiac ventricular myocytes, whereas it decreases such currents in rat cardiac ventricular myocytes. Diversity of PKC activity upon Ca^{2+} channel activity has also been noted. This topic has been extensively reviewed [28].

The fact that PKC has different effects upon apical K^+ channels in principal cells of the renal collecting duct and in MDCK cells may also reflect their different functional properties. Whereas K^+ channels in principal cells, as noted above, mediate K^+ secretion, the K^+ channels under investigation in MDCK cells are most likely involved in volume regulatory decrease (VDR), a phenomenon involving K^+ loss and secondary shrinking of kidney cells following cell swelling. Macroscopic current measurements indicate the involvement of K^+ channel activation in MDCK cells following hypotonic shock. After exposing cells to a Ringer solution whose osmolality is reduced by one third, the K^+ conductance of the cell membrane rises 64-fold [17]. Additional studies will be necessary to demonstrate whether PKC and Ca^{2+} are involved in the activation of K^+ channels in VRD.

In conclusion, we demonstrate in fused MDCK cells an inwardly rectifying Ca^{2+} dependent K^+ channel with properties similar to K^+ channels in single MDCK cells. The open probability of this K^+ channel is low but can be activated by the K^+ channel opener $MxSO_4$. An analysis of the mechanism by which $MxSO_4$ activates the K^+ channel shows that an increase of cell Ca^{2+} and PKC act synergetically to phosphorylate a channel site that activates channel activity by dramatically increasing its Ca^{2+} sensitivity.

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