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Activation of K^+ and Cl^- Channels in MDCK Cells during Volume Regulation in Hypotonic Media

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Summary. Single-channel patch-clamp experiments were performed on MDCK cells in order to characterize the ionic channels participating in regulatory volume decrease (RVD). Subconfluent layers of cultured cells were exposed to a hypotonic medium (150 mOsm), and the membrane currents at the single-channel level were measured in cell-attached experiments. The results indicate that MDCK cells respond to a hypotonic swelling by activating several different ionic conductances. In particular, a potassium and a chloride channel appeared in the recordings more frequently than other channels, and this allowed a more detailed study of their properties in the inside-out configuration of the patch-clamp technique. The potassium channel had a linear I/V curve with a unitary conductance of 24 ± 4 pS in symmetrical K^+ concentrations (145 mM). It was highly selective for K^+ ions *vs.* Na^- ions: $P_{Na}/P_K < 0.04$. The time course of its open probability (P_o) showed that the cells responded to the hypotonic shock with a rapid activation of this channel. This state of high activity was maintained during the first minute of hypotonicity. The chloride channel participating in RVD was an outward-rectifying channel: outward slope conductance of 63.3 ± 4.7 pS and inward slope conductance of 26.1 ± 4.9 pS. It was permeable to both Cl^- and NO_3^- and its maximal activation after the hypotonic shock was reached after several seconds (between 30 and 100 sec). The activity of this anionic channel did not depend on cytoplasmic calcium concentration. Quinine acted as a rapid blocker of both channels when applied to the cytoplasmic side of the membrane. In both cases, 1 mM quinine reversibly reduced single-channel current amplitudes by 20 to 30%. These results indicate that MDCK cells responded to a hypotonic swelling by an early activation of highly selective potassium conductances and a delayed activation of anionic conductances. These data are in good agreement with the changes of membrane potential measured during RVD.

Key Words MDCK cells · patch clamp · volume regulation · potassium channels · chloride channels

Introduction

It is well known that several factors, such as alterations in the extracellular medium osmolarity or in the equilibrium between solute efflux and influx, can induce important changes in cellular volume owing to the large permeability of cellular membranes to water. Many cell types show the capacity of recovering their regular size after a change in their volume induced by a modification of their environment or of their transport functions (reviewed by Grinstein et al., 1984; Eveloff & Warnock, 1987). A cell usually reacts to swelling with an extrusion of osmolytes from the cytoplasm, in order to force an osmotic water efflux and to re-establish a normal cell volume. On the other hand, cell shrinkage can stimulate the absorption of osmolytes from the extracellular medium which will be accompanied by an osmotically driven water influx. Such behaviors are commonly termed *regulatory volume decrease* (RVD) and *regulatory volume increase* (RVI), respectively. RVD is principally ruled by the loss of potassium and chloride ions from the cell. Several different transport mechanisms have been found to participate in the extrusion of such ions depending on the cell types studied: a K^+-Cl^- cotransport on Ehrlich cells (Thornhill & Laris, 1984), on sheep erythrocytes (Dunham & Ellory, 1981) and on duck erythrocytes (Kregenow, 1981), a system of coupled K^+-H^+ and Cl^--HCO_3^- exchangers on *Amphiuma* erythrocytes (Cala, 1980; Cala, Mandel & Murphy, 1986) and distinct K^+ and Cl^- conductances on Ehrlich cells (Hoffmann, Lambert & Simonsen, 1986), on human lymphocytes (Grinstein et al., 1982*a*; Grinstein, Dupre & Rothstein, 1982*b*) and on HeLa cells (Tivey, Simmons & Aiton, 1985).

Volume regulation is very important in epithelial cells because they can be exposed to large variations in transport rates or important changes in medium osmolarities. For this reason volume regulation has been extensively studied on epithelial tissues: frog skin (Ussing, 1985), frog urinary bladder (Davis & Finn, 1985), *Necturus* gallbladder (Larson & Spring, 1984), and mammalian kidney (Gagnon et al., 1982; Macknight, 1983; Welling, Linshaw & Sullivan, 1985). Madin-Darby canine kidney (MDCK) cells

are frequently used in the study of the properties of renal epithelial tissue since they retain the characteristics of intercalated cells of the cortical collecting duct of canine kidney (Valentich, Tschao & Leighton, 1981; Steigner et al., 1990*a*). Studies on volume regulation have also been performed on these cells (Simmons, 1984; Roy & Sauvé, 1987; Paulmichl et al., 1989; Rothstein & Mack, 1990). Roy and Sauvé (1987) have performed a detailed study of the behavior of MDCK cells exposed to anisotonic media. Cellular volume, membrane potential and cellular contents were measured in order to characterize their changes during volume regulation in a hypotonic medium. Monolayers of MDCK cells partly regulate their volume after a hyposmotically induced swelling, and, from the measurement of cellular contents, it was shown that RVD occurs with a loss of K^+, Cl^- and amino acids. The changes in cellular potential observed during RVD show that cells rapidly hyperpolarize in the first phase of the process and subsequently depolarize towards their control resting potential. The time evolution of membrane potential suggests that the initial hyperpolarization could reflect an early activation of potassium conductances, whereas a delayed increase in anionic conductances could account for the subsequent depolarization. Rothstein and Mack (1990) have produced further evidence that RVD in MDCK cells involves the activation of distinct conductive K^+ and Cl^- pathways.

Our purpose in this work was to determine if the activation of separate K^+ and Cl^- channels could be observed during volume regulation in MDCK cells. The patch-clamp technique was used to measure the membrane currents during RVD at the single-channel level. These patch-clamp studies were performed on subconfluent layers of cultured cells. In cell-attached experiments, it was found that up to four different ionic channels can be activated in these cells by a hypotonic shock. Two of these channels could be studied in details in cell-attached and inside-out configurations. One was a highly selective potassium channel, and the other one was a chloride channel with low selectivity for anions. The current-voltage relations of these two channels were measured, and the time course of their activation after the hypotonic shock was obtained.

Materials and Methods

Cell Cultures

The MDCK line was obtained from the American Type Culture Collection starting at the 59th serial passage. Cells were used for patch-clamp experiments up to the 80th serial passage. Cells were seeded at medium density and grown until confluence in MEM (Gibco 410–1100) in plastic bottles (Falcon 3023). The medium contained 10% fetal bovine serum (Gibco) which was changed every two days. Cultures were maintained at 37°C in a humid air atmosphere, and the Falcon bottles were kept closed. Subculture was performed by detaching the cells from the bottles using a trypsin-EDTA solution containing 0.05% trypsin and 0.5 mM EDTA-Na. Cells were then transferred onto glass microscope slides at low density (1.35×10^4 cells/cm^2) and used for patch-clamp experiments one to four days later. Under these conditions the experiments were performed on subconfluent cultures, allowing easy gigaohm seal formation. It must be pointed out that in general the formation of good giga-seals took several minutes, and sometimes the release of suction was necessary to obtain tight seals.

Solutions

The standard bathing solution was an Earle's medium containing (in mM): 121 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 6.0 $NaHCO_3$, 1.0 NaH_2PO_4, 5.5 glucose, 25.0 HEPES, 10.0 NaOH (pH: 7.3, osmolarity: 290 mOsm/kg). The hypotonic bathing medium used for volume regulation experiments was obtained by diluting 1 volume Earle's solution with the same volume of water. This procedure led to a twofold dilution of salt concentration. The compositions of the different pipette solutions are shown in the Table. In all of these solutions pH was adjusted to 7.3 with NaOH or KOH, and the osmolarity was 290 mOsm/kg. As shown in the Table neither Ca^{2+} nor EGTA were added in the preparation of these solutions, except the one containing K-gluconate. Therefore they could contain a small amount of calcium ions deriving from the impurities in the different compounds. This amount was evaluated to be of the order of 5 to 10 μM. The bath solutions used in the inside-out experiments were the same as the ones given in the Table. The eventual addition of particular agents to these solutions is specified in the text in the description of the particular experiment.

Patch-Clamp Technique

Single-channel experiments were carried out in the cell-attached and inside-out configurations of the patch-clamp technique, as described by Hamill et al. (1981) and by Corey and Stevens (1983). The resistance of the patch pipettes ranged from 5 to 10 MΩ in the presence of the K-Cl solution. The patch-clamp amplifier was a PC-501, Warner Instrument (Hamden, CT). The patch-clamp signal was filtered at 1 kHz with a four-pole low-pass Bessel filter and then recorded on magnetic tape with a Betamax video recorder (Sony SL-810D), after having been converted to a video signal by a Neuro-corder DR-384 (Neuro Data Instruments, New York).

Data Analysis

To perform data analysis, the recorded signal was digitized with a 12-bit A/D converter (MacADIOS M411, GW Instruments, Cambridge, MA) at a frequency of 5 kHz. The digitized signal was then loaded on the memory of a MacIntosh SE microcomputer (4 Mbytes) and stored on 800-kbytes disks. Data acquisition and analysis were performed with self-made computer programs in Quick Basic exploiting a software library supplied by GW Instru-

Table. Composition of pipette solutions (concentrations expressed in mM)

	K-Cl	Na-Cl	Chol-Cl	$Na\text{-}NO_3$	K-Gluc-EGTA
KCl	133				10
NaCl		133			
$NaNO_3$				136	
Choline-Cl			145		
$MgCl_2$	3	3	3	3	3
KOH	13				13
NaOH		12	8.5	11	
HEPES	25	25	15	25	25
K-gluconate					125
EGTA					1

ments. Routines from the GW library were typically used to calculate current-amplitude distribution histograms, to evaluate distribution integrals and to determine the average current in a recording. The analysis of the patch-clamp recordings was done on segments of the signal with a duration of 20 sec. Several independent measures of the current amplitude were taken at different points of such a recording, and the average of the measures ± SD represented the final value. The values of single-channel currents obtained with this procedure at different fixed pipette potentials (V_p) were used to determine the current-voltage relations of the channels. For the correct interpretation of I/V curves, the following conventions must be taken into account: (i) the voltage used as abscissa in the I/V plots ($-V_p$) is the reverse of the pipette potential in order to represent the membrane potential (V_m) with respect to the cytoplasmic side of the membrane, (ii) in a cell-attached recording, the cellular potential (V_c) must be added to $-V_p$ in order to obtain the actual PD across the membrane under the patch ($V_m = -V_p + V_c$), and (iii) positive currents flow from the cytoplasmic to the extracellular side of the membrane.

The channel open probability (P_o) was generally determined in two ways, and the results from the two measurements were compared. The first method was based on the analysis of current-amplitude histograms. In this case P_o is given by the following equation:

$$P_o = \frac{1}{N} \sum_{n=1}^{N} (n\, t_n) \tag{1}$$

where N is the total number of channels under the patch in conditions of maximal activity, n indicates the number of channels simultaneously open for a given current amplitude and t_n is the integral of the distribution of current amplitudes related to n open channels. The second method to determine P_o was based on the evaluation of the average current of a recording. Given the equation:

$$\langle I \rangle = N\, i\, P_o \tag{2}$$

where $\langle I \rangle$ is the average current and i the current through a single open channel. P_o is easily obtained after the measurement of the single-channel current and the determination of N. Both methods were used on a few cases and gave similar results. The values of P_o given in our results have been obtained with the first of these methods.

Results

A series of experiments in the cell-attached configuration was carried out in order to establish the MDCK cell response to a hypotonic shock at the single-channel level. These experiments were performed with pipettes containing the K-Cl solution. Figure 1*a* shows the continuous recording of a current signal from cells exposed to the hypotonic medium. The activity of a previously quiescent channel was immediately stimulated after exposure to this medium. In Fig. 1*b* the amplitude histograms under isotonic and hypotonic conditions are given, showing the presence of a single channel with an average current amplitude of 2.6 pA. The histograms were closely fitted with Gaussian probability distributions. From the histograms, the channel open probability was calculated and was found to increase from 0.01 to 0.9.

Channel current recordings after stimulation with a hypotonic medium were not always like the one shown on Fig. 1*a*. They frequently produced a multitude of channel opening events, and different current levels could be observed. In some cases, separate single-channel openings could be seen and four different amplitudes could be clearly distinguished. Figure 2 shows two current segments recorded during a hypotonic shock, and four different kinds of single-channel activity are clearly visible. With an applied pipette potential $V_p = +50$ mV, the channel openings measured on the recording had amplitudes of approximately 2.5 pA (α), 1.2 pA (β), 5 pA (γ) and 9 pA (δ). In the recording segment shown on Fig. 2, the opening events identified by α, γ and δ are most of the time superimposed on a baseline corresponding to the open state of activity β which closes only for very short periods. The histogram analysis of the complete recording (Fig. 3) shows more clearly the amplitudes and the relative opening probabilities of these channels. Clearly,

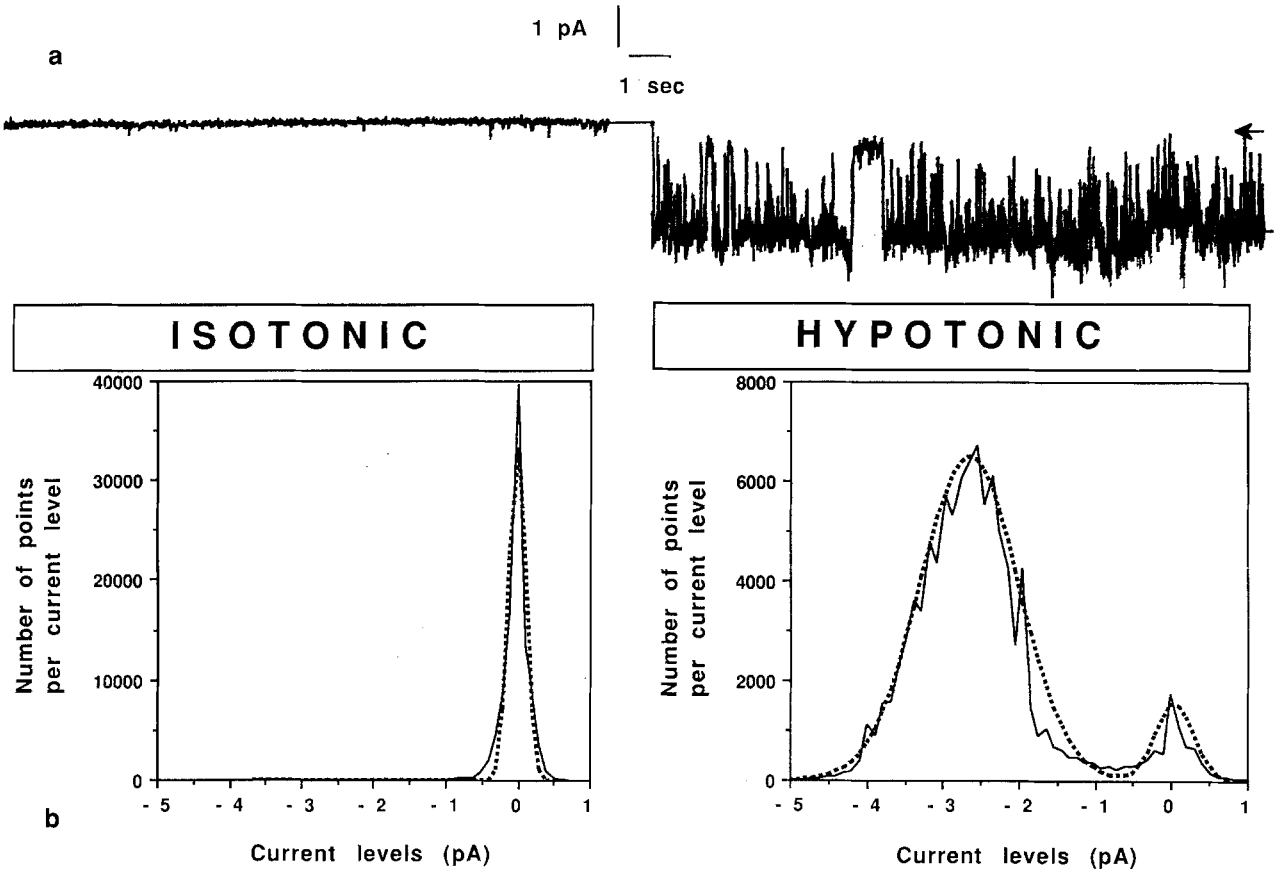

Fig. 1. Effect of a hypotonic medium (50%) on MDCK cells at the single-channel level. (*a*) Cell-attached recording showing channel activity before and during the hypotonic stress with an applied pipette potential $V_p = +40$ mV. Arrow indicates the closed-state level. (*b*) Current-amplitude histograms calculated from the total recording before and during hypotonic shock. Experimental histograms (continuous lines) have been fitted with Gaussian curves (dashed lines).

channel β is the most active followed by channel α, γ and δ. The amplitude of the last two channels is difficult to obtain from the histogram, because they remain open during very short times. It should be noted that channel α on Fig. 2 corresponds to the channel shown on Fig. 1*a*.

In our numerous recordings of current fluctuations triggered by the hypotonic medium, the presence of each of these channels was variable. The channels appearing most frequently were α and β, being observed in 60 and 21% of the recordings, respectively. They usually remained active for several minutes after the hypotonic shock, allowing enough time to perform a detailed analysis of their properties. Furthermore, activities like α and β did not stop after the excision of the patch to the inside-out configuration. The other two channels, γ and δ, were observed rarely (in 12 and 5% of the recordings, respectively), and, since their open duration was very short, it was not possible to analyze them in detail. Therefore, the following sections will describe the properties of channels α and β and their activation after a hypotonic shock.

Potassium Channel

In several different experiments with K-Cl pipettes, channel α alone was observed and the current voltage (I/V) curves were obtained in the cell-attached configuration, as shown in Fig. 4*a*. These experiments were performed on cells exposed to the isotonic medium, so K^+ concentrations in the pipette solution and in the intracellular medium were nearly equal; the channel was then exposed to almost symmetrical potassium concentrations. The Nernst equilibrium potential for potassium (E_K) was then expected to be around 0 mV. In a cell-attached experiment with a KCl-filled pipette, the reversal potential of a potassium current should be given by a pipette potential (V_p) equal to the cellular resting potential. The reversal potentials observed on the

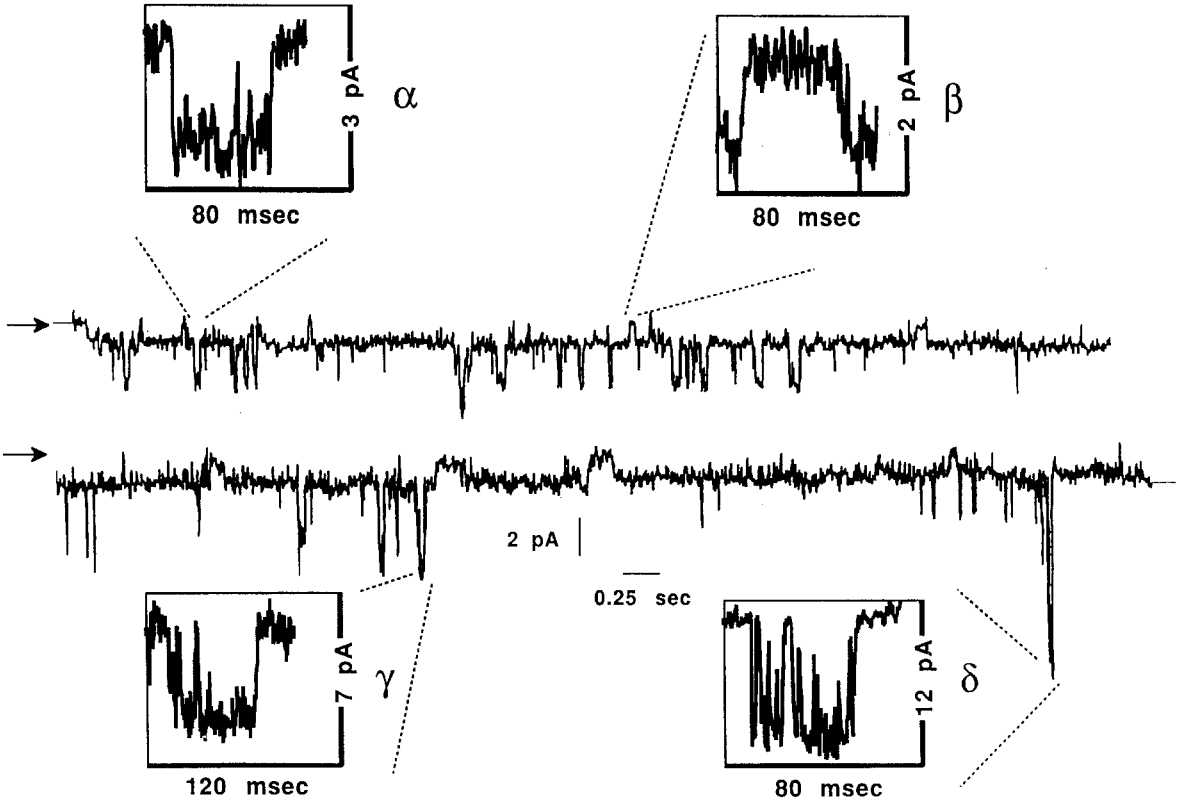

Fig. 2. Current recording segments from a cell-attached patch during hypotonic incubation with an applied pipette potential $V_p = +50$ mV. Arrows indicate the closed-state level. Channel openings at four different current levels can be observed. Insets show magnifications of single opening-closing events. Their current amplitudes are: $\alpha = 2.5$ pA, $\beta = 1.2$ pA, $\gamma = 5$ pA, and $\delta = 9$ pA.

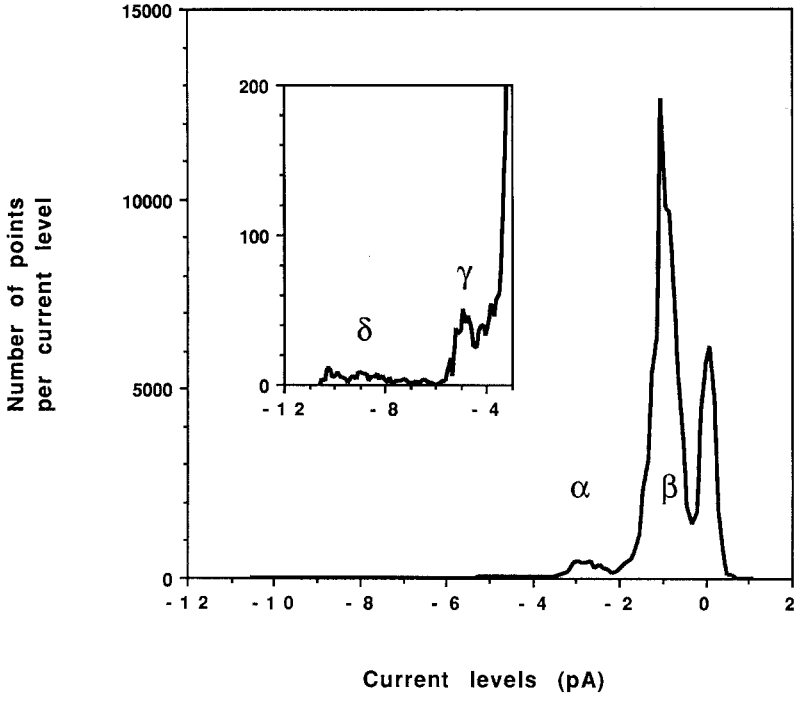

Fig. 3. Histogram of the distribution of current levels from a cell-attached recording containing several different channel activities stimulated by the hypotonic shock with a pipette voltage $V_p = +50$ mV. The histogram refers to the signal shown in Fig. 2.

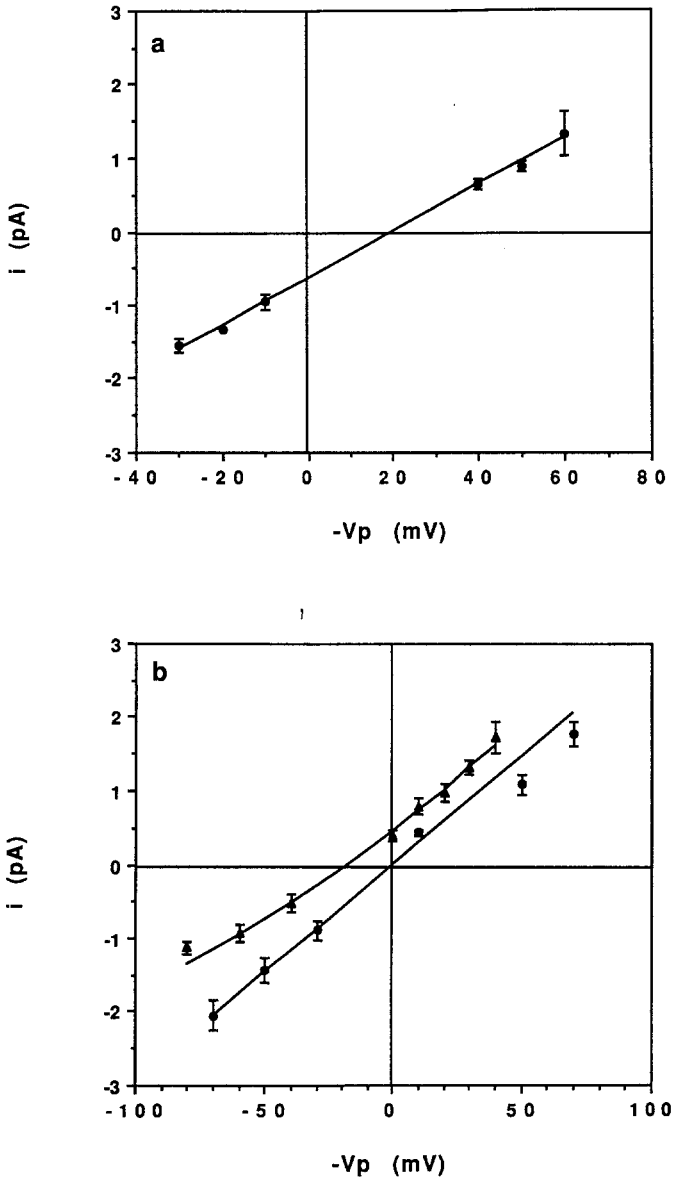

Fig. 4. Current-voltage relations for the potassium channel. Separate points represent single-channel current values i measured for different applied holding potentials V_p (*see* Materials and Methods for the conventions on voltages and currents). (*a*) Results from a cell-attached experiment. The pipette contained the K-Cl solution, and the cells were in the isotonic solution. The continuous line is a linear regression of the experimental points. (*b*) I/V curves from inside-out experiments. Circles were obtained in symmetrical K-Cl solutions, triangles for K-Cl in the bath and 50% K-Cl, 50% Na-Cl in the pipette solution. In both cases the experimental points have been fitted with GHK equations (continuous lines).

different curves were situated at values of $-V_p$ between +20 and +30 mV. These data are therefore consistent with I/V curves produced by a potassium current on cells at a resting potential $V_c \cong -25$ mV. Under these conditions the I/V curves showed no rectification and a slope conductance for the channel of 25.3 ± 4.6 pS ($n = 4$) was evaluated by means of linear regressions of the experimental data (the continuous line in Fig. 4*a*).

Inside-out experiments have also been performed on this channel in order to characterize better its selectivity. In a first series of experiments the K-Cl solution was used both in the pipette and in the bath. Under these conditions the current-voltage relation shown on Fig. 4*b* was still linear and had a conductance of 23.7 ± 4.3 pS ($n = 10$), which is consistent with the results in the cell-attached observations. In a second series of experiments, K-Cl was still the bath solution, while the pipette contained a 50% K-Cl, 50% Na-Cl solution. Under these conditions the I/V curve gave a reversal potential of approximately −17 mV. This implies a permeability ratio $P_{Na}/P_K < 0.04$, thus indicating that this channel is highly specific for K^+. The I/V curves of both experiments in Fig. 4*b* have been fitted with a Goldman-Hodgkin-Katz (GHK) equation:

$$I = P_K \frac{V_m F^2}{RT} \frac{[K]_i - [K]_o \exp(-V_m F/RT)}{1 - \exp(-V_m F/RT)} \qquad (3)$$

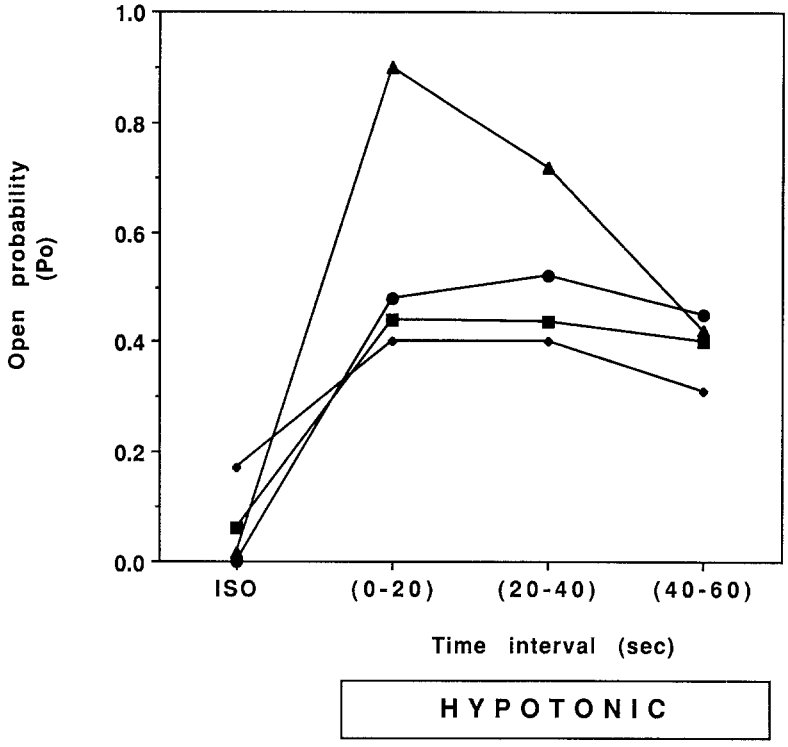

Fig. 5. Evolution of the open probability (P_o) of the potassium channel in 20-sec periods during the first minute of hypotonicity from four different cell-attached recordings.

where P_K = K^+ permeability, V_m = membrane potential, F = Faraday's constant, R = gas constant, T = absolute temperature, $[K]_i$ = bath K^+ concentration, and $[K]_o$ = pipette K^+ concentration. The good agreement between the experimental points and the GHK function in both situations implies that the deflection from linearity observed in the presence of NaCl in the pipette solution can be completely explained by the K^+ concentration gradient across the channel.

The recordings in which only one type of channel is present are particularly interesting since they allow an easy inspection of the time evolution of channel activities during the volume regulatory phase. Figure 1*a* shows an example of such recordings for the potassium channel. The left tracing shows no channel activity when cells were exposed to the isotonic medium. As soon as the bath was perfused with the hypotonic solution, a sudden, intense channel activity was recorded (right tracing). The analysis of current-amplitude distributions (Fig. 1*b*) revealed an increase in the channel open probability from 0.01 to 0.9 in the first 20 sec following the shock. Figure 5 shows the evolution of the channel's open probability (P_o) during the first minute of hypotonicity in four different experiments. It is interesting to point out that the hypotonic shock induced a large increase in the open probability of this potassium channel in the very first seconds of the hyposmotic stress. Generally this increase in P_o was sustained during the first minute of hypotonicity (circles, squares) or, in some cases, a certain decrease in P_o was observed (triangles).

The mechanism responsible for this activation has not been investigated yet; however, some inside-out experiments have been carried out in order to study the dependence of this activity on cytoplasmic calcium concentration. During these experiments the K-Gluc-EGTA solution with the addition of 1 mM $CaCl_2$ (giving 8 μM free-Ca^{2+} ions) was used as the pipette solution. The bath solutions were respectively: K-Gluc-EGTA (0 free Ca^{2+}), K-Gluc-EGTA + 1 mM $CaCl_2$ (8 μM free Ca^{2+}) and Earle's (1.8 mM free Ca^{2+}). Figure 6*a* shows three segments of a recording from an inside-out experiment, with three different bath calcium concentrations (V_p = +70 mV). In Fig. 6*b* the channel's open probability is plotted as a function of cytoplasmic calcium concentration as observed in two different experiments. The reduction of free Ca^{2+} ion concentration from 1.8 mM to 8 μM generally did not affect significantly the channel's activity; however, the open probability dropped to 0 when calcium ions were totally removed from the bath solution. The restoration of free Ca^{2+} ions at a concentration of 8 μM usually failed to reactivate the channel, only in a few cases

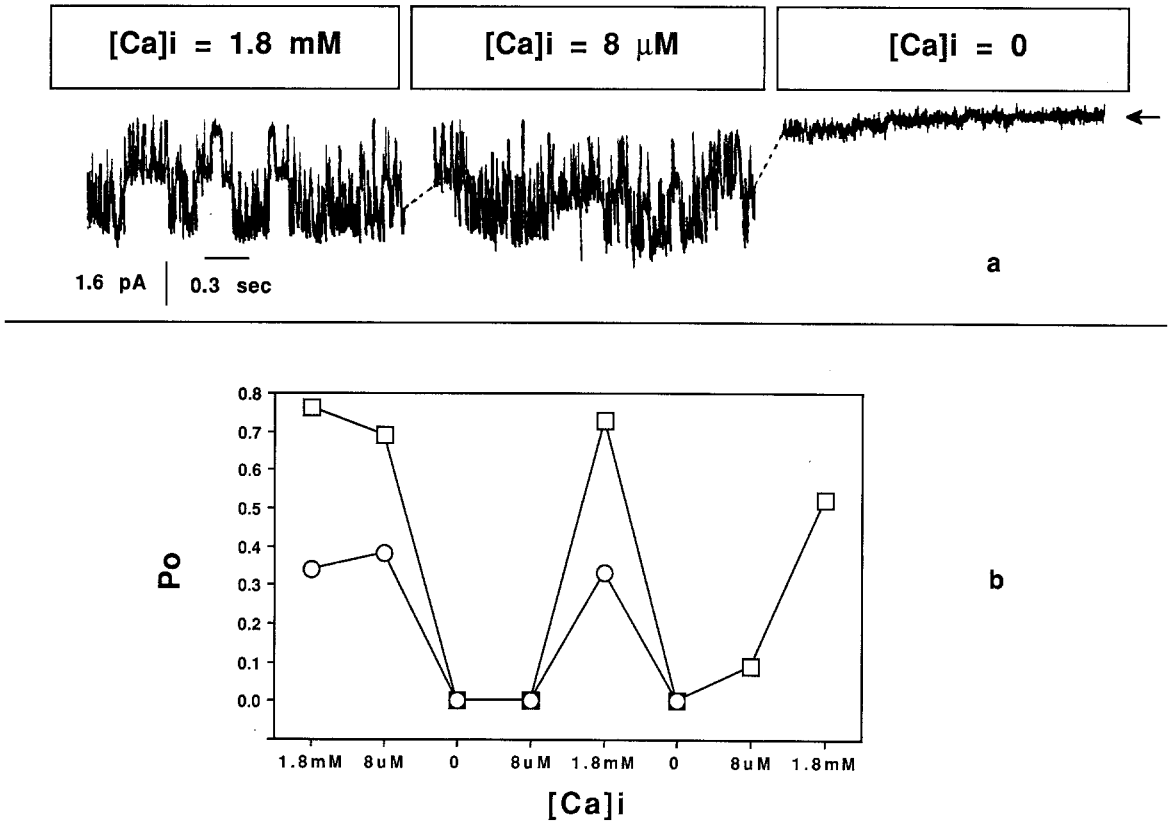

Fig. 6. Effect of cytoplasmic free calcium ion concentration on the activity of the potassium channel. (*a*) Inside-out recordings of channel activity with different bath calcium concentrations. The applied pipette potential was $V_p = +70$ mV. The arrow indicates the closed state. (*b*) Channel open probability expressed as a function of bath calcium concentration from two inside-out experiments. In both cases the pipette holding potential was $V_p = +70$ mV.

a small increase in P_o was recorded. When the Ca^{2+} concentration was raised to 1.8 mM, the open probability generally increased towards the control value.

Since it is established that quinine can inhibit RVD in MDCK cells (Simmons, 1984; Roy & Sauvé, 1987; Rothstein & Mack, 1990), inside-out experiments were performed in order to study the effect of quinine on the channel activity when applied to the cytoplasmic side of the membrane. In these experiments pipettes were filled with the K-Gluc-EGTA + 1 mM $CaCl_2$ solution. Either the Earle's medium or the K-Gluc-EGTA + 1 mM $CaCl_2$ were used as bath solution to which quinine was added. Similar results were obtained under both experimental conditions. Quinine was added at concentrations of 100 μM and 1 mM. Figure 7*a* illustrates the results of an inside-out experiment in which the Earle's medium was the bath solution and the pipette potential was +70 mV. In Fig. 7*b* single-channel currents were plotted as a function of bath quinine concentration, as observed in two experiments. The addition of 100 μM quinine did not produce large effects on the channel's activity (Fig. 7*a*). The observed changes in single-channel amplitude were always within the experimental errors, as seen in Fig. 7*b*. However when quinine concentration was raised to 1 mM a significant reduction in single-channel current by 18 ± 8% ($n = 9$) was observed and the effect was reversible. In addition, the application of 1 mM quinine significantly reduced open probability. In three different experiments carried out with the Earle's solution, the average P_o was 0.72 ± 0.07 in control, 0.68 ± 0.10 in presence of 100 μM quinine and 0.38 ± 0.12 with 1 mM.

Chloride Channel

To determine if anion-selective channels were involved during RVD, cell-attached experiments were carried out with a potassium-free pipette solution. In this case the Chol-Cl solution was used. Under these conditions the activity of channel β was the one most frequently observed at different pipette

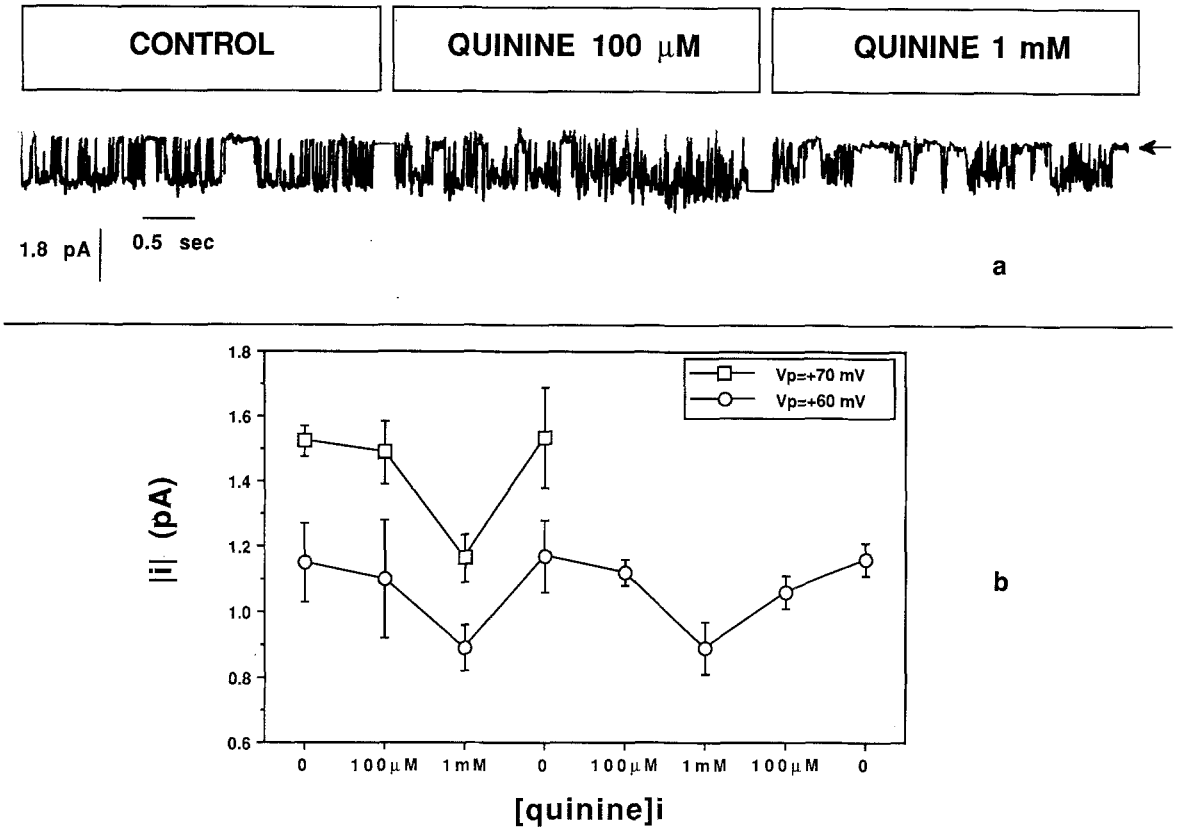

Fig. 7. Effect of quinine on the potassium channel activity. (*a*) Inside-out recordings with different bath quinine concentrations. The bath solution was Earle's medium, and the pipette potential was $V_p = +70$ mV. The arrow indicates the closed state. (*b*) Single-channel amplitudes i for different bath quinine concentrations from two inside-out experiments. Since the application of positive pipette potentials produced negative currents, the absolute value $|i|$ is plotted on the ordinate axis.

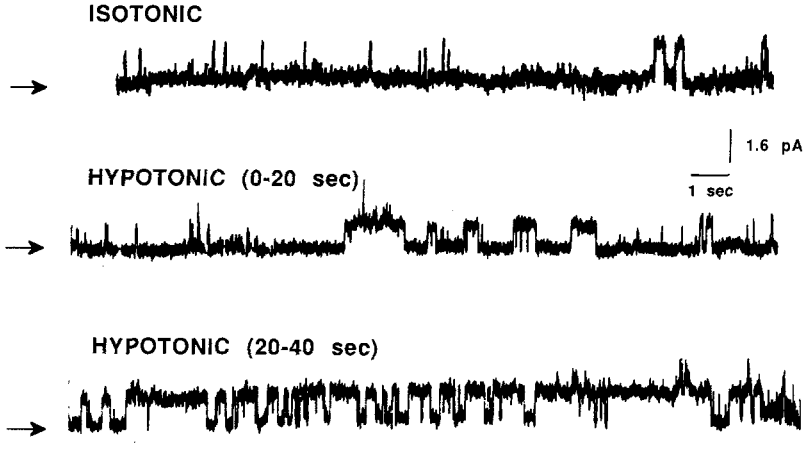

Fig. 8. Cell-attached recording showing the activity of the chloride channel before the hypotonic shock (upper trace), in the first 20 sec after the shock (middle trace) and during the second 20-sec period in hypotonicity (lower trace). Arrows indicate the closed state.

holding potentials, thus allowing the determination of the current-voltage curves. Figure 8 illustrates a typical example of the activity of this channel before and after the hypotonic shock. Figure 9*a* gives the *I/V* curve for this channel in cell-attached mode and the cell exposed to the hypotonic solution. Under these conditions we can calculate an approximate value of the Nernst equilibrium potential for chloride (E_{Cl}), taking into account that, with our hypotonic medium, the intracellular ionic concentrations are

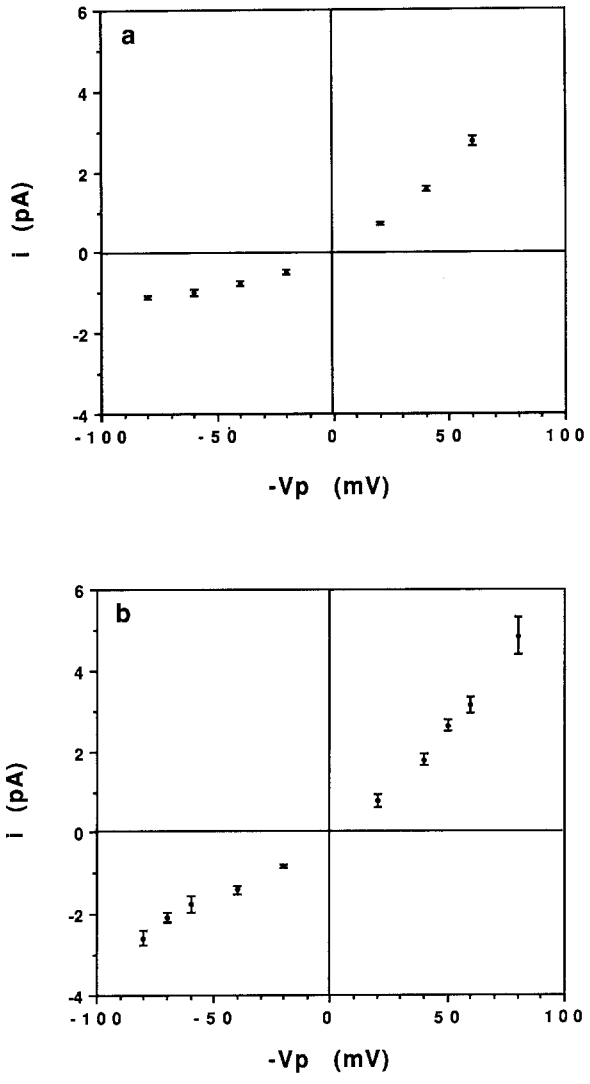

Fig. 9. Current-voltage relations for the chloride channel. (*a*) *I/V* curve obtained from a cell-attached experiment with the cells exposed to the hypotonic solution. The pipette contained the Chol-Cl solution. (*b*) *I/V* curve of the same channel after the excision of the patch to the inside-out configuration. The K-Cl solution was in the bath.

reduced by 50% because of cellular swelling. In this case E_{Cl} is expected to be at values of V_m around -35 mV, since the intracellular Cl^- concentration is approximately 30 mM (Roy & Sauvé, 1987). The Nernst potential for K^+ (E_K) is expected to be at very negative values of the membrane potential ($V_m < -70$ mV), because of the absence of K^+ in the pipette solution. The reversal potential of the *I/V* curve shown on Fig. 9*a* is around $-V_p = 0$ mV, meaning that the reversal potential is equal to the cell membrane potential, which is usually between -30 and -35 mV (Roy & Sauvé, 1987). Figure 9*b* shows the *I/V* curve of the same channel after excision of the patch to the inside-out configuration. These inside-out experiments were carried out with the pipette containing the Chol-Cl solution and with the K-Cl solution in the bath. Under these conditions the membrane was exposed to symmetrical Cl^- concentrations and to a strong cation concentration gradient. The reversal potential of the *I/V* curve is situated around 0 mV, thus indicating the high selectivity of this channel for chloride *vs.* potassium ions.

Both current-voltage curves of Fig. 9 show a marked outward rectification, which is more pronounced in the cell-attached condition because of the chloride concentration gradient. Fitting the positive and negative branches of the inside-out *I/V* curve separately with linear regressions, we evaluated an outward slope conductance of 63.3 ± 4.7 pS and an inward slope conductance of 26.1 ± 4.9 pS ($n = 6$). The zero current conductance has been calculated from the slope of the tangent at the reversal potential by fitting the experimental data to a 3rd degree polynomial. The value obtained with this method was 39.2 ± 3.4 pS ($n = 6$).

To determine the anionic selectivity of this channel, inside-out experiments were performed with the membrane exposed to a different anionic solution. In these experiments the pipette contained the Na-NO_3 solution and the bath solution was made of 50% Na-NO_3 and 50% Na-Cl. Figure 10 shows the results of these experiments where the separate points represent the experimental *I/V* curve under these conditions and the dashed line is the average *I/V* curve obtained with symmetrical chloride concentrations. It is evident that the experimental points fit the average curve quite well, thus indicating that NO_3^- is as permeable as Cl^- through this channel.

From the recording shown in Fig. 8 we can see that the hypotonic shock induced a gradual increase in the channel open probability and intense activity was observed only 20 sec after the shock. The time course of the open probability P_o was calculated during 20-sec segments of the recordings for the first minute of hypotonicity in four representative experiments. Figure 11 reveals some clear differences in the behavior of this chloride channel and of the potassium channel described in the previous section. The most important feature of the chloride channel was that it underwent a gradual increase in its activity, and the period of maximal activation was always reached with a certain delay after the hyposmotic shock. The observed delays could vary within a rather wide range: from 20–30 sec (as in Figs. 8 and 11) and up to 100–120 sec (*data not shown*). It seems that this chloride channel can express a variety of patterns of activity after the shock, changing from one cell to another.

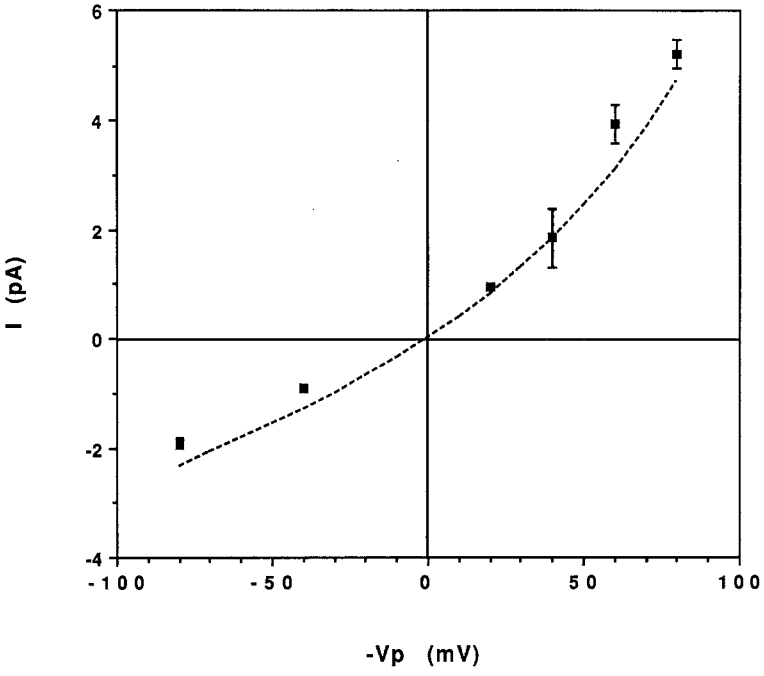

Fig. 10. Chloride channel permeability to nitrate. The separate points represent the *I/V* curve obtained in an inside-out experiment. The pipette contained the Na-NO_3 solution, and the bath solution was made of 50% Na-NO_3 and 50% Na-Cl. The dashed line is the average *I/V* curve of the channel when exposed to symmetrical chloride concentrations.

Since quinine was found recently to be a blocker of chloride channels (Gögelein & Capek, 1990), it was important to determine if it had a blocking effect on this channel. Inside-out experiments were performed, using the Chol-Cl solution both in the bath and in the pipette. It was observed, as shown on Fig. 12, that the application of 100 μM quinine to the solution bathing the cytoplasmic side of the membrane induced rapid transitions from the open to the closed state, thus producing a flickering in channel activity (Fig. 12*a*, upper trace). The addition of 1 mM quinine to the bath solution induced a reduction in single-channel current amplitude (Fig. 12*a*, lower trace). The amplitude reduction was totally reversible upon restoration of the quinine-free solution (Fig. 12*b*). Reversible current reductions were recorded at holding potentials (V_p) ranging from -40 to -80 mV, and it was found that quinine induces a decrease in single-channel amplitude by 37 ± 16% (n = 4). Figure 12*b* illustrates the current reduction as a function of bath quinine concentration, as observed in two of such inside-out experiments. These data indicate that quinine acts as a rapid blocker of this chloride channel.

In order to test whether the channel activity depended on cytoplasmic calcium concentration, inside-out experiments were performed using the Chol-Cl solution with 5 mM EGTA bathing the cytoplasmic side of the patch. The amplitude distribution histograms from one of these experiments are shown in Fig. 13*a* and *b*. It can be seen that both in absence (Fig. 13*a*) and in presence (Fig. 13*b*) of EGTA the channel activities do not exhibit significant differences. In a second series of experiments, the cytoplasmic Ca^{2+} concentration was raised up to 1.8 mM. In this case the Chol-Cl solution in the bath (Fig. 13*c*) was simply replaced by the Earle's solution (Fig. 13*d*). Once again the pattern of activity of the channel was not significantly affected by the change in cytoplasmic Ca^{2+} concentration. Further experiments have been carried out in which the calcium concentration was more rigorously controlled: 1 mM EGTA was added to the calcium-free control solution and 1 mM $CaCl_2$ was subsequently added as a test solution in order to obtain a free-Ca^{2+} activity of 8 μM. Also under these conditions the chloride channel behavior was unaffected by the presence of cytoplasmic free-Ca^{2+} ions.

Other possible messengers responsible for the activation of this channel have not been investigated yet. However, the observation that the excision of the patch to the inside-out configuration always increased the activity of the channel or the number of active channels under the patch, as shown on Fig. 14, may suggest the presence in the cytoplasm of some inhibitor, as recently demonstrated by Krick et al. (1991). It was also observed that when channels tended to inactivate, a rapid step to relatively high depolarizing voltages (100–150 mV) could re-establish a high level of activity, which persisted even

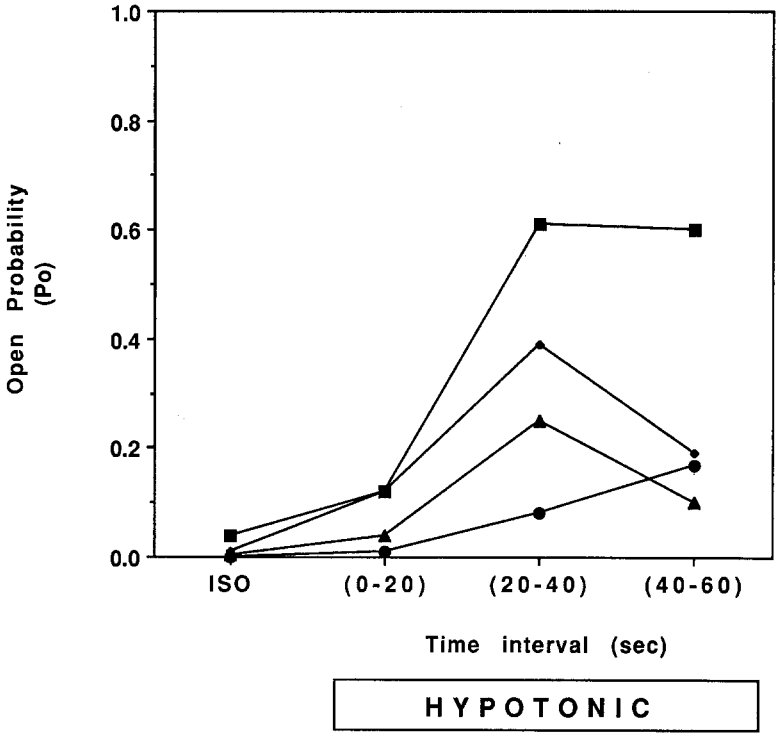

Fig. 11. Evolution of the open probability (P_o) of the chloride channel in 20-sec periods during the first minute of hypotonicity from four different cell-attached recordings.

when the potential returned to hyperpolarizing or less depolarizing values.

Discussion

Our study was intended to determine if some specific ion channels were activated by a hypotonic medium and if these channels could be involved in volume regulation. It was found that several (up to four) different membrane channels became active after a hypotonic shock. However, two particular channels, a K^+ and a Cl^- channel, have been observed more frequently than others in response to the hypotonic shock, and both remained active much longer than the other channels under these conditions. This indicates that they probably play a role of primary importance as mediators of the loss of KCl during the regulatory volume decrease. The evolution of open probabilities of these two channels (Figs. 5 and 11) indicates that after the hypotonic shock cells reacted with a rapid activation of potassium channels, whereas chloride channel activation took place after a delay of about 30 sec. A good agreement can be found between these results and a previous study in which the changes in membrane potential accompanying RVD have been investigated. Roy and Sauvé (1987) have shown that during volume recovery after a hypotonic stress, cells transiently hyperpolarized towards the potassium Nernst potential and then slowly depolarized back to their resting potential. The early activation of highly selective potassium conductances was correlated to the transient initial hyperpolarization, whereas the delayed activation of anionic conductances could contribute to the subsequent return of the cellular potential to its resting value. An initial increase in potassium conductance followed by a delayed increase in anionic conductances has also been reported by Ritter, Paulmichl and Lang (1991).

Both channels studied here and appearing to be the ones mostly involved in RVD have not yet been observed in MDCK cells. Three potassium channels and one anionic channel have been previously reported (reviewed by Lang et al., 1990): a large conductance (221-pS) calcium-activated potassium channel (K_{Ca}) characterized by a linear I/V relation (Bolivar & Cereijido, 1987), an inward-rectifying K_{Ca} (Friedrich et al., 1988), an outward-rectifying K_{Ca} (Kolb, Paulmichl & Lang, 1987) and a large conductance (460-pS) voltage-dependent anion channel (Kolb, Brown & Murer, 1985). It is possible that some of these channels are activated during volume regulation, being part of the numerous channels sometimes briefly observed as shown in Fig. 2, but they would not play a major role in RVD. The new

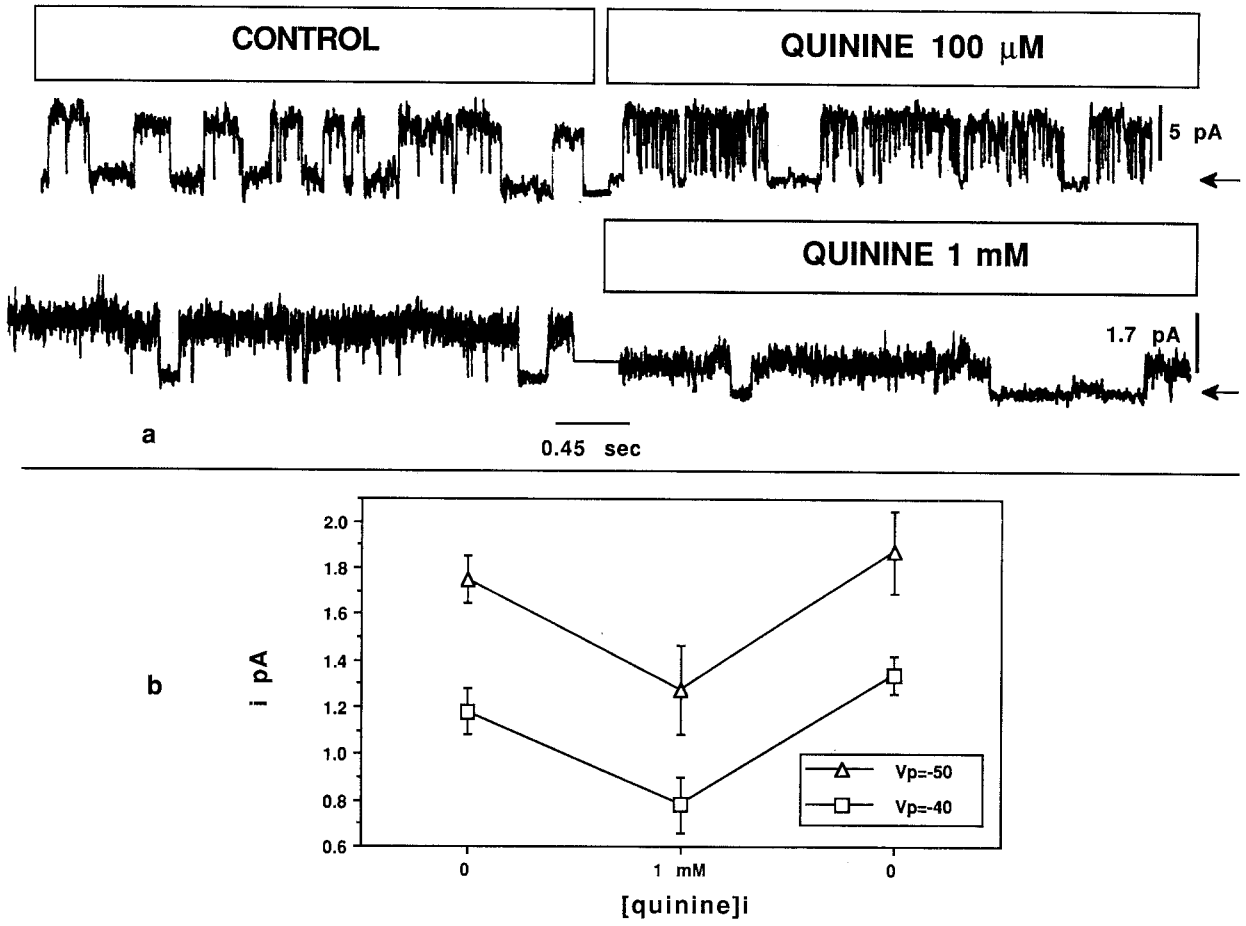

Fig. 12. Effect of quinine on chloride channel activity. (*a*) Inside-out recordings showing the response of the channel to different concentrations of bath quinine. (Upper trace) Behavior of the channel without (left) and with 100 μM quinine (right). Applied pipette potential $V_p = -80$ mV. (Lower trace) Behavior of the channel without (left) and with 1 mM quinine (right). Applied pipette potential $V_p = -40$ mV. Arrows indicate the closed state. (*b*) Plot of single-channel current *i* as a function of bath quinine concentrations from two representative inside-out experiments.

potassium channel has a small unitary conductance (24 pS), a linear *I/V* curve and a high specificity to potassium. The anion channel is an outward rectifier with a zero current conductance of 39 pS. This channel shows some similarities with other outward-rectifying chloride channels found in other epithelial and nonepithelial cells: human platelets (Mahaut-Smith, 1990), PANC-1 cells, T_{84} cells and primary cultures of sweat duct epithelium (Tabcharani et al., 1989), rat distal colon cells (Gögelein & Capek, 1990), human nasal epithelium (Jorissen et al., 1990) and airway epithelia (Schoumacher et al., 1987; Hwang et al., 1989; Li et al., 1989; McCann & Welsh, 1990).

The activities of both channels were affected by quinine. It was observed in fact, that the drug acted as a rapid blocker and, when applied at a rather high concentration (1 mM), it could significantly reduce but not abolish the current flow through each of them. These observations are in good agreement with previous results by Roy and Sauvé (1987) who have shown that, although quinine depolarizes the membrane potential, it does not prevent the hyperpolarization observed during RVD. Furthermore, the K^+ and Cl^- losses during RVD were only partly inhibited by the application of 1 mM quinine. Other outward-rectifying chloride channels found on rat distal colon cells (Gögelein & Capek, 1990) were observed to be blocked by quinine. Although the sensitivity of these channels to the drug is higher than that observed on MDCK cells, the effect of quinine is still a rapid block of the channel.

A study on the cellular mechanism that could activate these channels during RVD has not been performed yet; however, their sensitivity to intracellular calcium has been tested. The results of our inside-out experiments clearly show that the activity

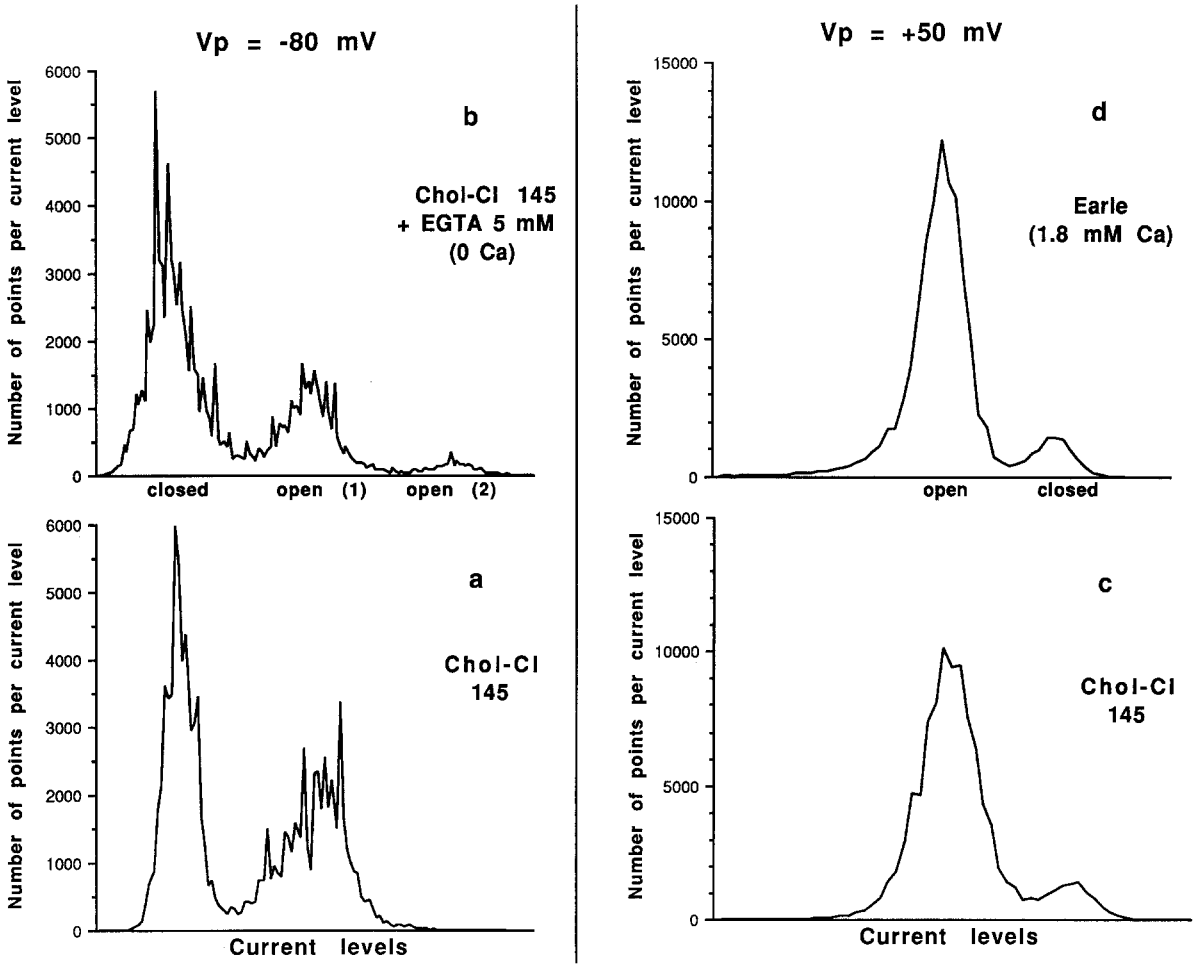

Fig. 13. Effect of Ca^{2+} on the chloride channel. (*a*) Histogram of current-level distribution for two chloride channels from an inside-out experiment. The membrane was exposed symmetrically to the nominally Ca^{2+}-free Chol-Cl solution and with $V_p = -80$ mV. The cytoplasmic side of the membrane is exposed to residual calcium ions at a concentration of about 5 to 10 μM. (*b*) Histogram of current-level distribution of the same channels as in *a*, after the free-Ca^{2+} ions were depleted from the bath solution by the addition of 5 mM EGTA. (*c*) Histogram of current-level distribution of a chloride channel as in *a*, except with $V_p = +50$ mV. (*d*) Histogram of the same channel as in *c*, with Earle's solution containing 1.8 mM Ca^{2+} replacing the Chol-Cl.

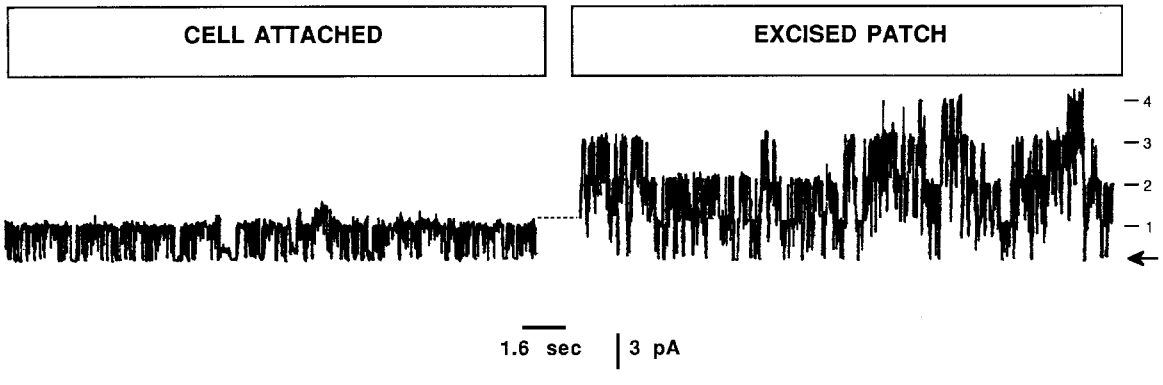

Fig. 14. Effect of excision of the patch on chloride channel activity. In the cell-attached configuration only one active channel could be observed. After patch excision up to four simultaneously active channels were observed. The pipette voltage was $V_p = -60$ mV. Arrow indicates the closed state.

of the anion channel is not affected by the absence or presence of cytoplasmic free calcium at different concentrations. On the other hand, the potassium channel is inactivated in absence of calcium ions in the cytoplasmic solution. The fact that this channel could not be reactivated by 8 μM Ca^{2+} in the cytoplasmic solution, but only with a much larger Ca^{2+} concentration, could mean that some changes in the Ca^{2+} sensitivity of this channel has occurred after the patch excision. A more detailed study of the Ca^{2+} dependence of this K^+ channel would be necessary.

An important feature observed on the outward-rectifying chloride channel was that the excision of the patch to the inside-out configuration generally increased its activity. A similar behavior of chloride channels has been reported by McCann and Welsh (1990) and Schoumacher et al. (1987) on airway epithelium. This indicates that the cytoplasm could contain an agent capable of inhibiting the channel's activity, whose concentration could be reduced during RVD. The hypothesis of the existence of cytoplasmic inhibitors of chloride channels has been recently tested by Krick et al. (1991) on a variety of epithelial tissues. The fact that the channels can be activated by depolarizing voltage pulses indicates that it could also be voltage operated, even if its open probability does not seem to be strongly voltage dependent. Steigner et al. (1990*b*) reported that fused MDCK cells regulate their volume after a hypotonic stress by means of a net efflux of K^+ and HCO_3^- and that bicarbonate extrusion could be mediated by a nonselective anion channel. In our experiments the permeability of the chloride channel to bicarbonate was not tested. However, Tabcharani et al. (1989) reported that their outward-rectifying chloride channels are also permeable to bicarbonate. We investigated instead the permeability of the MDCK chloride channel to nitrate. Roy and Sauvé (1987) had shown that nitrate could replace chloride during RVD in MDCK cells. The observation that nitrate ions were as permeable as chloride ions provides evidence for the rather low specificity of the channel. It could suggest that this channel might represent a pathway for the efflux of several different anionic compounds during regulatory volume decrease in MDCK cells.

In conclusion, we have tried to provide convincing evidence that two small ion channels, one for K^+ and one for Cl^-, and other anions play the major roles during the osmolyte losses induced by a hypotonic medium. Further studies will attempt to determine the cellular mechanism of activation of both the K^+ and the Cl^- channels during RVD, in order to give a more complete description of the phenomenon.

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