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Single-Channel Activities of the Human Epithelial Ca^{2+} Transport Proteins CaT1 and CaT2

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Abstract. The human epithelial channels, CaT1 and CaT2, were expressed in oocytes, and their single-channel characteristics were compared. In the presence of Na^+ and K^+ as charge carriers in the pipette solutions, channel activities were observed only when the the extracellular sides of the patches were exposed to nominally Ca^{2+}- and Mg^{2+}-free solutions. In patches of both CaT1- and CaT2-expressing oocytes, multiple channel openings were observed, but the current levels were higher in CaT2-expressing oocytes, particularly at more negative voltages. With K^+ as a charge carrier in patches of CaT1-expressing oocytes, the channel activity was low at −10 to −60 mV, but increased dramatically at more negative potentials. This voltage dependence was observed in the presence of both Na^+ and K^+. The channel activity with Na^+, however, was higher at all potentials. Differences between the voltage dependencies for the two cations were also observed in CaT2-expressing oocytes, but the channel activities were higher than those in CaT1-expressing oocytes, particularly in the presence of Na^+. We also found that low concentrations of extracellular Mg^{2+} (5–50 μM) elicited a strong inhibitory action on the CaT channels. Activation of the CaT1 and CaT2 channels by hyperpolarization and other factors may promote increased Ca^{2+} entry that participates in stimulation of intestinal absorption and renal reabsorption and/or other Ca^{2+} transport mechanisms in epithelial cells.

Key words: Calcium — Single channels — Voltage-dependence — Hyperpolarization-activated currents

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Introduction

In previous studies we have cloned two closely related epithelial calcium transport proteins, CaT1 and CaT2, from the duodenum of the gastrointestinal tract [26] and from kidney [25]. They comprise a novel subclass of Ca^{2+}-permeable channels that are homologous (~30% identities) to some types of nonselective cation channels including the capsaicin receptor VR1, which is gated by capsaicin and heat, its homologue VRL-1 (VRL-1 is also known as growth factor-regulated channel), the stretch-inhibitable channel (SIC) and the osmolarity-sensitive channel OTRPC4 [6, 30]. These channels are also distantly related to the transient receptor potential (TRP) and TRP-like family with limited homologies in the sixth transmembrane domain and in the pore region [37]. The CaT2 genes cloned from human and rat kidney are likely to be orthologs of the ECaC gene originally cloned from rabbit kidney [12].

In previous studies using methods to measure Ca^{2+} uptake and whole-cell currents, we as well as other investigators have characterized the electrophysiological properties of the CaT channels and found that they are highly permeable to Ca^{2+} and also to other alkaline-earth metals including Ba^{2+}, Sr^{2+} and transient metal Cd^{2+} [13, 14, 22, 27, 31, 34]. They are also permeable to Na^+ and K^+. In recent studies it was shown that the CaT1 channel activity is inhibited by a noncompetitive antagonist of the IP_3 receptor [33] and that CaT1 displays the properties of the Ca^{2+}-release-activated Ca^{2+} channel (I_{CRAC}) [36], suggesting that CaT1 is functionally coupled to the IP_3 receptor and can be activated by depletion of the intracellular Ca^{2+} stores. The physiological role(s) of CaT1 may also be related to apical Ca^{2+} entry in the gastrointestinal tract and other tissues involving transcellular calcium transport such as kidney, and placenta, as well as the exocrine systems of prostate, pancreas and

salivary gland. CaT2 may play a role in Ca^{2+} transport in the distal segment of the nephron where active calcium reabsorption takes place. Both CaT1 and CaT2 likely play key roles in Ca^{2+} homeostatic mechanisms in humans and other mammals. Without understanding the specific characteristics of the human orthologs of CaT1 and CaT2, however, it is impossible to clarify their contributions to these important physiological processes.

We examined and compared, therefore, the single-channel properties of human CaT1 and CaT2 when expressed in *Xenopus laevis* oocytes. We established that both types of channels display nonlinear current-voltage relationships and are strongly activated by hyperpolarization. Their conductances and voltage dependencies, however, differ in the presence of K^+ and Na^+. The kinetics of the CaT channels also vary in the presence of different divalent cation-chelating agents and are blocked by micromolar concentrations of Mg^{2+}.

Materials and Methods

Oocyte Preparation and Expression of CaT1 and CaT2 in Oocytes

Oocytes at stages V–VI were harvested from *Xenopus laevis* and defolliculated by treating them for ~2 hours at room temperature with 3 mg/ml collagenase (Boehringer Mannheim, Mannheim, Germany) in a Ca^{2+}-free solution containing Ca^{2+}-free modified Barth's solution. Oocytes were injected, on the same day (at least 4 hours after defolliculation) or on the following day, with 50 nl H_2O containing 50 ng of the synthetic RNA (cRNA) of either human CaT1 or CaT2, which were prepared by in vitro transcription as described previously. For comparison, in some experiments the same amounts of cRNA encoding rat CaT1 [26] or rat CaT2 [25] were injected into the oocytes in the same manner. Equal amounts of H_2O were injected into control oocytes as negative controls. Injected oocytes were then incubated at 18°C or 14°C in Barth's solution containing (in mM): 90 NaCl, 2 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 10 HEPES, 10 units/ml penicillin and 10 μg/ml streptomycin, pH 7.5.

Electrophysiological Measurements

Ion channel activities were recorded in cell-attached or excised membrane patches of CaT-expressing oocytes using standard patch-clamp techniques [11]. The micropipettes were pulled from borosilicate glass capillaries and fire-polished to a tip diameter of less than 1 μm. The pipette solution contained in mM (unless otherwise specified): 100 KCl, 0.1 $CaCl_2$, and 10 HEPES, pH, 7.5 or 100 KCl, 10 N-(2-hydroxyethyl) ethylene-diaminetriacetic acid (HEDTA), and 10 HEPES, pH, 7.4 adjusted with Tris base. In some experiments 100 mM KCl was substituted with 100 mM NaCl or higher concentrations (150 mM) of both salts were used as described in the figure legends When filled with one of these external solutions, the pipette tip resistances were 5–10 MΩ. Seals with resistances of >5 GΩ were employed in single-channel experiments, and currents were measured with an integrating patch-clamp amplifier. Single-channel currents were filtered at 3–10 kHz through an 8–pole Bessel filter. The bath solution contained in mM (unless otherwise specified): 100 KCl, 10 HEDTA, 10 HEPES, pH, 7.4 adjusted with Tris base or 100 KCl, 0.1 μM $CaCl_2$, and 5 ethylene glycol-bis(beta-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), pH, 7.5. Equations reported in a previous study [32] were used to adjust the concentrations of Ca^{2+} in solutions containing low Ca^{2+} (0.1–100 μM). In some experiments different amounts of $MgCl_2$ were added to adjust the final concentrations of free Mg^{2+} to 5 μM or 50 μM according to the Maxchelator program [2].

Data Acquisition and Analysis

Voltage stimuli were applied and single-channel currents digitized (20–150 μsec per point) and analyzed using a PC, a Digidata converter, and programs based on pClamp including pStat for statistical analysis (Axon Instruments, Foster City, CA). The baseline current was monitored frequently to ensure proper analysis of single-channel currents. In all the figures shown, downward deflections represent negative inward currents. The open state probability (*Po*) of the channels was calculated from 20 to 30-sec segments of current records in patches containing apparently only one functioning channel, since only one current level was observed in these recordings. However, only a small number of patches contained a single functioning channel with the main conductance level, and *Po* was estimated only in some experiments when comparing channel activity at two voltages, −50 and −100 mV, as described in the text. In most of the other recordings multiple-channel current levels were observed and for these experiments *nPo* was determined as shown in Figs. 5 and 6. Several hundred or more events were analyzed using half-amplitude threshold criteria for generating each data point. In recordings containing a large number of channels with multiple channel current levels, the current amplitudes were measured by averaging 8-sec intervals at each voltage. Statistical analysis of the data was carried out using the Fisher's least-significant-difference method of multiple comparisons utilizing ANOVA. A P value < 0.05 was considered to indicate a statistically significant result. The experiments were carried out at 23°C.

Results and Discussion

Our previous studies on CaT1- and CaT2-mediated Ca^{2+} uptake and whole-cell currents in *Xenopus* oocytes [25, 26, 27], served as a background to extend our investigations to examining the single-channel characteristics of the CaT channels under defined conditions. Ionic currents were measured in oocytes 2–6 days after injection with human CaT1 or CaT2 cRNAs.

In our previous studies of whole-cell currents [26, 27] we showed that CaT1 is permeable to Ca^{2+}, but we could not record single-channel currents when using high concentrations of Ca^{2+} (80–100 mM) or other divalent cations in the pipette solutions on the extracellular sides of the membrane patches. This was apparently due to the low unitary Ca^{2+} conductance of the CaT channels under these conditions, as has previously been observed with other types of Ca^{2+}-selective and other Ca^{2+}-permeable cation channels [9, 16]. Similar to the studies on these channels, we observed channel activities with a high probability of opening in the presence of both Na^+ and K^+ in pipette solutions that were free of divalent cations.

In the presence of Na^+ as the charge carrier in the pipette solution, long channel openings were observed in

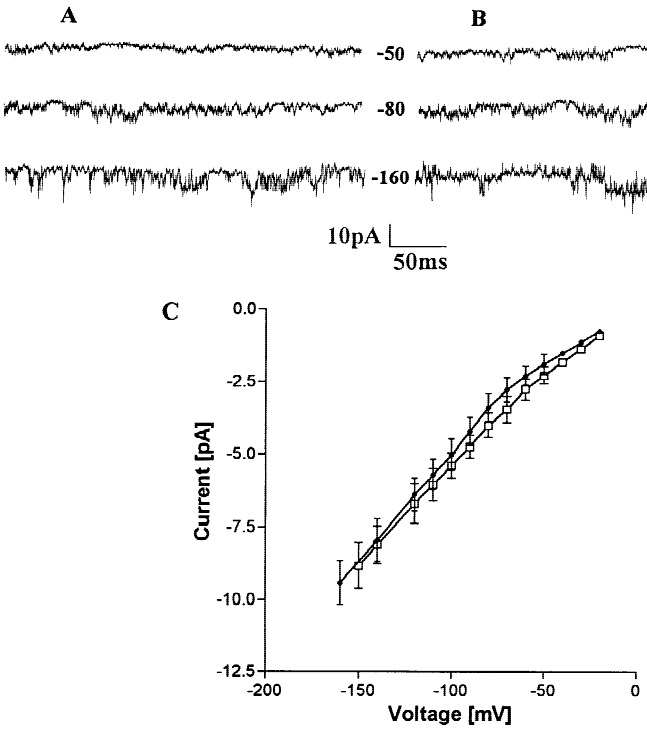

Fig. 1. Single-channel activities in the presence of Na^+ as a charge carrier in cell-attached patches of *Xenopus laevis* oocytes expressing human CaT1 (hCaT1) (*A*) or human CaT2 (hCaT2) (*B*). The current traces were taken at different voltages as indicated between each pair of traces. Downward deflections of the current traces represent inward currents. The current-voltage relationships are shown in *C*. The pipette solution contained 150 mM NaCl and other constituents as described in the Methods section. The bath solution contained in mM: 100 KCl, 10 HEDTA, 10 HEPES, pH, 7.4 adjusted with Tris base.

CaT1- or CaT2-injected oocytes only when the extracellular sides of the patches were exposed to nominally Ca^{2+}- and Mg^{2+}-free solutions (Fig. 1). Similar channel openings occurred in patches of oocytes expressing either human CaT1 or CaT2 (Fig. 1*A* and *B*). The unitary conductances determined from the slopes of the current-voltage relationships deviated from linearity for both CaT1 and CaT2 (Fig. 1*C*). For CaT1 a conductance of 38 +/− 4.3 pS (mean +/− SEM, $n = 12$) was measured at −10 to −60 mV, while at more negative voltages openings with a larger conductance (73.2 +/− 6.8 pS) were observed more frequently. For CaT2 the former conductance was larger but the latter conductance was lower in comparison to CaT1 (45.7 +/− 3.6 pS at −10 to −70 mV and 67.5 +/− 7.1, $n = 8$, respectively).

The differences between the nonlinear current-voltage relations of CaT1 and CaT2 can more clearly be seen in the presence of K^+ in the pipette solution (Fig. 2). The conductances at −10 to −70 mV were 34.2 +/− 3.7 pS ($n = 14$) for CaT1 and 47.6 +/− 5.3 pS ($n = 8$) for CaT2, while those measured at more negative voltages were 78.6 +/− 5.9 pS and 65.3 +/− 6.7 pS for CaT1 and CaT2, respectively. In some patches of oocytes expressing CaT1 or CaT2 we have also observed channel openings to subconductance levels corresponding to approximately 60% and 75% of the main conductance levels. All of the conductances displayed partial inward rectification. The outward currents at 30–120 mV were smaller than the inward currents at the respective negative voltages The conductances at 80–120 mV were 24.3 +/− 3.1pS ($n = 5$) for CaT1 and 21.4 +/− 2.8 pS (n = 4) for CaT2.

Other differences between CaT1 and CaT2 and their respective single-channel properties in the presence of the two charge-carrying monovalent cations were revealed in further studies on the voltage dependence of their channel activities (Figs. 3 and 4). In most of the patches of either CaT1- or CaT2-expressing oocytes, multiple channel openings were observed, but the current levels were at least 2- to 3-fold higher in CaT2-expressing oocytes, particularly at more negative voltages. With K^+ as a charge carrier in patches of CaT1-expressing oocytes, the channel activity was low at −10 to −60 mV but increased dramatically at more negative potentials (Fig. 3*A*). The CaT1 channel activity at −20 to −60 mV was higher in the presence of Na^+-containing pipette solutions, but it also increased at −70 mV and reached larger current levels at hyperpolarizing voltages (Fig. 3*B*). At these voltages the probability of opening was very high and channel closings to the basal level rarely occurred. Therefore, the change in the average current level, instead of the probability of opening as a function of voltage, was used as a criterion for assessing the voltage dependence of the CaT channel activities (Fig. 3*C*). Well pronounced voltage dependence is observed in the presence of both Na^+ and K^+, but the channel activity with Na^+ as charge carrier was higher at all potentials. Differences between the voltage dependencies for the two cations were also observed in CaT2-expressing oocytes, but the channel activities were also higher, particularly in the presence of Na^+ (Fig. 4*A*-*C*).

The above-described voltage-dependent channel activities were observed in 92 out of 108 patches of CaT1-

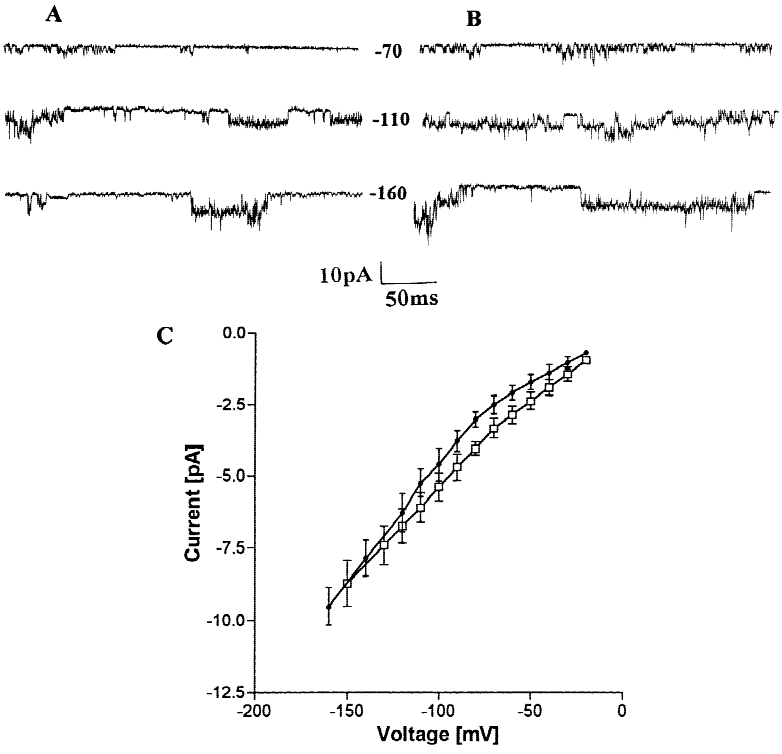

Fig. 2. Single-channel activities in the presence of K^+ as a charge carrier in cell-attached patches of *Xenopus laevis* oocytes expressing hCaT1 (*A*) or CaT2 (*B*). The current traces were taken at different voltages as indicated between each pair of traces. The current-voltage relationships are shown in *C*. The pipette solution contained 150 mM KCl and other constituents as described in the Methods section. The bath solution was the same as that described in the legend of Fig. 1.

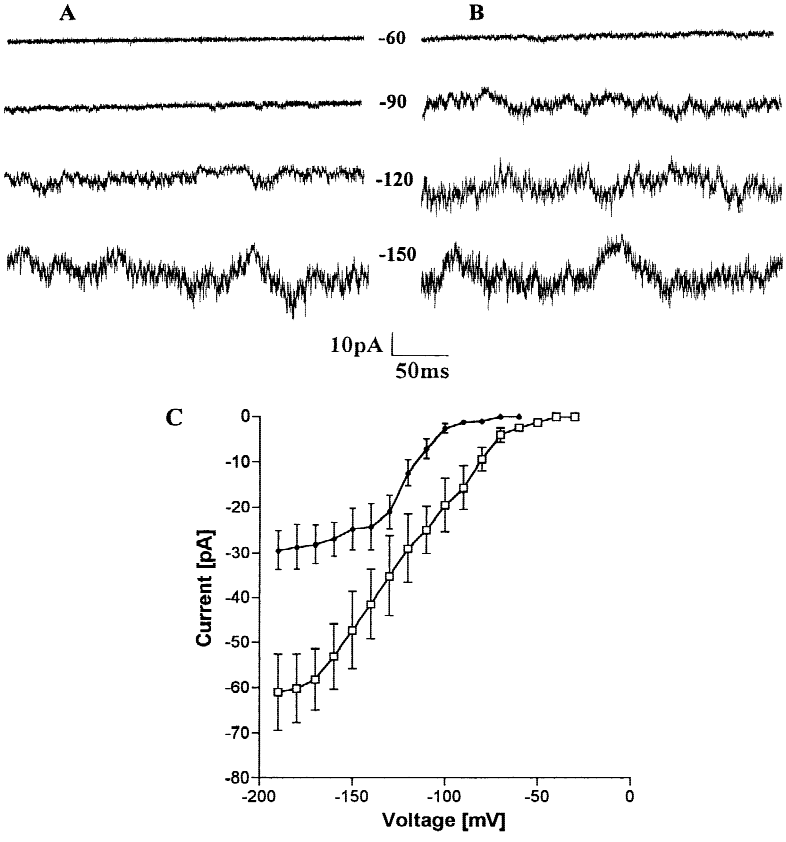

Fig. 3. CaT1 channel activities in the presence of 100 mM KCl (*A*) or 100 mM NaCl (*B*) in the pipette solution. The current traces were taken at various different voltages as indicated between each pair of traces. The average current amplitudes during 8-sec intervals were estimated at each voltage as shown in *C*. The bath solution was the same as that described in the legend of Figure 1.

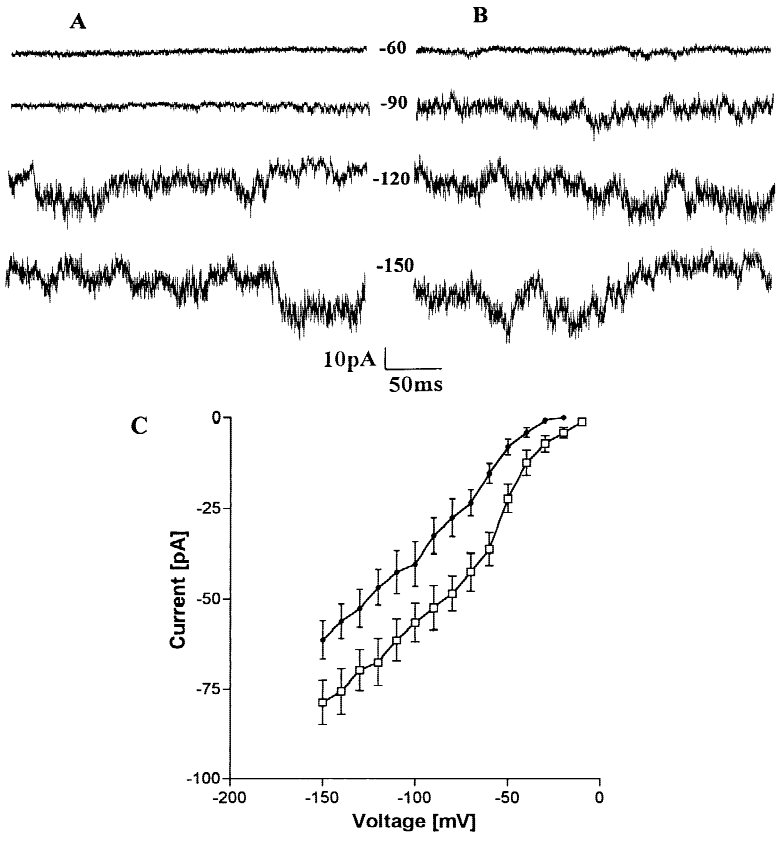

Fig. 4. CaT2 channel activities in the presence of 100 mM KCl (*A*) or 100 mM NaCl (*B*) in the pipette solution. The current traces were taken at different voltages as indicated between each pair of traces. The average current amplitudes during 8-sec intervals were estimated at each voltage as shown in *C*. The bath solution was the same as that described in the legend of Figure 1.

expressing oocytes and in 74 out of 83 CaT2-expressing oocytes. No channel activities with similar characteristics were found in water-injected oocytes ($n = 85$). The relatively small differences between the unitary conductances of the CaT1 and CaT2 channels cannot explain the large differences between their voltage-dependent activities, especially at hyperpolarizing potentials. They may be due to increases in open state probability (*Po*), mean open times and voltage-dependent changes in other channel characteristics. In some of the few patches apparently containing only one active channel, we found that for CaT1 *Po* was 58% higher at −100 mV than that at −50 mV with K^+ as a charge carrier. For CaT2 the difference between the values of *Po* measured at the same two voltages was 42%. Thus an increase in *Po* is at least one of the factors underlying the activation of the CaT channels by hyperpolarization.

Most of our studies on the activities of the CaT channels were carried out in the presence of pipette solutions containing high concentrations of EDTA or HEDTA, 0.25–10 mM, in order to chelate both Ca^{2+} and Mg^{2+}. In some of our initial studies, however, we used similar concentrations of EGTA, which chelates predominantly Ca^{2+}. When studying CaT1-expressing oocytes in the presence of 5 mM EGTA with K^+ as a charge carrier, we frequently observed channel activities that were characterized by bursts of brief openings (Fig. 5*A*). There were only a few openings at low voltages (−10 to −60 mV), but the channel activity increased at more negative potentials, particularly in the range of −90 to −180 mV. Figure 5*B* illustrates the open-state probability *Po* plotted as a function of voltage. It can be seen that *Po* is higher at hyperpolarizing voltages. A similar voltage dependence was also revealed by measurements of the dwell times as a function of voltage (*data not shown*). The voltage-dependent changes in these parameters are compatible with the results shown in Figs. 3 and 4, indicating that the same CaT channels display different kinetic behaviors in the presence of the two chelating agents.

Although no Mg^{2+} was added to the solutions used in the studies described in Fig. 5, it is possible that traces of Mg^{2+} in the micromolar range that are present in these EGTA-containing solutions contribute to the differences in the kinetic behavior observed in the presence of EGTA *vs.* EDTA or HEDTA. The bursts of brief openings shown in Fig. 5 could be due to a partial blocking effect of Mg^{2+} at micromolar concentrations. We have, therefore, tested the effects of 5–50 μM Mg^{2+} on CAT channel activities (Fig. 6). The channel activity was substantially reduced even at low concentration of Mg^{2+} (5 μM) (Fig. 6*A* and *B*), but a more significant blocking

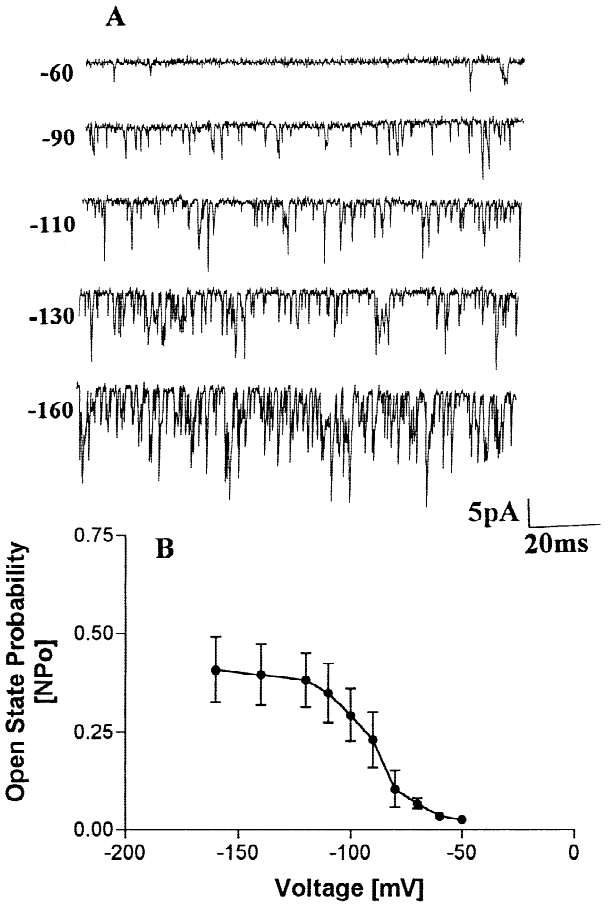

Fig. 5. CaT1 channel activities in the presence of 100 mM KCl, 100 μM $CaCl_2$, 5 mM EGTA and 10 mM HEPES (pH, 7.5) in the pipette solution. Current traces were taken at the voltages indicated on the left side of each trace (*A*). The changes in *Po* as a function of voltage are shown in *B*. The bath solution contained 100 mM KCl, 0.1 μM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES, pH, 7.5.

action was observed at 50 μM Mg^{2+} (Fig. 6*C*). Thus low μM concentrations of extracellular Mg^{2+} exhibit a strong inhibitory effect on the activities of the CaT channels.

To ascertain whether the channel activities described in the present study were specifically mediated by proteins translated from the injected CaT1/2 cRNAs, we treated the oocytes with actinomycin D (10 μg/ml) during the time between the injection of the cRNAs and the performance of electrophysiological studies and observed no effects on the characteristics of the channels. This suggests that the observed channel activities are unlikely to result from upregulation or modulation of channels endogenous to the oocytes.

The single-channel activities described above were observed in both cell-attached and inside-out patches when either Na^+ or K^+ was the charge carrier in the pipette solution, as long as EGTA and HEDTA/EDTA were present to chelate extracellular Ca^{2+} and Mg^{2+}. The channel activities in the present study were characterized by high *Po* and frequent fluctuations in the open state, particularly in the presence of K^+.

The kinetics shown in Fig. 5 and some of the conductance levels of the CaT channels are similar to those of transient receptor potential (TRP) and TRP-like (TRPL) channels, which are also characterized by bursts of brief openings and multiple conductance levels (35, 37.5, 66 and 69 pS) as described in several recent studies [7, 15, 20]. The CaT channels bear limited structural homologies with the TRP channels [26, 27, 37] but the latter type of channels is less selective for Ca^{2+} than the CaT channels. How these properties relate to their functions is not known.

There is evidence indicating that some types of TRP channels may be functionally coupled to the IP_3 and ryanodine receptor Ca^{2+} release channels [3, 17, 18, 21], although their role as potential store-operated channels in the plasma membranes remains debatable [1, 8, 23, 28, 29]. Therefore, the search for channels that may play similar functional roles continues [1, 4, 8, 19, 24, 29, 35, 38]. The CaT1 channels may also contribute to Ca^{2+} influx, but there is evidence that they are coupled to intracellular Ca^{2+}-release-channel mechanisms. Some of their properties, including their blockade by Mg^{2+}, are similar to channels suggested to play a role in such processes [9, 16]. It was shown that channels that are putatively coupled to the state of filling of Ca^{2+} stores are inhibited by 3 μM extracellular Mg^{2+} [16]. This low concentration of Mg^{2+} induced a partial channel blockade that resulted in bursts of brief openings similar to those described in the present study.

The inhibitory effects of micromolar concentrations of Mg^{2+} established in the present study at the single-channel level was substantial but not complete. It appears that the Mg^{2+}-mediated CaT channel blockade is only partial even at 1 mM Mg^{2+}, since this was the concentration we have used in our previous studies for measuring relatively large whole-cell currents in CaT-expressing oocytes. Thus Mg^{2+} at concentrations close to the physiological range does not fully inhibit the CaT channels. The physiological role of the blockade of the CaT channels elicited by Mg^{2+} as well as that related to the increase in extracellular Ca^{2+} is probably part of a protective mechanism limiting the extent of Ca^{2+} overload, similar to those described for other Ca^{2+}-permeable channels.

The CaT channels are similar to other Ca^{2+}-selective channels and lose their Ca^{2+} selectivity and become highly permeable to monovalent cations when extracellular Ca^{2+} (Ca_o) is removed (36). They also display anomalous mole-fraction behavior at very low concentrations of Ca_o. It was found that the voltage-dependent blockade of CaT1 by micromolar concentrations of Ca_o was similar to that of I_{CRAC} (36). The effects of extracellular Ca^{2+}, Mg^{2+} and other divalent cations on such

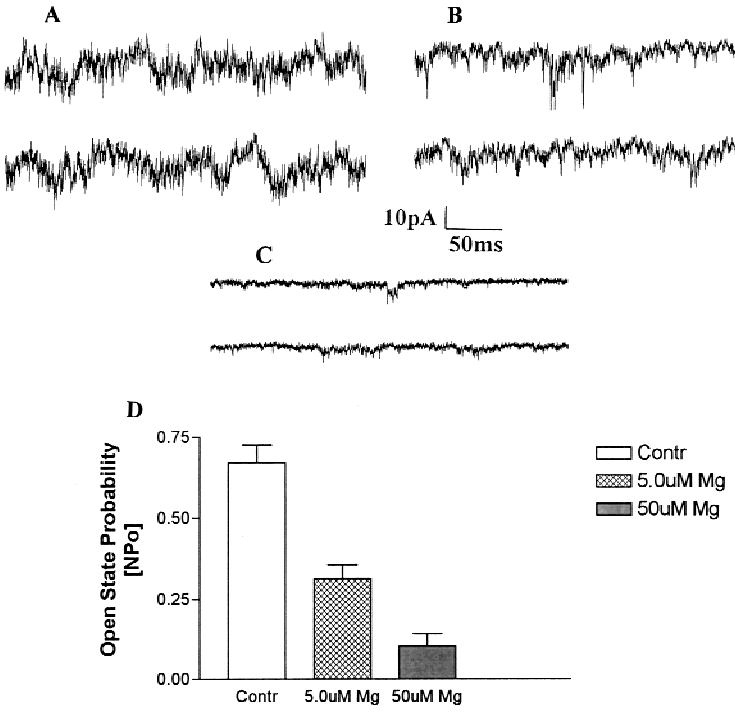

Fig. 6. Inhibition of CaT channels by micromolar concentrations of extracellular Mg^{2+}. Current traces were taken at −130 mV under control conditions in the absence of any added Mg^{2+} in the pipette solution (*A*) or in the presence of 5 μM Mg^{2+} (*B*), or 50 μM Mg^{2+} (*C*). The open-state probabilities measured in the absence of any added Mg^{2+} or at two different concentrations of Mg^{2+} are compared in *D*. The pipette solution contained in mM: 100 KCl, 10 HEDTA, 10 HEPES, pH, 7.4 adjusted with Tris base, and different amounts of $MgCl_2$ were added to adjust the final concentrations of free Mg^{2+} to 5 μM or 50 μM as described in the Methods section. The bath solution was the same as the pipette solution but no $MgCl_2$ was added.

types of Ca^2-selective channels should also be considered in terms of changes in permeation when replacing divalent with monovalent cations. It is also possible that the current-voltage relationships of the CaT1 and CaT2 channels could be different at physiological millimolar concentrations of extracellular Ca^{2+} and Mg^{2+}.

The activation of the CaT channels by hyperpolarization may contribute to the increased Ca^{2+} entry that occurs during intestinal Ca^{2+} absorption and/or renal tubular Ca^{2+} reabsorption [5, 10]. For example, it has been shown that Ca^{2+} influx through the apical membranes of renal tubule cells is mediated by Ca^{2+}-permeable channels activated by hyperpolarization [10]. The CaT channels could represent one of the conductances playing a key role in this process.

Thus the present study established that both CaT1 and CaT2 channels display nonlinear current-voltage relationships and are strongly activated by hyperpolarization. Their conductances and voltage dependencies, however, differ in the presence of K^+ and Na^+. The kinetics of the CaT channels also vary in the presence of different divalent cation chelating agents and are blocked by extracellular Mg^{2+} used at low (micromolar) concentrations.

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