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Inhibition of CaT1 Channel Activity by a Noncompetitive IP_3 Antagonist

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A newly cloned, human epithelial Ca^{2+} transport protein (CaT1) was expressed in *Xenopus laevis* oocytes, and its single channel characteristics were examined. The CaT1 channel shows a strong dependence upon hyperpolarizing voltages, being activated by very negative voltages. The probability of channel opening and mean open times increase substantially at more negative voltages in the range of −90 to −160 mV. In addition, CaT1 channel activity was markedly inhibited by micromolar levels of a noncompetitive antagonist of the IP_3 receptor originally isolated from a marine sponge, Xestospongin C. This inhibitory effect could be mediated indirectly via the binding of Xestospongin C to the inositol-trisphosphate (IP_3) receptor or, alternatively, by a direct action on the CaT1 channel itself. Independent of its mechanism of action in inhibiting CaT1, Xestospongin C will provide a useful tool for elucidating the physiological role(s) of this novel epithelial Ca^{2+} channel.

Key Words: **calcium; single channels; voltage-dependence; hyperpolarization-activated currents; IP_3.**

The epithelial calcium transport proteins, CaT1 and ECaC/CaT2, have been cloned from intestine (1) and kidney (2, 3), respectively. They represent a novel subclass of Ca^{2+}-permeable channels that bear limited homologies to the ligand-gated capsaicin receptor VR1 (4) and its homologue VRL-1, the stretch inhibitable channel SIC, the osmolarity-sensitive channel OTRPC4 (5), as well as the transient receptor potential (TRP) channels (6). In previous studies using measurements of Ca^{2+} uptake and whole-cell currents, we and others have characterized the electrophysiological properties of the CaT and ECaC channels and found that they are highly permeable to Ca^{2+} and other alkaline earth metal ions including Ba^{2+} and Sr^{2+} but not to Mg^{2+} (7–12). They are also permeable to Na^+ but inward currents generated by the permeation of K^+ through the channels at the single channel level have not previously been described. The physiological role(s) of the CaT channels may be related to intestinal Ca^{2+} absorption, transcellular Ca^{2+} transport in kidney and Ca^{2+}-dependent exocrine processes and proliferation. The identification of inhibitors of the CaT channels would be of great utility in regard to their physiological roles. The CaT1 channel has a much wider tissue distribution than CaT2 and could potentially serve as a Ca^{2+} influx pathway in the plasma membrane of a variety of epithelial cells. On the basis of its putative structural similarity to the TRP and TRP-like channels, which have been shown to interact in a functionally significant manner with IP_3 receptors and other Ca^{2+} release channels (13–17), we hypothesized that the CaT1 channel may be sensitive to agents modulating the activity of the IP_3 receptors.

The goals of this study, therefore, were twofold: (1) We investigated the single channel properties of CaT1 when expressed in *X. laevis* oocytes and found that its kinetics and conductances under specific conditions, particularly in the presence of K^+ as a charge carrier in Ca^{2+}-free solutions, are similar to those of some TRP isoforms. The single channel activities of the CaT1 channel are also strongly activated by hyperpolarization, similar to our previous results on whole-cell currents measured in human CAT1-expressing oocytes (12). (2) We also examined whether Xestospongin C (XC), an agent which has recently been described as a specific inhibitor of the IP_3 receptor (18), modulates the activity of CaT1. Indeed, XC can strongly inhibit CaT1 channel activity, raising the possibility that the IP_3 receptor, in addition to its modulatory actions on the

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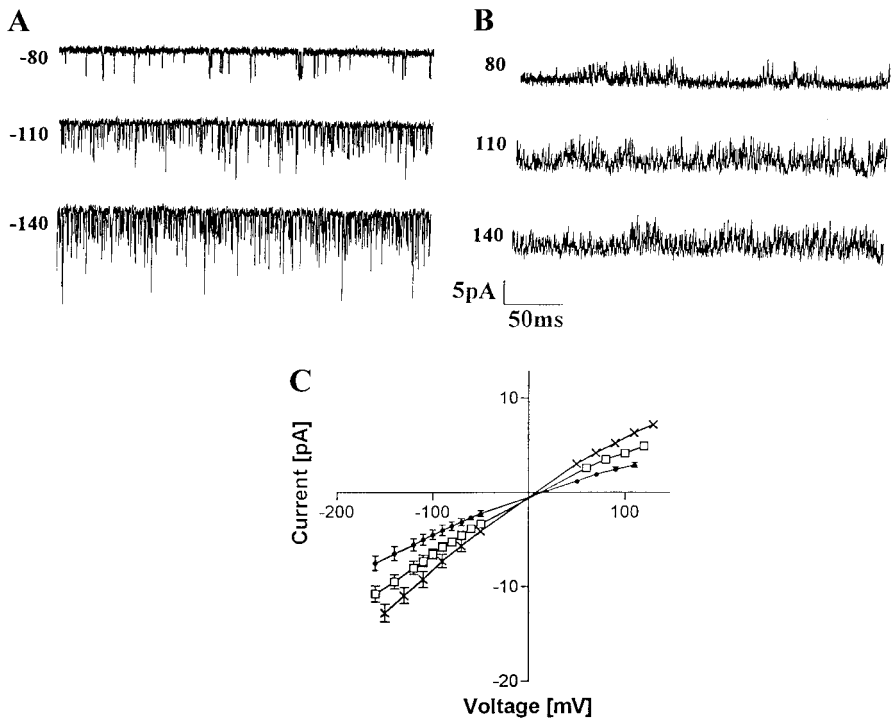

FIG. 1. Single channel activities in cell-attached patches of *Xenopus laevis* oocytes expressing rat CaT1. The current traces were taken at negative (A) or positive voltages (B) as indicated on the left side of each trace. Downward deflections of the current traces represent inward currents. The current–voltage relationships are shown in C. The pipet solution contained 100 mM KCl, 100 μM $CaCl_2$, 5 mM EGTA and 10 mM Hepes (pH 7.5), and the bath solution contained 100 mM KCl, 0.1 μM $CaCl_2$, 10 mM Hepes, and 5 mM EGTA, pH 7.5.

TRP channels, may participate in regulating CaT1, although a direct inhibitory action of XC on the CaT1 channel cannot be excluded.

METHODS

Expression of CaT1 in oocytes. Oocytes at stages V–VI were harvested from *X. laevis* and defolliculated by treating them for ~2 h at room temperature with 3 mg/ml collagenase (Boehringer-Mannheim, Mannheim, Germany) in a Ca^{2+} free modified Barth's solution. Oocytes were injected, on the same day (at least 4 h after defolliculation) or on the following day, with 50 nl H_2O containing 50 ng of the synthetic RNA (cRNA) of human CaT1, which was prepared by *in vitro* transcription as described previously. For comparison, in some experiments the same amounts of cRNA encoding the other isoforms of CaT channels, including human or rat CaT2 (3) or rat CaT1 (1), 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 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. Patch-clamp methodology was employed for measurement of the properties of single ion channels using cell-attached or excised membrane patches (19). Patch pipets were pulled from borosilicate glass capillaries and fire-polished to a tip diameter of less than 1 μm. Pipette solution contained in mM (unless otherwise specified): 100 KCl, 0.1 $CaCl_2$, and 10 Hepes, pH 7.5 or 100 mM KCl, 10 mM *N*-(2-hydroxyethyl) ethylenediaminetriacetic acid (HEDTA), 10 mM Hepes, pH 7.4 adjusted with Tris base. In some experiments 100 mM KCl was substituted with 100 mM NaCl or equal amounts of other salts. When filled with one of these external solutions, the pipet tip resistances were 5–10 MΩ. Seals with resistances of >10 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: 100 mM KCl, 10 mM HEDTA, 10 mM Hepes, pH 7.4 adjusted with Tris base or 100 mM KCl, 0.1 μM $CaCl_2$, 10 mM Hepes, and 5 mM ethylene glycol-bis (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 7.5. The concentrations of Ca^{2+} in solutions containing low Ca^{2+} (0.1–100 μM) were adjusted according to a previous study (20).

Data acquisition and analysis. Voltage stimuli were applied and single channel currents digitized (20–150 μs per point) and analyzed using a PC, a Digidata converter, and programs based on pClamp (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. Po was calculated from 20- to 30-s segments of current records in patches. Several hundred or more events were analyzed using half-amplitude threshold criteria for generating each data point. The experiments were carried out at 23°C. To estimate statistical differences, three or more experiments were performed for each condition.

RESULTS AND DISCUSSION

Based on our previous studies of CaT-mediated Ca^{2+} uptake and whole-cell currents in *Xenopus* oocytes (1, 3, 12), we extended our investigations to examining the single channel characteristics of the CaT1 channel un-

der defined conditions. In addition to studying the single channel properties of the previously cloned rat CaT1 channel, we expressed human CaT1 in oocytes. Ionic currents were measured in oocytes 2–6 days after injection with cRNA encoding full-length human or, in some cases, rat CaT1.

Although in our previous whole-cell current studies (1, 12) we showed that CaT1 is permeable to Ca^{2+}, no single channel currents could be recorded in the presence of 80–100 mM $CaCl_2$ in the pipette solution facing the extracellular sides of the membrane patches, apparently due to the low unitary Ca^{2+} conductance of the CaT1 channel under these conditions, as has previously been observed with other types of Ca^{2+}-permeable channels (21, 22). Similar to the studies on these other Ca^{2+}-permeable channels, however, we observed channel activities with a high probability of opening in the presence of both Na^+ and K^+ in a pipette solution that was free of divalent cations. In the present study we primarily used a K^+-containing external pipette solution, in the absence of any Na^+ and Ca^{2+}, to prevent any contributions of these cations to potential modulation on the intracellular side of the patch after their entry through the CaT1 channel. Previously described Ca^{2+}-permeable channels have been modulated by these cations on the intracellular sides of the patches.

In single channel studies, using K^+ as the charge carrier in the pipette solution in the presence of EGTA, which chelates primarily Ca^{2+}, we frequently observed channel activities (in 112 of 178 patches, 63%) with distinctive bursts of brief openings in CaT1-injected oocytes (Fig. 1A). No such activities were found in water-injected oocytes ($n = 85$).

Figure 1 shows that the currents were strongly activated by hyperpolarization. The channel activity increased substantially at more negative potentials, particularly at −90–150 mV. Several different current amplitudes could be measured at different voltages, suggesting that the channels have multiple conductances. Three different conductances were determined from the slopes of the current–voltage relationships (43.8 ± 3.7, 64 ± 5.8, and 82 ± 6.9 pS, mean ± SEM, $n = 8$), but owing to the difficulties inherent in measuring the current amplitudes of channel openings of such short durations, we cannot exclude the possibility that the largest conductance reflects a level resulting from the superimposition of smaller amplitudes. All of the conductances display inward rectification. The outward currents at 30–140 mV are substantially smaller than the inward currents at the respective negative voltages (Fig. 1C).

We have also studied CaT1 channel activities in the presence of two other chelating agents, EDTA and HEDTA, which chelate both Ca^{2+} and Mg^{2+}. HEDTA, in fact, preferentially chelates Mg^{2+}. The conductance of the CaT1 channels in the presence of these chelators

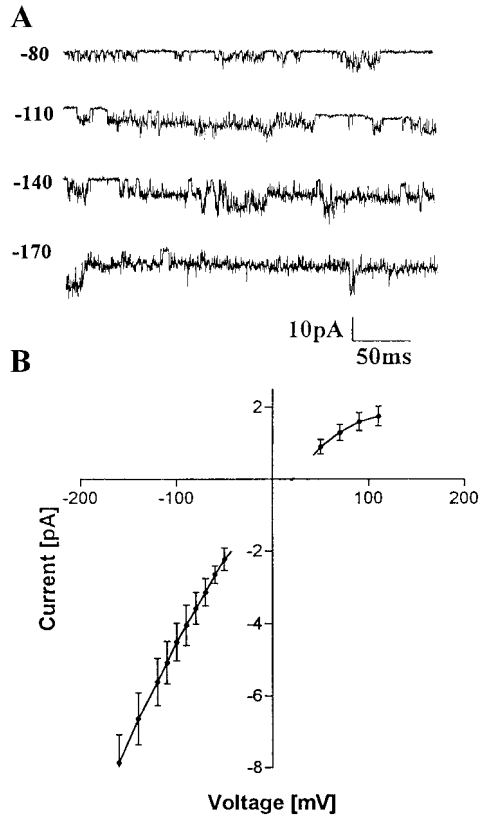

FIG. 2. Channel activities in the presence of HEDTA in the pipet solution in *Xenopus laevis* oocytes expressing human CaT1. Current traces were taken at the voltages indicated on the left side of each trace (A). The current–voltage relationships determined under these conditions are shown in B. The pipet solution was the same as the bath solution and contained 100 mM KCl, 10 mM *N*-(2-hydroxyethyl) ethylenediaminetriacetic acid (HEDTA), 10 mM Hepes, pH 7.4 adjusted with Tris base.

was 45.1 pS ± 0.52 ($n = 5$) in the voltage range from −30 to −90 mV but it showed nonlinear behavior and increased at more negative voltages (Fig. 2). The channel kinetics under these conditions changed substantially. The mean open times were longer and the probability of the open state was higher in HEDTA-containing pipette solutions than in the presence of EGTA. This suggested that traces of extracellular Mg^{2+} may partially block the CaT1 channel resulting in bursts of brief openings. Indeed in a separate study we found that micromolar concentrations of Mg^{2+} exert a significant channel blocking effect (data not shown), similar to its effects on store-operated channels coupled to IP_3 (21). To avoid this partial block of the channels by Mg^{2+}, therefore, we have used pipette solutions containing 10 mM HEDTA.

The kinetics and some of the conductance levels of the CaT1 channel are similar to those of the TRP channels, which are also characterized by bursts of brief openings and multiple conductance levels (35, 37.5, 66 and 69 pS) as described in recent studies (13,

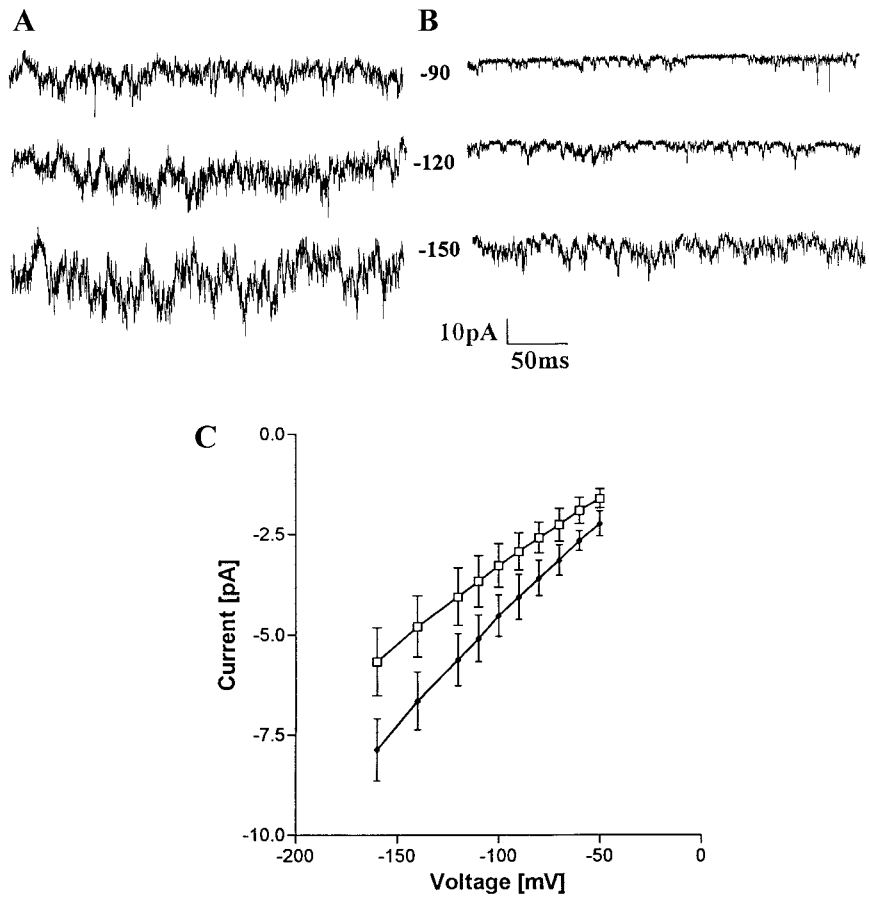

FIG. 3. Inhibition of CAT channels by 5 μM Xestospongin C (XC) added to the extracellular pipette solution. Current traces were taken at the voltages indicated in the middle between each pair of traces in the absence (A) or in the presence of 5 μM XC (B). The current–voltage relationships determined in the absence (closed symbols) or in the presence of 5 μM XC (open symbols) are shown in C. The pipet and the bath solutions were the same as described in the legend to Fig. 2 except the addition of XC to the solutions different from the control.

23, 24). Since CaT1 bears some structural homology to the TRP channels (1, 6, 12), it is possible that some of its functional roles may be related to similar physiological processes. Previous studies have shown that some isoforms of the TRP channels are modulated by agents that modulate the activity of the IP_3 receptors (15–17) but there are uncertainties about the functional interrelationships between the different TRP isoforms and the IP_3 receptors and their real contributions to the store-dependent Ca^{2+} influx (25–29).

To clarify the physiological functions of the CaT1 channel, it is also important to find inhibitors of its activity. We have, therefore, explored the effects of a recently described, non-competitive antagonist of the IP_3 receptor, Xestospongin C (XC) (18), on the activity of the CaT1 channel. Figure 3 shows that this agent exerts a strong inhibitory action on the single channel activity of CaT1. The CaT1 channel could not be activated even at very negative voltages in the presence of XC (Figs. 3A and 3B). The slope of the I-V curve was reduced (Fig. 3C) and the unitary conductance in the presence of XC was 72% of that of the untreated channels. A more substantial inhibition of channel activity was found in the presence of a higher concentration of this agent (Figs. 4A and 4B). The probability of opening was significantly reduced in the presence of 5 μM XC but a more drastic reduction was observed at 20 μM (Fig. 4C).

These inhibitory effects of XC on the CaT1 channel, however, were not observed in all patches of CaT1-expressing oocytes tested. The channel activity was reduced in 38 out of 57 patches (67%) of 27 oocytes from 5 different batches of oocytes. The lack of effect of XC in some patches may be due to a reduced degree or lack of functional coupling between the CaT1 channel and the IP_3 receptor. It is also possible that some clusters or individual CaT1 channels are coupled to other types of receptors, as has been suggested to be the case for some types of TRP channels. For example, in recent studies it was found that within the same cell the human TRP3 channel can couple to IP_3 or ryanodine receptors and that this coupling is mutually exclusive (16, 17).

In 18 experiments on CaT2-expressing oocytes we did not observe strong inhibition of CaT2 channel ac-

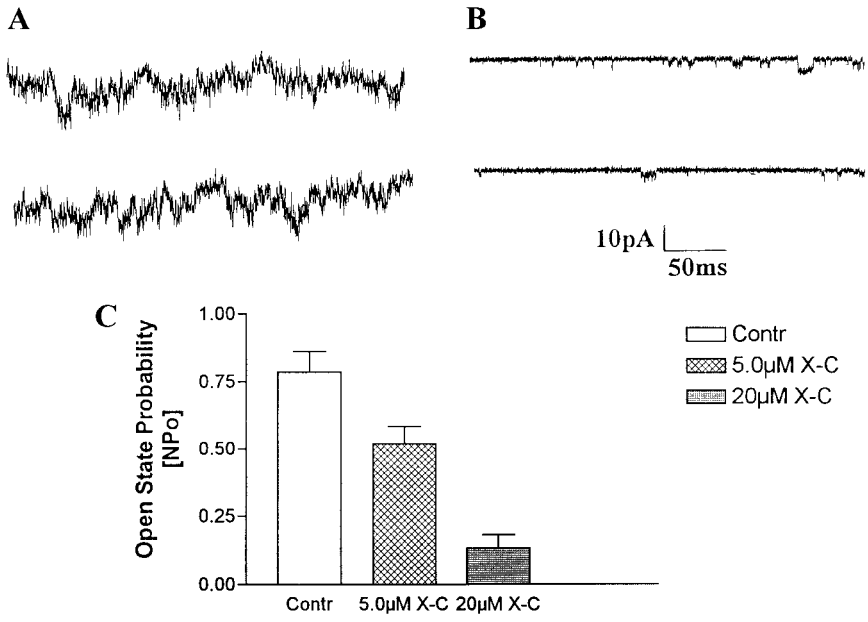

FIG. 4. Dose-dependent blockade of the CaT1 channel by XC. Current traces were taken at −150 mV in the absence (A) or in the presence of 20 μM XC (B). The open state probabilities measured in the absence of Xestospongin C or at the two different concentrations of the agent (5 and 20 μM) are compared in C. The pipet and the bath solutions were the same as described in the legend to Fig. 2 except the addition of XC to the solutions different from the control.

tivities by XC used at the same doses as in the studies on CaT1. CaT2 is expressed mainly in kidney, while the expression of CaT1 is higher in the gastrointestinal tract and several other tissue types. It is possible, therefore, that CaT2 does not share some of the functional interrelationships of CaT1 with other ubiquitously distributed proteins such as the IP_3 receptors.

In previous studies XC has been used as a specific, noncompetitive antagonist of the IP_3 receptor (18) indicating that its effects on CaT1 could be mediated indirectly via inhibition of the IP_3 receptor. We cannot exclude, however, the possibility that the inhibitory effect of XC observed in our studies could be mediated via direct inhibition of the CaT1 channel. Indeed, in a recent study of the effect of this agent on *Dictyostelium*, it has been suggested that XC can inhibit not only the IP_3-mediated Ca^{2+} release but also other Ca^{2+}-dependent events, although it was not established whether these effects were direct or indirect (e.g., mediated via actions on the IP_3 receptor) (30). Furthermore, the expression of CaT1 in *Dictyostelium* has not yet been examined. It was also found that treatment of cell suspensions of *Dictyostelium* for 30 min produced an elevation in the basal level of cytosolic Ca^{2+}. Therefore, it was suggested that XC may open, rather than close, the Ca^{2+} release channel in *Dictyostelium*. It is possible that this action of XC is only present in this species. Nevertheless, the latter observations raise the possibility that XC could affect, directly or indirectly, other types of channels involved in regulation of the levels of cytosolic Ca^{2+}, such as the channels controlling Ca^{2+} influx. The CaT1 channel probably serves such a role, and, therefore, might also potentially be directly modulated by XC. However, to evaluate the latter possibility, other approaches complementing the electrophysiological methods utilized here must be employed. Although, we have observed XC-mediated reductions in channel activity not only in cell-attached but also in excised patches, this does not exclude the possibility that the effect is mediated via the IP_3 receptor, since there is evidence that the latter resides in vesicles which can remain associated with the intracellular side of the plasma membranes after excision of membrane patches (13, 16).

Thus the activity of the CaT channels is strongly inhibited by an agent isolated from a marine sponge, Xestospongin C, which is known to be a membrane-permeable, noncompetitive antagonist of the IP_3 receptor. While its inhibitory action on the CaT1 channel may be mediated indirectly via the IP_3 receptor, we cannot exclude a direct effect on the CaT channel itself. Clarification of the mechanism of its inhibitory action in further investigations may provide insights into the manner in which the IP_3 receptor couples, directly or indirectly, to Ca^{2+} uptake channels in the plasma membrane and/or show XC to be a useful tool in studies on the physiological roles of the CaT channels.

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