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Pharmacological modulation of monovalent cation currents through the epithelial Ca^{2+} channel ECaC1

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1 The recent identification of the epithelial Ca^{2+} channel, ECaC1, represents a major step forward in our knowledge of renal Ca^{2+} handling. ECaC1 constitutes the rate-limiting apical Ca^{2+} entry mechanism of active, transcellular Ca^{2+} reabsorption. This unique highly selective Ca^{2+} channel shares a low but significant homology with transient receptor potential (TRP) channels and vanilloid receptors (VR).

2 We have studied the pharmacological modulation of currents through ECaC1 heterologously expressed in HEK 293 cells. Monovalent cation currents were measured by use of the whole cell patch clamp technique in cells dialysed with 10 mM BAPTA or 10 mM EGTA to prevent the fast Ca^{2+} dependent inactivation of ECaC1.

3 Several modulators were tested, including inorganic cations, putative store-operated Ca^{2+} entry (SOC) blockers, the vanilloid receptor (VR-1) blocker capsazepine, protein tyrosine kinase blockers, calmodulin antagonists and ruthenium red.

4 Ruthenium red and econazole appeared to be the most effective inhibitors of currents through ECaC1, with IC_{50} values of 111 nM and 1.3 μM, respectively, whereas the selective SOC inhibitor, SKF96365, was nearly ineffective.

5 The divalent cation current block profile for ECaC1 is $Pb^{2+} = Cu^{2+} > Zn^{2+} > Co^{2+} > Fe^{2+}$ with IC_{50} values between 1 and approximately 10 μM.

6 In conclusion, ECaC activity is effectively inhibited by various compounds including ruthenium red, antimycotic drugs and divalent cations, which might be useful tools for pharmacological manipulation and several disorders related to Ca^{2+} homeostasis could benefit from such developments.

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; C, concentration of inhibitor; CAPS, capsazepine; CHO, Chinese hamster ovary cells; Cont, control; CRAC, Ca^{2+} release activated Ca^{2+} channels; DADZ, dadzein; DMEM, Dulbecco's modified Eagle's medium; DynA, dynorphin A; DynA-F10, fragment 1-10 of dynorphin A; EC, econazole; ECaC, CaT, epithelial Ca^{2+} channel; EGTA, ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetra-acetic acid; GENIST, genistein; GFP, green fluorescent protein; HEK 293, Human Embryonic Kidney cells; HEPAR, heparine; HEPES, N-(hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid); IC_{50}, concentration for half-maximal inhibition; I_{CRAC}, Ca^{2+} release activated Ca^{2+} current; I_{norm}, current amplitude in the presence of inhibitor normalized to that in control conditions; ISOP, isoptin (verapamil); MC, miconazole; MDL 12330, acycloalkyl lactamimide; MIBEF, mibefradil; N, number of cells; n_H, Hill coefficient; Nif, niflumic acid; NMDG, N-methyl-D-glucamine; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinases; QUINID, Quinidine; R24571, calmidazolium; RbECaC, rabbit ECaC; RR, ruthenium red; SKF96365, 1-[-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenyl]-1H-imidazole hydrochloride; SOC, store-operated Ca^{2+} channel; SPERM, spermine; TEA, tetraethylammonium; TFP, trifluoperazine; Tris, Tris(hydroxymethyl)aminomethane; TRP, transient receptor potential; TYR A1, tyrphostin A1; TYR B46, tyrphostin B46; VR1, vanilloid receptor 1

Introduction

Ca^{2+} influx in non-excitable cells occurs through cation channels, which mainly belong to the TRP gene family. These cation channels are essential for a plethora of important cellular functions such as growth, proliferation, triggering of Ca^{2+} dependent signalling cascades, but also the molecular transduction of painful stimuli, osmoregulation and trans-epithelial ion transport (Harteneck *et al*., 2000; Hoenderop *et al*., 2000a). Distinctive members of this fast growing family of ion channels include vanilloid receptor 1 (VR1), transient receptor potential channels (TRP) and epithelial Ca^{2+} channels (ECaC1 and ECaC2, Barley *et al*., 2001; Hoenderop *et al*., 1999a; Müller *et al*., 2000a, b; Peng *et al*., 1999; 2000).

The recent cloning and characterization of the two highly homologous Ca^{2+} channels ECaC1 and ECaC2 (also

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known as CaT1) has led to the identification of various cell functions that are tightly controlled by these channels, such as transepithelial Ca^{2+} transport in kidney and small intestine, as well as their postulated function as store operated Ca^{2+} entry pathway (Hoenderop *et al*., 2000a, b; Yue *et al*., 2001).

The electrophysiological properties of ECaC have been extensively documented in several cell types heterologously expressing ECaC1 or ECaC2 including *Xenopus laevis* oocytes, Human Embryonic Kidney (HEK) 293 cells and CHO cells (Hoenderop *et al*., 1999b; Nilius *et al*., 2000; Vennekens *et al*., 1999; Yue *et al*., 2001). Both channels are highly Ca^{2+} selective, and show inward rectification and Ca^{2+}-dependent feedback inhibition.

Obviously, the increasing functional significance of these novel Ca^{2+} channels demands an intensive search for compounds that modulate currents through these channels. The central role of ECaC in active Ca^{2+} (re)absorption makes it a prime target for pharmacological manipulation and several disorders related to Ca^{2+} homeostasis could benefit from such developments. The aim of the present study was, therefore, to investigate a number of pharmacological tools that modulate ECaC1 currents carried by monovalent cations. This approach circumvents the fast inactivation of currents through ECaC1 carried by Ca^{2+}, which even occurs if intracellular Ca^{2+} is buffered by BAPTA or EGTA. We describe here for the first time a variety of inorganic and organic pharmacological drugs that modulate ECaC1 function.

Methods

Construction of mammalian expression vectors

The open reading frame of rbECaC was cloned as a *Pvu*II-*Bam*HI fragment in the pCINeo/IRES-GFP vector (Vennekens *et al*., 2000a). This bicistronic expression vector pCINeo/IRES-GFP/rbECaC was used to co-express rbECaC and enhanced green fluorescent protein (GFP) (Trouet *et al*., 1997).

Cell culture and transfection

Human embryonic kidney cells, HEK293, were grown in DMEM containing 10% (v v^{-1}) human serum, 2 mM L-glutamine, 2 u m^{-1} penicillin and 2 mg ml^{-1} streptomycin at 37°C in a humidity controlled incubator with 10% CO_2. HEK293 cells were transiently transfected with the pCINeo/IRES-GFP/rbECaC vector (Vennekens *et al*., 1999). Transfected cells were visually identified in the patch clamp set up. GFP was excited at a wavelength between 450 and 490 nm and the emitted light was passed through a 520 nm long-pass filter. The ECaC-expressing cells were identified by their green fluorescence and GFP negative cells from the same batch were used as controls.

Electrophysiology

Electrophysiology methods have been described previously in detail (Nilius *et al*., 2000). Whole-cell currents were measured with an EPC-9 (HEKA Elektronik, Lambrecht, Germany, sampling rate 0.2 ms, 8-Pole Bessel filter 10 kHz) or an L/M-EPC-7 (List Elektronics, Darmstadt, Germany) using ruptured patches. Electrode resistances were between 2 and 5 MΩ, capacitance and access resistances were monitored continuously, and series resistance was maximally compensated. The ramp protocol consisted of linear voltage ramps changing from −150 to +100 mV within 400 ms, applied every 5 s from a holding potential of +20 mV. The step protocol consisted of a series of 60 ms long voltage steps applied from a holding potential of +20 mV to voltages between +100 and −140 mV with a decrement of 40 mV. Current densities, expressed per unit membrane capacitance, were measured from the current at −100 mV during the ramp protocols.

Determination of transcellular Ca^{2+} transport

Confluent monolayers of rabbit cortical collecting system cells, grown on permeable filters, were washed twice and preincubated in physiological salt solution (PSS) containing (in mM): NaCl 140, KCl 2, K_2HPO_4 1, $MgCl_2$ 1, $CaCl_2$ 1, glucose 5, L-alanine 5 and HEPES/Tris (pH 7.4) 10 for 15 min at 37°C. Subsequently, the monolayers were incubated in PSS, 100 μl apical and 600 μl basolateral, for another 90 min to measure transepithelial Ca^{2+} transport. At the end of the incubation period, 25 μl samples were removed in triplicate from the apical compartment and assayed for Ca^{2+} concentration using a colorimetric assay kit (Boehringer). Under these experimental conditions, net apical-to-basolateral Ca^{2+} transport was linear with time for at least 3 h (Bindels *et al*., 1991). Ca^{2+} reabsorption is expressed in nmol h^{-1} cm^{-2}.

Solutions and experimental procedures

The standard extracellular solution ('Krebs') contained (in mM): NaCl 150, CsCl 6, $CaCl_2$ 1.5, $MgCl_2$ 1, HEPES 10 and glucose 10, pH 7.4 with CsOH. Nominally free Ca^{2+} solutions were buffered by 5 mM EGTA at a free [Ca^{2+}] below 1 nM, as calculated by the CaBuf program (G. Droogmans, KU Leuven, Belgium). The standard internal (pipette) solution contained (in mM): CsCl 20, Cs-aspartate 100, $MgCl_2$ 1, BAPTA 10, Na_2ATP 4, HEPES 10, pH 7.2 with CsOH. Cells were kept in a nominally Ca^{2+}-free medium to prevent Ca^{2+} overload and exposed for maximal 5 min to a Krebs solution containing 1.5 mM Ca^{2+} before sealing the patch pipette to the cell. All experiments were performed at room temperature (20–22°C).

The following compounds were used: econazole (Sigma), miconazole (Sigma), ruthenium red (Fluka), the cycloalkyl lactamimide MDL 12330A (RBI), 2-aminoethoxydiphenyl borate 2-APB (Calbiochem), capsazepine (TOCRIS), dynorphin A and the fragment 1–10 of dynorphin A (Sigma), 1-[-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl]-1H-imidazole hydrochloride (SK&F96365, Sigma), tyrphostin B46 (Calbiochem), genistein (Sigma), tyrphostin A1 (Calbiochem), dadzein (Sigma), trifluoperazine TFP (Sigma), calmidazolium R24571 (Janssen Pharmaceuticals, Beerse, Belgium), isoptin (verapamil, Knoll AG, Ludwigshafen, Germany). Tetraethylammonium (TEA), quinidine, niflumic acid (Nif), spermine, heparin, (all from Sigma), and $PdCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $FeCl_3$ (all from Merck).

Statistical analysis

In all experiments the data are expressed as mean ± s.e.mean. Overall statistical significance was determined by analysis of variance. In case of significance ($P<0.01$), individual groups were compared by Student's *t*-test.

Dose-inhibition data were described by the equation

$$I_{norm} = \frac{1}{1 + (C/IC_{50})^{n_H}} \quad [1]$$

where I_{norm} is the current amplitude in the presence of the inhibitor normalized to that in control conditions, C the concentration of inhibitor, IC_{50} the concentration of half-maximal inhibition, and n_H the Hill coefficient. Data were fitted to this dose-response function using Origin software v 6.0 (Microcal Software, Northhampton, MA, U.S.A.).

Results

The present study investigates the effect of organic and inorganic modulators on ECaC1 currents carried by monovalent cations. This approach circumvents the fast inactivation of currents through ECaC1 carried by Ca^{2+}, which even occurs if intracellular Ca^{2+} is buffered by BAPTA or EGTA. Figure 1 shows the protocol applied for these studies. Seal formation and rupture of the patch membrane occur in the presence of 1.5 mM $[Ca^{2+}]_e$, after which the extracellular solution is changed to a divalent cation free solution. The monovalent cation current through ECaC1 immediately after breaking into the cell is therefore blocked, and develops with a certain delay in the divalent cation free solution because dialysis of the cell with the 10 mM BAPTA buffered pipette solution removes the Ca^{2+}-dependent inactivation of the channel induced by cytosolic calcium (Nilius *et al*., 2000; 2001). This current was then stable over a relatively long time period (usually more than 30 min), which allowed testing of modulator compounds in the same cell.

Block of currents through ECaC1 by inorganic cations

As shown in Figure 1A, application of 10 μM Co^{2+} inhibited the inward current at −100 mV by approximately 50%, whereas 100 μM Co^{2+} reduced the current amplitude to the level observed after substituting extracellular Na^+ with $NMDG^+$. Recovery of the current upon washout of the inhibitor was fast and reversible. Figure 1B shows the current voltage relationships measured at the corresponding points in Figure 1A. Half-maximal inhibition was obtained at a concentration of 9.5 ± 0.9 μM, $n_H = 1.3 \pm 0.5$ (three concentrations, data not shown). A similar protocol was used in all further experiments.

Figure 2 shows another example of a highly selective inhibitor of ECaC1. Pb^{2+} completely blocked ECaC1 currents at a concentration of 10 μM (Figure 2A), but the washout of the effect was delayed (Figure 2B). The IC_{50} value of inhibition was 1 μM (Figure 2C). Also Cu^{2+} and to a

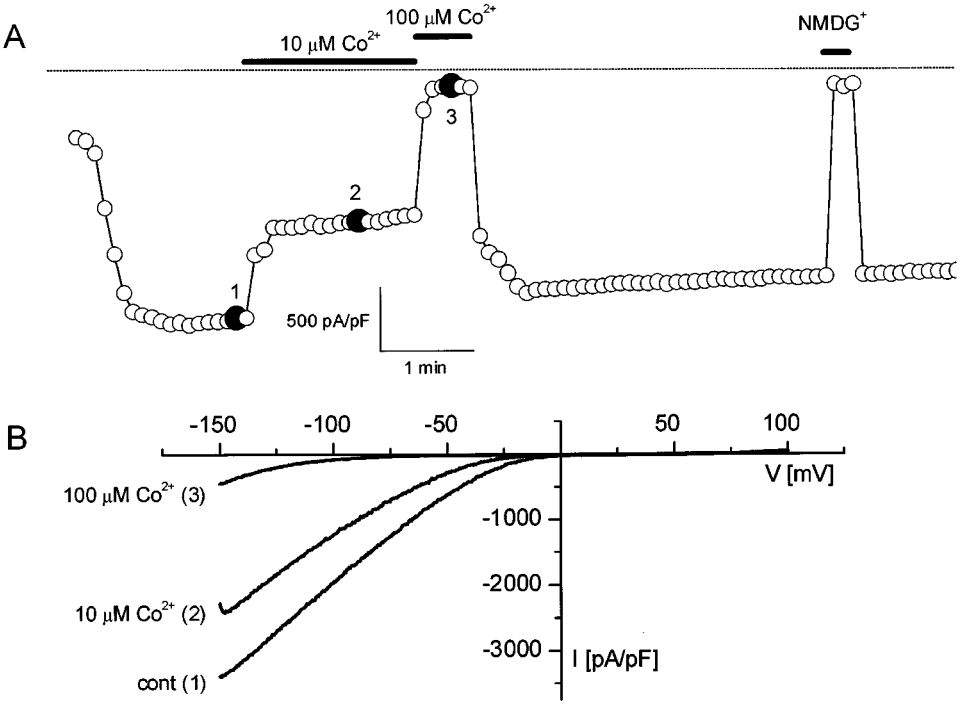

Figure 1 Effect of Co^{2+} on monovalent cation currents through ECaC1. (A) Currents were recorded after seal formation and membrane rupture (indicated by the arrow) in a solution containing 1.5 mM Ca^{2+}. Monovalent cation currents developed after removal of extracellular Ca^{2+} and dialysing the cell with the pipette solution containing 10 mM BAPTA. The amplitude of the current at −100 mV, as measured during repetitive voltage ramps, is displayed as a function of time. A similar protocol was used for all other blockers. Two concentrations of $CoCl_2$ (in μM) were applied at the times indicated. Substitution of all external cations by $NMDG^+$ abolished the inward current almost completely. Rabbit ECaC1 (rbECaC, accession number AJ133128) was expressed in HEK 293 cells. (B) Current voltage relationships measured at the corresponding times indicated in panel A.

smaller extent Zn^{2+} exerted a similar high sensitivity block of ECaC1 (Figure 3).

Fe^{2+} and Fe^{3+} appeared to be less efficient blockers of ECaC1 than all other tested divalent and trivalent cations. At a concentration of 100 μM, Fe^{2+} and Fe^{3+} blocked the ECaC1 monovalent cation current at -100 mV by $70\pm13\%$ (N=4) and $22\pm9\%$ (N=4), respectively (data not shown).

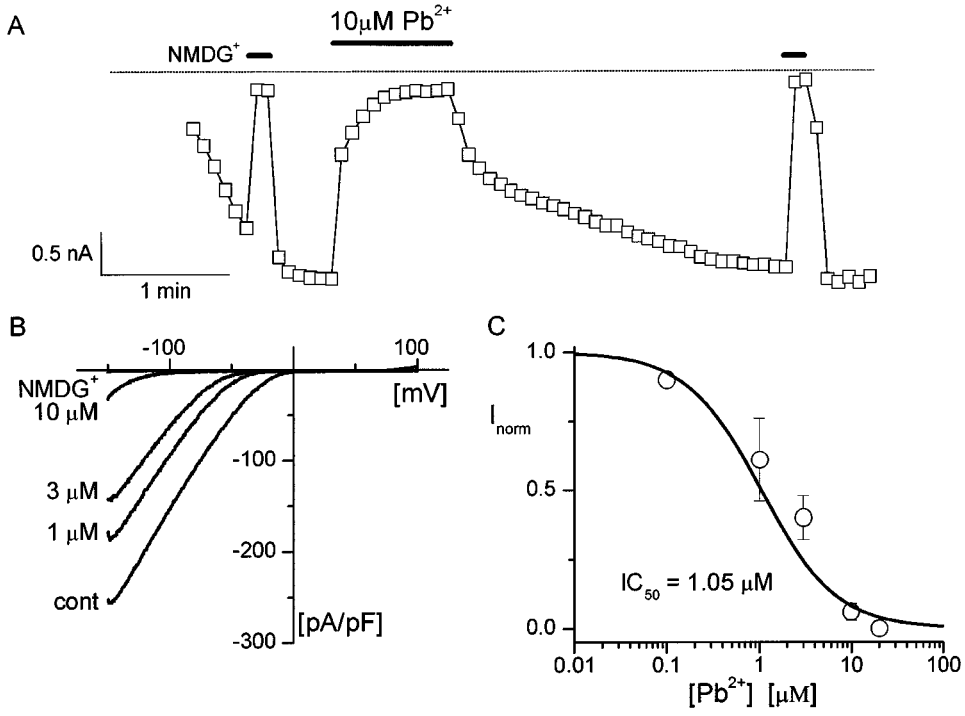

Figure 2 Inhibition of ECaC1 by Pb^{2+}. (A) Same screening protocol as in Figure 1A for inhibition of ECaC1 by Pb^{2+}. Note the slow recovery of the current after removal of 10 μM Pb^{2+}. Periods of perfusion with solution containing $NMDG^+$ are indicated. (B) Current voltage relationships from voltage ramps measured under control conditions (cont) and at three different concentrations of $PbCl_2$ (in μM). (C) Concentration response relationship for currents normalized to the control current before application of Pb^{2+} were fitted to equation 1. Data points are from 3 to 5 cells. Parameters of the fit are $IC_{50}=1.05\pm0.22$ μM, $n_H=1.07\pm0.12$.

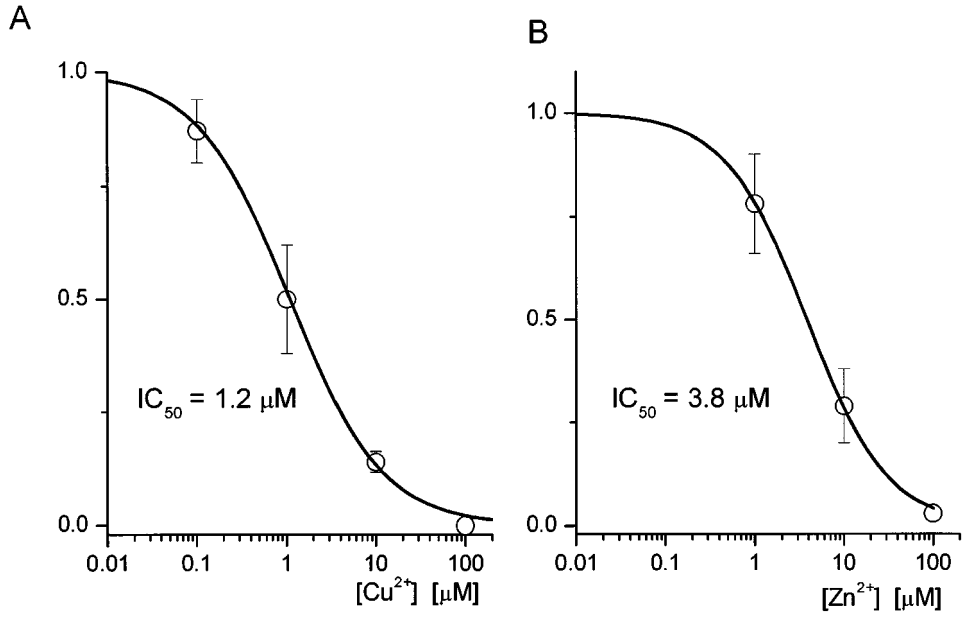

Figure 3 Inhibition of ECaC1 currents by Cu^{2+} and Zn^{2+}. Dose-response relationships of the current inhibition by Cu^{2+} (A) and Zn^{2+} (B). Current amplitudes at -100 mV derived from voltage ramps were normalised to the corresponding current amplitudes in the absence of the blocker. Each data point represents the average of 3 to 6 cells. Parameters of the fit to equation [1] for Cu^{2+} and Zn^{2+} were respectively IC_{50} 1.20 ± 0.38 μM, $n_H=0.89\pm0.14$, and 3.8 ± 1.56 μM, $n_H=0.97\pm0.32$.

Block of currents through ECaC1 by ruthenium red

The inorganic polycationic dye ruthenium red (RR), which binds to phospholipids and was therefore used for staining cells and tissues in electron microscopy, appears to be one of the most selective blockers of currents through the vanilloid receptor, VR1 (Tominaga *et al*., 1998), which shares around 30% homology with ECaC1 (Hoenderop *et al*., 2000b). Figure 4 illustrates the effects of RR on monovalent cation currents through ECaC1. Submicromolar concentrations of RR induced a substantial block of this current (Figure 4B). The current was completely inhibited at 0.3 μM (Figure 4A), but recovery from block was slow at this high concentration. During voltage steps to negative potentials, ECaC1 currents showed a time-dependent decay in the presence of high RR concentrations, which is consistent with a voltage-dependent component of block (Figure 5A). From the dose-inhibition curve shown in Figure 5B which was obtained from all the initial current at voltage steps and from ramp experiments, we derived an IC_{50} value of 111 ± 9 nM, indicating that RR is by far the most sensitive blocker of ECaC1 currents we have found so far. This inhibition by ruthenium red was confirmed on the transcellular Ca^{2+} transport in primary cultures of immunodissected rabbit connecting tubule and cortical collecting ducts. Ruthenium red added to the apical compartment inhibited transcellular Ca^{2+} transport dose-dependently with an IC_{50} value of 300 nM (Figure 5C).

Block of currents through ECaC1 by organic compounds

We have also tested a variety of compounds that have been described as weak selective blockers of store-operated Ca^{2+} channels (SOC) (Franzius *et al*., 1994). One of these compounds is the antimycotic drug econazole (EC). As shown in Figure 6, EC is a highly sensitive blocker of currents through ECaC1. Inhibition, which was not accompanied by a shift of the reversal potential (Figure 6A), was nearly complete at 10 μM and disappeared slowly after washing out (Figure 6B). The concentration for half-maximal inhibition was 1.3 μM (Figure 6C). The potency of current inhibition by miconazole (MC), another antimycotic imidazole derivative, was similar to that of EC (Figure 7A). However, its reversibility was much slower than that of EC (Figure 6B). Its IC_{50} value was 1.8 μM, as derived from the fit of the dose-inhibition curve to equation [1] (Figure 7C).

The well described blocker of SOC- and TRP-mediated Ca^{2+} entry, SK&F 96365 (Choi *et al*., 2001; Franzius *et al*., 1994; Inoue *et al*., 2001) was nearly ineffective on ECaC1 at concentrations up to 10 μM (Figure 8A). In addition, other SOC blockers, such as the adenylcyclase inhibitor MDL 12330A and 2-APB (van Rossum *et al*., 2000), did not remarkably affect the ECaC1-mediated current. Twenty μM MDL 12330A induced block of approximately 50%, whereas 2-APB, which has a biphasic effect on SOC causing activation at low concentrations (Lewis, personal communication) and inhibition with an IC_{50} of about 10 μM (van Rossum *et al*., 2000), was nearly ineffective and inhibited currents through ECaC1 by approximately 20% at a concentration of 100 μM (Figure 8A).

ECaC1 is only weakly affected by capsazepine, a selective blocker of VR1. The inhibition of ECaC1 by 100 μM capsazepine was less than 50% (Figure 8A). In addition, it is well known that arginine rich peptides, such as dynorphins, inhibit currents through VR1 (Planells Cases *et al*., 2000). ECaC1 is however resistant to both dynorphin A and the fragment 10 consisting of the first 10 amino acid residues of

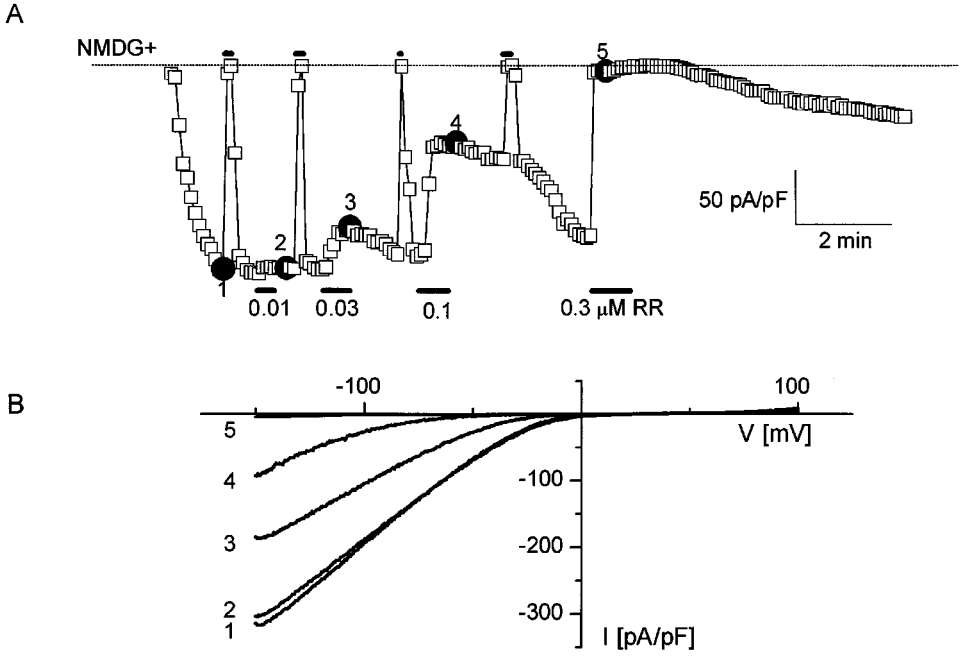

Figure 4 Inhibition of ECaC1 currents by ruthenium red (RR). (A) Time course of Na^+ currents through ECaC1 immediately after establishing whole cell configuration. Note the fast and reversible inhibition of the current at low concentrations of RR, but the extremely slow recovery at concentrations that induce complete block of ECaC1. (B) Current voltage relationships derived from voltage ramps (-150 to $+100$ mV). IV curves were taken at the times indicated in panel A.

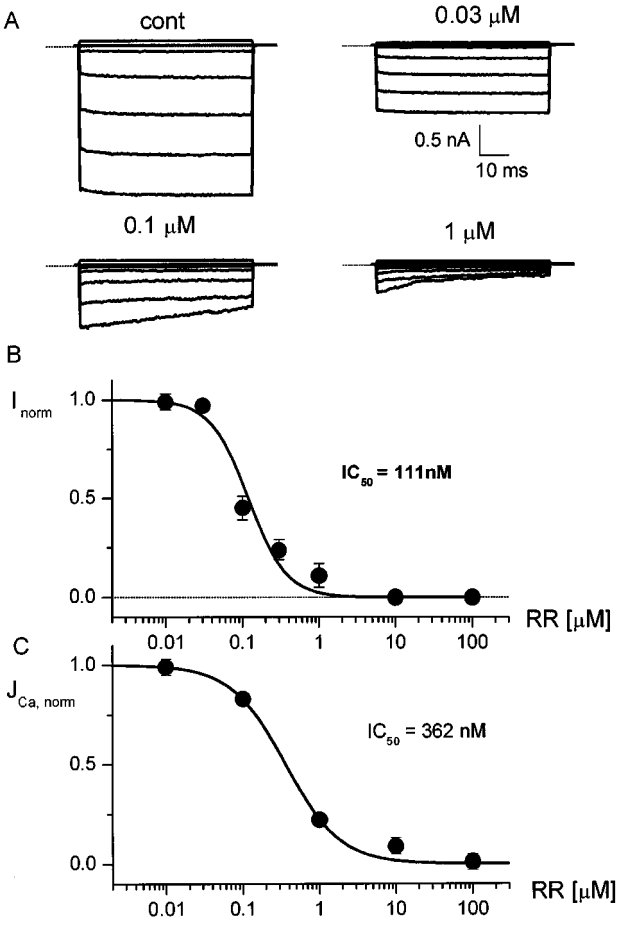

Figure 5 Block of ECaC1 currents by ruthenium red. (A) Voltage steps of 60 ms from +100 to −140 mV (decrement 40 mV, holding potential is +20 mV) under control conditions (cont) and at three concentrations of RR. Note the appearance of a time-dependent current decay at higher concentrations and negative voltages. (B) Concentration-current relationship at −100 mV from between four and nine cells per data point. Parameters of the fit to equation [1] are $IC_{50} = 111 \pm 10$ nM, $n_H = 1.7 \pm 0.2$. (C) Dose-inhibition curve of RR on transcellular Ca^{2+} transport, $J_{Ca,norm}$; across primary cultures of rabbit connecting tubules and cortical collecting ducts. Confluent monolayers were preincubated with the indicated RR concentrations (apical side only) for 10 min and subsequently Ca^{2+} transport was measured for 90 min and calculated as nmol Ca^{2+} h^{-1} cm^{-2}. The data presented are the mean±s.e.m. ($n=3$). Fit parameter: 362 ± 39 nM, $n_H = 1.2 \pm 0.1$.

dynorphin A. In contrast, these compounds block VR1 currents at concentrations of 5 μM by approximately 70 and 40%, respectively (Planells Cases *et al*., 2000) (see Figure 8A).

Blockers of protein tyrosine kinases (PTK) had also only a small effect on ECaC1. The blocking effect of Tyrphostin B46 and genistein was less than 50% at a concentration of 50 μM, and developed extremely slowly. In contrast, the PTK ineffective control compounds, tyrphostin A1 and dadzein had no effect at all (Figure 8A). We have not studied the effects of PKC and PKA inhibitors because both kinases are probably potent modulators of ECaC channels and will be described in detail elsewhere.

We have also investigated the effects of the calmodulin antagonists calmidazolium R24571 and trifluoperazine. Both compounds exerted a modest inhibition at 30 and 100 μM, respectively. However, these effects were very unstable and cells normally do not tolerate these concentrations for a long time.

Finally, other compounds, which were often used as potent blockers of various cation and anion channels were applied. Their effects are summarized in Figure 8B. Quinidine, a blocker of epithelial and endothelial K^+ and Cl^- channels (Voets *et al*., 1996; Yue *et al*., 2000), inhibited ECaC by approximately 50% at concentrations as large as 250 μM. All other blockers including the Ca^{2+} and/or Cl^- channel blocker mibefradil (Eller *et al*., 2000; Nilius *et al*., 1997), tetraethyl-ammonium (TEA), niflumic acid (Nif), spermine, heparin and verapamil (isoptin) were nearly ineffective on ECaC1 (Figure 8B).

Discussion

The cloning of epithelial Ca^{2+} channels (ECaC1 and ECaC2) has provided a molecular basis to study the process of Ca^{2+} entry in a variety of non-excitable and excitable cells. A complete understanding of the functional features of the channels from the ECaC family will significantly depend on the availability of selective modulators. We have, therefore, searched for compounds that might affect the current through one member of this family, ECaC1. Our screening was restricted to the modulation of monovalent cation currents through this channel, because these currents are very stable and provide, therefore, an excellent probe for studying the modulating effects of various compounds.

Previously, we have described that extracellular divalent cations effectively inhibit currents through ECaC1. The IC_{50} value for block by Mg^{2+} at −80 mV was 62 μM in the absence of extracellular Ca^{2+} and 328 μM at 100 μM $[Ca^{2+}]_e$, respectively. Ba^{2+} currents were blocked by La^{3+} and Gd^{3+} with IC_{50} values at − 80 mV of 4.6 and 1.1 μM respectively. Currents though ECaC1 in the presence of 100 μM $[Ca^{2+}]_e$ were inhibited by Cd^{2+} with an IC_{50} of 2.5 μM (Vennekens *et al*., 2000b). We add here novel cations which effectively block ECaC. The overall sequence of block is now $Pb^{2+} = Cu^{2+} = Gd^{3+} > Cd^{2+} > Zn^{2+} > La^{3+} > Co^{2+} > Fe^{2+} >> Fe^{3+}$.

ECaC has been proposed recently as a possible constituent of store operated or Ca^{2+} release activated Ca^{2+} channels (CRAC) (Putney, 2001; Vassilev *et al*., 2001; Yue *et al*., 2001), whereas this channel has been demonstrated to be constitutively active and regulated by $[Ca^{2+}]_i$ in previous studies from our group (Nilius *et al*., 2001; Vennekens *et al*., 2000a, b). Pharmacological intervention and comparison will help to resolve this discrepancy and to identify the role of ECaC channels in store-operated Ca^{2+} signalling. The present study demonstrates that ECaC1 and I_{CRAC} have different sensitivities for selective I_{CRAC} blockers, including SK&F96365 and the cycloalkyl lactamimide MDL 12330A, suggesting that in line with our previous findings ECaC may not be involved in store-mediated Ca^{2+} influx (Vennekens *et al*., 2000a).

Besides its binding to phospholipids and fatty acids (Voelker & Smejtek, 1996), the dye RR has been reported to affect the function of many Ca^{2+}-binding proteins, the mitochondrial Ca^{2+} uniporter and of various Ca^{2+} channels (Cardoso & de Meis, 1993; Cibulsky & Sather, 1999; Kannurpatti *et al*., 2000). It is also a well documented antagonist of the ryanodine receptor (Lukyanenko *et al*.,

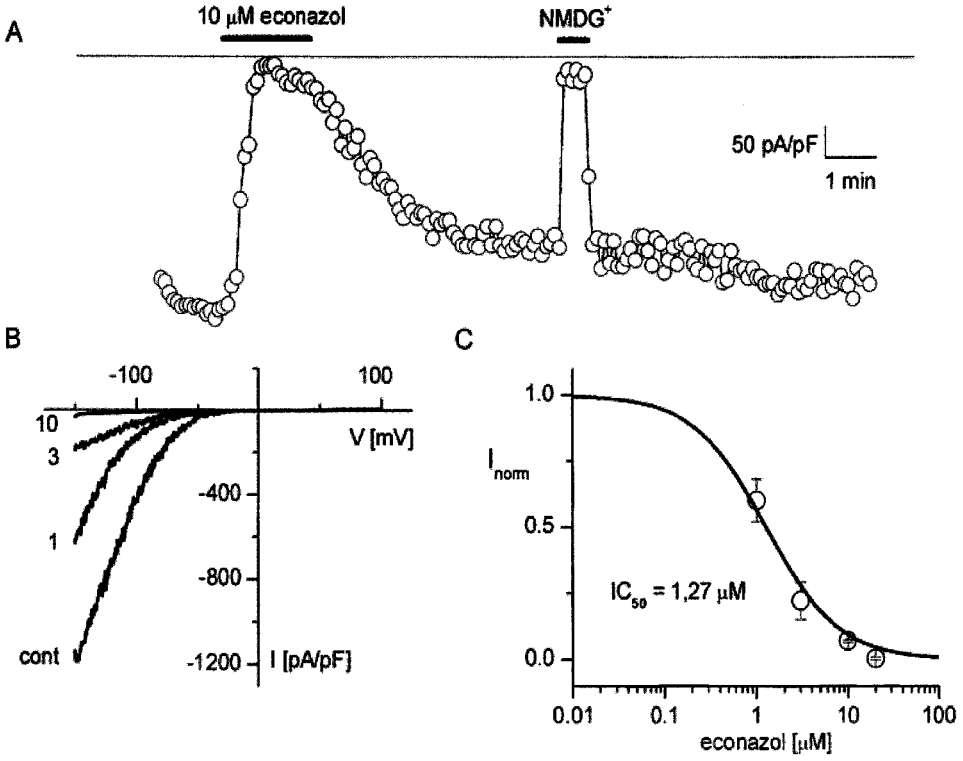

Figure 6 Block of ECaC1 currents by econazole. (A) Time course of the inhibitory action of econazole at −100 mV (same protocol as Figure 1). (B) Currents in response to voltage ramps from −150 to +100 mV (V_H = +20 mV; duration = 400 ms), applied every 5 s for ECaC-expressing HEK293 cells in the absence and presence of econazole (in μM). (C) Concentration-inhibition curve for the action of econazole on ECaC1. Data from between three and six cells. Parameters of the concentration – inhibition curve are $IC_{50} = 1.16 \pm 0.13$ μM, $n_H = 1.3 \pm 0.13$.

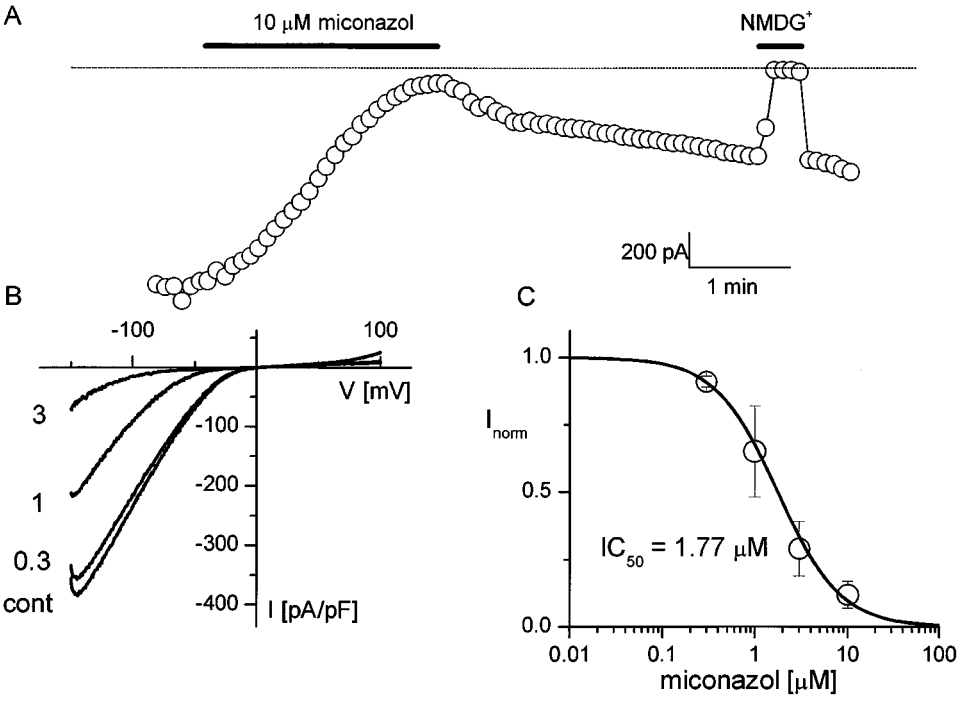

Figure 7 Effect of miconazole on Na^+ currents through ECaC1. (A) Time course of miconazole effects. Note the delayed recovery. (B) Currents in response to voltage ramps as in Figure 6. Three concentrations of miconazole were tested (in μM). (C) Concentration inhibition curve for miconazole measured from 3–6 cells per data point. Parameters of the inhibition – concentration curve are $IC_{50} = 1.8 \pm 0.3$ μM, $n_H = 1.24 \pm 0.14$.

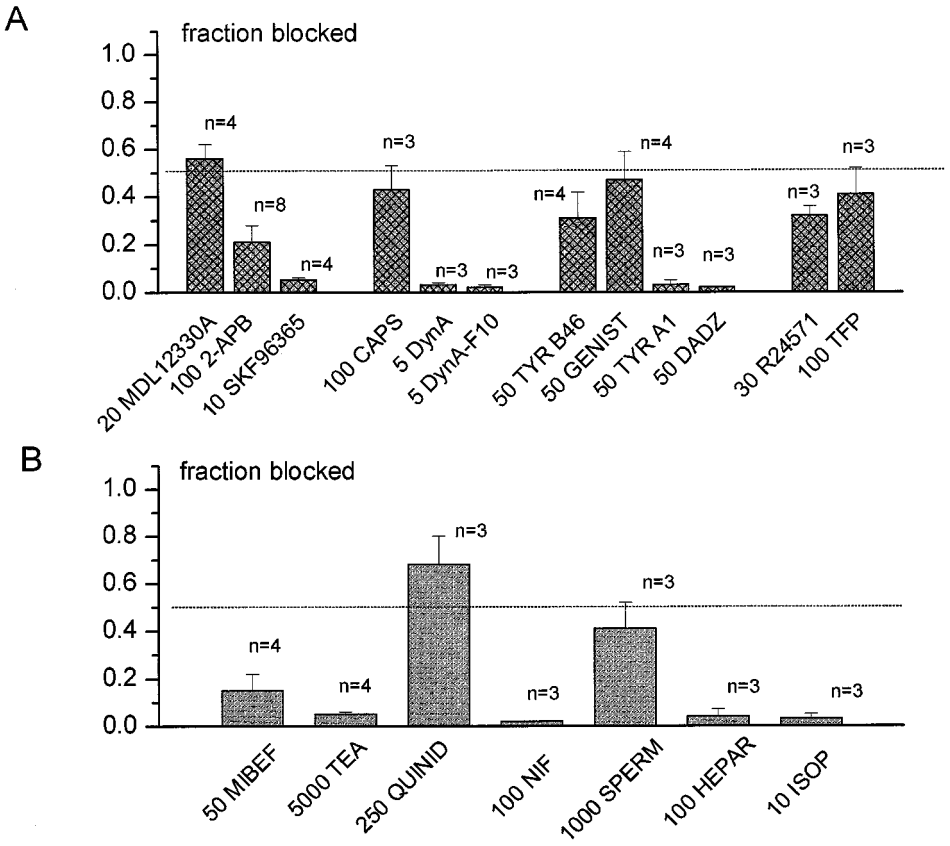

Figure 8 Synopsis of the various inhibitors on ECaC1 currents. (A) Fraction of the blocked current (inhibition) at −100 mV by various substances (concentrations μM). The various compounds were grouped as (a) possible SOC blockers, (b) VR1 blocker (capsazepine), (c) protein tyrosine kinase blockers (tyrphostin B46, A1, genistein, dadzein), (c) calmodulin antagonists (calmidazolium, trifluoperazine). (B) Fraction of the blocked current (inhibition) at −100 mV by various unrelated substances, such as mibefradil (MIBEF), tetraethylammonium (TEA), quinidine (QUINID), niflumic acid (NIF), spermine (SPERM), heparin (HEPAR), and isoptin (ISOP, verapamil).

2000; Stoyanovsky & Cederbaum, 1998), and has recently also been identified as a selective blocker of the VR1, a close homologue of ECaC1, which is 30% identical at the amino acid level. This block depends on an aspartate residue in the VR1-pore (Garcia-Martinez *et al*., 2000). Because this negatively charged residue is also present in the ECaC1 pore, we were interested in the modulating action of RR on ECaC1-currents. We found that RR is one of the most efficient inhibitors of ECaC1 with an IC_{50} of around 100 nM. One obvious aim to search for efficient ECaC1 blockers is to evaluate the function of these channels under more physiological conditions in different native systems. The present study demonstrates that Ca^{2+} reabsorption in primary cultures of distal tubule cells of rabbit kidney is like the recombinant expressed ECaC1 channel sensitive to block by ruthenium red, consistent with an important physiological role of this Ca^{2+} channel in Ca^{2+} reabsorption as proposed in previous studies (Hoenderop *et al*., 2000a).

The central role of ECaC1,2 in active Ca^{2+} (re)absorption makes it a prime target for pharmacological manipulation and several disorders related to Ca^{2+} homeostasis, including idiopathic hypercalciuria, osteoporosis, could benefit from such developments. In addition, it was demonstrated recently that overexpression of ECaC2 channels seems to play a crucial role in the de-differentiation of prostate epithelial cells into cancer cells (Peng *et al*., 2001; Tsavaler *et al*., 2001; Wissenbach *et al*., 2001). Likely, selective blockers of ECaC may help to slow down this process and might have the potential of novel anticancer drugs.

Excessive Ca^{2+} entry, probably mediated *via* SOCs, has been shown to play a decisive role in the pathogenesis of acute pancreatitis (Parekh, 2000; Raraty *et al*., 1999; 2000). Because members of the ECaC family are abundantly expressed in pancreatic acinus epithelium (Wissenbach *et al*., 2001), it is intriguing to speculate that blockers of ECaC1 could be an important therapeutic tool for treatment of pancreatitis.

In conclusion, we have described various inhibitors of ECaC1 Ca^{2+} channels which may play an important role in outlining the role of these novel channels in diverse cell functions, and which might also constitute important tools for the inhibition of Ca^{2+} entry in a variety of diseases.

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