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The Ca^{2+}-inactivated Cl^- Channel at Work: Selectivity, Blocker Kinetics and Transport Visualization

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Abstract. Removal of extracellular divalent cations activated a Cl^- channel in the plasma membrane of *Xenopus laevis* oocytes. This so-called Ca^{2+}-inactivated Cl^- channel (CaIC) was present in every oocyte and was investigated using two-electrode whole-cell voltage clamp and single-channel patch-clamp techniques. Beside other Cl^- channel inhibitors, anthracene-9-carboxylic acid (9-AC) and 3′azido-3′deoxythymidine (AZT), a nucleoside analogue commonly used as an antiviral drug, blocked at least partly the CalC-mediated currents.

Using the Cl^--sensitive dye 6-methoxy-N-(sulfopropyl)quinolinium (SPQ) we could visualize the transport of Cl^- from the oocyte cytoplasm to the surrounding medium after activation of the CaIC by Ca^{2+} removal. In the absence of external Cl^- and Ca^{2+}, the emission intensity of SPQ declined continuously, indicating a quenching of fluorescence by the efflux of Cl^- in the millimolar range. In the presence of external Ca^{2+}, no emission changes could be observed during the same time period. Chelating external Ca^{2+} in absence of Cl^- immediately activated Ca^{2+}-inactivated Cl^- channels leading to subsequent emission decrease of SPQ.

Investigations on the selectivity of the CaIC revealed only poor discrimination between different anions. With single-channel measurements, we found an anion selectivity sequence $I^- > Br^- > Cl^- \gg$ gluconate as it is also typical for maxi Cl^- channels.

Contrary to the majority of all other transport systems of the *Xenopus* oocyte, which show reduced activity due to membrane depolarization or endocytotic removal of the transport protein from the plasma membrane during oocyte maturation, the CaIC remained active in maturated oocytes. Single-channel measurements on maturated oocytes, also known as eggs, showed the presence of Ca^{2+}-inactivated Cl^- channels. However, this egg CaIC revealed an altered sensitivity to external Ca^{2+} concentrations.

All these data confirm and extend our previous observations on the CaIC and give clear evidence that this channel is peculiar among all Cl^- channels described up to now.

Key words: *Xenopus* oocytes — Cl^- channel — Calcium — Selectivity

Introduction

Oocytes of the South African clawed toad *Xenopus laevis* are a widely used model system for the investigation of membrane transport systems. These cells endogenously exhibit a whole orchestra of ion channels (*for review see* Dascal, 1987), cotransporters (Weber, Schwarz & Passow, 1990) and ion pumps (Vasilets & Schwarz, 1994). The fully grown *Xenopus* oocyte is a large cell of about 1.2 mm in diameter. This size and the convenient handling of these cells make manipulation easy and permit the application of several different techniques on a single cell. Especially, combinations of electrophysiological techniques with biochemical, molecular or optical methods allow the detection and profound characterization of transport systems.

All these advantages made the oocytes the favored expression system for foreign mRNA. Moreover, oocytes have a high capacity for translation, synthesizing more than 20 ng of protein per hour. In recent years this expression system was successfully used for the enlightenment of various transport systems ranging from ion channels of a wide variety of animal and human tissues and even of plant proteins (Sigel, 1990).

However, if one has the intention of using the oocyte expression system, it is necessary to know whether or not

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the oocyte endogenously already possesses the transport system under investigation (Weber et al., 1991). Though many believed that almost all transport systems of the oocyte had been explored, again and again new reports about hitherto unknown transporters and channels emerged. Recently, there were some reports about ion channels in the oocyte membrane that had not been described up to now. One of these newly discovered transport systems endogenous to the oocytes was an amiloride-sensitive Na^+ conductance, which shares some properties with expressed epithelial Na^+ channels (Weber et al., 1992), but could be clearly distinguished from these epithelial Na^+ channels by its insensitivity for benzamil and phenamil (Weber, Liebold & Clauss, 1995). Two other recently discovered transport proteins are a Na^+/Ca^{2+} exchanger (Schlief & Heinemann, 1995) and a Ca^{2+}-inactivated monovalent cation conductance (Arellano, Woodward & Miledi, 1995).

Another new unraveled transport system of the *Xenopus* oocyte plasma membrane, a Ca^{2+}-inactivated Cl^- channel, was described recently (Weber et al., 1995*a*,*b*). This Cl^- channel is blocked by micromolar doses of external Ca^{2+} and is inhibited by several known Cl^- channel blockers, such as flufenamic acid, niflumic acid and DPC while DIDS has further activating potencies. Single-channel analysis in the cell-attached configuration revealed a Cl^- channel with a conductance of about 90 pS and slightly outward rectification. Contrary to all other known transport systems of the oocytes, this Cl^- channel is not downregulated when the oocyte undergoes maturation, arguing for a role of these channels in the further developmental fate of the oocyte.

Here we report some more details about this Ca^{2+}-inactivated Cl^- channel, also termed CaIC (Weber et al., 1995*a*). We show that the CaIC is also inhibited by an antracene derivate and by an antiviral drug used to fight HIV-related symptoms. We also demonstrate that the transport capacity of this channel is high enough to become visible with optical methods by quenching a Cl^--sensitive dye. Single-channel analysis revealed only poor selectivity for Cl^- over other anions. All the data on the CaIC available up to now clearly indicate that this channel is peculiar among all Cl^- channels investigated so far.

Materials and Methods

Oocytes

Defolliculated oocytes of *Xenopus laevis* were obtained following experimental protocols described in more detail previously (Weber, Schwarz & Passow, 1990). South African clawed toads (purchased from African Xenopus Facility, Noordhoek, South Africa) were hypothermally anaesthetized and small pieces of ovary were removed. The oocytes containing lobes were cut into small pieces mechanically and bathed for 5 hr in oocyte Ringer (ORi, *see below*) containing collagenase (1 mg/ml, Serva, Heidelberg, Germany). After washing, the oocytes were placed for 10 min in Ca^{2+}-free ORi. If necessary larger clusters of remaining follicle cells were removed manually with small forceps. Defolliculated oocytes were stored at 18°C in ORi supplemented with penicillin (20 mg/l), streptomycin (25 mg/l) and pyruvate (2.5 mM) until the oocytes were used for the experiments. Measurements were performed with oocytes between one and five days after preparation. Only healthy-looking, full grown oocytes (stage V or VI, Dumont, 1972) were used for the experiments, which were performed at room temperature (22–25°C).

In vitro maturation was induced by incubating the oocytes in progesterone (0.1 μM) for 6 hr and were considered maturated when the so-called ''white spot'' appeared, which is a visible sign for germinal vesicle breakdown (Weber et al., 1995*a*).

Solutions

The composition of ORi was (in mM): 90 NaCl, 1 KCl, 2 $CaCl_2$ and 5 N-2-hydroxylethylpiperazine-N′-ethanesulfonic acid (HEPES), pH was set at 7.4. Ca^{2+}-free solutions were buffered with 0.5 mM ethylenediamine tetraacetic acid (EDTA) or ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). For Na^+-free solutions Na^+ was substituted by tetramethylammonium (TMA^+) or N-methyl-D-glucamine ($NMDG^+$). All substances, if not stated otherwise, were purchased from Sigma (Deisenhofen, Germany).

For patch-clamp experiments on oocytes the pipette was filled with a divalent ion-free solution containing NMDGCl or NaCl (100 mM) with EGTA (1 mM). Selectivity experiments were accomplished with different bath media: NMDGCl, NaCl, NaBr, Nal or Na-gluconate (100 mM respectively). For measurements on eggs, solutions with low ionic strength (pond water imitate, ≈5 mM NMDGCl) were used for bathing the oocytes. In this case Ca^{2+}-containing and Ca^{2+}-free solutions were buffered with EGTA (1 mM) supplemented with mannitol (200 mM) and adjusted to pH = 7.4 with $NMDG^+$ and HCl.

Two-electrode Voltage Clamp

Individual oocytes were placed in a small plexiglas chamber (1 ml volume) and were superfused constantly with a flow rate of about 2 ml/min. Conventional two-microelectrode voltage clamp was performed as described previously (Weber et al., 1992). Briefly, membrane potential and current-voltage relationships (*IV* curves) were determined with a voltage clamp amplifier (OC 725B, Warner Instruments, Hamden, CT). The voltage and the current microelectrodes were filled with 1 M and 3 M KCl, respectively and had resistances from 1 to 5 MΩ. The bath electrode was an Ag/AgCl pellet. If not stated otherwise oocytes were clamped to a holding potential of −60 mV and the holding current was recorded with a strip chart recorder. For determination of current-voltage relationships, steady-state current was measured during the last 100 msec of 500-msec rectangular pulses to different potentials from −150 to 50 mV in steps of 10 mV. These pulses were applied with a frequency of 0.25 Hz using a personal computer connected via an interface (CED 1401, Science Products, Hofheim, Germany). The software for the pulse protocol and the data acquisition was kindly provided to us by Drs. L.A. Vasilets and W. Schwarz (Max-Planck-Institute for Biophysics, Frankfurt/Main, Germany).

Patch Clamp

Patch-clamp pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) on a two-stage puller (Narishige, Tokyo, Japan).

The pipette resistance was about 5 MΩ in symmetrical 100 mM NaCl solutions.

Patch-clamp measurements were accomplished on devitellinated oocytes in the cell-attached or inside-out mode using the LM-PC patch-clamp amplifier (List-electronic, Darmstadt, Germany). Data acquisition and analysis were achieved with a TL-1 interface (Axon Instruments, Foster City, CA) and a personal computer running pCLAMP version 5.5 (Axon Instruments, Foster City, CA). Current traces from ramp or rectangular pulse experiments were stored directly on hard disk with 512 samples per trace, whereas long time data were stored with −10 dB on a modified conventional DAT recorder (AIWA, Tokyo, Japan). An 8-pole Bessel filter (Frequency Devices, Haverhill, MA) was used for low pass filtering (550 or 1550 Hz) and amplification (10 dB) of the tape stored data. The data were digitized for computer analysis with at least 0.5 msec sampling time.

Mean currents of a patch ($I_{mean} = Q/\Delta t$) were determined by integrating current traces (Q) obtained by rectangular voltage-pulse experiments. Baseline currents that exhibited no detectable channels, were termed as background currents because they were also voltage and ion dependent (Weber et al., 1995*a*). Mean CalC currents (I_{CalC}) were calculated by integrating current traces with CalC openings minus the above-mentioned background currents. Membrane potential (V_m) was monitored as negative pipette potential in the inside-out mode and was calculated in the cell-attached mode from measured resting membrane potential minus pipette potential.

For minimization of diffusion potentials, an agar bridge filled with 0.5 M NMDGCl was used as a reference electrode in the bath. Liquid junction potentials were measured by exposing the patch pipette to the different bath solutions and corrected. Membrane potentials (V_m) of oocytes and eggs were recorded by penetrating the cells with a microelectrode filled with NMDGCl solution (100 mM) free of divalent cations. The microelectrode was connected to a patch-clamp amplifier (L/M-PC). Electrode offset was adjusted in the search mode and V_m was measured in the current clamp mode using the current-monitor output of the amplifier and stored on a strip chart recorder.

Current Convention

Flow of negative charge (i.e., Cl^-) from the cytosol of the oocytes is conventionally termed inward current and plotted downwards in all graphs.

Selectivity Measurements

The relative permeabilities were calculated by applying the Goldman Hodgkin Katz (GHK) equation (Goldman, 1943). For monovalent anions A with respect to Cl^- the GHK equation can be written in the form:

$$V_{rev} = RT/F \ln ((P_A/P_{Cl}) [A_i] + [Cl_i]) / ((P_A/P_{Cl} [Ao] + [Cl_o])) \quad (1)$$

where P are the permeability coefficients for the respective ions A and Cl; F = Faraday constant, R = gas constant, T = temperature = 298 K), i is the cytoplasmic, o the extracellular side and V_{rev} is the reversal potential. When Cl_i was completely substituted by an anion A_i and A_o was zero, Eq. (1) could be simplified to:

$$V_{rev} = RT/F \ln ((P_A/P_{Cl}) [A_i] / [Cl_o]) \quad (2)$$

and for monovalent cations C with respect to Cl^- the equation could be modified to:

$$V_{rev} = RT/F \ln ((P_C/P_{Cl}) [C_o] + [Cl_i] / ((P_C/P_{Cl}) [C_i] + [Cl_o])) \quad (3)$$

Optical Measurements

Oocytes were placed in a small plastic chamber on an inverted microscope attached to laser confocal microscope (MRC 1000, Biorad, Hemel Hempstaed, UK). NaCl in the bathing solution was substituted by Na-gluconate and $CaCl_2$ by $CaSO_4$, which contained at least 1.8 mM free Ca^{2+}, respectively. 10 mM of the Cl^--sensitive dye 6-methoxy-N-(sulfopropyl)quinolinium (SPQ, Molecular Probes, Eugene, OR) was added to detect chloride ions which left the oocyte through Ca^{2+}-inactivated Cl^- channels in Ca^{2+}-free solution, which contained at the most 1 μM free Ca^{2+}, or after Ca^{2+} chelating by addition of EDTA (final concentration 5 mM). Measured was the decline in fluorescence intensity caused by the quenching effects of chloride ions on the fluorescence dye SPQ.

Statistics

Where applicable, data are expressed as arithmetic means ± SEM; n is the number of oocytes and N is the number of female donors. Statistical analysis was made by t-test where appropriate: significant difference was assumed at $P < 0.05$.

Results

Selectivity of the CaIC Revealed from Single-Channel Experiments

For single-channel analysis currents induced by removal of external Ca^{2+}, as described throughout this paper, were recorded from oocytes after removal of the viteline layer, indicating that the transport system under investigation is located in the oocyte plasma membrane. Depletion of extracellular divalent cations evoked outwardly rectifying CaIC currents in whole-cell two-microelectrode voltage-clamp and cell-attached patch-clamp experiments. Comparison of current-voltage relationships (*IV* curves) obtained with both techniques exhibited a similar voltage dependence of CalC currents (Fig. 1*A* and *B*). Whole-cell currents through Ca^{2+}-inactivated Cl^- channels and single-channel fluctuations (cell-attached mode) reversed in the range of the expected reversal potential for Cl^- in oocytes. The Cl^- reversal potential varies within the range of −18 to −35 mV according to the intracellular Cl^- concentration of individual oocytes (Dascal, 1987). The two-electrode voltage clamp *IV* relationship reflects steady-state ion fluctuations evoked by divalent cation depletion while patch-clamp currents represent ion fluxes through the CalC without background currents (*see* Materials and Methods).

For selectivity studies on the CaIC with the patch-clamp technique, inside-out patches were used so that the cytosolic side of a single patch could easily be exposed to different solutions. However, patch excision elicited frequently enhanced single-channel conductances of the CalC (up to 300 pS) and the simultaneous loss of recti-

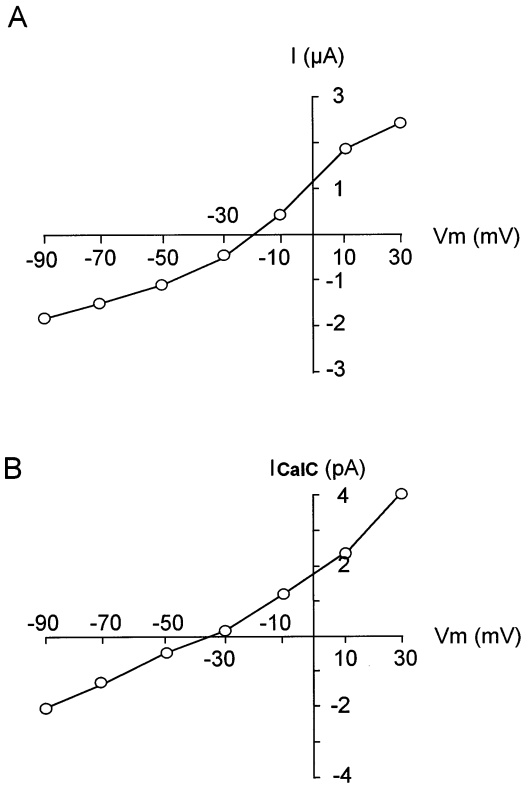

Fig. 1. *IV* relationships of the CalC during Ca^{2+} depletion. Shown are typical traces out of a pool of several measurements as indicated below. Traces are difference curves in the presence and absence of Ca^{2+}. (*A*) Two-electrode voltage clamp ''steady-state'' whole-cell currents (n = 7, N = 3). (*B*) Mean CalC patch currents in the cell-attached mode obtained from rectangular pulse experiments (n = 3, N = 3).

fying properties in symmetrical solutions. In cell-attached measurements the main conductance state of the CaIC was about 90 pS and showed moderate outward rectification in channel amplitude and mean current, for the selectivity analysis we used only this conductance state. Inside-out patch-clamp experiments with rectangular voltage pulses are shown in Fig. 2*A* and *B* using symmetrical solutions (NMDGCl:100 mM) with a subsequent bath solution change to a lower NMDGCl concentration (10 mM, adjusted to 200 mosm with mannitol). The same experiments were performed with voltage ramps, but the results showed that the current responses allowed only a qualitative determination of V_{rev} (*data not shown*) whereas *IV* curves constructed from current traces of rectangular pulse experiments permitted reliable identification of reversal potentials (Fig. 2*A* and *B*). V_{rev} was about 0 mV in symmetrical solutions and exhibited a hyperpolarizing shift of ≈23 mV in an unsymmetrical (10:1) solution. Using Eq. (3) (*see* Materials and Methods) a relative permeability ratio of P_{NMDG^+}/P_{Cl^-} of 0.31 was calculated indicating that CaIC discriminated poorly between $NMDG^+$ and Cl^-. Sur-prisingly, exchange of bath NMDGCl with NaCl (both 100 mM) caused a depolarizing shift of V_{rev} of about 7 mV. This fact might point out the possibility that the CaIC had a lower permeability for Na^+ than $NMDG^+$ possibly caused by different selectivity mechanisms.

Subsequent substitution experiments with NaBr and Nal solutions led to further depolarizing shifts of V_{rev} (10.1 and 13.6 mV, respectively) whereas substitution with Na-gluconate elicited a hyperpolarizing shift to −20 mV (Fig. 3). The table gives the parameters of a single oocyte representative for several experiments (n = 3, N = 3). All reversal potentials given in the table were calculated relative to V_{rev} measured with NaCl in the bath solution. V_{rev} of the curves in presence of NaCl was normalized to 0 mV before calculating the permeability ratios according to Eq. (2).

CaIC During Maturation

Up to now, only data on the fate of the CaIC during maturation obtained from experiments with two-electrode voltage clamp were available (Weber et al., 1995). To extend these observations we now applied the patch-clamp technique to maturated oocytes, termed eggs, and investigated the role of the CaIC in these eggs when exposed to pondwater imitate where they are normally spawned. Eggs did not depolarize after exposure to divalent cation-free pondwater as oocytes did when exposed to divalent-free Ringer solutions (Weber et al., 1995*b*). Prick activation of eggs was also uninfluenced by divalent cation-free pondwater imitate, indicating the absence of CaIC activation. V_m measurements on eggs were accomplished in pondwater imitate in the presence and absence of Ca^{2+} after insertion of a potential electrode. This pricking frequently evoked egg activation after a short delay of 1–3 min. Resting membrane potentials and V_m changes during prick activation were comparable in high (2 mM; Fig. 4*A*), low (10 μM; Fig. 4*B*) and Ca^{2+}-free (Fig. 4*C*) pondwater imitate.

The presence of a high conductance Cl^- current was proved in attached patches on partly devitellinated eggs (Fig. 5). A high ionic pipette solution (NaCl: 100 mM, Ca^{2+}: 100 nM) was used and the bath solution was pondwater imitate with Ca^{2+} (2 mM). Under these conditions, CaIC-like channels exhibited a slope conductance of approximately 200 pS, a reversal potential of about −20 mV and gating properties which were similar to the CaIC in oocytes.

Blocker Kinetics of the CaIC in Whole Cell Recordings

Using conventional two-microelectrode voltage-clamp, oocytes were held at holding potentials of −60 mV and the resulting clamp currents were recorded. The Ca^{2+}-

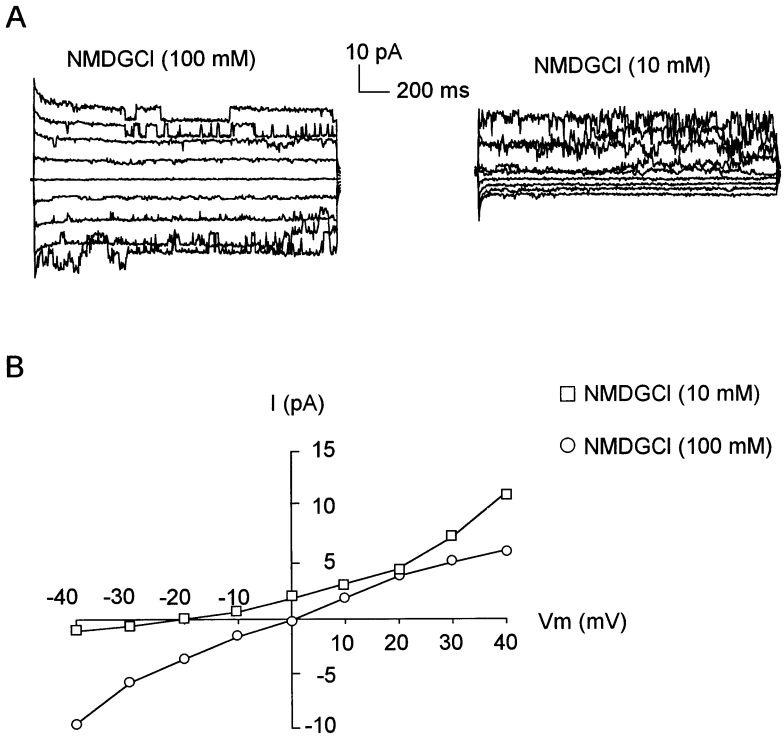

Fig. 2. CaIC sensitivity between the anion Cl^- and the cation $NMDG^+$ measured in the inside-out mode. (*A*) Rectangular voltage pulse experiments with symmetrical NMDGCl solutions (100 mM) in the pipette and bath (left) and a subsequent change of the bath solution to 10 mM NMDGCl. (*B*) Corresponding *IV* relationships derived from the current traces shown in (*A*). Reducing the bath concentration of NMDGCl from 100 mM to 10 mM resulted in a hyperpolarizing shift of −23 mV.

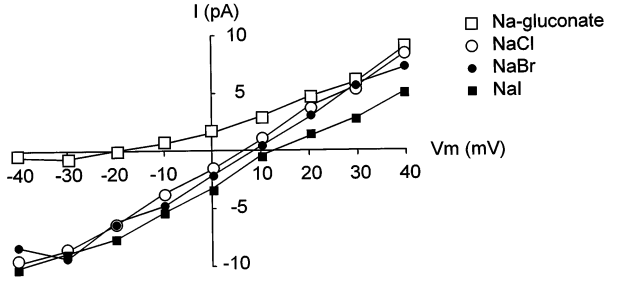

Fig. 3. CalC discrimination between the anions gluconate, Cl^-, Br^- and I^- measured on one patch in the inside-out mode. The pipette contained NMDGCl throughout (100 mM) and different sodium salt solutions (100 mM) were applied to the cytosolic side of the patch.

Table. Selectivity of the CaIC

	I^-	Br^-	Cl^-	Gluconate
V_{rev} (mV)	6.6	3.1	0	<−20
P_{anion}/P_{Cl^-}	1.30	1.14	1.00	<0.35
Conductance (pS)	232	275	275	≈ 265

inactivated Cl^- channel is blocked by several well-known Cl^- channel inhibitors as DPC, flufenamic acid and niflumic acid (Weber et al., 1995*a*,*b*). To further characterize this Cl^- channel, we tested two other compounds for their ability to block the Ca^{2+}-inactivated Cl^- channel. Oocytes responded on removal of external Ca^{2+} with large inward currents carried by Cl^-. These inward currents could be partly blocked by anthracene-9-carboxylic acid (9-AC). *IV* curves in presence of this blocker showed markedly reduced currents (Fig. 6) indicating the inhibitory potency of 9-AC on CaIC-mediated currents.

The antiviral drug 3′azido 3′-deoxythymidine (AZT) was recently shown to inhibit swelling-induced Cl^- channels in NIH 3T3 fibroblasts (Gschwentner et al., 1995). This thymidine nucleoside analogue also exhibited inhibitory potency on the Ca^{2+}-inactivated Cl^- channel. Figure 7 depicts that the holding currents induced by removal of external Ca^{2+} were at least partly blocked by AZT (200 μM).

Optical Measurements

Investigating the movement of chloride through cell membranes is seriously hampered by the limitations of most of the current techniques: Methods based on measurements of $^{36}Cl^-$ have poor sensitivity because of the low specific activity of this isotope and Cl^--sensitive microelectrodes have meager chloride ion selectivity. Therefore, we used a recently developed fluorescent indicator to make visible how chloride ions are transported by Ca^{2+}-inactivated Cl^- channels in the absence of external Ca^{2+} or after removal of Ca^{2+} from the bath solution. The fluorescence intensity of 6-methoxy-N-(sulfopropyl)quinolium (SPQ) is quenched upon collision with

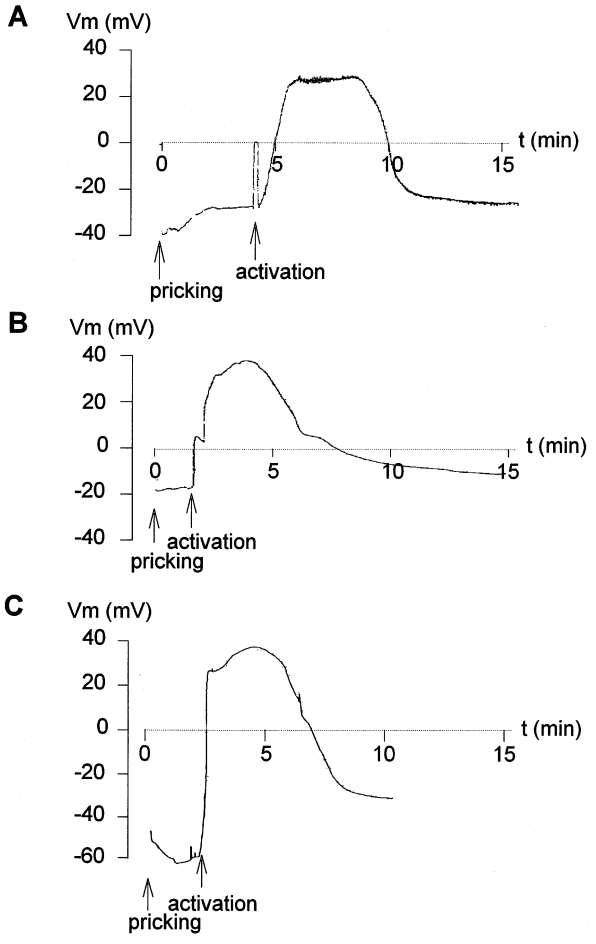

Fig. 4. Membrane potential measurements and prick activation on eggs in low ionic media (pondwater imitate) with and without Ca^{2+}. (*A*) 2 mM Ca^{2+} (*B*) 10 μM Ca^{2+} and (*C*) Ca^{2+}-free.

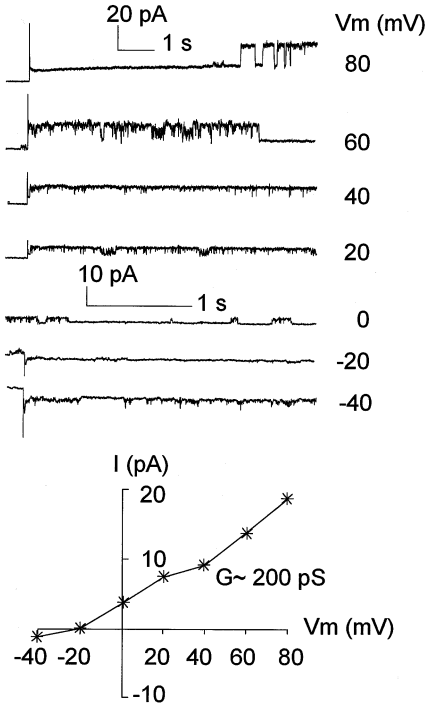

Fig. 5. Attached measurement on an egg exhibiting a CaIC-like channel of about 200 pS. The pipette solution contained NaCl (100 mM) and Ca^{2+} (100 nM); the bath solution was pondwater imitate.

Cl^- and undergoes a 50% decrease in emission intensity in 10 mM chloride (Verkman, 1990). When oocytes were kept in Cl^--free solution in presence of 2 mM Ca^{2+} and 10 mM SPQ no changes in the emission intensity of the Cl^--sensitive dye were observed for more than 75 min (Fig. 8*A*, upper trace). This experimental protocol clearly showed that during the measured time no detectable quenching of the fluorescence could be found meaning that no appreciable loss of Cl^- from the oocytes occurred. However, when oocytes were bathed in Cl^-- and Ca^{2+}-free solutions, the emission intensity of the Cl^--sensitive fluorescent dye SPQ declined continuously to 60.7 ± 2.2% (n = 16, N = 3) of the initial intensity value within 75 min (Fig. 8*A*, lower trace). These data give strong evidence for a transport of chloride ions in the millimolar range from the inside of the oocytes to the bath medium via the Cl^- channels, which were activated by the removal of external Ca^{2+}.

In an additional set of experiments we activated the Ca^{2+}-inactivated Cl^- channels by chelating Ca^{2+} during the experiment. Oocytes were placed in Cl^--free solution in presence of 2 mM Ca^{2+} and 10 mM SPQ. In the first part of the experiments the emission intensity of SPQ remained constant (Fig. 8*B*, left part). Instantly after addition of EDTA (final concentration 5 mM) the emission intensity decreased indicating an activation of Ca^{2+}-inactivated Cl^- channels and an accumulation of Cl^- in the bath medium (Fig. 8*B*, right part). In control experiments in the absence of oocytes, addition of EDTA had no detectable effect on the emission intensity of SPQ. This fact gives clear evidence that the observed increase in extracellular Cl^- is due to a Cl^- transport from the inside of the oocytes to the bath medium mediated via the CalC.

Discussion

The results of this paper confirm and extend our previous observations that oocytes of *Xenopus laevis* contain a Cl^- channel, which is inactivated by submillimolar doses of external Ca^{2+} and is immediately activated upon lowering the external Ca^{2+}-concentration below a certain threshold (Weber et al., 1995*a*,*b*). In the present study, we used one of the outstanding advantages that the *Xenopus* oocyte offers: the possibility to combine different techniques on a single cell. Concretely, we supplemented electrophysiological measurements with data ob-

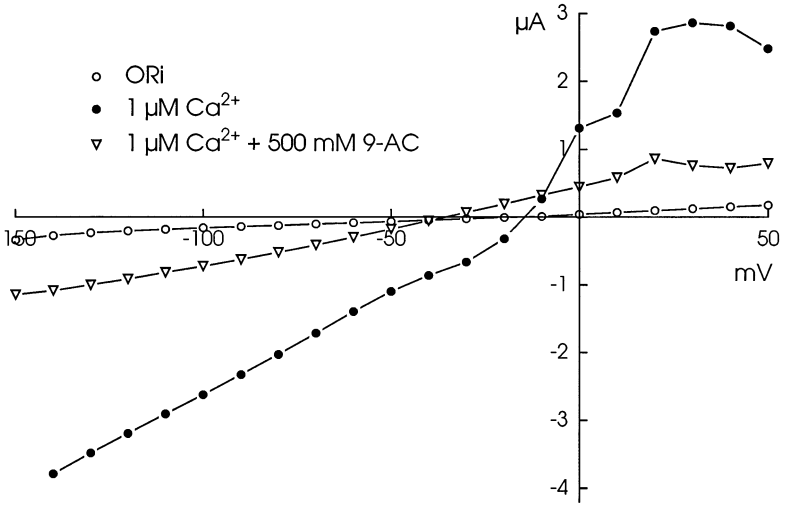

Fig. 6. Effect of 9-AC on the CalC current. *IV* curves in absence (filled circles) and presence (open triangles) of 9-AC under Ca^{2+}-free conditions and with 2 mM Ca^{2+} in the bath solution (open circles). The curves are typical for 6 oocytes of 2 animals.

tained by optical methods. By using a recently developed Cl^--sensitive dye (Verkman, 1990) we were able to visualize the activation of the Ca^{2+}-inactivated Cl^- channel. Unfortunately, our present equipment did not allow us to monitor fluorescence changes under voltage-clamp conditions.

The Cl^--sensitive dye that we utilized (6-methoxy-N-(sulfopropyl)quinolinium, SPQ) has several advantages over traditional Cl^--detection techniques. Beside the high sensitivity and its strong selectivity for Cl^-, SPQ and related analogues are highly polar and membrane impermeant (Verkman, 1990). So, we could be sure that the observed effects were not attributable to an uptake of the dye into the oocytes. Moreover, the dye is stable over a long period (>2 hr) and shows no detectable loss of fluorescence during the measured time interval. Interactions with Cl^- channels could also be excluded because studies of $^{36}Cl^-$ uptake in the presence and absence of SPQ showed that SPQ itself did not inhibit or activate chloride transport (Verkman, 1995).

Under Ca^{2+}-free conditions, the intensity of the Cl^--sensitive dye SPQ decreased within 75 min by about 40%. With 10 mM Cl^- in the solution one would obtain about 50% decrease in emission intensity (Verkman, 1990), indicating that the Ca^{2+}-inactivated Cl^- channels translocated Cl^- in the millimolar range from the oocytes cytoplasm to the surrounding bath solution during the measured time interval. Although fluorescence intensity measurements showed useful results, the sensitivity of this method is quite lower than the resolution of electrophysiological techniques and yielded only raw valuations about the transport capacity of the CalC. Therefore direct comparison of optical and electrophysiological data is not possible.

In an attempt to further characterize the Ca^{2+}-inactivated Cl^- channel, we evaluated the responses of this channel to two further Cl^- channel blockers. Both

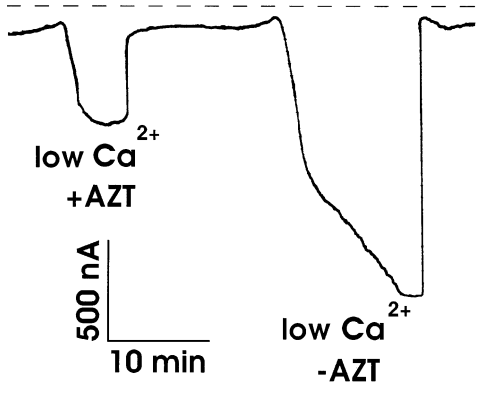

Fig. 7. Effect of AZT on the CalC current. Holding current of an oocyte clamped to −60 mV. Removal of Ca^{2+}-evoked large inward currents (right part of the trace) which could be blocked partly by AZT (200 µM; left part of the trace). The broken line indicates zero current level. Shown is a typical trace representative of 9 oocytes from 3 animals.

inhibitors used for the present study showed only incomplete blocking of the CalC. 9-AC reportedly blocked Cl^- channels in several different cell types with lower efficacy than other Cl^- channel inhibitors (Gögelein, 1988; Koumi, Sato & Aramaki, 1994; Jentsch, 1994). This low affinity or weak specifity could also be seen for the CaIC: High concentrations of the blocker (200 µM) resulted only in 80% inhibition of the total currents mediated by the CaIC. Even higher concentrations had no further effect.

Azidothymidin (AZT), a nucleoside analogue used in the treatment of viral infections was recently demonstrated to act also as a Cl^- channel blocker (Gschwentner et al., 1995). The drug which is utilized in the therapy of human acquired immunodeficiency could block the CaIC at least partly, though quite high concentrations of about 250 µM were necessary.

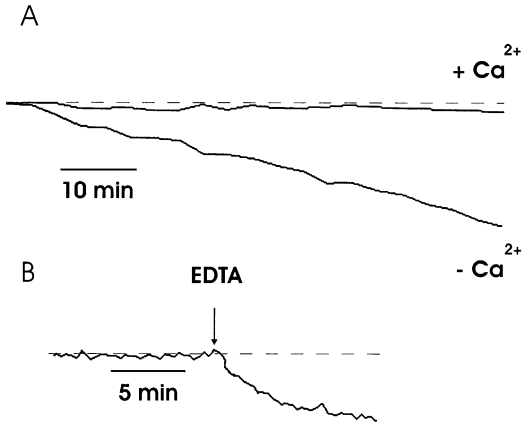

Fig. 8. Optical measurements with SPQ. Shown are relative changes in the emission intensity of SPQ. Downward reflections represent accumulation of Cl^- in the bath solutions, broken lines indicate zero fluorescence levels. (*A*) SPQ in presence of Ca^{2+} showed no change in emission over the whole experimental periode (upper trace). In the absence of Ca^{2+} the CalC of the oocytes was active and transported Cl^- to the bath medium demonstrated by a decrease in the emission intensity of SPQ (lower trace). Shown are typical traces out of a pool of experiments with 16 oocytes ($N = 3$). (*B*) Again in presence of Ca^{2+} the emission intensity of SPQ stayed constant (left part of the trace). Immediately after chelating the extracellular Ca^{2+} with EDTA, the CalC was activated and measured as an accumulation of Cl^- in the bath solution (right part of the trace). The results shown are representative of 8 oocytes ($N = 3$). Note the different time scales in *A* and *B*.

From the comparison of all substances used to block the CaIC (Weber et al., 1995a) it becomes obvious that flufenamic acid is the inhibitor with the highest affinity for this channel. Therefore, if an experimental design requires the absence of divalent cations in the solution surrounding an oocyte and an activation of the CaIC is unwelcome, the experimenter should add flufenamic acid to all solutions. In this case, 80 μM are able to block 99% of all Ca^{2+}-inactivated Cl^- channels present in the oocyte. If only Ca^{2+} has to be substituted the presence of 1 mM Mg^{2+}, Ba^{2+} or Sr^{2+}, respectively, prevents an undesired activation of the CaIC. During our experiments with different blockers we also found that TEA (tetraethylammonium, 20 mM) and ouabain (20 μM) had absolutely no effect on the CaIC.

Combining single-channel data with results from whole-cell recordings we calculated that one oocyte possesses on average about 10^6 CaIC molecules. Given that the oocyte surface is about 2×10^7 μm^2 (Vasilets et al., 1990) the average channel density is nearly one channel molecule per 20 square microns. That is approximately the area of the patch pipettes that we used for the present investigation and demonstrates that the opportunity to find a channel in the patch is quite high, and one CaIC might be present in nearly every patch.

Single-channel data in the cell-attached mode and results from whole cell recordings exhibited slightly outwardly rectifying properties of the CaIC currents in the range from −90 to 30 mV. The small differences of CaIC patch currents in contrast to whole cell measurements may reflect further nonselective cation conductances which are activated by depletion of extracellular Ca^{2+}. Such conductances, which could not be further resolved, were already identified in patches as background currents (Weber et al., 1995*a*). However, both techniques revealed an identical sensitivity to external Ca^{2+} and a reversal potential near the Cl^- equilibrium potential (−18 to −35 mV). Because of the large conductance and high density in oocytes this channel is responsible for the giant currents in two-electrode whole-cell voltage clamp recordings in absence or divalent cations in the extracellular medium.

When shifts of the reversal potential in dependence of the extracellular Cl^- concentration were measured, the CaIC appeared to be perfectly selective for the anion Cl^- in whole cell experiments (Weber et al., 1995*b*). However, inside-out patch-clamp investigations revealed a rather weak discrimination between the anion Cl^- and the cation $NMDG^+$ and yielded a ratio of 1:0.31 (Cl^-:$NMDG^+$). Based on all the above given observations the CaIC can be classified as a maxi Cl^- channel with only poor selectivity for Cl^- over gluconate and $NMDG^+$. Such quite unselective Cl^- channels had also been described from rat skeletal muscle (Blatz & Magleby, 1985) and rat hippocampal neurons (Franciolini & Nonner, 1987).

Recently, an inwardly rectifying current carried by cations was investigated in *Xenopus* oocytes, which could be activated by removal of external divalent cations or by applying positive voltages (Arellano, Woodward & Miledi, 1995). Contrary to the results described in that paper we found no such inwardly rectifying currents upon depletion of divalent cations in the extracellular medium. This might depend on the different methods for constructing *IV* curves. Arellano et al. (1995) analyzed peak currents immediately after application of the test pulse whereas our measurements refer to steady-state currents recorded during the last 100 msec of the 500 msec test pulses (*see also* Materials and Methods). Moreover, the cation-mediated current described by Arrellano et al. (1995) is inactivated during maturation whereas the anion-carried CaIC remains active in eggs.

The oocyte plasma membrane was reported to be poorly permeable for the big cation $NMDG^+$ (Costa et al., 1989). However, in case of the CaIC $NMDG^+$ was even more permeable than the small cation Na^+. Analysis of CaIC amplitudes in the presence of different anions revealed an anion selectivity sequence I^- (1.3) > Br^- (1.14) > Cl^- (1.0) ⩾ gluconate (0.35). This selectivity sequence reflects the hydrated size of the utilized halides (Dascal, 1987) and was reported from several other Cl^- channels (Frizzell & Halm, 1990; Dixon &

Martin, 1993; Cahalan & Levis, 1994). A similar selectivity pattern was also found for Ca^{2+}-activated anion channels of the *Xenopus* oocyte incorporated into lipid bilayers (Young et al., 1984) and for anion currents investigated in *Xenopus* eggs (Webb & Nuccitelli, 1985).

Eggs of anuran amphibians are extruded into pondwater for fertilization (Sardet & Chang, 1987). Although the eggs are surrounded by jelly coat known to retain high Ca^{2+} concentrations (Ishihara et al., 1984), exposition to media of such low ionic strength and rather low Ca^{2+} concentrations requires the presence of transport systems for volume regulation and ion maintenance, which should be largely independent of external ion concentrations. On the other hand, an activation potential elicited by fertilization or by pricking requires the fast activation of large Cl^- conductances, which might involve CaIC-like channels (Cross & Elinson, 1980; Webb & Nuccitelli, 1985). Therefore, it is likely that low ionic media induce modifications of the CaIC in eggs thereby preventing the loss of intracellular Cl^- in large amounts. As a test for this theory, we measured the resting potential of in vitro maturated oocytes and their ability to respond to prick activation in pondwater imitate. Even in completely divalent-free pondwater imitate no membrane potential breakdown or decreased prick activation responses were found, arguing for different regulation of Ca^{2+}-inactivated Cl^- channels under such conditions. Our results from single-channel measurements with 100 mM solutions in the pipette showed the presence of a Ca^{2+}-inactivated Cl^- channel in eggs, which seemed to differ from the CaIC in oocytes at least with respect to the extracellular Ca^{2+} sensitivity. These altered features of the CaIC in eggs might reflect conformational changes of the channel protein during the maturation or an altered regulation of the same channel in the different developmental stages of the amphibian oocyte.

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