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Further Characteristics of the Ca^{2+}-inactivated Cl^- Channel in *Xenopus laevis* Oocytes

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Abstract. Removal of extracellular Ca^{2+} activates ion channels in the plasma membrane of defolliculated oocytes of the South Africa clawed toad *Xenopus laevis.* At present, there is controversy about the nature of the Ca^{2+}-inactivated ion channels. Recently, we identified one of these channels as a Ca^{2+}-inactivated Cl^- channel (CaIC) using single channel analysis. In this work we confirm and extend previous observations on the CaIC by presenting a decisive extension of the regulation and inhibition profile. CaIC current is reversibly blocked by the divalent and trivalent cations Zn^{2+} (half-maximal blocker concentration, $K_{1/2} = 8$ μM), Cu^{2+} ($K_{1/2} = 120$ μM) and Gd^{3+} ($K_{1/2} = 20$ μM). Furthermore, CaIC is inhibited by the specific Cl^- channel blocker NPPB ($K_{1/2} \approx 3$ μM). Interestingly, CaIC-mediated currents are further sensitive to the cation channel inhibitor amiloride (500 μM) but insensitive to its high affinity analogue benzamil (100 μM). An investigation of the pH-dependence of the CaIC revealed a reduction of currents in the acidic range. Using simultaneous measurements of membrane current (I_m), conductance (G_m) and capacitance (C_m) we demonstrate that Ca^{2+} removal leads to instant activation of CaIC already present in the plasma membrane. Since C_m remains constant upon Ca^{2+} depletion while I_m and G_m increase drastically, no exocytotic transport of CaIC from intracellular pools and functional insertion into the plasma membrane is involved in the large CaIC currents. A detailed overview of applicable blockers is given. These blockers are useful when oocytes are utilized as an expression system for foreign proteins whose investigations require Ca^{2+}-free solutions and disturbances by CaIC currents are unwanted. We further compare and discuss our results with data of Ca^{2+}-inactivated cation channels reported by other groups.

Key words: Ca^{2+}-inactivated Cl^- channel — Inhibition profile — Capacitance measurements

Introduction

The oocyte of the South-African clawed toad *Xenopus laevis* is widely used as an expression system for the investigation of membrane transport proteins. The possibility of application of several different biochemical and electrophysiological techniques to a single cell has led to its employment in many membrane transport research laboratories. Using the oocytes as an expression system revealed the mechanisms of a vast number of channels and other transport systems as primarily and secondarily active transport systems and facilitative transporters (Sigel, 1990).

If the oocyte is used for heterologous expression of ion channels or other transport proteins it is mandatory to investigate whether oocytes endogenously possess the system that is to be expressed. Oocytes are endowed with a large variety of ion channels and transporters. One example is the high density of Na^+/K^+-ATPase proteins (Vasilets & Schwarz, 1993, 1994). Furthermore, a large number of ion channels in *Xenopus laevis* oocytes have been described up to now thoroughly reviewed by Dascal (1987) and more recently by Weber (1999*a*). Nevertheless, newly revealed ion conductances emerge with almost constant regularity. To those recently discovered new ion channels of the oocyte belongs an amiloride-sensitive Na^+ conductance (Weber, Liebold & Clauss, 1995), that can be clearly distinguished from epithelial Na^+ channels (ENaC) by its insensitivity for benzamil and phenamil. Even ion channels that have

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been extensively investigated in the oocytes were shown recently to exhibit hitherto unknown properties, namely stretch-independent activation of mechanosensitive cation channels (Reifarth, Clauss & Weber, 1999).

To the long list of newly discovered conductances belong further nonselective cation channels apparently inactivated by extracellular Ca^{2+} (Arellano, Woodward & Miledi, 1995; Zhang, McBride & Hamill, 1998) and endogenous hemi-gap-junctional channels originally described by Ebihara (1996). In contrast with these findings, we discovered and described an endogenous Ca^{2+}-inactivated Cl^- channel that we termed CaIC (Weber et al., 1995, Reifarth et al. 1997). Initial experiments showed a fast depolarization and large currents upon removal of divalent ions from the bath solution. First interpreted as an unspecific, irreversible leak current (Raditsch & Witzemann, 1994), the fast reversibility of this effect gave a first hint of a physiological process mediated by opening and closing of a channel population. Systematic investigation of the underlying phenomenon employing the two-electrode voltage-clamp technique, the patch-clamp technique and optical measurements revealed a Cl^- channel that is inhibited by micromolar doses of extracellular Ca^{2+} ($K_{1/2} \approx 20$ μM) (Weber et al., 1995).

The channel exhibits a selectivity sequence of $I^- > Br^- > Cl^-$ > gluconate >> $Na^+ = K^+$ which reflects the hydrated sizes of the employed halides. Under extracellular Ca^{2+}-free conditions, the CaIC shows a single channel slope conductance of 90 pS with a high spontaneous open probability and at least two substates. Even in the absence of any permeable cations in the extracellular bath, the channel is activated upon Ca^{2+} withdrawal. Previous studies furthermore revealed a large quantity of channel proteins in the membrane that were strongly inhibited by a series of common Cl^- channel blockers (Weber et al., 1995). An unexpected property of CaIC is a strong activation upon perfusion of the oocyte with low doses of SITS, usually also known as a Cl^- channel blocker. Surprisingly, the channel is not internalized or downregulated during oocyte maturation as is the fate for the majority of endogenous transport systems. These data reveal a new Cl^- channel that does not fit into one of the currently known Cl^- channel classes as reviewed recently (Jentsch & Günther, 1997). It is more likely that this new channel represents a new family of Cl^- channels that is only distantly related to the established Cl^- channel classes.

In this paper we extend and confirm our previous observations on the CaIC. We demonstrate that the di- and trivalent cations Cu^{2+}, Zn^{2+} and Gd^{3+} and the organic Cl^- channel blocker NPPB effectively inhibit CaIC with different affinities. We show that external protons seem to interact with CaIC and that increasing $[H^+]_o$ inhibits the channels. CaIC activity is independent of regulation by PKA but dependent on PKC as demonstrated by the use of PKA or PKC blockers. Currents activated by Ca^{2+} removal are sensitive to amiloride but insensitive to its high-affinity analogue benzamil. Furthermore, we introduce a newly developed technique for simultaneously measuring membrane current and conductance together with capacitance; enlighting changes of membrane surface area that enables monitoring of endo- and/or exocytotic events.

In a second part we compare and discuss these data and other recent data from our laboratory with those reported by three other groups on putative Ca^{2+}-inactivated nonselective cation channels in *Xenopus laevis* oocytes (Arellano et al., 1995; Ebihara, 1996; Zhang et al., 1998). Parts of the results were presented at the annual meeting of the Germany Physiological Society and have been published in abstract form (Amasheh, Clauss & Weber, 1999).

Materials and Methods

Oocyte Preparation

Females of the African clawed toad *Xenopus laevis* were purchased from African Xenopus Facility (Knysna, Republic of South-Africa). Oocytes were obtained following experimental protocols described in more detail previously (Weber, Schwarz & Passow, 1990). Briefly, animals were anesthetized hypothermically. A small cut of approximately 5 mm length in the lateral abdomen allowed the removal of ovarian lobes containing oocytes of the stages I to VI (Dumont, 1972). Small pieces of the ovaries were incubated in Ringer solution (ORi, *see below*) containing collagenase (1 mg/ml, Serva, Heidelberg, Germany) for 2 hr. Subsequently, oocytes were washed in Ca^{2+}-free ORi for 10 min to remove the follicle cell layer. Healthy-looking, full-grown oocytes of the stages V and VI (Dumont, 1972) were selected for the experiments and measurements were performed within 7 days after removal. Only oocytes with initial membrane potentials of −40 mV or more negative were used for our studies. All experiments were performed at room temperature (22–24°C) over the period from March 1997 to August 1999. Apparently no seasonally dependent effects on the results were observed. Injection procedures were identical with those reported elsewhere (Weber et al., 1992).

Voltage Clamp

Conventional two-electrode voltage-clamp technique was performed as described previously (Amasheh et al., 1997). Briefly, a personal computer was connected to an oocyte clamp amplifier (Warner Oocyte Clamp OC-725C, Warner Instruments, Hamden, CT) via an interface (CED1401, Science Products, Hofheim, Germany). The voltage electrode was filled with 1 M KCl, the current electrode with 3 M KCl. Two Ag/AgCl pellets were used as bath electrodes. Clamp current was recorded constantly while oocytes were clamped to a membrane potential of −60 mV. Oocytes were superfused continuously with a flow rate of 10 ml/min.

Capacitance Measurements

Simultaneous measurements of membrane current and conductance in parallel with capacitance were performed as described in detail earlier

(Weber et al., 1999). Briefly, two Digital Signal Processing (DSP) boards (Model 310B, Dalano Spry, Rochester, NY) were used for impedance and capacitance measurements. The boards were equipped with 2 high speed (300 kHz) analog-to-digital converters (14 bit) and 2 digital-to-analog converters (12 bit). The DSP boards were connected to the oocyte voltage-clamp via an interface consisting of anti-aliasing filters, programmable gain amplifiers and digital control circuits that were controlled by the DSP boards. One DSP board was used to record membrane conductance (G_m) and clamp current (I_m). G_m was measured by imposing a sine wave with a frequency of 0.25 Hz and an amplitude of 5 mV to the oocyte membrane. Current and voltage changes evoked by the sine wave were sampled with a frequency of 625 Hz. We used regression analysis to calculate G_m from the amplitudes of the current and voltage sine waves in a way that enabled the system to update G_m values every 15 sec. The use of a low-pass filter with a cutoff frequency of 3 Hz for the current and voltage signals enabled us to simultaneously record G_m and I_m while the high-frequency membrane capacitance (C_m) was measured with 5 sine waves with frequencies (f) ranging from 73 to 293 Hz. Since membrane capacitance is absolutely independent of the frequency for $f <$ 200 Hz, only the 3 lowest frequencies from 73 to 146 Hz were used for data interpretation. Figure 5 in this paper illustrates records at $f =$ 73 Hz.

Solutions

The composition of Oocyte Ringer solution (ORi) was (in mM): NaCl (90), KCl (1), $CaCl_2$ (2) and N-5-hydroxyethylpiperazine-N′-ethanesulfonic acid (HEPES, 5). In Ca^{2+}-free solutions, Ca^{2+} was chelated by ethylenglycol bis (β-aminoethyl ether)-N,N,-N′,N′-tetraacetic acid (EGTA, 5 mM). pH was set at 7.4 if not stated otherwise. For measurements investigating the pH-dependence, EGTA was replaced by the less pH-sensitive 1,2-bis (2 aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA). Oocytes storage solution (KulORi) additionally contained penicillin (20 mg/l), streptomycin (25 mg/l) and pyruvate (2.5 mM). All chemicals were obtained by Sigma (Deisenhofen, Germany) if not indicated otherwise.

Statistics

Where applicable, results are expressed as arithmetic means ± SEM, with n indicating the number of oocytes and N indicating the number of donors. Statistical analysis was made by t-test where appropriate. Significant difference was assumed at $P < 0.05$.

Results

Superfusion of defolliculated *Xenopus* oocytes with Ca^{2+}-free, EGTA or BAPTA buffered oocyte Ringer led in all experiments to a strong and consistent depolarization of the cell membrane. Under voltage-clamp conditions, Ca^{2+} removal activated currents in the range of several microamperes. Rarely, currents in the magnitude of only several hundred nanoamperes were observed (exemplarily demonstrated in Fig. 5). This variability between oocytes of different donors is probably due to variations in the individual degree of channel expression rather than the size of the oocytes. Current-voltage relationships in earlier experiments revealed a reversal potential around −10 mV, arguing for a Cl^- current according to the Nernst equation (intracellular [Cl^-]: 54 mM (Lotan et al., 1982)). Activation of CaIC upon Ca^{2+} removal was obtained independently of the presence of extracellular permeable cations. In this series of experiments the only extracellular cation was the impermeable tetramethylammonium (TMA^+). Therefore, we could be sure that the observed current was due to efflux of Cl^- as also demonstrated elsewhere (Weber et al., 1995; Weber et al., 1995; Reifarth et al., 1997).

A series of measurements investigating the influence of extracellular pH, regulation by intracellular messengers and the blocking capacity of the ion channel blockers NPPB, amiloride, benzamil, and several metal ions were accomplished. If not stated otherwise, the substances were applied after reaching a stable maximal outwardly directed Cl^- current under Ca^{2+}-free conditions. Additionally, in a series of experiments simultaneous measurements of membrane current (I_m), conductance (G_m) and capacitance (C_m) were performed. Continuous C_m recordings allowed us to monitor changes in cell surface area evoked by exocytosis or endocytosis.

Block of the CaIC by Cu^{2+}, Zn^{2+} and Gd^{3+}

Conventional two-electrode voltage-clamp techniques were used to study the effects of Cu^{2+}, Zn^{2+} and Gd^{3+} on steady-state CaIC-mediated currents at a holding potential of −60 mV. Both heavy metals and the lanthanide showed maximal inhibitory effects on CaIC currents at concentrations of 1 mM (Fig. 1*A* and *B*) and 0.5 mM (Fig. 1*C*), respectively. The inhibition occurred immediately on application and was fully reversible in all cases. Plotting the inhibition of CaIC as a function of the respective blocker concentrations yielded typical Michaelis-Menten kinetics with half-maximal blocker concentrations ($K_{1/2}$) of 8.4 ± 2 μM for Zn^{2+}, 123.6 ± 37.6 μM for Cu^{2+}, and 21.6 ± 5.5 μM for Gd^{3+}. Dose-response curves for the three blockers are shown in Fig. 2.

Inhibition of the CaIC by NPPB

NPPB is known as a Cl^- channel blocker (Greger, 1990). Dose-response relationships were measured superfusing buffer solutions with increasing concentrations NPPB. Blocker concentrations ranged from 0.7 to 200 μM. Michaelis-Menten kinetics revealed a half-maximal blocker concentration of 2.9 ± 0.5 μM (Fig. 3*A; n* = 5, *N* = 3). The quite low $K_{1/2}$ shows that NPPB is the blocker with the highest affinity for the CaIC so far known (*see also* Table 1). Complete block of CaIC was achieved with 200 μM NPPB. A representative current trace is shown in Fig. 3*B.* After the highest NPPB concentration subsequent washout of the blocker did not restore the initial

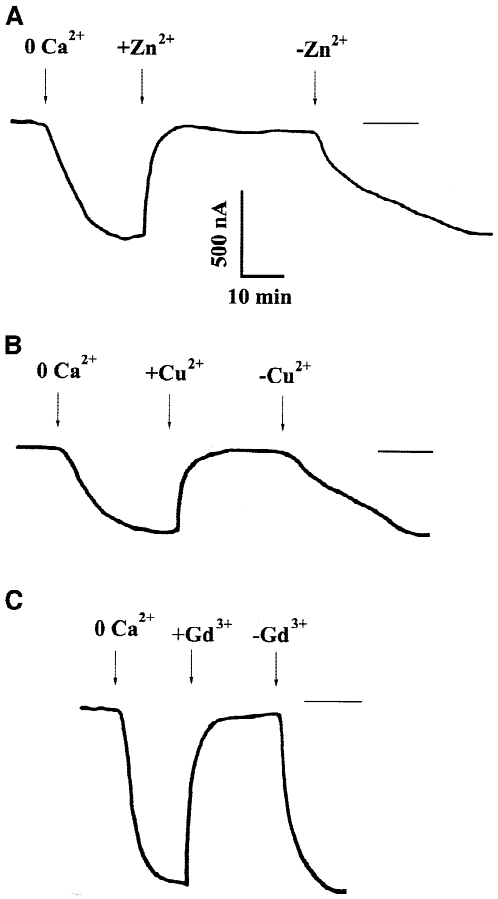

Fig. 1. Inhibition of the CaIC by Cu^{2+}, Zn^{2+} and Gd^{3+}. Removal of extracellular Ca^{2+} immediately activated large CaIC currents in voltage-clamped oocytes. Subsequent addition of the respective ion under Ca^{2+}-free conditions inhibited CaIC-mediated currents. Washout of the respective ion restored nearly the initial values. Shown are typical current traces representative of 6 oocytes from 5 different donors. Horizontal bars indicate zero current level. The concentration of the divalent ions was 1 mM for Zn^{2+} (*A*), 1 mM for Cu^{2+} (*B*), and 500 μM for Gd^{3+} (*C*).

clamp current, demonstrating partial irreversibility of NPPB or slight damage of the oocyte membrane.

Effects of Amiloride and Benzamil

The potassium-sparing diuretic amiloride and its high-affinity analogue are used to block epithelial Na^+ channels and nonselective cation channels (Kleyman & Cragoe, 1988). Zhang et al. (1998) recently reported block of nonselective cation channels activated by Ca^{2+} removal in *Xenopus* oocytes. In an attempt to compare the results of Zhang et al. (1998) with our data we investigated the effects of amiloride and benzamil on CaIC-mediated currents. Surprisingly, amiloride (500 μM) blocked I_m and G_m after CaIC activation by 88.2 ± 4.2% and 86.5 ± 4.4%, respectively (Fig. 3; $n = 8$, $N = 2$). Lower concentrations of amiloride (i.e., 10 and 100 μM) had no inhibiting effects on CaIC current. However, the high affinity analogue benzamil (100 μM) had no detectable effect on CaIC currents, indicating the lack of inhibitory potency (Fig. 4).

pH-dependence of the CaIC

The effect of external pH on maximal CaIC currents was investigated. In the acidic pH-range, an influence on CaIC-mediated currents could be detected: pH values more acidic than 7.1 showed dose-dependent inhibition of CaIC-mediated currents. This set of measurements was stopped at pH 6.5 to prevent artifacts evoked by influences of more acidic pH on the chelation capacity of BAPTA (Fig. 5). In the pH range between 7.1 and 8.0 external H^+ concentration had no significant influence on CaIC currents.

Effects of Intracellular Messengers

To assess possible regulatory pathways we investigated the influence of protein kinases on CaIC, namely protein kinase A (PKA) and C (PKC). For this purpose oocytes were either incubated or injected with PKA- or PKC-inhibiting compounds prior to the experiments. To investigate the influence of PKA, oocytes were injected with the potent PKA inhibitor PKA-622-amide (2.5 nM final intra-oocyte concentration). Subsequent measurements of CaIC-mediated current showed no significant alterations in current amplitudes. For the investigation of effects on CaIC evoked by PKC, oocytes were incubated for 12 hr in oocyte Ringer containing the membrane permeant PKC blocker staurosporine (1 μM). Under Ca^{2+}-free conditions, significant reduction of CaIC current was detected (staurosporine: 167 ± 75 nA, $n = 6$, $N = 2$; control: 443 ± 70 nA, $n = 6$, $N = 2$), indicating an influence of PKC on channel regulation.

Capacitance Measurements

Activation of ion channels can be achieved by increasing the single channel conductance, the open probability and/or delivery of former silent channel proteins to the plasma membrane and functional insertion into it (Bradbury & Bridges, 1994; Wright et al., 1997). The latter mechanism involves exocytotic delivery of new membrane material to the plasma membrane, causing expansion of membrane surface area measurable as increasing membrane capacitance. Simultaneous measurements of I_m, G_m and C_m after Ca^{2+} removal showed that C_m re-

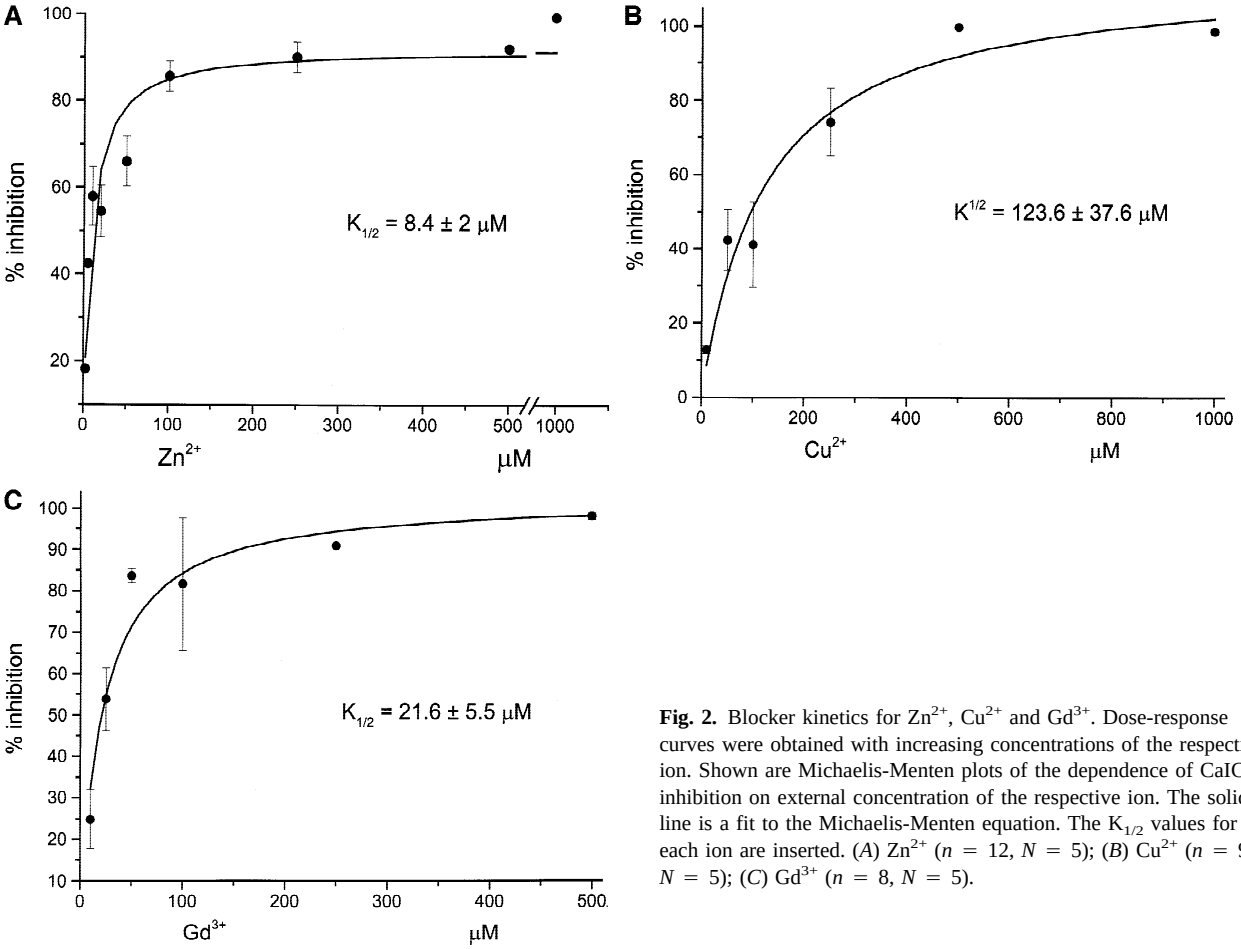

Fig. 2. Blocker kinetics for Zn^{2+}, Cu^{2+} and Gd^{3+}. Dose-response curves were obtained with increasing concentrations of the respective ion. Shown are Michaelis-Menten plots of the dependence of CaIC inhibition on external concentration of the respective ion. The solid line is a fit to the Michaelis-Menten equation. The $K_{1/2}$ values for each ion are inserted. (*A*) Zn^{2+} ($n = 12$, $N = 5$); (*B*) Cu^{2+} ($n = 9$, $N = 5$); (*C*) Gd^{3+} ($n = 8$, $N = 5$).

mained constant while I_m and G_m increased drastically (Fig. 5). These results demonstrate that CaIC activation occurs probably via other mechanism(s) than functional insertion of newly delivered channels into the plasma membrane.

Discussion

Perfusion of epithelia with Ca^{2+}-free solutions results in disintegration and subsequent irreversible damage of the tissues. Until recently it was believed that removal of extracellular Ca^{2+} could cause "leak currents" in *Xenopus laevis* oocytes (Raditsch & Witzemann, 1994). Common opinion was also that oocytes could not survive more than some minutes in Ca^{2+}-free solutions that were thought to induce irrevocable leakages in the oocyte membrane. However, in 1995 two groups independently showed that withdrawal of extracellular Ca^{2+} activates ion channels that are silent in the presence of Ca^{2+} (Weber et al., 1995; Arellano et al., 1995). These reports were confirmed and extended in the following years (Ebihara, 1996; Reifarth et al., 1997; Zhang et al., 1998). Although the authors of these reports disagreed about the ionic nature of the phenomena evoked by removal of extracellular Ca^{2+} (*see below*), they agreed on the reversibility of the observed effects. In the following we want to further characterize endogenous Cl^- channels (CaIC) of the oocyte that are inactivated by micromolar concentrations of extracellular Ca^{2+}. In a second part of the discussion we will compile the data that are available on Ca^{2+}-inactivated ion channels. Since there is an ongoing controversy about the ionic identity of the Ca^{2+}-inactivated ion channels we will compare the data and discuss possible explanations for the obvious discrepancies.

The Inhibition Profile of the CaIC

To further characterize CaIC and to complete the list of CaIC blockers we investigated the effects of some substances previously described as Cl^- channel blockers. NPPB, a known inhibitor of Cl channels, reportedly in-

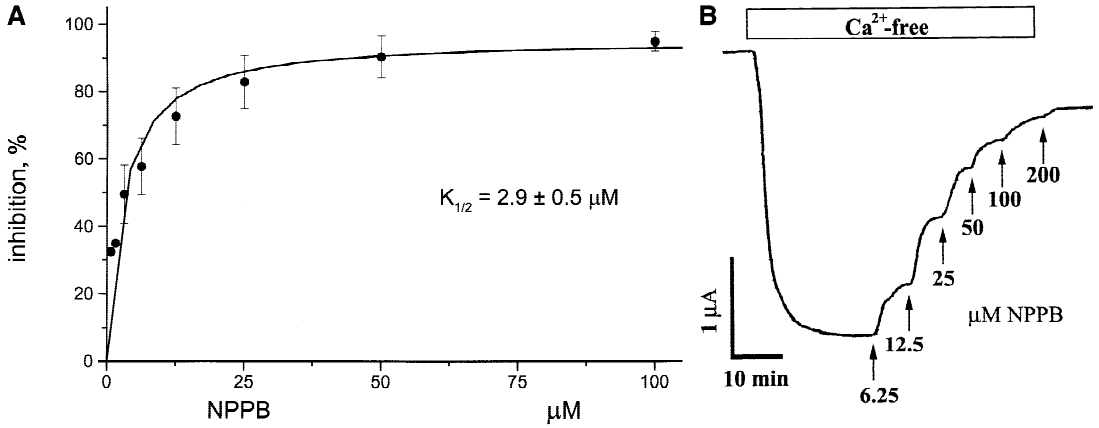

Fig. 3. Dose-dependent inhibition of the CaIC by NPPB. CaIC were activated by withdrawal of extracellular Ca^{2+}. (*A*) Dose-response curve for the inhibition of CaIC by NPPB. $K_{1/2}$ is inserted. Data are from 5 oocytes derived from 3 donors. (*B*) Increasing concentrations of NPPB led to successive inhibition of CaIC. Shown is a typical experiment out of a pool of 5 oocytes ($N = 3$).

hibits endogenous Ca^{2+}-activated Cl^- channels in the oocytes (Wu & Hamill, 1992; Quamme, 1997). NPPB inhibited the current activated by removal of extracellular Ca^{2+} in a concentration-dependent fashion and with high affinity. This finding further supports the notion that the Ca^{2+}-inactivated current is carried by Cl^-. However, NPPB showed the tendency to impair oocytes when applied for longer periods. The inhibition of the CaIC by NPPB possibly indicates structural conformities with other chloride channels. Recently, the exploration of a hypotonicity-activated Cl^- conductance endogenous to the oocyte membrane has been reported (Ackerman, Wickman & Clapham, 1994). This conductance shares some characteristics with the CaIC. One of these characteristics is an affinity constant for NPPB in the same range (i.e., 10 μM).

The trivalent cation Gd^{3+} inhibited Ca^{2+}-activated Cl^- channels of *Xenopus* oocytes in an almost irreversible manner (Tokimasa & North, 1996). Gd^{3+} was also effective in blocking CaIC-mediated currents, yet in a fully reversible way. Since Gd^{3+} is also known as an inhibitor of cation channels i.e., nonselective and stretch-activated cation channels (Zhang et al., 1998; Yang & Sachs, 1989), it might not be well suited for the discrimination between Ca^{2+}-inactivated ion channels. As reported from *Xenopus* kidney cells (Li et al., 1998), it could be possible that Gd^{3+} is able to enter the oocyte thereby evoking several cellular responses. From our current experiments we cannot exclude this possibility, yet the fast reversibility of the Gd^{3+}-mediated effects argue against a possible entrance of Gd^{3+} into the cells.

Zn^{2+} was reported to have no blocking effect on

Table 1. Inhibition profile of the CaIC

Compound	Inhibition	$K_{1/2}$ (μM)	Maximum inhibition (μM)	Reversibility	Reference
NPPB	+	3	Complete (200)	Partly	This paper
Flufenamic acid	+	6	Complete (150)	Complete	(Weber et al., 1995)
Niflumic acid	+	22	80% (400)	Partly	(Weber et al., 1995)
DPC	+	74	90% (400)	Complete	(Weber et al., 1995)
DIDS, SITS	−		Activation	Complete	(Weber et al., 1995)
AZT	+	100	60% (200)	Complete	(Reifarth et al., 1997)
9–AC	+	125	80% (500)	Complete	(Reifarth et al., 1997)
Amiloride	+	n.a.	90% (500)	Complete	This paper
Benzamil	−		No inhibition	Complete	This paper
Mg^{2+}	+	n.a.	Complete (1000)	Complete	(Weber et al., 1995)
Ba^{2+}	+	n.a.	Complete (1000)	Complete	(Weber et al., 1995)
Cu^{2+}	+	120	Complete (500)	Complete	This paper
Zn^{2+}	+	8	Complete (1000)	Complete	This paper
Gd^{3+}	+	20	Complete (500)	Complete	This paper

Abbreviations: +, observed; −, not observed; 9-AC, anthracene-9-carboxylic acid; AZT, 3′azido-3′deoxythymidine; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPC, diphenylamine-2-carboxylate; $K_{1/2}$, half-maximal blocker concentration; n.a., data not available; NPPB, 5-nitrophenyl-aminopropyl-benzoic acid; SITS, 4-4′-acetoamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid

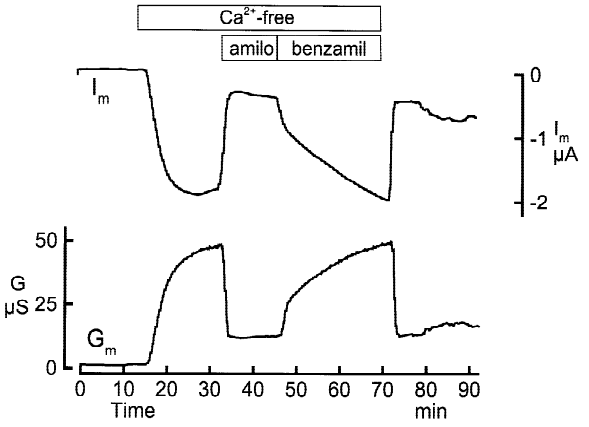

Fig. 4. Effects of amiloride and benzamil. Large increases in membrane current (I_m, upper trace; inward currents are conventionally displayed as downward deflections) and conductance (G_m, lower trace) were induced by removal of extracellular Ca^{2+}. Amiloride (500 µM) inhibited the major portion of the CaIC-mediated current reflected by decreasing values for I_m and G_m. Replacing of amiloride by benzamil (100 µM) abolished the CaIC inhibition ($n = 8$, $N = 2$).

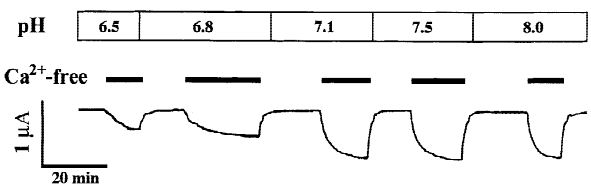

Fig. 5. pH-dependence of the CaIC. Maximum CaIC currents were measured as a function of extracellular pH. With decreasing H^+ concentrations beginning at pH = 6.5 the CaIC-mediated current increased, demonstrating inhibitory potency of H^+. pH values above 7.1 did not change CaIC current significantly. The example given is representative of 7 oocytes from 3 donors.

Ca^{2+}-activated Cl^- channels in oocytes (Tokimasa & North, 1996). However, as with other divalent and trivalent cations, CaIC-mediated current was nearly totally blocked by Zn^{2+} with quite high affinity ($K_{1/2} \approx 8$ µM). This differential inhibitory behavior of Zn^{2+} allows further discrimination between Ca^{2+}-activated and -inactivated Cl^- channels.

Copper is a trace element essential for life whose deficiency or excess cause severe pathological consequences. In our experiments Cu^{2+} in micromolar concentrations also proved to be a potent and fully reversible blocker of CaIC current. Yet, the affinity of Cu^{2+} with a $K_{1/2}$ of about 120 µM was considerably lower than that observed for Zn^{2+}.

It has been reported recently that Cu^{2+} competes with amiloride, a highly specific positive charged blocker of epithelial Na^+ channels (ENaC), for the same negative charged binding site on the Na^+ channel (Flonta et al., 1998). Therefore, and since Zhang et al. (1998) reported inhibition of Ca^{2+}-inactivated ion channels in

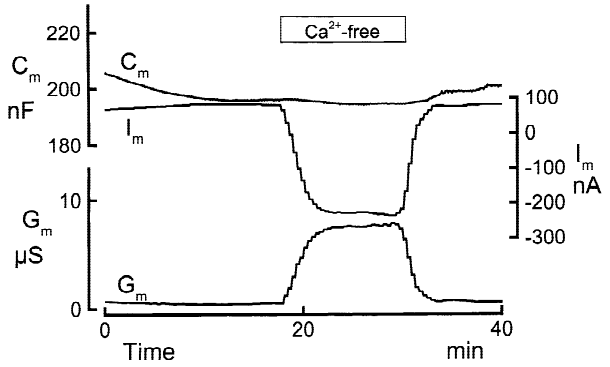

Fig. 6. Effects of Ca^{2+} removal on I_m, G_m and C_m. After an initial stabilization phase membrane current (I_m, middle trace), conductance (G_m, lower trace), and capacitance (C_m, upper trace) remained stable. Removal of extracellular Ca^{2+} instantly activated CaIC reflected by steep increases in I_m and G_m. Only C_m showed no change, indicating the lack of exocytotic translocation of membrane material into the plasma membrane. Please note also that this experiment is one of the rare cases with comparatively small CaIC current. The traces are representative of 10 oocytes from 3 toads.

Xenopus oocytes by amiloride, we further tested the effects of the diuretic on CaIC. We found that a high amiloride concentration (500 µM) inhibited large parts but not all of the current mediated by CaIC while lower concentrations of the diuretic (i.e. 10 and 100 µM) had no effects on CaIC currents. However, the amiloride analogue benzamil with even far higher affinity for the epithelial Na^+ channel (Kleyman & Cragoe, 1988) failed to inhibit CaIC current. Since benzamil is uncharged in the pH range that we used, the lack of inhibitory potency supports the hypothesis that positively charged compounds could compete with Ca^{2+} for a putative negatively charged binding site on the CaIC. Another possible hint for this theory is the observed decline of the CaIC-mediated current with increasing $[H^+]_o$, although we cannot rule out that changing the extracellular pH could have influences on the intracellular pH which could be responsible for the observed effects. As the oocytes possess an effective regulation machinery to keep the intracellular pH sufficiently constant (Amasheh et al., 1997), this explanation probably can be excluded.

The competition for a possible negatively charged binding site on CaIC could be a common feature of some but not all blockers that we investigated and which are summarized in Table 1. However, due to the lack of knowledge on the primary sequence of CaIC, a blocking mechanism by unspecific interaction of the metal ions with membrane domains cannot be ruled out.

The Ca^{2+}-inactivated Cl^- channel of the *Xenopus laevis* oocyte shows a unique inhibition and activation profile. We can now present a state-of-the-art list of applicable blockers of the CaIC (Table 1). The experimenter who wants to keep the CaIC from being active during expression experiments or investigations of en-

dogenous ion channels can be guided by the list of these substances. The Cl^- channel blocker NPPB had the strongest inhibitory effect on the CaIC. Despite this fact we recommend flufenamic acid (50–80 μM) to be employed. This substance showed complete inhibition (i.e. > 95% (Weber et al., 1995)), but furthermore did not cause any detectable damage to the oocyte membrane, as often observed with NPPB.

Regulation by Intracellular Messengers

The results shown in this paper indicate an important role of protein kinase C for the regulation of the CaIC, while PKA seems not to be directly involved in positive regulation of the channel. These data confirm former results concerning the involvement of PKC in possible regulation mechanisms: activation of the CaIC by the PKC-activator phorbol-12-myristate-13-acetate, 4-O-methyl (PMA) has been shown recently (Weber et al., 1995). Inhibition of PKC in turn leads to immediate downregulation of the CaIC-mediated current. It has been shown that cAMP is able to activate the CaIC (Weber et al., 1995). Therefore, a possible intracellular regulation mechanism could be a synergistic effect of cAMP and/or PKC-dependent signal transduction cascades, leading to phosphorylation and subsequent activation of Cl^- channels following removal of extracellular Ca^{2+}. However, as discussed in more detail below, PKC-mediated exocytotic delivery and insertion of new channels into the plasma membrane can be definitively excluded. Our results furthermore indicate that only PKC as a minimum crucial factor is responsible for channel downregulation.

Capacitance Measurements

Regulation of channel activity can be achieved via different pathways. An example for distinct pathways leading to activation of a channel is the cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR). CFTR already present in the plasma membrane but silent under unstimulated conditions, respond immediately upon cAMP-induced, PKA-independent phosphorylation with large increases in conductance. The second pathway involves the activity of cAMP-dependent PKA that induces, under availability of intracellular Ca^{2+}, exocytotic delivery of membrane material containing functional CFTR to the plasma membrane thereby increasing the cell surface area (Weber, Clauss & Van Driessche, 1999). For the CaIC only the first pathway seems to exist. Simultaneous measurements of membrane current (I_m), conductance (G_m) and capacitance (C_m) revealed that removal of extracellular Ca^{2+} induced large increases in I_m and G_m, but not in C_m. The capacitance of the membrane reflects accurately the cell surface area (Vasilets et al., 1990; Zampighi et al., 1995). Since the surface area of the oocyte remains stable upon Ca^{2+} withdrawal no exocytotic delivery of new membrane material containing additional CaIC channels seems to occur. All Cl^- channels that are activated by Ca^{2+} removal are already present in the plasma membrane. The constant presence of CaIC proteins in the oocyte, as well as after maturation in the egg membrane, argues for important functions of the channel protein and the necessity of the possibility of a fast and immediate channel regulation by Ca^{2+}. However, the functions of the CaIC for the physiology of the oocytes remain to be elucidated.

Comparison with other Ca^{2+}-inactivated Ion Channels

Since the first report on Ca^{2+}-inactivated Cl^- channels of *Xenopus laevis* oocytes early in 1995 (Weber et al., 1995) several other publications on Ca^{2+}-inactivated ion channels followed, listed here chronologically (Arellano et al., 1995; Weber et al., 1995; Ebihara, 1996; Reifarth et al., 1997; Zhang et al., 1998). While we identified the responsible mechanism as a Ca^{2+}-inactivated Cl^- channel, Ebihara (1996) attributed the Ca^{2+}-effect to a hemi-gap-junctional channel. Two other groups (Arellano et al., 1995; Zhang et al., 1998) independently came to the conclusion that Ca^{2+} removal activates nonselective cation channels in the oocyte membrane. How can these apparent contradictory findings be explained?

Firstly, the channel described by Ebihara (1996) seems to be completely different from the channels described by the three other groups. This channel population needs depolarization to be activated and produces only small currents in the range of 100 nA. Moreover, the single channel conductance of the hemi-gap-junctional channel is an order of magnitude lower than that reported by Weber et al. (1995) for the CaIC (i.e., 1.5 pS and 90 pS, respectively). However, the fact that the channel is active in presence of Mg^{2+} and even 200 μM Ca^{2+} clearly shows that there exist two channel populations that can be easily distinguished. We therefore restrict ourselves in the following on the comparison of the remaining three groups. The available data on the Ca^{2+}-inactivated ion channels are compiled in Table 2.

The most obvious difference in the above-mentioned investigations is the composition of the Ca^{2+}-free solutions that were used. While we always chelated Ca^{2+} with EDTA, EGTA or BAPTA (each 5 mM), both of the other groups used only virtually Ca^{2+}-free solutions, i.e., without Ca^{2+} chelator. In contrast to a previous report (Zhang et al., 1998), we found that CaIC activation is enhanced in Ca^{2+}-free solutions containing additionally Ca^{2+} chelators. Another difference in the experimental design could be seen in the membrane potential to which the oocyte membrane was clamped. Arellano et al. (1995) reported prevailing activation with depolarized

Table 2. Compilation of available data on Ca^{2+}-inactivated ion channels in native *Xenopus laevis* oocytes

Channel property	Reference		
	(Arellano et al., 1995)	(Weber et al., 1995) (Weber et al., 1995; Reifarth et al., 1997)	(Zhang et al., 1998)
Ca^{2+}-dependence at V_m = −60 mV, $K_{1/2}$, μM	~60	20–30	n.a.
V_m-dependence of $K_{1/2}$ for Ca^{2+}, μM	61 at V_m = −60 mV; 212 at V_m = 0 mV	−	n.a.
Selectivity	$Na^+ = K^+ >> Cl^-$	$I^- > Br^- > Cl^- >> Na^+ = K^+$	$Na^+ = K^+ >> Cl^-$
Permeability to large organic cations and anions	n.a.	n.a.	+
Transport direction at physiological V_m	Cation absorption	Cl^- secretion	Cation absorption
Inhibition by Cl-channel blockers	n.a.	+ (FFA, NFA, DPC, NPPB, AZT 9-AC, SITS)	+ (FFA, NFA) −(9-AC, SITS)
Activation by DIDS	n.a.	+	n.a.
Sensitivity to cation channel blockers	n.a.	+ (Amiloride) − (Benzamil)	+ (Amiloride)
Activity in absence of extracellular permeant cations	n.a.	+	n.a.
Sensitivity to Zn^{2+}, Cu^{2+}	n.a.	+ (This paper)	n.a.
Sensitivity to Gd^{3+}	+	+ (This paper)	+
Reversal potential, mV	−8.2 ± 3.8	−12.2 ± 0.8	−9.5 ± 3
Conductance, pS	n.a.	90	n.a.
Activity in eggs	−	+	−
Activation by cyclic nucleotides	n.a.	+ (cAMP, cGMP)	n.a.
Regulation by connexin	n.a.	n.a.	+
Methods applied	Voltage clamp	Voltage-clamp, patch-clamp, optical Cl^-efflux measurements, capacitance measurements (this paper)	Voltage- clamp

Abbreviations: +, observed; −, not observed; 9-AC, anthracene-9-carboxylic acid; AZT, 3′azido-3′deoxythymidine; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPC, diphenylamine-2-carboxylate; $K_{1/2}$, half-maximal blocker concentration; n.a., data not available; NPPB, 5-nitrophenyl-aminopropyl-benzoic acid; SITS, 4-4′-acetoamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid

membranes (i.e., membrane potential ≤−10 mV). Under these conditions the driving force for anions is reduced. Around the physiological membrane potential (i.e., between −50 and −70 mV, *see also* (Weber, 1999*b*)) the CaIC is independent of the membrane voltage.

By comparing all the available data, compiled in Table 2, two possible explanations for the phenomena caused by removal of extracellular Ca^{2+} seem to be possible. At first, one population of poorly selective ion channels could exist in oocytes that are able to operate in different modes according to the ionic environment. Depending on the ionic composition of the surrounding medium the channel responds to Ca^{2+} removal with cation influx or anion efflux. This capability could make sense since the oocytes are seriously challenged during development by changing ionic compositions of the environment after being spawned into hypo-osmotic pond water. However, the broad spectrum of ion channel inhibitors that are effective in blocking the Ca^{2+}-inactivated ion channels argue against the one-channel-different-modes option.

In our opinion, a better explanation for all the discrepanies described by the several groups is the existence of at least two different channel populations that are inactivated by extracellular Ca^{2+}. In this model more or less specific ion channels could change their selectivity according to the environmental demands. The relatively low ion specificity reported from single channel analysis by Weber et al. (1995) and from whole cell studies by Arellano et al. (1995) argue for this kind of explanation. Further, it could be possible that these channels use a putative common extracellular Ca^{2+}-sensing receptor as described from other cells (Chattopadhyay, Mithal & Brown, 1996). Another possibility is that the Ca^{2+}-inactivated ion channels use extracellular Ca^{2+} and Cl^- as regulative cofactors and that the respective channel population becomes active upon the availability of these ions. During development oocytes or eggs, respectively, are exposed to varying ionic environments causing severe changes in membrane voltage. Depending on the membrane voltage the respective Ca^{2+}-sensitive channel population could be active: at more negative voltages the CaIC and at more positive voltages the nonselective cation channel(s). As summarized in Tables 1 and 2, Ca^{2+}-inactivated ion channels are sensitive to a wide variety of ion channel blocking mechanisms. This is another po-

tential argument in favor of the existence of at least two different ion channel populations. As shown in this paper and also reported by Zhang et al. (1998) typical blockers of nonselective cation channels as well as Cl^- channel blockers were found to inhibit Ca^{2+}-inactivated ion channels of the oocytes, further arguing for the parallel existence of Ca^{2+}-sensitive cation and anion channels.

However, from the current state of the art, it is impossible to dispel all discrepancies that have come up in the last years on Ca^{2+}-inactivated ion channels of *Xenopus laevis* oocytes. However, since at least the CaIC is not downregulated during maturation and can still be found actively in eggs, it should be clear that there is a physiological role for the channel in the developmental fate of the oocyte.

General Conclusions

In the last two decades, a large number of different Cl^- channels have been discovered. For many of them, structure and function were investigated. Recently, the different Cl^- channel families known to date have been thoroughly reviewed (Pusch & Jentsch, 1994). The five families classified up to date are (i) the voltage-gated C1C channel family, (ii) ligand-gated Cl^- channels including the GABA and glycine receptor, (iii) cAMP-dependent Cl^- channels (e.g., CFTR), (iv) volume-activated Cl^- channels, and (v) Ca^{2+}-activated Cl^- channels. However, the Ca^{2+}-inactivated Cl^- channel does not fit without further ado into this classification. Furthermore, without the knowledge about the structural features of the CaIC it is impossible to integrate this channel into a family of known Cl^- channels.

The breakthrough in research of this channel could be the cloning and sequence analysis of the protein to compare it with the structure of other cloned transport systems. The fact that the CaIC was detected in every oocyte that we tested up to now, and the fact that it is not inactivated during maturation shows a high potency of the channel to be essential for development up to the involvement in physiological processes in early embryonic stages.

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