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The Ca^{2+}-induced Leak Current in *Xenopus* Oocytes is Indeed Mediated through a Cl^- Channel

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Abstract. Defolliculated oocytes of *Xenopus laevis* responded to removal of external divalent cations with large depolarizations and, when voltage clamped, with huge currents. Single channel analysis revealed a Cl^- channel with a slope conductance of about 90 pS at positive membrane potentials with at least four substates. Single channel amplitudes and mean channel currents had a reversal potential of approximately −15 mV as predicted by the Nernst equation for a channel perfectly selective for Cl^-. Readdition of Ca^{2+} immediately inactivated the channel and restored the former membrane potential or clamp current. The inward currents were mediated by a Ca^{2+} inactivated Cl^- channel (CaIC). The inhibitory potency of Ca^{2+} was a function of the external Ca^{2+} concentration with a half maximal blocker concentration of about 20 μM.

These channels were inhibited by the Cl^- channel blockers flufenamic acid, niflumic acid and diphenylamine-2-carboxylate (DPC). In contrast, 4,4′-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonicacid (SITS), another Cl^- channel blocker, led to activation of this Cl^- channel. Like other Cl^- channels, the CaIC was activated by cytosolic cAMP. Extracellular ATP inhibited the channel while ADP was without any effect. Injection of phorbol 12-myristate 13-acetate (PMA), a protein kinase C activating phorbol ester, stimulated the Cl^- current. Cytochalasin D, an actin filament disrupting compound, reversibly decreased the clamp current demonstrating an influence of the cytoskeleton.

The results indicate that removal of divalent cations activates Cl^- channels in *Xenopus* oocytes which share several features with Cl^- channels of the CLC family. The former so-called leak current of oocytes under divalent cation-free conditions is nothing else than an activation of Cl^- channels.

Key words: *Xenopus* oocytes — Cl^- channel — Divalent cations — Leak current

Introduction

Several cell systems have been developed for the molecular expression of chloride channels, the most favored of which is based on injection of foreign RNA into oocytes of the South African clawed toad *Xenopus laevis* (Jentsch, 1994, Pusch & Jentsch, 1994). This method provides both a decisive proof of successful RNA preparation, and a useful tool for studying function and regulation of Cl^- channels. Two properties make this expression system particularly attractive. First, oocytes promiscuously translate and process injected foreign RNA and insert the functional channels in their plasma membrane (Sigel, 1990). Second, oocytes are extremely suitable for the study of membrane transport. Their large size makes it possible to apply various electrophysiological and biochemical techniques to one single cell. However, the oocytes possess a lot of endogenous transport systems such as ion pumps (Lafaire & Schwarz, 1986), cotransport systems for a wide variety of substrates (Richter & Schwarz, 1991), and ion channels (Dascal, 1987). Besides K^+ channels (Dascal, Lotan & Lass, 1987; Parker & Ivorra, 1990), Ca^{2+} channels (Dascal, 1987), mechanosensitive cation channels (Lane, McBride & Hamill, 1991), and more or less selective cation channels (Burckhardt & Frömter, 1992), they exhibit different anion channels most of them being Cl^- channels (Dascal, 1987). The best described Cl^- channels in the oocyte membrane are the Ca^{2+} activated Cl^- channels which transport Cl^- from the external side to the cyto-

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plasm of the oocyte (Miledi & Parker, 1984). Increase of intracellular Ca^{2+} concentration leads to activation of this class of Cl^- channels thereby hyperpolarizing the oocyte membrane. They are more or less specifically inhibited by different Cl^- channel blockers such as flufenamic acid (FFA), niflumic acid (NFA), diphenylamine-2-carboxylate (DPC), 4,4′-diisothiocyanatostilbene-2,2′-disulfonicacid (DIDS), and 4,4′-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonicacid (SITS) (White & Aylwin, 1990; Schwiebert, Lopes & Guggino, 1994; Greger, 1994). When considering the use of the oocytes as an expression system for foreign Cl^- channels one should be aware of the existence of those endogenous Cl^- channels.

In a previous paper (Weber et al., 1995), we already demonstrated that *Xenopus* oocytes possess an additional class of Cl^- channels which were so far unknown. These channels are inactivated by small amounts of extracellular divalent cations, such as Ca^{2+}, Mg^{2+} or Ba^{2+}, and are specifically inhibited by flufenamic acid. Using the patch clamp technique we could identify a Cl^- channel type, which is immediately inactivated when small amounts of Ca^{2+} are added to the extracellular medium.

In some earlier reports, the currents observed after removal of external Ca^{2+} were attributed to so-called leakage or leak currents (Raditsch & Witzemann, 1994). It was even speculated, that removal of divalent cations could cause irreversible damage to the oocyte membrane. In the present paper, we extend our previous findings and we give detailed proof, that the "leak current," induced by removal of external divalent cations, indeed is mediated through a channel. This channel, which we named Ca^{2+} inactivated Cl^- channel (CaIC), is inhibited by FFA, NFA, and DPC, but not by SITS and DIDS. The CaIC is regulated via a cAMP-dependent mechanism and is inhibited by extracellular ATP. The current mediated by the CaIC is further increased by an activation of the protein kinase C. Contrary to all transport systems of the oocyte hitherto described the CaIC remains active even when the oocyte undergoes maturation. Single-channel analysis showed a conductance of 90 pS at positive membrane potentials, four substrates, and a reversal potential of about −15 mV. All these data argue against a so-called leakage current and give clear evidence that removal of external Ca^{2+} activates a hitherto unexplored Cl^- channel in the plasma membrane of *Xenopus* oocytes. Part of the results has been presented at the spring meeting of the German Physiological Society and has been published in abstract form in *Pfluegers Arch* **429:**R66, 1995.

Materials and Methods

Oocytes

The following methods to obtain defolliculated oocytes were identical to those described in more detail previously (Weber, Schwarz & Passow, 1990). South African clawed toads (purchased from African Xenopus Facility, Noordhoek, South Africa) were hypothermally anaesthetized, small pieces of ovary were removed and bathed for five hours in oocyte Ringer (ORi, *see below*) containing collagenase (1 mg/ml, Serva, Heidelberg, Germany). Oocytes were washed for 10 min in Ca^{2+}-free ORi and 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 (day 1 to day 5 after removing from the ovary). 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).

Solutions

The composition of ORi was (in mM): 90 NaCl, 20 tetraethylammoniumchloride, 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). Solutions for patch clamp experiments contained N-methyl-D-glucamine (NMDGCl, 100 mM) and EGTA (1 mM).

Electrophysiology

Single 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 were determined with a voltage-clamp amplifier (OC 725B, Warner Instruments, Hamden). The voltage and the current microelectrode were filled with 1 mM and 3 mM KCl, respectively and had resistances from 1 to 5 MΩ. The bath electrode was an Ag/AgCl pellet. 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 −120 to 60 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 by Dr. W. Schwarz (Max-Planck-Institute for Biophysics, Frankfurt/Main, Germany).

Patch clamp pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) on a two-stage puller (Narishige, Greenvale). The pipette resistance was about 5 MΩ in symmetrical 100 mM NaCl solutions. Pipette perfusion was performed as described in detail earlier (Reifarth, Weiser & Bentrup, 1994) and improved by using in- and outflow tubes connected to a 10-role peristaltic pump (Gilson, Villiers, France). The stability of oocyte patches allowed perfusion pump handling in the attached mode, i.e., without need of excision.

Patch clamp measurements were accomplished on devitellinated oocytes in the attached- or inside out mode using the new developed LM-PC patch clamp amplifier (List, Darmstadt, Germany). Data acquisition and analysis were achieved with a TL-1 interface (Axon, Foster City) and a personal computer running pCLAMP version 5.5 (Axon, Foster City). Current traces from 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) 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 load fluctuations (Q = 1 t) of a patch were analyzed by integrating current traces obtained by rectangular voltage-pulse experiments. Current traces which exhibited no detectable channels, were termed as background currents due to their voltage- and ion dependence. Mean load fluctuations of CaIC currents were calculated by integrating current traces with CaIC openings minus background currents. Membrane potential (V_m) was determined 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 NMDGCI was used as a reference electrode in the bath.

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

Statistics

Results, when not stated otherwise, are expressed as means ± SEM, with n the number of oocytes and N the number of animals.

Blocker Kinetics

To describe blocker kinetics, the following equation was fitted to the data shown in Figs. 1*B* and 2*A–C*

$$k = \frac{k_{max} \cdot (S)}{K_i + (S)} \quad (1)$$

were k_{max} represents the total current in absence of Ca^{2+}, K_i is the half-maximal blocker concentration and (S) stands for the respective blocker concentration.

Results

Dependence on External Ca^{2+}

The inhibitory potency of extracellular Ca^{2+} on the CaIC mediated current is a function of the external Ca^{2+} concentration. Removing external Ca^{2+} led to a drastic increase of the clamp current. As depicted in Fig. 1*A*, subsequent increasing concentrations of external Ca^{2+} decreased the holding current step by step. 500 μM Ca^{2+} showed the maximal inhibition. Plotting the inhibition as a function of the external Ca^{2+} concentration yielded a Michaelis-Menten kinetic with a half maximal blocker concentration (K_i) of 21 ± 9 μM Ca^{2+} (Fig. 1*B*). There was no dependence on intracellular Ca^{2+} since injection of EGTA (5 mM, 50 nl per oocyte) or Ca^{2+} (5 mM, 50 nl per oocyte) into the oocytes had no visible effects on the Cl^- currents induced by removal of extracellular Ca^{2+}.

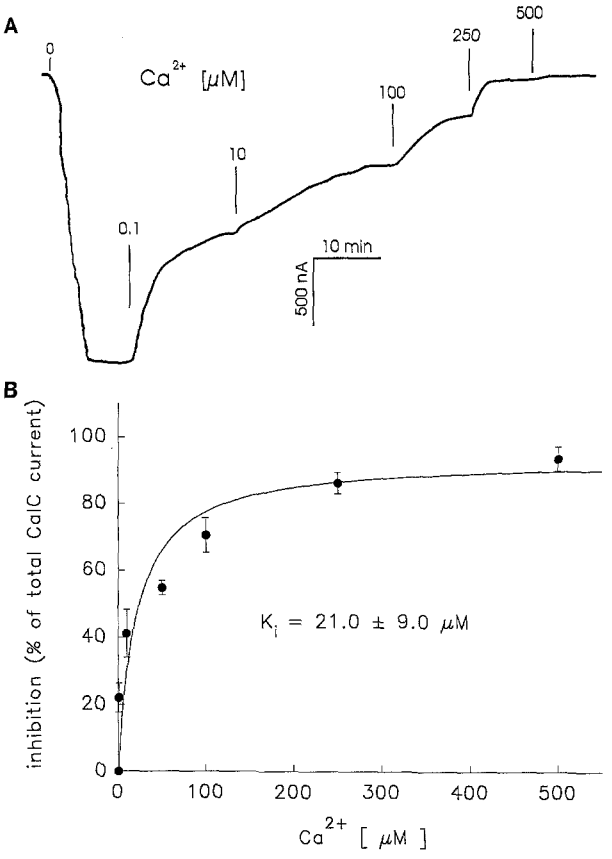

Fig. 1. Dependence of current on external Ca^{2+}. (*A*) Oocytes were clamped to –60 mV and the clamp current was recorded. Removal of Ca^{2+} led to a drastic current increase that was successively decreased by subsequently increasing external Ca^{2+} concentrations. Downward deflections indicate inward currents due to Cl^- secretion. Shown is a typical experiment representative of 8 experiments (N = 4). (*B*) Michaelis Menten plot of the dependence of inhibition of CaIC on external Ca^{2+} concentration following Eq. 1 (*see* Material and Methods). Data represent mean values ± SEM from 8 experiments (N = 4).

Blocker Kinetics

The Ca^{2+} inactivated Cl^- channel in *Xenopus* oocytes was found sensitive to several Cl^- channel blockers such as flufenamic acid, niflumic acid and DPC. However, the Cl^- channel had different affinities for the blockers. Flufenamic acid had the highest affinity indicated by a half maximal blocker concentration of 6.1 ± 0.9 μM (Fig. 2*A*, n = 9, N = 3), and with 150 μM being sufficient to block all Cl^- channels induced by removal of extracellular Ca^{2+}. Niflumic acid had a lower affinity (K_i = 22.0 ± 4.2 mM), and even blocker concentrations of 400 μM blocked only 80% of the CaIC (Fig. 2*B*, n = 9, N = 5). The lowest affinity showed DPC with a K_i of 74.0 ± 12.0 μM (Fig. 2*C*, n = 11, N = 4). All blockers were fully reversible, however, niflumic acid in higher concentrations sometimes caused oscillations of the membrane potential and even led to irreversible damage of some oocytes.

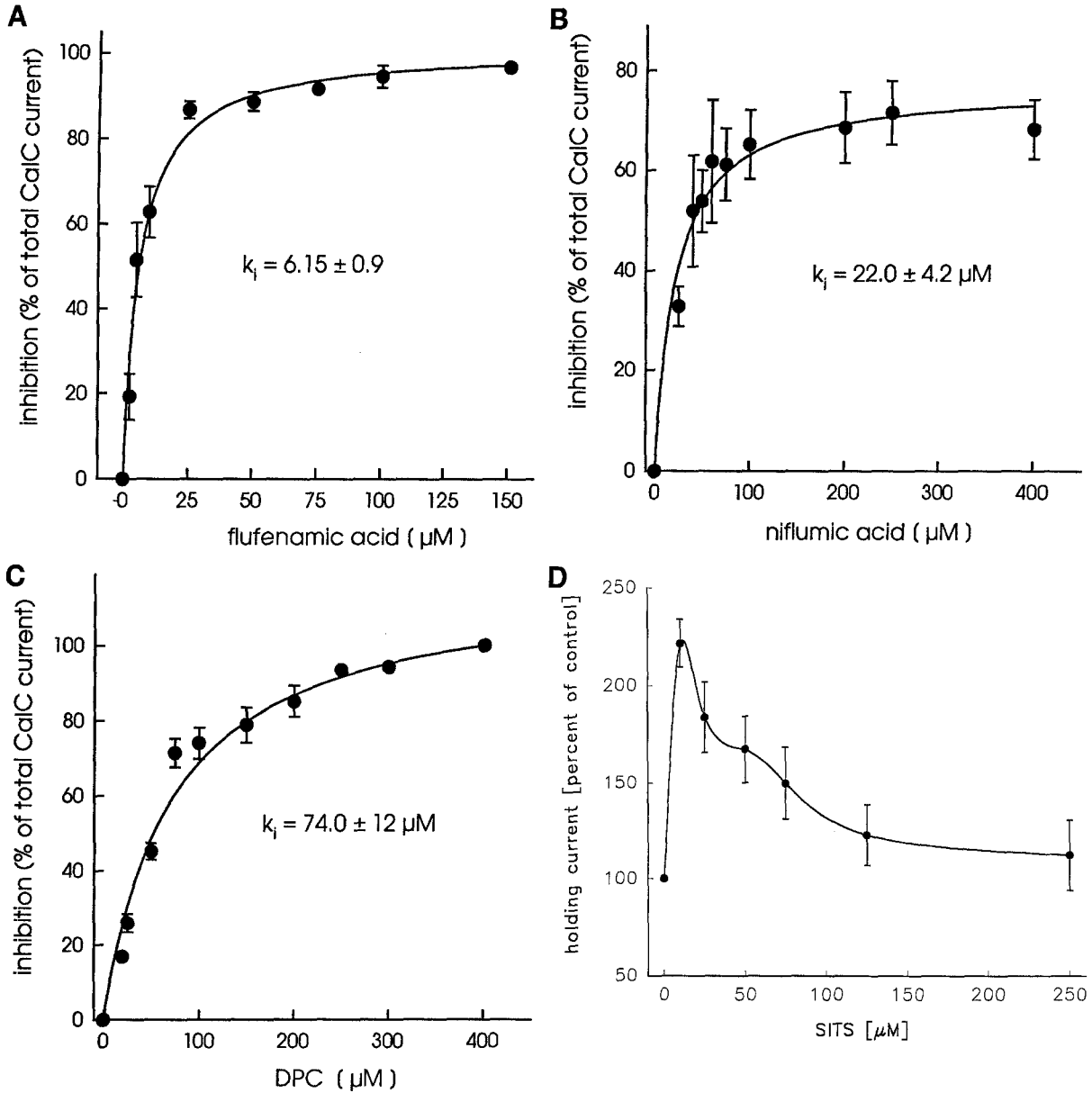

Fig. 2. Blocker kinetics for several Cl^- channel blockers. In *A* to *C* Michaelis Menten kinetics (Eq. 1) are shown: (*A*) Flufenamic acid, (*B*) Niflumic acid, (*C*) DPC. (*D*) Holding currents are normalized to percent of control in the presence of external Ca^{2+}. Immediately after addition of SITS (10 µM) the CaIC-mediated current increased. Subsequent increasing SITS concentrations reduced the SITS induced activation (means ± SEM, $n = 7$, $N = 3$).

SITS, another known Cl^- channel blocker had no inhibitory effect on the CaIC. Surprisingly, this Cl^- channel blocker in turn activated Cl^- conductances after Ca^{2+} removal. Under Ca^{2+}-free conditions the holding current in this series of experiments was −627 ± 81 nA. Application of 10 µM SITS nearly doubled the holding current to −1185 ± 69 nA (Fig. 2*D*, $n = 7$, $N = 3$). Lower concentrations of SITS had even stronger activating effects. However, we could not investigate these effects further because the currents became too high and exceeded the hardware limitations of our equipment. With increasing concentrations of SITS the activation of the current became lower as with 10 µM SITS, but all concentrations that we tested (up to 250 µM) had activating effects on the CaIC.

Effects of Cyclic Monophosphates

cAMP is known to modulate channel properties directly or via cAMP-dependent protein kinases (McCann &

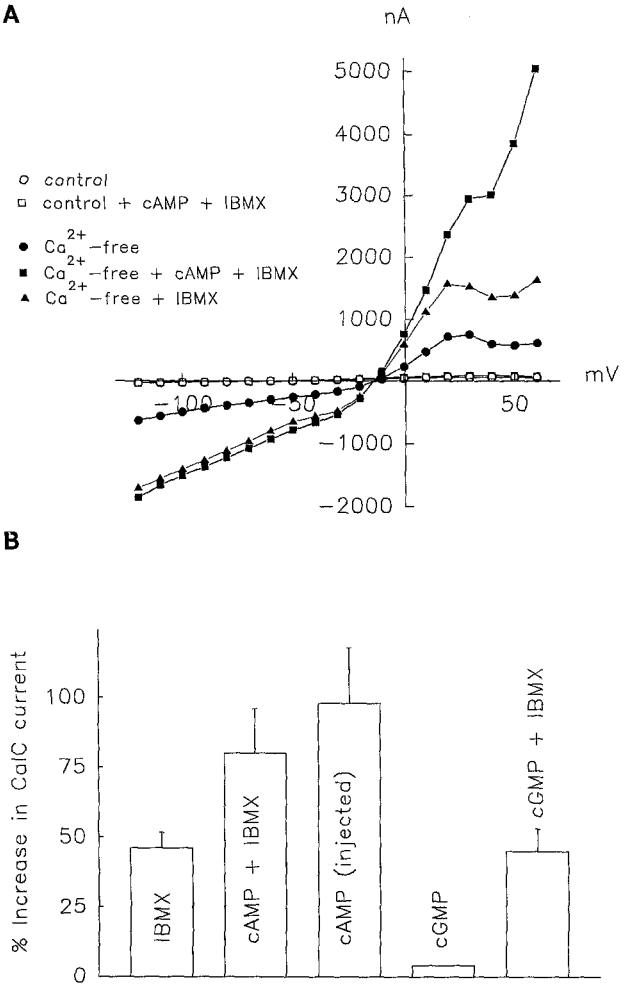

Fig. 3. Effects of cAMP, cGMP and IBMX. (*A*) *IV* curves were recorded in the absence and presence of external Ca^{2+} and with and without cAMP and cAMP + IBMX, respectively. The curves are representative of 7 experiments (N = 3). (*B*) Stimulation of CaIC by external IBMX, cAMP + IBMX and by injected cAMP. The data are normalized to the current value before the application of the respective substance. Shown are the means ± SEM from 5 to 8 oocytes (N = 3).

Welsh, 1990). We applied 100 μM 8-(4-chlorophenylthio)-cAMP, a permeable cAMP analogue, together with 1 mM IBMX, a phosphodiesterase inhibitor, to prevent fast cAMP degradation. Shortly after application of this "cocktail" under Ca^{2+}-free conditions the holding current at −60 mV increased by 80 ± 16% (n = 9, N = 4). *IV*-relationships in absence and presence of cAMP yielded the cAMP-activated Cl^- currents, which were stimulated over the entire voltage range (Fig. 3*A*).

Addition of impermeable cAMP (free acid) in concentrations up to 1 mM showed only small effects on the holding current in the absence of Ca^{2+}, indicating that the permeable cAMP analogue enrolled its activating potency mainly from the cytoplasmic side of the membrane. Injection of cAMP under voltage clamp conditions to a final internal concentration of approximately 50 to 100 μM nearly doubled the holding current (98 ± 19.8%, n = 6, N = 5). Extracellular application of IBMX alone (1 mM) in order to elevate intracellular cAMP concentration led to an increase of holding current by 47 ± 6%.

cGMP, applied as the permeable analogue N^2,2′-O-dibutyryl cGMP (100 μM) together with IBMX (1 mM) under Ca^{2+}-free conditions, led to a 45 ± 8% increase of the holding current while cGMP alone had virtually no detectable effect (n = 7, N = 5). To evaluate whether cAMP (50 μM) and cGMP (50 μM) might have cumulative effects, we applied first cAMP and then additionally cGMP. In these experiments, cAMP alone showed maximal activation, cGMP had no additional effect. Figure 3*B* summarizes the results from the different experimental protocols.

Both compounds showed no or only small effects on the holding current when they were applied in the presence of Ca^{2+}.

Effects of External Nucleotides

Paulmichl and coworkers (1992) identified a novel Cl^- channel in *Xenopus* oocytes after injection of mRNA derived from MDCK cells. This channel could be inhibited by extracellular nucleotides such as ATP and ADP. ATP (1 mM) had the same inhibitory effect on the CaIC: the current induced by Ca^{2+} removal was reduced 41 ± 5% (n = 8, N = 4). However, ADP (1 mM) showed no effect on the CaIC demonstrating that this channel is regulated in a different way than the Cl^- channel described earlier (Paulmichl et al., 1992). Both nucleotides had only minor influences on the membrane currents when applied in the presence of extracellular Ca^{2+} under voltage clamp conditions. Injection of the nonhydrolyzable GTP analogue GTP-γ-S (final concentration in the oocyte 170 μM) had no detectable effect on the CaIC (*data not shown*).

Effects of Adenylyl Cyclase Mediators

Cholera toxin, the secretory product of *Vibrio cholerae*, can persistently activate adenylyl cyclase and thereby initiate cell-specific responses involving cAMP (Sternweis & Pang, 1990). Oocytes were preincubated in the presence of cholera toxin (250 ng/ml) for 2 hr at room temperature. Preincubated oocytes responded to Ca^{2+} removal with more than doubled CaIC currents (148 ± 25%, n = 39, N = 5). In IV relationships, colera toxin induced elevated currents over the whole potential range (Fig. 4). The reversal potential of the cholera toxin induced current was nearly −10 mV as expected for a current mediated by a selective Cl^- channel.

Pertussis toxin, the secretory product of *Bordetella*

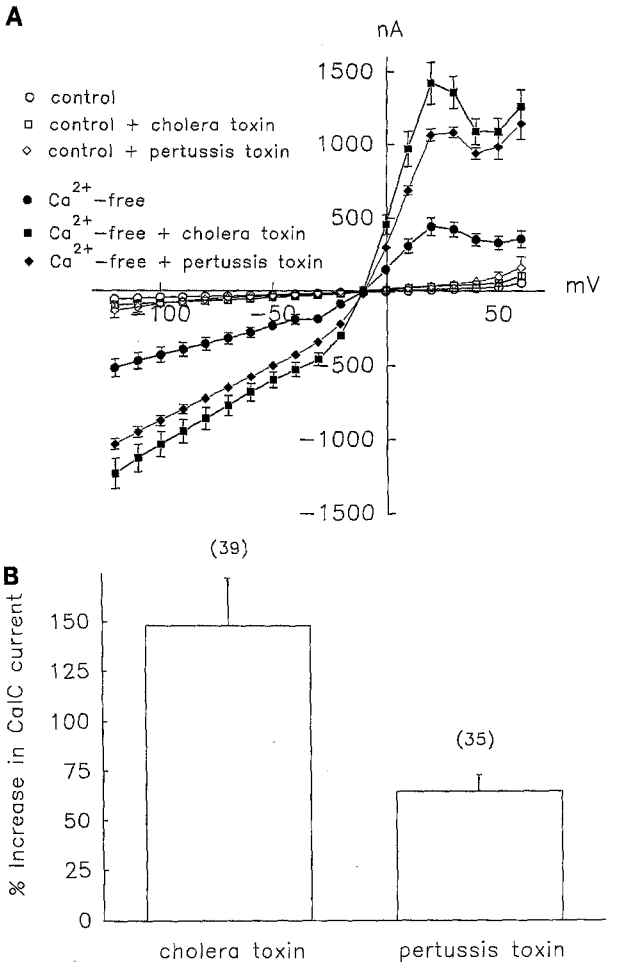

Fig. 4. Effects of cholera toxin and pertussis toxin. (*A*) *IV* curves were recorded in the absence and presence of external Ca^{2+} and with and without preincubation with cholera toxin (250 ng/ml) or pertussis toxin (250 ng/ml), respectively. The curves are representative of 6 experiments ($N = 4$). (*B*) Stimulation of CaIC by preincubation with cholera toxin (250 ng/ml) or pertussis toxin (250 ng/ml), respectively. The data are normalized to the current value before the application of the respective substance. Numbers in brackets give the numbers of individual oocytes.

pertussis, interferes with inhibitors of the adenylyl cyclase thereby increasing intracellular cAMP levels (Moriarty et al., 1990). Incubation of oocytes in pertussis toxin (250 ng/ml for 2 hr) resulted also in elevated currents mediated by the CaIC. However, the effect of pertussis toxin on the CaIC was quite lower than the effect of cholera toxin. The current was increased only by 63 ± 8% ($n = 35$, $N = 4$). Pertussis toxin led to increased currents over the whole potential range (Fig. 4). Again, the reversal potential of the pertussis toxin activated current was about −10 mV indicating that a selective Cl^- channel was opened. Figure 4*B* sums up the effects of cholera toxin and pertussis toxin on the clamp current at −60 mV.

Effects of Protein Kinase C Activation

Phorbol esters like PMA (phorbol 12-myristate 13-acetate) have been demonstrated to activate protein kinase C (PKC) in *Xenopus* oocytes thereby influencing several characteristic features of the oocyte membrane (Vasilets et al., 1990). Injection of PMA into the oocytes to a final concentration of about 140 nM led to an increase of the Cl^- channel activity induced by the removal of external Ca^{2+} (46.9 ± 7.8%, $n = 6$, $N = 4$). The same experimental protocol caused no significant changes in the electric properties of the oocyte membrane when Ca^{2+} was present in the bath solution.

Influence of the Cytoskeleton

To investigate the influence of the cytoskeleton on the activity of the CaIC, we superfused oocytes with cytochalasin D (5 μg/ml) under voltage-clamp conditions. This compound is known to disrupt actin filaments, which are in some way involved in the regulation of ion channels (Hesketh, 1994). Cytochalasin D reduced the currents mediated by the CaIC in Ca^{2+}-free solution about 15.3 ± 4.7% ($n = 8$, $N = 3$). Shortly after application of cytochalasin D the holding current dropped. Subsequent removal of cytochalasin D restored the former holding current after a longer lasting washout period.

Fate of the CAIC During Oocyte Maturation

Nearly all transport systems of the oocyte are regulated down by several mechanisms during maturation (Schmalzing et al., 1990). In vitro maturation was induced by incubating the oocytes in progesterone (0.1 μM) for 6 hr. Oocytes were considered maturated when the so-called "white spot" appeared, the visible sign for germinal vesicle break down. Contrary to most transporters of the oocyte membrane, the CaIC was still active in maturated oocytes also called eggs. Eggs, voltage clamped to −60 mV, had CaIC currents of −2052 ± 299 nA ($n = 5$, $N = 3$) compared with −3294 ± 369 nA ($n = 32$, $N = 13$) in progesterone untreated oocytes. The small difference between these two values might be due to maturation, seasonal changes or the wide variability of the magnitude of CaIC currents in individual oocytes (Weber et al., 1995). The dependence of the inactivation of the CaIC on external Ca^{2+} concentrations of eggs showed only moderate differences from the results obtained with oocytes. The K_i values for Ca^{2+} of oocytes and eggs are not significantly different. Again, the tiny difference could be explained by the above mentioned reasons (*see also* Discussion).

Single-Channel Data

Depletion of divalent cations in the pipette led to activation of Cl^- channels. Figure 5 shows CaIC channels

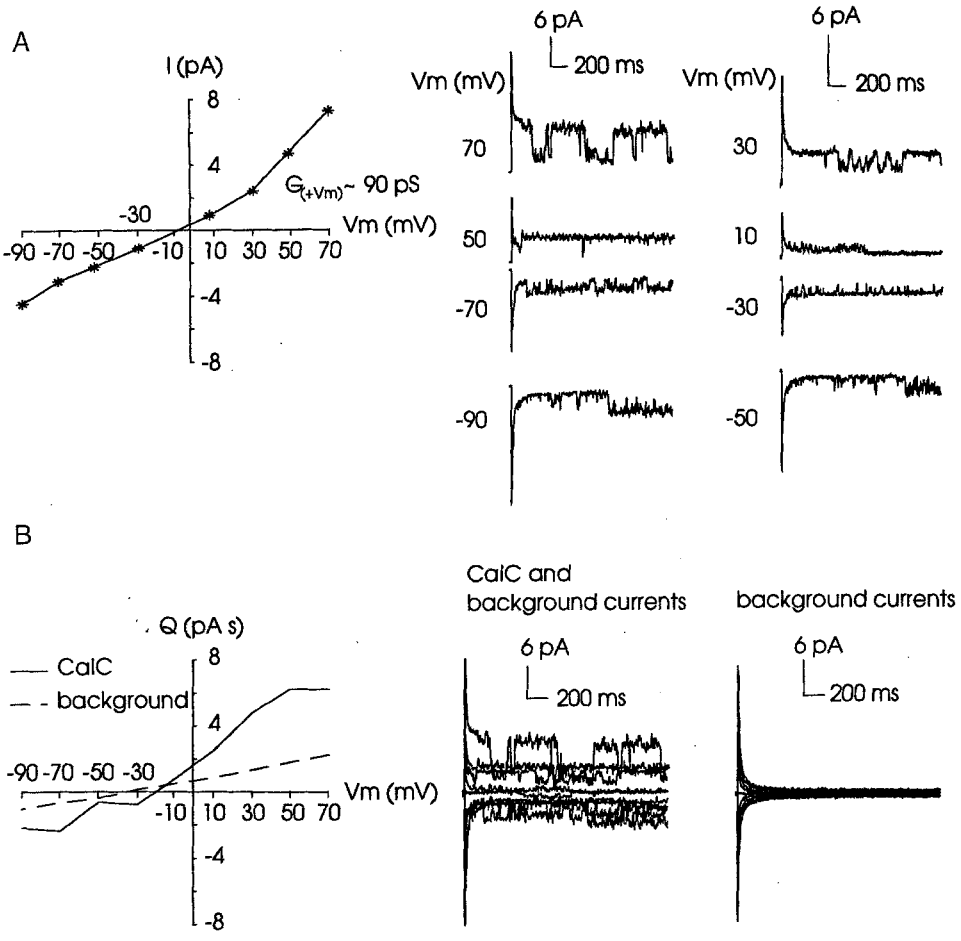

Fig. 5. Single channel conductance of CaIC. Measurements were performed in the attached mode with NMDGCI (100 mM) and EGTA (1 mM) in the pipette. *I-V* relationships from rectangular voltage pulse experiments. The corresponding current traces are shown in the right panel. (*A*) Slope conductance of the major open amplitude was about 90 pS at positive V_m, reversed at −10 mV and exhibited a slightly outward rectification. (*B*) Mean load fluctuation (closed line) *vs.* background currents (broken line) were derived from integrated current traces (integration time = 2 sec). The Q-V relationship exhibits pronounced outward rectification and a reversal potential similar to whole cell voltage clamp experiments (≈ −15 mV).

measured in the attached mode without divalent cations in the pipette solution (100 mM NMDGCI). These channels exhibited spontaneous openings at resting membrane potential (between −30 and −60 mV).

Single-channel analysis in the attached mode yielded a slope conductance of the major open state of about 90 pS at positive membrane potentials. Channel amplitudes reversed close to the expected Cl^- reversal potential of about −15 mV (Fig. 5*A*). The Ca^{2+} inactivated chloride mean load fluctuation of a whole patch ($Q = I \cdot t$) shown in Fig. 5*B* reversed also near −15 mV. The curve was resolved from integrating CaIC currents with and without background currents. Background (baseline) currents appeared to be mostly carried by Na^+ and K^+ and were strongly reduced when substituted by $NMDG^+$ on either side of the patch. As shown in Fig. 6*B*, depletion of Ca^{2+} influenced both background currents and CaIC amplitudes.

Long-lasting depolarizing voltages ($V_m \approx 30$ mV) stabilized the CaIC open dwell time and the probability that the channel remained in its full open state. Prolonged hyperpolarization induced substates and decreased the channel open probability. Especially during rectangular voltage-pulse experiments in the inside out mode CaIC amplitudes tended to disappear at negative V_m ($V_m < V_{rest}$). Addition of Ca^{2+} (2 mM) to the extracellular face of the membrane via pipette perfusion (Reifarth, Weiser & Bentrup, 1994) caused complete channel deactivation. Continuous single channel recording directly after pipette perfusion with Ca^{2+} (2 mM) is illustrated in Fig. 6*A*. CaIC amplitudes disappeared suddenly when Ca^{2+} was present on the extracellular side of the patch. Only a small channel of about 10 pS remained active. Further records on the same patch 10 min after pipette perfusion with Ca^{2+} (indicated by an arrow in Fig. 6*A*) showed no CaIC activity. CaIC deactivation and background current reduction by Ca^{2+} were additionally analyzed using voltage ramp experiments.

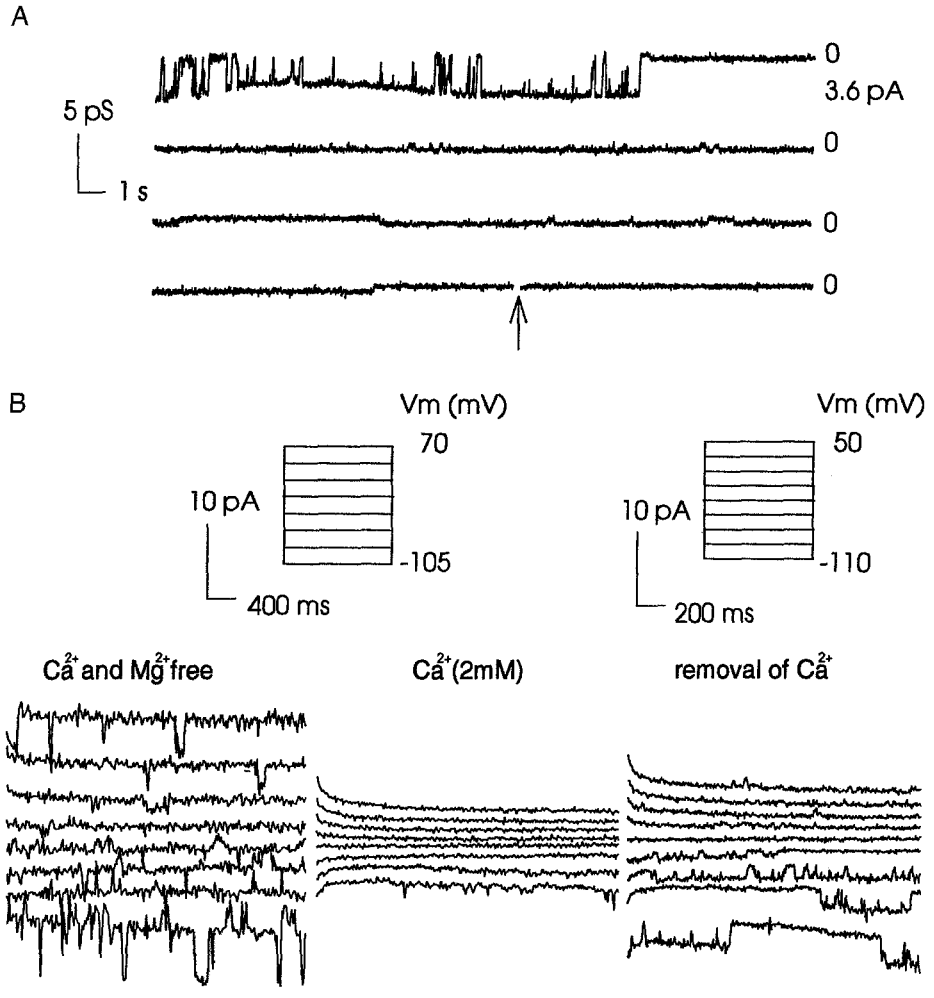

Fig. 6. Inhibition of CaIC activity by Ca^{2+}. In cell-attached patches with NMDGCI (100 mM) pipette solution CaIC activity and background currents were present only in absence of external divalent cations. (*A*) Continuous current recordings immediately after pipette perfusion with Ca^{2+} (2 mM). Ca^{2+} induced a brusque closing of CaIC, however a small channel about 10 pS remained active up to 3 min. The arrow indicates the restart of recording ten min after Ca^{2+} incubation. The membrane potential was identical with the resting potential. (*B*) CaIC activities under Ca^{2+}-free conditions(left panel). Addition of Ca^{2+} via pipette perfusion immediately abolished the CaIC activity (center). Washout of Ca^{2+} from the pipette solution resulted in only partial reactivation of the CaIC, probably due to imperfect Ca^{2+} removal from the pipette solution. Note that background (baseline) currents increase when Ca^{2+} is not present. Divalent cation free pipette solution was buffered with EGTA (1 mM).

Subsequent removal of Ca^{2+} induced only a substate conductance of the channel at hyperpolarizing V_m, but fully restored the background currents. Partly, return of channel activity might reflect an incomplete washout of Ca^{2+} (Fig. 6*B*).

In excised experiments, the CaIC was independent on cytosolic Ca^{2+}. The presence of Ca^{2+} (1 mM) or complete Ca^{2+} depletion did not influence the channel open probability, however, Ca^{2+} removal frequently reduced the seal lifetime.

CaIC gating, open probability and the presence of substates can vary considerably in the attached configuration. CaIC amplitudes frequently exhibited several conductance states. A record with a pronounced substate behavior is depicted in Fig. 7. The amplitude histogram in Fig. 7 shows typical subconductances of the CaIC measured in the attached mode at oocyte membrane resting potential ($V_m = -45$ mV). The substate currents did not exhibit integer fractions of the major open state.

Open- and closed histograms were achieved in the range of V_m −60 mV up to 0 mV in the attached- and inside out mode from measurements with minor substates ($n = 5, N = 3$). The half-maximal threshold method was used to analyze the CaIC dwell times. A representative recording of an attached measurement from which the distributions were obtained is shown in Fig. 8*A*. Only the major open state was manually analyzed for calculating open- and closed duration histograms which could be fitted with two exponentials. The short open time constants resulted from short-term transitions and had values between 1.2 and 6.4 msec strongly dependent on filter settings and sampling rate. The long

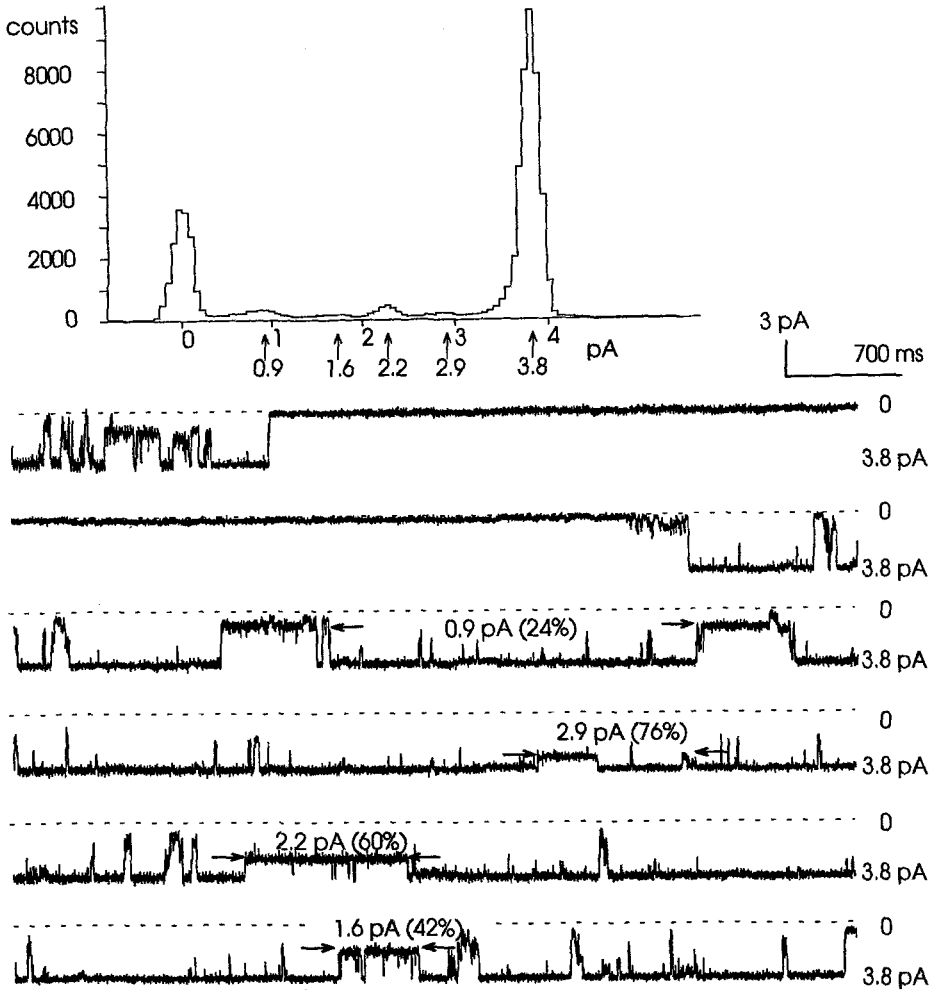

Fig. 7. CaIC gating with pronounced substate behavior. Amplitude histogram and corresponding single channel traces of an attached measurement at $V_m = -45$ mV. The full open state and four substates are marked with arrows. Amplitude distributions were made by subsequent software filtering (150 Hz) of shown single-channel recordings (550 Hz).

time constants reflected long lasting burst- and interburst sequences which varied strongly from 14 to 210 msec in dependence of the number of analyzed events with corresponding dwell times.

Discussion

The results of the present paper confirm and extend our previous observation, that oocytes and eggs of the South African clawed toad *Xenopus laevis* contain a Cl^- channel, which is inactivated in the presence of external divalent cations (Weber et al., 1995). Removal of external divalent cations, especially Ca^{2+}, immediately leads to activation of these channels, followed by large depolarizations and giant currents over the plasma membrane under voltage clamp conditions. These currents can easily amount to more than 10 microamperes at a holding potential of −60 mV, thereby sometimes breaking the limitations of our voltage-clamp equipment. The Ca^{2+} inactivated Cl^- channel (CaIC) is selective for Cl^- as can be demonstrated by the reversal potential of −15 mV (Weber et al., 1995). This reversal potential is predicted by the constant field equation (Goldman, 1943) for a perfectly selective Cl^- channel. The present data indicate that the rate of CaIC's inactivation is dependent on the external Ca^{2+} concentration, and that the channel is blockable by some but not all Cl^- channel blockers. CaIC is regulated by cAMP and ATP and is influenced by the cytoskeleton. In addition, the CaIC, which is unaffected by defolliculation, is the first transport system of the oocyte plasma membrane that is not downregulated during maturation. Single channel analysis gives strong evidence for the existence of a Cl^- channel with a conductance of about 90 pS and four discrete substates. Because the effects were similar for other divalent cations, such as Mg^{2+}, Ba^{2+}, and Sr^{2+} we focus here on Ca^{2+}, as the typical representative for the effects of divalent cations on the CaIC.

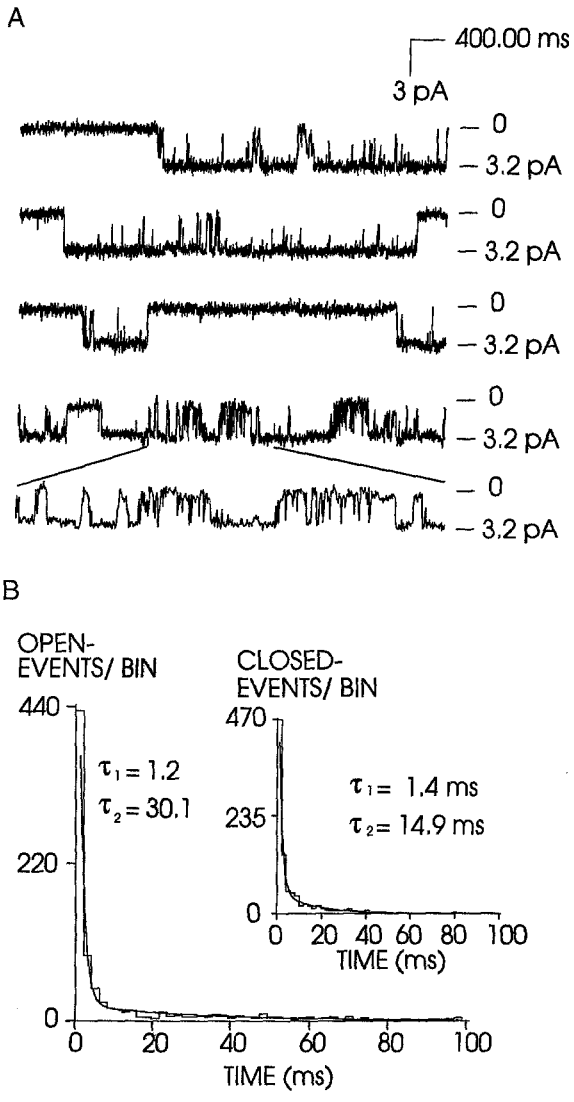

Fig. 8. Typical CaIC gating. (*A*) Representative current traces obtained from cell attached measurement at $V_m \approx -50$ mV. The bottom traces shows flickery openings of the CaIC. The traces were filtered at 300 Hz. (*B*) Open- and closed durations achieved with the half-maximal threshold method from the traces shown in Fig. 8*A*. Close fit achieved two exponentials (binning = 2 msec). For data analysis, a filter frequency of 1550 Hz was used and stored data were sampled at 140 μs.

Effects of Ca^{2+}

External Ca^{2+} concentrations higher than 500 μM completely inactivate the CaIC. When the external Ca^{2+} falls beneath that value the Ca^{2+} inactivated Cl^- channels respond with spontaneous openings. The magnitude of the Cl^- mediated current increases further with decreasing Ca^{2+} concentration. The Cl^- currents reach their maximum when the bath solution is Ca^{2+}-free thereby depolarizing the membrane to values near −10 mV.

The CaIC is completely independent from cytosolic Ca^{2+} concentrations. We blocked endogenous Ca^{2+} channels with verapamil (250 μM) and with quinidine (1 mM) to exclude possible influences on the internal Ca^{2+} concentration by external Ca^{2+} withdrawal. We increased or decreased the cytosolic Ca^{2+} concentration artificially by injection of Ca^{2+} or EGTA, respectively. All these maneuvers could not induce any change in the activity of the CaIC after removing of Ca^{2+} from the perfusate. This independence of intracellular Ca^{2+} clearly distinguishes the CaIC from other Cl^- channels of the *Xenopus* oocyte which are regulated via changes of cytosolic Ca^{2+} (Boton et al., 1989). Moreover, these Ca^{2+}-activated Cl^- channels produce only small currents in the range of a few hundred nanoamperes (Barish, 1983).

The procedure of Ca^{2+} removal is completely reversible and can be repeated several times with one single oocyte. It has been argued that withdrawal of external divalent cations leads instantaneously and inevitably to irreversible damage of the oocyte membrane by inducing irreparable "leak currents" (Raditsch & Witzemann, 1994). One point that emerges from this study is that removal of external divalent cations does no detectable harm to the plasma membrane of *Xenopus* oocytes and that the so-called "leak current" is nothing else than a Cl^- channel which is activated when the outer divalent cation concentration is lowered.

Effects of Cl^- Channel Blockers

Although Cl^- channels seem to be a very heterogeneous class of ion channels there exist some Cl^- channel blockers which show at least a certain specificity. The nonsteroidal anti-inflammatory agents FFA and NFA are reported to be potent inhibitors of endogenous Cl^- channels in the oocyte membrane (White & Aylwin, 1990), which are blocked by these compounds with apparent inhibition constants (K_i) of 28 μM and 17 μM respectively. The blockers show different characteristics for the inhibition of the CaIC. In this case, FFA with a K_i of about 6 μM shows higher affinity for the binding site on the channel than NFA (K_i = 22 μM). In general, both blockers seem to have slightly higher affinities for the CaIC than for the Ca^{2+}-activated Cl^- channels of the oocyte, which are regulated by cytosolic Ca^{2+}.

DPC, a Cl^- channel blocker known to be efficacious on Cl^- channels of various tissues (Distefano et al., 1985), also exhibits inhibitory potency on the CaIC. However, with an apparent blocker constant of 74 μM the affinity of this compound on the CaIC is somewhat lower than for the above-mentioned inhibitors. DPC, like NFA, blocks only about 80% of the total CaIC while FFA blocks nearly the complete current mediated by the CaIC.

SITS can block cAMP-stimulated chloride fluxes and other Cl^- channels in rather low doses (Greger,

1990). Surprisingly, SITS has absolutely no inhibitory potency on the CaIC in *Xenopus* oocytes. Moreover, SITS in low concentrations stimulated the CaIC-mediated Cl^- current. The maximal stimulation with 10 μM SITS doubled the current; subsequent increasing "blocker" concentrations leads to declining current amplitudes. However, even the highest concentration of SITS, that we tested (500 μM), showed slight activation but no inhibition of the current at all. Therefore, the CaIC can be clearly distinguished from the hyperpolarization activated Cl^- current in the oocyte which is completely inhibited by SITS (Kowdley et al., 1994).

We showed, that from the four investigated Cl^- channel blockers FFA is the inhibitor of choice if one wants to block the CaIC selectively.

Regulation

External ATP in millimolar doses causes depolarization of the oocyte membrane (Lotan et al., 1982) thereby increasing the inward currents which flow over the membrane at the resting potential (Kupitz & Atlas, 1993). Under Ca^{2+}-free conditions, the oocyte membrane is completely depolarized (Weber et al., 1995), and addition of ATP cannot induce further depolarization. The CaIC-mediated currents are inhibited by extracellular ATP while ADP shows no detectable effect. Therefore the CaIC seems to be different from the ATP and ADP sensitive mammalian chloride channel which has been cloned recently (Paulmichl et al., 1992) and which is characterized by a sensitivity for both nucleotides.

Cyclic monophosphates, such as cAMP and cGMP, play an outstanding role in the regulation of ion channels. At least one class of Cl^- channels is regulated by cyclic monophosphates with the cystic fibrosis transmembrane conductance regulator (CFTR) as the most prominent representative (for review *see:* Welsh et al., 1994). cAMP-dependent Cl^- channels are often activated through phosphorylation by cAMP-dependent protein kinase A (Greger, 1994). The activation of some other Cl^- channels might involve a molecular mechanism where cAMP interacts directly with the channel protein. The calcium inactivated Cl^- channel of *Xenopus* oocytes is activated by cAMP. We showed, that elevation of cytosolic cAMP concentrations leads to activation of the CaIC, independent of the method with which the elevation was achieved. Even indirect increasing of cytosolic cAMP concentration via inhibition of cAMP degradation showed clear activation. Neither extracellular cGMP nor injection of cGMP had any effect on the CaIC. From these results, we conclude that cGMP-dependent kinases do not participate in the regulation of the CaIC. In this respect, the CaIC behaves like an apical membrane chloride conductance described form the *Necturus* gallbladder (Heming, Copello & Reuss, 1994), which is also activated by cAMP but unaffected by cGMP.

The A subunit of cholera toxin activates adenylyl cyclase by catalyzing the transfer of the ADP-ribose moiety of NAD^+ to $G_{s\text{-}\alpha}$. The ADP-ribosylation prolongs the lifetime of the $G_{s\text{-}\alpha}$-GTP species which in turn activate adenylyl cyclase thereby increasing the cytosolic cAMP concentration. In oocytes pretreated with cholera toxin, the subsequent increase of intracellular cAMP leads to stimulation of the CaIC.

The effect of treatment with pertussis toxin is to diminish or abolish the efficacy of $G_{i\text{-}\alpha}$ to inhibit the activity of the adenylyl cyclase. The consequences are rising cytosolic cAMP levels which in turn lead to activation of the CaIC.

To investigate a possible activation of the CaIC by protein kinase C, we used PMA, a phorbol ester which activated PKC (Vasilets et al., 1990). Injection of PMA stimulates the CaIC. However, from our results, we cannot come to a conclusion on the mechanism of this stimulation. PMA could mediate its effect via an increase of cytosolic cAMP or by a PKC-mediated phosphorylation of the CaIC protein or a related regulatory molecule. PMA-induced activation of a Cl^- channel was also reported in the *Necturus* gallbladder (Heming, Copello & Reuss, 1994).

Cytoskeleton

The cytoskeleton is a complicated network consisting of microfilaments, intermediate filaments, and microtubules. Several lines of evidence support a role for F-actin, the main component of the microfilaments, in regulating ion channels in various cell lines (Mills & Mandel, 1994). It is widely accepted that components of the actin polymerization/depolymerization process could alter the activity of plasma membrane channels (Suzuki et al., 1993). Depolymerization of actin with cytochalasin D leads to lower CaIC activity in a reversible manner. This effect might be mediated by direct interaction of actin with the channel protein. It could also be mediated via an interaction of actin with one or more elements of the signal transduction apparatus that controls the activity of channels in the oocyte plasma membrane.

Maturation Events

Full-grown oocytes of stage VI (Dumont, 1972) are arrested in the prophase of the first meiotic division. They undergo maturation after gonadotropin-induced progesterone release from the surrounding follicle cells. This event causes some profound changes in the electrical properties of the maturated oocytes, so-called eggs. Membrane potential is completely depolarized leading to the downregulation of nearly all oocyte membrane transport processes. The Na^+/K^+-ATPase is internalized onto inner membranes (Schmalzing et al., 1990), other trans-

port systems like the Na^+/glucose cotransporter are downregulated through the depolarization of the membrane (Weber, Schwarz & Passow, 1990). The only transport system that seems to be functional after oocyte maturation is the CaIC. Eggs show nearly the same CaIC activity following Ca^{2+} removal as the oocytes do. Even the K_i values for the Ca^{2+} inactivation show no great difference, although the currents in eggs are a little bit lower. In addition to the above-mentioned reasons, this could be due to a lower cAMP content of eggs. The binding of progesterone to its receptor leads to an inhibition of the adenylyl cyclase resulting in a drop in the level of intracellular cAMP (Wasserman, Houle & Samuel, 1984). This lower cAMP content of the eggs on the other hand might cause slowly lower CaIC activity in *Xenopus* eggs.

Patch Clamp Analysis

Our results show that depletion of external divalent cations evoked Cl^- channel openings in cell-attached patches of devitellinated oocytes. This channel could be reversibly inhibited by extracellular Ca^{2+} but was shown to be independent of cytosolic Ca^{2+}. One possible explanation of this result is a regulatory extracellular binding side which functions as a Ca^{2+} sensor which might be physiologically necessary when eggs are spawned into hypo-osmotic pond water. Ca^{2+} removal on both sides of the patch caused no irreversible leak current, but increased background currents and reduced the seal life time. Contrary to background currents, which were nearly linear, the CaIC offered a slightly outwardly rectifying Cl^- flux at resting potential up to +50 mV.

The CaIC exhibits a relatively large slope conductance of about 90 pS in NMDGCI solution (100 mM, attached mode) which is higher than for previously reported endogenous channel conductances in *Xenopus* oocytes (Dascal, 1987). In comparison with well-investigated epithelial Cl^- channels (Gögelein, 1988) the CaIC can be classified as a large conductance channel rather than a so-called epithelial intermediate conductance channel (ICOR channels, (Greger, 1994)). Especially in inside-out patches the CaIC showed conductances similar to large Cl^- channels (up to 250 pS). Nevertheless, the CaIC share some similarities with these ICOR channels which are summarized below. The channel exhibits a slightly outward rectification both in channel amplitudes and load fluctuation. The open probability (P_o) is voltage dependent especially in the inside-out mode. Positive V_m stabilized P_o whereas hyperpolarizing potentials tended to inactivate the CaIC. The channel open probability could be regained at positive V_m. Two exponentials were needed for close fitting of the open- and closed duration histograms which indicate complex channel kinetics. In addition, substates may reflect further distinct functional states of CaIC gating. Contrary to ICOR channels, CaIC was also active in the cell-attached mode and SITS augmented CaIC currents in whole cell experiments.

CaIC exhibited several substates as also described for other Cl^- channels (Frizzel & Morris, 1994; Frizzel & Halm, 1994) and the major open state did not exclude the possibility of long-lasting lower amplitudes. Especially inside-out measurements showed Cl^- channels with conductances up to 250 pS which could reflect simultaneous gating of several CaIC. In all the above-mentioned features, the single channel patch data are consistent with the whole cell results obtained with microelectrodes.

In conclusion, *Xenopus* oocytes possess Cl^- channels that are activated when external divalent cations are removed. In this and most of their other identified features, these Ca^{2+} inactivated channels (CaIC) differ from Ca^{2+}-activated Cl^- channels in the oocytes. The present data show that the supposed "leak current" of *Xenopus* oocytes that occurs after removal of divalent cations is not mediated through unspecific leaks but through a new class of Cl^- channels.

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