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The *Alphavirus* 6K Protein Activates Endogenous Ionic Conductances when Expressed in *Xenopus* Oocytes

Anne-Frédérique Antoine · Claire Montpellier · Katia Cailliau · Edith Browaeys-Poly · Jean-Pierre Vilain · Jean Dubuisson

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Abstract The *Alphavirus* Sindbis 6K protein is involved in several functions. It contributes to the processing and membrane insertion of E1 and PE2 viral envelope glycoproteins and to virus budding. It also permeabilizes *Escherichia coli* and mammalian cells. These viroporin-like properties have been proposed to help virus budding by modifying membrane permeabilities. We expressed Sindbis virus 6K cRNA in *Xenopus* oocytes to further characterize the effect of 6K on membrane conductances and permeabilization. Although no intrinsic channel properties were seen, cell shrinkage was observed within 24 h. Voltage-clamp experiments showed that 6K upregulated endogenous currents: a hyperpolarization-activated inward current (I_{in}) and a calcium-dependent chloride current (I_{Cl}). 6K was located at both the plasma and the endoplasmic reticulum membranes. The plasma membrane current upregulation likely results from disruption of the calcium homeostasis of the cell at the endoplasmic reticulum level. Indeed, 6K cRNA expression induced reticular calcium store depletion and capacitative calcium entry activation. By experimental modifications of the incubation medium, we showed that downstream of these events cell shrinkage resulted from a 6K -induced KCl efflux (I_{Cl} upregulation leads to chloride efflux, which itself electrically drives potassium efflux), which was responsible for an osmotic water efflux. Our data confirm that 6K specifically triggers a sequential cascade of events that leads to cytoplasmic calcium elevation and cell permeabilization, which likely play a role in the Sindbis virus life cycle.

Keywords Alphavirus · Viroporin · Chloride current · Calcium release · Store-operated calcium entry · Cell volume regulation

A.-F. Antoine (✉) · K. Cailliau · E. Browaeys-Poly · J.-P. Vilain
Equipe d'Accueil 4020, Institut Fédératif de la Recherche 147, Université de Lille, 59655 Villeneuve d'Ascq Cedex, France
e-mail: anne-frederique.antoine@univ-lille1.fr

C. Montpellier · J. Dubuisson
Centre National de la Recherche Scientifique—Unité Propre de Recherche 2511, Institut de Biologie de Lille-Institut Pasteur de Lille, 59021 Lille Cedex, France

Introduction

Alphaviruses are all transmitted by arthropods and include numerous medically significant pathogens, which can induce encephalitis, arthritis or rash (reviewed in Griffin, Byrnes and Cook 2004). They are enveloped viruses with a positive single-stranded RNA genome of ~11.7 kb (reviewed in Strauss and Strauss 1994). The 5′ two-thirds of the genome (7.6 kb) encodes a single polyprotein containing the polypeptides implicated in transcription and replication. The 3′ one-third (4.1 kb) codes for another polyprotein (capsid-PE2-6K-E1) containing the polypeptides involved in particle formation. Several regions of this polyprotein are cotranslationally translocated across the endoplasmic reticulum (ER) membrane, and the polyprotein is ultimately cleaved by cellular signal peptidases and the autoprotease activity of the capsid protein. PE2 and E1 are the envelope glycoproteins, and 6K is a membrane polypeptide. PE2 and E1 glycoproteins associate as a heterodimer, and during its transport through the secretory pathway, the N-terminal region of PE2 is cleaved to produce the mature E2 protein (reviewed in Garoff et al. 1994).

6K is a small (58–61 amino acids) hydrophobic and acylated protein. Due to the presence of a signal for

reinitiation of translocation at the C terminus of PE2, the 6K protein is cotranslationally translocated across the ER, with its N terminus facing the ER lumen followed by two transmembrane domains. 6K associates with a PE2-E1 heterodimer soon after its synthesis and is thereafter transported to the site of viral assembly at the plasma membrane. The 6K protein contains a signal for reinitiation of translocation at its C terminus, which is responsible for the translocation of the ectodomain of E1 into the ER lumen. Surprisingly, only a small fraction of 6K is incorporated in the viral particles. 6K has also been shown to play an important role in virus budding, and its expression is important for the correct assembly of fully infectious particles (reviewed in Gonzalez and Carrasco 2003).

However, the role of 6K protein in the virus life cycle remains poorly characterized. Most interestingly, 6K might facilitate virus budding by modifying the permeability of membranes (Carrasco 1995). Indeed, 6K is structurally similar to numerous small viral proteins: M2 protein of influenza A (Pinto, Holsinger and Lamb 1992); NB and M2 proteins of influenza B (Paterson et al. 2003; Sunstrom et al. 1996); Vpr and Vpu of human immunodeficiency virus 1 (HIV-1) (Ewart et al. 1996; Piller et al. 1998; Schubert et al. 1996); Kcv, a K^+ channel protein of chlorella virus (Plugge et al. 2000); 3A and 2B proteins of poliovirus (Lama and Carrasco 1992a, b; Plugge et al. 2000); and p7 of hepatitis C virus (Carrere-Kremer et al. 2004; Griffin et al. 2003; Pavlovic et al. 2003; Premkumar et al. 2004). These are all short (50–120 amino acids) integral membrane proteins that increase membrane permeability (Carrasco 1995). They are named ''viroporins'' because they form membrane pores which allow the passage of ions with poor ionic selectivity and almost no gating mechanisms (reviewed in Kelly et al. 2003).

Accordingly, 6K forms cation-selective ion channels when inserted in planar lipid bilayers (Melton et al. 2002). To better understand the functional role of 6K, it is important to test its activity in the environment of the cell. It is often difficult to record specifically viroporin channel activity in infected cells because it is necessary to distinguish it from endogenous channel activities. Furthermore, when expressed in either mammalian cells or *Escherichia coli*, 6K induces cell permeabilization (Sanz et al. 2003; Sanz, Perez and Carrasco 1994), but rapid cell death prevented any further electrophysiological analysis. Therefore, we expressed Sindbis virus 6K in *Xenopus* oocytes, a well-known heterologous expression system for studying the properties of membrane proteins. In this heterologous system, 6K presented no intrinsic channel properties but disrupted calcium homeostasis and induced cell shrinkage followed by cell death. Our results indicate that 6K induced a sustained elevation of cytosolic calcium ($[Ca^{2+}]_i$) through reticular calcium store depletion and capacitative calcium entry. $[Ca^{2+}]_i$ elevation in turn upregulated an endogenous calcium-dependent chloride current (I_{Cl}). Resulting KCl-driven water efflux led to cell shrinkage, while cell death might be a direct consequence of $[Ca^{2+}]_i$ elevation. Taken together, these results show that 6K does indeed induce membrane permeabilization in *Xenopus* oocytes, although through an indirect mechanism, involving $[Ca^{2+}]_i$ elevation.

Materials and Methods

Plasmid Constructs

Sindbis virus sequences were amplified from plasmid pToto1101 (kindly provided by C. M. Rice, Rockefeller University, New York, NY, Rice et al. 1987). 6K was cloned into pCS2, a vector allowing *in vitro* mRNA synthesis (Roth, Zahler and Stolk 1991). The 6K sequence was also cloned into plasmid pCS2+MT (Myc tag), which allows translation of a fusion between 6K and the c-Myc epitope (Roth et al. 1991); the resulting plasmid was named ''p6K-myc.''

6K cRNA Preparation and Expression in *Xenopus* Oocytes

Plasmids were linearized with *Not*I endonuclease and then purified. Capped mRNAs were transcribed from linearized plasmids with the SP6 mMESSAGE mMACHINE kit (Ambion, Austin, TX). Experiments were performed on defolliculated oocytes obtained from adult *Xenopus laevis* anesthetized with 2 g • l^{-1} MS 222 (Sandoz, Holzkirchen, Germany). Ovaries were stored in ND96, pH 7.5 (Table 1), and oocytes were isolated by microdissection after 45-min incubation in 1 mg • ml^{-1} collagenase. Oocytes were microinjected in the equatorial region with either the cRNAs (30 ng) or an equivalent volume of water (controls). They were incubated for 24 h in ND96, pH 8.5 (unless differently specified), at 19°C before use. These conditions offered better viability.

Detection of 6K cRNA Expression

Expression of 6K in oocytes was confirmed by immunoprecipiation of oocyte homogenates. For control oocytes and for 24 h 6K cRNA-expressing oocytes, 20 oocytes were homogenized at 4°C in 200 µl of PY 25 mM MOPS pH 7.2, 60 mM β glycerophosphate, 15 mM paranitrophenyl phosphate, 15 mM EDTA, 15 mM $MgCl_2$, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylphosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinine, 10 µg/ml soybean trypsin inhibitor, 10 µM benzamidine (Caillian et al. 2005) and 0.5% Triton X-100 and

Table 1 Solutions used

	NaCl (mM)	Na Isethionate (mM)	KCl (mM)	$MgCl_2$ (mM)	$CaCl_2$ (mM)	pH
ND7.5	96	-	2	1	1.8	7.5
ND7.5, high K	78	-	20	1	1.8	7.5
ND8.5	96	-	2	1	1.8	8.5
ND8.5, low Cl	-	96	2	1	1.8	8.5
ND8.5, zero Ca	85	-	2	5	-	8.5
ND8.5, high K	78	-	20	1	1.8	8.5

The solutions were buffered by 5 mM HEPES, and the pH was adjusted with NaOH

centrifuged at 4°C for 15 min at 13,000 turns/min. Supernatant (150 µl) was collected and incubated with mouse anti-Myc antibodies (CRL-1725, dilution 1/1,000; ATCC, Rockville, MD) overnight at 4°C. Protein A-Sepharose beads (50%; Transduction Laboratories, Lexington, KY) were added for 1 h at 4°C. Immune complexes were collected by centrifugation, rinsed three times and resuspended in 60 µl Laemmli sampling buffer.

After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% polyacrylamide gels, protein preparations were transferred to nitrocellulose membranes (Hybond-ECL; Amersham, Arlington Heights, IL) by use of a Trans-Blot apparatus (Bio-Rad, Richmond, CA) and detected with an anti-Myc monoclonal antibody (ATCC CRL-1725, dilution 1/250 of antibody produced in a MiniPerm apparatus from Heraeus, South Bend, IN), followed by rabbit anti-mouse (Dako, Carpinteria, CA) immunoglobulin conjugated to peroxidase (dilution 1/1,000). The proteins of interest were analyzed by enhanced chemiluminescence detection (Amersham, Pittsburgh, PA) as recommended by the manufacturer.

Indirect Immunofluorescence Microscopy

Oocytes expressing 6K-Myc for 48 h were fixed in Bouin's fixative overnight and embedded in paraffin for serial sections. Sections were collected on gelatin-coated slides, paraffin was removed and sections were saturated with phosphate-buffered saline (PBS) containing 6% bovine serum albumin, incubated with the anti-Myc antibody (dilution 1/250 in PBS added with 0.05% Triton X-100) for 2 h and revealed with an anti-mouse ALEXA-conjugated antibody (Molecular Probes, Eugene, OR). Immunofluorescence was detected with a Zeiss (Thornwood, NY) microscope equipped with epifluorescence optics.

Electrophysiological Measurements

The oocyte was impaled by two glass microelectrodes pulled from thin-walled glass capillary tubing (Clark Electromedical, Reading, UK) and filled with 3 M KCl (electrode resistance <5 MΩ). The bath was grounded by an Ag/AgCl electrode through a 3 M KCl/agar bridge. A homemade conventional two-electrode voltage-clamp circuit was used. Generation of clamp command voltage, data acquisition and analysis were done using pCLAMP 6 (Axon Instruments, Burlingame, CA). All experiments were performed at room temperature in ND, pH 7.5 (ND7.5), unless otherwise specified. I_{Cl} and I_{in} were recorded with a modification of a previously described protocol (Kuruma and Hartzell 1999, 2000; Yao and Tsien 1997).

To inhibit calcium release from the reticulum through inositol 1,4,5-trisphosphate ($InsP_3$) receptors, 20 nl of a 5 mg/ml heparin solution was microinjected 45 min before 6K RNA microinjection. For evaluation of ER calcium store depletion, 0.5 µM A23187 was applied to oocytes in modified ND7.5 containing 0 mM Ca^{2+}, 5 mM Mg^{2+} and 0.1 mM ethyleneglycoltetraacetic acid (EGTA) (Kuruma and Hartzell 1999). To measure the intensity of the peak current as accurately as possible, the triple-step protocols were applied at 5-s intervals.

For measurements of capacitative calcium entry (I_{SOCE}), oocytes were injected with 7 nmoles 1,2-bis(*o*-aminophenoxy)etane-*N*-*N*′-*N*′-*N*′-tetraacetic acid (BAPTA, final concentration of ~7 mM) to buffer intracellular Ca^{2+}. Recordings were made in modified ND7.5 containing 55 mM NaCl and 30 mM $CaCl_2$. I_{SOCE} was recorded with a 2.5-s voltage ramp from –140 to +60 m*V*. The protocol was applied before and after 100 µM La^{3+} addition. I_{SOCE} was measured as the La^{3+}-inhibited current (Machaca and Haun 2000).

Values are given as mean ± standard error of the mean (SEM), with $n \geq 6$, where n is the number of oocytes. Each experiment was performed with at least three batches of oocytes, isolated from different females.

Results

Expression of 6K in *Xenopus* Oocytes

To achieve proper insertion in the ER membrane, Sindbis virus 6K was expressed downstream of the C terminus of PE2, which contains a signal peptide function and allows translocation of the N terminus of 6K in the ER lumen

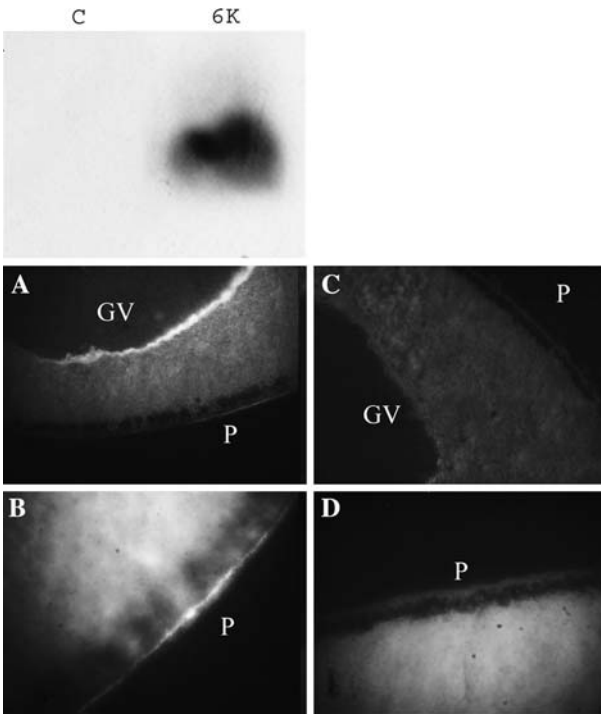

Fig. 1 Expression of 6K in *X. laevis* oocytes. (*Top*) Western blot of 6K in *X. laevis* oocyte extracts. Control oocytes (C) and oocytes injected with 30 ng 6K-Myc cRNA and incubated for 48 h in ND96 at 19°C (6K). (*Bottom*) Immunocytochemical localization of the 6K-Myc fusion protein expressed in *Xenopus* oocyte. Thin sections were prepared from oocytes injected with 30 ng 6K-Myc cRNA (*A, B*) or water (*C, D*) and incubated for 48 h in ND96 at 19°C. (*A*) Labeling is intense in the perinuclear region (germinal vesicle, *GV*). (*B*) Higher magnification showing labeling at the plasma membrane (*P*). (*C, D*) No labeling is observed on control oocytes. (*A, C*) x150, (*B, D*) x300

(Garoff et al. 1994; Liljestrom and Garoff 1991). We failed to raise a selective antibody against 6K. Thus, to detect 6K expression in *Xenopus* oocytes, a Myc epitope tag was fused at the C terminus of 6K. 6K-Myc mRNAs were microinjected into full-grown oocytes. 6K-Myc expression was confirmed by Western blotting (Fig. 1). In addition, subcellular localization of 6K-Myc was analyzed by immunofluorescence. The tagged polypeptide was detected at the plasma membrane and in a granular fashion in the perinuclear region, a region dense in ER membranes in *Xenopus* oocytes (Fig. 1). Interestingly, the labeling was brighter at the animal pole, which is richer in ER.

6K Expression Stimulates Endogenous Currents

Using the conventional two-electrode voltage-clamp system, we studied the effects of 6K expression on endogenous currents in *Xenopus* oocytes. At 24 h after RNA microinjection, 6K expression induced membrane depolarization and a membrane resistance decrease at the holding potential (–35 m*V*), both indicating membrane permeabilization of the oocyte (Table 2). Voltage ramp stimulation showed that 6K induced a conductance increase for every potential tested (–150 to +100 m*V*, Fig. 2).

Xenopus oocytes have endogenous calcium-activated chloride currents (I_{Cl}) composed of three different components revealed with a triple-step protocol (Kuruma and Hartzell 1999, 2000; Yao and Tsien 1997). Oocytes were held at –35 m*V* and stepped to +40 m*V* for 1 s, to –150 m*V* for 1 s and then back to +40 m*V* before return to the holding potential (Fig. 3). Each step allows, respectively, the recording of a noninactivating outward current ($I_{Cl1\text{-}S}$), a slow inward current (I_{Cl2}) and a transient outward current ($I_{Cl1\text{-}T}$). All three components of I_{Cl} are carried by chloride. Outward $I_{Cl1\text{-}S}$ was measured at the end of the first pulse at +40 m*V*. $I_{Cl1\text{-}T}$ was calculated by measuring the transient current during the second pulse at +40 m*V* and subtracting $I_{Cl1\text{-}S}$. I_{Cl2} was measured at the end of the –150-m*V* pulse.

It was shown that the expression of small exogenous proteins often upregulated a native hyperpolarization-activated current. This current is a mixture of a calcium-dependent chloride current and a cationic nonselective current that is permeable to calcium. I_{in} is both calcium- and pH-sensitive (Kuruma, Hirayama and Hartzell 2000). The hyperpolarization-activated current was measured with a similar protocol to that for I_{Cl}. The sole difference was the second step, which was made at –200 m*V*. I_{in} was measured at the end of the 1-s step made at –200 m*V*. I_{in} is a time-dependent inward current activated by strong hyperpolarization. The outward current (I_{out}) measured upon repolarization corresponds to the transient peak current measured during the following 1 s at –40 m*V* (Fig. 3).

When oocytes were incubated in ND8.5 and perfused in ND7.5, expression of 6K consistently enhanced endogenous calcium-activated chloride currents, with a differential effect on each of the three components of I_{Cl}. I_{in} and I_{out} were very strongly upregulated; notice that I_{out} resulted from the slow closure of the channels opened during the hyperpolization step (*see* Fig. 3 and Table 3). Thus, both I_{Cl} and I_{in} were upregulated but I_{in} was more drastically enhanced than I_{Cl}.

The effect of 6K upon I_{Cl} and I_{in} upregulation was dependent upon extracellular pH since I_{Cl} and I_{in} oocytes were more drastically upregulated when incubated in ND7.5 and perfused in ND7.5 compared to incubation in ND8.5 (*see* Tables 3 and 4).

We studied the effect of external chloride substitution and some inhibitors known to display a potent blocking effect on I_{Cl} in *Xenopus* oocytes (Kuruma and Hartzell 1999, 2000). Substitution of external chloride by isethionate greatly reduced all outward currents, $I_{Cl1\text{-}S}$, $I_{Cl1\text{-}T}$ and I_{in} (Table 5). As for endogenous I_{Cl} and I_{in}, 6K-activated currents were sensitive to different inhibitors of chloride currents in *Xenopus* oocytes: 2.8 mM barium, 0.15 mM

Table 2 Effect of 6K upon viability, apparent diameter, membrane potential (E_m) and membrane resistance (R_m): effect of incubation medium

	Incubation medium	Viability (%)	Apparent diameter (%)	E_m (m*V*)	R_m (MΩ)
Control	ND8.5	100	100	–61 ± 7	5.3 ± 1.9
6K	ND7.5	35	79	–29 ± 1	0.2 ± 3.0
	ND8.5	73	85	–41 ± 4	0.6 ± 2.8
	ND8.5, 50 μM BAPTA-AM	61	101	–50 ± 1	1.7 ± 3.0
	ND8.5, 5 mM caffeine	100	98	–51 ± 5	3.0 ± 1.1
	ND8.5, heparin microinjection	95	100	–80 ± 2	7.0 ± 1.9
	ND8.5, 0 Ca, 10 mM thapsigargin	83	93	–54 ± 1	5.6 ± 1
	ND8.5, 0.15 mM niflumate	89	94	–65 ± 8	3.2 ± 2.7
	ND8.5, 2.8 mM $BaCl_2$	92	92	–41 ± 8	33.7 ± 8.5
	ND7.5, high K	87	102	–50 ± 5	8.4 ± 4.3
	ND8.5, high K	92	102	–32 ± 2	2.9 ± 1.7

All measures were made in ND7.5. Viability indicates the percent of oocytes that survived after 24-h incubation. Cells were considered dead when the current-voltage relationship was linear. Apparent diameters were chosen as an easy and quick approximation of cell volume (since they had a spherical shape when the measurement was made). Apparent diameters were normalized since from one batch of oocytes to the other the mean diameter of control oocytes could vary. Membrane resistance was measured at the holding potential (–35 m*V*). % indicates the relative diameter compared to control oocytes

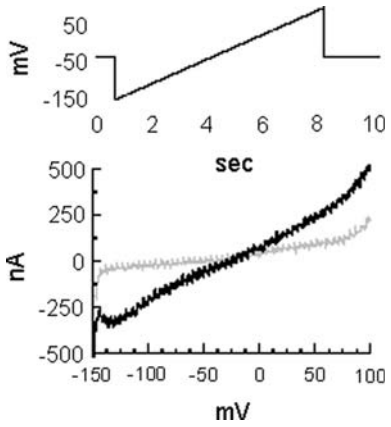

Fig. 2 6K induces a membrane conductance increase for all potentials between –150 and +100 m*V*. Whole-oocyte currents elicited by a ramp protocol for 6K cRNA-expressing oocytes (*black line*) and for control oocytes (*gray line*). Each trace is the average of at least six oocytes belonging to the same batch

niflumate and 10 mM gadolinium (Table 5). Taken together, these results show that 6K stimulated endogenous currents in *Xenopus* oocytes: both a calcium-dependent chloride current and a hyperpolarization-activated inward current were upregulated.

6K-Dependent Current Activation Results From A $[Ca^{2+}]_I$ Increase

I_{Cl} is now widely used as a reliable indicator of calcium fluxes in *Xenopus* oocytes (Kuruma and Hartzell 1999, 2000; Yao and Tsien 1997): $I_{Cl1\text{-}S}$ is activated by calcium release from internal stores, while I_{Cl2} and $I_{Cl1\text{-}T}$ are activated by capacitative calcium influx. Our results suggest that 6K expression might induce sustained calcium release and sustained calcium influx, thus resulting in all three components of I_{Cl} and I_{in} upregulation. This was confirmed by buffering the intracellular calcium by incubating oocytes in the presence of 50 μM BAPTA tetra-acetoxymethyl ester (BAPTA-AM). These incubation conditions reduced both I_{Cl} and I_{in} upregulation (Table 4). Thus, 6K may enhance two calcium-dependent currents by triggering a $[Ca^{2+}]_i$ increase by calcium release from the ER and/or by calcium entry from the extracellular medium.

We verified that I_{Cl} and I_{in} upregulation in oocytes expressing 6K indeed resulted from a raised $[Ca^{2+}]_i$ compared to control oocytes. Oocytes were incubated in ND8.5, and currents were first measured in ND7.5 and then with different known inhibitors of calcium flux to determine whether sustained calcium release and/or calcium influx are required for I_{Cl} and I_{in} activation. In this case, preventing a potential $[Ca^{2+}]_i$ increase should instantaneously reduce the intensities of both I_{Cl} and I_{in} (both are calcium-dependent) without interfering with the potential long-term modifications induced by 6K expression. Application of 5 mM caffeine, a known inhibitor of calcium release through $InsP_3$ receptors, reduced both I_{Cl} and I_{in} (Table 5), indicating that calcium release is needed for the upregulation of I_{Cl} and I_{in} in 6K-expressing oocytes. Reciprocally, current measurement in nominal zero calcium medium showed that $I_{Cl1\text{-}S}$, I_{in} and I_{out} were reduced while $I_{Cl1\text{-}T}$ and I_{Cl2} were surprisingly slightly increased (this may result from the activation of a calcium-inhibited current, see Amasheh and Weber 1999; Weber et al. 1995a, b; Zhang, McBride and Hamill, 1998). Thus, nominal zero calcium conditions are a slightly awkward means to test the implication of extracellular calcium. Nonetheless, we could

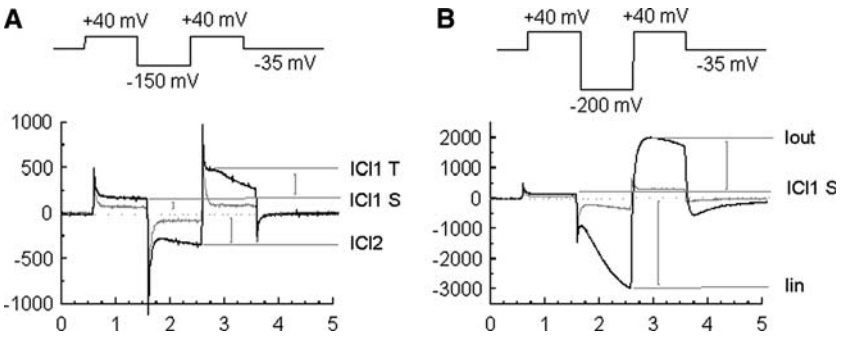

Fig. 3 6K-dependent activation of endogenous currents of *Xenopus* oocytes. Currents in 6K cRNA-expressing oocytes (*black line*) and control oocytes (*gray line*). (*A*) The three types of chloride currents are revealed by a triple-step protocol in control oocytes (*top*). Each step allows, respectively, the recording of a noninactivating outward Cl current ($I_{Cl1\text{-}S}$), a slow inward Cl current (I_{Cl2}) and a transient outward Cl current ($I_{Cl1\text{-}T}$). Outward $I_{Cl1\text{-}S}$ is measured at the end of the first pulse at +40 m*V*. $I_{Cl1\text{-}T}$ is calculated by measuring the peak current during the second pulse at +40 m*V* and subtracting $I_{Cl1\text{-}S}$. I_{Cl2} is measured at the end of the –150-m*V* pulse. (*B*) The hyperpolarization-activated inward current (I_{in}) is revealed by a step at –200 m*V*. Upon repolarization, a time-dependent outward current (I_{out}) is revealed. I_{in} and I_{out} were measured in a similar fashion to I_{Cl2} and $I_{Cl1\text{-}S}$. Each trace is the average of at least six oocytes belonging to the same batch

Table 3 Effect of incubation medium upon 6K-dependent upregulation of endogenous calcium-dependent chloride currents (I_{Cl})

Incubation medium	Incubation medium	$I_{Cl1\text{-}S}$	$I_{Cl1\text{-}T}$	I_{Cl2}	$I_{Cl\ in}$	$I_{Cl\ out}$
Control		69 ± 1	46 ± 1	–76 ± 2	–343 ± 2	273 ± 1
6K	ND8.5	169 ± 1	314 ± 1	–343 ± 3	–2957 ± 5	1851 ± 6
6K	ND7.5	197 ± 1	563 ± 1	–640 ± 1	–3495 ± 5	1759 ± 5

All measures were made in ND7.5. Values are given in nA for $n = 6$ oocytes

Table 4 Effect of incubation medium upon 6K-dependent upregulation of endogenous calcium-dependent chloride currents (I_{Cl})

Incubation medium	$I_{Cl1\text{-}S}$	$I_{Cl1\text{-}T}$	I_{Cl2}	$I_{Cl\ in}$	$I_{Cl\ out}$
ND8.5	100	100	100	100	100
ND7.5	116	179	186	118	95
ND8.5, 50 μ M BAPTA-AM	48	67	35	30	55
ND8.5, 5 mM caffeine	114	74	88	76	34
ND8.5, heparin microinjection	54	24	36	20	18
ND8.5, 0 Ca, 10 mM thapsigargin	85	29	75	38	19
ND8.5, 0.15 mM niflumate	92	74	43	12	6
ND8.5, 2.8 mM $BaCl_2$	126	77	94	4	8
ND8.5, high K	126	92	89	75	74
ND7.5, high K	20	21	34	102	83

All measures were made in ND7.5. Currents were normalized against cells expressing 6K and incubated in ND8.5 since from one batch to the other the 6K-dependent upregulation of I_{Cl} could vary. Current values are given as percentages of the currents recorded on oocytes incubated in ND8.5

confirm the implication of a calcium influx in I_{Cl} and I_{in} upregulation with lanthanum, which is a potent inhibitor of calcium currents. Application of 1 mM $LaCl_3$ drastically reduced both I_{Cl} and I_{in} (Table 5). Again, the reduction of $I_{Cl1\text{-}S}$ suggests that lanthanum has a more complex effect than simple inhibition of calcium influx channels.

The effect of 6K can start as soon as the RNA is translated in the cell. Thus, we investigated whether incubation in conditions that reduced either calcium release or calcium influx could reduce I_{Cl} and I_{in} upregulation. Cells were incubated in different conditions interfering with calcium release or calcium entry, and currents were recorded in ND7.5 medium. Currents were compared to the currents measured in oocytes incubated in ND8.5. In this case, preventing a potential $[Ca^{2+}]_i$ increase during incubation should result in a lesser upregulation of I_{Cl} and I_{in}. Implication of calcium release was confirmed by the effect of incubation with 5 mM caffeine and by the effects of microinjecting the cells with heparin (a known inhibitor of $InsP_3$ receptors). In both cases, I_{Cl} and I_{in} upregulation was reduced (Table 4). Implication of both a sustained calcium release and a sustained calcium influx was confirmed by experiments where the cells were preincubated for 1 h with 10 mM thapsigargin in zero calcium medium before injection of 6K cRNA; incubation was performed in the same medium. This protocol, which empties calcium stores and prevents their replenishment, leading to a decrease of the total oocyte calcium, also reduced both I_{Cl} and I_{in} upregulation (Table 4).

Table 5 Effect of external bathing composition upon 6K-dependent upregulation of endogenous calcium-dependent chloride currents (I_{Cl})

Bath	$I_{Cl1\text{-}S}$	$I_{Cl1\text{-}T}$	I_{Cl2}	$I_{Cl\ in}$	$I_{Cl\ out}$
ND 7.5	100	100	100	100	100
ND7.5, low Cl	83	13	61	43	3
ND7.5, 2.8 mM $BaCl_2$	67	78	69	17	19
ND7.5, 0.15 mM niflumate	82	40	58	15	22
ND7.5, 10 mM $GdCl_3$	69	3	14	16	29
ND7.5, 5 mM caffeine	63	19	55	62	77
ND7.5, zero Ca	90	101	108	81	29
ND7.5, 1 mM La Cl_3	61	5	35	3	12
ND8.5	127	59	93	82	88
ND6.5	60	100	110	121	107
ND5.5	43	110	110	116	112

All measures were made on oocytes incubated in ND8.5. Currents were normalized against control oocytes injected with water and incubated for 24 h in ND8.5 since from one batch to the other the 6K-dependent upregulation of I_{Cl} could vary. Current values are given as percentages of the intensities of the currents recorded in the ''standard recording medium'' i.e., ND7.5

Taken together, these results suggested that 6K induced a sustained depletion of the intracellular calcium stores and a long-lasting activation of the store-operated calcium (SOC) channels. Both can trigger upregulation of I_{Cl} and I_{in}. Our results show that instantaneously I_{Cl} and I_{in} are upregulated by a $[Ca^{2+}]$ increase. In the long term, 6K expression induces a higher level of I_{Cl} and I_{in} activity through a calcium-related mechanism. I_{in} upregulation in itself can also enhance the $[Ca^{2+}]$ increase: I_{in} can drive calcium influx since this current is carried by both chloride and cations.

$[Ca^{2+}]$ Elevation Results From Both Sustained Calcium Release and Calcium Influx

To check whether 6K induces calcium store depletion, we measured the intensity of $I_{Cl1\text{-}S}$ after application of the calcium ionophore A23187 in zero calcium medium. In these conditions, A23187 triggers only calcium release from the ER, the extracellular calcium entry being negligible. Thus, A23187 application in zero calcium medium resulted in activation of $I_{Cl1\text{-}S}$ without activation of $I_{Cl1\text{-}T}$ and I_{Cl2} and the intensity of $I_{Cl1\text{-}S}$ reflected the amount of calcium release by the ER (there is no calcium entry that would also increase $I_{Cl1\text{-}S}$). As illustrated in Figure 4, application of 0.5 μM A23187 induced an increase of $I_{Cl1\text{-}S}$ of only 58% of the one in control oocytes (425 ± 34 and 734 ± 100 nA, respectively), suggesting that the calcium stores were depleted in 6K cRNA-expressing oocytes. We could not perform the same experiment with I_{in} because of the strong current rundown.

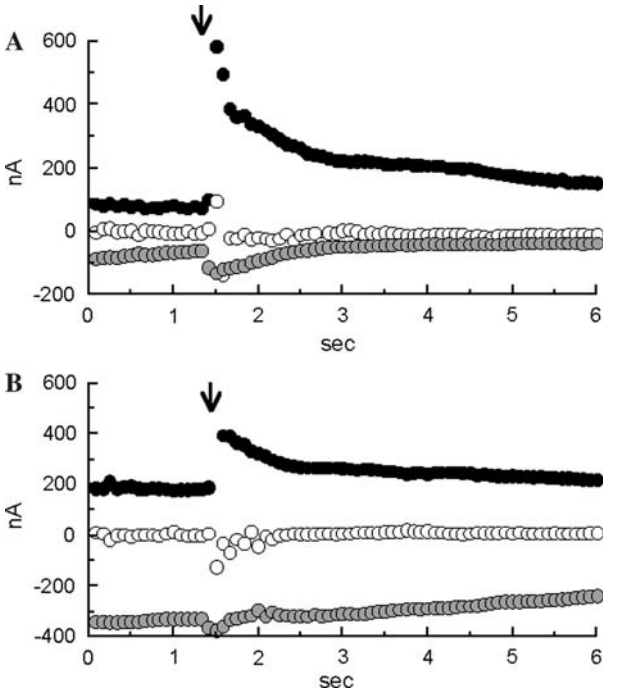

Fig. 4 6K-dependent depletion of the reticular calcium store. A23187 application (*arrow*) in calcium-free medium reveals that 6K induces depletion of reticular calcium stores. The intensity of the $I_{Cl1\text{-}S}$ current is indicative of the repletion state of the ER. (*A*) Control oocytes. (*B*) 6K cRNA-expressing oocytes. *Black circle*, $I_{Cl1\text{-}S}$; *white circle*, $I_{Cl1\text{-}T}$; *gray circle*, I_{Cl2}. Representative traces are illustrated

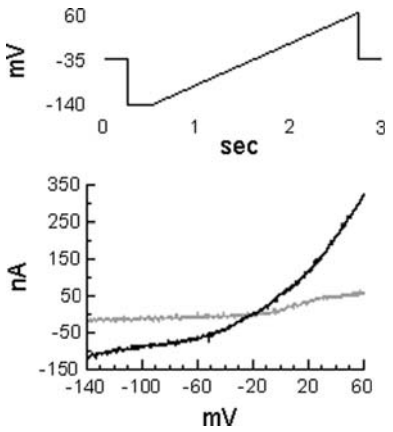

Fig. 5 6K-dependent activation of I_{SOCE}. I_{SOCE} in 6K cRNA-expressing oocytes (*black line*) and control oocytes (*gray line*). This trace is the average of seven oocytes belonging to the same batch. The protocol used is depicted at the top of the figure

We also measured I_{SOCE}, normally activated by calcium store depletion, with the previously established protocol (Machaca and Haun 2000). I_{SOCE} measured at –140 m*V* for oocytes expressing 6K and for control oocytes were –117 ± 22 and –9 ± 6 nA, respectively (Fig. 5), indicating that 6K indeed depletes the ER store and triggers I_{SOCE}.

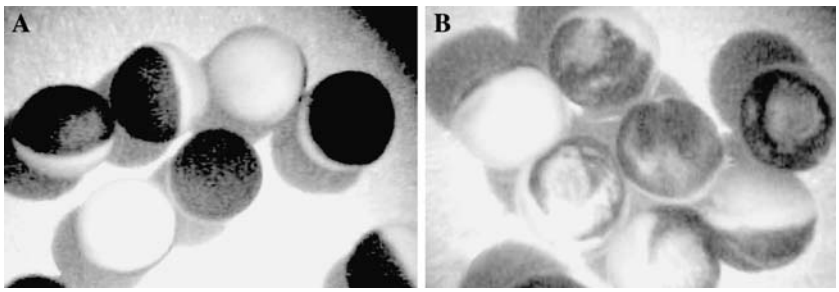

Fig. 6 Expression of 30 ng 6K cRNA induces strong cytological disruption of oocytes. (*A*) Control oocytes injected with an equivalent volume of water. (*B*) Oocytes show strong disorganization of the pigments at the animal pole after 32-h incubation at 19°C

$[Ca^{2+}]$ Elevation Contributes to 6K-Induced Cell Death in *Xenopus* Oocytes

Xenopus oocytes exhibited upregulated I_{Cl} and I_{in} currents 24 h after 6K cRNA microinjection when incubated in ND7.5 or ND8.5 medium. However, the high lethality observed in ND7.5 led us to preferentially incubate oocytes in ND8.5 medium, which minimized the deleterious effects of 6K expression in oocytes, even though from 24 h expression oocytes often had a spotty appearance (Fig. 6) associated with cell death. Simultaneously, electrophysiological recordings showed leaky membranes. We first asked whether 6K was cytotoxic to *Xenopus* oocytes.

Oocytes showed a time-dependent volume decrease from 24 h 6K expression onward. This volume decrease was pH-dependent: oocytes incubated in ND7.5 had a diameter of 79% of the initial diameter (0.92 ± 0.2 mm compared to 1.16 ± 0.1 mm) but ND8.5 oocytes had 85% of the initial diameter (0.99 ± 0.3 mm, Table 2). In addition, after 48 h of incubation, multiple black spots appeared over the animal pole of 6K-expressing oocytes. After 72 h of incubation, no cell survived.

The viability of the oocytes was evaluated by measuring the apparent diameter, membrane potential and membrane resistance values. Electrophysiologically, 6K-expressing oocytes were characterized by depolarized values of their resting membrane potential in a pH-dependent manner. In ND7.5, the resting membrane potential was drastically depolarized to –29 ± 1 m*V*, while in ND8.5 it was depolarized to only –41 ± 4 m*V* (control oocytes –61 ± 7 m*V*). In addition, 6K-expressing oocytes had very low membrane resistance values (Table 2). Thus, expression of 6K exerts significant cytotoxic effects in *Xenopus* oocytes, and the viability was better in ND8.5 incubation medium than in ND7.5 medium.

Long-lasting calcium increases are notorious for their cytotoxic effects. To check whether 6K-induced calcium elevation may contribute to the cytotoxic effects, we analyzed the effects of the buffering of any $[Ca^{2+}]_i$ elevation. Even though viability was less in BAPTA-AM solution, apparent diameter, membrane potential and resistance values were less affected by 6K expression and I_{Cl} and I_{in} upregulations were less drastic. These results indicate that the buffering of intracellular calcium significantly diminished but did not abolish the cytotoxic effects of 6K expression in *Xenopus* oocytes. Since we observed that the calcium elevation results from both sustained calcium release and calcium influx, we compared the effects of incubation in conditions where either one or the other was inhibited. Viability was increased in conditions reducing either calcium release (caffeine application, heparin microinjection and zero calcium in the presence of thapsigargin; *see* Table 2) or calcium influx (nominal zero calcium). Unfortunately, lanthanum, which inhibits I_{SOCE}, proved toxic during long-term incubation (even at as low a concentration as 0.1 mM); thus, we could not test the effects of direct inhibition of I_{SOCE} during incubation. Along with the lesser increase of the intensities of I_{Cl} and I_{in} currents, in all these experiments apparent diameter, membrane potential and resistance values were closer to control values (Table 2). These results indicate that in the absence of calcium release or calcium influx, the cytotoxic effects of 6K expression in *Xenopus* oocytes were reduced.

Finally, direct inhibition of I_{Cl} and I_{in} upregulation by incubation in the presence of inhibitors of these currents (niflumate, barium and gadolinium proved to be toxic at a concentration as low as 0.1 mM) also improved viability and reduced the permeabilization of the membrane. Thus, I_{Cl} and I_{in} upregulation in themselves are also involved in the cytotoxic effects of 6K, suggesting that I_{in} is indeed responsible for an autoamplification loop.

Cell Shrinkage Is a Secondary Consequence of 6K-Dependent Current Activation

Our data suggest that 6K primarily induces both a calcium store depletion and a sustained I_{SOCE} activation. This leads to a permanent leakage of calcium into the cytosol and, thus, to a $[Ca^{2+}]_i$ elevation, which likely upregulates endogenous chloride currents. In turn, this current activation is not without consequences. Indeed, cell shrinkage might be the result of a chloride efflux. In 6K cRNA-expressing oocytes, the chloride current is inward at the membrane potential measured at 24 h (at –40 m*V*, the ramp

protocol gives a current of –40 ± 08 nA for the experiment depicted in Fig. 2). This chloride efflux may then induce a potassium efflux and consequently a water efflux, which results in cell shrinkage. In this hypothesis, 6K would indirectly induce cell shrinkage through the calcium-dependent activation of chloride efflux.

To test this hypothesis, we first verified whether cell shrinkage is prevented when the chloride efflux is inhibited. Incubation with 10 mM $GdCl_3$ proved to be toxic to the cell, but incubation with either 0.15 mM niflumate or 2.8 mM $BaCl_2$ greatly reduced the 6K-dependent activation of $I_{Cl1\text{-}T}$ and I_{Cl2} (Table 5). Both incubation conditions, which reduced chloride efflux, also reduced 6K-induced cell shrinkage (Tables 2 and 4). We also checked whether cell shrinkage might be reduced when chloride efflux is activated but potassium efflux is prevented. When the cells were incubated in a medium reducing the potassium driving force (ND8.5 high K), the 6K-dependent activation of endogenous chloride currents was observed (even though the intensities measured were smaller than when the cells were incubated in ND8.5) but cell shrinkage, membrane depolarization and membrane permeabilization were reduced even when cells were incubated in ND7.5, a condition which worsens the effect of 6K expression (Table 2). Thus, 6K-dependent activation of endogenous chloride currents can be uncoupled from cell shrinkage. Interestingly, if cell shrinkage was reduced, cell death always occurred within 48 h, which suggests that cell death may be independent of both KCl efflux and cell shrinkage but may be another consequence of 6K expression or of 6K-dependent $[Ca^{2+}]_i$ elevation.

Discussion

$[Ca^{2+}]_I$ Elevation Is an Early Consequence of 6K Expression

When expressed in *Xenopus* oocytes, 6K induced both membrane depolarization and conductance increase. The endogenous calcium-dependent chloride currents (I_{Cl}) and the hyperpolarization-activated inward current (I_{in}) are strongly upregulated. I_{in} upregulation has been frequently observed in oocytes expressing small exogenous proteins (Kuruma et al. 2000; Shimbo et al. 1995; Tzounopoulos, Maylie and Adelman 1995). The very strong upregulation of I_{in} can explain the global permeabilization of the oocyte (as shown by membrane resistance and membrane potential decreases). I_{in} upregulation can mean the upregulation either of a cationic nonselective current or of a calcium-dependent chloride current (Kuruma et al. 2000). We favor the hypothesis that I_{in} upregulation results from a 6K-dependent $[Ca^{2+}]_i$ increase since I_{Cl} is also upregulated.

In our experimental conditions, i.e., incubation medium at pH 8.5 and perfusion solution pH 7.5, the transient outward current ($I_{Cl1\text{-}T}$) and the slow inward current (I_{Cl2}) were strongly upregulated. The effect was more drastic when cells were incubated in ND7.5. Both I_{Cl} and I_{in} were blocked by potent inhibitors of calcium-activated chloride currents: niflumate, gadolinium and barium.

Upregulation of endogenous conductances have been observed in *Xenopus* oocytes expressing various viral proteins (Schubert et al. 1996), but clear association to $[Ca^{2+}]_i$ variations and cell viability were not investigated. I_{Cl} upregulation is now considered a reliable indicator of calcium signaling. Our data support the fact that 6K leads to both sustained calcium release and calcium influx. Indeed, all three components of I_{Cl} are upregulated: $I_{Cl1\text{-}S}$ increase indicates calcium release (confirmed by evaluation of the state of replenishment of calcium stores), while $I_{Cl1\text{-}T}$ and I_{Cl2} increases indicate capacitative calcium entry (confirmed by I_{SOCE} measurement, *see* Kuruma and Hartzell 1999, 2000; Yao and Tsien 1997). Since $I_{Cl1\text{-}T}$ and I_{Cl2} are more strongly upregulated than $I_{Cl1\text{-}S}$, the calcium influx contribution to the $[Ca^{2+}]_i$ elevation would be stronger than the calcium release from the ER (especially since we have shown that it is depleted). However, the slight $I_{Cl1\text{-}S}$ upregulation suggests that the ER has lost its capacity to buffer any $[Ca^{2+}]_i$ elevation since it continuously leaks calcium inside the cytosol.

A long-lasting $[Ca^{2+}]_i$ elevation due to 6K expression was confirmed by the reductions of the different electrophysiological and cytological consequences of 6K expression when $[Ca^{2+}]_i$ elevation is prevented (by incubation in the presence of BAPTA-AM) and when calcium release and calcium influx are inhibited (by caffeine, heparin, thapsigargin and zero calcium treatments).

These data suggest that expression of 6K could thus upregulate I_{Cl} and I_{in} by triggering a sustained $[Ca^{2+}]_i$ elevation. Furthermore, upregulation of I_{in} can increase any $[Ca^{2+}]_i$ elevation since one of the components of I_{in} is a cationic nonselective current that readily draws calcium inside the cell (Kuruma et al. 2000). An initial 6K-dependent $[Ca^{2+}]_i$ elevation could thus be amplified. This autoamplification loop appears to be disrupted when the cells are incubated in the presence of niflumate or barium, two inhibitors of both of I_{Cl} and I_{in}. As predicted, both currents remained small in such conditions, but the other consequences of 6K expression (membrane permeabilization, conductance increase, lethality and cell shrinkage) were also reduced, showing that the global effects of 6K are diminished. One should not underestimate the importance of ''side effect'' calcium fluxes. For instance, the MEC-4(d) sodium channel is responsible for calcium-dependent neurotoxicity. This sodium channel is also permeable to calcium; its upregulation is sufficient to induce a calcium

influx which itself induces calcium release form the ER. The resulting $[Ca^{2+}]_i$ elevation contributes to necrosis activation (Bianchi et al. 2004).

The link between 6K expression and $[Ca^{2+}]_i$ elevation is still elusive. When inserted in planar lipid bilayers, 6K protein from another alphavirus (Ross River virus) showed conductances between 400 and 800 pS, suggesting that the protein is capable of oligomerization (Melton et al. 2002) and formed cation nonselective channels ($Na^+>K^+>Ca^{2+}>>Cl^-$) (Fischer et al. 2000). The sustained calcium influx revealed by $I_{Cl1\text{-}T}$ and I_{Cl2} upregulation could directly result from the insertion of 6K proteins in the plasma membrane. However, we detected no intrinsic channel properties for 6K when expressed in *Xenopus* oocytes. The intrinsic channel properties of 6K might be masked by the stronger upregulation of endogenous channels. Such an experimental drawback was observed for two viral proteins, the M2 protein of influenza virus (Kelly et al. 2003) and Vpu of HIV-1 (Coady et al. 1998), and for two nonviral proteins, the cellular protein IsK and the synthetic protein SYN-C (Shimbo et al. 1995).

Nonetheless, we favor another explanation that does not preclude a direct contribution of 6K to membrane permeabilization but that takes into account the fact that 6K is not only inserted in the plasma membrane during the infection. 6K channel activity could otherwise permeabilize the ER membrane. Indeed, during viral infection, 6K is essentially inserted in the ER membrane (Lusa, Garoff and Liljestrom 1991). 6K could lead to calcium leakage from the ER either by regulating the endogenous ER channel (as the NCC and CaBPs proteins, which interact with $InsP_3$ receptor [Burgoyne et al. 2004]) or by carrying a novel conductance (as supposedly wolframin, which, when expressed in *Xenopus* oocytes, induces a $[Ca^{2+}]_i$ elevation through the appearance of a novel cation-selective channel activity in the ER membrane [Osman et al. 2003]). This second hypothesis appears more probable since planar lipid bilayer experiments showed that 6K forms calcium-permeable cationic channels. Indeed, $I_{Cl1\text{-}T}$ and I_{Cl2} activation are characteristics of the activation of I_{SOCE} currents (Kuruma and Hartzell 1999, 2000; Yao and Tsien 1997). Long-lasting I_{SOCE} activation was confirmed by direct measurement and by the effects of $LaCl_3$. However, long-lasting calcium influx is surprising since I_{SOCE} activation is classically a transient phenomenon, brought to an end by the replenishment of the intracellular calcium store. This paradox could be explained by a permanent leakage of calcium from intracellular stores due to the insertion of 6K proteins in the ER membrane, which would activate I_{SOCE}. $I_{Cl1\text{-}S}$ upregulation suggested permanent calcium release, which was confirmed by its inhibition by incubation in the presence of different inhibitors of calcium release. Sustained calcium release and subsequent calcium store depletion were verified by the experiment using A23187 in zero calcium medium. Consequently, 6K induced continuous calcium leakage from both the intracellular and the extracellular stores. These calcium influxes would induce a $[Ca^{2+}]_i$ elevation that, in turn, activates I_{Cl} and I_{in}. Direct implication of $[Ca^{2+}]_i$ elevation on I_{Cl} and I_{in} upregulation was finally confirmed by the buffering experiments with BAPTA-AM and by experiments where cells were incubated in zero calcium in the presence of thapsigargin (which empties the calcium stores and prevents their replenishment). Taken together, these experiments confirmed that $[Ca^{2+}]_i$ elevation is an early event induced by 6K expression due to the putative viroporin properties of 6K.

Cell Shrinkage and Lethality are Secondary Consequences of 6K Expression

Later events induced by 6K are cell shrinkage and ultimately cell death. Cell shrinkage is a likely osmotic consequence of I_{Cl} and I_{in} upregulation. Indeed, at the resting membrane potential measured in 24 h 6K-expressing oocytes, both I_{Cl} and I_{in} upregulation induced an inward current. In turn, this net chloride efflux can electrochemically induce potassium efflux. KCl loss osmotically drives a water efflux and, thus, cell shrinkage. A similar cascade of events was observed in *Xenopus* oocytes expressing heterologous chloride channels: this heterologous expression increased chloride efflux, resulting in KCl-dependent cell shrinkage (Cooper and Fong 2003). In 6K-expressing oocytes, by reducing external chloride concentration in the incubation medium and by incubating the cells in the presence of either niflumate or barium, we showed that inhibition of the chloride efflux reduced cell shrinkage. Furthermore, dissociation of chloride fluxes from potassium fluxes (by increasing external potassium concentration) also reduced cell shrinkage. Thus, KCl-driven water fluxes could be dissociated from I_{Cl} activation. Cell shrinkage would thus be an indirect consequence of 6K expression.

Cell death appears independent of cell shrinkage since it persists when cell shrinkage is prevented. Cell death might result directly from disruption of calcium homeostasis since increased $[Ca^{2+}]_i$ is notoriously lethal (Tang et al. 2005). Conditions that decreased the $[Ca^{2+}]_i$ elevation (as reported by I_{Cl} levels) delayed cell death without abolishing it.

Indeed, viability was improved when cells were incubated at pH 8.5 rather than at pH 7.5. The smaller increase of I_{Cl} and I_{in} in more alkaline pH would suggest a lesser $[Ca^{2+}]_i$ elevation in these conditions. Since I_{in} is also pH-dependent, its intensity being reduced in alkaline pH, incubation in ND8.5 would reduce the autoamplification loop. Furthermore, this better viability could also result

from the marked decrease of I_{SOCE} intensity for alkaline extracellular pH (Gillo et al. 1996), thus reducing the calcium influx.

A $[Ca^{2+}]_i$ elevation has often been observed during infection by numerous viruses (Cheshenko et al. 2003; Irurzun et al. 1995; Nokta et al. 1987; Perez et al. 1999; Sanderson et al. 1996; Shainkin-Kestenbaum et al. 1993; van Kuppeveld et al. 1997). In a mammalian or insect context, direct association between expression of a viral protein and a $[Ca^{2+}]_i$ elevation was established only for the A38L protein of vaccinia virus (Sanderson et al. 1996), the 2B protein of coxsackievirus (van Kuppeveld et al. 1997) and the 2BC protein of poliovirus (Aldabe, Irurzun and Carrasco 1997). In these experiments, expression of recombinant viral proteins induced both a $[Ca^{2+}]_i$ elevation and cell death. The $[Ca^{2+}]_i$ elevation is a probable culprit for cell cytotoxicity, but no clear demonstration was performed. Here, we have shown in *Xenopus* oocytes that experiments leading to a lesser 6K-dependent $[Ca^{2+}]_i$ elevation also reduced the cytotoxic effect, thus suggesting that the $[Ca^{2+}]_i$ elevation is indeed responsible for cell death.

Therefore, we propose that 6K induces calcium homeostasis disruption, through calcium store depletion and subsequent I_{SOCE} activation. $[Ca^{2+}]_i$ elevation in turn activates I_{Cl} and I_{in}, resulting in KCl efflux inducing cell shrinkage. Ultimately, the sustained $[Ca^{2+}]_i$ elevation would lead to cell death.

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