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ORIGINAL ARTICLES

Calcium Dynamics During Physiological Acidification in *Xenopus* Oocyte

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Abstract Interplays between intracellular pH (pHi) and calcium ($[Ca^{2+}]_i$) variations remain unclear, though both proton and calcium homeostasis changes accompany physiological events such as *Xenopus laevis* oocyte maturation. In this report, we used NH_4Cl and changes of extracellular pH (pHe) to acidify the cytosol in a physiological range. In oocytes voltage-clamped at −80 mV, NH_4Cl triggered an inward current, the main component of which is a Ca^{2+}-dependent chloride current. Calcium imaging confirmed that NH_4Cl provoked a $[Ca^{2+}]_i$ increase. The mobilized sources of calcium were discriminated using the triple-step protocol as a means to follow both the calcium-activated chloride currents (ICl-Ca) and the hyperpolarization- and acid-activated nonselective cation current (I_{In}). These currents were stimulated during external addition of NH_4Cl. This upregulation was abolished by BAPTA-AM, caffeine and heparin. By both buffering pHi changes with MOPS and by inhibiting calcium influx with lanthanum, intracellular acidification, initiated by NH_4Cl and extracellular acidic medium, was shown to trigger a $[Ca^{2+}]_i$ increase through both calcium release and calcium influx. The calcium pathways triggered by pHe changes are similar to those activated by NH_4Cl, thus suggesting that there is a robust signaling mechanism allowing the cell to adjust to variable environmental conditions.

Keywords *Xenopus* · Oocyte · Calcium homeostasis · Intracellular pH

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Introduction

Intracellular acidification could be associated with an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (Siskind et al. 1989; Wakabayashi and Groschner 1997), but on the contrary, calcium variations have been coupled with alkalization (Dube and Epel 1986; Kim and Smith 1988; Kottgen et al. 1994; Naccache et al. 1988). Moreover, changes of intracellular pH (pHi) may mobilize calcium from various sources. For example, in rat inner medullary collecting duct cells, an NH_4Cl-induced pHi decrease facilitates inositol 1,4,5-triphosphate (InsP3) synthesis, leading to increase calcium release (Slotki et al. 1993). However, the mechanisms driving interplay between Ca^{2+} and pHi homeostasis remain unclear.

Mitogenic signaling triggers changes of pHi and modifications of intracellular calcium mobilization (Lipskaia and Lompre 2004; Munaron et al. 2004; Whitaker 2006). These ionic events occur at crucial transition phases of the cell cycle, in particular during M-phase entry (Whitaker 2006). The *Xenopus* oocyte provides a useful model system to study the interconnection between pHi changes and Ca^{2+} variations. Full-grown oocytes are physiologically arrested in the prophase of the first meiotic division. They resume meiosis upon hormonal stimulation by progesterone. The pioneering work of Lee and Steinhardt (1981) described a transient alkalization during *Xenopus* oocyte meiosis. Maturing oocytes alkalize by an average 0.2–0.4 pH unit, then pHi decreases back to almost its original value just before germinal vesicle breakdown (GVBD). This appears to be a common process since it was also observed in urodele. The injection of M-phase promoting factor (MPF, the complex cyclin B/cdK1) into *Xenopus* oocytes induces a similar pattern of pHi changes, and the microinjection of the conserved peptide sequence—PSTAIR—of cdK1 induces a

calcium transient current as in starfish oocytes (Picard et al. 1990). Thus, there could be a strong link between pH and calcium dynamics, both being necessary for meiosis resumption. Indeed, it has been demonstrated that calcium signals regulate the timing of meiosis entry of *Xenopus* oocytes by negatively regulating the mitogen-activated protein kinase (MAPK)-MPF cascade and that calcium is required for the completion of meiosis I (Sun and Machaca 2004). During meiosis, the mechanisms underlying the regulation of calcium homeostasis are modified: MPF blocks the coupling between store depletion and store-operated Ca entry (SOCE) (Machaca and Haun 2002).

We previously reported that during *Xenopus* oocyte meiosis the pHi rise was coupled to cellular events such as germinal vesicle migration towards the apex of the cell and that it finely regulate the early molecular events driving G_2/M transition of the cell cycle (Flament et al. 1996). On the contrary, intracellular acidification delayed the MAPK pathway induced by progesterone. Moreover, when stimulated by the active form of MAPK, this intracellular acidification would not promote M-phase entry (Sellier et al. 2006).

In *Xenopus* oocytes, pHi can be modulated either by changing extracellular pH (pHe, which triggers a reflecting pHi change) or with different compounds (Roos and Boron 1981). Weak bases are often used, even though in most cells they induce intracellular alkalization. These weak bases increase pHi after membrane permeation and subsequent cytosolic protonation of their deprotonated form (Burckhardt et al. 1992). Such is the case in sea urchin eggs activated by NH_4Cl or procaine (Lopo and Vacquier 1977; Shen and Steinhardt 1978). Contrarily, in *Xenopus* oocytes, primary, secondary and tertiary amines such as NH_4Cl, trimethylamine (Burckhardt and Thelen 1995) and procaine (Rodeau et al. 1998) were found to trigger a slow pHi acidification associated with a large depolarization and membrane resistance decrease. These two events have different kinetics: The NH_4Cl-induced acidification is slow and reverses in about 20 min, while the electrophysiological changes are instantaneously reversed after NH_4Cl washout (Boldt et al. 2003; Burckhardt and Burckhardt 1997; Burckhardt and Fromter 1992; Burckhardt and Thelen 1995; Cougnon et al. 1996).

In this study, we used NH_4Cl and pHe changes as a means to vary pHi and recorded the endogenous calcium-activated chloride currents that can be used as real-time indicators of cytosolic calcium concentration (Hartzell et al. 1997; Machaca and Hartzell 1998, 1999a, b). These conditions induced the upregulation of all of the calcium-activated chloride currents studied and led to the intracellular increase of Ca^{2+} as measured by fluorescent dyes. The slow and lingering decrease of pHi induced by NH_4Cl happened simultaneously with a rapid and reversible calcium release from the intracellular stores, followed by calcium influx from the extracellular medium. $[Ca^{2+}]_i$ variations appear to result directly from the pHi decrease since they were induced by both NH_4Cl applications and changes of pHe.

Materials and Methods

Solutions and Chemicals

Except fluorescent dyes, which came from Invitrogen (Cergy Pontoise, France), all chemicals were purchased from Sigma (Milwaukee, WI). ND96 medium composition was as follows (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$ and 1.8 $CaCl_2$ (pH 7.4 adjusted with NaOH). NH_4Cl solutions were made by replacing an equivalent amount of NaCl by NH_4Cl to have iso-osmotic solution. Low-Cl^- solution was made by replacing NaCl by Na-isothionate; thus, the final Cl^- concentration was reduced from 103.6 to 7.6 mM.

Xenopus Oocyte Isolation

Experiments were performed on defolliculated oocytes obtained from adults *Xenopus laevis* anesthetized by immersion in MS 222 solution (2 g l^{-1}). Ovaries were stored in ND96 medium. Oocytes [stage VI according to Dumont's (1972) classification] were arrested at prophase I. Isolation and defolliculation were done by manual dissection with forceps after 45-min incubation in 1 mg ml^{-1} collagenase, and cells were then stored for up to 96 h in ND96 medium (pH 7.5) at 14°C before use.

Electrophysiological Measurements

Oocytes were voltage-clamped at room temperature using a custom-made two-electrode amplifier in a Perspex chamber (diameter 4 mm) containing ND96 medium. Oocytes were impaled by two 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. Experimental protocols were controlled by pCLAMP 6 software through a Digidata 1200B interface (Axon Instruments, Foster City, CA).

The current recorded on oocytes voltage-clamped at −80 mV (I_{-80}) is a long-used and reliable mean of recording calcium-dependent chloride currents activated both by calcium release from the intracellular stores and by calcium influx from the extracellular medium (Miledi and Parker 1984).

For I–V relationships, voltage ramps were done between −140 and +100 mV before and just after pH modifications. In voltage-clamp experiments, oocytes were held at −40 mV and stepped to +40 mV for 1 s, to −140 mV for 1 s and then back to +40 mV. This triple-step protocol, modified from Kuruma and Hartzell (1999), allows respectively the recording of a noninactivating outward current (ICl1-S, measured at the end of the first pulse at +40 mV), a slow inward current (ICl2, measured at the end of the −140-mV pulse) and a transient outward current (ICl1-T, calculated by measuring the peak transient current during the second pulse at +40 mV and subtracting ICl1-S). All three components of the calcium-dependent chloride current (ICl-Ca) are carried by chloride.

The inward hyperpolarization-activated current (I_{In}) was measured with a similar triple-step protocol, where the second step was made at −200 mV instead of −140 mV (Figs. 4, 5). Thus, ICl1-S was again measured at the end of the first pulse at +40 mV. I_{In} was measured at the end of the −200-mV pulse, and I_{Out} was calculated by measuring the transient current during the third pulse at +40 mV and subtracting ICl1-S. When ICl-Ca or I_{In} currents were measured over time, to measure the current intensities as accurately as possible, while reducing current rundown, triple-step protocols were applied at 5-s intervals. Repeated applications of the same NH_4Cl concentration reduced the current intensity; therefore, experiments were preferentially performed on different oocytes except when explicitly stated.

Variations of $[Ca^{2+}]_i$ were inhibited by 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM, 50 μM) incubation at least 1 h before NH_4Cl application. To test whether the calcium rise involved calcium release from the endoplasmic reticulum through InsP3 receptors, oocytes were incubated for 1 h with caffeine (5 mM) or microinjected with 20 nL of a 50 mg/ml heparin solution 1 h before NH_4Cl application. ND nominal 0-Ca^{2+} medium or $LaCl_3$ (1 mM) was used to block calcium entry. In some experiments, we microinjected 50 nl per oocyte of a morpholino propane sulfonic acid (MOPS) buffer solution [400 mM, pH 6.9 (Flament et al. 1996)] to buffer pHi changes. Distilled water was used as control for microinjection experiments.

Data and Statistics

Recording data are shown typically for an experiment run on one oocyte, but the findings were always confirmed by recordings on at least three oocytes belonging to the same batch (isolated from a single female). This was repeated for at least three batches.

Values are given as the mean ± standard error of the mean (SEM) with $n \geq 9$, where n is the number of oocytes, and $N \geq 3$, where N is the number of batches. Statistical significance was assessed using Student's paired t test versus control and accepted as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

pH and Calcium Imaging

For pH imaging, oocytes were incubated in ND96 medium containing acetoxymethyl-ester derivative of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (the pH-sensitive fluorescent probe BCECF-AM, 5 μM). Excitation was performed at 440 and 490 nm, with 530 nm for emission. For calcium imaging, oocytes were loaded with the calcium-sensitive nonratiometric fluorescent probe Fluo4 (acetoxymethyl-ester derivative, Fluo4-AM, 25 μM; excitation performed at 494 nm and emission at 516 nm). The dyes were allowed at least 1 h (BCECF-AM) or 5 h (Fluo4-AM) to equilibrate within the oocytes.

Fluorescence experiments were performed at room temperature (18–20°C) in ND96 solution with or without acidification challenge. Acquisitions were done on the animal hemisphere of the oocytes using an epifluorescence scanning system fitted to a Nikon (Paris, France) microscope. Images were collected with Nikon software and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Fluorescence was expressed in arbitrary units, and background fluorescence was subtracted. Data obtained from these experiments were from multiple oocytes (at least three batches and three oocytes for each).

Results

NH_4Cl Triggers an Inward Current in *Xenopus* Oocytes Voltage-Clamped at −80 mV

In a first set of experiments, NH_4Cl addition in medium containing *Xenopus* oocytes voltage-clamped at −80 mV always triggered a sustained inward current, often preceded by a transient current (29 out of 58 oocytes, $N = 8$). The activation of this NH_4Cl-induced inward current was dose-dependent (respectively, −13 ± 4, −50 ± 6 and −104 ± 14 nA for 1, 5 and 10 mM NH_4Cl, $n = 6$) (Fig. 1a).

The slow inward current was sustained as long as NH_4Cl was present and was reversed in seconds following NH_4Cl washout (Fig. 1).

As shown in Fig. 1a, currents induced by NH_4Cl could be superimposed with oscillations (12 out of 58 oocytes, $N = 8$). The amplitude of such oscillations increased with the length of NH_4Cl application (Fig. 1b). For batches with oscillating oocytes, the mean sustained current was −124 ± 8 nA ($n = 7$), while for batches of nonoscillating oocytes,

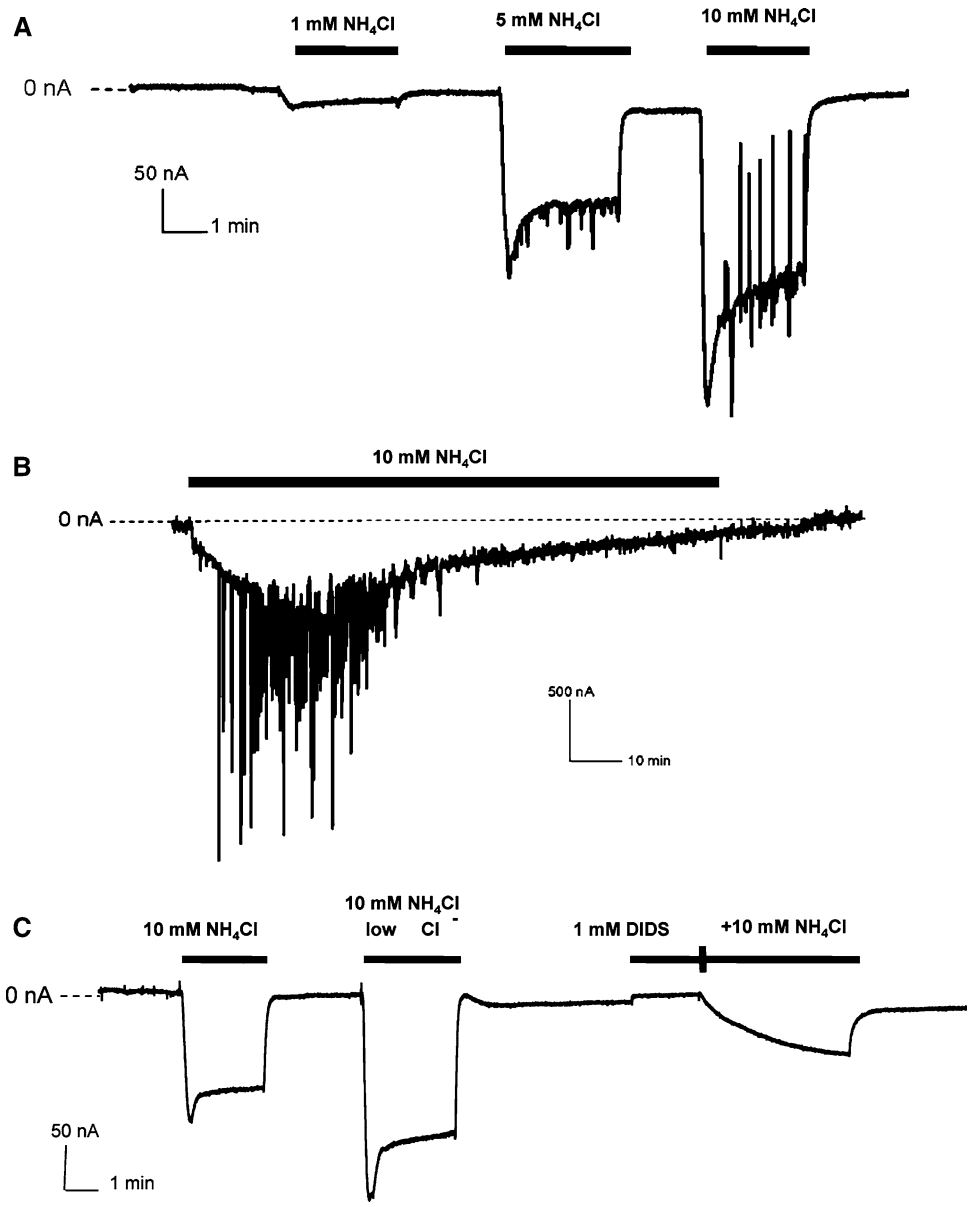

Fig. 1 Effect of NH_4Cl on I_{-80}. **a–c** Inward currents were measured on oocytes voltage-clamped at −80 mV. Representative recordings are depicted. **a** Solutions of NH_4Cl (1, 5 and 10 mM, *black line*) were successively perfused in the chamber. **b** Long-term application of NH_4Cl, responsible for an inward current superimposed with oscillations. **c** The oocyte was successively exposed to 10 mM NH_4Cl in normal ND96, in a medium depleted in chloride (low Cl^-, 7.6 mM chloride) and in 1 mM DIDS alone and with 10 mM NH_4Cl

the sustained current was -76 ± 9 nA ($n = 7$; t test $P < 0.01$). A positive correlation appeared between highly sustained, high-transient currents and oscillations. In *Xenopus* oocytes such transient currents and oscillations are usually associated with activation of the endogenous Ca^{2+}-activated chloride channels. Thus, we checked whether the activation of calcium-dependent chloride currents could be implicated in the NH_4Cl-induced inward current.

Voltage-ramp experiments (I–V plots, Fig. 2) showed reversal potentials (E_{rev}) in various experimental conditions. In the presence of NH_4Cl, E_{rev} shifted from −40 mV to a mean value of −25 mV ($n = 12$; Fig. 2a, b). It is also important to note that BAPTA-AM incubation and MOPS injection did not affect this potential (Fig. 2a). This potential value was close to the inversion potential of chloride ions (between −14 and −28 mV, see Weber 1999). When we used ND96 pH 5.5, again, E_{rev} ranged between −25 and −15 mV ($n = 10$, Fig. 2c). In addition, the same voltage-ramp experiments carried out in chloride-depleted medium (low Cl^-) revealed a reverse potential value of –10 mV, which shifted to +20 mV with 10 mM NH_4Cl (Fig. 2d; E_{inv} Cl^- in low-Cl^- medium was about +27 mV). Taken together, these results were consistent with the activation of a Ca^{2+}-dependent chloride current by intracellular acidification with either NH_4Cl or ND96 pH 5.5.

Moreover, the use of the Cl^- transport blocker 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) and chloride-depleted ND96 (ND low Cl^-) suggested that the Cl^- current was an important contributor to the NH_4Cl-dependent

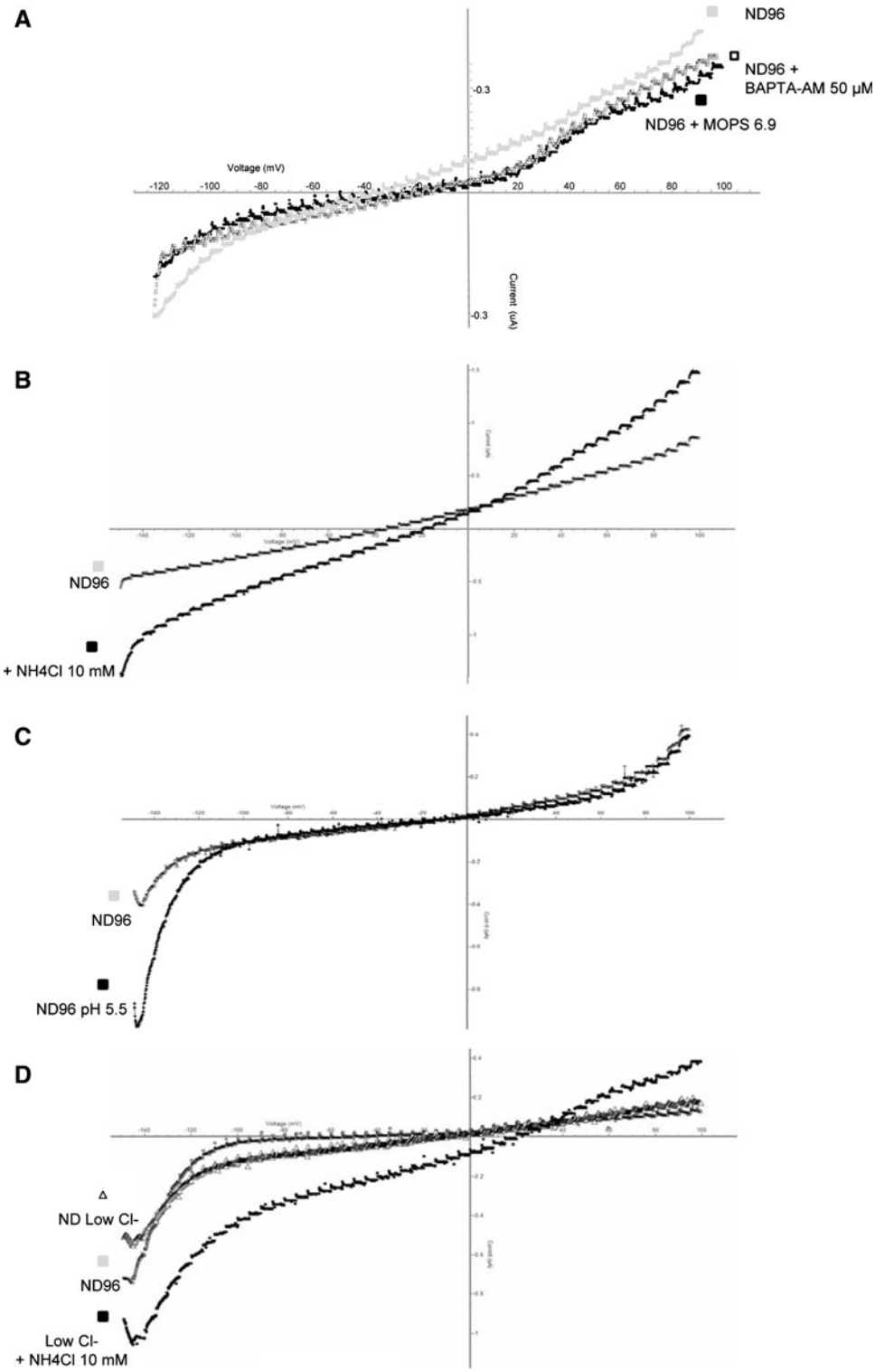

Fig. 2 I–V relationships. **a–d** Typical I–V relationships are depicted ($N \geq 3$). **a** I–V curves in ND96 (*gray squares*), BAPTA-AM (*white squares*) and MOPS pH 6.9 (*black squares*). **b** I–V curves in ND96 (*gray squares*) and after NH_4Cl 10 mM addition (*black squares*). **c** I–V curves in ND96 (*gray squares*) and in ND96 pH 5.5 (*black squares*). **d** I–V curves in ND96 (*gray squares*), ND low Cl^- before (*white triangles*) and after (*black squares*) NH_4Cl 10 mM addition

inward current (Fig. 1c). In ND96, the steady-state inward current induced by 10 mM NH_4Cl was -81 ± 4 nA ($N \geq 3$). When chloride was replaced by sodium isothionate, this chloride-depleted conditions increased the steady-state inward current to -101 ± 9 nA; when 1 mM DIDS was added to the normal bath solution, the steady-state inward current was reduced by a mean factor of 53% (-38 ± 3 nA, $n = 6$, Fig. 1c). Preincubation of oocytes with the calcium chelator BAPTA-AM (50 µM) or the injection of heparin or caffeine (inhibitors of IP3 calcium release) reduced dramatically the NH_4Cl-induced inward current (respectively, -30 ± 10, -10 ± 10 and -20 ± 10 nA, $n = 9$; Fig. 7).

These results suggested that this inward current was composed by Ca^{2+}-dependent chloride currents associated with at least one other uncharacterized component.

Extracellular NH_4Cl Induces an Intracellular Calcium Increase in *Xenopus* Oocytes

The Ca^{2+}-dependent chloride component described above could be initiated by an intracellular calcium rise triggered by NH_4Cl. To test this hypothesis, we first monitored pHi and intracellular calcium variations during NH_4Cl treatment by loading oocytes with BCECF-AM or Fluo4-AM. In a second set of experiments, we explored ICl-Ca using the electrophysiological approach. We also monitored I_{In}, which is a hyperpolarization and acid-activated nonselective cation current, stimulated by cytosolic acidification and permeable to Ca^{2+} ions.

Addition of 10 mM NH_4Cl to the bath, at pHe 7.5, provoked a BCECF fluorescence decrease, which is consistent with a cytosolic acidification (Fig. 3a). During washout, BCECF fluorescence came back to the control level in 5–25 min (in 7/12 oocytes, $N = 3$). The calcium fluorescence instantaneously increased after external application of 10 mM NH_4Cl. Figure 3b shows a typical response, with the nonratiometric fluorescent dye Fluo4 ($N = 3$, $n = 9$). These direct recordings with fluorescent dyes clearly confirmed that NH_4Cl not only decreased pHi but also simultaneously increased intracellular calcium. In addition to these experiments, as depicted in Fig. 3c and d, the superfusion of an acidic external medium (pH 5.5) promoted both a pHi decrease and an intracellular calcium fluorescence rise. Taken together, these imaging results strongly suggested that intracellular acidification triggered intracellular calcium modifications.

In order to discriminate the mobilized sources of calcium (either from endoplasmic reticulum stores or from extracellular medium) during intracellular acidification-dependent Ca^{2+} increases, the ICl-Ca kinetics was followed with a triple-step protocol (Fig. 4a), revealing the two different triggers: ICl1-S is activated by calcium release from internal stores, ICl2 by calcium influx and ICl1-T both by calcium release from stores and by calcium influx (Hartzell 1996) (Fig. 4).

A typical response of the oocyte to NH_4Cl (10 mM) is depicted in Fig. 3a and b. The magnitude of the three components of ICl-Ca first transitorily increased and then stabilized at a steady-state value, which was augmented by a mean factor of 2.5 (respectively, ×2.9 [ICl1-S], ×2.5 [ICl2], ×2.6 [ICl1-T]; Figs. 4b, 5, 7). The rise of the three components of ICl-Ca was consistent with the signatures of a $[Ca^{2+}]_i$ increase through both calcium release and calcium influx. The hyperpolarization-activated inward current I_{In} and I_{Out} were also augmented by a factor 2.2 (Figs. 5, 7). Interestingly, physiological acidification, using ND96 pH 5.5, more potently upregulate ICl-Ca and I_{In} currents (Figs. 4c, 7; respectively, by a factor ×7 [ICl2] and ×8.8 [ICl1-T]). These results suggested that the extracellular acidification more effectively influenced Ca^{2+} influx from the extracellular medium than NH_4Cl. Kuruma et al. (2000) reported that extracellular acidification produces a dramatic increase of I_{In}. Thus, the superfusion of the oocytes with the acidic solution ND96 pH 5.5 triggered a strong augmentation of I_{In} and more slightly I_{Out} (Figs. 5, 7). This confirmed the pH dependence of I_{In}.

The evidence for relationships between intracellular acidification and calcium fluxes comes from experiments where cytosolic calcium was buffered by incubating oocytes for 1 h in the presence of 50 μM BAPTA-AM prior to NH_4Cl application (Fig. 6a). The current upregulations induced by 10 mM NH_4Cl were impaired except for ICl2, which was only slightly reduced compared to untreated oocytes (Fig. 7).

To efficiently block calcium release from the endoplasmic reticulum, we used well-known inhibitors of InsP3 receptors. Incubation in the presence of 5 mM caffeine led to surprising observations: The effects of NH_4Cl were often amplified (in 57% of cells, $n = 24$, $N \geq 5$), while caffeine incubation prevented ICl-Ca and I_{In} upregulation in the other cells. In those cases, ICl1-S and ICl1-T upregulations were abolished (Fig. 7). Inhibition of IP3R calcium release by heparin injection significantly reduced the 10 mM NH_4Cl-dependent upregulation of ICl1-S, ICl2 and I_{In} and the current intensities were comparable to those in untreated oocytes (Fig. 7a, b).

Calcium influx from the extracellular medium may be reduced by incubating cells in a Ca^{2+}-depleted medium. Nominal 0-Ca^{2+} medium reduced NH_4Cl-induced ICl-Ca and I_{In} upregulations (Fig. 7a, b). Furthermore, lanthanum, a prototypic inhibitor of I_{SOCE}, was used to block calcium influx. Incubation in $LaCl_3$ effectively blocked the rise of ICl-Ca and I_{In} currents induced by NH_4Cl treatment (Fig. 7a, b). In both conditions that reduced calcium influx (nominal 0-Ca^{2+} medium and 1 mM $LaCl_3$), 10 mM NH_4Cl-dependent upregulations of ICl1-S, ICl2, ICl1-T and I_{In} were significantly reduced and the current intensities were comparable to those in control oocytes. Thus, NH_4Cl induces a calcium influx inside the oocyte, which increases ICl2, ICl1-T and I_{In} (Fig. 7).

These results strongly suggested that NH_4Cl induces calcium release through IP3R stimulation, which in turn upregulates ICl1-S and I_{In}. Upregulation of ICl2, ICl1-T and I_{In} would be then induced by the depletion of calcium store that triggers capacitative calcium entry.

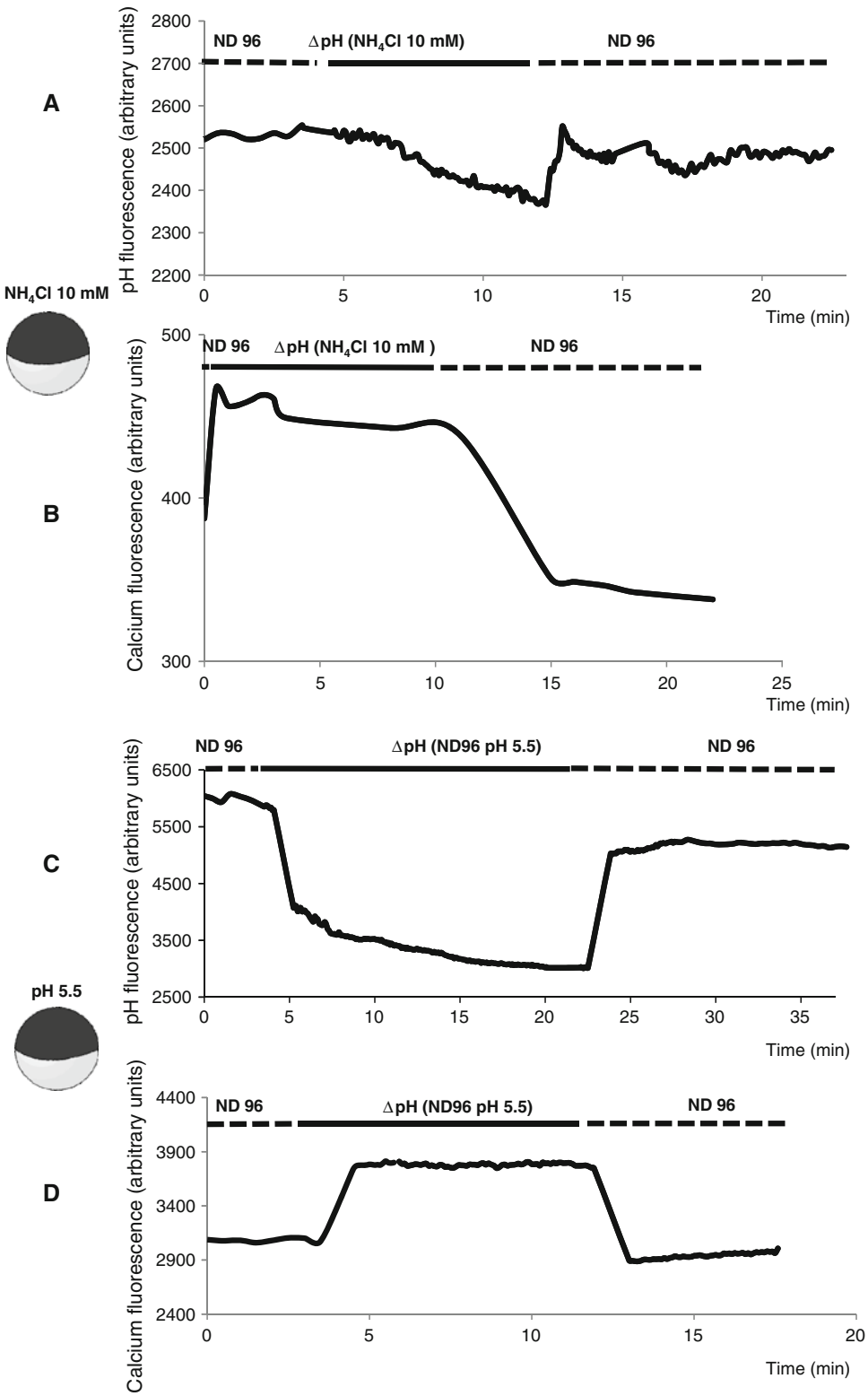

Fig. 3 pH and calcium imaging in *Xenopus* oocytes. Effects of 10 mM NH_4Cl (**a**, **b**) and ND96 pH 5.5 (**b**, **c**) superfusion (*top black line*) on intracellular pH (**a**, **c**) and Ca^{2+} (**b**, **d**). Typical results are depicted. Fluorescence was expressed in arbitrary units and background and autofluorescence were subtracted. **a**, **c** *Xenopus* oocytes were incubated with BCECF-AM (5 μM). **b**, **d** Other *Xenopus* oocytes were incubated with Fluo4-AM (25 μM). NH_4Cl and acidic pHe were responsible for acidification and the Ca^{2+} rise

Interdependence of $[Ca^{2+}]_i$ Rise and Acidification

The effects of NH_4Cl upon $[Ca^{2+}]_i$ could result either from a specific effect of NH_4Cl or from an interdependence between pHi and $[Ca^{2+}]_i$ changes. We ruled out the specific effect of NH_4Cl since we could mimic the effects of NH_4Cl with procaine, another weak base that is also described as inducing a pHi decrease when applied to *Xenopus* oocytes (Rodeau et al. 1998). We also checked the interdependence between pHi and $[Ca^{2+}]_i$ by inducing changes in pHi with another effector,

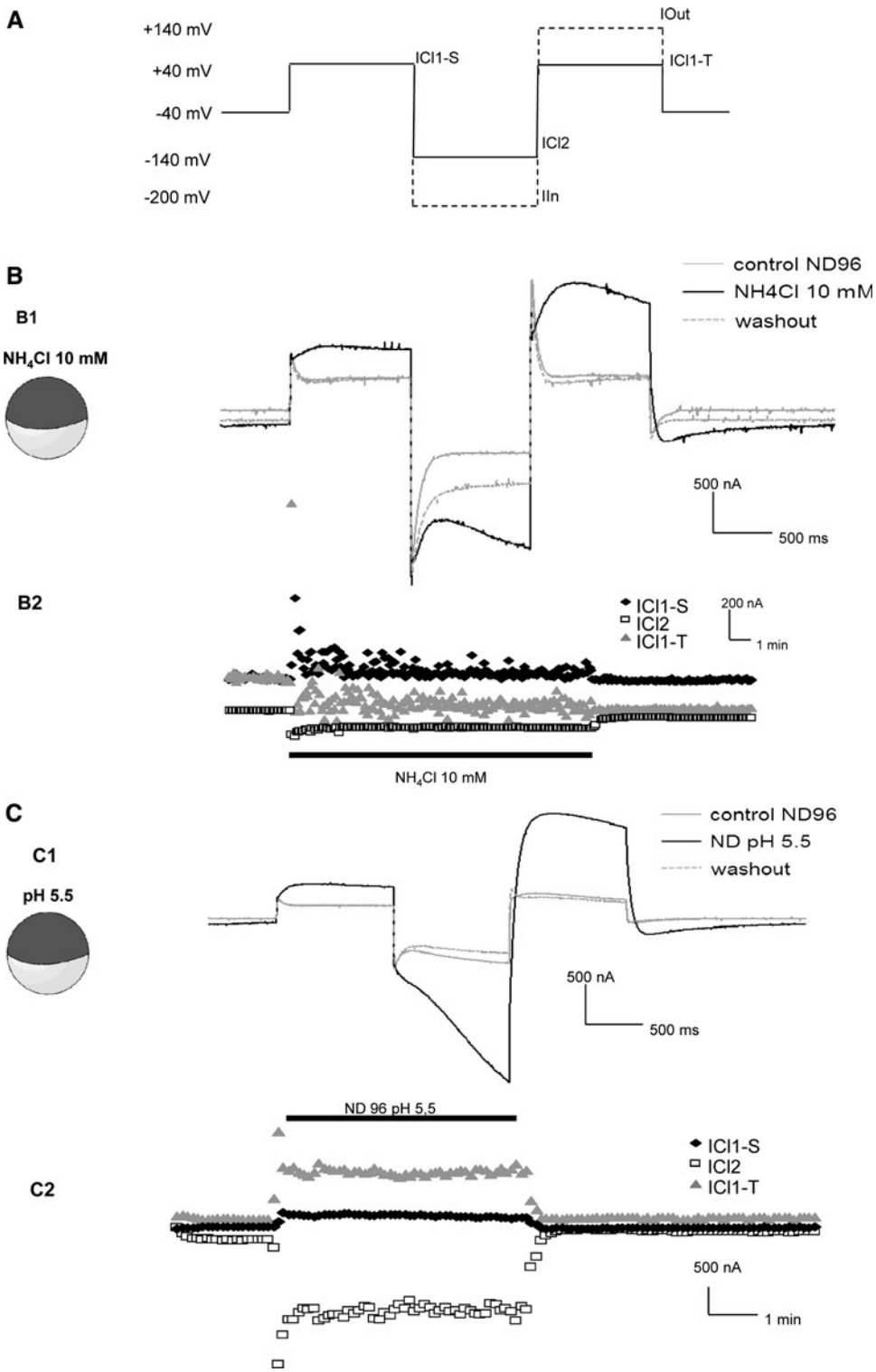

Fig. 4 Effect of physiological pHi acidification on ICl currents. **a** Triple-step protocol (see Materials and Methods section). **b** Effect of NH_4Cl on ICl currents. **b1**, **b2** Representative traces of ICl recordings (**b1**, control ND96, *gray line*; during NH_4Cl treatment, *black line*; washout, *dotted line*) and ICl1-S, ICl2 and ICl1-T (**b2**) currents during NH_4Cl application (ICl1-S, *black squares*; ICl2, *white squares*; and ICl1-T, *gray triangles*). **c1**, **c2** Representative recordings of ICl currents (same marks as in **b**) during external ND96 pH 5.5 perfusion. Note that ICl1-S, ICl2 and ICl1-T intensities increase during NH_4Cl or pH 5.5 treatment

namely changes of pHe. Indeed, changes of pHe are mirrored by corresponding changes in pHi since H^+ readily permeates the oocyte through Na^+/K^+-ATPase (Sasaki et al. 1992). In previous experiments, superfusion of *Xenopus* oocytes with a medium at pH 5.5 triggered an inward calcium-dependent chloride current with superimposed oscillations (Hayashi et al. 2002; Woodward and Miledi 1992). In accordance with these studies, a decrease of pHe from pH 7.4 to pH 5.5 induced a large increase of ICl-Ca and I_{In} with superimposed oscillations (Figs. 4b, 5, 7).

Consequently, intracellular physiological acidification by NH_4Cl would be responsible for a chain reaction: NH_4^+

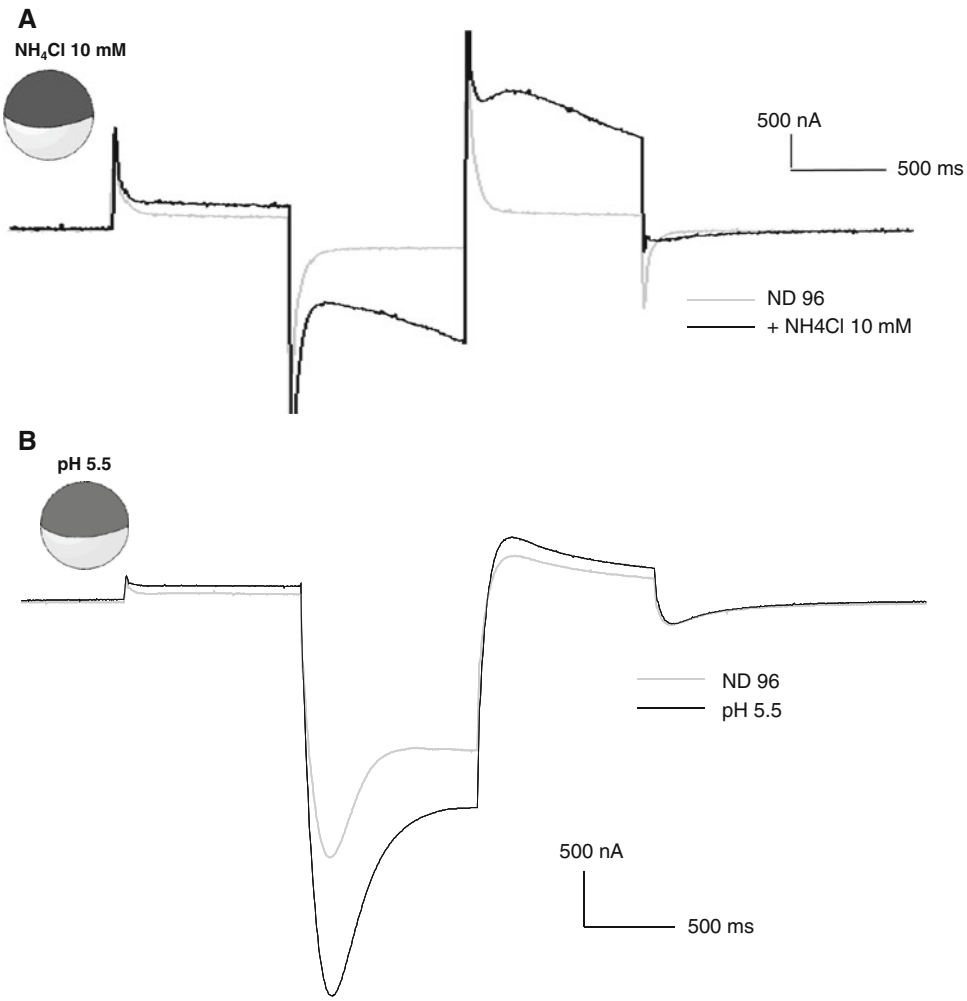

Fig. 5 Effect of physiological acidification on hyperpolarization and acid-activated cation current (I_{In}). **a**, **b** Characteristic traces are depicted. **a** Effect of NH4Cl (*black line*) superfusion. **b** Effect of ND pH 5.5 (*black line*) application

influx inside the oocyte would trigger a cytosolic acidification, which in turn would trigger a $[Ca^{2+}]_i$ increase. This was verified by buffering pHi by microinjection of 50 nL MOPS buffer (pH 6.9) prior to NH_4Cl or ND (pH 5.5) application (Musa-Aziz et al. 2009). MOPS buffer microinjection did not alter ICl1-S but abolished its upregulation upon NH_4Cl addition or ND pH 5.5 superfusion, while an instantaneous and durable increase of ICl2 was still observed, even though it was reduced compared to untreated oocytes (Figs. 4, 6b, 7). These results suggest that while the buffering of pHi by MOPS microinjection inhibits acidification-induced calcium release, it only partially inhibits calcium influx.

Discussion

According to numerous works on *Xenopus* oocytes (Burckhardt and Burckhardt 1997; Burckhardt et al. 1992; Cougnon et al. 1996; Sasaki et al. 1992), NH_4Cl induced an intracellular acidification associated with a dose-dependent inward current increase when oocytes were clamped at −80 mV. NH_4Cl evoked different patterns of currents: a transient current associated with a sustained inward current or a slow inward current accompanied, in some cases, by oscillations. DIDS, an inhibitor of chloride currents, reduced this current, while low-Cl^- conditions increased it, suggesting a chloride component. This is consistent with the reverse potential of this current: $E_{rev} = -25$ mV, near E_{Cl} (ranging between −14 and −28 mV according to different authors [Sasaki et al. 1992; Weber 1999]). The usual means to inhibit a calcium rise in the oocyte drastically reduced but did not abolish the NH_4Cl-induced inward current, which reversed, furthermore, at a potential value of $E_{rev} = -25$ mV, near E_{Cl} [ranging between −14 and −28 mV according to different authors (see Weber 1999)]. Taken together, these results strongly suggest that a calcium-dependent chloride current would be the major component of the NH_4Cl-induced inward current. The contribution of NH_4^+ to this inward current cannot be ruled out since it has been proposed that the shift of E_{rev} in the presence of NH_4Cl might also result from a calcium conductance, from NH_4^+, via K^+ channels or various transporters (Burckhardt et al. 1992; Roos and Boron 1981;

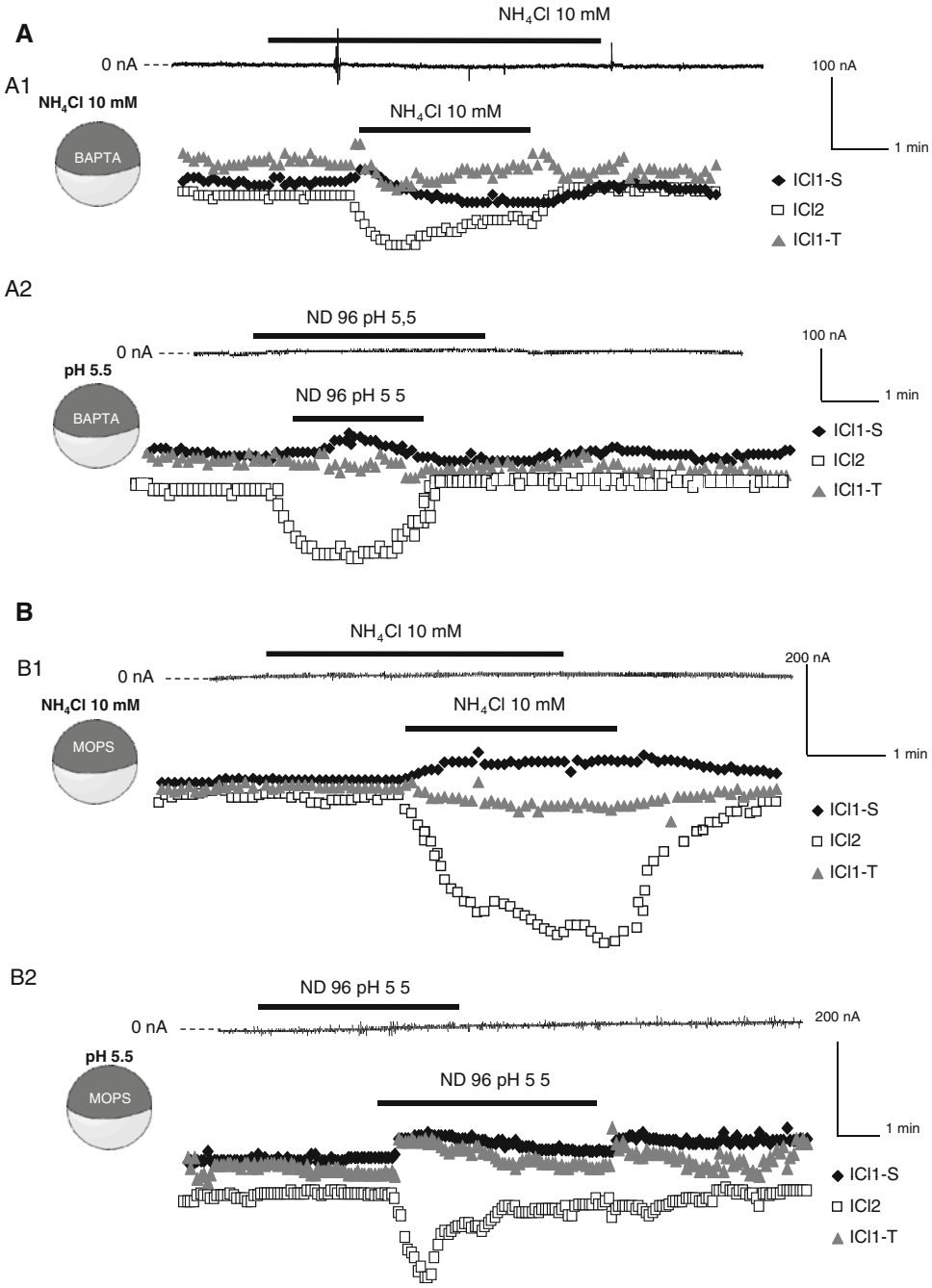

Fig. 6 Modulation of calcium-activated chloride currents. Representative recordings are depicted. Oocytes were incubated with 50 μM BATA-AM (**a**) or injected with 50 nl MOPS pH 6.9 (**b**). Effect on I_{-80} and ICl currents of NH_4Cl application (**a1**, **b1**) or ND 96 pH 5.5 (**a2**, **b2**)

Shen and Steinhardt 1978) or from a Ca^{2+}-dependent Na^+ channel identified in *Xenopus* oocytes (Charpentier and Kado 1999).

These first observations indicate that, even if there is a variability between oocytes, NH_4Cl induced a rise of intracellular Ca^{2+} that, in turn, activated ICl-Ca, which could contribute to the NH_4Cl-induced inward current when the oocytes were clamped at −80 mV. The rise of $[Ca^{2+}]_i$ induced by NH_4Cl was by directly measured pHi and intracellular Ca^{2+} variations with fluorescent indicators. We demonstrated in this report that NH_4Cl has a dual effect in *Xenopus* oocytes: a cytosolic acidification and a rise in intracellular calcium. NH_4Cl initiated simultaneously both a pHi decrease and a $[Ca^{2+}]_i$ increase, which followed different kinetics. It is important to note that, in accordance with Rodeau et al. (1998), intracellular Ca^{2+} returned to its basal level rapidly during washout, even if the cytosolic acidification was persistent minutes after washout (5–20 min). Moreover, according to Nagaraja and Brookes (1998), who worked on astrocytes, NH_4Cl

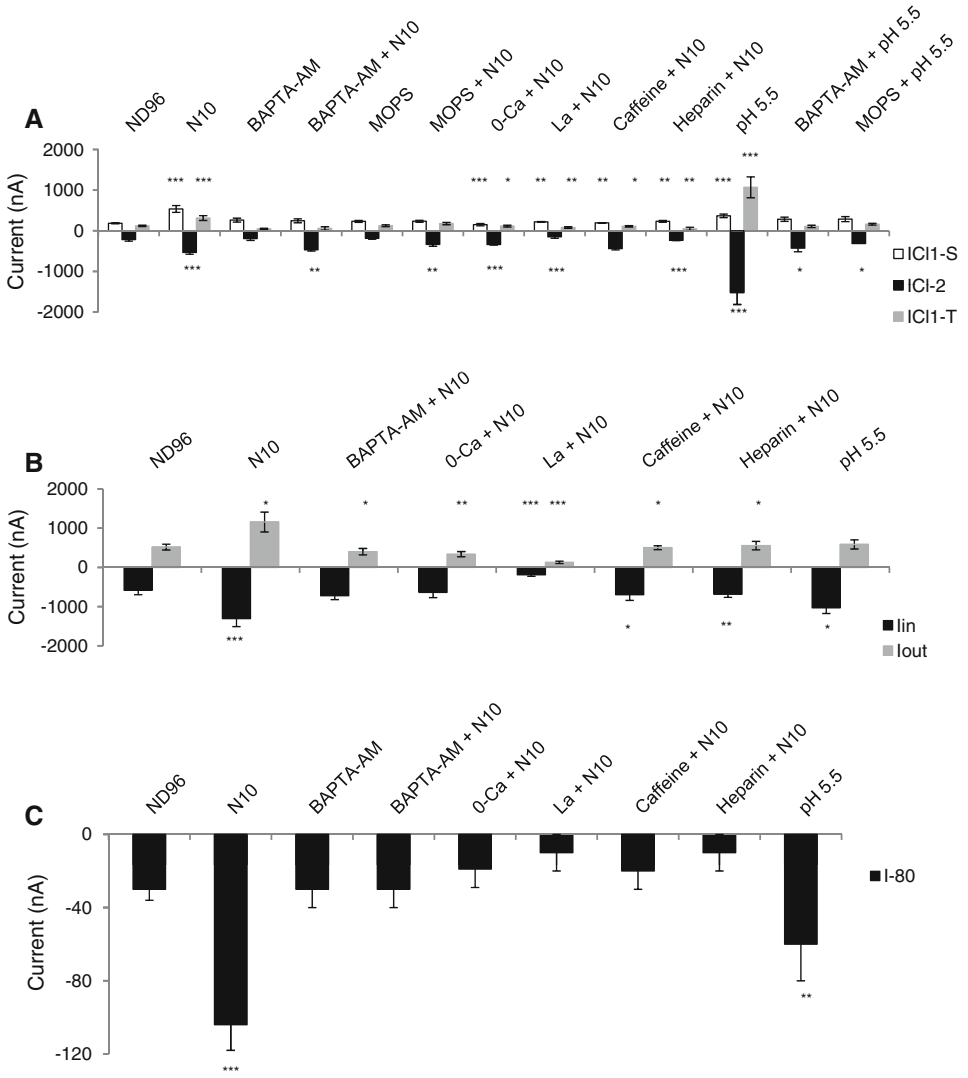

Fig. 7 Currents intensities during calcium and pH variations. **a–c** Data are given in nanoamperes (±SEM). Statistical significance was obtained with *t*-test versus control conditions (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). Experiments were conducted with $n \geq 9$ and $N \geq 3$. **a** Intensities of ICl1-S (*white*), ICl2 (*black*) and ICl1-T (*gray*). **b** Intensities of I_{In} (*black*) and I_{Out} (*gray*). **c** Intensities of I_{-80}

superfusion was first responsible for a rapid and transient alkalization (due to NH_3 entry) during the first 5 s. Our recording technique did not allow us to see this transient effect as our washout took up to 35 s.

The triple-step stimulation protocol developed by Parekh (1995) allows discrimination between various sources of calcium. ICl1-S is activated by calcium release from intracellular stores, ICl2 by calcium influx and ICl1-T both by calcium release from stores and by calcium influx. Our results showed that NH_4Cl induced an immediate increase of ICl1-S and ICl2, indicating a $[Ca^{2+}]_i$ elevation through both calcium release (inhibited by caffeine and heparin) and calcium influx (inhibited by nominal 0-Ca^{2+} medium and lanthanum). The inhibition of calcium release by heparin also inhibited calcium influx, which is unsurprising if we consider that calcium influx is a consequence of calcium store depletion. On the contrary, caffeine was able to only partially inhibit calcium influx since ICl2 was not totally abolished. This effect of caffeine could result from the dual effect of this compound. Indeed, caffeine was able to increase chloride currents evoked by capacitative calcium entry, as demonstrated by Hague et al. (2000).

The $[Ca^{2+}]_i$ rise induced by NH_4Cl was assessed by chelating intracellular calcium with BAPTA-AM (50 µM): These conditions abolished ICl-1S and ICl-T but only reduced ICl2. It appears that while ICl1-S would be strictly calcium-dependent, ICl2 would be both calcium- and pH-dependent. The amplitude of ICl2 was measured at −140 mV, and the acid-activated nonselective cation current I_{In} was expressed at −200 mV. It has been demonstrated that acidification increased the amplitude of I_{In} at all potentials and shifted its activation potential to more positive values. I_{In} is composed of a Ca^{2+}-activated chloride current associated with a small inward Ca^{2+}-independent chloride current (Kuruma et al. 2000). In our conditions of calcium chelation, we suppressed the NH_4Cl-induced Ca^{2+}-activated Cl^- current of ICl2, but there would remain the residual current Ca^{2+}-independent

Cl^- current, which would be also triggered at the more positive potentials of ICl2 measurement.

Consistent with this last observation and with the work of Kuruma et al. (2000), the acidification by NH_4Cl dramatically increased I_{In} when oocytes were stimulated at −200 mV. All tested conditions to prevent the $[Ca^{2+}]_i$ rise, i.e., caffeine and heparin, nominal 0-Ca^{2+} medium and $LaCl_3$, abolished the I_{In} increase, confirming that I_{In} is sensitive to $[Ca^{2+}]_i$ elevations without discrimination between the source of calcium.

In addition, pHi changes affect the calcium transduction pathway since IP3Rs are notoriously known as being pH-sensitive: Affinity for InsP3 would increase with pHi alkalization (Joseph et al. 1989; Worley et al. 1987). All these results strongly suggest that NH_4Cl induces calcium release from the reticulum through IP3R channels. The resulting depletion of the calcium store would in turn activate a capacitative calcium influx through I_{SOCE} channels. However, we cannot rule out the idea that calcium influx was not only due to I_{SOCE} activation but could result from another calcium influx pathway, a noncapacitative, pH-dependent calcium influx such as the pHi-dependent calcium influx pathway described in capacitated mouse sperm (Santi et al. 1998). In rat aortic smooth muscle cells, the NH_4Cl-induced pHi decrease triggered calcium influx through non-voltage-dependent plasma membrane calcium channels, similar to SOCE channels, while the contribution of a calcium influx was negligible (Eto et al. 2003).

The hypothesis of a specific effect of NH_4Cl on calcium increase was discarded since extracellular acidification (from pH 7.5 to 5.5) induced a $[Ca^{2+}]_i$ increase, as testified by the rise of all the components of ICl-Ca and I_{In}. These results are consistent with those of Woodward and Miledi (1992), who showed that pHe acidification activates an inward calcium-dependent chloride current. In our hands, a decrease in pHe triggered a $[Ca^{2+}]_i$ increase through both calcium release and calcium influx (as shown by ICl1-S and ICl2 increases). One can note that extracellular acidification has a stronger effect on calcium influx than NH_4Cl. This might be due to a dual effect of acidic external medium: (1) an extracellular effect on channels responsible for calcium entry and (2) an intracellular acidification responsible for calcium release from stores (and consequently for calcium influx), the sole pathway activated by NH_4Cl application.

Moreover, microinjections of MOPS buffer were used to inhibit intracellular acidification. In these conditions, the effects of application of either NH_4Cl or ND pH 5.5 were prevented and ICl1-S and I_{In} upregulations abolished. However, only a part of ICl-2 was inhibited. In the same way as in experiments carried out with BAPTA, a part of ICl2 might be pH-independent.

During *Xenopus* oocyte meiosis, high Ca^{2+} delays meiosis entry by negatively regulating the initiation of the MAPK–MPF cascade (Machaca and Haun 2002; Sun and Machaca 2004). In a previous work we reported that physiological intracellular acidification inhibits G_2/M transition (Sellier et al. 2006). The increase of calcium by the acidification can explain these events. It has been reported that pHi oscillations are associated with cleaving *Xenopus* embryos (Grandin and Charbonneau 1990; Webb and Nuccitelli 1981). The cyclic oscillations of pHi and $[Ca^{2+}]_i$ would be associated with the cycling activity of MPF (Grandin and Charbonneau 1991; Keating et al. 1994). However, the connections between pHi, $[Ca^{2+}]_i$ and MPF are far from being established and the modalities must be different between meiosis and mitosis of the first cleavage.

To conclude, this study provides strong evidence that intracellular acidification, obtained by external application of NH_4Cl or through changes of pHe, induced a $[Ca^{2+}]$ increase. The calcium pathways triggered by NH_4Cl changes were similar to those activated by external acidification and are hypothesized to be relevant to allow cell adjustment to environmental conditions.

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