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Hydrogen peroxide-induced current in *Xenopus* oocytes: current characteristics similar to those induced by the removal of extracellular calcium

Myung-Jun Kim, Jin-Kwan Han*

Division of Molecular and Life Science, Department of Life Science, Pohang University of Science and Technology, San 31, Hyoja-Dong, Nam Gu, Pohang 790-784, South Korea

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Abstract

The effects of hydrogen peroxide (H_2O_2) exposure on *Xenopus* oocytes were examined. An application of 1 μL of 10% H_2O_2 to oocytes voltage-clamped in 1 mL of Modified Barth Saline (MBS: final concentration of 0.01% H_2O_2) induced a transient ionic current. This H_2O_2-induced current, however, was not transient but long-lasting in a Ca^{2+}-free medium. The H_2O_2-induced current was independent of increases in intracellular calcium. Intriguingly, the H_2O_2-induced current was similar in signature to one stimulated by the removal of extracellular calcium (Ca_o^{2+}-inactivated current). Both currents (a) were inactivated by 1.5 mM $LaCl_3$, $GdCl_3$, $CdCl_2$, $NiCl_2$, $CaCl_2$, or $MgCl_2$, but not by LiCl or KCl, (b) exhibited reversal potential shifts to more positive values with increasing external NaCl, (c) showed linear voltage–current (*I–V*) relationships, and (d) were reversibly inhibited by two chloride channel blockers, 200 μM 5-nitro-2-(3-phenylpropylamino)-benzoic acid and 250 μM niflumic acid. Additionally, H_2O_2 was still able to induce current in oocytes loaded with either catalase or *N*-acetyl-L-cysteine, H_2O_2 scavengers. These results imply that H_2O_2 induces this ionic current possibly through the activation of Ca_o^{2+}-inactivated channels by an extracellular mechanism. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: H_2O_2; Ca_o^{2+}-inactivated current; Voltage-clamping; Reversal potential; Cl^- channel blocker; *Xenopus* oocyte

1. Introduction

H_2O_2, a member of ROS, has been shown to exert various biological effects in both animals and plants. Although initial studies concentrated on its roles in mediating pathological conditions, H_2O_2 is now recognized as a signaling molecule at subtoxic levels [1]. As a signaling molecule, H_2O_2 has been known to control diverse biological processes, including gene expression, by protein phosphorylation and transcription factor activation [1,2]. Recently, the role of H_2O_2 as a second messenger has been demonstrated in various signal transduction systems stimulated by diverse ligands including cytokines and peptide growth factors acting through tyrosine kinases and G-protein-coupled receptors [3–6]. Besides the stimulation of protein phosphorylation and transcription factor activation, H_2O_2 is known to modulate K^+ channels expressed in *Xenopus* oocytes [7,8]. H_2O_2 is also known to be able to regulate Ca^{2+} signaling [9,10]. One of the ways that H_2O_2 is able to modulate the intracellular Ca^{2+} level is to induce Ca^{2+} influx by directly activating Ca^{2+} channels on the plasma membrane [11,12].

Xenopus oocytes are widely used as an expression system for cloned ion channels and receptors. However, it is important to consider the possible involvement of the endogenous current when one interprets electrical signals attributed to heterologously expressed ion channels or receptors. Various ion channels have been reported to exist in *Xenopus* oocytes. Endogenous channels of *Xenopus* oocytes are well summarized in a review article by Weber [13]. Among the endogenous ion channels of *Xenopus* oocytes, several types of Cl^- channels are predominant. Ca^{2+}-activated Cl^- channels are especially important in

* Corresponding author. Tel.: +82-54-279-5542; fax: +82-54-279-2199.
E-mail address: jkh@postech.ac.kr (J.-K. Han).

Abbreviations: ROS, reactive oxygen species; LPA, lysophosphatidic acid; *I–V*, voltage–current; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; NFA, niflumic acid; NAC, *N*-acetyl-L-cysteine; RuRed, ammoniated ruthenium oxychloride.

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the study of heterologously expressed receptors activating Ca^{2+} signaling because of their ability to sense the level of intracellular Ca^{2+}. *Xenopus* oocytes also possess hypotonicity-activated Cl^- channels that are distinct from previously known stretch-activated nonselective cation channels and Ca^{2+}-activated Cl^- channels [14]. Recently, three groups have reported that removal of extracellular Ca^{2+} elicited current [15–17]. There are some discrepancies about the ionic nature of the so-called Ca_o^{2+}-inactivated current. Arellano *et al.* and Zhang *et al.* have shown a requirement for monovalent cations in the Ca_o^{2+}-inactivated current, whereas Weber *et al.* have proposed that Cl^- carry the current. To explain these discrepancies, it has been suggested that the Ca_o^{2+}-inactivated current is generated through the activation of a single population of poorly selective channels that barely discriminate between anions and cations. Another explanation is that the removal of extracellular Ca^{2+} can activate two types of ion channels, cationic and anionic in nature, which can mask each other depending upon environmental conditions [13].

Schlief and Heinemann [18] have shown that H_2O_2 can induce a Cl^- current in *Xenopus* oocytes by elevating the level of intracellular Ca^{2+} through the activation of the Na^+–Ca^{2+} exchanger. They recorded electrical signals elicited by H_2O_2 in Na^+-free K^+-Ringer solution. In the present study, we examined the effect of H_2O_2 exposure on *Xenopus* oocytes in the presence of a physiological concentration of Na^+. We found that H_2O_2 induces a current in *Xenopus* oocytes that has the same characteristics as the Ca_o^{2+}-inactivated current.

2. Materials and methods

2.1. Materials

LPA, NFA, NPPB, NAC, catalase [from *Aspergillus niger* suspended in 3.2 M $(NH_4)_2SO_4$ solution, pH 6.0], heparin (MW 6000), *N*-methyl-D-glucamine, RuRed, and gentamicin sulfate were purchased from the Sigma Chemical Co. Cation chloride salts ($GdCl_3$, $LaCl_3$, $NiCl_2$, $CdCl_2$, $MgCl_2$, $CaCl_2$, LiCl, NaCl, and KCl) also were purchased from Sigma. H_2O_2 was obtained from the Junsei Chemical Co. LPA was dissolved in aqueous 1% fatty acid-free BSA to a stock concentration of 1 mM, and was kept at −20° in small aliquots.

2.2. Oocyte preparation

Mature wild-type female *Xenopus laevis* purchased from Xenopus I were maintained at 18–24°. Individual oocytes at stage VI were manually dissected from their outer follicles using watchmaker's forceps and stored in Modified Barth Saline [MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM Na-HEPES, pH 7.4] at 19° and were used within 1 day after isolation. Ca^{2+}-free ND96 solution contained: 96 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 5 mM HEPES (adjusted to pH 7.4 with NaOH).

2.3. Microinjection

Microinjection was performed using a Nanoliter Injector (WPI). Twenty-five nanoliters of each solution was injected into oocytes unless otherwise specified. The final concentration of the injection solution (heparin, 50 μM; RuRed, 50 μM; and EGTA, 300 μM) in the oocyte was calculated based upon the assumption that the actual accessible cytoplasmic volume of the oocyte is 500 nL since half of the oocyte volume is estimated to be occupied by membrane-bound yolk platelets [19]. Poor impalements caused leakage of the cytoplasm, and these oocytes were discarded.

2.4. Voltage-clamp recording

Current was recorded using the standard double electrode voltage-clamp technique. A single oocyte was placed in a recording chamber filled with 1 mL MBS. Microelectrodes were pulled in one step through capillary glass (Borosilicate Glass Capillaries with an inner filament, Cat. No. GT12, Warner Corp.) using a micropipette puller (model 700C; David Kopf Instruments), and the tips were broken to a diameter of ∼10 μm. They were filled with 3 M KCl, and tip resistance was usually 1–5 MΩ. The cell was voltage-clamped using a two-microelectrode voltage clamp amplifier (oocyte clamp OC725A; Warner Corp.), connected to a data acquisition system, MacLab/4e (AD Instruments Pty Ltd.) running on a Power Macintosh computer. Membrane current was sampled at 16.7 Hz. One microliter of 10% H_2O_2 was applied over a 1- to 2-s period using a hand-held micropipette positioned ∼5 mm from the oocyte. For the determination of current-voltage relations, steady-state current was measured during the last 100 ms of 500-ms rectangular pulses applied in 20-mV increments from −80 to +60 mV. Voltage ramps of 500 m from −120 to +40 mV were applied to determine the relationships between reversal potential and extracellular concentration of NaCl. Zero or low-NaCl solutions (0 and 16 mM NaCl) were prepared by reducing the amount of NaCl in MBS and replacing it with an isomolar amount of *N*-methyl-D-glucamine and by adjusting the pH with methanesulfonic acid.

2.5. Statistics

The maximal current amplitudes are expressed as means ± SEM. A Student's *t*-test was used for comparing individual treatments with their respective control values. A probability of $P < 0.05$ was accepted as denoting a significant difference.

3. Results

3.1. H_2O_2-induced current in Xenopus oocytes in the presence of a physiological concentration of Na^+

Administration of H_2O_2 to an oocyte voltage-clamped at a holding potential of −60 mV in MBS containing a physiological concentration of Na^+ activated a transient inward current (H_2O_2-induced current). The current consisted of a fast inward component, followed by a relaxation over some tens of seconds, on which small fluctuations were superimposed (Fig. 1A). In 35% of the oocytes tested, the fast and relaxation components were separated (Fig. 1B). The ability of the oocytes to activate the H_2O_2-induced current diminished as a function of the time elapsed since their surgical removal from the animal. Three days after surgical removal, the oocytes could no longer activate the H_2O_2-induced current (data not shown). To minimize the variations in the magnitude of currents resulting from the difference of the time elapsed since removal, all experiments were done using oocytes within 1 day after removal.

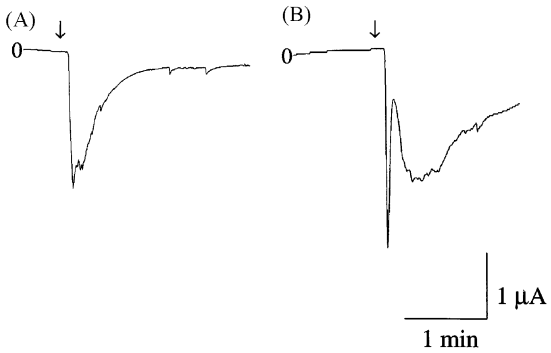

Fig. 1. H_2O_2-induced current in *Xenopus* oocytes. An oocyte was voltage-clamped at a holding potential of −60 mV in a recording chamber filled with 1 mL of MBS. Administration of 1 μL of 10% H_2O_2 to the oocyte using a hand-held pipette positioned ∼5 mm from the cell induced transient inward currents. (A) Fast inward component followed by immediate relaxation with small fluctuations. (B) In about one-third of the cases the fast and relaxation components were well separated. Arrows indicate the point of application of H_2O_2. Each trace is representative of at least 15 experiments using oocytes from three different animals.

3.2. Calcium independence of the H_2O_2-induced current

It has been shown in other cell systems that H_2O_2 elevates intracellular calcium [9,10]. To examine whether the H_2O_2-induced current was dependent upon elevation of intracellular Ca^{2+}, we tested oocytes for their responsiveness to H_2O_2 following intracellular injection of (final cytosolic concentration) 50 μM heparin or 50 μM RuRed or 300 μM EGTA. Heparin is known to be an inhibitor of IP_3-induced Ca^{2+} release, and RuRed is a competitive inhibitor of the ryanodine receptor that is responsible for Ca^{2+}-induced Ca^{2+} release [20,21]. Intracellular injection of these reagents did not affect the H_2O_2-induced current (Fig. 2A), suggesting that Ca^{2+} release from IP_3- or ryanodine-sensitive pools was not involved in the current generation by H_2O_2 in oocytes. Injection of 300 μM EGTA, a chelator of Ca^{2+}, also did not inhibit the generation of the H_2O_2-induced current (Fig. 2A), further demonstrating that this current is independent of intracellular calcium.

To verify that both heparin and EGTA are effective in inhibiting the elevation in intracellular Ca^{2+}, we injected oocytes with the same concentrations of heparin or EGTA and tested for responsiveness to LPA. LPA is known to elevate intracellular Ca^{2+} through the generation of IP_3 [22,23]. The LPA response was inhibited greatly in the oocytes whether injected with heparin or EGTA (Fig. 2B). This result implies that heparin and EGTA effectively inhibited the elevation of intracellular Ca^{2+}.

3.3. Possible involvement of Ca_o^{2+}-inactivated channels in the generation of the H_2O_2-induced current

The above data indicate that the H_2O_2-induced current in *Xenopus* oocytes is independent of intracellular Ca^{2+}. To elucidate the role of extracellular Ca^{2+} in the H_2O_2-induced current, we recorded the H_2O_2 response of oocytes in Ca^{2+}-free medium (Ca^{2+}-free ND96, see Section 2).

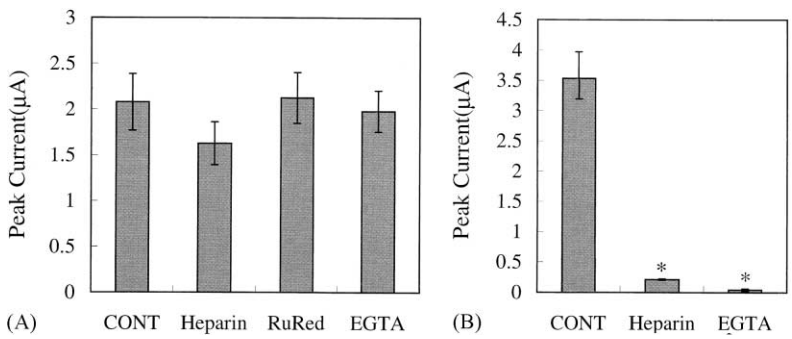

Fig. 2. Effects of injection of oocytes with heparin, RuRed, or EGTA on the response to H_2O_2. Oocytes were injected with 50 μM heparin (Heparin), 50 μM ruthenium red (RuRed), or 300 μM EGTA (EGTA), and 30 min later 1 μL of 10% H_2O_2 (A) or 1 μM LPA (B) was applied. Maximal peaks of H_2O_2- or LPA-induced currents were evaluated and averaged. Data are means ± SEM of 3 independent experiments using 5–6 oocytes per condition in each experiment. In each experiment oocytes from different animals were used. Key: (∗) $P < 0.01$, compared with the control.

H_2O_2 still elicited current in Ca^{2+}-free ND96 (data not shown). Surprisingly, however, the current generated in Ca^{2+}-free ND96 was not inactivated and lasted for at least 20 min (data not shown). Because the H_2O_2-induced current was not inactivated in a Ca^{2+}-free medium, we postulated the involvement of the so-called Ca_o^{2+}-inactivated channels in the generation of the H_2O_2-induced current. Three groups have demonstrated that removal of extracellular calcium (Ca_o^{2+}) from the bathing solution results in a huge and non-inactivating current in *Xenopus* oocytes [15–17].

Since there are reports that the Ca_o^{2+}-inactivated current is blocked by extracellular Ca^{2+}, Mg^{2+}, and Gd^{3+} [16,17], we first compared the effects of extracellular di- and trivalent cations on the long-lasting H_2O_2-induced current with those on the Ca_o^{2+}-inactivated current. We induced the Ca_o^{2+}-inactivated current in a *Xenopus* oocyte by adding 1.25 mM EDTA to a recording chamber containing normal MBS. To elicit the H_2O_2-induced current in a Ca^{2+}-free condition, we applied 1.25 mM EDTA and 1 μL of 10% H_2O_2 sequentially. A huge and slowly activating current was induced by the addition of 1.25 mM EDTA, and the subsequent application of H_2O_2 further increased the current. The long-lasting nature of the H_2O_2-induced current in Ca^{2+}-free ND96 was retained (Fig. 3A). The current elicited by the addition of 1.25 mM EDTA was inactivated to a basal level by the subsequent addition of 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, $NiCl_2$, $MgCl_2$, or $CaCl_2$ (Fig. 3B, Table 1). The EDTA/H_2O_2-induced current, which is mediated by the sequential addition of EDTA and H_2O_2, was also inactivated by the same concentration of tri- or divalent cations used to inactivate the Ca_o^{2+}-inactivated current (Fig. 3C). The addition of these cations reduced the EDTA/H_2O_2-induced current by more than 90%. $GdCl_3$, $LaCl_3$, $CdCl_2$, and $NiCl_2$ were equally effective in inactivating Ca_o^{2+}-inactivated or EDTA/H_2O_2-induced currents (Table 1). Monovalent cations (1.5 mM LiCl or KCl) had no effect on Ca_o^{2+}-inactivated and EDTA/H_2O_2-induced currents (Table 1). These data support the idea that the H_2O_2-induced current may be mediated through the Ca_o^{2+}-inactivated channels. Like the H_2O_2-induced current (Fig. 2A), the Ca_o^{2+}-inactivated current was independent of intracellular calcium (data not shown).

We next examined if pretreatment with 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, or $NiCl_2$ could block the generation of the

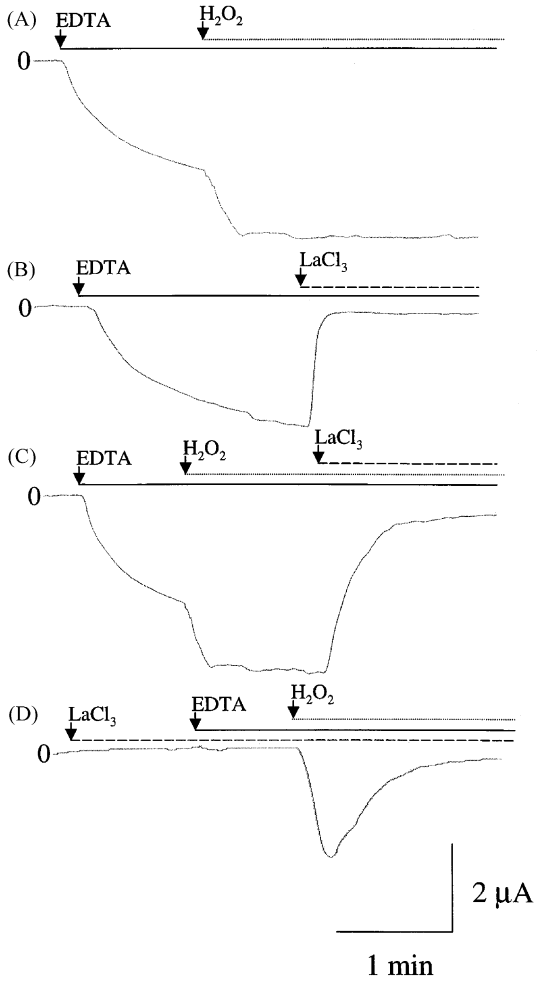

Fig. 3. Effects of various cations on H_2O_2-induced and Ca_o^{2+}-inactivated currents. (A) Oocytes were voltage-clamped at −60 mV, and 1.25 mM EDTA and 1 μL of 10% H_2O_2 were applied sequentially. The current generated by the addition of EDTA and H_2O_2 was not inactivated and lasted at least for 20 min. The trace is representative of nine experiments using oocytes from three different animals. (B and C) Oocytes were voltage-clamped at −60 mV, and EDTA; (B) or sequentially EDTA and H_2O_2; (C) were applied. About 2 min after the application of EDTA, 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, $NiCl_2$, $MgCl_2$, or $CaCl_2$ was added to the recording chamber. (D) Oocytes were voltage-clamped at −60 mV, and 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, or $NiCl_2$ was added to the chamber. Approximately 1 min after the addition of the cation, EDTA was applied. One minute later H_2O_2 was added. The traces for La^{3+} in B, C, and D are representative of the results obtained with each cation. The experiments for each cation were performed 6–7 times on oocytes harvested from at least three different animals. Solid, dashed, and dotted lines indicate the presence of EDTA, $LaCl_3$, and H_2O_2, respectively.

Table 1
Inhibition of H_2O_2- and EDTA-induced currents by various cations and Cl^- channel blockers

Currents		Cations								Cl^- channel blockers	
		Gd^{3+}	La^{3+}	Cd^{2+}	Ni^{2+}	Ca^{2+}	Mg^{2+}	Li^+	K^+	NPPB	NFA
EDTA-induced	Initial	++	++	++	++	ND	ND	−	−	++	++
	Long-lasting	++	++	++	++	++	++	−	−	ND	ND
EDTA/H_2O_2-induced	Initial	−	−	−	−	ND	ND	−	−	++	+
	Long-lasting	++	++	++	++	++	++	−	−	ND	ND

ND, not determined; ++, complete inhibition; +, partial inhibition; −, no inhibition.

H_2O_2-induced current. When 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, or $NiCl_2$ was added to the recording chamber before the application of EDTA and H_2O_2, it inhibited the EDTA-induced Ca_o^{2+}-inactivated current but failed in blocking the current generation induced by the subsequent application of H_2O_2 (Fig. 3D). However, the EDTA/H_2O_2-induced current was transient in the presence of 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, or $NiCl_2$ (Fig. 3D). This result is consistent with the finding that the H_2O_2-induced current in normal MBS containing the divalent cations Ca^{2+} and Mg^{2+} was transient (Fig. 1A and B). Gentamicin, known to block mechanically gated (MG) channels, inhibited Ca_o^{2+}-inactivated and H_2O_2-induced currents only slightly. Both currents were reduced by $32 \pm 10\%$ by 50 μg/mL of gentamicin (not shown).

3.4. Ionic nature of Ca_o^{2+}-inactivated and H_2O_2-induced currents

The ionic basis of the Ca_o^{2+}-inactivated current is controversial. Arellano *et al.* [16] and Zhang *et al.* [17] have concluded that the Ca_o^{2+}-inactivated current is mainly mediated by monovalent cations (Na^+, K^+), whereas Weber *et al.* [15] have argued for Cl^- involvement in this current. To characterize the ionic nature of H_2O_2-induced and Ca_o^{2+}-inactivated currents, we have approached the problem in two ways. First, we have investigated the effects of changing the external NaCl concentration on the reversal potential (V_{rev}) values; second, we have examined the effects of NPPB and NFA, blockers of the chloride channel, on these currents.

To obtain reversal potential values as a function of the external NaCl concentration, we applied voltage ramps of 500 ms (from −120 to +50 mV) to an oocyte after the addition of EGTA and the subsequent application of H_2O_2. Because the Ca_o^{2+}-inactivated current induced by the addition of EDTA was so large (it hardly reached a steady-state level), we added 2.5 mM EGTA instead of EDTA to the recording chamber to generate this current. The reversal potential was determined as the membrane potential at a point where current of an oocyte reached zero (Fig. 4A). Experiments were repeated with 0, 16, 88, and 154 mM concentrations of external NaCl, and the averaged reversal potentials were plotted as a function of external NaCl concentration (Fig. 4B). The reversal potentials of both currents shifted to more positive values as the external NaCl concentration increased. Although the data did not show linear relationship between reversal potential and external NaCl concentration, they clearly indicate that both the Ca_o^{2+}-inactivated and H_2O_2-induced currents are more permeable to cations than anions.

When oocytes were pretreated with 200 μM NPPB, Ca_o^{2+}-inactivated and H_2O_2-induced currents were abolished completely in these cells (Fig. 4C). NFA also inhibited both currents (Fig. 4D). The Ca_o^{2+}-inactivated current was abolished in 75% of oocytes treated with 250 μM NFA

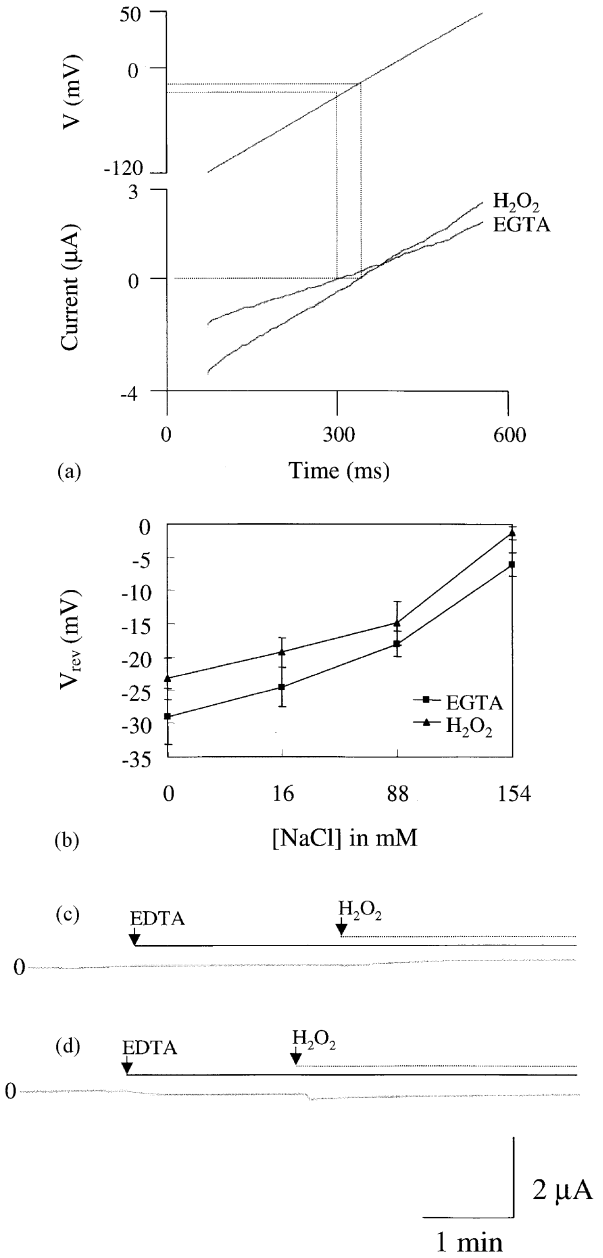

Fig. 4. Reversal potential as a function of external NaCl concentration and effects of chloride channel blockers. (A) A typical trace of current of an oocyte in response to an applied voltage ramp. An oocyte was held at −60 mV, and a voltage ramp of 500 ms (from −120 to +50 mV) was applied after the addition of 2.5 mM EGTA and the subsequent application of H_2O_2. Experiments were repeated with 0, 16, 88, and 154 mM concentrations of external NaCl. The dotted lines show how the reversal potential was determined. (B) The reversal potential was averaged and plotted as a function of membrane potential. Data are means ± SEM of four independent experiments using 7–8 oocytes per condition in each experiment. Oocytes harvested from different animals were used for each independent experiment. (C and D) Oocytes were preincubated with 200 μM NPPB (C) or 250 μM NFA (D) for 15 min and were subjected to a voltage-clamp recording in the presence of the same chloride channel blocker as used in the preincubation. In panels C and D, 14 experiments using oocytes from three animals were done for each chloride channel blocker. Solid, dashed, and dotted lines indicate the presence of EDTA, $LaCl_3$, and H_2O_2, respectively.

for 15 min, and the H_2O_2-induced current was blocked in 60% of those oocytes. These data indicate that H_2O_2-induced and Ca_o^{2+}-inactivated currents have a similar susceptibility to chloride channel blockers. In addition, they indicate that the Cl^- ion is involved in the generation of these currents. The inhibition of H_2O_2-induced and Ca_o^{2+}-inactivated currents by the two blockers was reversible, judged from the fact that H_2O_2-induced and Ca_o^{2+}-inactivated currents were generated in pretreated oocytes when recording was done in a bath solution devoid of the pretreatment inhibitor (data not shown). The effects of various cations and chloride channel blockers on H_2O_2-induced and Ca_o^{2+}-inactivated currents are summarized in Table 1.

3.5. *I–V relations of Ca_o^{2+}-inactivated and H_2O_2-induced currents*

We did not observe any indications of rectification in the Ca_o^{2+}-inactivated and H_2O_2-induced currents in response to the applied voltage ramp (Fig. 4A). To investigate the *I–V* relations of Ca_o^{2+}-inactivated and H_2O_2-induced currents further, we measured the steady-state current during the last 100 ms of 500-ms rectangular pulses. Ca_o^{2+}-inactivated current was induced by adding 2.5 mM EGTA. The current was smaller than that of the EDTA-induced Ca_o^{2+}-inactivated current and reached a plateau in about 2 min (Fig. 5A). The EGTA-induced Ca_o^{2+}-inactivated and subsequently activated H_2O_2-induced currents were also inhibited by 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, or $NiCl_2$ (Fig. 5A).

We applied pulses in 20-mV increments from −80 to +60 mV to an oocyte voltage-clamped at −60 mV at three plateaus of the trace, before and after the activation of the Ca_o^{2+}-inactivated current and after the activation of H_2O_2-induced current. When the steady-state current was plotted vs. membrane potential, the *I–V* curves of both Ca_o^{2+}-inactivated and H_2O_2-induced currents were almost linear (Fig. 5B). The line representing the H_2O_2-induced current had a greater slope than that of the Ca_o^{2+}-inactivated current. Rectification was not observed at any membrane potential, which is consistent with the voltage ramp experiment.

3.6. *Extracellular site of action of H_2O_2*

We finally examined whether H_2O_2 acted intracellularly or extracellularly. To test this, we injected oocytes with 9.6 nL of 1 M NAC or 0.127 unit of catalase, H_2O_2 scavengers, and recorded responses to H_2O_2 in those injected oocytes. As shown in Fig. 6A, neither NAC nor catalase had an effect on H_2O_2-induced currents under these conditions. NAC and catalase at higher concentrations than were used above were toxic to the oocytes. As an alternative method to microinjection, oocytes were incubated with 778.8 units/mL of catalase for 1 hr, and H_2O_2 responses were recorded in catalase-free MBS. As with the injection procedure, incubation of oocytes with catalase did not affect the H_2O_2-induced current (Fig. 6B). From these data, we conclude that H_2O_2 acts on the extracellular surface of oocytes possibly by oxidizing an extracellular domain of a plasma membrane protein.

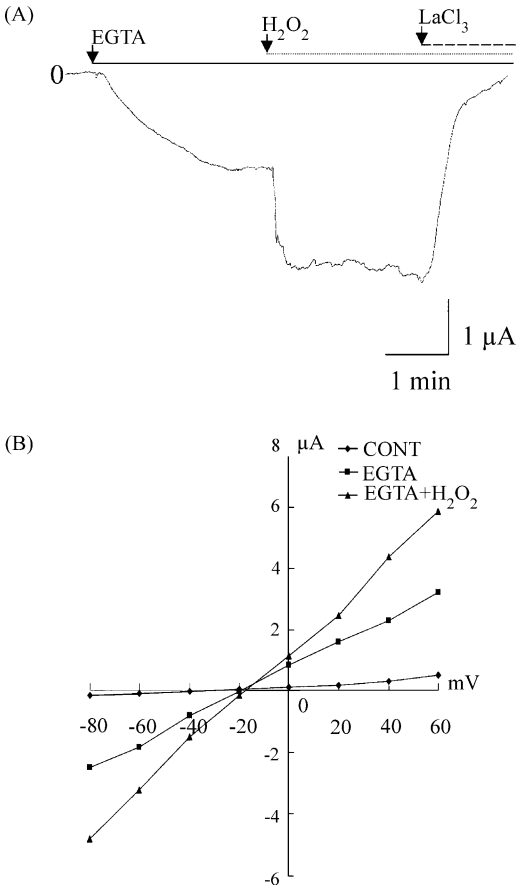

Fig. 5. *I–V* relations of the current induced by EGTA or EGTA plus H_2O_2. (A) Oocytes were voltage-clamped at −60 mV, and 2.5 mM EGTA, 1 μL of 10% H_2O_2, and 1.5 mM $GdCl_3$, $LaCl_3$, $CdCl_2$, or $NiCl_2$ were applied sequentially. Five experiments using oocytes from different animals were performed for each cation. A trace for La^{3+} is presented as representative of the results obtained with the other cations. The solid, dashed, and dotted lines indicate the presence of EGTA, $LaCl_3$, and H_2O_2, respectively. (B) Oocytes were voltage-clamped at a holding potential of −60 mV, and 500-ms rectangular pulses (from −80 to 60 mV in 20-mV increments) were applied before and after the addition of 2.5 mM EGTA and after the subsequent application of 1 μL of 10% H_2O_2. Steady-state current was measured during the last 100 ms of 500-ms pulses. The curves are from the same oocyte and are representatives of 12 experiments using oocytes from three animals.

4. Discussion

In this study, we have shown that H_2O_2 elicited current in MBS containing a physiological concentration of Na^+ in *Xenopus* oocytes. The current was independent of intracellular calcium and was not inactivated in a Ca^{2+}-free medium. This current is distinct from that demonstrated by Schlief and Heinemann [18]. In their study, Schlief and

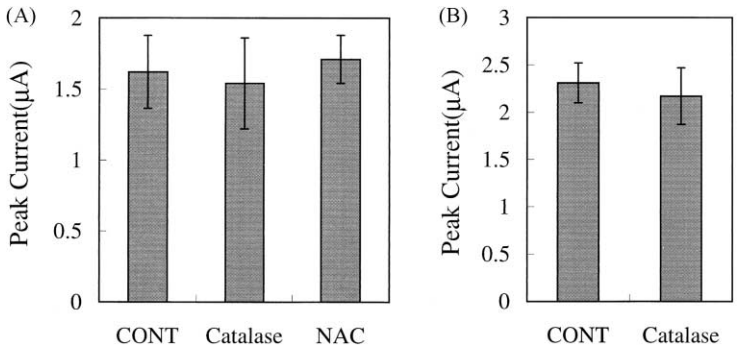

Fig. 6. Extracellular site of action of H_2O_2. (A) Oocytes were injected with 9.6 nL of 1 M of NAC (NAC) or 0.127 unit of catalase (Catalase), and 30 min later responses to H_2O_2 were recorded. Control oocytes (CONT) were injected with an equal amount of denatured catalase. (B) Oocytes were incubated with 778.8 units/mL of catalase (Catalase) or an equal amount of denatured catalase (CONT) for 1 hr, and responses to H_2O_2 were recorded in catalase-free MBS. Maximum peaks of the H_2O_2-induced current were evaluated and averaged. Data are means ± SEM of three independent experiments using 4–5 oocytes per condition in each experiment. Oocytes harvested from different animals were used for each independent experiment.

Heinemann observed that the current induced by H_2O_2 in K^+-Ringer solution was blocked when Na^+ was substituted for K^+. Furthermore, they showed that the current was abolished completely in the absence of extracellular Ca^{2+}, demonstrating the involvement of the Na^+–Ca^{2+} exchanger in the generation of the current. We interpret this paradoxical situation as a result of the ability of H_2O_2 to elicit two types of currents, one shown by us and another by Schlief and Heinemann. The type of current produced is dependent upon the concentration of H_2O_2 used. Schlief and Heinemann prepared solutions of H_2O_2 up to a concentration of 0.24% before application to the oocyte. In contrast, we introduced 1 μL of 10% H_2O_2 to the oocyte using a hand-held pipette positioned ~5 mm from it, which might create a much higher local concentration of H_2O_2 at the cell surface than the 0.24% H_2O_2 administered by Schlief and Heinemann around the oocyte. Therefore, it appears that 0.24% H_2O_2 generates current through the activation of the Na^+–Ca^{2+} exchanger whereas a higher localized concentration of H_2O_2 may elicit current by the activation of Ca_o^{2+}-inactivated channels. Supporting this idea, exchanging the bath solution with MBS containing 0.24% H_2O_2 did not induce current (data not shown).

Both H_2O_2-induced and Ca_o^{2+}-inactivated currents were inactivated by various di- and trivalent cations (1.5 mM), but not monovalent cations. They also were inhibited by treatment with NAF and NPPB but were independent of changes in intracellular calcium concentration. Finally, both H_2O_2-induced and Ca_o^{2+}-inactivated currents showed linear *I–V* relationships. From these similar characteristics between H_2O_2-induced and Ca_o^{2+}-inactivated currents, we concluded that the H_2O_2-induced current occurred by the activation of Ca_o^{2+}-inactivated channels. However, it should be noted that there are some discrepancies between the H_2O_2-induced and Ca_o^{2+}-inactivated currents. NFA was more potent in blocking the Ca_o^{2+}-inactivated current than the H_2O_2-induced current. In addition, pretreatment with 1.5 mM $LaCl_3$, $GdCl_3$, $CdCl_2$, or $NiCl_2$ did not inhibit the generation of the H_2O_2-induced current, although it completely blocked the Ca_o^{2+}-inactivated current (Table 1). It is likely that these inconsistencies are the result of the diverse mechanisms involved in activating Ca_o^{2+}-inactivated channels in the presence of H_2O_2 and the absence of extracellular calcium. The fact that H_2O_2 can further increase the current induced by the removal of extracellular calcium supports the hypothesis that H_2O_2 and the absence of extracellular calcium differentially activate Ca_o^{2+}-inactivated channels. Equally possible is that H_2O_2 and removal of external calcium have different potencies in activating Ca_o^{2+}-inactivated channels. Recently, it has been hypothesized that Ca_o^{2+}-inactivated Cl^- currents could be elicited by the activation of at least two different channel populations, cationic and anionic channels [24]. Since the H_2O_2-induced current shares some properties with the Ca_o^{2+}-inactivated Cl^- channel, it is possible that the H_2O_2-induced current could be mediated by at least two different populations of ion channels.

Several laboratories have reported that the removal of extracellular calcium elicited current with a different ionic make-up [15–17]. In this paper, we have shown that H_2O_2-induced and Ca_o^{2+}-inactivated currents are more permeable to cations than anions. However, we have also shown the inhibition of H_2O_2-induced and Ca_o^{2+}-inactivated currents by the chloride channel blockers. We think that the complexity of the ionic nature of H_2O_2-induced and Ca_o^{2+}-inactivated currents reflects the heterogeneity of the channels involved in these currents. Another possibility is that H_2O_2-induced and Ca_o^{2+}-inactivated currents are mainly mediated by cation-selective channels whose activity is dependent upon the activity of Cl^- channels. Alternatively, it could also be possible that these currents are Cl^- currents that are gated by Ca^{2+}.

In conclusion, we have demonstrated that H_2O_2 induces current in *Xenopus* oocytes in the presence of a physiological concentration of NaCl, and that this current has characteristics that are similar to the Ca_o^{2+}-inactivated current. Further studies to clearly reveal the ion selectivity of the H_2O_2-activated channel are needed. It will also be of interest to study how H_2O_2-induced currents are regulated by various cytosolic factors.

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