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NOVEL EFFECT OF AZIDE ON SODIUM CHANNEL OF *XENOPUS* OOCYTES

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SUMMARY. Bath application of a few mM of sodium azide to a voltage clamped oocyte activated a voltage dependent Na^+ current. Characteristics of the azide-induced current were the same as those of already described Na^+ current induced by prolonged depolarization. The Na^+ current induced either by azide or depolarization was suppressed by the application of Ca^{2+} channel blockers (Diltiazem and La^{3+}). Azide is known to affect some metabolic processes (ATP and cGMP production *etc.*), but the present effect could not be attributed to metabolic actions.

Xenopus oocytes are widely used in the investigation of membrane receptors, ion channels and the mechanisms of signal transduction. The membrane of the *Xenopus* oocyte can be made to produce a long-lasting action potential, provided the cell has first been held at a positive potential for a certain time by injecting current (1,2). This action potential is attributed to the opening of putative voltage dependent Na^+ channels, which are "induced" by the prolonged depolarization. Functions of this kind of channels are still unknown. This paper describes the induction of voltage dependent Na^+ channels by an application of sodium azide, which is commonly used as a preservative for biological preparations such as antibodies and enzymes. Characteristics of the electrically "induced" Na^+ channel and those of the azide-induced Na^+ channel were found to be the same. Furthermore, induced Na^+ channels were blocked by some Ca^{2+} channel blockers (Diltiazem and La^{3+}) suggesting that azide and long depolarization affected the same putative Na^+ channels.

MATERIAL AND METHODS. Experiments were made on fully grown oocytes of *Xenopus laevis*. Oocytes were manually removed from a piece of excised ovary using a pair of fine scissors. Some oocytes were treated for 2 hours with 2 mg/ml of collagenase (Sigma type Ia) to remove surrounding follicular cells. The oocyte was placed in a bath continually perfused with Ringer's

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solution (composition in mM: NaCl 115,KCl 2,$MgCl_2$ 1,$CaCl_2$ 1.8, Tris/HCl 5, pH 7.5) at room temperature (18-25°C). Na^+ concentration was changed by substitution with choline$^+$. The oocyte was voltage clamped with a conventional two-electrodes system (NihonKohden CEZ-1100, Japan), whole cell currents were digitized at 5 msec intervals and stored on a hard disc by a computer (NEC PC9800, Japan) for further analysis.

RESULTS AND DISCUSSION. Current-voltage relations of an oocyte were obtained before and 1 minute after an application of 2 mM azide (Fig. 1A). Stepping the membrane potential of the oocyte from resting (about -60 to -90 mV) to various potentials for less than 1 second resulted in resistive currents and slowly rising outward current, when depolarized to more than 0 mV, which was due to the opening of Ca^{2+} dependent Cl^- channels (3). In the presence of azide, the oocyte displayed progressively decreasing outward currents when the membrane potential was stepped to +12 and +32 mV indicating the appearance of slowly-developing depolarization-induced inward currents. Furthermore, the application of azide decreased the membrane conductance from 1.36 to 1.09 μS. This effect of azide was not studied in detail because magnitudes of the conductance changes varied with each oocyte. The net azide-induced current (Fig. 1A bottom) was calculated by subtracting the control record from the record obtained in the presence of azide and was plotted as a function of membrane potential (Fig. 1B, open circles). Due to the azide-induced decrease in conductance, a linear current-voltage relation of -0.27 nA/mV was observed within the voltage range from -120 to -20 mV. When depolarized more, data points deviated from the linear relation in the inward direction. Similar azide-induced currents were observed from naked oocytes, follicular cells of which were removed by collagenase treatment.

Reversal potential of the inward current induced by azide was about +48 mV when the external Na^+ concentration was 115 mM, shifted to +33 mV by halving the Na^+ concentration and to +10 mV by reducing that to 10 mM (Fig. 1B). These changes in the reversal potential suggest that the azide-induced current was carried mainly by Na^+. However, assuming cytoplasmic Na^+ concentration of 10 mM (4), equilibrium potential for sodium should be +61 mV, more than 10 mV higher than the observed reversal potential. Probably, development of other voltage activated currents such as delayed rectifier and Ca^{2+} dependent Cl^- currents had obscured the Na^+ current of decreased driving force.

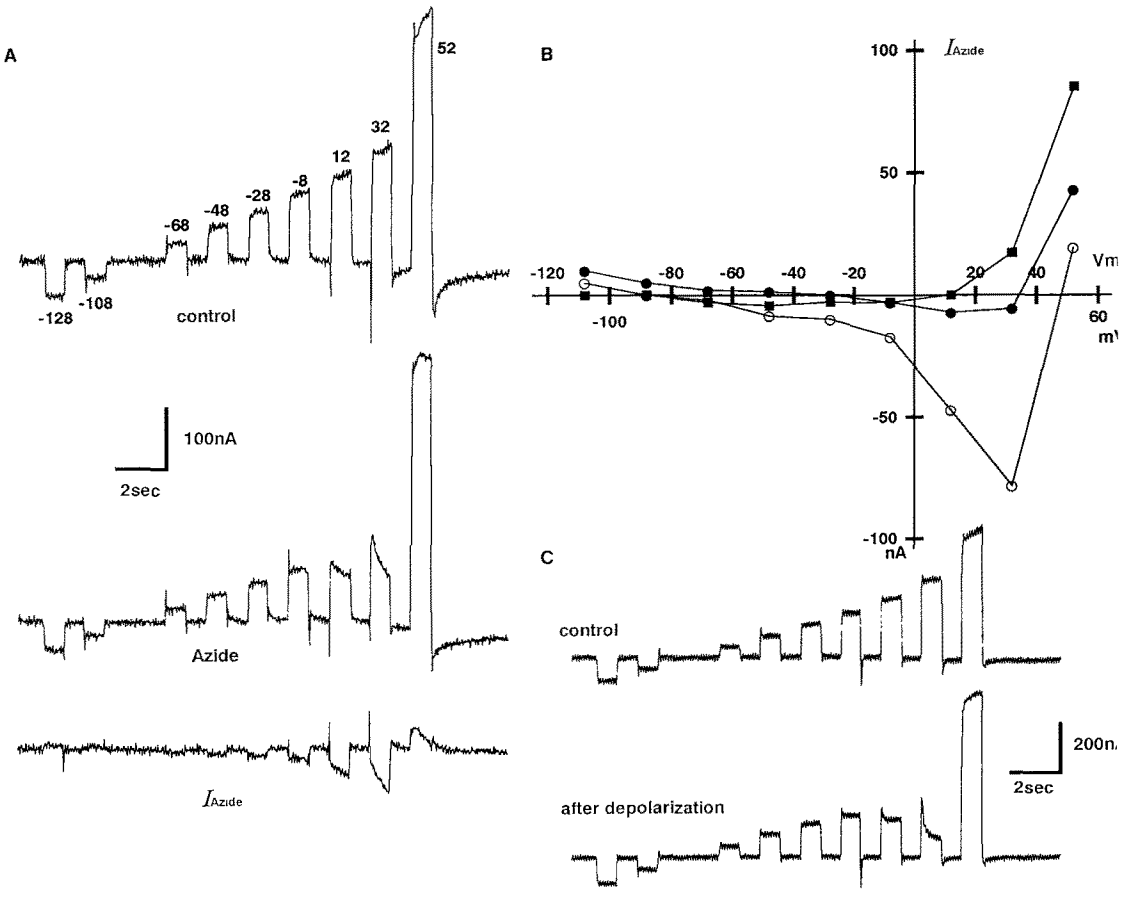

Figure 1. Voltage-dependent inward currents induced by azide and prolonged depolarization. (A): An oocyte was held at -88 mV in voltage-clamp and stepped to various potentials indicated (in mV, in the upper trace). Upper trace was obtained before the application of azide, middle trace was obtained 1 min after the application of 2 mM Azide. Bottom trace was obtained by subtracting the upper trace from the middle trace representing the azide-induced currents. (B): Effect of Na^+ concentration on current-voltage relations of the azide-induced currents. Currents were recorded as shown in the bottom trace of A and measured at the end of each voltage pulses. Sodium concentrations were 115 (o), 58 (●) and 10 mM (■). (C): Induction of voltage-dependent inward current by holding the membrane potential of an oocyte at +40 mV for 2 min. Lower trace was recorded 30 sec after the membrane potential was returned to -88 mV. Voltage steps applied were the same as those shown in A. Azide was not applied to this oocyte.

It has been shown that a long (several tens of seconds) depolarization of *Xenopus* oocyte "induces" electrically excitable Na^+ channels and once induced, the channels slowly ($t_{1/2}$ = 4 min) "disappear" back to a non-excitable state if the membrane is held at negative potentials. While the channels are still in the excitable state, rapid inward currents can be

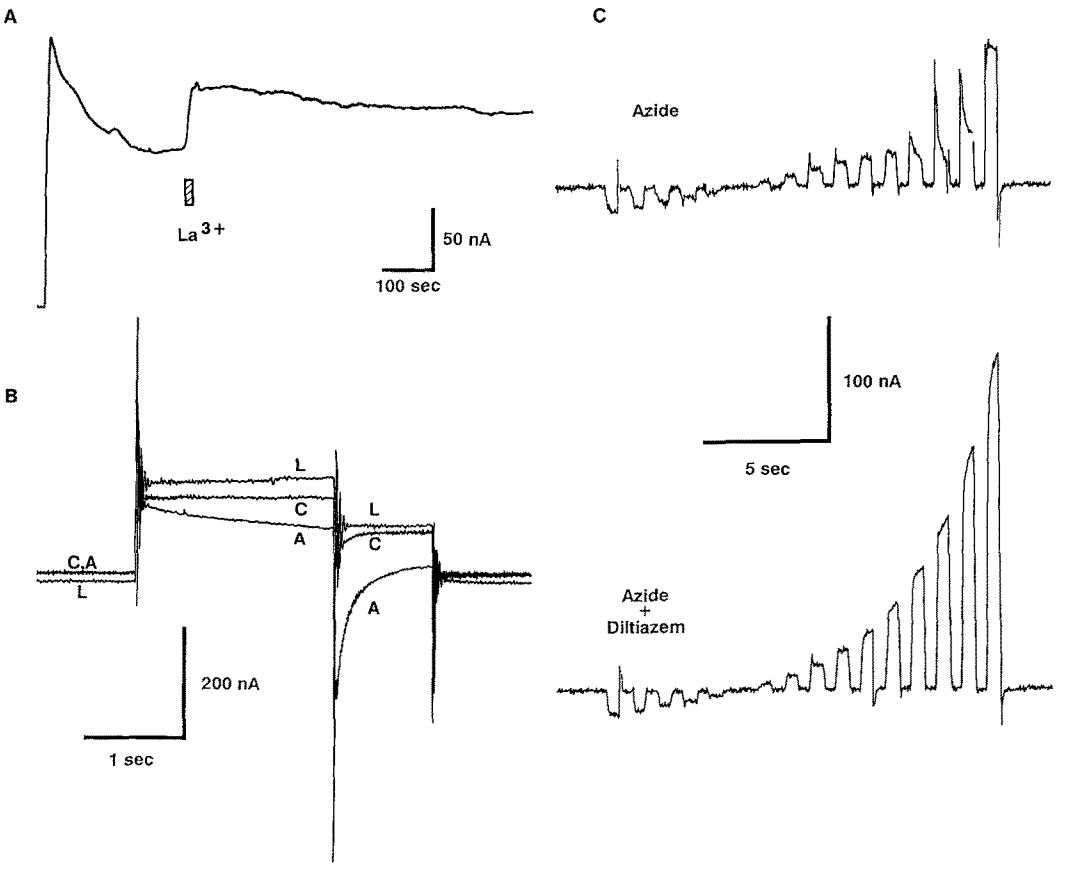

Figure 2. Effects of Ca^{2+} channel blockers. (A): Membrane potential of an oocyte was switched to +30 from -80 mV and kept at this potential until the end of recording. When the depolarization-induced inward current was stabilized, 0.1 mM La^{3+} was applied for indicated period. (B): An oocyte was held at -60 mV and stepped for 2 sec to +20 mV then to -10 mV for 1 sec. Three records obtained from a single oocyte were superimposed. Trace labeled C is a control record obtained in the normal solution, labeled A in the presence of 2 mM Azide, labeled L in the presence of both azide and 0.01 mM La^{3+}. Note the marked tail-current in trace A and complete suppression of that in trace L. (C): Effects of 0.01 mM Diltiazem. An oocyte was held at -80 mV and stepped to various potentials ranging from -130 to +20 mV in 10 mV steps. Upper trace was obtained in the presence of 1 mM azide, and lower trace was obtained in the presence of both azide and diltiazem.

obtained when the potential is stepped to -20 mV and above (1,2). Figure 1C demonstrates the "induction" of the Na^+ channels by depolarization to +40 mV for 2 minutes. Comparing the tracings in Fig. 1A and 1C, it is clear that voltage dependence, reversal potential and time course of the activation of the voltage-dependent inward currents induced either by azide or prolonged depolarization were almost the same. However, the azide-induced current was always smaller than that induced by long depolariza-

tion even higher dose of azide was used. Both inward currents were blocked by the application of Ca^{2+} channel blockers (described later) and tetrodotoxin of high dose. Results suggest that azide and the long-lasting depolarization affected the same type of Na^{+} channels.

In addition to the features described above, both Na^{+} channels induced by long-depolarization and azide were found to be blocked by Ca^{2+} channel blockers. When the membrane potential of an oocyte was stepped from -80 to +30 mV and then kept at this level, an inward current developed very slowly (Fig. 2A). A brief application of 0.1 mM La^{3+} blocked this inward current induced by long depolarization. Figure 2B demonstrates the same effect of La^{3+} on the azide-induced current. Before the application of azide, only passive currents were induced by the potential step from -60 to +20 mV followed by another step to -10 mV before returning to -60mV (Fig. 2B, trace marked C). In the presence of azide, slowly developing inward current was observed during the first step and a large inward tail current was observed immediately after the second step (trace A). The azide-induced tail current, another manifestation of the azide-induced opening of Na^{+} channels, was blocked completely by the additional application of 0.01mM $LaCl_3$ (trace L). Since applications of divalent cations such as Co^{2+} Cd^{2+} and Ni^{2+}, which are commonly used to block Ca^{2+} influxes, sometimes induce the current response by itself (5), their effects on the azide-induced Na^{+} current were not studied. Another Ca^{2+} channel blocker, diltiazem also suppressed the azide-induced inward currents at the concentration of 0.01 mM (Fig. 2C).

Azide is known to have various effects: 1) Inhibition of cytochrome oxidase (6). 2) Activation of guanylate cyclase (7). 3) Release of Ca^{2+} from mitochondria of smooth muscle (8). 4) direct interaction with membrane channels (9,10,11) and pumps (12). Some of these effects could account for the present results. However, applications of cyanide up to 1 mM failed to mimic the azide effects indicating that the inhibition of cellular respiration was not responsible for the azide-effects. Application of 1 mM sodium nitroprusside, known to activate the guanylate cyclase (7), also failed to induce any effect. Intracellular injection of EGTA (500 pmol/oocyte) showed little effects on the azide-induced currents. A defective proton pump is reported to be fully reactivated by direct modulating effect of azide (12) and some anion conductance of retinal pigment

epithelial cell is increased by azide through mechanisms unrelated to its role as a metabolic inhibitor (11). Therefore, azide may also have modulated the hypothetical Na^+ channel and transformed the channel from an original unexcitable state to an electrically excitable state.

Recently, there are many reports describing the "induction" of various ion channels in *Xenopus* oocytes by injecting mRNAs (reviewed in ref. 13). The present work demonstrated that the application of azide could also induce sodium channels suggesting that the appearance of a new channel activity by RNA injection does not necessarily indicate the production of functional channels.

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