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Sodium channels induced by depolarization of the *Xenopus laevis* oocyte

(membranes/excitability/egg/ionic pores)

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ABSTRACT **An electrically gated Na^+ channel can be made to appear in the membrane of the *Xenopus laevis* oocyte by simple depolarization. This membrane normally responds passively to imposed transmembrane currents with resting potentials around −60 mV, but when it is held depolarized to more than about +30 mV it becomes possible to obtain long-lasting regenerative depolarizations up to +80 mV; these depolarizations can last as long as 20 min. This potential is due to an "induction" of a Na^+-dependent channel that is electrically gated open and closed. Its threshold for opening is about −20 mV and it is selective for Na^+ over Cs^+ and choline$^+$ but is blocked by relatively small quantities of Li^+. When a long voltage clamp step to a positive potential under E_{Na} (+70 to +90 mV) is applied, an inward current is observed for many minutes, implying that this channel does not have an inactivation mechanism. The inward Na^+ current is blocked by 0.50 mM tetrodotoxin. When the membrane is held at or near resting potential, the excitability will disappear with time, but it can be made to reappear by again depolarizing the membrane.**

It has recently been reported that the membrane of the oocytes from the African frog *Xenopus laevis* can be made to produce a long-lasting action potential (1). To our knowledge, no regenerative responses to injected currents have previously been reported for this oocyte, although they have been demonstrated in many other eggs and oocytes (2). All of these are of the classical types found in adult excitable tissues involving Na^+ or Ca^{2+} channels and having short durations. The action potential reported in ref. 1 has two important characteristics: (*i*) it cannot be obtained until the membrane has been held positively polarized for some time; (*ii*) it can last for as long as 20 min at +80 to +60 mV. The first characteristic will be referred to as an "induction" of membrane excitability or of excitable channels. The word "induction" is used here as a purely descriptive term with no implications for the nature of the process.

Using voltage and current clamp conditions, we have further studied this phenomenon in an effort to compare it with other known channels. The long-lasting action potential reported in ref. 1 is shown to be due to a voltage-gated sodium channel in the membrane, which is not excitable in the normal state and which is induced by a long depolarization. Once induced, the channel opens and closes with potential, its threshold is about −25 mV, and it selectively allows Na^+ to pass. The channels will remain excitable for a limited time at resting potential, disappearing after about 10 min. We present here descriptive data on the voltage and temperature dependence of the slow processes of induction and disappearance under voltage and current clamp conditions. A more detailed report on the induction and disappearance will appear later. Data for the threshold, voltage dependence, selectivity, and some pharmacological properties of the channel itself are also presented. Preliminary results have been reported (3, 4).

MATERIALS AND METHODS

The materials used are oocytes from the African frog *Xenopus laevis* raised in laboratory holding tanks and kept on a 12-hr day/night cycle. Fully developed, Dumont stage VI (5), oocytes were manually removed with fine forceps from their follicular sacs in pieces of excised ovary after being treated for 2 hr with 40 mg of Dispase (protease, neutral grade II, Boehringer Mannheim, no. 165-859) in 100 ml of OR2 solution (6). The OR2 composition in mM was: NaCl, 82.5; KCl, 2.5; $CaCl_2$, 1.0; $MgCl_2$, 1.0; Na_2HPO_4, 1.0; pH 7.4 Hepes, 5.0. The donor females had no hormonal treatment of any kind. Oocytes at this stage of development are 1.2–1.4 mm in diameter and are covered with microvilli and invaginating crypts, which greatly increase their surface area (4, 7).

The two-electrode voltage clamping technique was used with a single low-voltage differential amplifier supplying the clamping current. The clamping current was measured by a current-to-voltage converter in the ground circuit of the chamber. The system was arranged to provide either voltage or current clamp conditions of the oocyte membrane. The oocyte was held on a nylon mesh by the microelectrodes in a constantly perfused temperature-controlled chamber with a washout time of about 30 s. Microelectrodes were pulled on a horizontal puller from thin-walled glass tubing containing a fiber. The voltage electrodes were filled with a 2 parts 3 M KCl/1 part 0.6 M K_2SO_4 solution with resistances of 10–20 MΩ, while the current electrodes were filled with 3 M KCl with resistances of 1 MΩ or less. The generally high input resistances of these oocytes ensured an adequate space clamp in spite of their size. Observations with a second voltage electrode showed that, over the accessible hemisphere, space clamp conditions were obtained. Potentials and currents were monitored with digital voltmeters, recorded with a Brush 280 inkwriter, and digitized and stored with a PDP 8 computer (Digital Equipment Corporation). Immediately upon defolliculation, the oocytes can have resting potentials of up to −90 mV with input resistances of 2–4 MΩ. The large resting potential is greatly diminished by 0.1 mM ouabain, suggesting a 20- to 30-mV contribution by a Na/K pump such as that previously reported for these oocytes (8). Such oocytes can be induced to produce prolonged action potentials lasting only a few minutes with peak potentials of about +50 mV. Dissected oocytes kept in the refrigerator in OR2 at about +5°C for 24 hr have resting potentials of around −60 mV and input resistances of 2–7 MΩ. They reliably undergo channel induction up to 3 days after dissection and sometimes to as

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long as 7 days after. In general, the peak depolarization did not reach the +60- to +80-mV level until the second day, when the duration of the action potential became very much longer. These effects are consistent with the hypothesis that a Na/K pump had been activated by the defolliculation, thus reducing the internal Na activity (8). The same results have been obtained with oocytes from females exposed to natural daylight and in all seasons of the year as well as in oocytes removed manually without any enzymatic treatment or defolliculated entirely by a collagenase treatment. It would appear, therefore, that the results were not a seasonal phenomenon or an artifact of defolliculation.

RESULTS

Channel induction and disappearance

Injected currents in these oocytes produced potential changes that were characteristic of a passive membrane. The membrane potential response to 5- to 10-s square current pulses was approximately exponential for both inward- and outward-directed currents. However, when the membrane was repeatedly depolarized more positively than about +35 mV the potential responses became modified. They began to continuously depolarize instead of reaching a plateau, as initially, and began to show a delayed return to resting or shouldering at the end of the current pulses. Once the shouldering had appeared, a succeeding pulse resulted in a large regenerative depolarization in membrane potential that did not return to resting for long periods. Fig. 1*A* shows an example of such an induction process and the large depolarization, which in this case lasted longer than 15 min. During this depolarization the input resistance was greatly decreased to as little as 1/100th of the initial value and was lowest at the time of the maximal depolarization. The potential attained amplitudes of +70 to +90 mV and then slowly declined to about +55 to +60 mV, from which it more rapidly returned toward resting in two steps. Passing a depolarizing current at this time rapidly produced another long depolarization, which generally did not last as long as the first. On the other hand, when the depolarizing pulse was applied more than a few minutes after repolarization, the entire series had to be repeated in order to again obtain the long depolarization, indicating that the channels were no longer present in the membrane.

Under voltage clamp, when the membrane potential was stepped from a holding potential at −60 mV to +55 mV and then kept at this level, the current was initially outward directed and proportional to the resting resistance of the membrane. This was followed, for a short time, by either no change or a small increase in the outward direction. A slow decrease in the outward current then began and the current became inward directed, reaching a plateau if the potential was maintained for a sufficiently long time. Returning the potential to −60 mV resulted in zero current following the capacitance and inward current transients, indicating that the channels responsible for the inward current were closed. Immediately depolarizing again to +55 mV resulted in a very rapidly established inward current of the same magnitude as that obtained at the end of the initial depolarization. Stepping the membrane potential to any level more positive than about −20 mV resulted in rapidly established currents. This indicated that the channels were opened and closed rapidly with the membrane potential—in other words, they were electrically gated. An example of such responses, obtained at 12°C, is shown in Fig. 1*B*. It can be seen in this figure that the inward current was maintained for a long time, indicating that the channels did not "inactivate." If more than about 10 min had elapsed after the long depolarization,

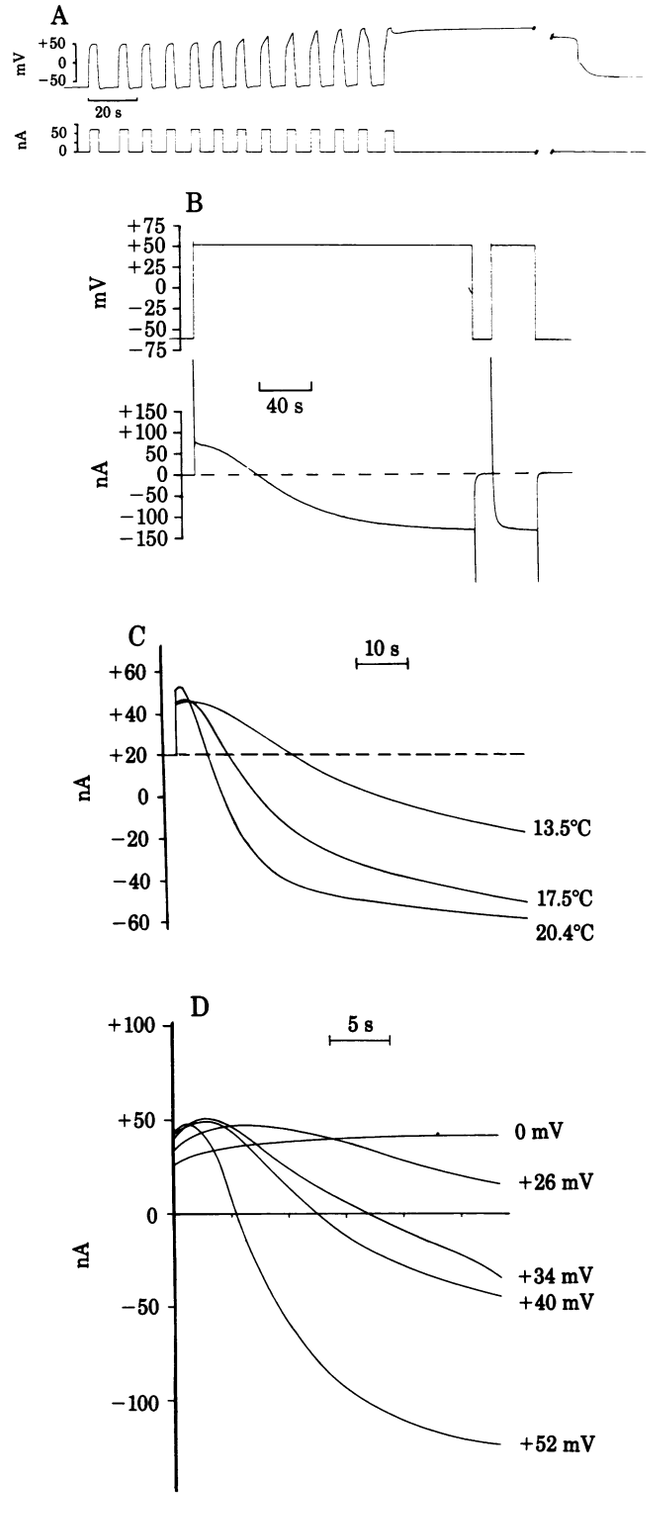

FIG. 1. (*A*) Membrane potential responses (upper trace) to 6-s injected current pulses (lower trace) at 12°C. The last pulse is smaller than the others. The break in the tracings represents a 15-min segment during which the potential diminished linearly to the level on the right. (*B*) Membrane current responses to voltage clamp steps, traces as in *A*. Asymmetries in the transients are due to chart edge pen blocking as the reference level was shifted to accommodate pen excursions on the chart paper. (*C*) Superposed tracings of membrane current records for a fixed voltage step to +50 mV from holding at −60 mV, at different temperatures as indicated on each tracing, in the same oocyte. (*D*) Superposed tracings of membrane current as in *C*, all at 19°C, stepping from holding at −60 mV to the potentials indicated at each step in one oocyte. All records shown are from different oocytes.

while holding at −60 mV, a subsequent depolarization again resulted in an outward-directed current, which again slowly became inward. Thus, as in the case for current clamp conditions, the effect of leaving the membrane repolarized for more than about 10 min was a disappearance of the channels and a reversion of the membrane to its initial state.

Properties of channel induction and disappearance

Channel induction and disappearance were examined in two ways. To test the effects of temperature, the holding potential was stepped from −60 mV to +60 mV in the same oocyte while it was maintained at different temperatures. Fig. 1*C* shows three tracings obtained as above at 13.5°C, 17.5°C, and 20.4°C. A great increase in the speed of establishing the inward current with temperature was evident even in this moderate range. By way of comparison, the increase in the initial outward current with temperature was quite small. To test the effects of the membrane potential on induction at a fixed temperature, the potential was stepped to various depolarized levels and held there until an inward current was established. The rate of channel induction was clearly dependent on the potential, as can be seen in Fig. 1*D*. The inward current at +40 mV was 5 times greater than that at +26 mV after 15 s and more than 10 times greater at +52 mV in the same time. These differences were not explained solely on the basis of differences in driving force or voltage dependence of the gating at these potentials (see Fig. 3*A*) and were interpreted as an increased rate of channel induction with greater depolarization.

Experiments to determine whether the maximal number of channels induced at different potentials and temperatures will eventually be the same if enough time is allowed under each condition have not yet been done. One reason is the slow appearance of an outward current when the membrane is held depolarized for long times at temperatures above about 12°C.

Once the channels had been maximally induced at +55 mV, returning the potential to around −60 mV caused them to slowly disappear. This process was followed by test pulses, which periodically returned the potential to +55 mV for a few seconds. The resulting inward current diminished for successive test pulses, as can be seen in Fig. 2*A*, and this diminution was interpreted as due to the disappearance of the channels. The disappearance was an S-shaped function of time, showing that even while the membrane was held at resting potential, channel excitability persisted for a few minutes at the same level. The test pulse responses for the data at 16°C in Fig. 2*A* are shown plotted in Fig. 2*B* to illustrate the time course of the disappearance process. The inward current did not asymptotically approach zero even at longer time. This was interpreted as an inward current due to channels induced during the test pulses. Other results (not shown) have shown that the initial number of channels was maintained for longer times at lower temperatures, as long as 3 min at 12°C.

Channel electrical properties

Because channel induction is not instantaneous, the currents obtained for short pulses of 0.1- to 1-s duration, in a previously nondepolarized oocyte, show little or no time-dependent change in magnitude after the capacitive transients. The curve with open circles in Fig. 3*A* was obtained by using 1-s pulses at 10- to 20-s intervals at 19°C. The current was measured at the end of the voltage step. No anomalous rectification type of conductance increase has been observed in any oocytes to about −120 mV. The membrane conductance remained almost purely resistive at all negative potentials, showing only a slight increase in slope from about −20 mV. From about +20 mV, channel induction usually decreased the slope until about +50 mV, where the slope again increased with more positive potentials.

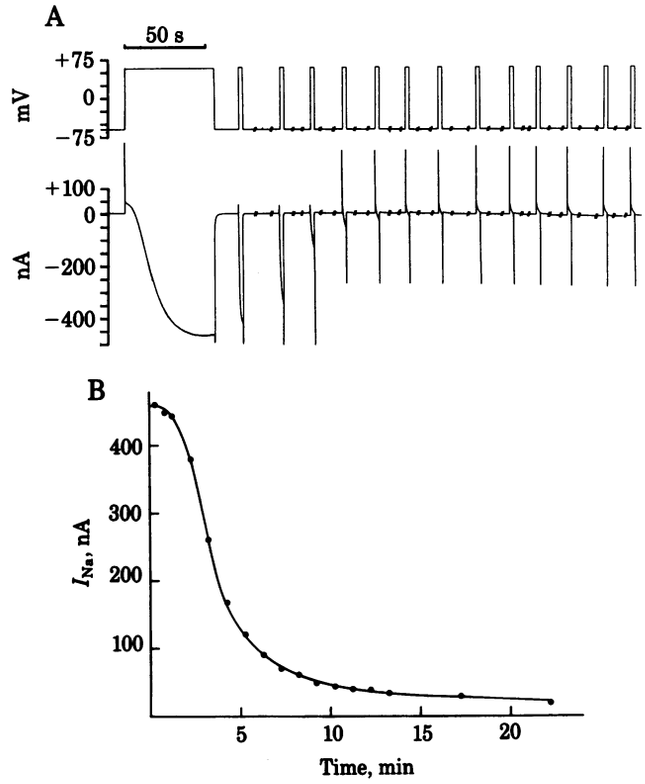

FIG. 2. (*A*) Voltage clamped membrane potential and current recordings as in Fig. 1. Two-second test pulses to +55 mV at 2-min intervals follow a long inducing step to the same potential at 16°C. Note the channel induction effects of the test pulses after about 10 min. (*B*) Inward currents at the ends of each of the test pulses measured from the level of the initial outward current.

However, as in the case shown in Fig. 3*A*, the number of channels induced seems to be small, as indicated by the inflection. This type of curve was therefore taken as the current–voltage (*I–V*) properties of the membrane without induced channels.

From the foregoing results it was evident that obtaining data for characterizing the ionic properties of these channels would be complicated by the fact that the number of channels will be varying. For example, channel induction at 19°C with +80 mV may be as rapid as channel opening at 55 mV at the same temperature. If so, the currents obtained at these two potentials may not be for the same number of channels. The question of the number of channels was again raised for the cases in which the test potential was progressively going more negative, due to slow disappearance. The following procedure was therefore adopted to ensure some kind of steady state in channel population during the test voltage steps. Because the channels do not disappear within a few seconds, they were first induced by a long depolarization at +55 mV until an inward current plateau was obtained. Before stepping to each test potential level, a conditioning pulse more positive than the equilibrium potential was applied for a few seconds. The large depolarizing potential should ensure that before each test step, the number of induced channels, if not maximal, was at least constant. Fig. 3*A* shows typical *I–V* curves obtained by the above procedure, using a +80-mV conditioning pulse. The noninduced and induced *I–V* curves were superposed from about −100 mV to about −25 mV, where the current for the induced case begins to deviate in the inward direction and smoothly approaches a peak inward current at between +50 and +60 mV. For more positive potentials, the inward current reverses direction and is considered to become outward at the potential where it crosses the current es-

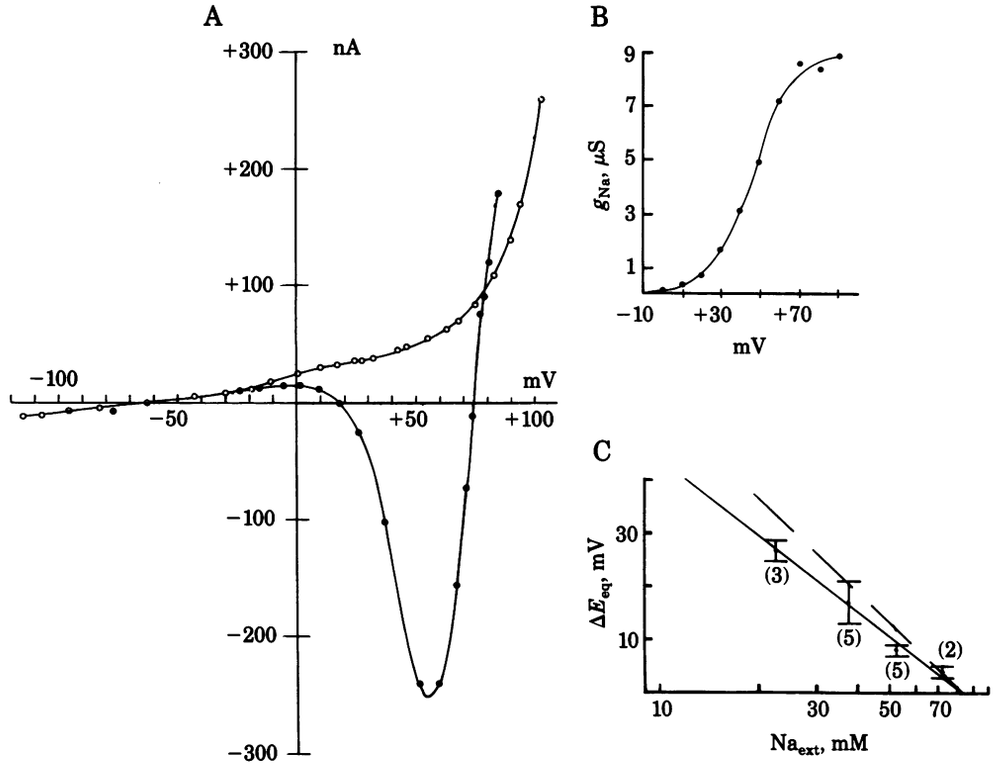

FIG. 3. (*A*) *I–V* relationships for the membrane without induced channels (○) and after induction using conditioning pulses as described in the text (●) at 19°C. (*B*) Chord conductances for an oocyte at 12°C, obtained from $\Delta I_m/(E_{Na} - V_m)$, in which ΔI_m is change in membrane current, E_{Na} is sodium equilibrium potential, and V_m is the membrane potential. (*C*) Differences in equilibrium potentials in normal and choline-substituted solutions, mean values ± SD, with the number of determinations in parentheses. The broken line is drawn for 59 mV/10× change in cation. See text for further explanation.

tablished without channel induction. The current during the test step to this potential was outward and showed no change, which was what may be expected if the noninduced outward current channels did not change with time and the induced inward current channel was at its ionic equilibrium potential. This potential also agrees very well with the maximal potential obtained during the long regenerative depolarization and therefore has been taken as the equilibrium potential for the current-carrying ions. Equilibrium potentials obtained in these ways imply cytoplasmic Na^+ activities of about 1–6 mM, which agrees with the 6 mM given for *Rana pipiens* oocytes (9). The very sharp increase in the slope of the *I–V* relationship beyond the peak inward current was a consistent feature of these curves and indicated a very large increase in total conductance at these potentials. Chord conductances have been determined from such *I–V* curves, and a typical example is shown in Fig. 3*B*. The chord conductance, in general, reached a maximum at about +80 mV.

The presence of an outward potassium current is not eliminated in these *I–V* curves, although preliminary experiments with tetraethylammonium-injected oocytes indicate that a delayed rectification type of potassium conductance may be small in these oocytes. The fact that this rectification may be small and appears at large depolarizations has been reported in other eggs and oocytes (see ref. 2).

Channel ionic properties

Because the equilibrium potential for the channel suggests that it may be a Na^+ channel, several monovalent cation substitutions have been tried. These experiments were done with normal OR2 controls before and after the substitution series. Substitution of the chloride salts of Cs^+ and $choline^+$ for NaCl in the bathing medium produced almost a Nernstian shift of the equilibrium potential and a decreased inward current with decrease in Na^+ in the medium. Therefore cesium and choline behave in this channel as in most other sodium channels, but cesium produced effects that changed the equilibrium potential in the control *I–V* curves after the test series. With choline the control curves were identical before and after the changes in the bathing medium. The data obtained for 15 oocytes with choline substitution are shown in Fig. 3*C*. The difference between the equilibrium potential in normal solution and in solutions in which part of the sodium had been replaced by choline is plotted on the ordinate. Each oocyte was tested only for one substitution. The plot gave a slope of 50 mV change for a 10-fold variation in the external sodium concentration.

Lithium substitution for sodium produced effects different from those seen in other sodium channels. Briefly, Li^+ produced no shifts in equilibrium potential even at full substitution, and channel conductance was reduced in a concentration-dependent way. Some preliminary results from further study of this effect of lithium have been presented (4).

Tetrodotoxin rapidly and reversibly blocked both inward and outward currents in this channel, but at 1.0 mM, in the one oocyte tested. At 0.50 mM most of the inward current was blocked but not the outward in three oocytes, and at 0.10 mM blocking effects were just detectable. In all cases, the introduction of tetrodotoxin in the chamber was accompanied by a membrane depolarization that was concentration dependent. These effects of tetrodotoxin did not appear to be related to the citrate that is usually found in the toxin preparation, because addition of citrate alone to 10 mM had no effects. Veratridine and toxin II from *Anemonia sulcata* (the latter was a generous gift of Laszlo Beress) have been shown to induce membrane

instability by activating channels in other nonexcitable cells (10). These substances also produced membrane instability in this oocyte but had no apparent effect on the induction or the gating of the present channels. Induction and gating of the channels were also obtained while oocytes were undergoing meiotic maturation induced by the application of progesterone.

Changes in external calcium resulted in shifts of the *I–V* curve along the voltage axis in the directions expected for a surface charge masking effect (11) accompanied by some changes in the peak inward current. Although the present results do not provide conclusive evidence, it would appear that Ca^{2+} is not a major current-carrying ion in this channel.

DISCUSSION

The foregoing results are consistent with the hypothesis that in the fully developed *Xenopus laevis* oocyte prolonged depolarization by an injected transmembrane current induces a channel that is electrically gated and selective for sodium. This channel shows many characteristics similar to those of other known sodium channels with respect to selectivity and voltage sensitivity but lacks an inactivation mechanism and seems to be pharmacologically different. The channel properties demonstrated above and the apparent lack of a large delayed rectification are sufficient to explain the long-lasting regeneratively obtained depolarization or action potential previously observed (1). An outward potassium current may have a role in the repolarization process from the prolonged depolarization, as indicated by the finding that tetraethylammonium injected into the oocyte reduced outward currents in the noninduced membrane and in one case extended the time of the long depolarization to 39 min.

This channel should not be confused with the channels responsible for the "activation" potential (see refs. 2 and 12 for references) obtained in fertilizable eggs for several reasons. Most importantly, the activation potential in anuran eggs is obtained only in 1:10 diluted media, where it may attain amplitudes of +10 to +20 mV. In the case of *Bufo bufo* (13) and *Rana pipiens* (12), it has been demonstrated that anion substitutions were effective in modifying the magnitude of the activation potential. In *Xenopus laevis* indirect evidence exists (14) that external chloride modifies the activation potential. Furthermore, the potential reported here attains a magnitude of +80 mV and is electrically activated. Fertilization potentials are generally smaller and reported not to be electrically activated (12). The present results have been obtained in fully developed but not meiotically matured oocytes, which in *Rana pipiens* are reported to be unable to produce a true activation potential (15). In view of this evidence, it is concluded that the channels reported here are not those that will eventually subserve the activation potential.

It is doubtful that this channel, in the induced form, exists in the normal oocyte membrane at this stage of development because there appears to be little likelihood of its experiencing the conditions necessary to induce the channels. It is more probable that some kind of sodium selective mechanism exists in the membrane and that the present results are a fortuitous finding that these mechanisms can be made to be voltage sensitive channels by a reduction or reversal of the membrane potential gradient.

It is interesting to speculate that this kind of channel may also exist in fully differentiated excitable as well as nonexcitable cell membranes. The facts that this channel exhibits many properties very similar to those found for other sodium channels but does not inactivate and is in a membrane lacking a large outward potassium rectification seem to recommend it for the study of channel properties. Unfortunately, the highly convoluted plasma membrane and the presence of a vitelline membrane will present a major problem in trying to apply the quantitative techniques such as single-channel recording and noise analysis.

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