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CALCIUM-DEPENDENT CHLORIDE TRANSIENT CURRENTS IN THE IMMATURE OOCYTE OF THE FROG, *Rana esculenta*

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INTRODUCTION

Interest in chloride currents in amphibian oocytes is related to the fact that they have a role in the genesis of fertilization potential in the mature egg-cell (10, 16, 7). Furthermore, chloride currents subserve the oocyte responses to acethylcholine (18, 3).

Ca-dependent, transient Cl-currents can be activated in immature oocytes by wide variations in membrane potential. Outward transients, activated by membrane depolarization, have been studied thoroughly in *Xenopus* (11, 1), and ascribed to entry of Ca^{2+} into the oocyte through voltage-operated Ca-channels. Inward transients, activated by membrane hyperpolarization, have been described both in *Xenopus* and in *Rana* (15, 17, 12, 14). However, the mechanism causing the intracellular Ca^{2+} concentration to rise has not been defined in this case.

The present paper reports that both inward and outward Cl-transients can be elicited in *Rana* oocyte by adequate voltage clamp protocols. Both transients reflect surges of intracellular Ca^{2+} concentration. Some observations indirectly suggest the existence of Ca-activated Ca-release in immature amphibian egg-cells.

METHODS

Oocytes at stages IV and V (5) were dissected from the ovaries of pithed frogs (*Rana esculenta*) and their outer envelopes were manually removed under the microscope using fine forceps.

All experiments were performed using two microelectrode voltage clamp; a detailed description of the recording conditions is given elsewhere (17). The clamp circuit (Dagan 8500) was modified by using a high voltage (± 210 V) power supply for current injection. This enabled us to reduce the duration of the capacitative transients to 1-2 msec.

Single oocytes were placed in a Plexiglas recording chamber having a volume of 2 ml, perfused with Ringer solution (containing, in mM/l, NaCl 108, KCl 2, $CaCl_2$ 1.8, Glucose 5.5, Tris-HCl 10; pH = 7.4), or other test solutions. Most of the experiments were performed in the presence of Tetraethyl Ammonium Chloride (TEA) 10 mM, to block the outward current of K^+ activated by membrane depolarization (17).

Intracellular injection of Ca^{2+} was carried out by ionophoresis using microelectrodes filled with .5 M $CaCl_2$.

The tracing were recorded on tape in FM. A subsequent analysis was performed using a HP/9816 after A/D conversion at 5 KHz.

RESULTS

Inward transient (I_{it})

As shown in Fig. 1A, an evident inward transient current, named I_{it}, can be elicited by a hyperpolarizing voltage step preceded by a conditioning depolarizing command. It should be noticed that I_{it} adds to an inward Cl current, named I_{ir} (17), which grows exponentially and does not inactivate.

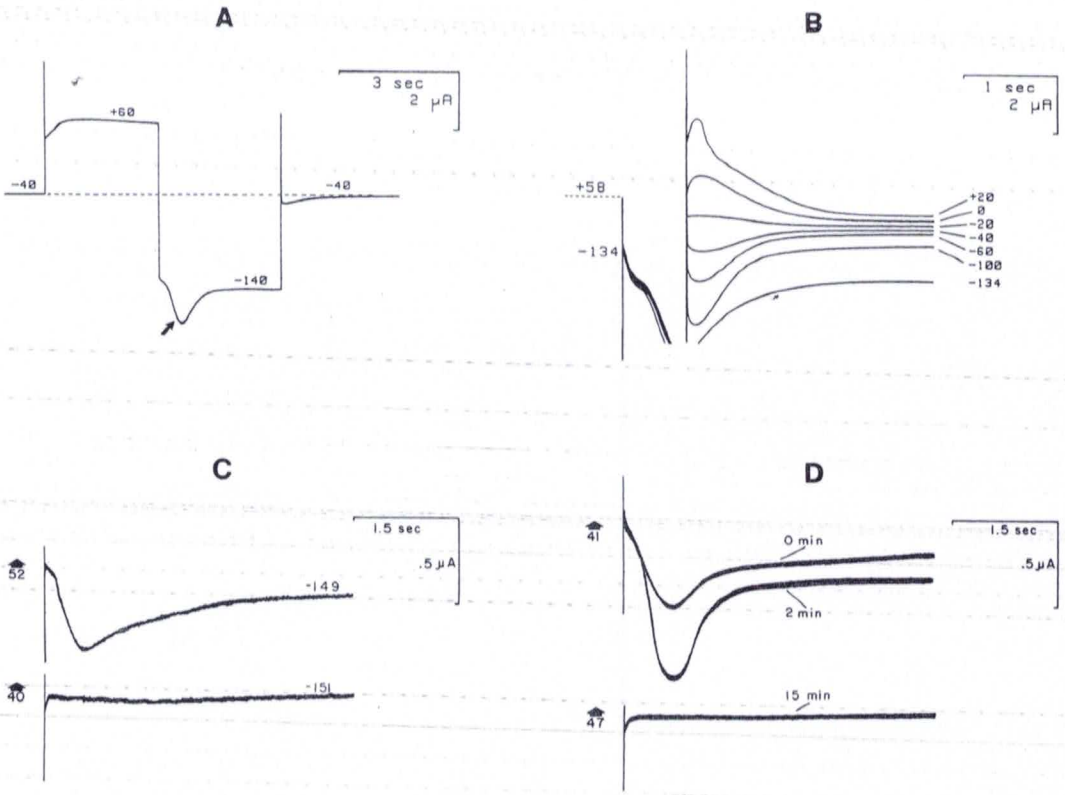

Fig. 1. – The inward chloride transient current (I_{it}). *A*: Example of inward Cl-transient current (arrow) in the frog oocyte. Holding, conditioning and test potentials are indicated in mV by the figures along the tracings. The time-dependent current activated by the conditioning potential is an outward current carried by K^+, subserving outward rectification in *Rana* oocyte; *B*: Examples of current relaxations from inward transients. The clamp potentials are indicated by figures near the traces; *C*: Membrane currents obtained from the same oocyte in standard Ringer (upper trace) and in Ringer containing SITS (lower trace). Figures adjacent to each trace indicate test potentials (right) and conditioning potentials (left, with arrows); the conditioning pulses lasted 3 sec; *D*: Membrane currents recorded during perfusion (1 ml/min) with Cl-free Ringer. Conditioning potentials indicated as in C; the test potentials are around —135 mV.

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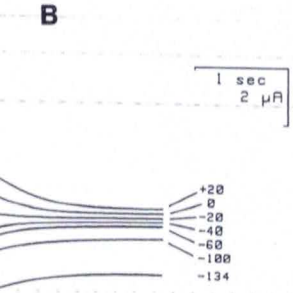

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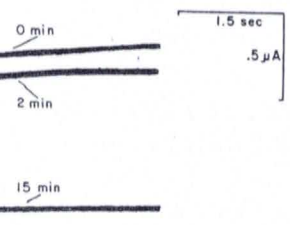

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In some cases, hyperpolarizing voltage steps starting directly from the resting potential (—30/—50 mV) were able to elicit inward transient without any conditioning pulse, though they were of much smaller amplitude (see below).

The reversal potential of the $I_{it}+I_{ir}$ current can be evaluated by stepping the membrane potential to different voltage levels at the peak of I_{it}, and was close to —20 mV (Fig. 1B). Since I_{ir} is reported to have a reversal potential at —20 mV (17), this implies that I_{it} has the same reversal potential. This is very near the equilibrium potential for Cl^- (E_{Cl}) in the amphibian oocyte (9, 11).

When Acetamido-Isothiocyanate-Stilbene-Disulfonic Acid (SITS), which has been shown to be a specific inhibitor of transmembrane chloride currents (8), was added to the bath, both currents were suppressed, as shown in Fig. 1C. I_{it} and I_{ir} increased when the chamber was perfused with a Cl-free Ringer solution, in which NaCl and $CaCl_2$ were replaced by Na-Methansulphonate and Ca-Propionate respectively (Fig. 1D). Then gradually decreased, and disappeared in about 15 min, probably paralleling the depletion of internal chloride.

All these observations seem to provide convincing evidence that a Cl-current is responsible for the inward transient I_{it} in the frog oocyte.

Fig. 2A shows sample records of I_{it}, as obtained by stepping the membrane to different test potentials starting from a constant conditioning level at +40 mV. We measured the amplitude of I_{it} by subtracting the steady state current from the corresponding peak current (in fact, I_{it} can be a little larger, when I_{ir} has not fully attained its steady value at the time when the transient displays its peak). The resulting I/V plot is reported in Fig. 2B. The inward transients are activated at potentials negative to —80/—90 mV, their peak amplitudes increasing with rising membrane hyperpolarization almost linearly beyond —100/—110 mV. The membrane conductance to chloride appears also to increase without saturation with membrane hyperpolarization, since the straight lines fitting the data beyond —100 mV do not intercept the voltage axis at E_{Cl}.

The amplitude of I_{it} proved to be related both to the amplitude and the duration of the conditioning potential.

Sample records obtained by stepping the membrane to a constant test potential of —140 mV starting from different conditioning potentials having the same duration are shown in Fig. 2C. Peak amplitudes of I_{it} are plotted vs. the amplitude of the conditioning potential in Fig. 2D, using oocytes in which small transients already were observed by stepping the membrane directly from the resting potential.

The dependence of I_{it} upon duration of the conditioning potential is illustrated in Fig. 3A and Fig. 3B: I_{it} gradually increased in amplitude as the duration of the conditioning potential increased, reaching a saturating level for pulse durations over 8-10 sec.

Finally, we observed that the onset of I_{it} is followed by a period of «refractoriness» during which the same test potential proved less effective in producing inward transients. In the case of Fig. 3C, complete recovery could only be achieved after a latency of about 30 sec.

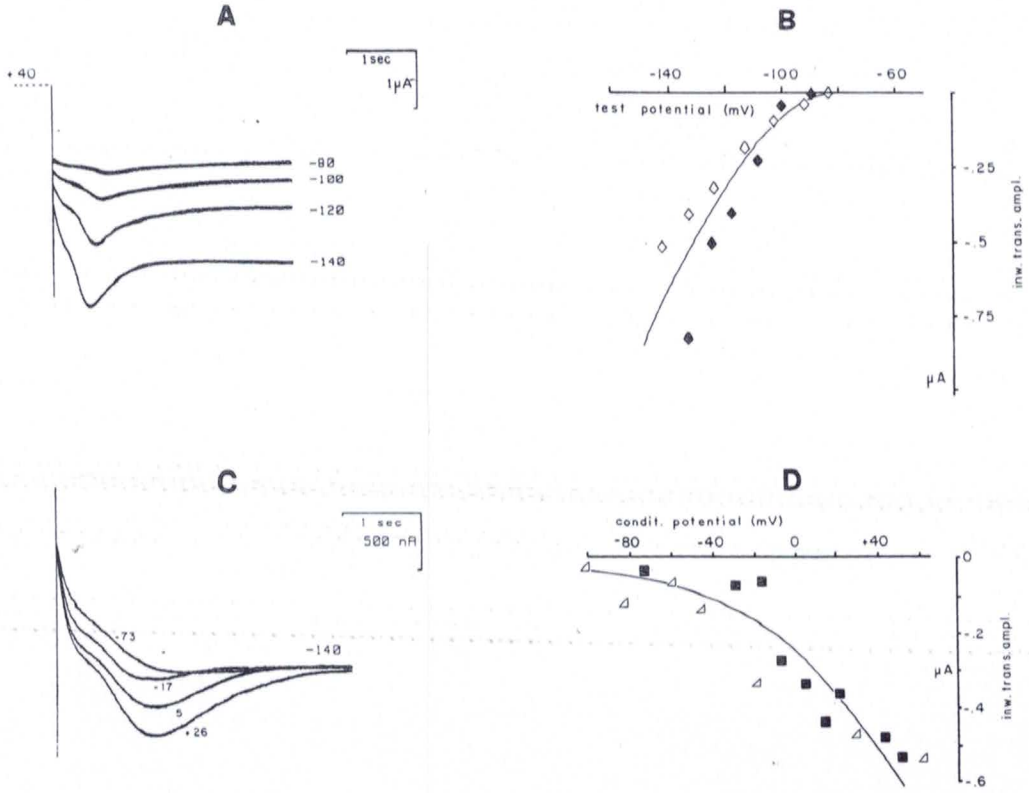
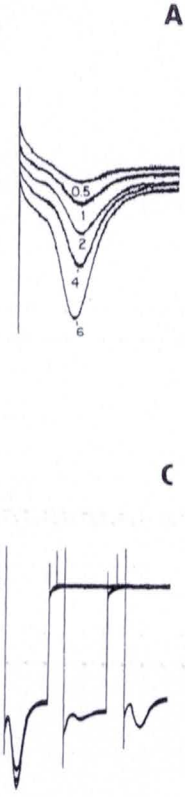


Fig. 2. – The dependence of I_{it} upon conditioning and test pulse amplitude. *A*: Sample records of inward transients, obtained with test command levels of increasing amplitudes, preceded by the same conditioning pulse to +40 mV; *B*: Plot of peak amplitude of I_{it} vs. test command potential. The symbols are examples of peak amplitudes measured in two experiments. The curve is the result of best fitting of data from six different oocytes to a 2nd order function. Amplitude and duration of the conditioning pulses were +40 mV and 3 sec respectively; *C*: Example of a series of inward transients, obtained with test command steps to the same membrane potential, starting from conditioning levels of increasing amplitudes, as indicated by figures adjacent to the traces; *D*: Plot of peak amplitude of I_{it} elicited by the same test command (—140 mV) vs. amplitude of preceding conditioning pulses. Symbols and curve as in B.



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Ca-dependence of I_{it}

I_{it} strongly increased in amplitude when the external Ca^{2+} concentration was raised and was abolished when Ca^{2+} was replaced by Ba^{2+} or when inorganic Ca-blockers such as Cd^{2+}, Co^{2+} or La^{3+} were present in the bath at 1mM concentration, even with 20 mM $[Ca^{2+}]_{out}$ (data not shown).

These observations suggest that inward transient currents of Cl^- may be related to the entry of Ca^{2+} from the external medium. To obtain further evidence, we injected Ca^{2+} into the cell by means of a third microelectrode. Following a pulse of Ca^{2+} into the oocyte, a transient current appeared (Fig. 4A) with the following properties:

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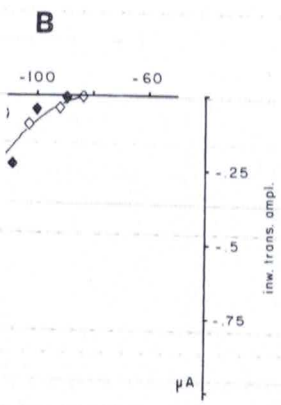

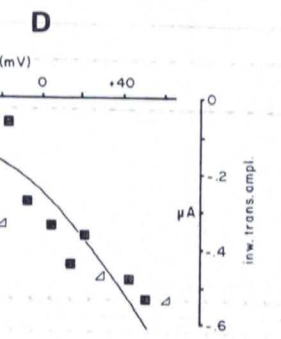

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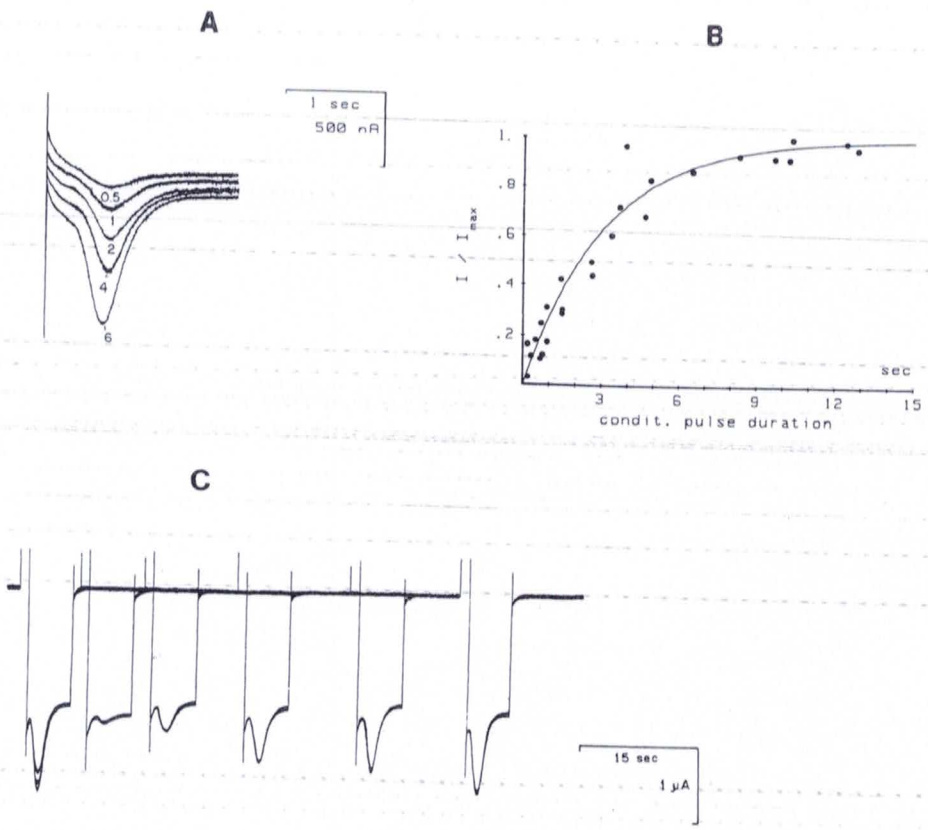


Fig. 3. – The dependence of I_{it} upon the conditioning pulse duration and upon the delay between two subsequent test pulses. *A*: Sample records of inward transients, obtained with test command steps to the same membrane potential (—140 mV), preceded by conditioning pulses having a constant amplitude of +40 mV but different durations, indicated (in sec) by figures adjacent to the traces; *B*: Effect of conditioning pulse duration on peak amplitude of I_{it}. The circles are examples of data obtained in two esperiments. The curve is the result of best fitting of data from four other oocytes to an exponential function; *C*: Superimposed tracing showing the time course of recovery of I_{it} after a delay. The holding potential was —40 mV; the recovery was studied by stepping to —140 mV for 5 sec, after a conditioning pulse to +40 mV for 1.5 sec, twice, with increasing delays, in different trials.

1) it reversed at about —20 mV;

2) it disappeared when SITS was added to the bath;

3) it disappeared when external NaCl and $CaCl_2$ were replaced by Na-Methansulphonate and Ca-Proprionate respectively.

Ca pulses are therefore able to elicit a transient current of chloride in the frog oocyte, without any preceding step to a conditioning potential. Pulses of Ca^{2+} repeated at relatively short intervals (1-2 sec) actually produced a sustained Cl-current, thus suggesting that the decay of I_{it} in voltage-evoked responses does not depend on inactivation of Cl-channels, but is related to the mechanism leading to the surge in intracellular Ca^{2+} concentration.

As shown in Fig. 4B, the amplitudes of the responses activated by single Ca^{2+}

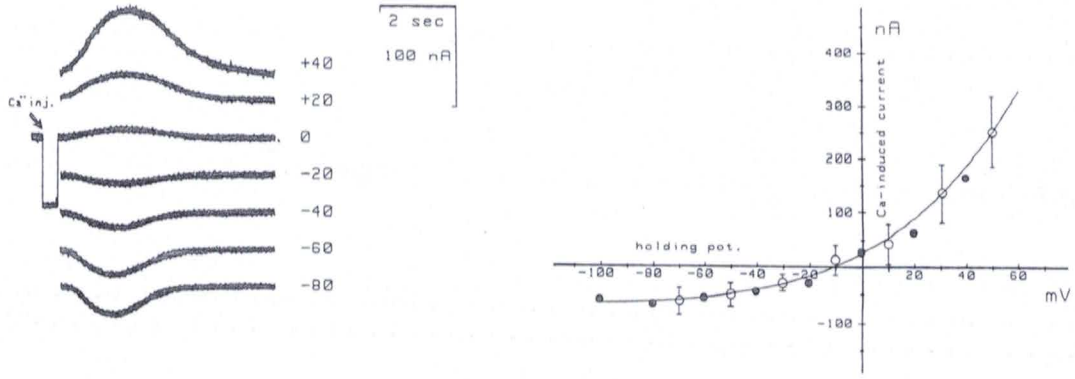


Fig. 4. – The effect of Ca^{2+} intracellular injection. *A*: Sample records of Cl-transients following pulse injection of Ca^{2+} at different holding potentials (indicated by figures near the traces); *B*: Shows a plot of Ca-induced current peak amplitues vs. holding potential. Dots are measurements taken during the experiment illustrated in A. Circles are mean values (±S.D.) from five other oocytes.

pulses were non-linearly related to the membrane holding potential. In the hypothesis that, as Miledi and Parker (12) have observed in *Xenopus*, the same amount of Ca^{2+} injected into the oocyte activates the same number of Cl-channels at all potentials, the non linear I/V relation in Fig. 4B suggests a remarkable outward rectification in single Cl-channel conductance of the frog oocyte.

Outward transient current (I_{ot})

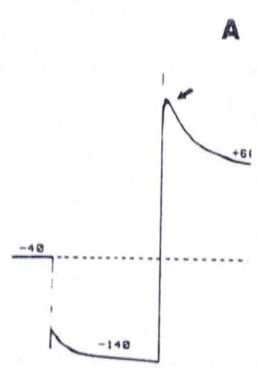
Depolarizing steps preceded by a conditioning pulse to a hyperpolarizing potential elicited an outward transient current, named I_{ot}. This is shown in Fig. 5A: when the cell was hyperpolarized starting from its resting potential, only the non-inactivating current I_{ir} was elicited in this case. When the potential was further stepped up to +60 mV, I_{ot} became manifest.

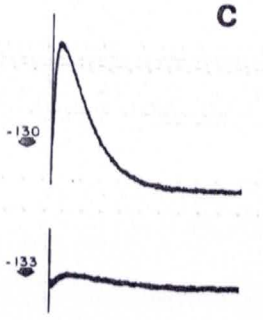
Unlike I_{it}, which followed the hyperpolarizing step with a latency of about 1 sec, I_{ot} rose soon after the depolarizing step.

The total outward current following the depolarizing step is actually the sum of two currents, the tail of I_{ir}, fading out from its peak value with a rather slow exponential time course, and the outward transient I_{ot}, which grows fairly quickly and inactivates within 1-2 sec. The difference between the peak amplitude of the outward current and the outward current at the end of the capacitive transient following the depolarizing step therefore gives an estimate of I_{ot} (see below) with a small negative error.

The same evidence reported for I_{it} suggests that the outward transient is due to an inward flux of Cl^-, namely:

1) a reversal potential close to —20 mV (Fig. 5B);
2) blockade by SITS (Fig. 5C);
3) abolishment of I_{ot} in Cl-free Ringer (Fig. 5D).





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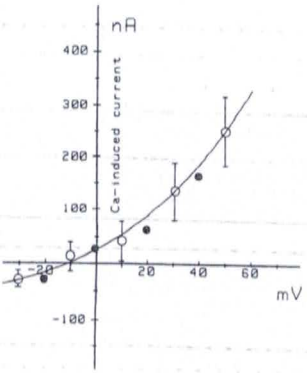

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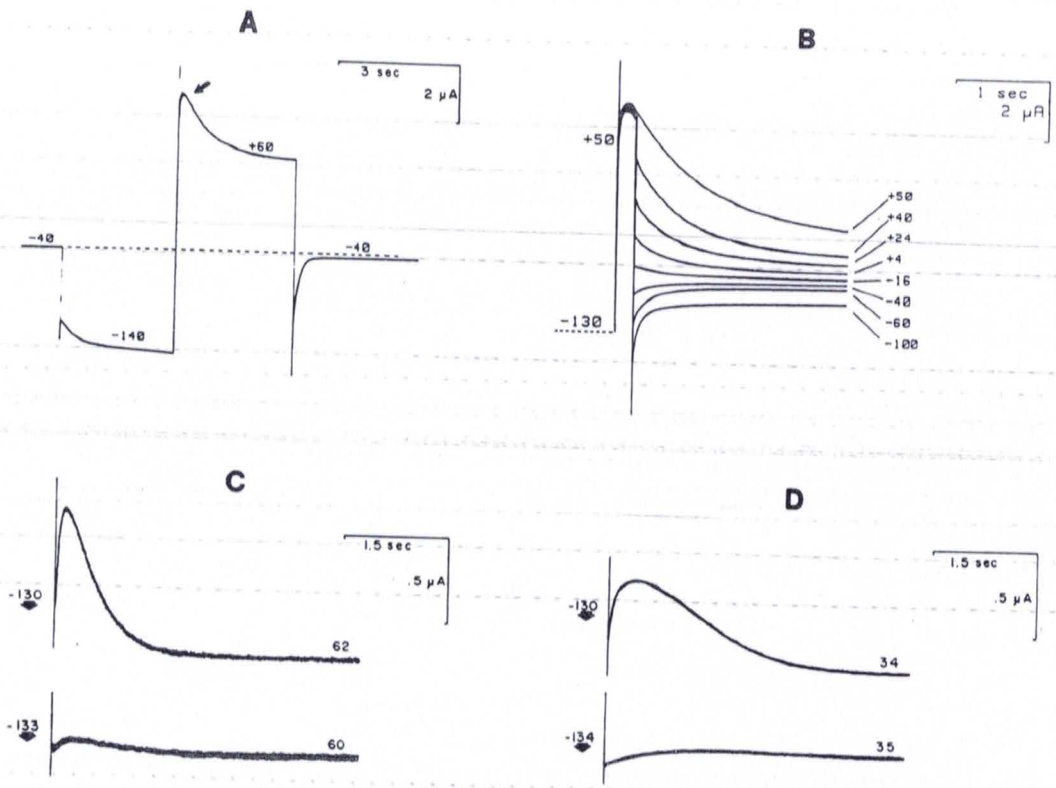


Fig. 5. – The outward chloride transient current (I_{ot}). *A*: Example of outward Cl transients (arrow) in the frog oocyte. Holding, conditioning and test potentials are indicated in mV by the figures along the tracings. The time-dependent current activated by the conditioning potential is an inward current carried by Cl^-, named I_{it}, subserving inward rectification in *Rana*; *B*: Examples of current relaxations from outward transients. The clamp potentials are indicated by figures near the traces; *C*: Membrane currents obtained from the same oocyte in standard Ringer (upper trace) and standard Ringer with SITS 1 mM (lower trace); figures adjacent to each trace indicate test potentials (right) and conditioning potentials (left, with arrow); the conditioning potentials lasted 3 sec; *D*: Membrane currents recorded from the same cell in standard Ringer (upper trace) and in Cl-free Ringer (lower trace); test and conditioning potentials are indicated as in C.

Fig. 6A shows sample recordings of outward currents obtained by stepping the membrane to different test potentials after a conditioning pulse to —140 mV. When the peak amplitudes of I_{ot} are plotted against the test potential (Fig. 6B), a monotonic dependence on membrane depolarization is evident. The conductance to chloride also appears to increase without saturation in the voltage range explored, as observed with I_{it} in relation to progressive membrane hyperpolarization (Fig. 2B).

The amplitude of I_{ot} depends upon both the amplitude and the duration of the conditioning potential. The plot of peak amplitudes of I_{ot} vs. conditioning command amplitude (Fig. 6C) suggests that the mechanism producing outward transient is inhibited at potentials positive to —40 mV, this inhibition being progressively removed by conditioning potentials at more negative potentials.

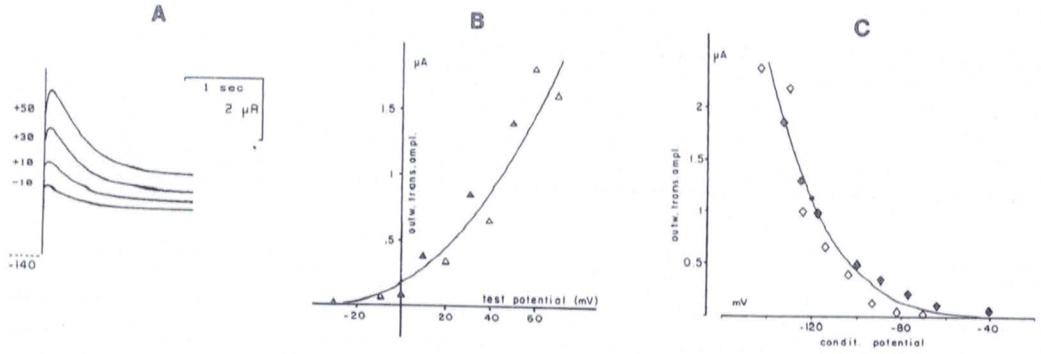


Fig. 6. – The dependence of I_{ot} upon the conditioning and test pulse amplitude. *A*: Sample records of outward transients, obtained with test command levels of increasing amplitudes, preceded by the same conditioning pulse to —140 mV, lasting 3 sec; *B*: Plot of peak amplitude of I_{ot} vs. test command potential. The symbols are examples of peak amplitudes measured in two oocytes. The curve is the result of a best fitting of data from 5 different oocytes to a 2nd order function. Amplitude and duration of the conditioning pulses were —140 mV and 3 sec respectively; *C*: Effect of conditioning command potential on peak amplitude of I_{ot} elicited by the same test command (+40 mV). Symbols and curve as in B.

The dependence of the outward transients on the conditioning potential duration is shown in Figs. 7A and 7B. Outward transients were almost negligible with conditioning pulses shorter than .5 sec and longer than 6-7 sec, and attained their maximum in a time window around 2 sec. The diagram closely resembles the time course of inward transients elicited by membrane hyperpolarization.

The outward transients were followed by a «refractory» period (Fig. 7C), and displayed a recovery time course qualitatively similar to that illustrated in Fig. 3C for I_{it}.

Ca-dependence of I_{ot}

Outward transients proved to be Calcium-dependent in the same way as the inward transients, i.e.:

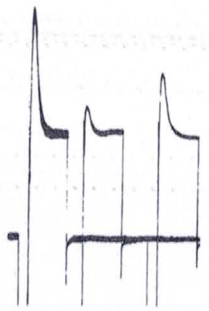
1) their amplitudes increased with increasing $[Ca^{2+}]_{out}$;

2) I_{ot} was absent when Ca^{2+} was replaced by Ba^{2+};

3) I_{ot} was abolished after Co^{2+}, La^{3+} or Cd^{2+} (1 mM) was added to the bath medium.

Further observations on the Ca-dependence of I_{it} *and* I_{ot}

In contrast with the results of Dascal et al. (3), whenever Ca^{2+} (up 20 mM) was replaced by Ba^{2+}, and therefore no Cl-transient could be activated, no inward, time-dependent current could be detected following membrane depolarization. Furthermore, neither I_{it} nor I_{ot} were affected by Verapamil at 1 mM concentration.



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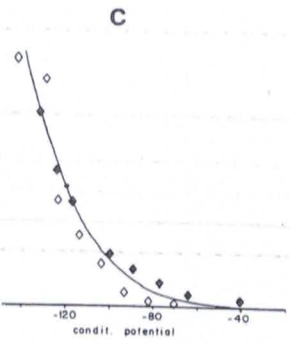

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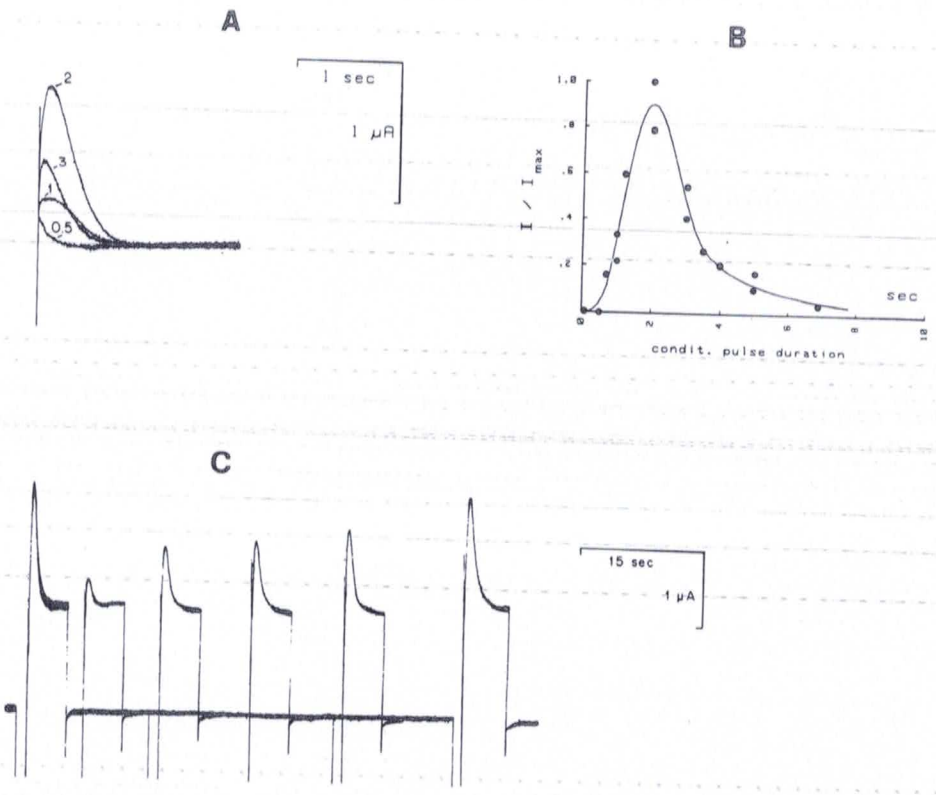

Fig. 7. – The dependence of I_{ot} upon the conditioning pulse duration and upon the delay between two subsequent test pulses. *A*: Example of a series of outward transients, obtained with test command steps to the same membrane potential (+40 mV), preceded by conditioning pulses having constant amplitude (—120 mV) but different durations, indicated (in sec) by figures adjacent to each trace; *B*: Effect of conditioning pulse duration on peak amplitude of I_{ot}. Dots are examples from two oocytes. The curve is the result of a best fitting of data from five oocytes to a 3rd order function; *C*: Superimposed tracings showing recovery of I_{ot} after a delay. The holding potential was —40 mV; the recovery was studied by stepping to +40 mV for 5 sec, after a conditioning pulse to —140 mV for 1.5 sec, twice, with increasing delays, in different trials.

These observations suggest that the Ca inflow eliciting Cl-transients in the frog oocyte does not involve the activation of specific and voltage dependent Ca-channels, in particular «high-voltage-activated» Ca-channels, which are known to be highly sensitive to Verapamil (see for example 6).

Another interesting observation on the Ca-dependence of I_{it} and I_{ot} is that both transients were abolished when the Na^+ in the bath was substituted with Li^+ or TEA^+, producing conditions which would be expected to block the Na/Ca exchange pump (see, for example, 13).

DISCUSSION

Our data show that, in *Rana* oocyte, hyperpolarization and depolarization can elicit an inward Cl-transient and an outward Cl-transient respectively, when an appropriate conditioning potential of opposite polarity is applied before the test potential.

Both transients progressively increased in amplitude as the test potential increased, without showing any saturation in membrane conductance (Fig. 2B and Fig. 6B). Since there is probably a marked outward-going rectification in single Cl-channel conductance, as suggested by Fig. 4B and by the paper of Miledi and Parker (12), the increase in membrane conductance for Cl^- during I_{it} indicates progressive activation of Cl-channels as hyperpolarization increases. The population of Cl-channels in the membrane of the frog oocyte must be quite numerous, since they cannot be fully activated even by extreme hyperpolarization. However, the increase in Cl-conductance during I_{ot} can be explained by the increase in single channel conductance as membrane depolarization increases, without involving progressive activation of Cl-channels. The rectification shown in Fig. 6B does in fact parallel that shown in Fig. 4B.

Both transients appear to be strictly dependent upon Ca^{2+} influx into the cell, and therefore they are probably subserved by the same type of Cl-channels. This influx of Ca^{2+} is unlikely to involve selective Ca-channels activated by depolarization alone, since: *a*) inward transients could be obtained in many oocytes by hyperpolarizing steps not preceded by conditioning depolarization; no Ca-channel activable by membrane hyperpolarization has been reported so far; *b*) Verapamil did not affect the transients; *c*) outward Cl-transients progressively increased as the cell was depolarized up to +60 mV or more, whereas the driving force for Ca^{2+} would be expected to be considerably reduced at such potentials; *c*) no voltage- and time-dependent, inwardly directed current was recorded following depolarizing voltage steps, even when Ca^{2+} was substituted with Ba^{2+} in the external medium.

Thus, the entry of Ca^{2+} needed to produce the transient currents of chloride studied here probably occurs through leakage channels, during cell hyperpolarization.

This Ca-influx may in principle be able to stimulate I_{it} directly. However, the slow activation kinetics, the lengthy period of «refractoriness» following the transients (Fig. 3C), and the facilitation exerted by a previous depolarization on I_{it} (Fig. 2D), argue against this possibility.

On the other hand, since amphibian oocytes have a powerful system for Ca-uptake/Ca-release (14), the present observations suggest that a Ca-activated Ca-release can take place, primarily induced by membrane hyperpolarization via a Ca-influx through non-selective leakage.

The model for Ca-activated Ca-release developed for the heart (4) does in effect account for many features of the inward Cl transients reported here. For example, Ca-activated Ca-realase induced by membrane hyperpolarization can be amplified by a previous conditioning depolarization. All the conditions causing the steady

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level of intracellular Ca^{2+} concentration to change, in particular the inhibition of the Na/Ca exchange pump, are expected by the model to interfere with the Ca-induced Ca-release.

With regard to I_{or}, its particular dependence on the duration of the preceding pulse to hyperpolarized potentials (Fig. 7B), lack of latency, and recovery after a delay quite similar to the time course of I_{it}, suggest that it is closely dependent upon the transient increase in intracellular Ca^{2+} concentration associated with membrane hyperpolarization.

A moderate Ca^{2+} influx through voltage-dependent Ca-channels activated by membrane depolarization could actually occur in the frog oocyte, even though we could not detect the inward current associated to such an influx. It would not be sufficient by itself to produce the outward transients investigated here, but it might contribute to the facilitating effect exerted by membrane depolarization on the responses to the subsequent hyperpolarization. This is quite different from the results of Barish (1), who however studied outward Cl-transient in *Xenopus* having amplitudes at least one order of magnitude lower than ours and induced by positive voltage steps starting directly from a constant holding level.

Future research will aim at investigating what mechanisms besides membrane hyperpolarization may be involved in eliciting Ca-release from intracellular stores in the frog oocyte.

SUMMARY

Transient currents of chloride were studied in the plasma membrane of immature frog oocyte in voltage clamp conditions.

The transients appeared to be activated by an influx of Ca^{2+} from the external medium. The mechanism leading to a surge of intracellular Ca^{2+} concentration needed at least 30 sec before full recovery. It was inhibited by substituting Ba^{2+} for Ca^{2+} in the external medium, or in the presence of La^{3+}, Co^{2+} and Cd^{2+}, or when external Na^{+} was replaced by Li^{+}. Verapamil proved ineffective.

The data suggest that an intracellular system of Ca-activated Ca-release is present in the frog oocyte, which can be primarily activated by membrane hyperpolarization *via* an influx of Ca^{2+} through non-selective channels.

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