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Ca-mediated activation of a K current at fertilization of golden hamster eggs

(periodic hyperpolarization/K conductance/intracellular Ca^{2+}/polyspermy block)

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ABSTRACT **Golden hamster eggs respond to fertilization with recurring hyperpolarizations [Miyazaki, S. & Igusa, Y. (1981) *Nature (London)* 290, 703–705]. We analyzed the ionic mechanism of the fertilization potential and examined whether the fertilization potential plays a role in polyspermy block. Each hyperpolarizing response (HR) during fertilization is found to be caused by an increase in the K conductance activated by an increase in the intracellular Ca^{2+} concentration. This conclusion is based on the following: (*i*) The reversal potential of the HR shifted with the Nernstian slope for K ions when the external K concentration was changed, whereas it was unaltered by the removal of Cl ions. (*ii*) The HR was blocked by the intracellular injection of EGTA. (*iii*) Injection of Ca^{2+} into an egg induced a hyperpolarization of the membrane similar to the HR. The Ca-activated K conductance shows an apparent outward rectification, which could be explained by an asymmetric distribution of K ions across the membrane. The HR associated with sperm entry into the egg occurred at any membrane potential between −160 and +50 mV. Therefore, a potential-dependent block of sperm entry does not occur in the hamster egg.**

The egg cell membrane has potential-dependent ionic permeabilities (see ref. 1 for review), the study of which is one of the important subjects of neurobiology. Another interesting ionic permeability produces the activation (or fertilization) potential (1), which is observed at fertilization of the egg. The fertilization potential of mammalian eggs has been recorded recently in the golden hamster (2). In these eggs the sperm induces recurring transient hyperpolarizations, a phenomenon that is quite different from the fertilization potential in eggs of other species (2). We have analyzed the ionic mechanism of the hyperpolarizing response (HR).

The transient depolarization of the fertilization potential that is induced by the interaction of the first sperm with the egg functions to block additional sperm entry in echinoderm (3, 4), echiuran (5), and frog (6) eggs. We have examined if the HRs of the hamster egg also provide an electrical polyspermy block.

MATERIALS AND METHODS

Eggs. Mature eggs were obtained from the oviducts of superovulated females treated with pregnant mare serum and human chorionic gonadtropin (7); the eggs were freed from cumulus cells by treatment with 0.1% hyaluronidase (P-L Biochemicals) in the culture medium (see below) for 5–8 min at 23–26°C. The zona pellucida was dissolved by applying 0.1% trypsin (GIBCO) for 4 min. The advantage of using zona-free eggs for fertilization *in vitro* (8) is that the sperm reach the egg plasma membrane soon after insemination. Treated eggs were transferred to a 0.4-ml drop of culture medium in a plastic Petri dish and covered with paraffin oil. The diameter of the egg is about 72 μm.

Capacitation of Sperm. Spermatozoa obtained from the cauda epididymis have to undergo a physiological change called "capacitation" to be capable of interacting with eggs (9). To accomplish this, sperm were incubated in modified Tyrode's solution containing 1% bovine adrenal gland extract and 20% human serum (10) for more than 4 hr at 37°C (gas phase, 5% CO_2 in air).

Insemination. Experiments were carried out at 30–32°C on a heated microscope stage. Eggs were used between 15 and 18 hr after injection of human chorionic gonadotropin. After penetration of an egg with a microelectrode, 5–20 μl of sperm suspension was added to the medium surrounding the egg by using a microsyringe. The final sperm concentration was $1–3 \times 10^4$ per ml. Sperm–egg interactions were observed with a phase-contrast inverted microscope.

Electrical Recordings. A glass microelectrode filled with 4 M potassium acetate was inserted into the egg. The resistance of the electrodes ranged between 40 and 100 MΩ. Current was passed through the intracellular electrode by means of a conventional bridge circuit. To avoid bridge unbalance, a second potassium acetate electrode was sometimes used for injecting currents greater than 1 nA. EGTA or Ca^{2+} was injected into an egg iontophoretically through an electrode filled with 0.25 M EGTA (K salt) or 0.5 M $CaCl_2$, respectively. Membrane potentials were recorded on a pen recorder and an oscilloscope.

Solutions. The culture medium for rat eggs (11) was primarily used. The composition is (mM): NaCl, 94.6; KCl, 4.8; $CaCl_2$, 1.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; sodium lactate, 22; sodium pyruvate, 0.5; glucose, 5.6; $NaHCO_3$, 25.1 (equilibrated with CO_2, pH 7.4). Bovine serum albumin (Calbiochem) was added before use (4 mg/ml). For convenience of changing ionic compositions, the following saline was sometimes used as the standard (mM): NaCl, 140; KCl, 5.5; $CaCl_2$, 1.7; $MgCl_2$, 1.2; glucose, 5.6; Hepes buffer at pH 7.4, 5. When K or Ca concentration was changed, the tonicity of the solution was adjusted by altering the Na concentration of the standard saline. For Cl free solution, propionate was substituted for Cl^-. Fertilization was not prevented in these modified salines.

RESULTS

Electrical Properties of Unfertilized Egg. The resting potential of unfertilized, mature eggs in the culture medium averaged −29 ± 6 mV (mean ± SD, n = 160), and the effective membrane resistance was 150 ± 66 MΩ (n = 135) in the linear portion of the current–voltage relationship (see Fig. 1). A larger membrane resistance was usually found in eggs showing larger negative resting potentials. This suggests that leakage due to microelectrode impalement is not negligible. The membrane

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Abbreviation: HR, hyperpolarizing response.

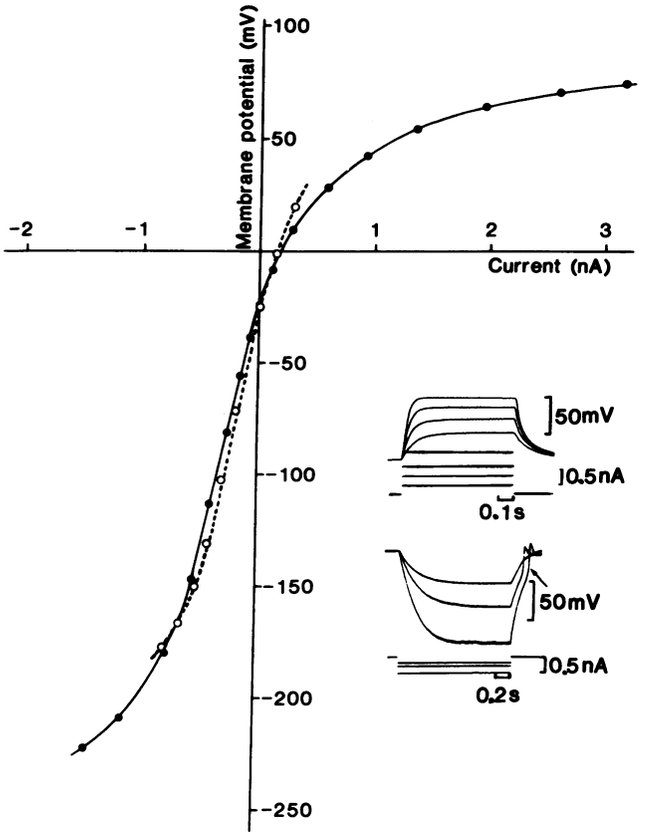

FIG. 1. Steady-state current–voltage relationship of unfertilized eggs in culture medium (○) and in 3 mM Ca saline (●). Membrane potentials at the end of current pulses of 1.5 s for hyperpolarization and 0.75 s for depolarization were plotted on the ordinate. The slope resistance is 220 MΩ in the linear portion. (*Inset*) Hyperpolarizations in culture medium and depolarizations in 3 mM Ca saline. Arrow indicates an off-response.

resistance increased when the external Ca^{2+} concentration was raised. This is probably due to reduction of the leakage. However, the resting potential did not become more negative than −45 mV, even in 20 mM Ca^{2+} saline. A 4-fold increase in the K concentration shifted the membrane potential by only 10 mV (see Fig. 2*B*). These findings suggest that nonspecific permeability is substantial as in mouse eggs (12, 13). The specific capacitance of the membrane was calculated to be 3.4 ± 0.6 $\mu F/cm^2$ (n = 10), assuming that the egg is a sphere of 72-μm diameter. The large value of the specific capacitance is likely to be due to underestimation of the surface area, because numerous microvilli on the egg surface were not considered in this calculation.

The broken curve in Fig. 1 is a steady-state current–voltage relationship (*I–V* curve) obtained with a single electrode experiment performed in the culture medium. The solid curve is also a steady-state *I–V* relationship obtained with two electrodes, with the saline containing 3 mM Ca^{2+} (instead of 1.7 mM Ca^{2+} in the culture medium). The *I–V* curve is linear at potentials between −10 and −150 mV. For larger hyperpolarizations the slope resistance became smaller, indicating the existence of an inward rectification. Another feature of the *I–V* relationship is the presence of outward rectification beyond −10 mV, especially at potentials more positive than 50 mV.

Hyperpolarizations to potentials more negative than −80 mV were followed by an off-response during potential return (*inset* of Fig. 1, arrow). For the following two reasons, the off-response is identified as a Ca-dependent action potential: (*i*) the potential level at the peak of the action potential changed with the Nernstian slope for changes in the external Ca concentration, and (*ii*) the action potential was blocked by Co^{2+}, whereas it was unaltered by the removal of Na^+. The S shaped *I–V* curve and the Ca action potential are qualitatively similar to those in the mouse egg (13).

Ionic Mechanism of the HR. Fig. 2*A* shows a typical fertilization potential in the zona-free egg, in which polyspermy usually occurs. To monitor changes in the membrane conductance, constant hyperpolarizing current pulses were applied repetitively. After the first sperm attached to the egg surface, the first HR was observed, and this approximately coincided with the end of active flagellar motion of the sperm. The single sperm induced several HRs, and a series of HRs appeared after additional sperm attachments. The HRs were associated with an increase in the membrane conductance (2).

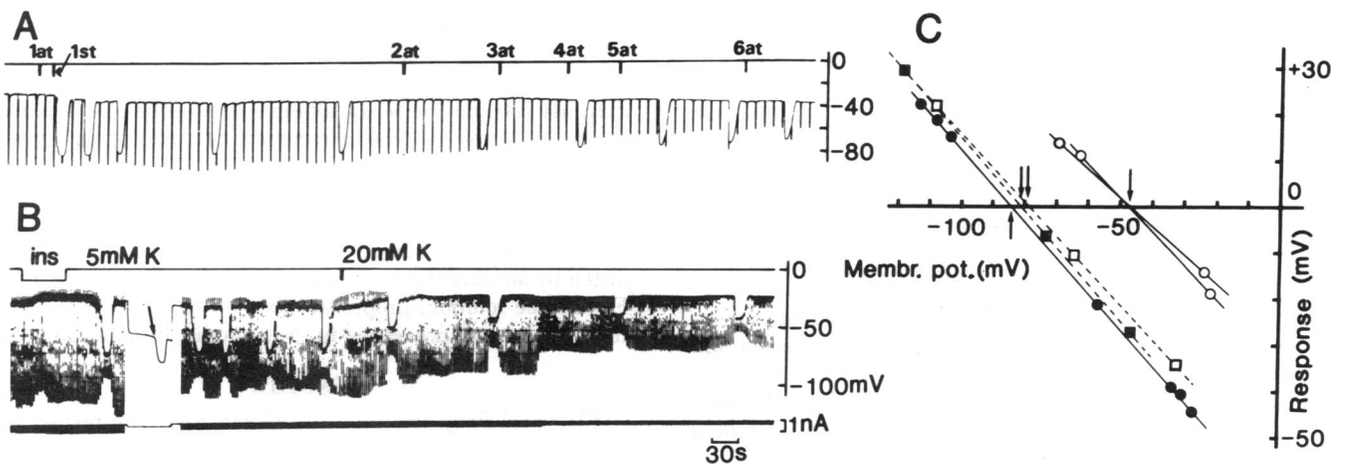

FIG. 2. (*A*) Typical fertilization potential. Constant current pulses of 0.25 nA were applied throughout the recording. 1–6at, Attachment of the first to sixth sperm to the egg surface. 1st, Stop of flagellar motion of the first sperm. These events were marked electrically by the observer. The 2–6st are omitted, because they were not marked accurately. (*B*) The egg was inseminated (ins) in 5 mM K saline. A perfusion of 20 mM K saline was started at the mark indicated. The small depolarization that appeared immediately after insemination is not induced by sperm but is caused by the sperm incubation medium (2). (*C*) Membrane potential–response relationships. The amplitude of the HR is plotted against the membrane potential both in the presence and absence of polarizing current. The solid line with filled circles was obtained from the first four HRs in 5 mM K saline and those with open circles from the last two HRs in 20 mM K saline. Broken lines represent data obtained in standard saline (filled squares) and in Cl-free saline (open squares).

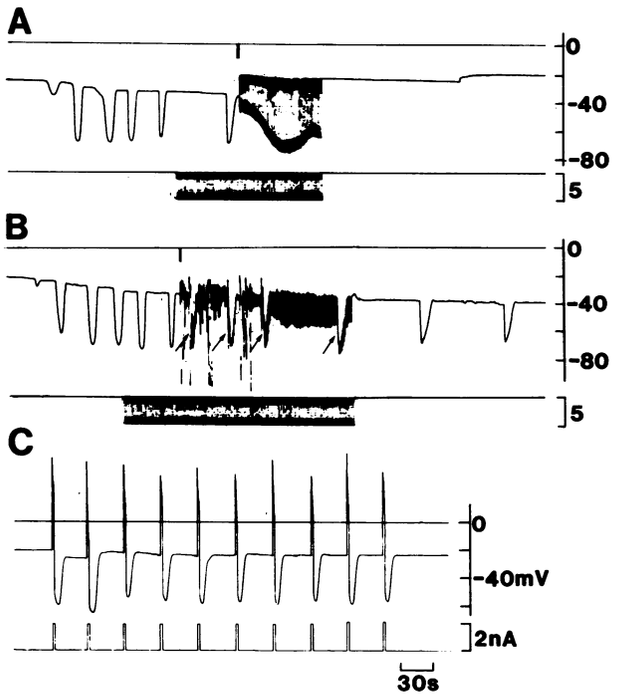

FIG. 3. Intracellular injection of EGTA or Ca^{2+}. EGTA electrode (*A*) or potassium electrode (*B*) was inserted at the mark indicated in the upper trace. Explanations are in the text.

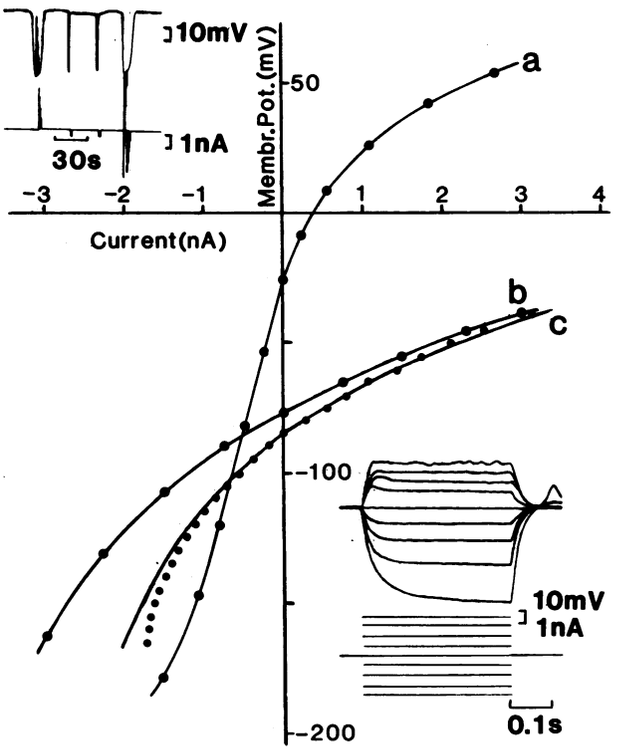

FIG. 4. Steady-state *I–V* curves before insemination (curve a) and at the peak of HRs (curve b). (*Insets*) Records for curve b on a pen recorder (*Upper Left*) and on an oscilloscope (*Lower Right*). The small filled circles along curve c were obtained by subtraction of curve a from curve b along the current axis every 5 mV. Curve c is the *I–V* curve based on the constant-field theory.

In Fig. 2*B* the egg was inseminated in 5 mM K saline, while repetitive current pulses were applied. The upper and lower envelopes of potential steps allow us to estimate the reversal potential of the HR. Fig. 2*C* illustrates the relationship between the amplitude of the HR and the membrane potential from which it started, both in the absence of the current pulse (negative amplitude) and during the current pulse (positive amplitude). The first, third, and fourth HR in Fig. 2*B* started from slightly different potentials and gave six points in Fig. 2*C* (filled circles). The second HR (arrow in Fig. 2*B*) was made to start from −57 mV by injection of dc current, and it gave one point in Fig. 2*C*. These seven points lie on a straight line.* The intersection of the line with the abscissa (arrow) indicates the reversal potential of −85 mV. When the same egg was perfused with 20 mM K saline, the resting potential became more positive by 10 mV, and the reversal potential became −48 mV (Fig. 2 *B* and *C*). The change of the reversal potential averaged 35.9 ± 1.8 mV (n = 5), which is consistent with the value of 36.3 mV calculated from the Nernst equation at 31°C. On the other hand, the reversal potential was unaltered by the removal of Cl^- (Fig. 2*B*, open squares). These findings indicate that the HR is associated with an increase in the K conductance.

In Fig. 3*A*, an EGTA electrode was inserted into the egg after several HRs had appeared. No HR appeared during or after the injection of EGTA. When the same procedure was performed with a potassium acetate electrode in another dish about 10 min later, HRs continued during (arrows) and after the injection (Fig. 3*B*), suggesting that HRs disappeared in Fig. 3*A* neither because the egg was damaged by the insertion of the second electrode nor because HRs ceased spontaneously. The result suggests that the HR is blocked when an increase in the concentration of intracellular Ca ions is suppressed by EGTA. In Fig. 3*C*, Ca ions were injected iontophoretically into the egg with 2-nA, 2-s pulses every 30 s. Transient hyperpolarizations similar to HRs were produced by each injection. The duration of Ca-induced HR was 10.1 ± 1.9 s (n = 60) and that of sperm-induced HR was 11.3 ± 2.3 s (n = 300); the reversal potential ranged between −80 and −85 mV for both HRs. It is concluded that the increase in the K conductance during the HR is mediated by an increase in the concentration of intracellular Ca^{2+}.

Ca-Induced K Conductance. The potential dependence of the Ca-induced K conductance was analyzed in Fig. 4. The experiment was carried out with two electrodes in the saline containing 3 mM Ca^{2+} and 5.5 mM K^+. *I–V* curves were obtained before insemination (curve a) and at the peak of the first two HRs (*Upper Left Inset*). The membrane resistance at times between the HRs was constant (see two pulses between HRs in the *Inset*). The *Lower Right Inset* shows superimposed oscilloscope traces at the peak of the HRs, and they give *I–V* curve b. The intersection of the two curves (a and b) corresponds to the reversal potential of the HR, and its averaged value in the culture medium was −82.7 ± 2.2 mV (n = 34).

Because curves a and b are steady-state *I–V* curves, the *I–V* relationship for only the Ca-induced K conductance (g_K) can be obtained by subtraction of the currents of curve a from the currents of curve b, if one assumes that the leakage conductance is unaltered. The series of filled circles in Fig. 4 represents the differences obtained every 5 mV. The *I–V* relationship for g_K showed an apparent outward rectification. Similar apparent outward rectification was observed also in the case of HRs induced by Ca^{2+} injection. This outward rectification was most

* This finding implies that the first four HRs occurred under the same conditions, the same magnitude of conductance increase superimposed on the same original membrane conductance. This was often the case for the first two to four HRs. Later HRs provide pairs of points that have different slopes but the same reversal potential on such a plot, as is the case for 20 mM K saline in Fig. 2*C*.

probably due to the asymmetry of K^+ concentration across the membrane and could be fitted by an *I–V* relationship calculated from the constant-field theory (14), using the value of internal K^+ concentration $[K^+]_i$ = 141.2 mM (estimated from outside K^+ concentration $[K^+]_o$ = 5.5 mM and K^+ equilibrium potential E_K = −85 mV) and assuming K^+ permeability P_K to be 6.64 × 10^{-13} cm/s from the g_K at the zero current. The calculated *I–V* relationship is shown by curve c in Fig. 4. A slight deviation of the points from the constant-field prediction seen at potentials more negative than −110 mV might mean that the nonspecific leakage current is smaller during the HR, because we have observed a decrease of membrane conductance after Ca^{2+} injection (unpublished result).

Fertilization Potentials Observed at Various Membrane Potential Levels. In echinoderm (3, 4), echiuran (5), and frog (6) eggs, an overshooting fertilization potential plays a role in blocking polyspermy. This conclusion is based primarily on the fact that sperm entry into the egg is blocked when the membrane potential is held at positive potentials (3–6). We examined whether the membrane potential plays a similar role in the hamster egg. The membrane potential was held between −160 and +50 mV by passing dc current through the second electrode, and then the egg was inseminated (Fig. 5). The initiation of sperm entry can be indicated by two events; the occurrence of the HR and the stop of flagellar motion of the sperm (2, 15).

HRs were induced by sperm at all resting potentials between −40 and −10 mV. With membrane potentials polarized by current, HRs also occurred at holding potentials between 0 and +48 mV in all 8 eggs examined and between −78 and −50 mV in 12 eggs. They were also observed with reversed polarity in 12 eggs at −160 to −100 mV, although the records were unstable (see legend to Fig. 5). Fig. 5 shows sample records obtained from 3 eggs on the same scale. In every case the first HR began several seconds before one of sperm stopped moving (not always the first sperm that had attached to the egg). Additional HRs, as well as sperm "stops," were observed. It is concluded that sperm entry is not blocked at any potential between −160 and +50 mV.

It seemed unlikely that the treatment with trypsin for dissolving the zona pellucida (see *Materials and Methods*) was responsible for the absence of an electrical block of sperm entry, because sperm entry was not blocked with polarized potentials even when the treatment was reduced to 40 s instead of 4 min. There is a possibility that sperm entry is delayed at certain potentials. The time between sperm attachment and stop was usually variable within about 90 s. The delay time was not changed significantly at any polarization of the potential. In Fig. 5 some sperm did not stop moving. This probably indicated that not all of the sperm were capacitated. Therefore, the possibility of potential-dependent delay seemed unlikely. At potentials more negative than −120 mV, the frequency of the occurrence of HRs tended to be lower than at more positive potentials, even though several sperm had stopped moving.

DISCUSSION

At the early stage of fertilization, sperm induce a dramatic increase in the intracellular Ca concentration ($[Ca^{2+}]_i$) of eggs, as has been shown in sea urchin eggs (16, 17). The Ca ionophore A23187 can activate echinoderm eggs (16) and, in tunicate eggs, it induces activation currents depending on Na^+, Ca^{2+}, and possibly K^+ (18). Fertilization potentials have an early depolarization phase, due to an increase in permeabilities to Na^+ and possibly Ca^{2+} in echinoderm (2), echiuran (19), and tunicate (18) eggs. The depolarizing phase persists for several minutes. In hamster eggs, if we take the HRs as the indication of $[Ca^{2+}]_i$, the increase in $[Ca^{2+}]_i$ during fertilization is quite different: the

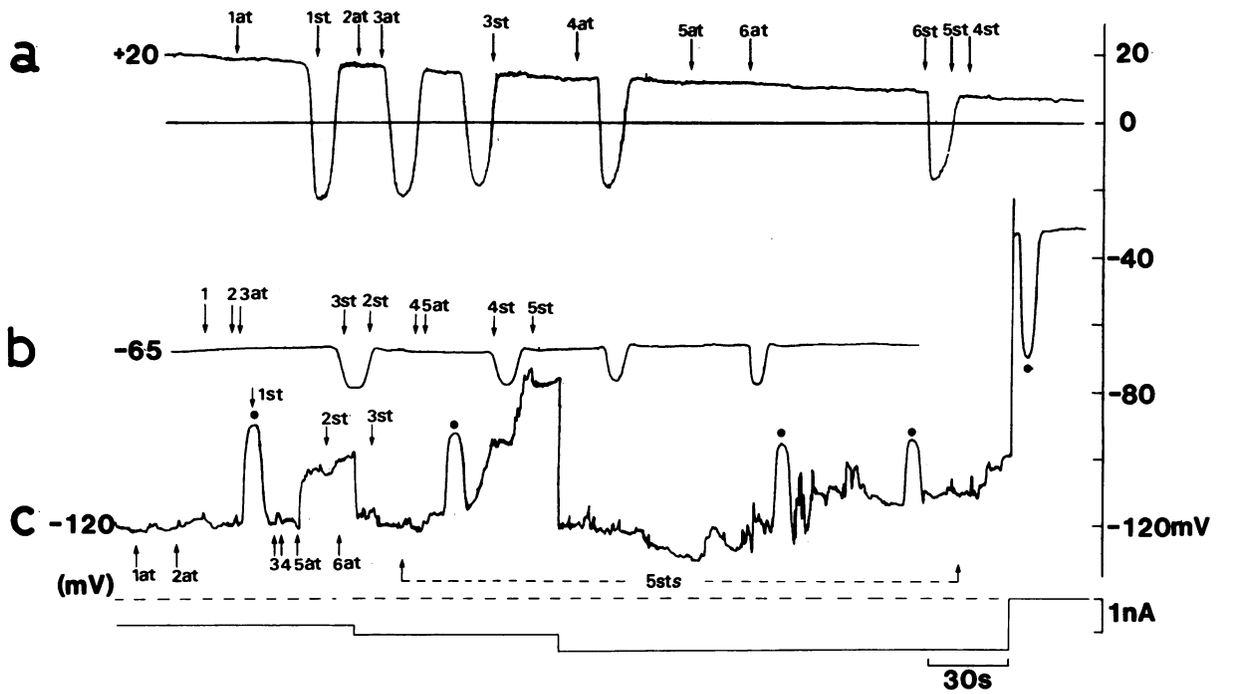

FIG. 5. Fertilization potentials obtained in the culture medium while polarizing the membrane potential with different currents. The initial potential level is indicated on the left. The figure with "at" or "st" indicates each sperm in the order of attachment; the at and st of the same number indicate the same sperm. In trace c, eight sperm stopped moving during the recording, although the timing of five sts was missed. Current trace at the bottom is only for trace c. Current was turned off near the end of the record. Note: The membrane potential showed fluctuations when it was shifted to −160 to −120 mV, even without insemination. However, reversed HRs could be identified by their shape (dots). In addition, the membrane resistance decreased so that the conditioning dc current had to be increased to keep the membrane potential at the original level (see current trace).

occurrence of HRs is periodic and the ionic permeability change during the HR is exclusively to K^+.

The HRs are based on a so-called Ca-induced K current, which has been found in a variety of nervous tissues (20). In many cells, it occurs together with voltage-gated K conductances (20). In hamster eggs, an outward rectification is seen at positive potentials, especially potentials more positive than +50 mV (Fig. 1), whereas the Ca-induced K current is induced by sperm at potentials, at least, between −160 and +50 mV. Furthermore, although the Ca-induced K conductance apparently shows an outward rectification, this could be explained by an asymmetric distribution of K ions across the membrane (Fig. 4). The present result shows that the Ca-activated K channels are essentially not voltage-gated and are distinct from voltage-gated K channels.

Mechanisms involved in the increase of $[Ca^{2+}]_i$ require further studies. So far, a depolarizing phase that might trigger the HR has not been observed (ref. 2; see also Fig. 5). A Ca influx through voltage-gated Ca channels seems unnecessary to generate the HR, because the response could be induced at −160 to −120 mV (Fig. 5), potentials at which the Ca channels are closed.

It remains to be elucidated what cellular functions the characteristic hyperpolarizing fertilization potential is related to. Our observations strongly suggest that the hyperpolarizing fertilization potential does not provide an electrical fast polyspermy block. In the hamster egg it is thought that the primary mechanism for polyspermy block resides in the zona pellucida, which becomes impenetrable to sperm on account of the material(s) released from cortical granules; that is, zona reaction (21, 22). Periodic hyperpolarizing responses have been found in fibroblastic L cells (23, 24), macrophages (25, 26), and sympathetic ganglion cells (27). They are suggested to be related to cell motility, such as phagocytosis of fibroblastic L cells (28) or the activation of chemotactic migration of macrophages (29). These observations are interesting, considering that the HRs occur during incorporation of sperm into the hamster egg (2).

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