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ON THE MECHANISM OF A pH-INDUCED RISE IN MEMBRANE POTASSIUM CONDUCTANCE IN HAMSTER EGGS

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SUMMARY

1. The effect of external pH (pH_o) on the membrane potential and resistance of unfertilized zona-free hamster eggs was investigated by intracellular recording techniques.

2. A hyperpolarization of the hamster egg membrane was induced by raising the extracellular pH above 8·0. This hyperpolarization was accompanied by a rise in membrane conductance and was reversible by washing the egg.

3. The estimated value of the reversal potential of the hyperpolarizing response to a solution with pH_o 9·5 was about −85 mV. The membrane potential changed linearly with log $[K^+]_o$ with a slope of 43 ± 2 mV (mean ± S.D.; $n = 4$) for a 10-fold change in $[K^+]_o$, while it was unaltered by the removal of Cl^- from the solution.

4. The amplitude of the pH_o-induced hyperpolarization decreased substantially as $[Ca^{2+}]_o$ was lowered from 20 to 1 mM. Sr^{2+} could substitute for Ca^{2+} in sustaining the response to high pH_o, whereas Ba^{2+} or Mg^{2+} could not.

5. Injection of the Ca^{2+} chelator EGTA into the egg prevented the pH_o-induced hyperpolarization suggesting that a rise in $[Ca^{2+}]_i$ is required.

6. The rate of rise of Ca^{2+} action potentials was reversibly enhanced by raising pH_o. However, influx through the voltage-gated Ca^{2+} channels is not involved in initiation and maintenance of the pH_o-induced response, as responses were not affected by the Ca^{2+} channel blocker La^{3+}.

7. The duration of the hyperpolarization evoked by intracellular Ca^{2+} injection in eggs bathed in normal solution or Na^+-free solution was greatly prolonged by raising pH_o.

8. It is suggested that a rise in external pH produces an increase in $[Ca^{2+}]_i$, activating a Ca^{2+}-mediated K^+ conductance which hyperpolarizes the egg membrane.

9. It is concluded that both a Na^+–Ca^{2+} exchange system and a Ca^{2+} pump are responsible for Ca^{2+} extrusion and that inhibition of the Ca^{2+} pump by high pH_o is the chief mechanism underlying the pH-induced hyperpolarization in hamster eggs. Although the Na^+–Ca^{2+} exchange system is facilitated at high pH_o, the effect of this facilitation of efflux is outweighed by the inhibition of the Ca^{2+} pump.

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INTRODUCTION

Fertilization of hamster eggs produces a series of transient hyperpolarizations of the egg plasma membrane (Miyazaki & Igusa, 1981). Each hyperpolarizing response results from an increase in the membrane K^+ conductance activated by a rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Miyazaki & Igusa, 1982). Ionophoretic injection of Ca^{2+} into eggs will activate this K^+ conductance, producing a transient hyperpolarization of the membrane (Miyazaki & Igusa, 1982; Georgiou, Bountra, Bland & House, 1983). The recovery phase of this transient hyperpolarization is prolonged by the application of La^{3+}, high $[Ca^{2+}]_o$ or quercetin (Georgiou, Bountra, McNiven & House, 1987*a*) which are known to inhibit the Ca^{2+} pump in red blood cells, mouse fibroblasts and toad retinal cells (Sarkadi, Szasz, Gerlocozy & Gardos, 1977; Okada, Tsuchiya & Yada, 1982; Cervetto & McNaughton, 1986). Raising the extracellular pH (pH_o) is known to inhibit the Ca^{2+} pump in squid giant axon (Dipolo & Beaugé, 1982). Thus it might be expected that raising the pH of the solution bathing a hamster egg should decrease the active efflux of Ca^{2+}. If this inhibition is sufficiently large, the resulting rise in $[Ca^{2+}]_i$ might activate the Ca^{2+}-mediated K^+ conductance hyperpolarizing the egg membrane.

Investigation of the effects of changes of pH_o on the electrical properties of mammalian eggs is of general interest because of the influence of pH on fertilization. The pH of the fluid in the reproductive tracts of several mammalian species lies in the range 7·10–8·04 (Hall, 1936; Blandau, Jensen & Rummery, 1958). In particular, Vishwakarma (1962) found that the pH of the fluid in the ligated oviduct of rabbits was about 7·9. It is possible that the mild alkalinity of the oviducal fluid favours sperm penetration. This idea is supported by the observation that optimal rates (93–99 % sperm penetration) of fertilization *in vitro* of hamster eggs occurred in the range 7·0–8·5 (Miyamoto, Toyoda & Chang, 1974). Moreover, accompanying fertilization is a marked rise in intracellular pH in the eggs of sea urchin (Shen & Steinhardt, 1978), sand dollar (Hamaguchi, 1982) and frog (Webb & Nuccitelli, 1981). This change in intracellular pH probably has an important regulatory role in a number of processes occurring in fertilized eggs (Busa & Nuccitelli, 1984).

In this paper we examine the effects of raising pH_o on the electrical properties of the hamster egg. Above a pH_o of 8·0 the cell hyperpolarizes and there is a fall in membrane resistance. The possible role of a pH_o-induced reduction in active extrusion of Ca^{2+} in this response is examined. A preliminary account of this work has been published (Georgiou, House, McNiven & Yoshida, 1987*b*).

METHODS

Eggs

The egg donors were mature (6–8 weeks) virgin female golden hamsters maintained under a controlled light–dark cycle (16 h light : 8 h dark). They were induced to superovulate by first injecting them intraperitoneally (I.P.) with 30 i.u. of pregnant mare's serum gonadotrophin (PMSG: Folligon; Intervet Labs Ltd, Cambridge) in the early evening, followed 48 h later by an I.P. injection of 45 i.u. of HCG (human chorionic gonadotrophin, CG-2, Sigma Chemical Co., St Louis, U.S.A.). The hamsters were killed by cervical dislocation 15–18 h after the HCG injection and eggs collected from their oviducts. The eggs were freed from cumulus cells by bathing them for 2–4 min in normal solution (see below) containing hyaluronidase (1 mg/ml; Type I-S, Sigma) at room temperature (21–24 °C). After washing, the eggs were kept in normal solution at 37 °C. When

an egg was required it was transferred to a normal solution containing trypsin (1 mg/ml; type III, Sigma) for 30–40 s in order to remove its zona pellucida. The trypsination was carried out at room temperature.

Solutions

The composition of the normal solution was (mM): NaCl, 136·6; KCl, 5·5; $CaCl_2$, 4·0; $MgCl_2$, 1·2; glucose, 5·6. The composition of the high-Ca^{2+} solution was (mM): NaCl, 112·6; KCl, 5·5; $CaCl_2$, 20·0; $MgCl_2$, 1·2; glucose, 5·6. Both solutions contained 10 mM-HEPES ($pK_a = 7.5$; 25 °C) and 10 mM-CHES (2-(*N*-cyclohexylamino)ethanesulphonic acid) ($pK_a = 9.3$; 25 °C) to cover the pH between 7·5 and 9·5 and 10 mM-HEPES plus 10 mM-MES (2-(*N*-morpholino)ethanesulphonic acid) ($pK_a = 6.1$; 25 °C) for the pH between 5·5 and 7·5; all buffers were obtained from Sigma. The pH was adjusted to desired values by NaOH. The total Na^+ concentration was kept constant by adjusting the amount of NaCl. Bovine serum albumin (Sigma) was added to all solutions (3 mg/ml). When the concentration of K^+, Ca^{2+}, Sr^{2+} or Ba^{2+} was altered, the tonicity of the solution was adjusted by changing the Na^+ concentration of the normal solution. Na^+-free solutions were obtained by equimolar replacement of NaCl with *N*-methyl-D-glucamine (NMDG) (Sigma), and the pH adjusted with HCl. Low-Na^+ solutions were obtained by equimolar replacement of 5/6 mM-NaCl with LiCl. Normal and high-Ca^{2+} solutions were mixed to obtain solutions having different concentrations of Ca^{2+} with the same tonicity. Methane sulphonate (Aldrich Chemical Co. Ltd) was substituted for Cl^- to obtain Cl^--free solution. Amiloride and ouabain were obtained from Sigma Ltd, and the tetraethylammonium (TEA) from BDH Chemicals. The pH of all solutions was checked just before use.

Electrical recordings

A single microelectrode (40–80 MΩ) filled with 2 M-potassium acetate was inserted into the egg for simultaneous monitoring of the membrane potential and input resistance. This microelectrode was connected to the input of a high-impedance preamplifier (DAGAN 8100) set in the bridge mode of operation. The electrode was inserted into the egg by over-compensation of the negative capacitance of the preamplifier. Current pulses from a Devices isolated stimulator (2533; Devices Ltd) driven by a Digitimer (D4030; Devices Ltd) were passed between the barrel of the microelectrode and an agar bath electrode (3 M-KCl). The resulting electrotonic potentials were monitored for the determination of the current–voltage relation of the cell. In some experiments where action potentials were evoked by depolarizing pulses the rate of change of membrane potential was measured using an electronic differentiator during action potentials.

Tape-recordings (Racal Store 4DS) of the experimental data were routinely made, and permanent experimental records for reproduction were obtained as pen recorder traces on a Graphtec servocorder (SR 6335) or as photographs from the screen of a storage oscilloscope (RM5113, Tektronix Ltd).

EGTA injection

EGTA (Sigma) was injected from a second electrode inserted into the egg. This electrode was filled with 0·25 M-EGTA (potassium salt) and had a resistance of 10–15 MΩ when filled with 3 M-KCl. The EGTA electrode was inserted into the cell in the same manner as the potassium acetate electrode. EGTA was applied intracellularly by leakage from the EGTA pipette.

Ca^{2+} injection

Ca^{2+} was injected ionophoretically from an electrode inserted into the egg. This electrode was filled with 1 M-$CaCl_2$ and had a resistance of 10–15 MΩ when filled with 3 M-KCl. The Ca^{2+} electrode was inserted into the egg in the same manner as the potassium acetate recording electrode. Current pulses from a Devices stimulator were delivered to the Ca^{2+} electrode using a preamplifier (M 707; WPI Instruments, U.S.A.). The actual injection current (I) flow through the egg was monitored using a virtual ground (i.e. current-to-voltage converter).

Reversal potential measurement

The reversal potential of the pH-induced hyperpolarization was measured according to the method described by Ginsborg, House & Silinsky (1974), using the equation:

$$E_r = \left(\frac{P}{P-p}\right)v + E_m,$$

where E_r is the reversal potential of the response, E_m is the resting potential, P is the amplitude of the electrotonic potential at rest, p is amplitude of the electrotonic potential at the peak of the response and v is the amplitude of the potential change from the resting potential to the peak of the response. This equation is only valid if the resting conductance is independent of the applied current and hence of the value of the membrane potential. Therefore the strength of the current pulses used was such that the resulting electrotonic potential fell within the linear region of the current–voltage relation.

All values in this paper are given in the form mean ± S.D.

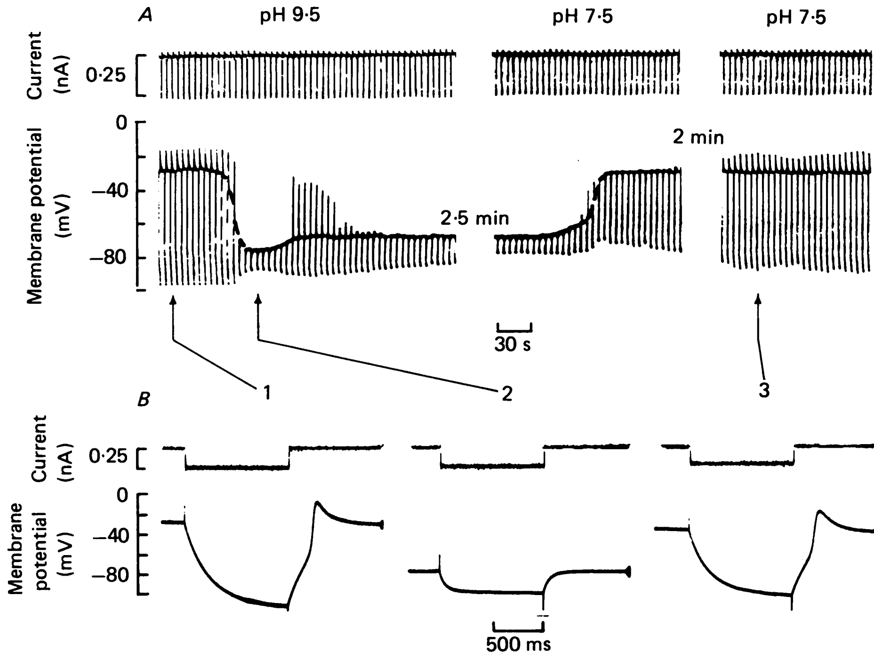

Fig. 1. Reversible effect of high pH_o (9·5) on the membrane potential and resistance of a hamster egg in solution containing 4 mM-Ca^{2+}. *A*, pen traces of current pulses (upper) and membrane potential (lower). *B*, corresponding oscilloscope pictures taken at the times indicated by arrows.

RESULTS

The effect of changes of pH_o on potential and resistance

When the pH of the normal solution bathing an egg at room temperature (21–24 °C) was raised from 7·5 to 8·0 a transient hyperpolarization lasting about 20 s was recorded occasionally. At pH_o values above 8·0 a transient hyperpolarization frequently was observed and it was followed by a further maintained hyperpolarization. The time course and amplitude of the responses to raised pH_o were not altered significantly by performing experiments at 35–37 °C ($n = 4$). Lowering the pH_o to 6·5 or 5·0 did not produce a hyperpolarization in these experiments. Instead a depolarization of about 10 mV was observed associated with a small reduction in input resistance.

Most responses were recorded at pH_o 9·5 because at that pH_o a large steady hyperpolarization of about 40 mV usually was observed; in eight cells out of twenty-eight a small hyperpolarization (< 10 mV) was recorded. The hyperpolarization was associated with a marked reduction in input resistance. A representative example of

an experiment shows that the hyperpolarization induced by pH_o 9·5 (Fig. 1*A*) was accompanied by a large fall in resistance from 310 to 90 MΩ at the peak of the response (Fig. 1*B*, compare electrotonic potentials at 1 and 2). An almost full recovery of potential and resistance was achieved by superfusing the egg with normal solution for 10 min (Fig. 1).

TABLE 1. Effects of high pH_o on membrane potential and input resistance

	4 mM-Ca^{2+}, pH 9·5	20 mM-Ca^{2+}, pH 9·5
Resting		
E_m (mV)	-27 ± 7 (10)	-30 ± 4 (15)
R_{in} (MΩ)	330 ± 130 (10)	400 ± 84 (15)
Peak		
E_m	-65 ± 9 (10)	-73 ± 5 (15)
R_{in}	84 ± 23 (10)	110 ± 31 (15)
Recovery		
E_m	-29 ± 6 (7)	-32 ± 13 (14)
R_{in}	320 ± 55 (7)	300 ± 130 (14)
Reversal potential, E_r (mV)	-80 ± 7 (10)	-87 ± 7 (15)

E_m, membrane potential; R_{in}, input resistance; E_r, estimated reversal potential (see Methods). Values are given in the form mean ± S.D.; number in parentheses is the number of eggs. Resting and recovery values were measured in solutions containing 4 mM- or 20 mM-Ca^{2+} with a pH of 7·5.

Table 1 gives the mean values of membrane potentials and input resistances measured before, at the peak of and after recovery from the hyperpolarization due to high pH (9·5) at 4 mM-Ca^{2+} in ten representative experiments. The mean membrane conductance increased by 8·9 nS during the response to high pH_o.

In another series of experiments ($n = 14$) made at a $[Ca^{2+}]_o$ of 20 mM elevation of pH_o from 7·5 to 9·5 always elicited a large steady hyperpolarization. In many cases the steady hyperpolarization was preceded by one or two transient hyperpolarizations. In some eggs a rise of pH_o from 7·5 to 8·0 was sufficient to evoke a transient hyperpolarization. The response to high pH_o could be reversed to a depolarization in eggs current clamped to potentials more negative than -100 mV. Mean values of potential and resistance measured before, during and after hyperpolarizing responses obtained in the presence of 20 mM-Ca^{2+} are given in Table 1. The mean membrane conductance increased by 6·6 nS during the response to high pH_o.

Rapid application of high-pH solution

Our perfusion system had a lag time of about 2–3 min before exchange of the bathing solution began. Hence, in order to examine the speed of onset of the hyperpolarization a high-pH solution was applied from a broken-tipped pipette placed close to an egg (Fig. 2*A*). A hyperpolarization could be evoked within 10 s of bringing the broken-tipped pipette into close proximity to the egg (Fig. 2*B*). The membrane potential recovered fully when the pipette was removed. Such transient hyperpolarizations could be evoked repeatedly in eggs by this method (Fig. 2*B* and *C*). Occasionally a burst of action potentials accompanied the recovery phase of the hyperpolarizations (Fig. 2*C*). The mean value of the latency of the pH_o-induced hyperpolarization elicited by rapid application of high-pH solution was $20·8 \pm 11·4$ s ($n = 6$). In these experiments the shortest latency was 6 s.

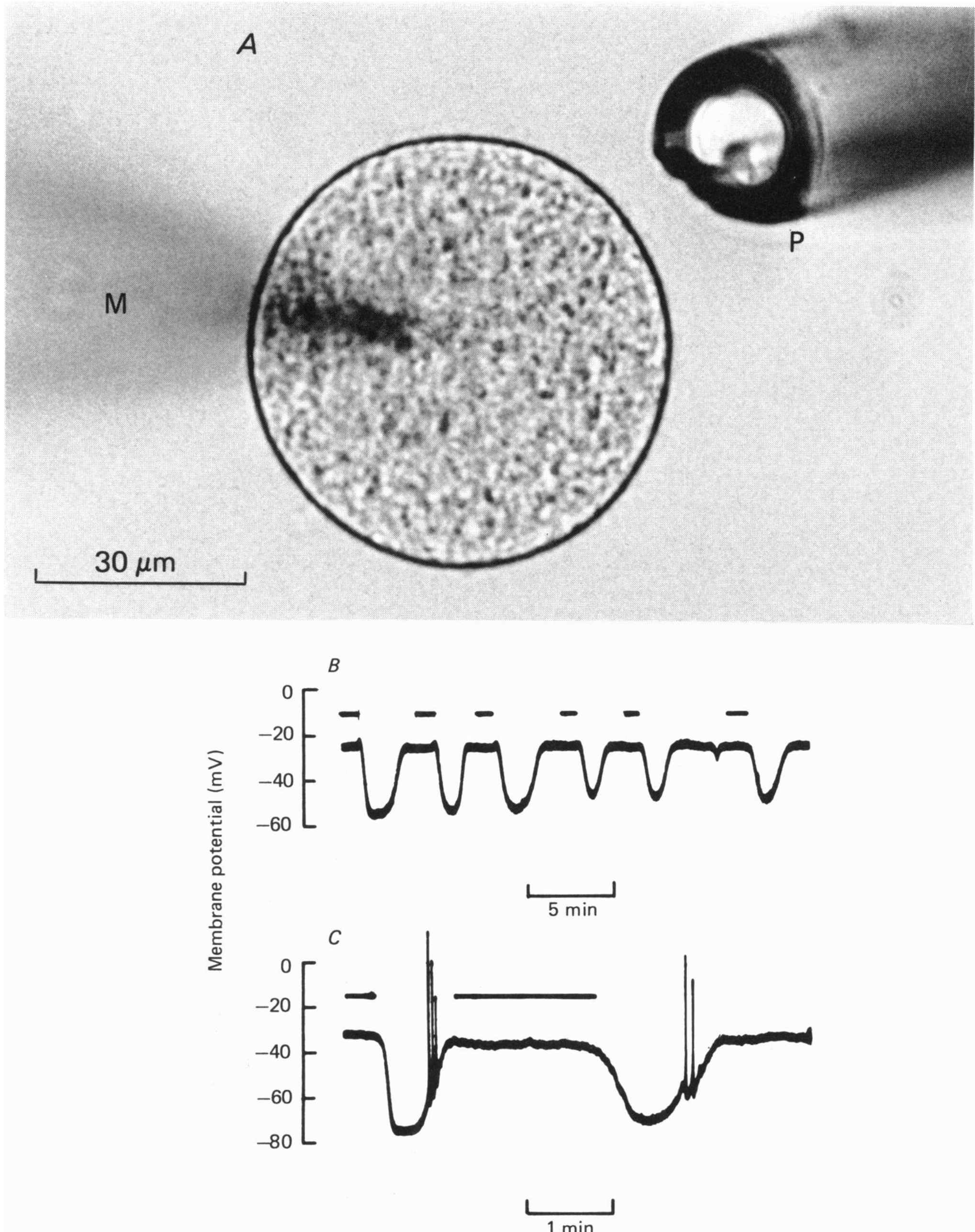

Fig. 2. Rapid application of high-pH solution to an egg. *A*, photograph showing the method. Membrane potential was monitored by a microelectrode (M). High pH (9·5) was applied to the egg from the broken-tipped pipette (P). The pipette was withdrawn as soon as the membrane started to hyperpolarize. The pH of the bathing solution was 7·5. *B*, the membrane hyperpolarized when the broken-tipped pipette was brought close to the egg and recovered on removal of the pipette. Applications are shown by bars. *C*, another example of the membrane potential change induced by the same method. Note action potentials on the recovery phase of the responses.

Ionic basis of the hyperpolarization

The estimated value of the reversal potential (see Methods) given in Table 1 of the response to high pH_o was compatible with the equilibrium potential of either K^+ or Cl^-. In three experiments substitution of external Cl^- with the impermeant anion methane sulphonate (Sharp & Thomas, 1981) had no effect on either the amplitude

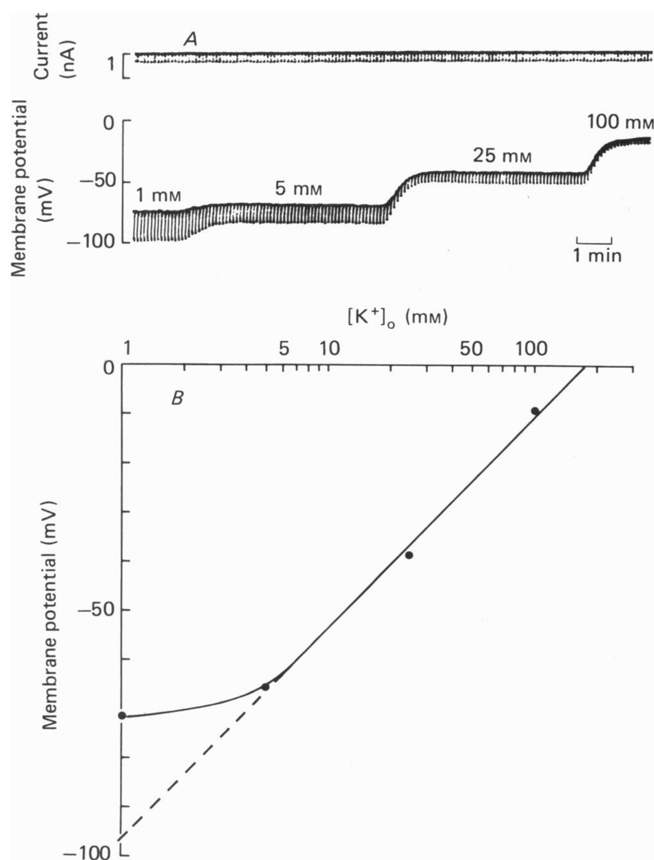

Fig. 3. Effect of $[K^+]_o$ (shown in mM) on the steady membrane potential at pH 9·5. *A*, pen traces of current pulses (upper) and membrane potential (lower). *B*, membrane potential plotted against log $[K^+]_o$. The linear part of the relation was fitted by regression analysis and had a slope of 43 mV for a 10-fold change in $[K^+]_o$.

of the pH_o-induced hyperpolarization or the reversal potential of this response. However, the membrane potential at pH_o 9·5 changed linearly with log $[K^+]_o$ between 5 and 100 mM (Fig. 3) with a slope of 43 mV for a tenfold change in $[K^+]_o$. The mean value of the slope obtained from four such experiments was 43 ± 2 mV. The dependence of the hyperpolarization on $[K^+]_o$ and the value of the reversal potential indicate that a rise in the membrane K^+ conductance is evoked by high pH_o.

Further support for the proposition that the hyperpolarization was due to an increase in the membrane K^+ conductance was obtained from three experiments where the effect of the K^+ channel blocker tetraethylammonium (TEA) was

investigated. External application of 20 mM-TEA caused a reduction in the amplitude of the pH_0-induced hyperpolarization concomitant with a fall in input conductance. The amplitudes of the hyperpolarizations in the presence of TEA were 50, 73 and 72% of the values in the absence of TEA.

Calcium requirement for the initiation and maintenance of the response

If the primary effect of high pH_0 were to release Ca^{2+} from intracellular stores, perhaps as a consequence of a rise in intracellular pH, then it would be expected that a hyperpolarization would be initiated in the absence of external Ca^{2+}. In three experiments where Ca^{2+} was replaced with Mg^{2+} (see Methods), a rise in pH_0 from 7·5

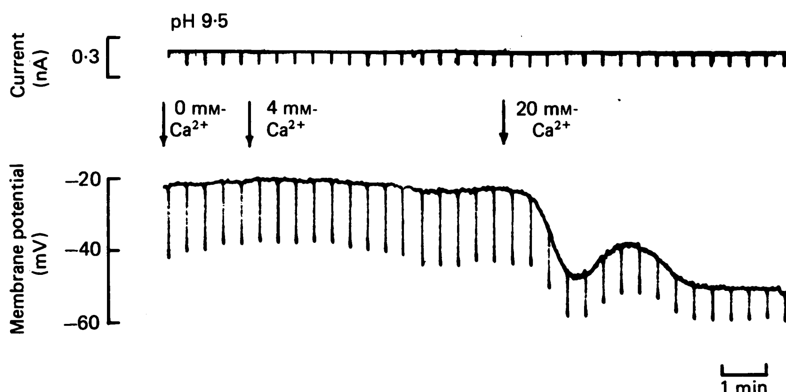

Fig. 4. Effect of $[Ca^{2+}]_0$ on the initiation of the hyperpolarizing response. Arrows indicate the time when the perfusing solution was changed in the bath. Membrane resistance change was monitored by observing the injected current (upper trace) and the membrane potential (lower trace).

to 9·5 never elicited a hyperpolarization. However, when $[Ca^{2+}]_0$ was raised to a sufficient level a hyperpolarizing response to high pH_0 was evoked (Fig. 4). This suggests that a transmembrane Ca^{2+} influx is involved in the initiation of the response.

If maintenance of the pH_0-induced hyperpolarization depended on Ca^{2+} influx then reducing $[Ca^{2+}]_0$ would be expected to cause a corresponding reduction in the amplitude of the hyperpolarization. Indeed, this was found to be the case in seven experiments. Moreover, the amplitude of the hyperpolarization induced by high pH_0 was dependent on $[Ca^{2+}]_0$; however, the potential recorded at pH_0 7·5 was insensitive to changes in $[Ca^{2+}]_0$ as is evident from Table 1. In each experiment raising the value of $[Ca^{2+}]_0$ was associated with an increase in the amplitude of the hyperpolarization induced by pH_0 9·5. The mean values of the potential at high pH_0 for a $[Ca^{2+}]_0$ of 2 and 20 mM were -53 ± 17 and -64 ± 17 mV respectively.

A pH_0-induced hyperpolarization could not be obtained from cells bathed in a solution where Ba^{2+} replaced Ca^{2+} in three experiments. Responses to high pH_0 were recorded, however, when Ca^{2+} was substituted by Sr^{2+} (five experiments). Although Sr^{2+} could be used as a substitute for Ca^{2+} the Sr^{2+} concentration had to be raised to 10 mM before a pH_0-induced hyperpolarization could be sustained by high pH_0 whereas Ca^{2+} was effective at 4 mM. The value of E_r for responses sustained by Sr^{2+}

was -78 ± 5 mV and the increase in mean membrane conductance was 7·8 nS. Both values are similar to those for responses sustained by Ca^{2+} (Table 1).

Intracellular application of EGTA

Our results suggest that the pH_o-induced hyperpolarization is caused by the opening of K^+ channels probably activated by a rise in the cytosolic Ca^{2+} concentration. A test, albeit indirect, of that hypothesis is to load eggs with the Ca^{2+} chelator EGTA (see Methods) before treatment with high pH_o in order to prevent a rise in $[Ca^{2+}]_i$. In five experiments loading eggs with EGTA prevented a pH_o-induced hyperpolarization. Moreover, trains of anode-break excitations could not produce hyperpolarizations in EGTA-loaded cells ($n = 5$). Because the EGTA electrodes had low resistance it was necessary to investigate the effect of the insertion of low-resistance electrodes on the pH-induced response. In a typical experiment a second low-resistance electrode filled with potassium acetate was inserted into an egg (*a*, Fig. 5*A*) to mimic the insertion of an EGTA electrode prior to application of high-pH_o solution. A normal response to high pH_o was obtained. Then the second electrode was removed and an EGTA electrode was inserted (*b*, Fig. 5*B*). After the insertion of the EGTA electrode there was sufficient leakage of EGTA into the cell to prevent a conspicuous hyperpolarization (Fig. 5*B*).

By applying an equivalent circuit model it is possible to estimate the membrane potential attained on the opening of K^+ channels by high pH_o. For example, in Fig. 5*A* the resting potential E_m is -20 mV and the conductance (g) is 4·5 nS. The opening of K^+ channels by high pH_o causes an expected increase of conductance (g_K) of 6·6 nS with an $E_r = -85$ mV (Table 1). The expected potential at high pH_o is given by:

$$\frac{E_m g + E_r g_K}{g + g_K} = \frac{(-20)(4{\cdot}5) + (-85)(6{\cdot}6)}{4{\cdot}5 + 6{\cdot}6} = -59 \text{ mV}.$$

In Fig. 5*B*, $E_m = -14$ mV and $g = 5{\cdot}3$ nS and so the expected potential at high pH_o is

$$\frac{(-14)(5{\cdot}3) + (-85)(6{\cdot}6)}{5{\cdot}3 + 6{\cdot}6} = -52 \text{ mV}.$$

Thus any increase in the leak impalement artifact produced by the insertion of the EGTA pipette is insufficient to account for the failure of high-pH_o solution to evoke a hyperpolarization.

Is passive Ca^{2+} influx though voltage-gated Ca^{2+} channels responsible for the pH_o-induced hyperpolarization?

Voltage-clamp studies (Okamoto, Takahashi & Yamashita, 1977) and intracellular recordings (Miyazaki & Igusa, 1982; Georgiou *et al.* 1983) have revealed the presence of voltage-gated Ca^{2+} channels in both mouse and hamster eggs. These channels are inactivated at the recorded resting potentials in our experiments (around -30 mV). It is possible, however, that the high pH_o can increase their open-state probability at these potentials and so cause an enhancement of the passive Ca^{2+} influx. An indirect estimate of the Ca^{2+} influx through the voltage-gated channels is obtained by measuring the maximum rate of rise of the Ca^{2+} action potential in 4 mM-Ca^{2+}

solution. To obtain such estimates we current-clamped cells at membrane potentials in the range −90 to −140 mV and evoked action potentials by depolarizing pulses. The mean maximum rate of rise at $pH_0 = 7{\cdot}5$ was $1{\cdot}7 \pm 0{\cdot}2$ V/s ($n = 12$). The maximum rate of rise of Ca^{2+} action potentials was reversibly increased to $2{\cdot}8 \pm 0{\cdot}6$ V/s at $pH_0 = 9{\cdot}5$ in the same cells (Fig. 6). In addition, the threshold of the

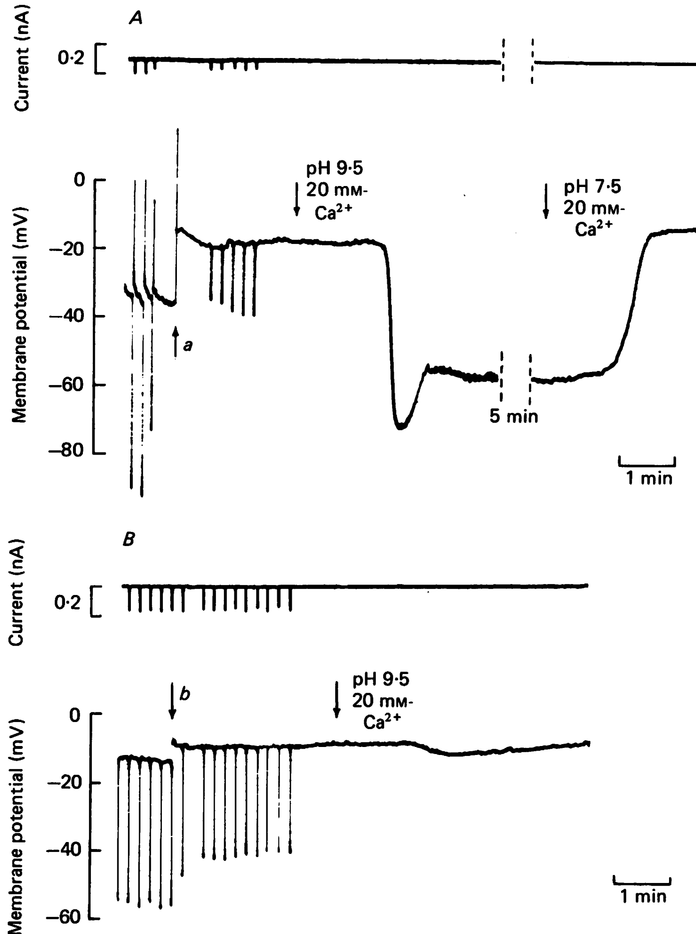

Fig. 5. Effect of an intracellular application of the Ca^{2+} chelator EGTA to an egg. Upper trace, current. Lower trace, membrane potential. *A*, a second electrode filled with potassium acetate was inserted into the egg (*a*) before the applications of high pH. *B*, a third electrode filled with 0·25 M-EGTA was introduced into the egg (*b*) after the withdrawal of the second. High-pH solution was then applied to the egg.

action potential shifted to more negative values by about 4 mV. This negative shift in threshold at high pH_0 further excludes the participation of the voltage-gated Ca^{2+} channels in the response to pH_0.

The rate of repolarization of the Ca^{2+} action potentials was also reversibly increased by the high-pH treatment in the same cells. The mean values of maximum rate of fall were $-0{\cdot}24 \pm 0{\cdot}10$ V/s at pH 7·5 and $-0{\cdot}42 \pm 0{\cdot}15$ V/s at 9·5 respectively ($n = 12$).

A further test of the possible role of the voltage-gated Ca^{2+} channels in the initiation and maintenance of the response to high pH_0 was carried out by adding the Ca^{2+} channel blocker La^{3+} to the bathing solution in eight experiments. La^{3+} was chosen as a Ca^{2+} channel blocker in preference to Co^{2+} or Cd^{2+} which precipitate in solutions with high pH. The presence of La^{3+} (0·5–1 mM) did not prevent the response to high pH_0 suggesting that the voltage-gated Ca^{2+} channels are not a major route

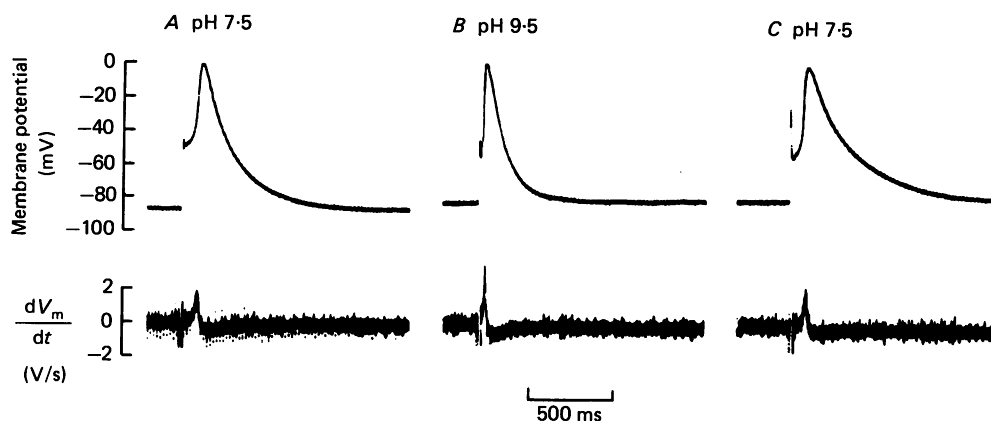

Fig. 6. Effects of high pH_0 on Ca^{2+} action potentials in a hamster egg current clamped at a membrane potential of about −90 mV. Oscilloscope picture of action potentials (upper) and their differentiated records (lower) in standard solution (*A*), in high-pH_0 solution (*B*) and after washing with standard solution (*C*).

for the necessary Ca^{2+} influx. Because La^{3+} is also an inhibitor of the Ca^{2+} pump it was used for that purpose in other experiments (see below).

The effect of pH_0 on hyperpolarizations evoked by Ca^{2+} injection

The dependence of the pH_0-induced hyperpolarizations on $[Ca^{2+}]_0$ suggests that continuous Ca^{2+} influx is probably required to maintain the response. High pH_0 might reduce the ability of eggs to regulate $[Ca^{2+}]_i$. Previous studies have shown that bath application of either La^{3+}, high Ca^{2+} or quercetin, which have been reported to inhibit active Ca^{2+} efflux (Dipolo & Beaugé, 1982; Okada *et al.* 1982; Baker & Dipolo, 1984) prolong the recovery phase of hyperpolarizations evoked by injections of Ca^{2+} (Georgiou *et al.* 1987*a*). La^{3+} also prolonged the hyperpolarizations recorded in hamster eggs at fertilization (P. Georgiou & A. I. McNiven, unpublished). A similar prolongation would be expected if active Ca^{2+} extrusion were inhibited by a rise in pH_0. To examine this possibility, Ca^{2+} was injected ionophoretically into eggs in bathing solutions of different pH_0 at room temperature. The values of pH_0 were chosen to minimize the induced hyperpolarization and thus avoid a large reduction in the amplitude of the Ca^{2+}-evoked hyperpolarization.

In five experiments, one of which is illustrated in Fig. 7, a rise in pH_0 from 7·5 to 8·5 caused in all cells but one an increase in the amplitude of the Ca^{2+}-evoked hyperpolarization. The measured amplitudes were 28, 25, 33, 27 and 47 mV in pH 7·5 and 35, 37, 42, 38 and 43 mV respectively in pH 8·5. In all cells the rise in pH_0 caused a substantial reduction in the recovery rate of the membrane potential. The

time of the decay of the potential ($T_{\frac{1}{2}(V)}$) from its peak value to half of that value increased approximately fourfold from 4·3, 4·2, 6·6, 5·0 and 4·2 s to 18·4, 21·0, 16·0, 19·0 and 27·0 s respectively. In three of the above experiments it was possible to obtain values for amplitude and $T_{\frac{1}{2}(V)}$ after recovery in normal solution; the values of amplitudes were 33, 33 and 51 mV and the values of $T_{\frac{1}{2}(V)}$ were 10·6, 5·6 and 4·5 s

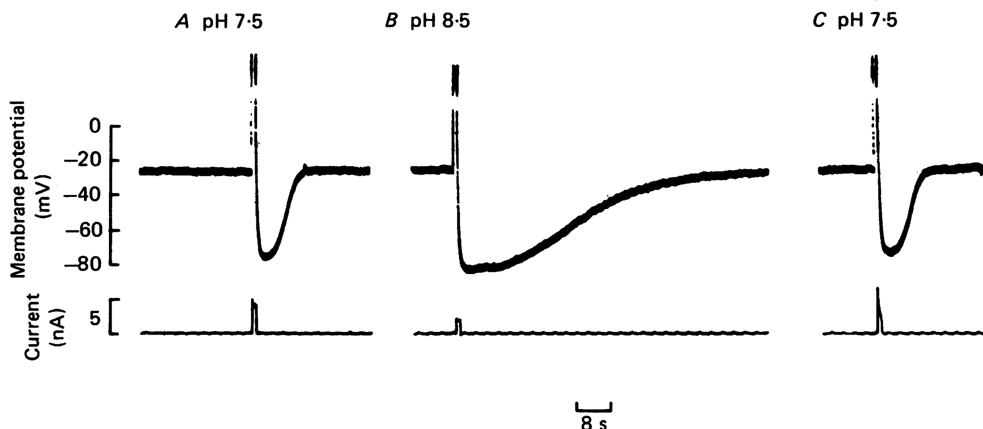

Fig. 7. Prolongation of the response induced by intracellular Ca^{2+} injection in high-pH solution. Note that the injected current in *B* is smaller than that in *A* and *C*. Upper traces show membrane potentials, and lower traces show injected currents.

respectively. In the other two experiments blocking of the Ca^{2+} micropipette prevented the induction of responses during recovery. A similar reversible prolongation was observed at the higher temperature of 35–37 °C ($n = 2$).

The effect of Na^+ removal on the properties of Ca^{2+}-evoked hyperpolarizations

Igusa & Miyazaki (1983) reported that the duration of the hyperpolarization evoked by intracellular injection of Ca^{2+} was markedly prolonged (i.e. by 3–4 times) in solutions where external Na^+ was substituted for either Li^+ or $Tris^+$. They tentatively attributed the increase in duration to the inhibition of the Na^+–Ca^{2+} exchange mechanism.

In six experiments where Li^+ was used as a partial replacement for Na^+ (see Methods) $T_{\frac{1}{2}(V)}$ was prolonged by a factor in the range 1·1–1·9. Because substitution of Na^+ by Li^+ resulted in a depolarization of about 10 mV and a rise in conductance Li^+ is possibly not a good substitute for Na^+ and therefore *N*-methyl-D-glucamine (NMDG) was chosen because it does not cause any depolarization (Nachshen, Sanchez-Armass & Weinstein, 1987). In experiments on four eggs conducted at 35–37 °C $T_{\frac{1}{2}(V)}$ was prolonged by 1·9 times from $1·9 \pm 0·5$ s ($n = 10$) in control solutions to $3·6 \pm 0·9$ s ($n = 10$) in Na^+-free solution. A similar prolongation was observed in additional experiments performed at room temperature (21–25 °C); in nine experiments $T_{\frac{1}{2}(V)}$ increased from $9·9 \pm 3·0$ s in control solution to $17·5 \pm 7·2$ s in Na^+-free solution.

Removal of Na^+ might have affected other systems such as a Na^+–H^+ exchanger or a Na^+–K^+ pump. Also high $[Ca^{2+}]_o$ is known to block the Na^+–K^+ exchanger (Putnam & Roos, 1986). Application of inhibitors of these systems such as amiloride

(1 mM) (Putnam, Roos & Wilding, 1986) and ouabain (10^{-5} M) did not produce any changes in the electrical properties of the eggs.

The effect of pH_0 in the absence of Na^+

The moderate increase in the duration of the hyperpolarizing response to Ca^{2+} injection at pH_0 7·5 in Na^+-free solution consisted of a prolongation of the plateau

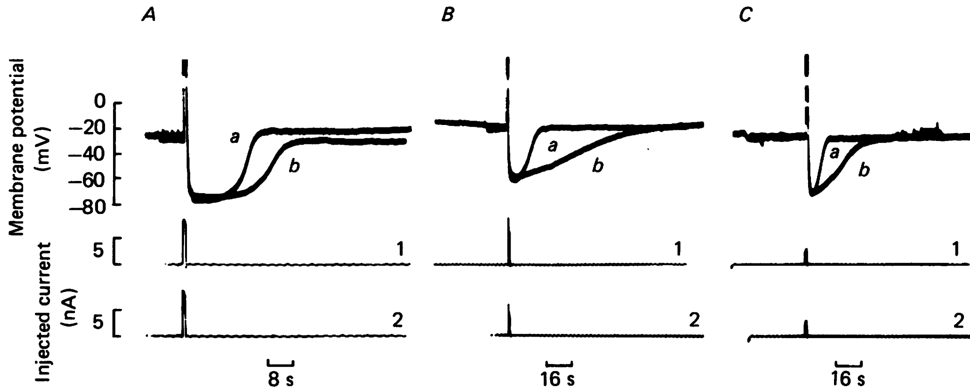

Fig. 8. Prolongation of the responses evoked by Ca^{2+} injection under Na^+-free conditions. Responses from the same cell are shown in superimposed traces. Injected currents are shown in lower traces. *A*, in the presence (trace *a* and current 1) and in the absence (trace *b* and current 2) of Na^+ in bathing solution at pH 7·5. *B*, in the presence of (trace *a* and current 1) and in the absence (trace *b* and current 2) of external Na^+ at pH 8·0. *C*, at pH 7·5 (trace *a* and current 1) and at pH 8·0 (trace *b* and current 2) in the absence of external Na^+.

phase and a slowing of the recovery phase (Fig. 8*A*). At higher pH_0, however, the response was greatly prolonged mainly due to the slowing of the recovery phase in the absence of Na^+ (Fig. 8*B*). There was little change in the shape of the responses evoked in the presence of external Na^+ at pH_0 7·5 and 8·0 (Fig. 8*A* and *B*); the recovery phase was slightly slowed at pH_0 8·0 in one experiment out of three. When the putative Na^+–Ca^{2+} exchange system was suppressed by application of Na^+-free solution, high pH_0 caused a marked slowing of the recovery phase (Fig. 8*C*). Our interpretation of the representative results in Fig. 8 is that high pH_0 produces two opposing effects on the recovery phase of the hyperpolarization. The first effect is an enhancement of Na^+–Ca^{2+} exchange and the second is an inhibition of active Ca^{2+} extrusion. The second effect exerts the greater influence on the recovery rate (compare differences between curves *a* and *b* in Fig. 8*A* and *B*).

If Na^+–Ca^{2+} exchange were operating during the response, then by augmenting the electrochemical gradient for Ca^{2+} in the absence of external Na^+ it might be possible to reverse the direction of the exchange so as to cause a rise in $[Ca^{2+}]_i$. In two experiments complete substitution of Na^+ with NMDG in the presence of a $[Ca^{2+}]_o$ of 20 mM resulted in the generation of two transient hyperpolarizations. The responses were accompanied by a large rise in membrane input conductance with a reversal potential close to E_K.

Transient hyperpolarization induced by La^{3+}

It has been reported in a previous study (Georgiou *et al.* 1987*a*) that the recovery phase of the Ca^{2+}-evoked hyperpolarization was considerably prolonged in the presence of external La^{3+}, a fact which is consistent with reports that La^{3+} inhibits active Ca^{2+} efflux (Sarkadi *et al.* 1972; Baker & Dipolo, 1984). Georgiou *et al.* (1987*a*) observed that the effect of La^{3+} on the passive electrical properties of hamster eggs was a steady hyperpolarization of about 20 mV. Because this hyperpolarization was accompanied by an approximately twofold rise in input resistance rather than a fall, as would be expected if channels were opened, it was attributed to an increase in the microelectrode seal resistance. Georgiou *et al.* (1987*a*) carried out those experiments in solutions where $[Ca^{2+}]_o$ was 4 mM. Since high $[Ca^{2+}]_o$ is also known to inhibit Ca^{2+} efflux (Dipolo & Beaugé, 1983), we examined the effect of La^{3+} (1 mM) in the presence of high Ca^{2+} (20 mM) to see whether the synergistic action of the two ions on Ca^{2+} efflux would lead to a hyperpolarization. In three experiments the combined effect of La^{3+} and high Ca^{2+} was the induction of transient hyperpolarizations similar to those induced by high Ca^{2+} in Na^+-free solutions. Again the input conductance rose approximately fourfold at the peak of these responses, indicative of channel opening.

DISCUSSION

The present study shows that raising the pH of the solution bathing hamster eggs can produce a reversible membrane hyperpolarization accompanied by a rise in membrane conductance. Reversal potential measurements suggest that high pH_o activates a potassium or a chloride conductance. Although the presence of Ca^{2+}-activated K^+ channels has been demonstrated in mammalian eggs (e.g. Miyazaki & Igusa, 1983; Georgiou *et al.* 1983) Cl^- channels also activated by calcium exist in *Xenopus* oocytes (Barish, 1982; Miledi & Parker, 1984) and so it was necessary to test for the latter type in hamster eggs. Methane sulphonate is a useful replacement anion for Cl^- as it is impermeant and does not alter intracellular pH (Sharp & Thomas, 1981). Because the amplitude of the hyperpolarization is unaffected by the removal of extracellular Cl^- while it varies with $[K^+]_o$ (Fig. 3) it is highly likely that the pH_o-evoked response is caused by the opening of K^+ channels. This interpretation is supported by the finding that TEA partially blocks the pH_o-induced rise in membrane conductance. The discrepancy between the observed relation between potential and log $[K^+]_o$ in Fig. 3 and that predicted by the Nernstian equation implies the presence of an additional conductance pathway.

Dependence of the response on Ca^{2+}

Calcium ions are required for the initiation and maintenance of the pH_o-induced hyperpolarization. The amplitude of the response decreased when $[Ca^{2+}]_o$ was lowered and was abolished when external Ca^{2+} was replaced by Mg^{2+} or Ba^{2+}. Moreover, Sr^{2+} was an effective substitute for Ca^{2+} (whereas Ba^{2+} was not) as in the case for the mediation of the hyperpolarizations occurring after fertilization or intracellular cation injection in hamster eggs (Miyazaki & Igusa, 1983; Georgiou *et*

al. 1987*a*). Those features of the Ca^{2+} dependence of the pH_o-induced response suggest that an influx of Ca^{2+} is necessary to initiate and sustain it.

A rise in $[Ca^{2+}]_i$ activates K^+ channels underlying the recurring hyperpolarizations seen during fertilization of hamster eggs (e.g. Igusa & Miyazaki, 1986). If the same channels are opened by high pH_o then preventing a rise in $[Ca^{2+}]_i$ should abolish the hyperpolarization as found when EGTA was injected into fertilized eggs (Miyazaki & Igusa, 1982). In our experiments (Fig. 5) the insertion of the EGTA-filled electrode might introduce an additional leak conductance which, if substantial, could itself reduce the amplitude of the pH_o-induced hyperpolarization. The insertion of a similar low-resistance electrode (filled with potassium acetate) in place of the EGTA electrode, however, failed to attentuate the response (Fig. 5*A*). Thus the abolition of the response by the intracellular application of EGTA (Fig. 5*B*) appears to be genuine and implicates intracellular Ca^{2+} as a mediator in the response to high pH_o. Possibly the effect of high pH_o on potassium conductance in hamster eggs is mediated at an external site on the membrane. The rapid onset of the response to raised pH_o (Fig. 2) is consistent with that view.

Direct effects of pH_o on channels

A possible mechanism underlying the suggested pH_o-induced rise in $[Ca^{2+}]_i$ could be a direct effect of high-pH_o on influx through voltage-gated Ca^{2+} channels (Miyazaki & Igusa, 1982). Altering pH_o affects current and gating of channels in skeletal muscle (Hille, 1968; Campbell & Hahin, 1984). Although high pH_o did directly affect voltage-gated Ca^{2+} channels in the egg (Fig. 6), preventing influx through these channels by La^{3+} did not block the response, indicating that they do not underlie the hyperpolarizing response. Thus it is likely that the proposed Ca^{2+} influx initiating and maintaining the response to high pH_o passes through non-voltage-gated channels (Fig. 9).

Effects of pH_o on hyperpolarizations evoked by Ca^{2+} injection

Alkaline pH_o is known to inhibit reversibly Ca^{2+} efflux via the uncoupled Ca^{2+} pump in squid giant axons (Dipolo & Beaugé, 1982). Consistent with this is our finding that the recovery phase of the Ca^{2+}-evoked response is reversibly slowed by high pH_o (Fig. 7). A similar reversible prolongation of the Ca^{2+}-evoked hyperpolarization has been observed on external application of La^{3+}, high Ca^{2+} and quercetin (Georgiou *et al.* 1987*a*), all known to block Ca^{2+} extrusion (Sarkadi *et al.* 1977; Dipolo & Beaugé, 1982; Okada *et al.* 1982). Thus high pH_o inhibits active Ca^{2+} efflux and thereby might cause a rise in $[Ca^{2+}]_i$ in the face of Ca^{2+} influx through non-voltage-gated channels (Fig. 9).

Evidence obtained at pH 7·5 (Fig. 8*A*) suggested that a Na^+–Ca^{2+} exchange was present in the hamster egg. It was then necessary to determine if raising pH_o had any effect on this extrusion system. Hodgkin & Nunn (1987) have reported that high pH_o facilitates the Na^+–Ca^{2+} exchange in salamander rods. Evidently the Na^+–Ca^{2+} exchange in the egg is also facilitated by high pH_o. It is concluded that both Na^+–Ca^{2+} exchange and a Ca^{2+} pump contribute to the recovery phase of the response to Ca^{2+} injection and that high pH_o inhibits active Ca^{2+} extrusion to a greater extent than it facilitates Na^+–Ca^{2+} exchange (Fig. 8).

Involvement of Ca^{2+} release

It is possible that the proposed pH_0-induced rise in $[Ca^{2+}]_i$ is amplified by a number of mechanisms reported in other studies. For example an initial rise in $[Ca^{2+}]_i$ may be amplified by a Ca^{2+}-induced Ca^{2+} release from intracellular stores in fertilized (Igusa & Miyazaki, 1983; Miyazaki, Hashimoto, Yoshimoto, Kishimoto, Igusa & Hiramoto, 1986) and unfertilized hamster eggs (Georgiou, House, McNiven & Yoshida, 1988) (see Fig. 9). Alternatively, a rise in $[Ca^{2+}]_i$ may promote the

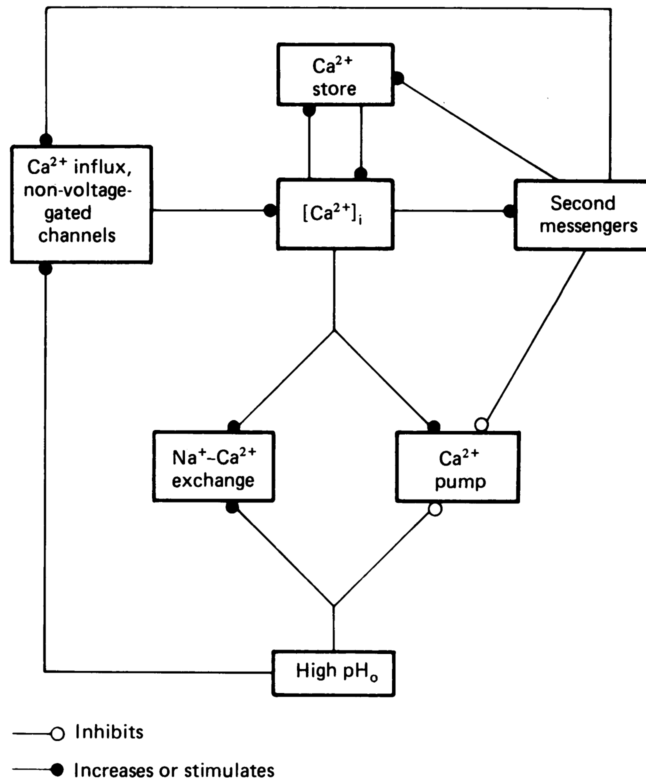

Fig. 9. Diagram of possible mechanisms involved in regulation of $[Ca^{2+}]_i$. The Ca^{2+}-dependent hyperpolarization induced by high-pH solutions can be ascribed to inhibition of the Ca^{2+} pump along with a possible stimulation of Ca^{2+} influx through non-voltage-gated channels. The resulting rise in $[Ca^{2+}]_i$ could be amplified through a system involving an intracellular Ca^{2+} store and production of second messengers (see text).

production of a second messenger such as inositol trisphosphate (IP_3) causing Ca^{2+} release from a store as proposed for eggs of a number of species such as sea urchin (Whitaker & Aitchison, 1985), frog (Parker & Miledi, 1987) and hamster (Miyazaki, 1987). Moreover, IP_3 might directly activate non-voltage-gated Ca^{2+} channels and thus augment Ca^{2+} influx as observed in T-lymphocytes (Kuno & Gardner, 1987). Yet another possibility is that IP_3 blocks Ca^{2+} extrusion as reported for vascular smooth muscle (Popescu, Hinescu, Musat, Ionescu & Pistritzu, 1986), an effect mimicked by high pH_0 in our experiments on hamster eggs.

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