J. Physiol. (1983), **340**, *pp.* 633–647
With 2 plates and 6 text-figures
Printed in Great Britain

PERIODIC HYPERPOLARIZING RESPONSES IN HAMSTER AND MOUSE EGGS FERTILIZED WITH MOUSE SPERM

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(*Received 16 August 1982*)

SUMMARY

1. The zona-free hamster egg allows multiple entries of heterologous as well as homologous sperm. The hamster egg inseminated with mouse sperm (M × H egg) showed recurring, transient hyperpolarizing responses (h.r.s) with the peak of −70 to −80 mV. They were superimposed on a hyperpolarizing shift of the resting potential (h.s.) which gradually reached −60 mV in 50 min after insemination.

2. Unlike the hamster sperm, the cessation of flagellar motion of the first mouse sperm ('1-stop') failed to induce the first h.r. but produced only a small hyperpolarizing 'step' of 3–7 mV. Similar steps occurred for each of additional sperm with a one-to-one correspondence, 4–50 sec ahead of the cessation of sperm motion.

3. In M × H eggs, the h.r. first appeared about 15 min after the '1-stop'. The intervals of the h.r.s thereafter were in the range between 2–10 min, in contrast to 30–45 sec in hamster eggs inseminated with hamster sperm (H × H eggs).

4. The h.r.s in M × H eggs were abolished by intracellular injection of EGTA, suggesting that they were caused by periodic increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as in H × H eggs.

5. The gradual h.s. in M × H eggs was considered to be due mainly to an increase in Ca-independent K permeability, since the resting potential beyond −60 mV at 50–70 min after insemination was changed by only 3–5 mV on the removal of Cl ions and on EGTA injection.

6. Histological observations revealed that the resumption of the second meiosis, the indication of egg activation, is delayed in M × H eggs by about 15 min, compared with that in H × H eggs. There was a good correlation between the delay of activation and that of the occurrence of the first h.r.

7. In M × H eggs, the probability of egg activation within 70 min was dependent on the number of sperm penetrations: 90 % for more than ten sperm while 20–30 % for less than five sperm. Eggs in which sperm penetration was not followed by activation showed no h.r.s.

8. The mouse egg inseminated with mouse sperm showed small h.r.s (3–4 mV) superimposed on the h.s. from −35 to −55 mV in 50 min after insemination. Both

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h.r.s and h.s. were associated with an increase in the membrane conductance. The h.s. was considered to be due mainly to a Ca-independent increase in K permeability.

9. Iontophoretic injection of Ca^{2+} into the unfertilized mouse egg could not increase the K conductance with injection currents up to 4 nA. However, the h.r.s were suggested to be resulted from a periodic increase in $[Ca^{2+}]_i$, since they were abolished by injection of EGTA.

INTRODUCTION

It has been proposed that the release of intracellular Ca^{2+} may be the universal factor promoting activation of egg metabolism at fertilization (Steinhardt, Epel, Carrol & Yanagimachi, 1974; Steinhardt, Zucker & Schatten, 1977). The dramatic increase in $[Ca^{2+}]_i$ has been demonstrated using the Ca^{2+}-sensitive photoprotein aequorin in eggs of the sea urchin (Steinhardt *et al.* 1977) and medaka fish (Gilkey, Jaffe, Ridgway & Reynolds, 1978). In mammalian eggs, it has been reported that parthenogenetic activation is induced by increasing the $[Ca^{2+}]_i$ with the Ca ionophore in the rat and hamster eggs (Steinhardt *et al.* 1974) or by injection of Ca^{2+} into the mouse egg (Fulton & Whittingham, 1978).

In mouse eggs, experiments with microinjected aequorin have shown oscillatory $[Ca^{2+}]_i$ transients following exposure to mouse sperm (Cuthbertson, Whittingham & Cobbold, 1981). In golden hamster eggs we have previously described periodic hyperpolarizing responses (h.r.s) superimposed on a slow hyperpolarizing shift (h.s.) of the membrane potential, following insemination by hamster sperm (Miyazaki & Igusa, 1981). The h.r. is due to Ca-activated K conductance (Miyazaki & Igusa, 1982), and it is probably mediated by the release of intracellular Ca^{2+} (Igusa & Miyazaki, 1983). The functional role of the h.r.s and underlying $[Ca^{2+}]_i$ transients in the activation of mammalian eggs is as yet unknown.

It is well known that the hamster egg freed from the surrounding zona pellucida allows multiple entries of heterologous as well as homologous sperm (Yanagimachi, 1972; Hanada & Chang, 1972). In the present paper we report changes in membrane potential observed when zona-free hamster eggs are inseminated by mouse sperm, in order to examine whether heterologous sperm produces similar or different responses, and how these responses may relate to activation, indicated by the resumption of a second meiotic division. The nature and the ionic basis of potential changes that follow fertilization of mouse eggs by mouse sperm were also analysed and compared with previous results in hamster eggs.

A preliminary account of this work has appeared elsewhere (Miyazaki, Yamashita & Igusa, 1982).

METHODS

Eggs. Mature eggs were collected from the oviducts of superovulated golden hamsters (Igusa & Miyazaki, 1983) and DDy mice (Okamoto, Takahashi & Yamashita, 1977). Hamster eggs were freed from the cumulus cells and zona pellucida, as described previously (Igusa & Miyazaki, 1983). The zona-free hamster eggs *in vitro* tend to undergo spontaneous activation, which is indicated by the formation of the second polar body (see below). Therefore, ten to fifteen eggs out of collected eggs were used as a control, kept in culture without insemination during each experiment. The rate of the spontaneous activation in the control eggs was usually less than 8%, when observed

histologically 60 min after insemination of test eggs. Since the rate of the spontaneous activation tended to increase with time, no observations for longer than 70 min after insemination were done in hamster eggs.

Zona-free mouse eggs were obtained mainly by mechanical techniques in combination with minimized enzymatic treatments, otherwise the fertility of the eggs is reduced (Wolf, Inoue & Stark, 1976). A mass of cumulus cells including eggs was torn into smaller pieces with a pair of fine needles, and the pieces were sucked into a capillary containing the standard medium (see below) with 0·05 % hyaluronidase. The eggs were freed from the cumulus cells during washing by repeated aspiration. Then, the eggs were sucked into a micropipette (i.d. 100–125 μm) containing 0·02 % α-chymotrypsin (Sigma) and immediately washed by repeated aspiration. These treatments were so mild that only about a half number of the eggs were freed from the zona pellucida. Zona-intact mouse eggs were used in some experiments.

Sperm and insemination. Hamster sperm were 'capacitated', as described in the preceding paper (Igusa & Miyazaki, 1983). Mouse sperm were incubated for 2–3 hr in the standard medium (Yamashita, 1982), and 3–10 μl. of the sperm suspension was added to the medium containing fifteen to thirty mouse or hamster eggs. The final concentration of hamster or mouse sperm was $5–10 \times 10^4$ cells/ml. Sperm–egg interactions were observed with a phase-contrast inverted microscope. All experiments were carried out at 32 °C in a 0·4 ml. drop of medium placed in a plastic dish and covered with paraffin oil. Experiments were performed at the following time after injection of human chorionic gonadtropin: hamster eggs, 15·5–17 hr; zona-free mouse eggs, 13·5–15·5 hr; zona-intact mouse eggs, 14–18 hr.

Electrophysiological recordings and solutions. An electrode filled with 4 M-K acetate was inserted into the egg. A change in the membrane conductance was monitored by applying constant current pulses through the electrode with a bridge circuit. In some experiments Ca^{2+} or EGTA was injected iontophoretically into the egg, using the second electrode filled with 0·5 M-$CaCl_2$ or EGTA (K salt) as described before (Igusa & Miyazaki, 1983).

For hamster eggs BWW medium (Biggers, Whitten & Whittingham, 1971) was mainly used. The composition was (mM): NaCl, 94·6; KCl, 4·8; $CaCl_2$, 1·7; $MgSO_4$, 1·2; KH_2PO_4, 1·2; Na lactate, 22; Na pyruvate, 0·5; glucose, 5·6; $NaHCO_3$, 25·1 (equilibrated with 5 % CO_2, pH 7·4). The standard medium described by Toyoda, Yokoyama & Hoshi (1971) was used for preparing mouse sperm and eggs. The composition was the same as that of the BWW medium except in the following (mM): NaCl, 119; Na lactate, 0; Na pyruvate, 1·0. Electrophysiological recordings in mouse eggs were performed in the following saline (5 mM-Ca saline) (mM): NaCl, 124; KCl, 6·0; $CaCl_2$, 5·0; $MgCl_2$, 1·2; Na pyruvate, 1·0; glucose, 5·6; HEPES-NaOH buffered at pH 7·4, 20. This saline was used because 5 mM-Ca^{2+} had the effect of reducing leakage due to micro-electrode impalement (Yamashita, 1982). Fertilization was not affected by the 5 mM-Ca saline. For Cl-free saline, methanesulphonate was substituted for Cl^- in the 5 mM-Ca saline for mouse eggs or 3 mM-Ca saline for hamster eggs (Igusa & Miyazaki, 1983). Bovine serum albumin was added to all solutions before use (4 mg/ml.).

Histological observations. After withdrawing the electrode, the impaled egg and the surrounding eggs in the same dish were transferred to a drop of medium on a slide glass, fixed and stained with 0·25 % lacmoid for visualization of egg chromosomes and sperm head (see Yamashita, 1982).

Freshly ovulated eggs are arrested at metaphase of the second meiotic division, having already released the first polar body (Austin, 1961). The penetration of a spermatozoon into an egg causes a series of rapid morphological and physiological changes, which is generally referred to as 'activation' of the egg. The earliest visible indications of egg activation in mammals are breakdown of the cortical granules, and the resumption of the second meiotic division (Austin, 1961). The penetration of the sperm is recognized histologically as the swelling of the head and the resumption of the second meiosis is recognized as the separation of egg chromosomes. In the present experiments at 32 °C, the former was recognized at least 40 min after the attachment of the sperm to the egg surface (see Pls 1 and 2) and the latter at least 15 min after the sperm attachment. In the present paper the term 'activation' was defined by the resumption of the second meiotic division, not simply by sperm penetration. The stage of the second meiotic division was defined as follows: metaphase (M), the stage at the arrest in the metaphase (Pl. 1, *A* and Pl. 2, *E*); early anaphase (EA), chromosomes have been just separated (Pl. 2, *B*); anaphase (A), chromosomes are separating toward two poles (Pl. 1, *B* and Pl. 2, *D*); telophase (T), the stage after chromosomes have been completely separated and assembled at the poles (Pl. 1, *F*), forming the second polar body

(Pl. 1, *C*). The midbody was usually visible at the equator line in eggs at the telophase (Pl. 1, *C* and *F*). The time course of the second meiotic division is illustrated in Fig. 3. Some hamster eggs resumed the second meiosis without insemination (Pl. 1, *F*)). These eggs were considered to be spontaneously activated. This was never found in the mouse egg.

Mouse eggs inseminated with hamster sperm. Attempts to fertilize zona-free mouse eggs with hamster sperm were unsuccessful. The attachment of sperm was poor and the eggs were not activated, even if the sperm concentration was raised. No enlarged sperm head was observed in the egg cytoplasm 1 hr after insemination.

RESULTS

Difference of the potential change between M × H eggs and H × H eggs

The resting potential of unfertilized hamster eggs ranged between −20 and −30 mV. Fig. 1*B* shows an example of the potential change in the M × H eggs. After insemination (ins.), four sperm attached to the egg surface at short intervals (1–4 t.) and, later, two more sperm attached (5 t., 6 t.). Identification of the timing for more than 5–6 sperm was usually abandoned because of its difficulty. Active flagellar motion decreased gradually and then stopped (hereafter described as 'stop'). The complete stop is indicated by 's.' in Figure.

In Fig. 1*B* a small hyperpolarizing step of 6 mV appeared at about the time of the 'stop' of flagellar motion of the first sperm (hereafter described as '1-stop'). The potential step was associated with an increase in the membrane conductance (see short potential responses to constant hyperpolarizing current pulses). Similar potential steps occurred, corresponding to the 'stop' of each sperm (2–7 s.). A large transient h.r. reaching −80 mV appeared abruptly at 18 min after the '1-stop' (dot). It was followed by the second h.r. 9 min later. The interval of the h.r.s thereafter became shorter progressively with time, but it was still 4 min at 50 min after the '1-stop'. These findings are quite different from the potential change in H × H eggs, in which a series of large h.r.s begins at about the time of the '1-stop' and persists at fairly constant intervals of 30–45 sec (Fig. 1*A*: also Miyazaki & Igusa, 1981).

In the M × H egg the resting potential became more negative, partly due to the summation of the hyperpolarizing steps, and it further shifted gradually to a steady level at about −60 mV in 50 min after insemination. Thus, the final steady level was more negative than in H × H egg (Fig. 1*A* and *B*). In some cases, a transient depolarization appeared sporadically (arrows in Fig. 1*B* and *C*). The egg in Fig. 1*B* was found to be at anaphase of the second meiotic division at 50 min after insemination.

The h.r.s occurred sooner and more frequently when large numbers of mouse sperm attached to the egg, as shown in Fig. 1*C*. However, there was still a delay of 7 min. The resting potential reached −60 mV in 7 min after the '1-stop'. The egg was at anaphase after 42 min (see Pl. 2*D*).

In summary, the potential change in M × H eggs differs from that in H × H eggs in the following respects: (1) only a small hyperpolarizing step occurs at the cessation of flagellar motion, (2) the occurrence of the first h.r. is delayed, (3) the frequency of h.r.s is much lower and (4) the resting potential eventually becomes more negative.

An increase in the membrane resistance was observed immediately after insemination but before sperm attachment (Fig. 1*B* and *C*). The supernatant of the centrifuged sperm suspension caused a similar change, and the significance was unclear.

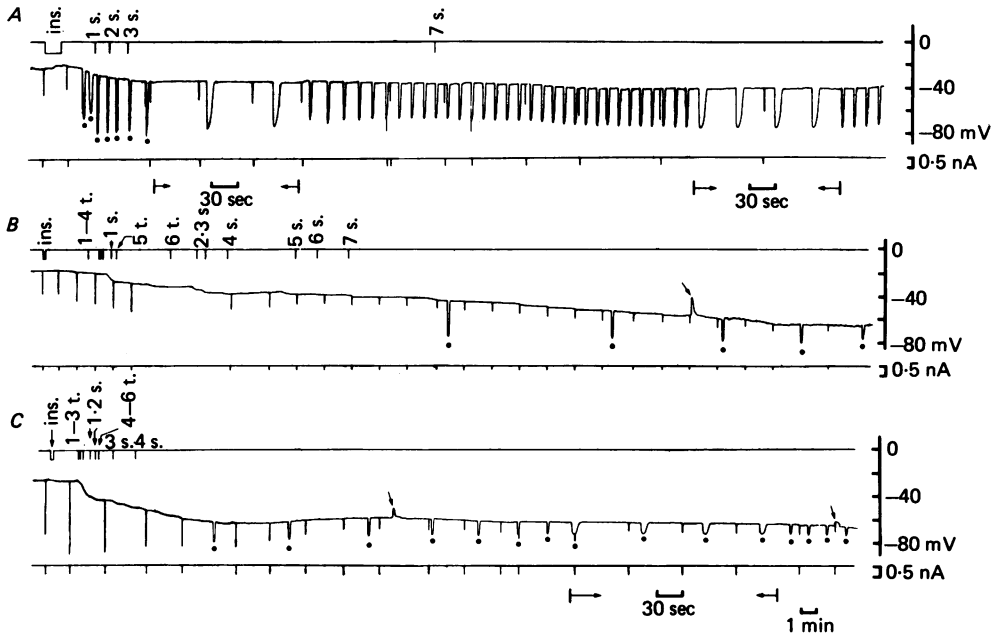

Fig. 1. Potential change in H × H egg (*A*) and M × H eggs (*B*, *C*). In *C* sperm concentration was about five times higher than usual. The first seven h.r.s in *A* and every h.r. in *B* are indicated by dots. Arrows indicate sporadic depolarizations. Chart speed was raised at the indicated periods in *A* and *C*. Bottom trace, constant current pulses. The upper trace indicates zero potential, and also shows events which were marked electrically by the observer: ins., insemination; t., attachment of sperm to the egg surface; s., cessation of flagellar motion of sperm ('stop'). The figure with 't.' or 's.' indicates each sperm in the order of attachment or 'stop', respectively. The 't.' and 's.' of the same number do not always indicate the same sperm. These indications are common to all other Figs. The timing of sperm attachments is omitted in *A*. 4–6 's.' in *A* and 7 't.' in *B* were missed.

Hyperpolarizing potential step

Electronmicroscopic studies have revealed that the membrane fusion between sperm and egg has already occurred in hamster eggs fixed 3 min after insemination with homologous sperm (Yanagimachi & Noda, 1970). The cessation of flagellar motion of the sperm is likely to correspond with the beginning of the membrane fusion (Yanagimachi, 1978). As shown in Fig. 2*A*, each hyperpolarizing step started a little prior to the complete 'stop' of each mouse sperm. The timing of the potential step is shown in Fig. 2*B* in correlation with the complete 'stop', which is taken as zero time (see inset). All of the potential steps preceded the 'stop' of sperm by 4–50 sec (18 sec in average). The result is based on the assumption of a one-to-one correlation between sperm and the potential step. This seemed true, because in some cases no additional step was observed until the next sperm stopped moving more than 5 min later. The amplitude of the first potential step was 4·6 ± 1·5 mV (mean ± S.D., $n = 18$). It became smaller for additional sperm and was undetectable at the later stage (Figs. 1*B*, 2*A*). This is presumably partly due to a progressive decrease in the membrane resistance, and partly due to the membrane potential approaching the equilibrium potential of K ions (E_K).

Hyperpolarizing response (h.r.)

In H × H eggs the first h.r. begins in most cases within 10 sec before or after the '1-stop' (Miyazaki & Igusa, 1981). In M × H eggs it appeared about 15 min after the '1-stop'. The delay time averaged 15·1 ± 2·4 min (n = 10) and ranged between 12 and 18 min. The delay was 7 min in Fig. 1*C* which was an exceptional case. The interval of succeeding h.r.s became shorter with time, and the mean interval at 40–60 min after insemination was about 4 min, ranging between 2 and 8 min.

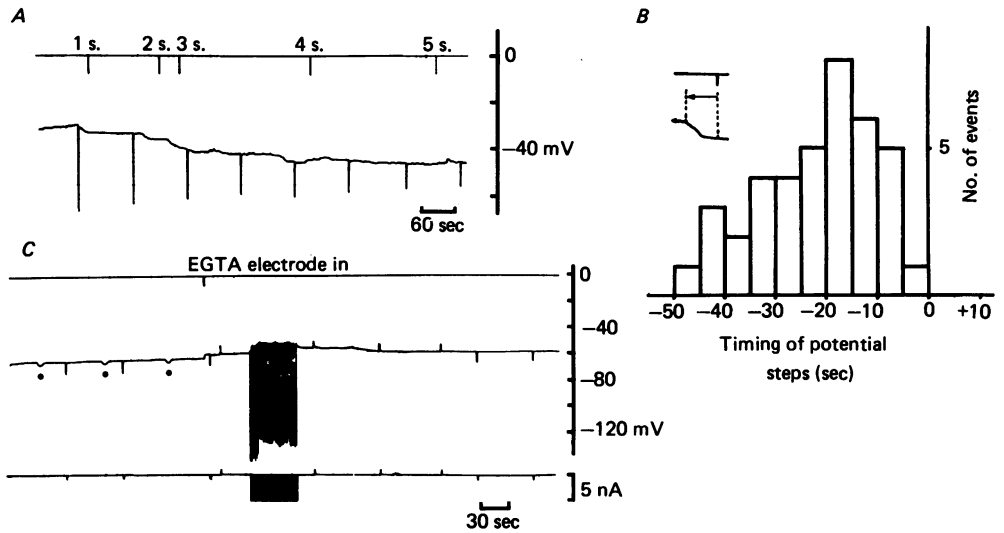

Fig. 2. *A*, expanded record of hyperpolarizing potential steps in M × H egg. The timing of insemination and sperm attachments is omitted. *B*, timing of the onset of the potential step in relation to the 'stop' of each sperm, based on 1:1 correspondence. The time when the flagellar motion of each sperm ceased was taken as zero time (see inset). The histogram was obtained from total thirty-nine sperm in eighteen M × H eggs (mean value, −18 sec). *C*, iontophoretic injection of EGTA into an activated M × H egg, through the second electrode inserted at the mark indicated in the upper trace. Injection pulses: 4 nA, 0·5 sec duration, 1 Hz. The egg was inseminated 70 min prior to the recording, and its activation was evidenced by the extrusion of the second polar body. H.r.s indicated by dots were small partly because of the large negative resting potential.

The property of each h.r. is essentially the same as that in H × H eggs. The amplitude of the h.r. appeared to be smaller, but this is probably due to the more negative resting potential. The duration of the h.r. was about 10 sec. The reversal potential was −82 mV (in three cases), obtained from the intersection of two steady-state current–voltage relations at the peak of the h.r. and just before or after the h.r. These values are consistent with E_K, as in H × H eggs (Miyazaki & Igusa, 1982). Iontophoretic injection of EGTA into the egg abolished the h.r. in four eggs examined. A sample record is shown in Fig. 2*C*. Therefore, it is considered that each h.r. is mediated by Ca-activated K conductance and the series of h.r.s indicates periodic increase in $[Ca^{2+}]_i$ as in H × H eggs.

Hyperpolarizing shift of the resting potential (h.s.)

After the last hyperpolarizing step in M × H eggs, the resting potential shifted gradually in the hyperpolarizing direction, associated with an increase in the membrane conductance, and reached a steady level in about 50 min. The steady level was -62 ± 3 mV ($n = 13$) in activated eggs. Even in unactivated eggs the resting potential reached -43 ± 6 mV ($n = 8$), 50 min after insemination. The final steady level of the resting potential was changed by only a few millivolts on the removal of external Cl^- ions (not shown), consistent with the shift being due to an increased K permeability (P_K). Injection of EGTA as in Fig. 2*C* altered the potential by only 3–5 mV. The large h.s. in M × H eggs is considered to be due mainly to Ca-insensitive P_K.

Comparison of the time course of egg activation

We speculated that the difference in the potential change between M × H eggs and H × H eggs may be correlated to possible differences in egg activation or sperm entry. Therefore, histological observations were performed by fixing eggs at various times after insemination, apart from electrophysiological studies. Fig. 3 represents the time course of the second meiotic division indicative of egg activation in H × H eggs (*A*) and M × H eggs (*B*) by the ratio of eggs at each stage (see Legend for detailed illustration and see Methods for the stages). The time when the first sperm attached to almost all eggs in a dish was taken as zero time. In H × H eggs the early anaphase appeared in the observation at 16 min (35 %), and 90 % of eggs were at anaphase at 27 min. The telophase appeared at 33 min (20 %), and 90 and 97 % of eggs were at telophase at 43 and 53 min, respectively. This time course may be a little slower than in physiological condition, because temperature was 32 °C. In M × H eggs, early anaphase first appeared in the observation at 31 min (20 %), and 80 % of eggs were at anaphase at 42 min. The telophase appeared at 42 min (3 %) or at 51 min (34 %), and 90 % of eggs were at telophase at 61 min. Compared with H × H eggs, the appearance of early anaphase was delayed by about 15 min. On the other hand, the time between the appearance of early anaphase and the attainment of telophase in the majority of eggs was 26 min in H × H eggs and 30 min in M × H eggs. This indicates that egg activation is delayed in M × H eggs by about 15 min, compared with that in H × H eggs. It should be noted that the delay has a good correspondence with the delay of the first h.r.s (Fig. 1*B*), $15{\cdot}1 \pm 2{\cdot}4$ min ($n = 10$). The h.r.s, therefore, is considered to be related to egg activation.

Sperm penetration, which is indicated by enlarged head (Pls. 1*D*, *E* and 2*C*), was recognized at 40 min both in H × H and M × H eggs. Apparently it was not retarded in M × H eggs. In Fig. 3 the mean number of enlarged sperm heads per an activated egg is shown in parentheses. In M × H eggs, activation was retarded in spite of more than double the sperm entries seen in H × H eggs.

Relation between the number of sperm entries and egg activation

Fukuda & Chang (1978) have described that, although 97 % of the zona-free hamster eggs had enlarged mouse sperm heads 30 min after insemination, only 45 % of eggs resumed their second meiosis 60 min after insemination. We confirmed that

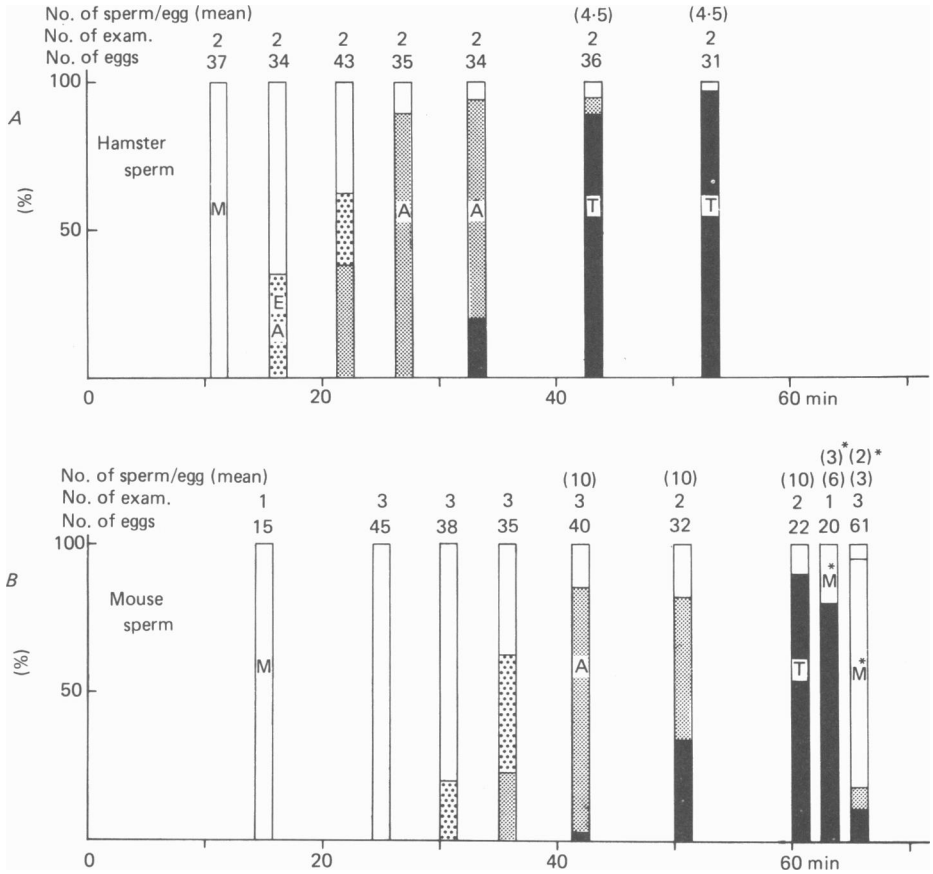

Fig. 3. Time course of the second meiotic division in H × H eggs (*A*) and M × H eggs (*B*). Abscissa: the time starting from the attachment of the first sperm in almost all eggs in a dish. Ordinate: the ratio of eggs at each stage of second meiosis; M, metaphase; EA, early anaphase; A, anaphase; T, telophase. Number of examinations and that of total eggs are shown at the top of each column. One experiment consisted of two to three examinations at different periods, by picking up twelve to twenty eggs out of forty to sixty eggs that were obtained from two animals and inseminated in the same dish. The figure in a parenthesis indicates the mean number of enlarged sperm heads per an activated egg, and that with an asterisk in *B* is for the egg that showed enlarged sperm heads but was arrested at metaphase (stage M*).

the probability of egg activation is lower in M × H eggs, especially when the sperm concentration is reduced. The probability depended on the number of sperm entries. In Fig. 3*B*, 80 % of eggs were activated by the penetration of six or more sperm within 65 min, but only 18 % by three sperm. Most of unactivated M × H eggs had one to four enlarged sperm head (stage M*). Fig. 4*B* represents the probability of activation as a function of the number of enlarged sperm heads. Eggs were observed histologically at 50–70 min after insemination. More than 70 % of eggs that had been penetrated by more than six sperm were activated, while 70–80 % of eggs that had been penetrated by less than five sperm were not activated.

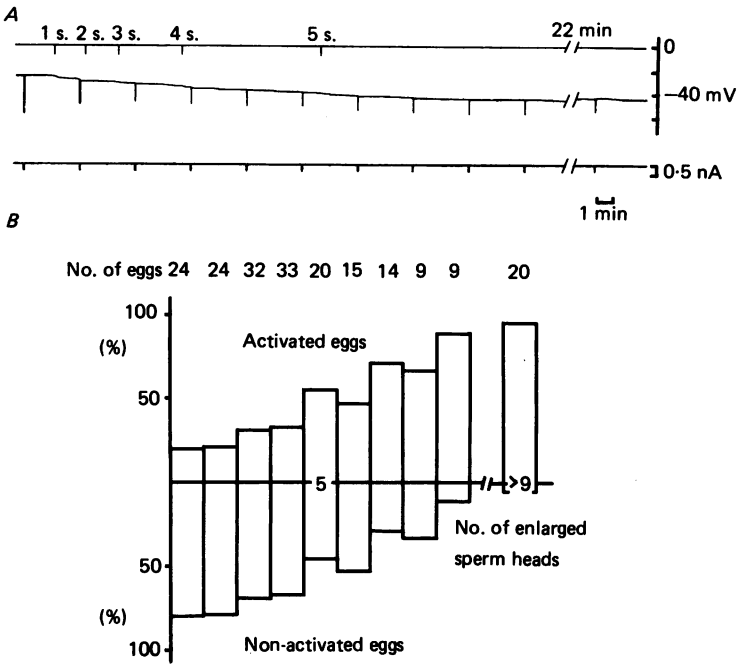

Fig. 4. *A*, potential change in M × H egg inseminated with diluted (1/5) sperm suspension. The egg showed five enlarged sperm heads but it was not activated at 60 min after insemination. The timing of sperm attachments is omitted. *B*, relation between the number of enlarged sperm heads in an egg and the probability of egg activation, examined at 50–60 min after insemination. Less than nine sperm entries were obtained by the dilution of sperm suspension usually used.

It is interesting to examine what kind of potential change occurs in the egg that has enlarged sperm head but is not activated. In Fig. 4*A* the egg was inseminated by sperm suspension diluted five times and was confirmed after the experiment to have been penetrated by five sperm but arrested at metaphase. Hyperpolarizing steps were observed in response to the 'stop' of each sperm. The resting potential reached −45 mV, less negative than in activated eggs. No h.r. appeared throughout the recording for 60 min. Similar results were obtained in all seven eggs examined. On the other hand, all of fifteen eggs, of which activation was confirmed, had shown h.r.s.

H.r.s and h.s. in mouse eggs inseminated with mouse sperm (M × M eggs)

The resting potential and input membrane resistance of unfertilized, zona-intact or zona-free mouse eggs in 5 mM-Ca saline was $-35{\cdot}0 \pm 5{\cdot}9$ mV ($n = 26$) and 140 ± 47 MΩ ($n = 19$), respectively. Fig. 5*Aa* shows the potential change in a zona-free M × M egg. After insemination, six sperm attached to the egg surface (1–6 t.), and some of them stopped their flagellar motion (the timing was missed except the '1-stop'). Hyperpolarizing steps seen in M × H eggs were not observed in the M × M egg. A small, transient hyperpolarization of 3–4 mV, which will be also described as h.r., appeared 10 min after the '1-stop'. The h.r.s occurred at a fairly constant interval of about 2·5 min (see dots in Fig. 5*Aa*). Magnified h.r.s are shown in

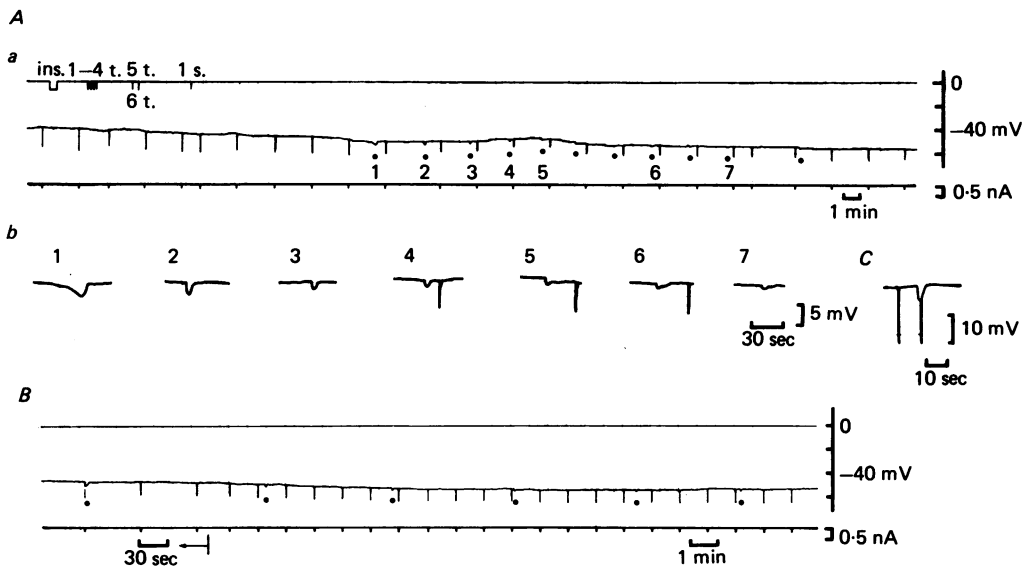

Fig. 5. *Aa*, potential change in zona-free M × M egg in 5 mM-Ca saline. H.r.s are indicated by dots. Seven h.r.s are shown in *b* with four-fold magnification. The egg was at anaphase at 45 min after the '1-stop'. The timing of the 'stop' of sperm was missed except for the first one. *B* potential change in zona-intact M × M egg. The egg was inseminated in standard medium, kept for 60 min, and then transferred to 5 mM-Ca saline immediately before the recording. The second polar body was visible. *C*, conductance increase at the peak of the h.r.

Fig. 5*Ab*. The resting potential shifted gradually from −35 to −57 mV in 40 min after the '1-stop'. Thus both h.r.s and h.s. were observed in the M × M egg and they were associated with an increase in the membrane conductance (Fig. 5*A* and *C*). This egg was at anaphase 45 min after the '1-stop'. Fig. 5*B* is the record from a zona-intact egg, starting at 60 min after insemination (see Legend). The series of small h.r.s had already appeared and persisted at intervals of 6–3 min. The h.s. further advanced to −55 mV in the record.

The potential change in M × M eggs is summarized as follows, from the results in eleven zona-intact or zona-free eggs of which activation was confirmed: (1) there is no substantial potential change at the initial stage of fertilization. (2) Small h.r.s were observed in nine out of eleven eggs. The amplitude was 3·3 ± 0·7 mV and the duration was 8·5 ± 3·1 sec. (3) The first HR appeared 9–12 min after the '1-stop' and h.r.s thereafter occurred at intervals of 2–6 min. The h.r.s were observed until at least 2 hr after insemination. (4) The h.s. reached a steady level of −55 ± 7 mV. More than half of zona-free M × M eggs were at anaphase at 40–45 min after the attachment of the first sperm. Activation of M × M eggs seemed to be delayed to some extent, compared with H × H eggs shown in Fig. 3*A*.

Dependence of h.r.s. and h.s. in M × M eggs on $[Ca^{2+}]_i$

H.s. Fig. 6*A* and *B* show depolarizing potential changes upon the removal of external Cl^- in an unfertilized egg and an activated egg at telophase of the second

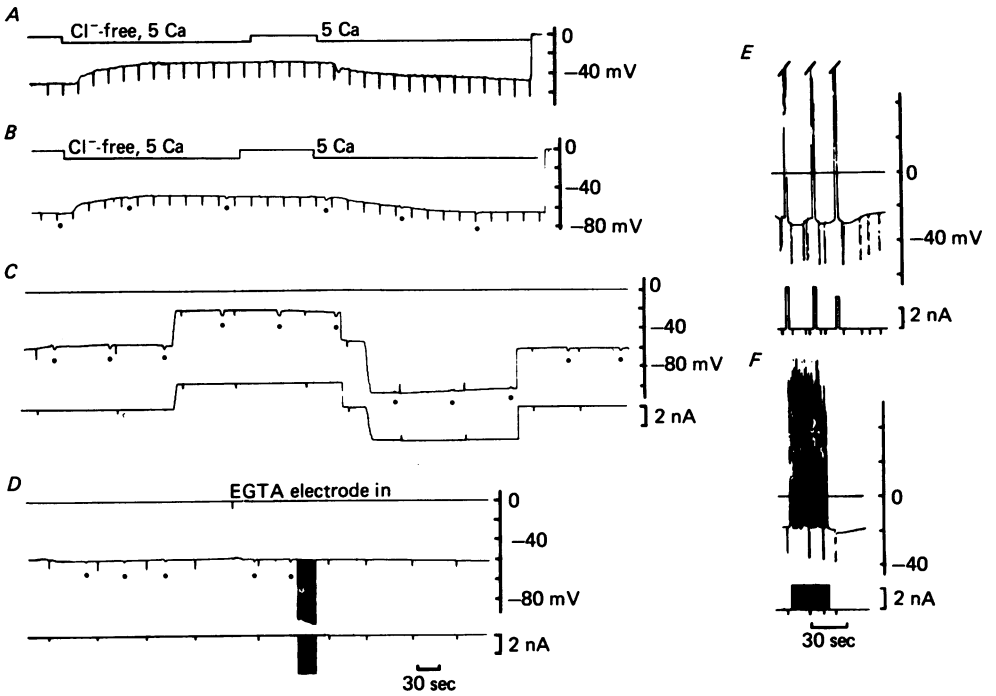

Fig. 6. *A* and *B*, potential change by the removal and recovery of external Cl^- in unfertilized (*A*) and fertilized (*B*) mouse egg. The period of perfusion is indicated by the downward hollow of the upper trace. The membrane resistance in 5 mM-Ca saline before the introduction of Cl^- free saline was 200 (A) and 100 MΩ (B). *C*, h.r.s at various membrane potentials held by d.c. current through the second 3 M-KCl electrode. *D*, iontophoretic injection of EGTA into the unfertilized mouse egg. The eggs in *B–D* were inseminated in standard medium, kept for 60 min, and then transferred to 5 mM-Ca saline before the recording. Activation was confirmed by the second polar body. *E* and *F*, injection of Ca^{2+} into unfertilized mouse eggs at 27 °C with a single 2 sec pulse or repetitive 0·5 sec pulses (1 Hz).

meiosis, respectively. The depolarization was 21 mV (from −48 to −27 mV) in the former and 15 mV (from −60 to −45 mV) in the latter, associated with an increase in the membrane resistance. The change was reversible in both cases. Thus the membrane potential was partly dependent on Cl permeability in both eggs. However, the decrease in the membrane conductance on the removal of Cl^- was comparable in both eggs, and the resting potential of the activated egg in Cl-free saline was more negative by 18 mV than that of the unfertilized egg. Therefore, the h.s. during fertilization is considered to be due mainly to an increase in P_K. Injection of EGTA into the fertilized egg produced no significant change in the membrane potential in three eggs (Fig. 6*D*), suggesting that the h.s. is due mainly to Ca-insensitive P_K.

H.r.s. The h.r.s were not abolished by the removal of Cl^- (Fig. 6*B*). Their amplitude was increased by depolarization with d.c. current through the second 3 M-KCl-filled electrode (Fig. 6*C*). The responses at −60 mV were hyperpolarizing whereas those at −100 mV had the reversed polarity (Fig. 6*C*), suggesting the reversal potential to be around −80 mV. Furthermore, the h.r. was blocked by intracellular injection

of EGTA in three eggs (see Fig. 6*D*). These findings suggest that the h.r. is due to Ca-activated K conductance increase.

In Fig. 6*E* and *F*, Ca ions were rejected iontophoretically into unfertilized mouse eggs. A small hyperpolarization of 3–4 mV was produced by either a single 2 sec pulse of 3–4 nA or repetitive (1 Hz) 0·5 sec pulses of 2·5 nA. The hyperpolarization, however, was associated with an increase in the membrane resistance, apparently due to a decrease in the leakage current. The Ca-activated K conductance increase was not observed in ten eggs examined with currents up to 4 nA, which was the upper limit of injection current through the $CaCl_2$-filled electrode.

DISCUSSION

The hamster egg inseminated with mouse sperm showed both h.r.s and h.s., as does the egg inseminated with homologous sperm. However, in M × H eggs the start of the h.r. series was delayed by about 15 min, corresponding well with the delay of egg activation. Eggs penetrated by mouse sperm but failed to be activated showed no h.r. These findings led to the conclusion that the h.r.s are closely related to the activation of the hamster egg. Although egg activation is delayed in M × H eggs, Fig. 3 suggests that the time interval from the occurrence of the first h.r. to the appearance of early anaphase is about 15 min, almost identical with that in H × H eggs. Since the first h.r. probably indicates the initial release of intracellular Ca^{2+} (Igusa & Miyazaki, 1983), the first h.r. seems to be an initial sign of egg activation.

In M × H eggs, the time interval from early anaphase to telophase was little retarded, while the frequency of h.r.s was much lowered. The biological significance of such a series of h.r.s that persists for more than 1 hr is still unclear. The swelling of the sperm head could occur without the occurrence of h.r.s in M × H eggs, suggesting that periodic increase in $[Ca^{2+}]_i$ is not indispensable to the penetration of, at least, the head of mouse sperm. Since the present experiment was limited to the observation of enlarged sperm heads, further study is necessary to find out any relation of the long-lasting h.r. series to the incorporation of entire sperm into the egg cytoplasm. Ca-mediated periodic hyperpolarizations have been found in other tissues and they are suggested to be related to cell motility, such as phagocytosis in fibroblastic L cells (Okada, Tsuchiya, Yada, Yano & Yawo, 1981) or the activation of chemotactic migration in macrophages (Gallin & Gallin, 1977).

Hyperpolarizing steps in M × H eggs

It has been shown in echinoderm eggs that the depolarizing fertilization potential includes step-like change(s) in the rising phase (Jaffe, 1976; Miyazaki & Hirai, 1979; DeFelice & Dale, 1979) and the first step precedes main depolarization (DeFelice & Dale, 1979; Hülser & Schatten, 1982). In polyspermic eggs each step is considered to be induced by each sperm. However, the step has not been recorded in combination with direct observation of the timing of each sperm–egg contact. Hamster or mouse sperm enable this type of experiment because of their large size. In M × H eggs small hyperpolarizing steps with a conductance increase were observed a little prior to the 'stop' of each sperm with a one-to-one correlation, irrespective of egg activation. The potential step seems likely to coincide with the beginning of the membrane fusion

between the acrosome of sperm and microvilli of egg (see Yanagimachi, 1978). In H × H eggs the small step was not clearly identified at each 'stop' of the sperm. It may be obscured by the series of h.r.s. In M × H eggs the potential step was separated from the delayed h.r. It seems that mouse sperm penetration cannot induce an early increase in $[Ca^{2+}]_i$, whereas homologous sperm can.

H.r.s and h.s. in M × H eggs

In the preceding paper (Igusa & Miyazaki, 1983), it was suggested that the frequency of h.r.s is dependent on an elevated Ca influx. Therefore, the longer interval of h.r.s in the activated M × H eggs may indicate that the heterologous mouse sperm cannot increase Ca influx as much as the homologous hamster sperm can. The delay in the occurrence of the first h.r. can be also interpreted on this basis because the delay was shortened when the highly concentrated suspension of mouse sperm was applied. Conversely, with diluted sperm suspension, it seems probable that small number of the mouse sperm penetrations cannot increase the Ca influx enough to cause Ca release and consequent activation of the hamster egg.

The resting potential in M × H eggs became more negative than that in H × H eggs. In the preceding paper (Igusa & Miyazaki, 1983), it was postulated that Ca-activated P_K as well as P_{Ca} contribute to the h.s. in H × H eggs. In M × H eggs, however, the gradual h.s. was considered to be due mainly to Ca-insensitive P_K. The less negative resting potential in H × H eggs may be due partly to relatively large P_{Ca}, while Ca-insensitive P_K is predominantly increased in hamster eggs activated by mouse sperm.

The transient depolarization was occasionally observed during the h.s. in M × H eggs (Fig. 1). Although the exact mechanism is unknown, this depolarization may reflect the episodic increase in P_{Na} and/or P_{Ca}, because it was accompanied with a conductance increase.

Fertilization potential in M × M eggs

Potential changes in the inseminated mouse egg was composed mainly of two changes; h.s. to about −55 mV and small periodic h.r.s. Jaffe, Sharp & Wolf (1983) have recorded a small oscillation (*ca.* 4 mV) of the membrane potential in M × M eggs at about 7 min after insemination. The small h.r.s were suggested to be based on a Ca-activated K current. However, compared with the hamster egg, h.r.s were much smaller, and in some occasions h.r.s were too small to be visible. In unfertilized mouse eggs no significant increase in K conductance was induced by Ca injection with currents up to 4 nA, while a 0·4 nA pulse caused a substantial increase in K conductance in unfertilized hamster eggs (Igusa & Miyazaki, 1983). In the mouse egg membrane, the density of Ca-activated K channels seems to be much lower than in the hamster egg. Therefore, the h.r. in the mouse egg would indicate a considerable increase in $[Ca^{2+}]_i$, although its amplitude is small. Cuthbertson *et al.* (1981) have demonstrated rapid $[Ca^{2+}]_i$ transients during fertilization of zona-free mouse eggs, using micro-injected aequorin. The Ca^{2+} transients appear after delays of between 5 and 90 min, and they occur once to six times in every 10 min, more frequently later in the series. The h.r.s possibly correspond to these Ca^{2+} transients. In aequorin signals a much larger Ca^{2+} rise has been shown after a delay of 100–150 min, although

the developmental stage of the inseminated egg is not described (Cuthbertson *et al.* 1981). In the present experiments, an extra large h.r. was not observed at least during the period from insemination to the end of the second meiotic division.

In addition to hamster and mouse eggs, small periodic h.r.s have been also found during fertilization of rabbit eggs (McCulloh, Rexroad & Levitan, 1981). The h.r.s may be a common feature of the fertilization potential of mammalian eggs.

We acknowledge Professor K. Maekawa for continuous encouragement throughout the work and Professor K. Takahashi for valuable comments on our manuscript. We also thank Misses Y. Hodota and S. Shinozaki for technical assistance. This work was supported by grants from the Ministry of Education, Science and Culture, Japan.

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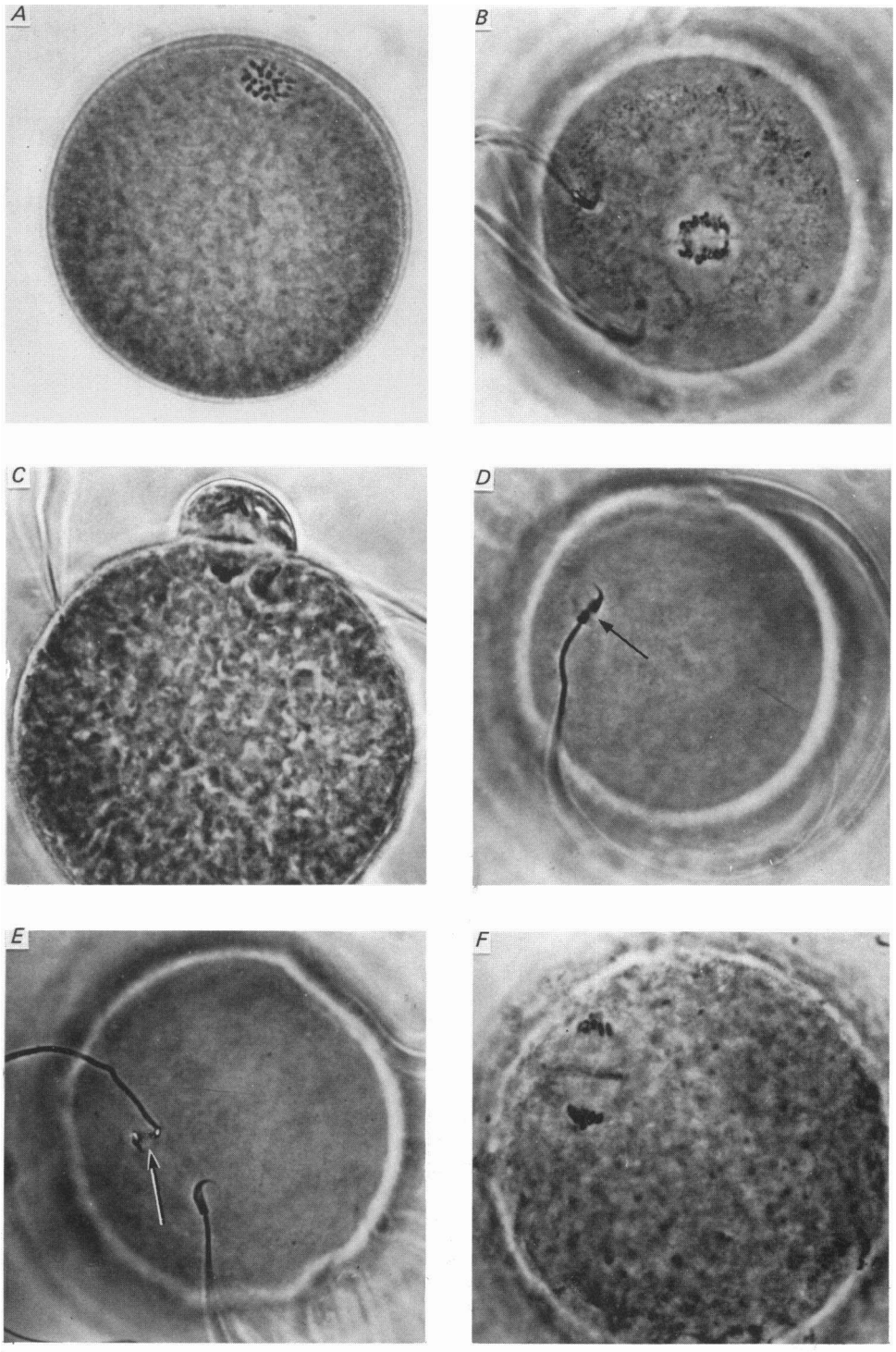

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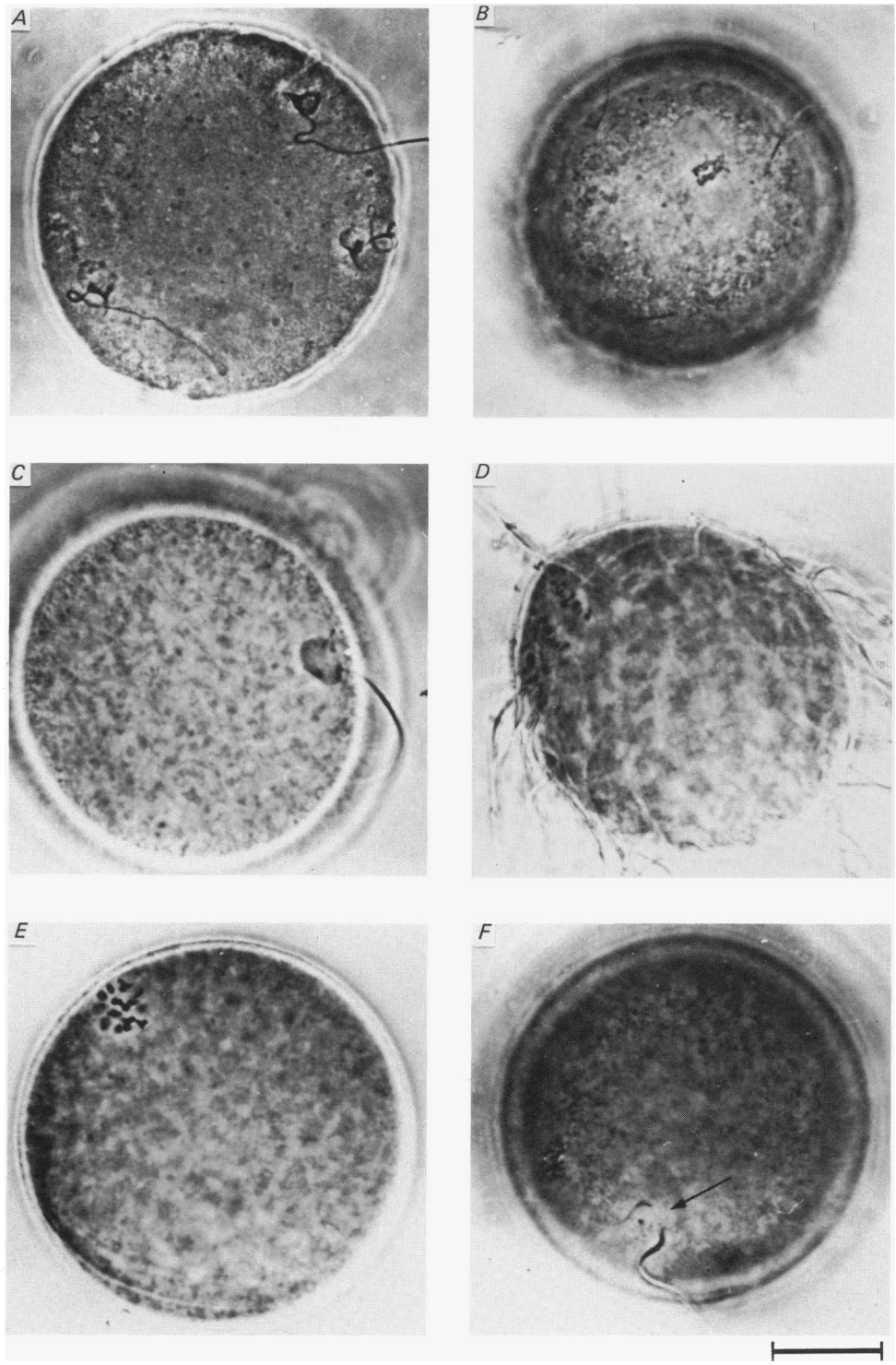
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EXPLANATION OF PLATES

PLATE 1

Phase contrast micrographs of zona-free hamster eggs during the second meiotic division. Prefixed with 5 % glutaraldehyde, fixed with 10 % formaldehyde, stained with 0·25 % lacmoid in 45 % acetic acid. The parenthesized time below indicates the period of fixing after the attachment of the first sperm to the egg surface. Scale, 20 μm. *A*, non-inseminated, unfertilized hamster egg. Chromosomes remain at metaphase (M). Examined 75 min after removing the zona pellucida. *B*, activated H × H egg (25 min). Chromosomes are separating (anaphase, *A*). *C*, activated H × H egg at telophase (T) (45 min). Chromosomes have been completely separated and assembled at the poles, forming the second polar body. The mid-body is clearly visible. *D*, *E*, enlarged heads of hamster sperm (arrows) (40 min). Note that the change of the sperm head begins at the middle portion. *F*, spontaneously activated hamster egg. Examined 60 min after removing the zona pellucida. Chromosomes are at telophase.

PLATE 2

Mouse and hamster eggs during the second meiotic division. Scale, 20 μm. *A*, activated M × M egg (trispermic egg) (40 min). The sperm heads are enlarged. *B*, early activated M × M egg (30 min). Chromosomes are just separated (early anaphase, EA). *C*, activated M × H egg (60 min). One enlarged sperm head is visible. The other sperm and the second polar body are out of focus. *D*, activated M × H egg at anaphase (40 min), the egg from which the record of Fig. 1*C* was obtained. *D*, *F*, unactivated M × H egg (60 min). Chromosomes remain at metaphase, while the sperm head is enlarged in the same egg (arrow).