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Stationary Outward and Transient Ca^{2+}-Dependent Currents in Hamster Oocytes

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The outward currents elicited in hamster eggs by depolarizing pulses were studied. The currents appeared to comprise at least two components, a transient outward component (I_{to}) and a steady-state outward component (I_{∞}). I_{to} was transiently followed by the cessation of inward Ca^{2+} current (I_{Ca}), and its current-voltage (I-V) relation was a mirror image of that of I_{Ca}. Either blockade of I_{Ca} by Co^{2+} or replacement of Ca^{2+} with Sr^{2+} abolished I_{to} without change in I_{∞}. Intracellular EGTA (10 mM) inhibited I_{to} but not I_{∞}. suggesting strongly that generation of I_{to} requires intracellular Ca^{2+}. Apamin (1 nM) abolished selectively I_{to}, indicating that I_{to} is Ca^{2+}-dependent K^+ current. On the other hand, I_{∞} was Ca^{2+}-independent. Both I_{to} and I_{∞} were completely inhibited by internal Cs^+ and external TEA. The estimated reversal potential of I_{to} was close to the theoretical E_K. Taken together, both outward currents were carried by K^+ channels. From these results, I_{to} is likely to be a current responsible for the hyperpolarizing responses seen in hamster eggs at fertilization.

Key Words: K^+ currents, Ca^{2+}-dependent K^+ current, Whole-cell current, Hamster oocytes

INTRODUCTION

Membrane hyperpolarizing responses (HR) occur at fertilization in hamster and mouse eggs due to the increase of the Ca^{2+}-activation K^+ conductance induced by a rise in intracellular free Ca^{2+} concentration (Miyazaki & Igusa, 1981, 1982; Georgiou et al, 1983; Igusa et al, 1983; Igusa & Miyazaki, 1983, 1986). The Ca^{2+}-activated K^+ channel has been found in excised membrane patches of unfertilized hamster eggs (Yosida et al, 1990), Several routes for Ca^{2+} elevation at fertilization have been suggested in hamster eggs (Miyazaki, 1988; Swann et al, 1989; Miyazaki, 1991; Miyazaki et al, 1990, 1992a, 1992b). Ca^{2+} might be released from inositol 1,4,5-triphosphate ($InsP_3$)-sensitive Ca^{2+} stores by $InsP_3$ stimulation ($InsP_3$-induced Ca^{2+} release, IICR) (Miyazaki, 1988, 1991; Berridge & Irvine, 1989; Miyazaki et al, 1990, 1992, 1992) or from Ca^{2+}-sensitive Ca^{2+} stores (Ca^{2+}-induced Ca^{2+} release, CICR) (Miyazaki & Igusa, 1982; Georgiou et al, 1983; Igusa & Miyazaki, 1983; Miyazaki et al, 1986, Press, 1990; Miyazaki, 1988, 1991; Galione et al, 1991). Otherwise, $[Ca^{2+}]_i$ might be raised by Ca^{2+} influx via voltage-dependent membrane channels (Igusa & Miyazaki, 1983; Miyazaki, 1988, 1991; Berridge & Irvine, 1989; Luckhoff & Clapham, 1992).

Recently Miyazaki et al (1992) asserted that at fertilization $[Ca^{2+}]_i$ increased only by IICR. Although IICR is an established Ca^{2+} source in oocytes, external Ca^{2+} is critical to the survival of embryo in the early developmental stage. Furthermore the presence of voltage-operated Ca^{2+} channels in hamster eggs is well documented (Georgiou et al, 1983; Igusa & Miyazaki, 1983). The membrane potential changes occurring at fertilization (hyperpolarizing responses, HRs) may serve to establish a feedback loop important for the long-term maintenance of the Ca^{2+} oscil-

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lations by cyclically reactivating the voltage-dependent Ca^{2+} channels. Therefore, an outward current activated by the Ca^{2+} influx should be required to control the membrane potential and to avoid the excessive Ca^{2+} influx via voltage-activated channels. In this light it is of interest to investigate whether Ca^{2+}-activated K^+ channels can be opened by the Ca^{2+} rise produced only by the Ca^{2+} influx through the voltage-dependent membrane channels.

METHODS

Preparation of eggs

Sexually mature female hamsters, older than 8 weeks, were used as donor of eggs. The animals were induced to superovulate with intraperitoneal injection of 20 units of pregnant mare serum gonadotropin (PMSG) and 20 units of human chorionic gonadotropin (hCG). The superovulated females were sacrificed by cervical dislocation. Cumulus cells surrounding the eggs were removed by treatment with 100 units hyaluronidase (Sigma Type I-S) for 2~3 min at room temperature and zona pellucida were subsequently freed with 1 unit protease (Sigma type VII) for 10~15 s at room temperature. After digestion of zona pellucida, eggs were washed several times with a medium containing (in mM): NaCl 140, Na-Pyruvate 0.1, Lactate 10, HEPES 20, $MgCl_2$ 1.2, $CaCl_2$ 2, KCl 6, and 2 mg/ml polyvinylpyrrolidone. Zona-free eggs were transferred to a 35 mm plastic Petri dish. All eggs were used within 8 hours after collection from the oviduct. They were kept in refrigerator before use ($<4^{\circ}C$).

Solutions

The control external solution contained (in mM): NaCl 140, Na-Pyruvate 0.1, Lactate 10, $MgCl_2$ 1.2, $CaCl_2$ 10, KCl 6, HEPES 20. In some experiments $CaCl_2$ was substituted with equimolar amounts of $SrCl_2$ or $BaCl_2$. Apamin was added to the external solution without changing composition. The pipette solution contained (in mM): KCl 140, $MgCl_2$ 2, ATP-Mg 1.0, phosphochreatine-ditris 5, HEPES 10. EGTA (10 mM) was dissolved and diluted with the normal pipette solution. In some experiment, KCl in the pipette solution was replaced by CsCl (120 mM) and TEA-Cl (20 mM). When K^+ or Ca^{2+} concentration was changed, the tonicity of the solution was adjusted by altering the extracellular Na^+ concentration. All solutions were buffered at pH 7.4.

Electrophysiology

Current traces were recorded by using the whole-cell voltage clamp technique (Hamill et al, 1981). The bathing solution was perfused at a rate of 1 ml/min. Eggs were left at least 5 min to equilibrate in bathing solution before beginning the experiment. The patch pipette resistance was 2~3 MΩ. After establishment of the giga-seal, the patch membrane was usually disrupted by suction. At this point the condition of eggs was examined visually. Eggs in which the cytoplasm was squeezed into the pipette were discarded.

Signals were digitized by an analog-to-digital converter (TL-1, Axon, USA) and stored in a personal computer. Stimulation, data acquisition and analysis were performed with pClamp software (V6.02, Axon, USA). The currents were amplified by patch clamp amplifier (EPC-7, List, Germany).

Two main voltage-clamp protocols were used; First, a series of 500 ms-long depolarization from a holding potential (V_h) of -80 mV and going from -50 mV to $+50$ mV (in 10 mV steps) was given. Second, a series of voltage pulses between -50 and $+50$ mV (500 ms, 10 mV steps) was preceded by a prepulse lasting 40 ms from $V_h=-80$ mV to -20 mV. All experiments were performed at room temperature. Data represent mean$\pm$S.D. with number of observations. Current traces are leak subtracted.

RESULTS

Outward currents of the hamster egg

It has been well known that depolarizations from -80 mV to potentials more positive than -50 mV elicit large and transient Ca^{2+} currents (I_{Ca}) in hamster oocytes (Haan et al, 1994). In our experiments, in addition to this kind of depolarization and following I_{Ca}, a distinctive outward phase of the whole cell current could be observed (Fig. 1A). Typically this outward current increased transiently and then decreased to reach a steady state level by the end of the 500 ms-long depolarization. It is conceivable that current could be carried by K^+ through the Ca^{2+}-

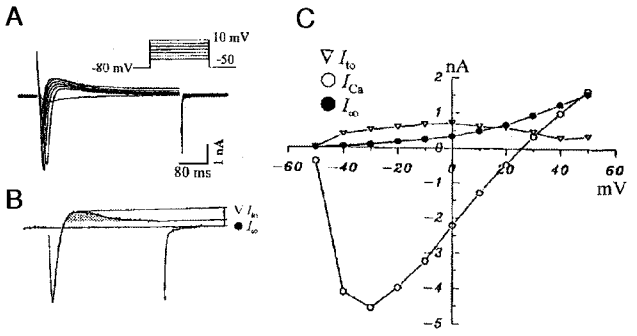

Fig. 1. Currents elicited by depolarization in the unfertilized egg of the hamster. A: transient outward currents following the inward transient Ca^{2+} current. B: definition of the two components of the outward current. Transient outward current (I_{to}) is distinguished from steady-state outward current (I_∞) by its time course and peak amplitude. C: The I-V relationships of the peak Ca^{2+} current (I_{Ca}, ○), the peak transient outward current (▽) and the steady-state outward current (●).

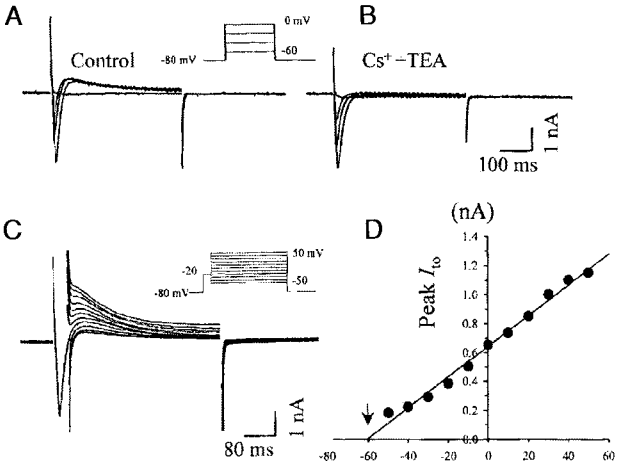

Fig. 2. Blockade of the outward current and I-V relation of the transient outward component. A: Effect of the replacement of internal K^+ (140 mM) with Cs^+ (120 mM) and TEA (20 mM) by internal perfusion. 120 mM Cs^+ and 20 mM TEA was perfused through the pipette using internal perfusion technique. Note that the outward current was completely blocked, while no change was seen in the inward Ca^{2+} current. C: Voltage-dependence of I_{to}. Outward currents in response to depolarizing test pulses from −50 to +50 mV following a prepulse 40 ms-long to −20 mV. D: I-V relationship of the peak I_{to}. The peak amplitude was measured as the difference of the peak outward current and steady-state outward current at each potential. The arrow indicates the estimated reversal potential by the extrapolated regression line.

activated channels responsible of HRs and, therefore, we concentrated our attention on it. We decided to arbitrarily divide the outward current into two components: a transient outward component and a steady-state outward component. The transient outward current (I_{to}) appeared earlier following the inward Ca^{2+} current (shaded area in Fig. 1B). While the steady-state outward current (I_∞) was defined as the current remaining after the decay of I_{to}.

Fig. 1C shows the voltage-dependency of these three currents. First component, I_{Ca}, reached its maximum at −30 or −20 mV (4.22±0.63 nA, n=6) and showed the reversal potential (E_r) around +30 mV; the transient outward current (I_{to}) began to be activated at −40 mV and reached the peak at 0 mV (345.4±82.7 pA, n=6), and I_∞ increased with depolarization and showed a slight outward rectification. To find the ionic nature of these two outward components, intracellular 140 mM K^+ was replaced with 120 mM Cs^+ and 20 mM tetraethylammonium (TEA) to block K^+ current. In these conditions both outward I_{to} and I_∞ entirely disappeared (Fig. 2A & 2B), suggesting that outward currents be carried by K^+.

The transient component is a K^+ current

To investigate the voltage-dependency of I_{to}, a two-step pulse protocol was used (Fig. 2C). With this protocol I_{to} can be recorded at the various test pulse voltages without differences in peak of the inward I_{Ca} elicited by a fixed prepulse. Also, at the end of the 40 ms-long prepulse, I_{Ca} was almost completely inactivated, minimizing its contribution to the currents recorded during test pulses. The values of peak I_{to} during test pulses were plotted as shown in Fig. 2D. The current-voltage (I-V) relation of I_{to} appeared essentially linear and its extrapolation indicated that its E_r is around −60.5 mV indicated by arrow. This value agreed reasonably with the theoretical K^+ equilibrium potential (E_K) of −66.5 mV.

This last observation indicates therefore that K^+ carried the most of transient outward current. To further investigate the ionic nature of I_{to}, currents were recorded in the extracellular solution containing 20 mM TEA. In this condition, I_{to} completely disappeared below 0 mV and decreased more than 90% above 0 mV, and then reappeared upon reperfusion with a TEA-free solution.

Ca^{2+}-dependency of the outward transient current

In order to examine the relationship between the inward Ca^{2+} current and the outward K^+ currents described above, various extracellular solutions affecting I_{Ca} were tested. First, extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was reduced from 10 mM to 2.5 mM (Fig. 3). For the lower Ca^{2+}, both I_{Ca} and I_{to}, were significantly decreased. These reduction of both currents were repeated in three oocytes, although amounts of the decrement in amplitude were different at each trial. These reductions of I_{to} by lowering $[Ca^{2+}]_o$ were remarkable over the entire voltage range tested (Fig. 3B). This finding indicates that I_{to} was dependent on I_{Ca} and could be activated by the preceding Ca^{2+} influx. On the contrary, I_∞ did not seem to change in $[Ca^{2+}]_o$ as shown in Fig. 3C.

This requirement of external Ca^{2+} for the generation of I_{to} was confirmed by substituting Ca^{2+} with other divalent cations. Substitution of Ca^{2+} with Co^{2+}, divalent Ca^{2+} channel blocker, led to the complete disappearance of both I_{Ca} and I_{to} (Fig. 4A). On the contrary to the effect of Co^{2+} on both current components, the replaced Sr^{2+} blocked only the component of I_{to} (Fig. 4B). In the presence of Sr^{2+}, the amplitude of inward I_{Ca} was not changed, but interestingly I_{to} was almost completely blocked over the entire voltage ranges (refer to Fig. 4C). A similar result was obtained when Ca^{2+} was substituted with Ba^{2+}.

Throughout experiments shown in Fig. 3 & Fig. 4, the I_{to} component was likely to be Ca^{2+}-dependent. To further investigate the Ca^{2+}-dependency of I_{to}, we observed whether I_{to} might be changed under the condition of the intracellular Ca^{2+} deprivation. In the presence of high amount of EGTA (10 mM) which chelates Ca^{2+} in the cytoplasm, I_{to} was abolished in spite of the increases in I_{Ca}, indicating that internal Ca^{2+} is essential to generate I_{to}. Since the amplitude of I_{to} did not exceed 1 nA (refer to Fig. 1C and Fig. 4C), this I_{to} might be carried via a small conductance Ca^{2+}-activated K^+ (SK) channel. As shown in Fig. 5, addition of 1 nM apamin, a SK channel blocker, to the extracellular medium resulted in suppression of I_{to} without significantly affecting the inward Ca^{2+} current. All the preceding results indicate that I_{to} is a Ca^{2+} activated K^+ current ($I_{K,Ca}$) activated by the increased $[Ca^{2+}]_i$ resulting from the Ca^{2+} influx via voltage-activated Ca^{2+} channel.

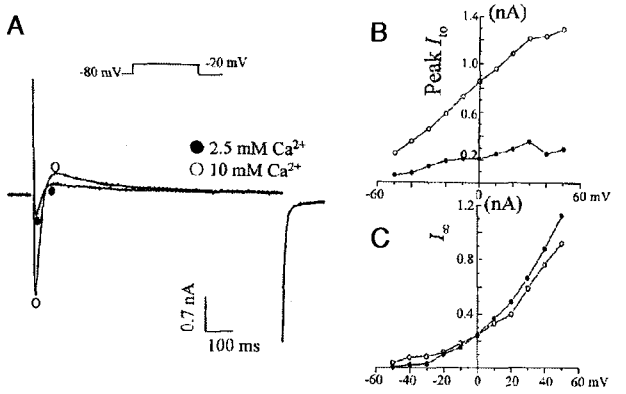

Fig. 3. Effects of extracellular Ca^{2+} concentration changes on I_{to} and I_∞. A: Representative traces of the inward current recorded under the condition of 10 mM $[Ca^{2+}]_o$ (○), and 2.5 mM $[Ca^{2+}]_o$ (●). These were elicited by −20 mV depolarization from holding potential (V_h) of −80 mV shown as inset. B and C: I-V relationship for I_{to} (B) and I_∞ (C) recorded by using the two-step protocol shown in Fig. 2. Open and closed circle stands for currents measured at 10 mM and 2.5 mM $[Ca^{2+}]_o$, respectively.

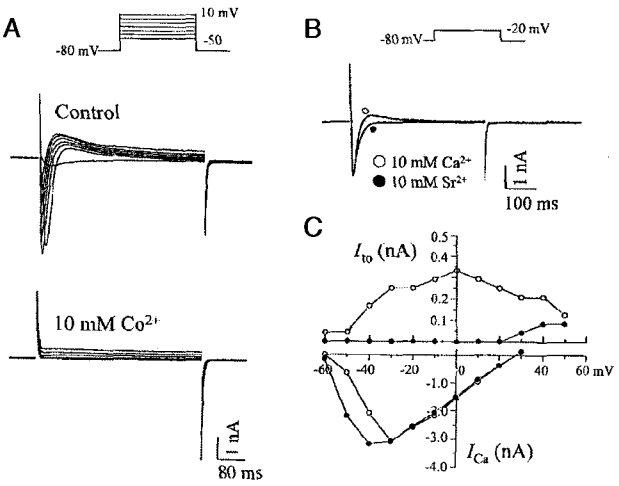

Fig. 4. Effect of divalent cations on I_{to}. A: Effect of the Ca^{2+} channel blocker, Co^{2+} on whole cell currents. Current traces before and after adding 10 mM Co^{2+} to the bathing solution were shown in upper and lower panel, respectively in the same egg. B: Disappearance of I_{to} by substitution of Sr^{2+}. Current traces elicited by step depolarization of −20 mV (inset) recorded in the presence of 10 mM Ca^{2+} and 10 mM Sr^{2+} were overlapped as indicated by open circle and close circle, respectively. C: I-V relations of both currents after switching to the equimolar Sr^{2+} from 10 mM Ca^{2+}. Both currents with a same voltage range were displayed at different current scale. Circles are same as in B.

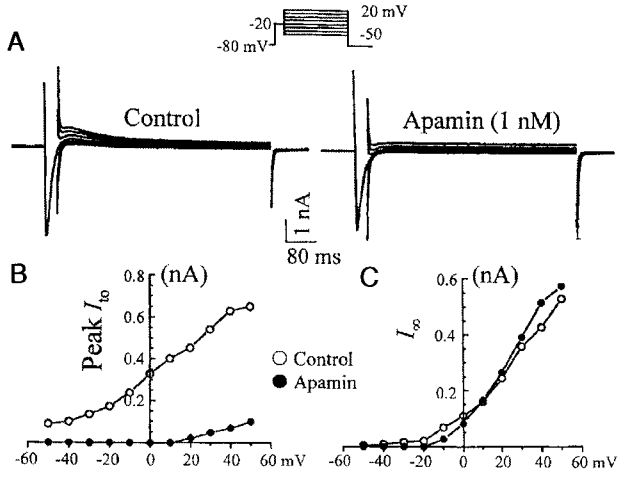

Fig. 5. Effect of apamin on I_{to} and I_∞. A: Reduction in I_{to} by application of 1 nM apamin to the bathing solution. B: I-V plots of the peak I_{to} and I_∞ elicited by two-step voltage pulses before (○) and after adding apamin (●).

Steady-state outward current

From the observations in Fig. 3C, the steady-state outward component, I_∞ was not sensitive to extracellular Ca^{2+} but disappeared when internal K^+ was replaced by Cs^+ and TEA (Fig. 2). In addition, I_∞ was not affected by 1 nM apamin as shown in Fig. 5C. Indeed I_∞ was not even affected by the presence of 100 nM apamin (not shown). Therefore, I_∞ appears to be a K^+ current carried through channels different from the Ca^{2+}-activated K^+ channels.

DISCUSSION

In the present study, three kinds of ionic currents were recorded in hamster eggs. One inward current carried respectively by Ca^{2+} (for the preceded one) and by K^+ (for the last two). Several evidences in this study indicate that the outward current was composed of at least two types of K^+ currents, which we named transient outward current (I_{to}) and steady- state outward current (I_∞). I_{to}, appears to be Ca^{2+}-dependent K^+ currents ($I_{K,Ca}$), which is very likely to be the current responsible for the hyperpolarization response (HR) observed at fertilization in these cells (Igusa & Miyazaki, 1983; Igusa et al, 1983). On the contrary, I_∞ does not seem to be dependent on Ca^{2+}. These characteristics of the two currents were assessed by various experiments affecting either the Ca^{2+} currents (or the intracellular Ca^{2+} concentration) or the K^+ currents.

All treatments led to the reduction or block of the Ca^{2+} current, such as the extracellular Ca^{2+} reduction. The block by Co^{2+} also affected the transient outward current, I_{to}, in the same way. This effect was also obtained when Ca^{2+} was replaced by Sr^{2+} or Ba^{2+}, indicating interestingly that they cannot substitute Ca^{2+} in activating K^+ channels, although these ions are able to pass through Ca^{2+} channels. Conversely, these treatments did not influence the steady-state I_∞, indicating that it is Ca^{2+}-insensitive.

Both time course and voltage dependency of I_{to} are in agreement with the interpretation that it is a current activated by preceding Ca^{2+} influx. Hence, I_{to} could be visible following the rapid decay of I_{Ca} and also transient unless intracellular Ca^{2+} lasts at a high level. Although the voltage-dependent inactivation mechanism cannot be excluded, the decline phase is consistent with the expected $[Ca^{2+}]_i$ decrease due to sequestration of Ca^{2+} into intracellular stores and/or by binding to Ca^{2+} buffers. The I-V relationship of I_{to} has the maximum at about 0 mV (see Figs. 1 & 4) and shows a decline at more positive potentials. One could simply expect I_{to} to be zero at a potential corresponding to E_r (about 30 mV) of the inward current. This is not the case, however, in our experiments. This apparent contradiction is easily explained by remembering that channels are permeable to monovalent cations and carry mixed currents on entering Ca^{2+} ions and exiting K^+ ions at positive potential (Tsien et al, 1987; Press et al, 1988). Indeed the theoretical Ca^{2+} equilibrium potential in the present conditions exceeds +100 mV and, therefore, at E_r we may still expect a substantial Ca^{2+} influx. On the other hand, under conditions enough to influence K^+ channels in general, such as the replacement of internal K^+ with Cs^+ and TEA, both I_{to} and I_∞ were strongly blocked, indicating that K^+ is the main charge carrier in these currents. This is also supported by the close E_r of I_{to} (*ca.* −61 mV) to the estimated E_K of −67 mV (Fig. 2).

The experiments shown in Fig. 2 give a linear I-V relation. This suggests that I_{to} belongs to the SK class of Ca^{2+}-activated K^+ channels (Rudy, 1988). The pharmacological evidence that 1 nM apamin blocked it completely reinforces this suggestion. However, Yoshida et al (1990) working on the same preparation obtained outwardly rectifying I-V relation based on single channel current measurements. The disparity between these two sets of results may indicate that conditions of

the channel may be modified in the excised patch.

If, as we propose, I_{to} plays a role in producing repetitive HRs at fertilization, the main source of Ca^{2+} for the activation of HRs would be supplied by an internal store rather than by influx of the extracellular environment because channels responsible for the inward Ca^{2+} current were soon inactivated. It is more probable that I_{to} channels constitute a part of the complex signalling network linked to the fertilizing process. The outward currents including I_{∞} presented in this study may be an useful tool estimating for the function of ionic channels involved in the fertilization and the early stage of development, especially in relation to regulation of other components of these complex process.

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