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Expression of sperm Ca^{2+}-activated K^+ channels in *Xenopus* oocytes and their modulation by extracellular ATP

H.C. Chan[a,*], W.L. Wu[b], Y.P. Sun[b], P.S. Leung[a], T.P. Wong[a], Y.W. Chung[a], S.C. So[a], T.S. Zhou[a], Y.C. Yan[b]

[a] *Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China*
[b] *Shanghai Institute of Cell Biology, Academia Sinica, Shanghai 200031, China*

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Abstract **Ionic fluxes across the sperm membrane have been shown to be important in the initiating process of sperm activation and gamete interaction; however, electrophysiological investigation of the ion channels involved has been precluded by the small size of the sperm, especially in mammalian species. In the present study sperm ion channels were expressed in *Xenopus* oocytes by injection of RNAs of spermatogenic cells isolated from the rat testes. The RNA-injected oocytes responded to ATP, a factor known to regulate sperm activation, with the activation of an outwardly rectifying whole-cell current which was dependent on K^+ concentrations and inhibitable by K^+ channel blockers, charybdotoxin (CTX) and tetraethylammonium (TEA). The ATP-induced current could be mimicked by a Ca^{2+} ionophore but suppressed by a Ca^{2+} chelator applied intracellularly, indicating a Ca^{2+} dependence of the current. Single-channel measurements on RNA-injected oocytes revealed channels of large conductance which could be blocked by CTX and TEA. Co-injection of germ cell RNAs with the antisense RNA for a mouse gene encoding *slowpoke* 'Maxi' Ca^{2+}-activated K^+ channels resulted in significant reduction of the ATP- and ionomycin-induced current. The expression of the 'Maxi' Ca^{2+}-activated K^+ channels in sperm collected from the rat epididymis was also confirmed by Western blot analysis. These results suggest that sperm possess Ca^{2+}-activated K^+ channels which may be involved in the process of sperm activation.**

Key words: Sperm; K^+ channel; Ca^{2+}; Rat; *Xenopus* oocyte

1. Introduction

Ion channels have been implicated in various sperm functions including sperm activation and sperm-egg interaction. Elevation of intracellular Ca^{2+} has been observed in various reproductive events including acrosome reaction and gamete interaction [1]. K^+ efflux, measured by $^{86}Rb^+$, was found to be associated with sperm motility initiation while the K^+ channel blocker, tetraethylammonium (TEA), inhibited motility initiation [2]. Recent evidence has indicated a role of Ca^{2+}-activated K^+ channels in hamster sperm acrosome reaction [3] and the initiation of sperm motility in salmonid fishes [4]. However, direct characterisation of sperm ion channels has not been carried out to any significant extent, especially in mammalian species. This is largely due to the small size of the sperm, which renders electrophysiological approaches infeasible. Attempts have been made to investigate sperm ion channels in a reconstituted system using the planar lipid bilayer technique. Non-selective cation channel, Ca^{2+} channel as well as K^+ and Na^+ channels have been characterised [5–10]. However, the artificial membrane system limits further studies on channel regulation.

In the present study, we expressed sperm ion channels in *Xenopus* oocytes by injection of total RNAs from spermatogenic cells, the spermatogonia, spermatocytes and spermatids, which are the precursors of the spermatozoa. The spermatogenic cells, instead of mature spermatozoa, were used as the source of RNAs because spermatozoa are highly differentiated cells lacking the machinery for protein synthesis and thus, many long-lived mRNAs that encode sperm proteins are made during spermatogenesis.

In the present study, we have identified for the first time the Ca^{2+}-activated K^+ channel expressed in *Xenopus* oocytes injected with RNAs from spermatogenic cells. Modulation of this channel by extracellular ATP, a factor known to regulate sperm activation [11,12], has been demonstrated and the expression of the channels in epididymal sperm is also confirmed, indicating a potential role of the Ca^{2+}-activated K^+ channel in sperm activation.

2. Materials and methods

2.1. RNA extraction

Sexually mature Sprague-Dawley rats (8–10 weeks old, 320–350 g) were used. The rats were killed by cervical dislocation. Total germ cells were isolated from the testes using the method described by Meistrich et al. [13]. RNA extraction, using the method described by Monstein et al. [14], was performed on germ cell preparations which had been determined to be free of Leydig, peritubular and Sertoli cells by histochemical staining of 3β-hydroxysteroid-dehydrogenase, alkaline phosphatase and oil Red O [15,16]. Previous studies using flow cytometry have also shown predominant haploid nuclei of the isolated cells [17].

2.2. Antisense and antibody production

A full-length cDNA for the mouse *slowpoke* gene subscribed into a pBluescript-based plasmid vector [18] was kindly provided by Dr. Aguan Wei. Plasmid DNA was linearised with *Eco*RI and *Cla*I, and capped transcripts (mRNA and antisense RNA for the mSlo gene) were synthesised in vitro with T3 and T7 RNA polymerase as previously described [19].

A polyclonal antiserum was raised against residues 913–926 of *slowpoke* [18] using the sequence VNDTNVQFLDQDDD. The immunogenic peptide was synthesised on a lysine core linked to a solid-phase peptide synthesis support. After cleavage, 2 μM of peptide emulsified in complete Freund's adjuvant was injected into two rabbits. The procedure was repeated 2 weeks later, and serum was collected 2 weeks later. Production of antibodies was monitored by ELISA.

2.3. Protein extraction and Western blotting

Rat epididymal sperm were extracted at 4°C in PBS, 1:9 (w/v) containing 10 mM EDTA and 1 mM PMSF. The homogenate was

*Corresponding author. Fax: (852) 26035022.
E-mail: hsiaocchan@cuhk.edu.hk

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centrifuged at 1000×*g* for 15 min at 4°C and the pellet was discarded. The supernatant was re-centrifuged at 30 000×*g* for 30 min at 4°C. Proteins from the resultant pellet were determined for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins (5–10 μg/lane) were subjected to electrophoresis on a 12% polyacrylamide gel in SDS. Proteins were then subject to electroblotting to polyvinylidene difluoride (PVDF) membrane. The blotted membrane was saturated with 5% (w/v) of skimmed milk in PBS, pH 7.4 and 0.1% (v/v) of Tween 20 for 1 h at room temperature. The PVDF membrane was incubated with rabbit anti-Ca^{2+}-activated K^+ channel serum (1:3000) overnight at 4°C, followed with a peroxidase-labelled anti-rabbit IgG antibody (1:500) for 1 h at room temperature. After washing, the positive band from the membrane was detected using ECL Western blotting reagents and exposed to autoradiography film (Amersham, UK).

2.4. Electrophysiological studies

Xenopus oocytes, treated with collagenase (2 mg/ml) to remove the follicle cell layer, were injected with 50 nl total RNA (1 μg/μl) extracted from germ cells of the rat testes. Currents were recorded 2–5 days after injection under two-electrode voltage-clamped configuration. The pipette solution contained 3 M KCl. The bath solution contained (mM): NaCl, 95; KCl, 1; $MgCl_2$, 1; $CaCl_2$, 1; HEPES, 5. In experiments testing the dependence of reversal potential on K^+ concentrations, NaCl in the bath was fixed at 35 mM and KCl was varied from 1 to 60 mM with corresponding NMDG-Cl of 59 to 0 mM.

For single-channel recording, the vitelline envelope of the oocytes was removed after hypertonic treatment [20]. Patching experiments using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA) were performed 30 min after the removal of the vitelline

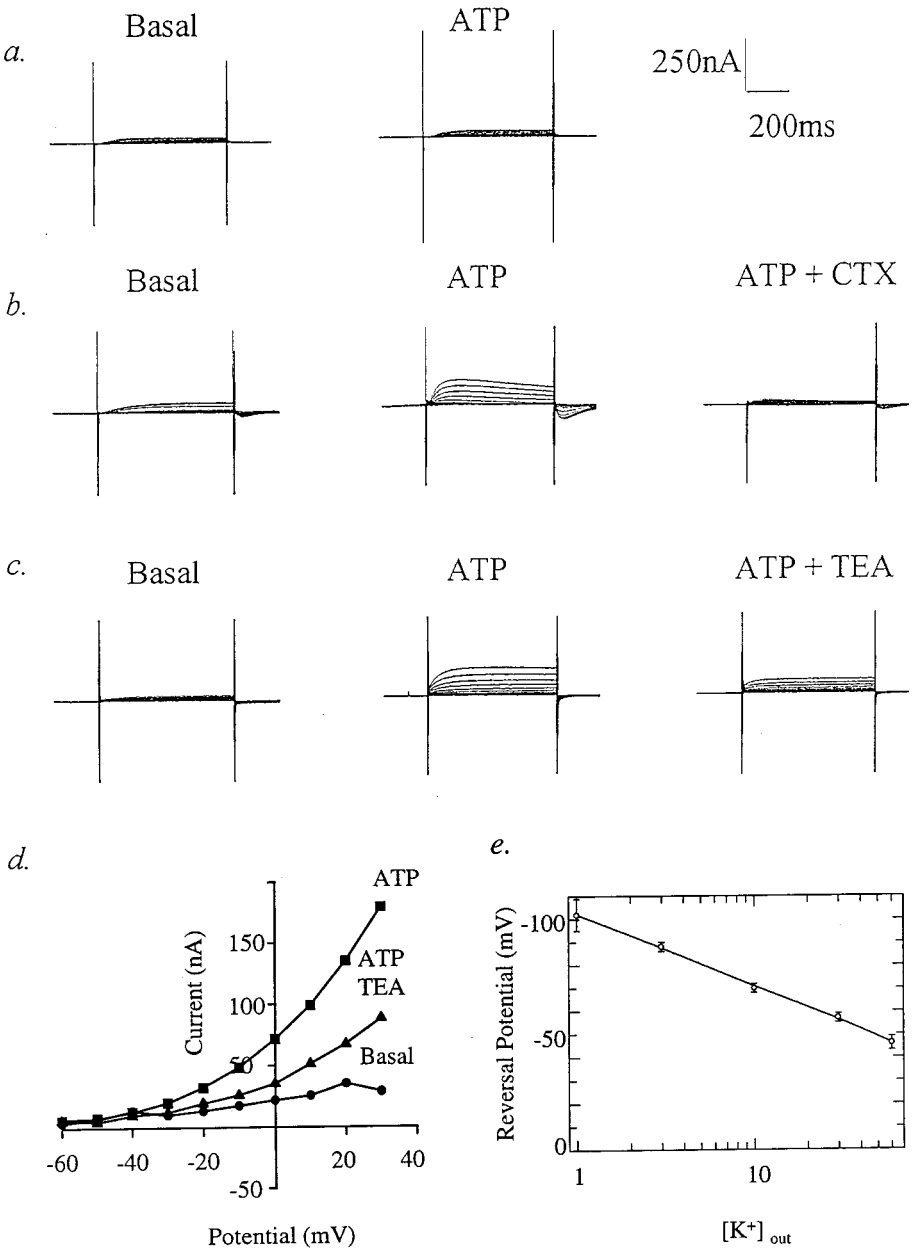

Fig. 1. Current activation in germ cell RNA-injected *Xenopus* oocytes. a: Current recording from a mock-injected and ATP (1 mM)-stimulated oocyte. b: ATP-activated current recordings with subsequent inhibition by CTX (300 nM) obtained from germ cell RNA-injected oocyte. c: Inhibition of ATP-activated current by TEA. Currents were elicited by voltage steps from −60 mV to +30 mV with 10 mV increment. d: I-V curves corresponding to experiments depicted in c. e: Dependence of the reversal potential of the ATP-activated current on external $[K^+]$. Reversal potentials, measured by the tail currents, are plotted against K^+ concentrations in the bath ($[K^+]_{out}$).

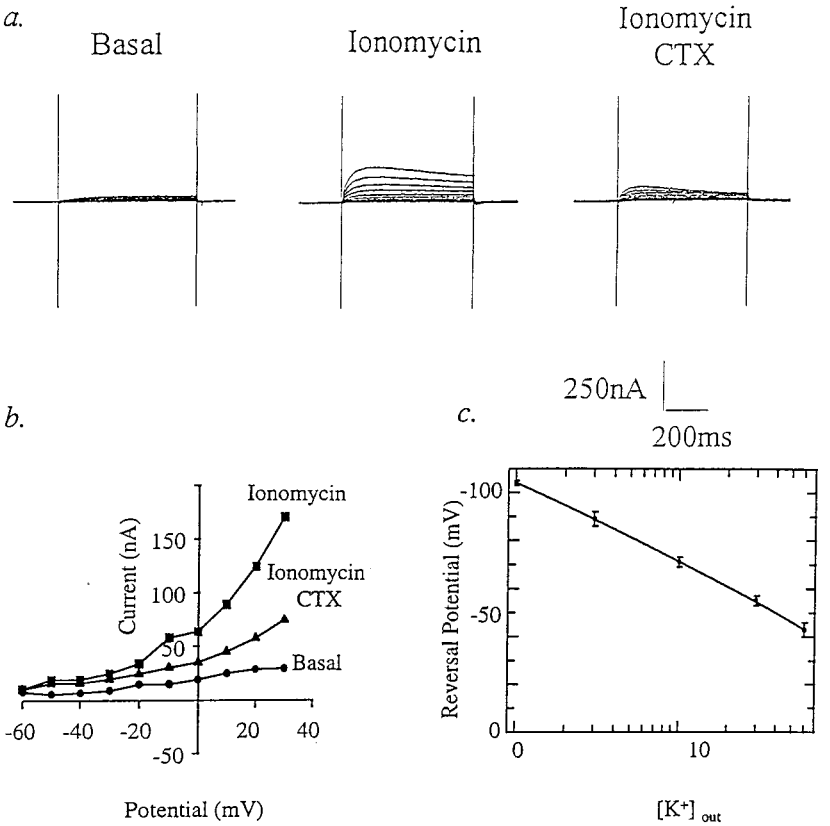

Fig. 2. Ca^{2+} ionophore-induced current in RNA-injected oocytes. a: Ionomycin (0.5 μM)-activated current recordings with subsequent inhibition by CTX (300 nM) obtained from germ cell RNA-injected oocyte. b: Corresponding I-V curves. c: K^+ dependence of the reversal potentials.

envelope. Pipette solution contained (mM): KCl, 140; EGTA, 2; $CaCl_2$, 1.3 (free Ca^{2+}, 100 nM); $MgCl_2$, 1 and HEPES, 10. Bath solution was the same as described for two-electrode voltage-clamped experiment (see above).

3. Results

3.1. Expression of Ca^{2+}-activated K^+ channel in *Xenopus* oocytes

In 85% of oocytes ($n = 40/47$) 2–5 days after injection of germ cell RNAs, outwardly rectifying whole-cell currents were elicited, under two-electrode voltage-clamped configuration, by extracellular ATP (1 mM), a known stimulus of sperm activation, with an average increase of 73 ± 10 nA at +30 mV (Fig. 1b). No significant current increase was observed in response to the same concentration of ATP in mock-injected oocytes ($n = 9$, Fig. 1a), excluding the possibility that the ATP-induced current observed under the experimental conditions was due to the activation of endogenous channels [21]. The ATP-activated current, which exhibited an outward rectification in the current-voltage (I-V) relationship (Fig. 1d), could be substantially inhibited by Ca^{2+}-dependent K^+ channel blockers, CTX [22] (300 nM, $n = 5$, Fig. 1b), and TEA [23] (1 mM, $n = 6$, Fig. 1c), with 70% and 55% current inhibition, respectively. The reversal potential the ATP-activated current, measured by the tail currents, shifted to the new K^+ equilibrium upon changes in external K^+ concentration, as indicated by a linear dependence on the $[K]_o$ (Fig. 1e), further indicating possible activation of K^+ channels upon ATP stimulation.

The ATP-activated current was mimicked by the Ca^{2+} ionophore, ionomycin (0.5 μM) in RNA-injected oocytes (Fig. 2), but not in mock-injected oocytes (not shown). A current increase of 145 ± 15 nA ($n = 64$, Fig. 2a) was induced by ionomycin, which exhibited a sensitivity to CTX (61%, $n = 9$) and TEA (45%, $n = 13$), an outwardly rectifying I-V relationship (Fig. 2b) and a K^+ dependence (Fig. 2c) similar to that exhibited by the ATP-activated currents. The ATP-activated currents were suppressed when a Ca^{2+} chelator, EGTA (10 mM), was included in the pipette solution ($n = 6$, not shown). These results suggested the involvement of intracellular Ca^{2+} which may in turn activate Ca^{2+}-dependent K^+ channels upon stimulation by ATP.

3.2. Channel identification by single-channel recordings

To further identify the type of channels expressed in *Xenopus* oocytes injected with germ cell RNAs, patch-clamp experiments were also conducted. Under cell-attached configuration, very little channel activity which could be attributed to endogenous stretch-activated channels [20] was observed in mock-injected oocytes ($n = 5$) in the presence of ATP (1 mM, Fig. 3a). However, much increased channel activity with maximum single-channel conductance of 150 pS was observed in RNA-injected oocytes ($n = 5$) in the presence of ATP (1 mM, Fig. 3b). This channel activity was suppressed in the presence of CTX (300 nM) as evidenced by long quiescent intervals (Fig. 3c), a characteristic of CTX blockade of 'Maxi' K^+ channels [24]. Similar inhibition was also observed with TEA (1 mM, $n = 4$). Amplitude histograms were also obtained to indicate an increase in current magnitude as

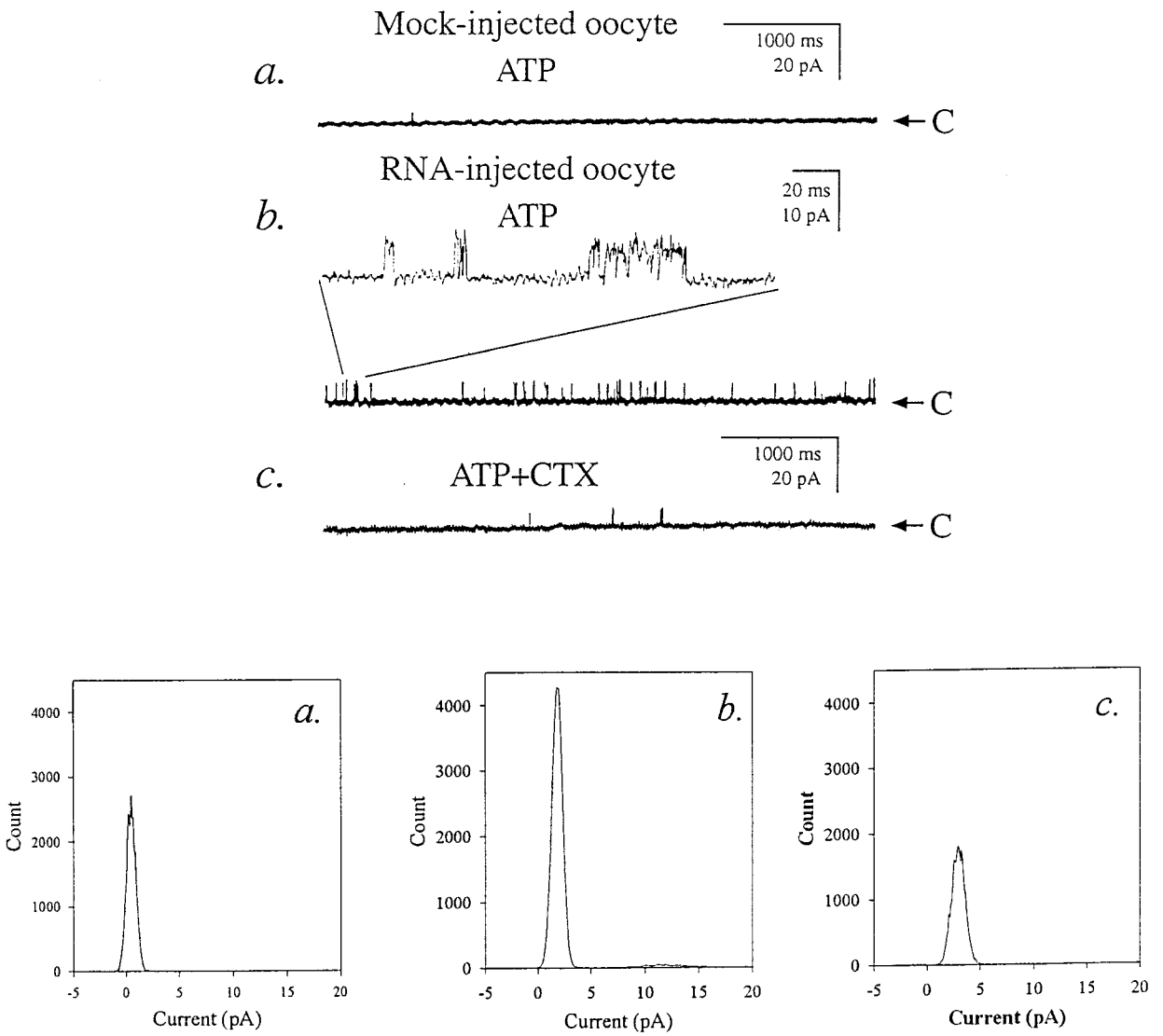

Fig. 3. Identification of Ca^{2+}-activated K^+ channel by patch-clamp technique. Single-channel recordings obtained from cell-attached patches on mock-injected oocyte in the presence of ATP (1 mM, a), germ cell RNA-injected oocyte in the presence of ATP (b, inset displays expanded portion of the recording) and in the presence of both ATP and CTX (300 nM, c). Lower panel: Corresponding amplitude histograms. Note the shift in amplitude and frequency (count). The channels observed in germ cell RNA-injected oocytes resembled other 'Maxi' Ca^{2+}-dependent K^+ channels in their large conductance and sensitivity to CTX.

well as open probability (as indicated by the increased frequency) in the presence of ATP and their suppression by CTX (Fig. 3). The large conductance and the sensitivity of the channel to CTX and TEA suggest that it was likely to be a 'Maxi' Ca^{2+}-activated K^+ channel.

3.3. Expression inhibition by antisense

The above data indicated possible expression of a 'Maxi' Ca^{2+}-activated K^+ channel, which was sensitive to both CTX and TEA, in *Xenopus* oocytes injected with RNAs of rat spermatogenic cells. The sensitivity to TEA, in addition to CTX, suggests the involvement of *slowpoke* 'Maxi' K^+ channels since in contrast to another 'Maxi' K^+ channel family, *Shaker*, *slowpoke* has been shown to have high affinity for TEA [23]. This notion was tested by co-injecting germ cell RNAs with an antisense RNA of a mouse *slowpoke* gene encoding 'Maxi' Ca^{2+}-activated K^+ channels [18] into the oocytes. Currents elicited by ATP in co-injected oocytes were only 26 ± 9 nA ($n = 29$), significantly smaller than that in oocytes injected with germ cell RNAs alone ($P < 0.01$). Much reduced current activation in response to ionomycin (92 ± 13 nA, $n = 35$, $P < 0.01$) was also observed in co-injected oocytes as compared to that observed in RNA-injected oocytes. The reduction in the magnitude of current activation indicated suppressed expression of the Ca^{2+}-activated K^+ channels in the presence of the antisense to the gene encoding *slowpoke* 'Maxi' Ca^{2+}-activated K^+ channels.

3.4. Expression in epididymal sperm

Western blot analysis was performed to demonstrate the presence of the Ca^{2+}-activated K^+ channel in sperm collected from the rat epididymis. The antibody raised against the α subunit of the Ca^{2+}-activated K^+ channel detected a protein band about 70 kDa (Fig. 4) which was consistent with the predicted value for the α subunit of the Ca^{2+}-activated K^+ channel as previously reported [25]. This result confirmed that

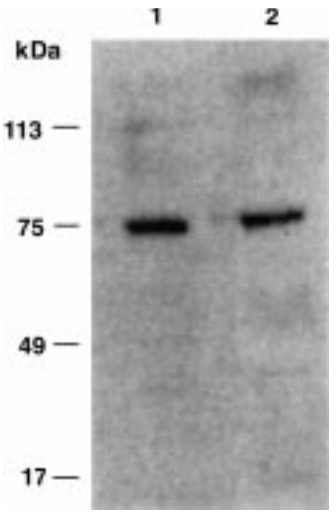

Fig. 4. Western blot analysis of Ca^{2+}-activated K^+ channel in rat sperm. Epididymal sperm homogenate of 10 μg (lane 1) and 5 μg (lane 2) were subject to SDS-PAGE and analysed subsequently by western blotting. A major band of about 70 kDa was detected.

the Ca^{2+}-activated K^+ channel is present in sperm, which may be important for sperm functions.

4. Discussion

We have demonstrated for the first time the expression of a sperm Ca^{2+}-activated K^+ channel in *Xenopus* oocytes. First, the channel could be activated by a Ca^{2+}-mobilising agent, ionomycin, but suppressed by a Ca^{2+} chelator, EGTA. Secondly, large single-channel conductance and sensitivity to TEA and CTX are consistent with the presence of a *slowpoke* Ca^{2+}-activated K^+ channel. This notion is supported by the inhibition of channel expression in *Xenopus* oocytes by co-injection of germ cell RNAs with an antisense RNA of a mouse *slowpoke* gene encoding 'Maxi' Ca^{2+}-activated K^+ channel. In addition, immunoblotting analysis (Western blotting) of the Ca^{2+}-activated K^+ channel has also demonstrated its presence in spermatozoa collected from the rat epididymis. All the evidence strongly indicates the presence of a Ca^{2+}-activated K^+ channel in the rat sperm.

The present study also indicates a potential role of the Ca^{2+}-activated K^+ channel in sperm functions. The activation of a sperm Ca^{2+}-activated K^+ channel by ATP demonstrated in the present study suggests possible involvement of the channel in sperm activation since extracellular ATP has been shown to be a rapid and potent activator of the acrosome reaction in non-capacitated human spermatozoa [11,12]. The demonstrated presence of the Ca^{2+}-activated K^+ channel in sperm also lends support to the recent implication of Ca^{2+}-activated K^+ channels in the hamster sperm acrosome reaction [3] and the initiation of sperm motility in salmonid fishes [4]. Interestingly, K^+ channels, which were shown to be sensitive to TEA, in rat and human sperm membranes have also been demonstrated using the planar lipid bilayer technique [10].

It should be noted that RNAs of spermatogenic cells instead of mRNAs were used in the present study. The observed expression of Ca^{2+}-activated K^+ channels under this condition indicates the abundance of expression of these channels. Insufficient mRNAs present in the RNA preparations may explain why other sperm channels, e.g. the cyclic-nucleotide-gated channel [26] and T-type Ca^{2+} channels [27], demonstrated by cloning methods, were not observed. However, it should be noted that the above studies [26,27] used starting materials from the testis rather than from spermatogenic cells directly as in the present study. Another reason for not observing other channels in our expression system may be that channel expression could be affected by other factors during sperm maturation; therefore, some of the channels expressed in sperm may not be expressed in the *Xenopus* oocytes. Nevertheless, the present study has demonstrated the feasibility of applying molecular biology to probe ion channels in spermatogenic cells and/or spermatozoa which are normally not accessible by electrophysiological techniques. This may enable us to further elucidate different roles of ion channels in the processes of spermatogenesis, sperm functions and sperm-egg interaction.

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