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# Forskolin stimulates porcine sperm capacitation by increasing calcium uptake

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Using the fluorescent calcium indicator fura-2, forskolin was found to dose-dependently cause an immediate increase in the concentration of intracellular free calcium of porcine cauda epididymal sperm. This stimulatory effect of forskolin is due to the enhancement of $Ca^{2+}$ uptake by the verapamil-sensitive transporter on the sperm plasma membrane and results in the promotion of the sperm capacitation and subsequent acrosome reaction.

Calcium transport; Capacitation; Acrosome reaction; Porcine sperm

## 1. INTRODUCTION

Calcium ions are known to be necessary for the capacitation and the subsequent acrosome reaction of mammalian sperm [1,2]. The increase in the intracellular $Ca^{2+}$ levels during capacitation and the concomitant acrosome reaction was directly measured with the fluorescent calcium indicator fura-2 [3,4], while the mechanism of $Ca^{2+}$ entry into the sperm remains unknown.

In porcine sperm, a relatively high level of calcium (4.7 mM) is required for the induction of capacitation in vitro and for successful fertilization [5]. It is likely that the procedures stimulating $Ca^{2+}$ entry into sperm improve the results of the attempts for fertilization of porcine eggs in vitro.

Recently, we found that forskolin, a potent activator of adenylylcyclase in somatic cells but not in sperm, stimulated $Ca^{2+}$ uptake by sperm [6]. In addition, the preliminary observation by Nagai et al. [7] that forskolin enhanced porcine sperm penetration into egg in vitro, prompted us to examine the effect of forskolin on porcine sperm capacitation. This is the first report that shows that forskolin directly acts on the verapamil-sensitive $Ca^{2+}$ transporter on the sperm plasma membrane to increase cytosolic free $Ca^{2+}$ levels and stimulates the acrosome reaction.

## 2. MATERIALS AND METHODS

### 2.1. *Sperm preparation*

Porcine cauda epididymal sperm were collected by perfusion of the ductus epididymidis with Pyr-Glu buffer (130 mM NaCl, 5 mM KCl, 5 mM glucose, 3 mM sodium pyruvate, 20 mM Tris-HCl, pH 7.4) and were washed three times by centrifugation. Washed sperm were suspended in the same buffer ($10^9$ cells/ml) and stored at room temperature until use.

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### 2.2. *Measurement of intracellular free $Ca^{2+}$*

Sperm was incubated with 1 $\mu$M fura-2/AM in Pyr-Glu buffer containing 1.5 mM $CaCl_2$ for 1 h at 37°C. The extracellular unloaded fura-2 was removed by centrifugation three times with Pyr-Glu buffer at $2000 \times g$, for 5 min. Washed sperm was resuspended in Pyr-Glu buffer containing 1.8 mM $CaCl_2$ and 0.1% fatty acid-free BSA (the capacitation medium according to Nikolopoulou et al. [8]) to a concentration of $2 \times 10^7$/ml and incubated at 37°C for an appropriate time in the dark. 3-ml aliquots of the incubation mixture were used for spectrofluorometry and 10 $\mu$l aliquots for the determination of the acrosome reaction. Fluorescence was monitored using a Hitachi F-2000 spectrofluorometer at excitation wavelengths of 340 and 380 nm, respectively, and an emission wavelength of 510 nm, each with 10 nm slits. At the end of the experiments, sperm was lysed with Triton X-100, and then calcium was depleted by the addition of 25 mM EGTA. Intracellular $Ca^{2+}$ concentrations were calculated according to Grynkiewicz et al. [9].

### 2.3. *Determination of $^{45}Ca^{2+}$ uptake*

Sperms ($2 \times 10^8$/ml) were incubated in Pyr-Glu buffer containing 1 mM $^{45}CaCl_2$ at 30°C for 20 min. 100-$\mu$l aliquots were added to 5 ml of the washing buffer (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4), layered on GF/C filter and immediately filtrated. The filter was washed twice more with 5 ml of the same buffer. After the filter was dried, radioactivity on the filter was counted in the scintilation cocktail.

### 2.4. *Determination of acrosome reaction*

Acrosome status was assessed using the triple-stain technique of Talbot and Chacon [10]. After staining the sperm, the percentage of live and acrosome reacted sperms were estimated under a light microscope. Three persons independently examined the sperm; over 200 sperms per each person.

2.5. *Materials*

Forskolin, fatty acid free BSA, Bismark brown, and Rose Bengal were purchased from Sigma. Fura-2/AM was obtained from Wako Pure Chemical Industry, Ltd., Japan. $^{45}CaCl_2$ was purchased from New England Nuclear.

## 3. RESULTS

In the preliminary experiment [6], 0.24 mM forskolin was found to enhance $^{45}Ca^{2+}$ uptake by porcine cauda epididymal sperm without affecting the $^{45}Ca^{2+}$ efflux. As shown in Fig. 1, forskolin stimulated $^{45}Ca^{2+}$ uptake in a dose-dependent manner. A significant stimulation was observed at 0.05 mM.

Fig. 2 shows the effect of forskolin on the levels of intracellular free $Ca^{2+}$. Forskolin very rapidly increased the concentration of cytosolic free $Ca^{2+}$ within the same concentration range that stimulated $^{45}Ca^{2+}$ uptake. The $Ca^{2+}$ uptake by sperm was inhibited by verapamil but not by diltiazem [6]. Consistent with this result, the increase in the levels of the cytosolic free $Ca^{2+}$ by forskolin was inhibited by verapamil but not by diltiazem (Fig. 3).

As shown in Fig. 4, in the Pyr-Glu buffer containing 0.1% fatty acid-free BSA and 1.8 mM $CaCl_2$, which had been used for the induction of the boar sperm acrosome reaction [8], sperm gradually underwent the acrosome reaction. About 18% of the sperms finished the acrosome reaction after 4 h of incubation at 37°C. Forskolin clearly stimulated the acrosome reaction. The concentrations of the intracellular $Ca^{2+}$ slightly increased during the incubation and forskolin was found to enhance them in a similar time course to that of the stimulation of the acrosome reaction.

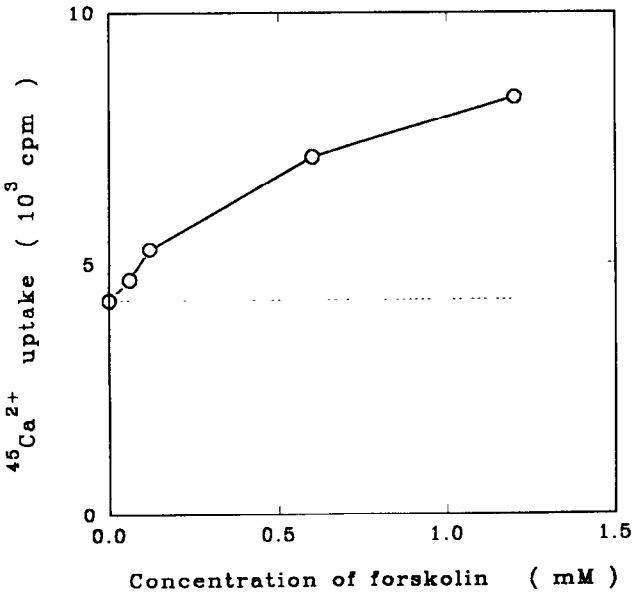


Fig. 1. The effect of forskolin on $^{45}Ca^{2+}$ uptake by porcine cauda epididymal sperm. Porcine cauda epididymal sperm was incubated with various concentrations of forskolin and 1 mM $^{45}CaCl_2$ in Pyr-Glu buffer and the activity of $^{45}Ca^{2+}$ uptake was determined as described in section 2. Data are means from 4 determinations.

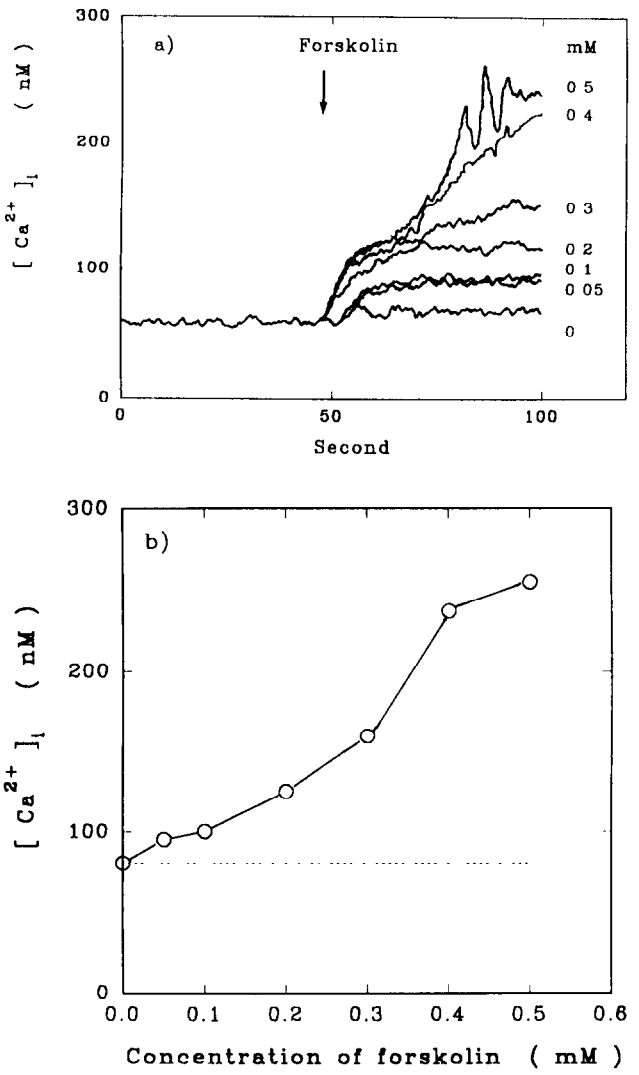


Fig. 2. The effect of forskolin on the concentration of intracellular free $Ca^{2+}$ in sperm. (a) Time course of the forskolin effect. The concentration of intracellular free $Ca^{2+}$ was monitored as described in section 2. Various concentrations of forskolin were added to the incubation mixture at 50 s. (b) Dependency of the concentration of intracellular free $Ca^{2+}$ on forskolin. The concentrations of intracellular free $Ca^{2+}$ at 100 s in (a) were plotted against the concentration of forskolin. Representative data are shown in both (a) and (b).

## 4. DISCUSSION

In a previous study, we have demonstrated the presence of the verapamil-sensitive $Ca^{2+}$ transporter on the porcine sperm plasma membrane [6]. The present report shows for the first time that forskolin stimulates $Ca^{2+}$ uptake and enhances intracellular free $Ca^{2+}$ levels in porcine cauda epididymal sperm. Both effects of forskolin occurred in the same concentration range. Vera-

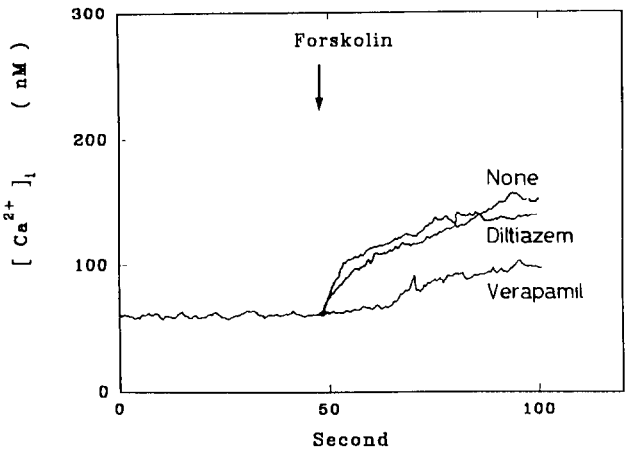


Fig. 3. The effect of $Ca^{2+}$ transport inhibitors on the increase in intracellular free $Ca^{2+}$ levels induced by forskolin. Fura-2 loaded sperm was incubated with 1 mM diltiazem or verapamil at 37°C for 20 min. Then the concentration of the intracellular free $Ca^{2+}$ level was monitored before and after the addition of 0.25 mM forskolin.

pamil, by which the $Ca^{2+}$ influx into porcine sperm was inhibited [6], decreased the forskolin effect on the intracellular $Ca^{2+}$ concentration. It seems that forskolin directly acts on the $Ca^{2+}$ transporter, because the effects of forskolin appeared without a lag time, as shown in Fig. 2. These results strongly suggest that the increase in intracellular $Ca^{2+}$ concentrations elicited by forskolin is due to the direct stimulation of the $Ca^{2+}$ influx mediated by the verapamil-sensitive transporter.

On the other hand, forskolin is known to be a potent and direct activator of adenylylcyclase in somatic cells [11]. But the sperm enzyme does not interact with forskolin [12], indicating that cyclic AMP is not concerned with the forskolin action on $Ca^{2+}$ entry. It is likely that forskolin directly interacts with the verapamil-sensitive $Ca^{2+}$ transporter. In this respect, it is very interesting that brain adenylylcyclase has similar topographical characteristics to various plasma membrane channels and transporters [13]. In addition, forskolin has also been shown to interact directly with the glucose transporter [14,15]. This suggests the possibility that the $Ca^{2+}$ transporter on the sperm plasma membrane can be detected by forskolin photoaffinity labeling [14]. The purification of verapamil-binding protein from the extract of purified plasma membranes of porcine sperm is in progress by using forskolin affinity chromatography.

For mammalian sperm, capacitation and the subsequent acrosome reaction are obligatory processes to acquire the full ability for fertilization [16]. External $Ca^{2+}$ is an essential requirement for both processes [17] and the increases in $Ca^{2+}$ influx during capacitation and at the initial step of the acrosome reaction were reported [3,4,16,18,19]. So, it is likely that agents which stimulate $Ca^{2+}$ influx also promote both processes [20]. In the present study, for the first time folskolin was clearly

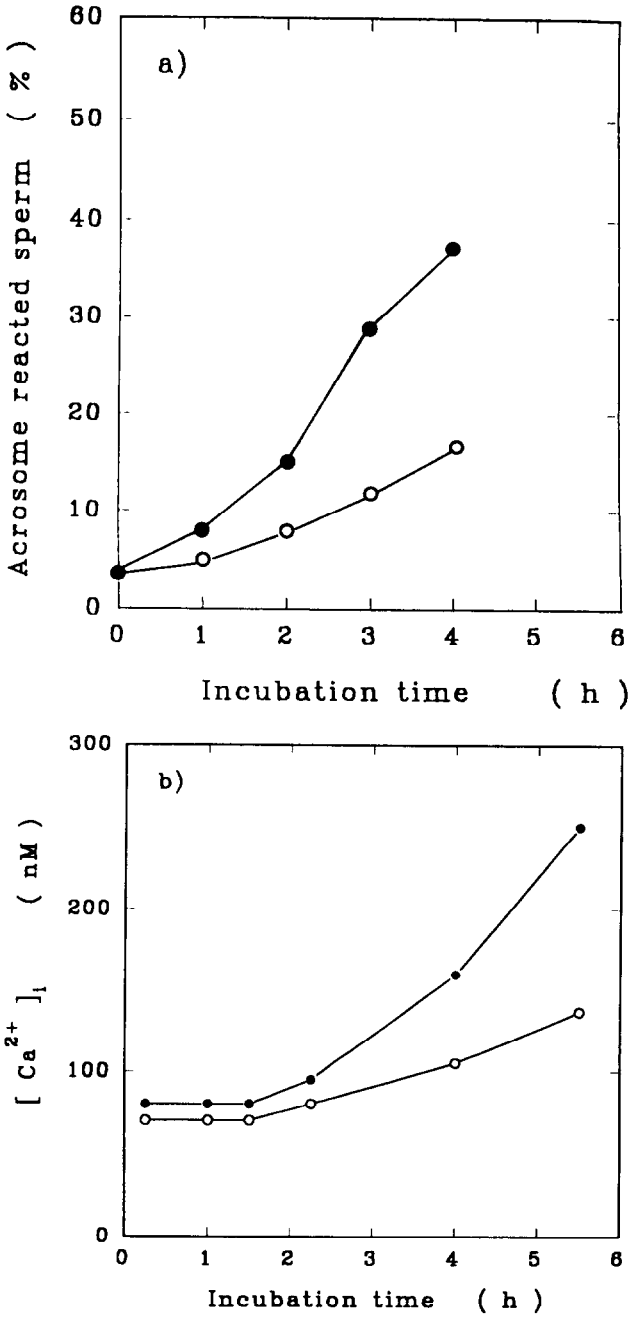


Fig. 4. The effect of forskolin on the induction of the acrosome reaction in vitro. Sperm was incubated in the capacitation medium in the presence (●) or the absence (○) of 0.25 mM forskolin at 37°C for an appropriate time as described in section 2. 10 μl and 3 ml aliquots were taken out for the determinations of the acrosome status (a) and the concentration of intracellular free $Ca^{2+}$ (b), respectively. Data are means from duplicate determinations of a representative experiment.

shown to promote the capacitation and induce the acrosome reaction through increasing the intracellular free $Ca^{2+}$ levels. Although forskolin is not a physiological substance in the in vivo system, it can be used as an

exogenous promoter in the fertilization system in vitro [9].

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