The Journal of Experimental Biology 210, 4053-4064
Published by The Company of Biologists 2007
doi:10.1242/jeb.007013

Protein kinase C is an important signaling mediator associated with motility of intact sea urchin spermatozoa

Daniel White*, Eve de Lamirande and Claude Gagnon

Urology Research Laboratory, Royal Victoria Hospital, McGill University Health Center and Faculty of Medicine, McGill University, Montréal, H3A 1A1, Canada

*Author for correspondence (e-mail: dan7white@yahoo.ca)

Accepted 4 September 2007

Summary

Numerous kinases and phosphatases are most likely implicated in sperm motility initiation and maintenance. Data on these signaling molecules were mostly obtained from studies conducted on *in vitro* demembranated–reactivated sperm models but are not necessarily representative of the *in vivo* situation. We therefore investigated the effect of a variety of cell-permeable chemicals, mostly kinase inhibitors, on the motility initiation and maintenance of intact sea urchin spermatozoa. Among the 20 substances tested, the protein kinase C (PKC) inhibitor chelerythrine was the most potent, arresting motility at concentrations starting from 1.5–2 μmol l^{-1}. Motility was also inhibited by two other PKC inhibitors as well as staurosporine. Furthermore, these inhibitors prevented the motility-associated increase in phosphorylation of at least four PKC substrates. These phospho-PKC target proteins, as assessed with an antibody specific to phosphorylated motifs of PKC substrates, were found to be associated with the flagellum, either in the Triton X-100 soluble portion or the axoneme (Triton X-100 insoluble). A phosphorylated PKC-like enzyme was also detected by immunoblotting in the flagellum, as well as a significant 50 kDa PKC cleavage product. Taken together, the data strongly indicate for the first time that, *in vivo*, which means on intact spermatozoa, PKC is a key signaling mediator associated with the maintenance of sea urchin sperm motility.

Key words: sperm motility, kinase inhibitors, protein kinase C, protein kinase M, protein phosphorylation, axoneme, sea urchin, *Lytechinus pictus*.

Introduction

Reproduction of freshwater and seawater organisms like fishes and echinoderms is accomplished by external fertilization. Spermatozoa of males are stored quiescent in gonads until their release in the external fluid environment, where they rapidly acquire motility in order to reach the freshly spawned eggs.

Initiation of sperm motility of these organisms is naturally triggered by the sharp differences between the physico-chemical characteristics of water and the internal gonad environment (Gagnon and de Lamirande, 2006). In the case of sea urchins, the high K^+ concentration (30–50 mmol l^{-1}) and CO_2 tension in the gonad seminal fluid make the internal pH of spermatozoa stay between 6.6 and 7.2 (Lee et al., 1983; Christen et al., 1982; Christen et al., 1983). Under these conditions, axonemal dynein ATPases responsible for powering flagellar movement are kept inactive and the respiration system is down. When sperm is released in the external medium, the lower K^+ content and CO_2 tension of seawater will activate a sperm membrane Na^+/H^+ exchanger to release H^+ ions, which will in turn allow the raise of the internal pH to ~7.5–7.6 and eventually lead to the activation of the dynein ATPases for sperm motility initiation (Lee, 1984; Darszon et al., 2001). Upon subsequent increase in ADP following the massive utilization of ATP by the dynein enzymes, the mitochondrial respiration system will be stimulated to produce more ATP to maintain the level of ATP stores high enough to sustain motility for several hours (Christen et al., 1983).

Numerous signaling molecules are most likely implicated in sperm motility initiation and maintenance. The presence of kinases, phosphatases and many phosphorylated proteins has been described in spermatozoa of various teleost and invertebrate species including sea urchins (Tash and Bracho, 1994; Inaba, 2003; Gagnon and de Lamirande, 2006). For instance, the contribution of the cAMP-dependent protein kinase (PKA) as a regulator of sperm motility is well known (Inaba, 2003; Brokaw, 1987) but cAMP-independent phosphorylation of axonemal proteins during motility initiation has been reported as well (Hayashi et al., 1987; Chaudhry et al., 1995; Morita et al., 2003; Nakajima et al., 2005). Most of these studies though have been conducted on *in vitro* demembranated–reactivated spermatozoa and therefore do not tell much about the upstream signaling molecules leading to motility initiation and maintenance in intact cells. Although giving a direct access to the axonemal structure, the detergent treatment itself applied to prepare these sperm models may potentially activate or deactivate some of the signaling molecules by eliminating kinase–substrate interactions created by lost compartmentalization (Tash, 1989).

Investigations on the signaling molecules associated with the motility activation of intact spermatozoa are scarce. In one study done on sea urchin spermatozoa, flagellar proteins phosphorylated on a serine or threonine residue have been linked to the initiation of motility. However, the correlation between these phosphorylations and motility (percentages, time-course, etc.) was partial, and the nature of the kinases involved was only speculative (Bracho et al., 1998).

The aim of our study was to find out which kinases could be implicated in the motility initiation and maintenance of intact sea urchin spermatozoa. First, the effect of inhibitors specific for a variety of kinases was determined. As our initial results pointed to protein kinase C (PKC) as a key signaling element, we investigated sperm protein phosphorylation changes (correlation with motility, time course, cellular localization) with antibodies specific to phosphorylated motifs of PKC substrates as well as PKC itself.

Materials and methods

Materials

The following reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA): N^6,2^1-o-dibutyryl cAMP (dbcAMP), 3-isobutyl-1-methylxanthine (IBMX), Rp-adenosine-3′,5′-monophosphorothionate (Rp-cAMPs), poly-glutamine-tyrosine (poly-Glu-Tyr), H-7, phospho-Serine (Ser), phospho-Threonine (Thr) and phospho-Tyrosine (Tyr). The following were obtained from Calbiochem, EMD Biosciences Inc. (La Jolla, CA, USA): AKT inhibitor, okadaic acid, β-glycerophosphate, H-89, KT5720, chelerythrine, calphostin C, Gö 6976, wortmannin, tyrphostin A47, tyrphostin A1, genistein, PP2, herbymicin A and staurosporin. Competitors of SH2 domain of Grb2, CGP85793 and CGP78850, were a generous gift from Novartis Pharma Inc. (Basel, Switzerland). Percoll was bought from Amersham Biosciences Inc. (Baie-d'Urfé, QC, Canada, part of GE Healthcare). All other chemicals were at least of reagent grade. Whenever possible, stock solutions of chemicals were prepared in distilled water. Stock solutions of other kinase inhibitors were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the incubation media never exceeded 1% (v/v), a condition that does not affect sperm motility.

Primary rabbit antibodies and their respective blocking agents used in the immunoblotting and immunolocalization procedures were obtained from Cell Signaling Technology (Beverly, MA, USA). D66, which is an anti β-tubulin mouse monoclonal IgG_1 antibody, was developed in our laboratories (Audebert et al., 1999). Secondary horseradish peroxidase (HRPO) or streptavidin-conjugated antibodies, as well as the porcine and goat sera used as blocking agents, were from Cedarlane Laboratories Ltd (Hornby, ON, Canada). The positively charged slides used in the immunofluorescence procedure were bought from Fisher Scientific (Nepean, ON, Canada; Fisherbrand Superfrost/Plus, catalog number 12-550-15). The fluorescent reagent Alexa Fluor 555, as well as the antibleaching product Prolong Gold, were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Electrophoresed sperm proteins were blotted on a 0.2 μm pore size supported nitrocellulose membrane (Osmonics, Inc., Westborough, MA, USA). Molecular mass standards used in immunoblotting experiments were from Fermentas Canada Inc. (Burlington, ON, Canada). The enhanced chemiluminescence (ECL) detection was performed using Lumi-Light reagents (Roche Diagnostics, Laval, QC, Canada) and Fuji Super RX films (Fuji Photo Film Co. Ltd, Tokyo, Japan).

Sea urchin maintenance and sperm collection

Gravid *Lytechinus pictus* Verrill sea urchins were purchased from Marinus Scientific (Garden Grove, CA, USA) and sustained alive for several months at our laboratory in a 300-liter aquarium tank filled with artificial seawater (ASW) (Crystal Sea™, Marine Enterprises International, Inc., Baltimore, MD, USA) maintained at 13°C.

Using a disposable syringe with an 18-gauge needle, urchins were showered in the area of the five gonadal openings with 20 ml of immotility medium (IM; 20 mmol l^{-1} Tris-HCl pH 6.0, 300 mmol l^{-1} NaCl, 150 mmol l^{-1} K^+-acetate, 25 mmol l^{-1} $MgSO_4$ and 1 mmol l^{-1} DTT) in order to displace residual seawater that could come into contact with ejaculated sperm. Urchins were then induced to spawn by intracoelomic injections of 0.5–1 ml of 0.5 mol l^{-1} KCl at five different spots around the mouth. Semen was collected devoid of any seawater ('dry' collection) in small Petri dishes kept on ice, then gently transferred to microtubes and concentrated by centrifugation at 2300 ***g*** for 5 min at 4°C. After spinning, the top fluid was removed by gentle aspiration and the white concentrated sperm layer transferred to a clean tube, leaving the colored pellet containing phagocytes and large debris behind. At this stage, the preparation contained more than 99.9% spermatozoa and the sperm concentration was $\cong 60\times10^9$ cells ml^{-1}.

Sperm motility evaluation

The concentrated sperm suspension kept on ice was first diluted 1/150 in IM containing a kinase inhibitor. After a 3 min incubation at 20°C, spermatozoa were further diluted 1/150 into ASW containing the inhibitor at the same concentration. A 20 μl aliquot was then rapidly transferred to a clean microscopic slide, overlaid with a cover slip with silicone grease on its edge for sealing, to prevent evaporation of the fluid. Sperm motility in the presence or absence of specific kinase inhibitors was evaluated at room temperature (20°C) by videomicroscopy using a dark-field illumination at 200× magnification. Number of motile spermatozoa was determined at three time intervals: 0.5–2.5, 5–7, 13–15 min after the sperm dilution in ASW, counting 100–200 sperm cells at each time interval. Sperm curvilinear velocity (total distance traveled divided by time) measurements on spermatozoa were obtained by videomicroscopy using a computer-assisted semen analyzer (CASA) (SpermVision™; Penetrating Innovations, Ingersoll, ON, Canada). Controls consisted of spermatozoa incubated with 1% DMSO and were done at the beginning and end of the day. At this final concentration, DMSO alone did not have any significant effect on intact sea urchin motility (percentage of motility and velocity).

Detection of sperm protein phosphorylation by immunoblotting

For these experiments, the concentrated sea urchin sperm stock suspension was first diluted 1/10 in IM in the presence or absence of a specific kinase inhibitor. After a 3 min incubation

at 20°C, samples were further diluted 20-fold in ASW containing the inhibitor at the same concentration. Following an additional 5 min incubation at 20°C, spermatozoa were centrifuged at 10 000 ***g*** for 30 s. The supernatants were gently aspirated and the sperm pellets resuspended in electrophoresis sample buffer supplemented with 20% Percoll (to prevent DNA decondensation) and a cocktail of phosphatase inhibitors (1 mmol l^{-1} sodium fluoride, 4 mmol l^{-1} β-glycerophosphate, 0.1 mmol l^{-1} sodium vanadate and 20 nmol l^{-1} okadaic acid). The samples were boiled for 3 min and centrifuged at 21 000 ***g*** for 15 min at 20°C. The supernatants containing the dissolved sperm proteins were stored in clean microtubes for the immunoblotting analysis.

To determine cellular localization of the phosphorylated proteins, spermatozoa, before and after motility initiation, were subjected to dissociation of heads and tails by passing samples 10 times through a 23-gauge syringe needle (Cosson and Gagnon, 1988). Dissociated sperm samples were then loaded on a 20% Percoll layer (prepared with ASW plus the above-mentioned phosphatase inhibitor cocktail) and centrifuged at 10 000 ***g*** for 15 min at 4°C. Flagella were recuperated in the top layer while heads were found at the bottom of the tubes. A second round of passages of the sperm heads through the 23-gauge needle was performed to remove most of the remaining non-dissociated flagella. Finally, both purified head and flagellar fractions (≅96% and 99% pure, respectively, by microscopic count) were treated with 0.1% Triton X-100 to refine the localization of the targeted proteins.

Prepared samples were either immediately electrophoresed on 10% SDS–polyacrylamide gels or stored overnight at –20°C prior to loading. Proteins were then transferred to nitrocellulose membranes according to Laemmli's procedure (Laemmli, 1970). Membranes were blocked with 10% porcine serum in a Tris pH 7.8 buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TTBS) for 1 h at room temperature before incubating them overnight at 4°C with the primary antibody diluted 1/1000 (0.1 μg ml^{-1}) in the same blocking buffer. The next morning, membranes were washed extensively with TTBS and incubated with the HRPO secondary antibody conjugate (1/3000 in blocking buffer) for 30 min at room temperature. Extensive washing with TTBS was repeated and membranes soaked into the ECL substrate according to the manufacturer's instructions before exposure to autoradiography films. At the end of each experiment, blots were silver-stained according to the method of Jacobson and Karsnäs (Jacobson and Karsnäs, 1990) to ascertain that the amount of proteins loaded in each well was the same.

The specificity of the primary antibodies was verified by pre-adsorbing them with their respective antigenic blocking peptide for 2 h at 20°C before conducting the immunoblotting procedure. As a control, the phospho-specific antibodies were also incubated with a mixture of phospho-Ser, phospho-Thr and phospho-Tyr at a phospho-amino acid:antibody molar ratio of 10 000:1 to eliminate the possibility of non-specific binding to single phospho-amino acid.

Immunofluorescence on intact spermatozoa

Immunolocalization of phospho-PKC and PKC phospho-substrates was performed on immotile or motile intact spermatozoa following a procedure previously described (Gagnon et al., 1994) and slightly modified. Briefly, 25 μl of sea urchin spermatozoa was diluted twofold with 4% paraformaldehyde in the same medium on positively charged slides. Following a 6 min incubation period, they were permeabilized, while fixation continued, by the addition of two volumes of 1% Triton X-100 in TBS for another 6 min. Slides were then gently but extensively rinsed with TBS plus 0.1% Triton X-100 before a 15 min blocking step at 20°C with 2.5% goat serum in TBS. The specimens were then incubated overnight at 4°C with 20 μl of primary antibodies (10 μg ml^{-1}) in blocking buffer. After rinsing the slides as above, 20 μl of the appropriate biotinylated secondary antibody, diluted 1/300 in blocking buffer, was added for a 30 min incubation at 20°C. Slides were rinsed again before the addition of the Alexa Fluor 555 streptavidin conjugate (1/500 w/v in TBS) and incubated for 10 min in the dark at 20°C. Following a final wash, slides were mounted with a drop of Prolong Gold antifade reagent and observed under a Carl Zeiss (Oberkochen, Germany) Axiophot microscope (excitation filter 546) at a magnification of 400×. Background fluorescence was assessed using the secondary antibody plus the Alexa Fluor 555 conjugate. Images were captured with a monochrome Retiga 1300 CCD camera (QImaging, Surrey, BC, Canada) and digitized on a computer using the Northern Eclipse version 6.0 program.

Results

Motility of intact L. pictus spermatozoa in the presence of various kinase inhibitors

Concentrated dry sea urchin spermatozoa diluted in seawater become actively motile within several seconds. To properly evaluate the role of kinases in the initiation of motility of intact spermatozoa, we first had to find a medium in which these cells could remain completely immotile during a few minutes at 20°C without harmful effects so that the subsequent dilution in seawater would allow motility. We tested two media previously reported to keep sea urchin quiescent (Bracho et al., 1997; Cheung, 1995). These media did not meet the above-mentioned criteria in our hands. In one case, a low percentage of spermatozoa (≅3 to 5%) initiated motility two minutes after dilution in the medium even though the latter had a pH of 6 and contained 50 mmol l^{-1} KCl (Bracho et al., 1997). The other medium was a pH 7.9 Na^+-free seawater where choline chloride was substituted for NaCl (Cheung, 1995). A reduced motility was always readily initiated in about 5–10% of spermatozoa resuspended in that solution. We therefore devised a simple Tris-based medium at pH 6 containing 150 mmol l^{-1} K^+-acetate (IM). Completion with 300 mmol l^{-1} NaCl plus 25 mmol l^{-1} $MgSO_4$ contributed to give an osmolarity similar to that of seawater and thus preserve the shape and viability of spermatozoa. Microscopic observations confirmed the sustained total immotility of the intact sperm cells in IM at 20°C and, after a 3 min incubation in that medium, the subsequent initiation of motility upon dilution in ASW. Furthermore, the percentages and duration of motility upon dilution in ASW was comparable to the motility of concentrated spermatozoa immediately resuspended into ASW.

Inhibitors of several types of kinases were tested (Table 1). Because the specificity of inhibitors is never perfect, and various

Table 1. *Effect of various kinase inhibitors on the motility of intact sea urchin spermatozoa*

Kinase or pathway targeted	Substance	IC_{50} (μmol l^{-1})	Type of action or target	Concentration range tested	Inhibition of motility
AKT	AKT inhibitor	5	Phosphatidyl-inositol ester analog	1–10 μmol l^{-1}	None
ERK pathway	CGP85793	ND	Grb2-SH2 domain competitor	1–100 μmol l^{-1}	None
	CGP78850	ND	Grb2-SH2 domain competitor	1–100 μmol l^{-1}	Partial at 100 μmol l^{-1}
PKA	H-89	0.048	ATP-site competitor	1–100 μmol l^{-1}	Total at $\geqslant$30 μmol l^{-1}
	KT 5720	0.056	ATP-site competitor	0.1–10 μmol l^{-1}	None
	dbcAMP	NA	Activator	1–10 mmol l^{-1}	None
	IBMX	NA	Activator	0.1–1 mmol l^{-1}	None
	Rp-cAMPS	11	cAMP competitor	0.2–2 mmol l^{-1}	None
PKC	Chelerythrine	0.66	Inhibits PKC translocation	0.5–50 μmol l^{-1}	Total at $\geqslant$2 μmol l^{-1}
	Calphostin C	0.05	Diacylglycerol competitor	0.15–15 μmol l^{-1}	Total at $\geqslant$10 μmol l^{-1}
	Gö6976	0.002–0.006	Ca^{2+} dependent PKC α1and β1	0.05–50 μmol l^{-1}	Partial at $\geqslant$0.5 μmol l^{-1}
PI3K	Wortmannin	0.005	Catalytic subunit	1–100 nmol l^{-1}	None
PTK	Tyrphostin A47	2.4	Receptor type PTK	1–50 μmol l^{-1}	Partial at $\geqslant$10 μmol l^{-1}
	Tyrphostin A1	>100	Inactive analog	0.1–100 μmol l^{-1}	None
	PP2	0.005	Non-receptor type PTK	0.2–2 μmol l^{-1}	None
	Herbymicin A	8–12	Non-receptor type PTK	10 μmol l^{-1}	None
	Genistein	2–6	ATP-site competitor	100 μmol l^{-1}	None
	poly-Glu-Tyr	NA	Competitor for Tyr phosphorylation	10 μg ml^{-1}	None
Broad spectrum	Staurosporine	0.001–0.03	ATP-site competitor	0.2–2 μmol l^{-1}	Total at $\geqslant$1 μmol l^{-1}
	H-7	3–6	ATP-site competitor	10–100 μmol l^{-1}	None

Immotile concentrated spermatozoa were first diluted in IM in the absence or presence of kinase inhibitor and then in ASW with or without the inhibitor at the same concentration. Motility was assessed by light microscopy with a dark-field illumination as detailed in the Materials and methods, and evaluations of motility inhibition reported here are for the end of the 15-min observation period. ND, not determined; NA, not applicable.

isoforms of kinases exist, we tested different inhibitors acting by different mechanisms, some activators, a competitor of Tyr phosphorylation (poly-Glu-Tyr) and an inactive analogue (tyrphostin A1). Only seven inhibitors out of the 20 substances had significant effects on the percentage of motile cells over the measurement period of 15–20 min; three of these are known to act on PKC, one on protein kinase A (PKA), one on protein tyrosine kinase (PTK) and one on Grb2 (ERK pathway). The other inhibitor, staurosporin, is considered as a broad spectrum inhibitor having proven effects on many kinases (Davis et al., 1989). The effects of the PKC inhibitors were clearly time- and dose-dependant, as shown in Fig. 1. Within the range of concentrations tested, and despite pre-incubation of spermatozoa with the inhibitor in IM, none of the seven effective chemicals totally prevented motility initiation upon dilution in ASW. Even when a high concentration (15 μmol l^{-1}) of chelerythrine was used, for instance, 5–10% of slowly progressive cells were always recorded within the first 2 min, before spermatozoa went to a complete arrest. Nevertheless, the most dramatic decreases of *L. pictus* sperm motility were observed with chelerythrine and calphostin C, PKC inhibitors, causing total inhibition after 15 min at a final concentration of 5 and 10 μmol l^{-1}, respectively. Gö6976, the other PKC inhibitor tested, also caused noticeable reduction in the number of motile spermatozoa, although never achieving 100% motility inhibition (Table 1, Fig. 1A). CGP78850, H-89 and tyrphostin A47 were also effective in reducing sea urchin sperm motility, but their effect was not corroborated by that of the other ERK pathway, PKA or PTK inhibitors or activators tested. Furthermore, in the case of H-89, the inhibition within the first 5–7 min was observed at $\geqslant$30 μmol l^{-1}, concentrations at which PKC is also affected (Chijiwa et al., 1990).

Mean velocity of sea urchin spermatozoa incubated in the presence of PKC inhibitors was determined with a computer-assisted semen analyzer system (Fig. 1B). Reduced spermatozoa curvilinear velocities were recorded with time in the presence of a low and a high concentration of chelerythrine (0.5 and 5 μmol l^{-1}), calphostin C (1 and 10 μmol l^{-1}) or Gö6976 (0.5 and 5 μmol l^{-1}), chelerythrine and calphostin C having a stronger effect than Gö6976. These results indicated that motility inhibition by these substances was most likely consecutive to a decrease in flagellar beat frequency, which in turn is directly dependent on the activity of the axonemal dynein arms.

Phosphorylation level of PKC substrate associated with motility

Since chelerythrine was found to be the most potent kinase inhibitor to affect sea urchin sperm motility, we investigated the phosphorylation of PKC protein targets. Using an antibody specific to a phosphorylated epitope of PKC substrates (motif recognized: Arg or Lys-X-Ser^{phos}-Hyd-Arg or Lys), we observed an increase in the phosphorylation level of several proteins when quiescent spermatozoa resting in IM medium were transferred to ASW (Fig. 2). Control spermatozoa maintained immotile by transfer in IM always had a basal level

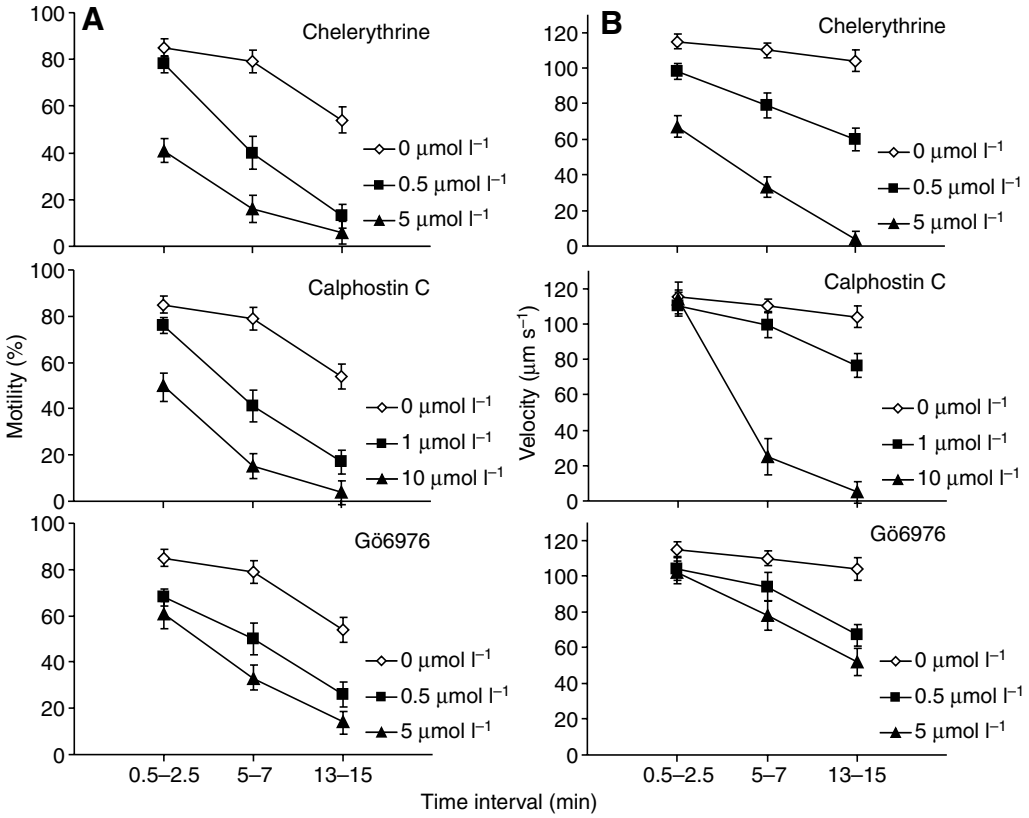

Fig. 1. PKC inhibitors dose-dependently inhibit the motility of intact sea urchin spermatozoa. *Lytechinus pictus* spermatozoa were pre-incubated in IM medium with one of the following inhibitors – chelerythrine, calphostin C or Gö6976 – and then diluted in ASW containing the same amount of inhibitor. The percentage of motile cells (A) and the spermatozoa velocity (B) were evaluated over a 15 min period as described in the Materials and methods. Values are means ± s.e.m. from three different experiments.

of phosphorylation on several protein bands, which was more prominent on two of the bands (M_r of 120 and 45 kDa). Nevertheless, the phosphorylation intensity of all these bands was notably higher in the ASW-motile sperm counterparts in most instances. In particular, the phosphorylation level of four of these proteins (indicated by arrowheads in Fig. 2; M_r of 200, 100, 65 and 28 kDa) was found higher in all the experiments. Therefore, emphasis will be given to these four protein bands in the subsequent description of results.

The anti-phospho-PKC substrate antibody was specific since pre-incubation with a large amount of its antigenic phospho-peptide (#2261=Arg-Lys-Arg-Ser^{phos}-Arg-Lys-Glu) totally blocked the detection of the bands. Moreover, incubation with a similar amount of either a mixture of phospho-Ser, phospho-Thr and phospho-Tyr or of another phospho-peptide (#9621=Arg-Thr-Trp-Thr^{phos}-Leu-Cys-Gln) did not cause any significant difference in the intensities of the bands from the original untreated antibody (Fig. 2).

We then proceeded to evaluate the change of that PKC substrate phosphorylation with time (Fig. 3). It is clear that the increase in phosphorylation was very rapid and occurred concomitantly to the sperm motility initiation, being already stronger at 30 s after sperm transfer in ASW. Moreover, this phosphorylation plateaued at about 6 min and was maintained to a similar level for at least an hour, whereas the percentage of motile cells was also constant at 65–70% over that period. Sperm motility slowly decreased by ~50% during the next 4 h, which was associated with a reduction in phospho-PKC substrates levels. For the subsequent experiments, a 5 min

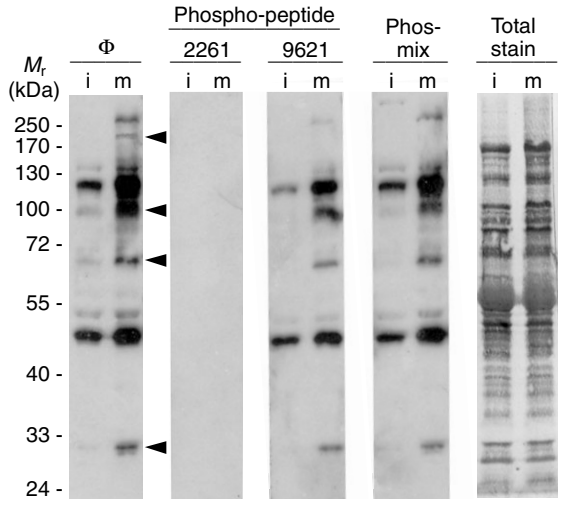

Fig. 2. Increase in phospho-PKC substrate following motility initiation of intact sea urchin spermatozoa. The phosphorylation level, as detected by immunoblotting with an anti-phospho-PKC substrate antibody, of several proteins was markedly increased when immotile (i) *L. pictus* spermatozoa were diluted into ASW to initiate their motility (m) (Φ). The increase in phosphorylation of four of these proteins (arrowheads at 200, 100, 65 and 28 kDa) was observed in all the experiments. The specificity of the antibody for these proteins is demonstrated by the disappearance of bands when the antibody was pre-incubated with its antigenic phospho-peptide (2261) but not after pre-incubation with a mixture of phospho-amino acids (phos-mix) or a different phospho-peptide (9621). A blot representative of two other blots is presented.

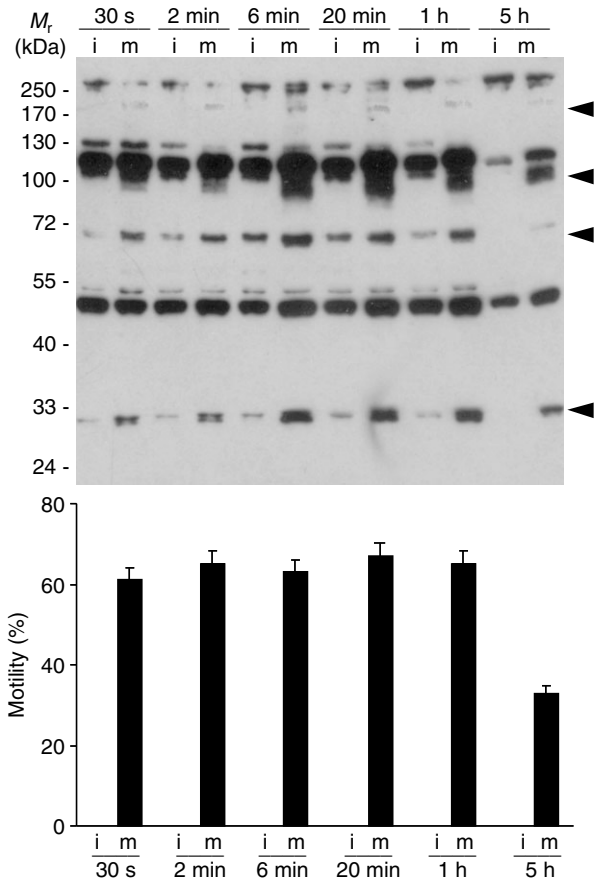

Fig. 3. Time course of the phospho-PKC substrate levels following motility initiation of intact sea urchin spermatozoa. The phosphorylation level of the proteins detected with an anti-phospho-PKC substrate antibody increased rapidly (30 s) after transfer of immotile (i) *L. pictus* spermatozoa into ASW to initiate their motility (m). Protein phosphorylations further increased to a maximum at around 6 min and stayed almost at this level for up to an hour before making a significant drop after 5 h. The arrowheads indicate the position of the 200, 100, 65 and 28 kDa bands. A blot representative of two other blots is presented.

incubation time in ASW was chosen, which leaves sufficient time for the sperm to become fully motile and for any kinase inhibitor added in the medium to act.

The increase in the levels of phospho-PKC substrates was prevented when the PKC inhibitor chelerythrine was introduced in the IM and ASW media. At concentrations of 5 μmol l^{-1}, and corresponding to the inhibitory concentration on sperm motility (Fig. 1), levels of phospho-PKC substrates were similar or even lower to those found in the control (immotile) spermatozoa (Fig. 4). A similar effect on phospho-PKC substrates was also observed with calphostin C at 10 μmol l^{-1}, except for the 28 kDa band, for which the phosphorylation level did not return to the immotile control level. Addition of Gö6976 (up to 5 μmol l^{-1}) reduced the level of two of the phospho-PKC substrates (65 and 28 kDa). Accordingly, this latter PKC inhibitor did not cause such a drastic effect on sperm motility as chelerythrine and Calphostin C (Table 1, Fig. 1). Interestingly, prevention of the increase in phospho-PKC substrates was also observed when sea urchin spermatozoa were incubated with ⩾30 μmol l^{-1} of PKA inhibitor H-89 but not in the presence of up to 50 μmol l^{-1} of the PTK inhibitor tyrphostin A47 or 100 μmol l^{-1} of the Grb2 competitor (SH2 domain) CGP78850 (data not shown).

Fractionation of L. pictus spermatozoa and phospho-PKC substrate proteins

To localize the phosphorylated PKC substrates associated with motility of sea urchin sperm, we mechanically separated heads and flagella and then solubilized the membranes and cytosolic components of each sperm cell part using the neutral detergent Triton X-100. Most of the phospho-PKC substrates, including the 200, 100 and 28 kDa bands, were recovered in the flagella (Fig. 5). The 200 and 28 kDa proteins were found in the Triton X-100-insoluble (axonemes) portion whereas the 100 kDa protein was found in the detergent-soluble flagellar fraction. Reduced phosphorylation of several protein bands,

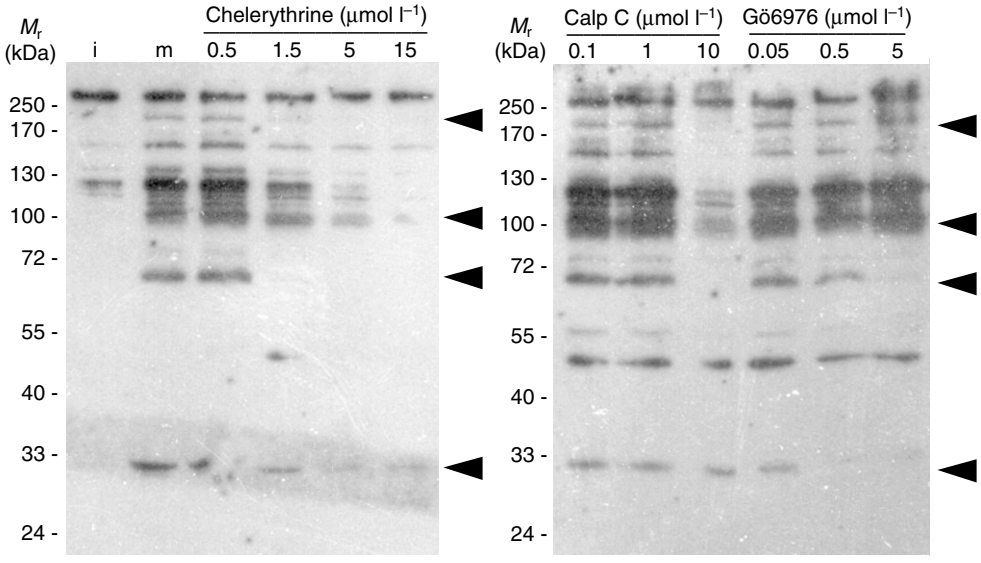

Fig. 4. Effect of various PKC inhibitors on the phospho-PKC substrate levels following motility initiation of intact sea urchin spermatozoa. The increase in phospho-PKC substrates, as detected with an anti-phospho-PKC substrate antibody, observed during motility (m) initiation was prevented when chelerythrine was present in IM and ASW at 1.5 μmol l^{-1} or more, with calphostin C at 10 μmol l^{-1} and with Gö6976 at 5 μmol l^{-1} (only bands at 65 and 28 kDa were affected in the latter case). The arrowheads indicate the position of the 200, 100, 65 and 28 kDa bands. i, immotile; m, motile. A blot representative of two other blots is presented.

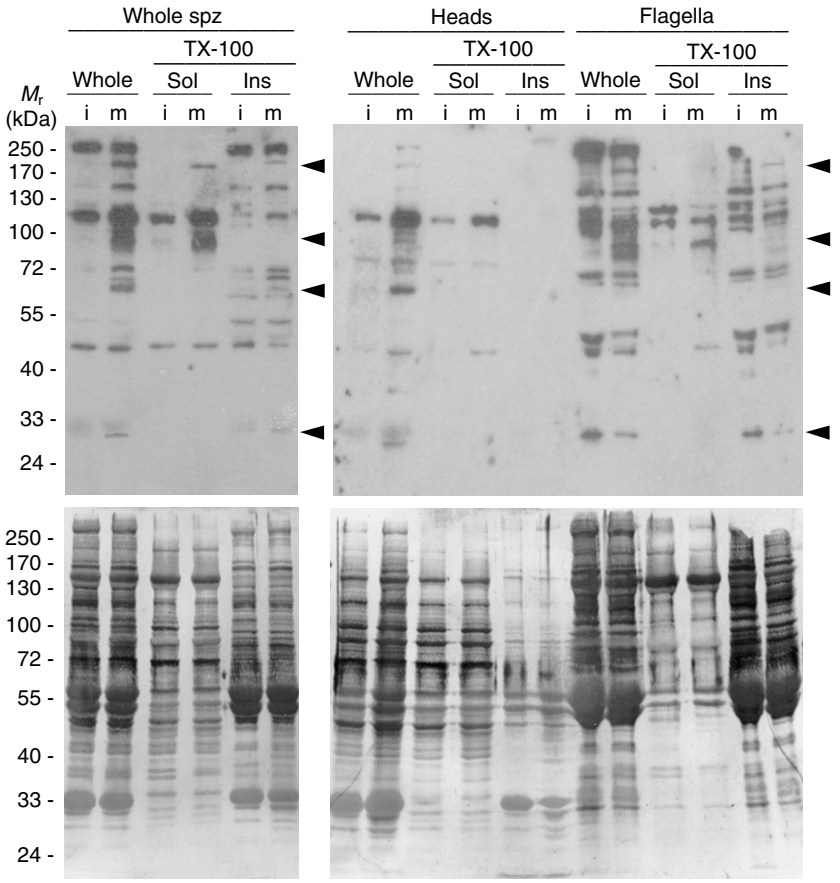

Fig. 5. Cellular localization of phospho-PKC substrates. Sea urchin spermatozoa were mechanically separated into heads and flagella and then detergent fractionated with Triton X-100. The soluble phospho-PKC substrate proteins of spermatozoa, as detected with an anti-phospho-PKC substrate antibody, were predominantly found in the sperm heads while those recovered in the Triton X-100-insoluble sperm fraction were mainly associated with the flagella. The arrowheads indicate the position of the 200, 100, 65 and 28 kDa bands. i, immotile; m, motile; sol, Triton X-100 soluble; insol, Triton X-100 insoluble. A blot representative of two other blots is presented.

including the 28 kDa one, were apparent in flagellar fractions of motile *versus* immotile sperm. Changes in protein phosphorylation patterns were likely due to head/tail mechanical dissociation plus Triton X-100 solubilization treatments. The 65 kDa protein was among the few phospho-PKC substrate bands associated with the sperm head. The latter were essentially all Triton X-100 soluble, but the 65 kDa phospho-PKC substrate was undetectable after that treatment, suggesting a weaker phosphorylation stability of this protein in the presence of the detergent.

Detection of PKC in intact sea urchin spermatozoa

The presence of PKC itself in sea urchin sperm was investigated by immunoblotting and immunolocalization using an antibody against phophorylated PKC (Figs 6 and 7) (phospho-PKC pan detects several PKC isoforms only when phosphorylated at a carboxy-terminal residue homologous to Ser^{660} of PKC βII).

On immunoblots (Fig. 6), two protein bands with an M_r of 135–140 and 50–52 kDa were detectable with that antibody in immotile spermatozoa. When the immotile spermatozoa were transferred into ASW to initiate their motility, the higher band was not detected anymore whereas the 50–52 kDa band was still clearly visible with no significant change in phosphorylation intensity. The molecular mass of the 135–140 kDa band is about 60% higher than that of the PKC isoforms usually detected in mammalian cells (78–85 kDa) but is close to the 132 kDa value of Pkc1, a more primitive PKC found in the yeast *Saccharomyces cerevisiae* (Mellor and Parker, 1998). The 50–52 kDa protein band is a regularly detected cleavage product, corresponding to the catalytic portion of PKC, also known as protein kinase M (PKM) (Pontremoli et al., 1990). Additional sperm fractionation results (D.W., unpublished) indicated that the 135–140 kDa PKC protein was found in the head and flagellum and was solubilized by Triton X-100 while the 50–52 kDa cleavage product was clearly associated with the axonemal fraction of the flagellum (Triton X-100 insoluble).

Immunolocalization experiments confirmed the presence of both PKC and phospho-PKC substrates on sperm head and flagellum (Fig. 7). In the presence of the anti-phospho-PKC pan antibody, fluorescence was observed mostly along the sperm flagellum and also as a spot at the tip of the head (acrosomal region). The discontinuous fluorescent pattern was noticeably

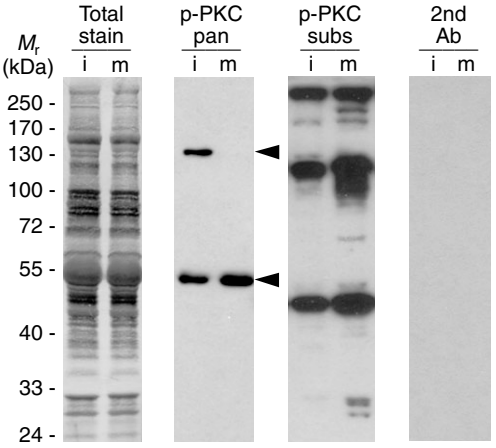

Fig. 6. PKC phosphorylation change following the initiation of motility in intact sea urchin spermatozoa. The presence of PKC and its catalytic cleavage product (arrowheads) was evidenced by immunoblotting with an anti-phospho-PKC antibody (pan). The protein phosphorylation profile obtained with the anti-phospho-PKC substrate antibody (subs) is presented for comparison. i, immotile; m, motile. A blot representative of two other blots is presented.

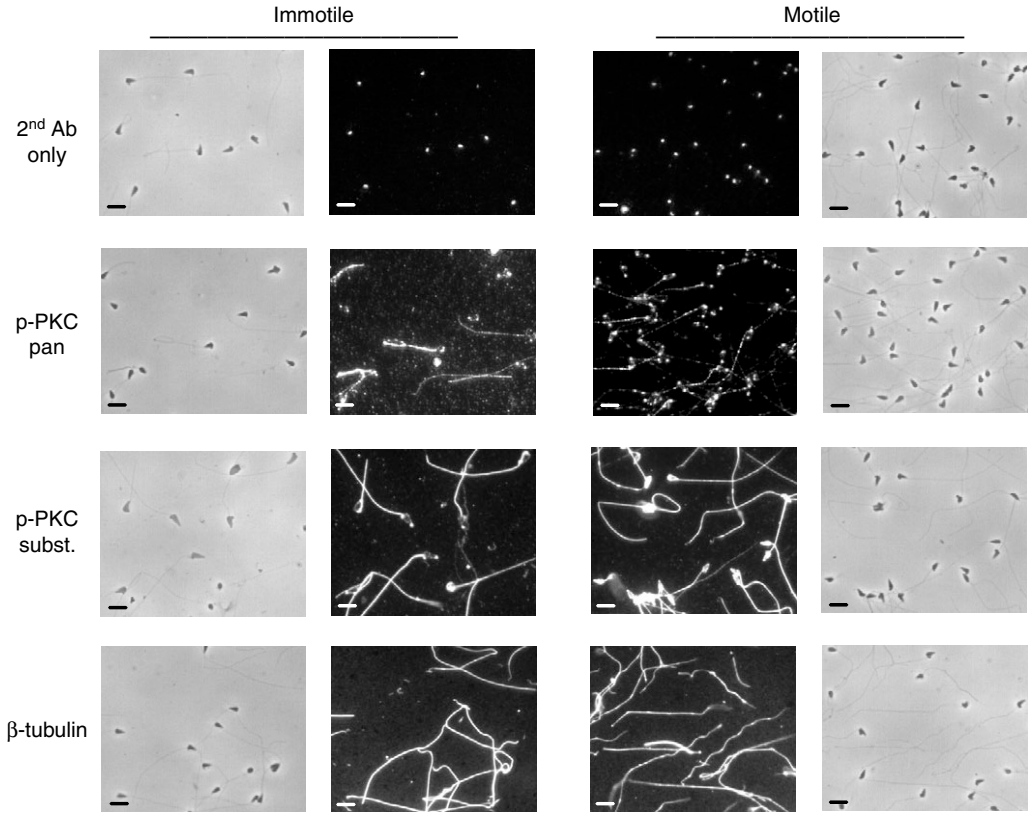

Fig. 7. Immunolocalization of PKC and phospho-PKC substrates on sea urchin spermatozoa. The presence of PKC and its phosphorylated substrates were evidenced by immunofluorescence with an anti-phospho-PKC antibody (p-PKC pan) and an anti-phospho-PKC substrate (p-PKC subst.) antibody in both immotile and motile spermatozoa (central columns). Labeling of flagellar β-tubulin with a specific monoclonal antibody (see Materials and methods) is also shown for comparison purposes. In controls without any primary antibody (2nd Ab only), a weak spot of fluorescence was present at the base of sperm heads. The outer columns show similar images to those in the middle columns but viewed by phase-contrast. Scale bars, 10 μm.

more pronounced in motile spermatozoa compared with the immotile ones and may suggest a redistribution of PKC along the flagellum upon motility initiation. On the other hand, the fluorescence observed after labeling with the anti-phospho-PKC substrates was more intense and uniformly distributed. In this case, stronger fluorescence intensity was observed on sperm head of motile spermatozoa compared with the immotile ones. No significant difference was noted at the flagellum level, and the fluorescence intensity there was comparable to that found on flagella of spermatozoa incubated with the anti-β-tubulin antibody D66. The visible small fluorescent spot surrounding the base of the sperm head was due to unspecific marking, as shown in controls without the primary antibody.

Discussion

Using a series of protein kinase inhibitors, we have shown for the first time that PKC has a key role in the signal transduction mechanisms during initiation and maintenance of motility in intact sea urchin spermatozoa. Strong inhibitory effects on motility were observed with the three PKC inhibitors tested, chelerythrine being the most potent, arresting motility at concentrations starting from 1.5–2 μmol l^{-1} (Fig. 1). We also demonstrated that the increase in phosphorylation of several PKC substrate proteins observed during motility initiation was prevented in the presence of those inhibitors (Fig. 4). The majority of the phospho-PKC substrate proteins, and PKC itself, were found to be associated with the flagellum, both in the Triton X-100-soluble portion (membrane plus cytosol) and the cytoskeleton (axoneme).

Results obtained with the kinase inhibitors (Table 1) could also indicate that PKA, PTK and the adaptor protein Grb2 are potentially involved in intact sea urchin sperm motility, but suggest at the same time that these proteins are not major signaling transducers in this process. Among the PKA inhibitors or modulators tested, only H-89 at ⩾30 μmol l^{-1} demonstrated a significant inhibitory effect on the motility of intact sea urchin spermatozoa. Moreover, at such a concentration range, H-89 has been reported to act on other kinases, including PKC (Chijiwa et al., 1990). The other substances that reduced the motility of intact sea urchin sperm were tyrphostin A47, a PTK inhibitor, and CGP78850, a Grb2-SH2 competitor affecting the ERK pathway (Table 1). However, even at concentrations reported to block the PTK or ERK pathway on intact cells (Thundathil et al., 2002; de Lamirande and Gagnon, 2002; Liguori et al., 2005), the sperm movement was never completely arrested within the 15–20 min observation period (~30% decrease of the percentage of motile spermatozoa). As for PKA inhibition, none of the other PTK or Grb2 inhibitors tested was effective in modifying intact sea urchin sperm motility (Table 1), suggesting again that PTK and kinases of the ERK pathway are not key modulators in the initiation and maintenance of intact sea urchin sperm motility. On the other hand, staurosporine, a broad-spectrum kinase inhibitor, was as effective as chelerythrine in blocking sea urchin sperm motility (Table 1) and the corresponding increase of phospho-PKC substrate phosphorylation (data not shown), while H-7 failed to do so. Considering that PKC appeared to be a central signaling molecule for the motility of intact sea urchin sperm, this discrepancy may be partly explained by the fact that staurosporine is at least 1000 times

more efficient than H-7 as a PKC inhibitor (Table 1) (Schächtele et al., 1988). As a matter of fact, both chelerythrine and staurosporine are chemically related microbial alkaloids that directly exert their action on the catalytic domain of PKC (Herbert et al., 1990; Tamaoki et al., 1986).

PKA and its cofactor cAMP have been implicated in motility regulation of numerous flagellated and ciliated cells, including sea urchin spermatozoa (Brokaw, 1987; Tash, 1989; Chaudhry et al., 1995; Inaba, 2003; Gagnon and de Lamirande, 2006). PTK is also involved in the initiation of flagellar movement in rainbow trout spermatozoa (Hayashi et al., 1987). However, most of these studies were performed using *in vitro* models where cells are first demembranated in the presence of a neutral detergent (usually Triton X-100) prior to artificial reactivation of movement following the addition of exogenous ATP. Due to the disruption of kinase/substrate compartmentalization at the membrane level, molecular signaling features that are not necessarily representative of the *in vivo* situation likely occur. Moreover, it has also been reported that detergent alone could have a major stimulatory effect on reactivation of dog sperm (Tash, 1989) and activate PKA towards the phosphorylation of certain bovine sperm membrane proteins (Noland et al., 1984). This may explain why PKA, PTK or Grb2 inhibitors were inoperative or not as effective as the PKC inhibitors in reducing motility of intact sea urchin sperm. In agreement with this, some experiments done on Triton X-100 demembranated sea urchin spermatozoa indicated that they could reactivate normally despite the presence of chelerythrine in the final reactivation medium (D.W. and J. Cosson, unpublished), suggesting that the targeted PKC of this inhibitor has been removed or modified by the detergent treatment. Nevertheless, even in those sperm demembranation–reactivation systems, investigators have also found cAMP-independent protein phosphorylation of axonemal proteins and suggested the involvement of kinases other than PKA in the regulation of flagellar motility (Chaudhry et al., 1995; Morita et al., 2004; Nakajima et al., 2005).

Corresponding with the rapid initiation of sea urchin sperm motility in ASW, we found an increase in the level of at least four phospho-PKC substrates (M_r of 200, 100, 65 and 28 kDa) that also always occurred within seconds. Interestingly, in a previous study using anti-phospho-Ser and anti-phospho-Thr antibodies, increased phosphorylation on Ser residues of a few sea urchin flagellar proteins (29, 32 and 45 kDa) was observed during the motility activation of intact spermatozoa (Bracho et al., 1998). Allowing for slight differences in SDS–PAGE and molecular mass standards, it is very likely that the 28 kDa phospho-PKC substrate and the 29 kDa Ser phosphorylated protein are identical since both of these were recovered in the axonemal fraction of the sperm flagella. While we observed an apparent dephosphorylation of the 28 kDa protein from immotile to motile sperm (Fig. 5), Bracho et al. reported no change in the phosphorylation level of the 29 kDa one (Bracho et al., 1998). At least five other phospho-PKC substrates were similarly affected in that axonemal fraction. Whether this decrease in phosphorylation is real or a consequence of the manipulations (high-speed pelleting, washing and demembranation) inflicted on the flagella is unknown at this point. Modified phosphorylation of some flagellar protein bands has previously been reported following fractionation and differential centrifugation and/or solubilization treatment (Chaudhry et al., 1995; Bracho et al., 1998). The lack of detection of the head-associated 65 kDa phospho-PKC substrate after detergent solubilization probably denotes phosphorylation instability consecutive to that treatment as well. We also detected a phospho-PKC substrate with a M_r of 45 kDa, but no definitive correlation between its phosphorylation level and motility initiation was observed in our case. This latter discrepancy might be explained by the different sensitivity of antibodies used in both studies to detect the phospho-proteins. In the report of Bracho et al. (Bracho et al., 1998), this 45 kDa protein was not seen in whole sperm samples but only detected in isolated flagella or axonemes. While we have to be cautious when comparing with demembranation–reactivation models (for the reasons discussed earlier), it is interesting to note that a phosphorylation increase was shown on three axonemal proteins, among which was a 45 kDa polypeptide, during the motility reactivation of demembranated starfish sperm (Nakajima et al., 2005). This rise in phosphorylation was induced by an increase in intracellular pH independently of cAMP. In freshwater-acclimated tilapia fish, Ser/Thr dephosphorylation has been observed on two low-molecular-mass axonemal proteins upon reactivation of demembranated spermatozoa (Morita et al., 2003). Intracellular Ca^{2+} release was required to reactivate motility but, again, the presence of cAMP was not necessary. In hypertonic conditions (seawater-acclimated tilapia fish), those two phospho-proteins remained phosphorylated following sperm motility reactivation but still dependent on the presence of Ca^{2+} (Morita et al., 2004). Even though the nature of the kinases involved was not investigated in the above two examples, PKC and/or its PKM cleavage product (see discussion below) certainly appear as potential candidates according to our results with sea urchin sperm. Moreover, activation of sperm flagellar motility in sea urchin has been linked to increases in intracellular pH and Ca^{2+} (Christen et al., 1982; Lee et al., 1983; Brokaw, 1987). It is known that conventional PKC isoforms have a requirement for Ca^{2+} for their activation (Newton, 1995) as well as calpain, the enzyme that cleaves PKC into PKM, a catalytic active form of PKC (Melloni and Pontremoli, 1989; Pontremoli, 1990).

Phosphorylation levels of phospho-PKC substrates further rose to a maximum at around 6 min and stayed almost at this level for up to an hour before decreasing a few hours later (Fig. 3). Moreover, the percentage of sperm motility remained relatively constant during the first 60 min and then dropped significantly after 5 h. This correlation between motility and the level of phospho-PKC substrates, as well as the inhibitory effect of chelerythrine, calphostin C and Gö6976 on these two phenomena (Figs 1 and 4), strongly suggests that PKC activation and phosphorylation of its target proteins are tightly associated with or may even be an absolute requirement for the maintenance of motility in intact sea urchin spermatozoa. It is reasonable to speculate that some of these phospho-PKC substrates are most likely exerting their ultimate effect on axonemal dyneins, the molecular motors powering motility, considering that the majority of these were recovered in the flagella (Triton X-100-soluble and -insoluble flagellar fractions) (Fig. 5). Strong reduction of sea urchin sperm velocities

observed in the presence of chelerythrine and calphostin C (Fig. 1B) is also in agreement with the previous statement. It is known that reduced dynein activity is mechanically translated by a decrease in flagellar beat frequency and therefore in slower swimming of spermatozoa (Porter and Sale, 2000).

PKC detection in the flagellum (Figs 6 and 7) is of particular interest and is consistent with the fact that PKC inhibitors can rapidly stop intact sea urchin sperm movement. Moreover, molecular redistribution of PKC upon motility initiation is suggested by immunolocalization experiments (Fig. 7) where a clear patchy fluorescence distribution was observed along the flagellum of motile spermatozoa compared with immotile sperm. This is in agreement with reports linking the presence of PKC to human sperm flagellar motility (Rotem et al., 1990; Kalina et al., 1995) because of the direct correlation between the number of PKC-stained sperm cells and the number of motile spermatozoa. It was later shown by electron microscopy and the immunogold technique that PKC was ultrastructurally localized in patches along the mid-, principal and end pieces of the flagellum, demonstrating a close association of PKC with flagellar axonemes and outer dense fibers in human spermatozoa as well as in the acrosome, equatorial segment and post-acrosomal region of the sperm head (Kalina et al., 1995). As a matter of fact, we also found a PKC fluorescent spot in the acrosomal region at the tip of the sea urchin sperm head. Moreover, a role for PKC has been suggested in the acrosome reaction of mammalian spermatozoa (De Jonge et al., 1991; Breitbart et al., 1992; Lee et al., 1987). Even though weakly visible, the PKC band was also detectable by immunoblotting in the sea urchin sperm head with the anti-phospho-PKC pan antibody (D.W., unpublished results).

Although the M_r (135–140 kDa) of the PKC protein is about 60% higher than those of the majority of PKC isozymes usually detected in mammalian cells (78–85 kDa) (Newton, 1995), it may just represent a specific sea urchin PKC isoform. For instance, the molecular mass of Pkc1, a PKC found in the yeast *S. cerevisiae*, is 132 kDa (Antonsson et al., 1994; Mellor and Parker, 1998). PKC represents a large family of enzymes with at least 10 isoforms (Mellor and Parker, 1998). In the sea urchin, four different PKC genes have been found in the recently released *Strongylocentrotes purpuratus* genome (Bradham et al., 2006). The PKC inhibitors, as well as the antibodies used herein, can act (or react) on several mammalian PKC isoforms, and not necessarily with the same potency on each isoform. These chemicals were not equally effective in inhibiting intact sea urchin sperm motility, which may suggest the participation of one or more specific PKC isoforms. Nevertheless, any attempt at this point to discriminate between PKC isoforms remains totally speculative. Differentiating between PKC isozymes as well as establishing their distribution in sea urchin sperm cells are definitely questions to be addressed in the future.

In mammalian cell systems, it is known that PKC undergoes three sequential phosphorylation steps (the last two being autophosphorylations) to acquire its full activation (Newton, 1995). The anti-phosphorylated PKC antibody used in our study (phospho-PKC pan) (Fig. 6) specifically recognizes the phosphorylated carboxy-terminal residue Ser^{660} of PKC, which is the last phosphorylation step allowing the release of the mature enzyme into the cytosol and its eventual translocation to the inner membrane anchoring sites and most likely other cellular locations, including cytoskeletal elements (Keranen et al., 1995; Keenan and Kelleher, 1998). The immunoblotting results presented in Fig. 6 showed that the phosphorylation level of the 135–140 kDa band (likely to be the full PKC protein) was much higher in immotile compared with motile sperm cells whereas that of the 52 kDa band also detected by the anti-phospho-PKC antibody was not significantly different in motile spermatozoa. Although not investigated in the present study, the 52 kDa protein may represent the free catalytic PKC subunit known as PKM, a constitutively active enzyme of 50 kDa resulting from the cleavage of PKC by calpain (Pontremoli et al., 1990). Since the anti-phospho-PKC pan antibody is directed against an epitope located on the catalytic region of the molecule, it seems logical not to find as much of the 135–140 kDa band if its cleavage is stimulated following motility initiation of sea urchin spermatozoa. In this context, one can hypothesize that the PKC catalytic fragment PKM, liberated from its membrane association, could therefore diffuse freely into the cytosol to target and phosphorylate various axonemal structures and particularly dynein arm components. Interestingly, fractionation of sea urchin spermatozoa (D.W., unpublished results) indicated that the 135–140 kDa PKC-like protein was found in the Triton X-100-soluble fraction (containing membrane + cytosol) of the head and flagellum while the 50–52 kDa degradation product was clearly associated with the axonemal fraction only (Triton X-100 insoluble) and would agreed with such a cascade of events. Moreover, the presence of calpain, a calcium-activated protease (Melloni and Pontremoli, 1989), has been reported in human (Rojas et al., 1999), mouse (Ben-Aharon et al., 2005) and fowl spermatozoa (Ashizawa et al., 1994), and even in this latter case, a role for calpain and PKC in the regulation of sperm motility was suggested. Similar activation scenarios, with PKC converted to PKM, were demonstrated for the phosphorylation of myosin in the sustained contraction mechanism of the smooth muscle cell (Andrea and Walsh, 1992) and, more recently, for the enhanced transmission of dopamine from specific rat neurons (Liu et al., 2007). Moreover, PKM, with the PKC N-terminal regulatory domain removed, was shown to have long-lasting phosphorylation capabilities (Andrea and Walsh, 1992), which, in this regard, would represent a strong asset for sperm cells to maintain their motility until fertilization occurs. Nevertheless, the precise role of that PKC cleavage product in sea urchin sperm motility remains to be clarified.

Taken together, the data presented here strongly indicate for the first time that, *in vivo*, which means on intact sea urchin spermatozoa, PKC, most likely through its cleavage into the active catalytic product PKM, is a central signaling mediator associated with the maintenance of sperm motility. PKC inhibitors such as chelerythrine and calphostin C, as well as staurosporine, were found to rapidly arrest the motility of sea urchin spermatozoa freshly released into seawater. At the same time, these inhibitors prevented the motility-associated increase in phosphorylation of several PKC substrates. Sperm fractionation and immunolocalization further indicated a clear association of the PKC-like enzyme with the flagellum and a

tight link between the majority of phospho-PKC substrates and the flagellar axoneme.

List of abbreviations

ASW	artificial seawater
dbcAMP	dibutyryl-cAMP
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ERK	extracellular signal-regulated kinase
Grb2	growth factor receptor-bound protein 2
HRPO	horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IM	immotility medium
PKA	protein kinase A
PKC	protein kinase C
PKM	protein kinase M
PTK	protein tyrosine kinase
Rp-AMPs	Rp-adenosine-3′,5′-monophosphorothionate
TTBS	Tris-buffered saline with Tween 20

We are grateful to Drs Jacky Cosson, Philippe Huitorel and Gérard Prulière (Université P. et M. Curie) for revising the manuscript and their helpful comments. This work was realized with the financial support of the Canadian Institute for Health Research to C.G.

References

Andrea, J. E. and Walsh, M. P. (1992). Protein kinase C of smooth muscle. *Hypertension* **20**, 585-595.

Antonsson, B., Montessuit, S., Friedli, L., Payton, M. A. and Paravicini, G. (1994). Protein kinase C in yeast. Characteristics of the *Saccharomyces cerevisiae* PKC1 gene product. *J. Biol. Chem.* **269**, 16821-16828.

Ashizawa, K., Katayama, S., Kobayashi, T. and Tsuzuki, Y. (1994). Possible role of protein kinase C in regulation of flagellar motility and intracellular free Ca^{2+} concentration of fowl spermatozoa. *J. Reprod. Fertil.* **101**, 511-517.

Audebert, S., White, D., Cosson, J., Huitorel, P., Eddé, B. and Gagnon, C. (1999). The carboxy-terminal sequence Asp427-Glu432 of β-tubulin plays an important function in axonemal motility. *Eur. J. Biochem.* **261**, 48-56.

Ben-Aharon, I., Brown, P. R., Etkovitz, N., Eddy, E. M. and Shalgi, R. (2005). The expression of calpain 1 and calpain 2 in spermatogenic cells and spermatozoa of the mouse. *Reproduction* **129**, 435-442.

Bracho, G. E., Fritch, J. J. and Tash, J. S. (1997). A method for preparation, storage, and activation of large populations of immotile sea urchin sperm. *Biochem. Biophys. Res. Commun.* **237**, 59-62.

Bracho, G. E., Fritch, J. J. and Tash, J. S. (1998). Identification of flagellar proteins that initiate the activation of sperm motility *in vivo*. *Biochem. Biophys. Res. Commun.* **242**, 231-237.

Bradham, C. A., Foltz, K. R., Beane, W. S., Arnone, M. I., Rizzo, F., Coffman, J. A., Mushegian, A., Goel, M., Morales, J., Geneviere, A. M. et al. (2006). The sea urchin kinome: a first look. *Dev. Biol.* **300**, 180-193.

Breitbart, H., Lax, J., Rotem, R. and Naor, Z. (1992). Role of protein kinase C in the acrosome reaction of mammalian spermatozoa. *Biochem. J.* **281**, 473-476.

Brokaw, C. J. (1987). Regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation. *J. Cell. Biochem.* **35**, 175-184.

Chaudhry, P. S., Creagh, S., Yu, N. and Brokaw, C. J. (1995). Multiple protein kinase activities required for activation of sperm flagellar motility. *Cell Motil. Cytoskeleton* **32**, 65-79.

Cheung, L. H. (1995). Isolation of flagella and their membranes from sea urchin spermatozoa. In *Methods in Cell Biology, Vol. 47, Cilia and Flagella* (ed. W. Dentler and G. Witman), pp. 43-46. New York: Academic Press.

Chijiwa, T., Mishimu, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990). Inhibition of Forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthetized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.* **265**, 5267-5275.

Christen, R., Shackmann, R. W. and Shapiro, B. M. (1982). Elevation of intracellular pH activates respiration and motility of sperm of the sea urchin, *Strongylocentrotus purpuratus*. *J. Biol. Chem.* **257**, 14881-14890.

Christen, R., Shackmann, R. W. and Shapiro, B. M. (1983). Metabolism of sea urchin sperm. Interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration. *J. Biol. Chem.* **258**, 5392-5399.

Cosson, M. P. and Gagnon, C. (1988). Protease inhibitor and substrates block motility and microtubule sliding of sea urchin and carp spermatozoa. *Cell Motil. Cytoskeleton* **10**, 518-527.

Darszon, A., Beltran, C., Felix, R., Nishigaki, T. and Trevino, C. L. (2001). Ion transport in sperm signaling. *Dev. Biol.* **240**, 1-14.

Davis, P. D., Hill, C. D., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. and Wilkinson, S. E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Lett.* **259**, 61-63.

De Jonge, C. J., Han, H. L., Mack, S. R. and Zaneveld, L. J. D. (1991). Effect of phorbol diesters, synthetic diacylglycerols, and a protein kinase C inhibitor on the human sperm acrosome reaction. *J. Androl.* **12**, 62-70.

de Lamirande, E. and Gagnon, C. (2002). The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. *Mol. Hum. Reprod.* **8**, 124-135.

Gagnon, C. and de Lamirande, E. (2006). Controls of sperm motility. In *The Sperm Cell: Production, Maturation, Fertilization, Regeneration* (ed. C. De Jonge and C. Barratt), pp. 108-133. Cambridge: Cambridge University Press.

Gagnon, C., White, D., Huitorel, P. and Cosson, J. (1994). A monoclonal antibody against the dynein IC1 peptide of sea urchin spermatozoa inhibits the motility of sea urchin, dinoflagellate, and human flagellar axonemes. *Mol. Biol. Cell* **5**, 1051-1063.

Hayashi, H., Yamamoto, K., Yonekawa, H. and Morisawa, M. (1987). Involvement of tyrosine protein kinase in the initiation of flagellar movement in rainbow trout spermatozoa. *J. Biol. Chem.* **262**, 16692-16698.

Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrand, J. P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **172**, 993-999.

Inaba, K. (2003). Molecular architecture of the sperm flagella: molecules for motility and signaling. *Zool. Sci.* **20**, 1043-1056.

Jacobson, G. and Karsnäs, P. (1990). Important parameters in semi-dry electrophoretic transfer. *Electrophoresis* **11**, 46-52.

Kalina, M., Socher, R., Rotem, R. and Naor, Z. (1995). Ultrastructural localization of protein kinase C in human sperm. *J. Histochem. Cytochem.* **43**, 439-445.

Keenan, C. and Kelleher, D. (1998). Protein kinase C and the cytoskeleton. *Cell. Signal.* **10**, 225-232.

Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995). Protein kinase C is regulated by three functionally distinct phosphorylations. *Curr. Biol.* **5**, 1394-1403.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of bacteriophage T4. *Nature* **227**, 680-685.

Lee, H. C. (1984). Sodium and proton transport in flagella isolated from sea urchin spermatozoa. *J. Biol. Chem.* **259**, 4957-4963.

Lee, H. C., Johnson, C. and Epel, D. (1983). Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. *Dev. Biol.* **95**, 31-45.

Lee, M. A., Kopf, G. S. and Storey, B. T. (1987). Effects of phorbol esters and diacylglycerol on the mouse sperm acrosome reaction induced by the zona pellucida. *Biol. Reprod.* **36**, 617-627.

Liguori, L., de Lamirande, E., Minelli, A. and Gagnon, C. (2005). Various protein kinases regulate human sperm acrosome reaction and the associated phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif. *Mol. Hum. Reprod.* **11**, 211-221.

Liu, Y., Doré, J. and Chen, X. (2007). Calcium influx through L-type channels generates protein kinase M to induce burst firing of dopamine cells in the rat ventral tegmental area. *J. Biol. Chem.* **282**, 8594-8603.

Melloni, E. and Pontremoli, S. (1989). The calpains. *Neuroscience* **12**, 438-444.

Mellor, H. and Parker, P. J. (1998). The extended protein kinase C superfamily. *Biochem. J.* **332**, 281-292.

Morita, M., Takemura, A. and Okuno, M. (2003). Requirement of Ca^{2+} on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus*. *J. Exp. Biol.* **206**, 913-921.

Morita, M., Takemura, A. and Okuno, M. (2004). Acclimation of sperm motility apparatus in seawater-acclimated euryhaline tilapia *Oreochromis mossambicus*. *J. Exp. Biol.* **207**, 337-345.

Nakajima, A., Morita, M., Takemura, A., Kamimura, S. and Okuno, M. (2005). Increase in intracellular pH induces phosphorylation of axonemal

proteins for activation of flagellar motility in starfish sperm. *J. Exp. Biol.* **208**, 4411-4418.

Newton, A. C. (1995). Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* **270**, 28495-28498.

Noland, T. D., Olson, G. E. and Garbers, D. (1984). Protein phosphorylation of plasma membranes from bovine epididymal spermatozoa. *Biol. Reprod.* **31**, 185-194.

Pontremoli, S., Michetti, M., Melloni, E., Sparatore, B., Salamino, F. and Horecker, B. L. (1990). Identification of the proteolytically activated form of protein kinase C in stimulated neutrophils. *Proc. Natl. Acad. Sci. USA* **87**, 3705-3707.

Porter, M. E. and Sale, W. S. (2000). The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility. *J. Cell Biol.* **151**, F37-F42.

Rojas, F., J,., Brush, M. and Moretti-Rojas, I. (1999). Calpain-calpastatin: a novel, complete calcium-dependent protease system in human spermatozoa. *Mol. Hum. Reprod.* **5**, 520-526.

Rotem, R., Paz, G. F., Homonnai, Z. T., Kalina, M. and Naor, Z. (1990). Protein kinase C is present in human sperm: possible role in flagellar motility. *Proc. Natl. Acad. Sci. USA* **87**, 7305-7308.

Schächtele, C., Seifert, R. and Osswald, H. (1988). Stimulus-dependent inhibition of platelet aggregation by protein kinase C inhibitors polymyxin B, H-7 and staurosporine. *Biochem. Biophys. Res. Commun.* **151**, 542-547.

Tamaoki, T., Nomoto, H., Takahashi, T., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**, 397-402.

Tash, J. S. (1989). Protein phosphorylation: the second messenger signal transducer of flagellar motility. *Cell Motil. Cytoskeleton* **14**, 332-339.

Tash, J. S. and Bracho, G. E. (1994). Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. *J. Androl.* **15**, 505-509.

Thundathil, J., de Lamirande, E. and Gagnon, C. (2002). Different signal pathways are involved during human sperm capacitation induced by biological and pharmacological agents. *Mol. Hum. Reprod.* **8**, 811-816.