Various protein kinases regulate human sperm acrosome reaction and the associated phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif

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Acrosome reaction (AR) is an exocytotic process of fundamental importance for the spermatozoon to fertilize the oocyte. The mechanisms mediating this process are only partially defined. The aim of the present study was to investigate the role of various kinases and the extracellular signal-regulated kinase (ERK) pathway in the induction of the AR and associated phosphorylation of tyrosine (Tyr) residues and of the threonine-glutamic acid-tyrosine (Thr-Glu-Tyr) motif that occurs in 80 and 105 kDa proteins (p80/p105). Human spermatozoa were capacitated and AR was induced with lysophosphatidylcholine in the presence of inhibitors of various kinases and of the ERK pathway. Phosphorylation of Tyr and of Thr-Glu-Tyr peaked 15 min after the induction of the AR. Both phosphorylations were prevented by inhibitors of protein kinase C, MEK, phosphoinositide 3-kinase and Akt but not by protein kinase A inhibitors. Phosphorylation of Thr-Glu-Tyr, but not Tyr, was decreased by inhibitors of protein tyrosine kinase and Grb2-SH2. All the inhibitors prevented lysophosphatidylcholine-induced AR, indicating the involvement of PKC, PTK, PI3K, Akt and the ERK pathway. These results show that phosphorylation of Tyr and Thr-Glu-Tyr are associated with the AR and are differently regulated by the various kinases emphasizing the complexity of this process.

Key words: lysophosphatidylcholine/protein phosphorylation/signalling transduction/spermatozoa/sperm function

Introduction

Mammalian spermatozoa acquire their fertilizing ability either during transit in the female reproductive tract or during incubation in a suitable in vitro media (Yanagimachi, 1994; de Lamirande et al., 1997; Visconti et al., 2002). Such conditioning, called capacitation, renders possible the interaction between spermatozoa and the zona pellucida surrounding the oocyte and the induction of the acrosome reaction (AR) (de Lamirande et al., 1997). AR is an exocytotic process by which lytic enzymes are released from the sperm acrosome and digest the zona pellucida so that spermatozoa can reach and fertilize the oocyte (Yanagimachi, 1994; de Lamirande et al., 1997). Capacitation and AR are triggered by many, but different, effectors and involve different signal transduction pathways. Their molecular basis is still only partially known.

Sperm capacitation is a multistep process that occurs over hours and involves several biochemical and ultrastructural changes such as modifications of membrane lipid composition, increased permeability to ions (Visconti et al., 2002), acquisition of hyperactivated motility (de Lamirande et al., 1997), and phosphorylation of proteins on tyrosine (Tyr), serine (Ser) and threonine (Thr) residues (de Lamirande et al., 1997; Baldi et al., 2000; de Lamirande and Gagnon 2002; Urner and Sakkas, 2003). A cAMP/protein kinase A (PKA)-dependent increase in Tyr phosphorylation of two fibrous sheath proteins, p80 and p105, antigenically related to A-anchoring proteins (AKAPs), is one of the prominent events associated with capacitation (Aitken et al., 1995; Leclerc et al., 1996, 1997; de Lamirande et al., 1997; Baldi et al., 2000; Visconti et al., 2002; Urner and Sakkas, 2003). Components of the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases are present in spermatozoa and involved in capacitation (Luconi et al., 1998; de Lamirande and Gagnon, 2002). There is a gradual increase in the double phosphorylation of the Thr-Glu-Tyr motif, characteristic of activated kinases such as ERK1, 2 and 5 in two proteins of 80 and 105 kDa in capacitating human spermatozoa (de Lamirande and Gagnon, 2002; Thundathil et al., 2002, 2003).

Sperm AR occurs within minutes, cannot be reversed once it is induced and can be triggered in vitro by different inducers, such as zona pellucida (Yanagimachi, 1994), progesterone (Harrison et al., 2000), calcium ionophores (ex: A23187), lysophosphatidylcholine (LPC, a membrane disturbing agent) (de Lamirande et al., 1997), follicular fluid (De Jonge et al., 1993) and ATP (Luria et al., 2002). Sperm AR takes place after fusion between the acrosome and the overlying plasma membrane and involves calcium influx, actin polymerization, rise in intracellular pH, protein activation (phospholipases, kinases, G proteins, etc.), etc. (Yanagimachi, 1994; Baldi et al., 2000).

While protein phosphorylation is regarded as a hallmark of capacitation, the role of protein kinases and phosphorylation has not been fully characterized. The aim of the present study was to investigate the role of various kinases and the extracellular signal-regulated kinase (ERK) pathway in the induction of the AR and associated phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif that occurs in 80 and 105 kDa proteins (p80/p105). Human spermatozoa were capacitated and AR was induced with lysophosphatidylcholine in the presence of inhibitors of various kinases and of the ERK pathway. Phosphorylation of Tyr and of Thr-Glu-Tyr peaked 15 min after the induction of the AR. Both phosphorylations were prevented by inhibitors of protein kinase C, MEK, phosphoinositide 3-kinase and Akt but not by protein kinase A inhibitors. Phosphorylation of Thr-Glu-Tyr, but not Tyr, was decreased by inhibitors of protein tyrosine kinase and Grb2-SH2. All the inhibitors prevented lysophosphatidylcholine-induced AR, indicating the involvement of PKC, PTK, PI3K, Akt and the ERK pathway. These results show that phosphorylation of Tyr and Thr-Glu-Tyr are associated with the AR and are differently regulated by the various kinases emphasizing the complexity of this process.
been as well studied in sperm AR. Observations that cAMP and PKA activity increase during AR (Lefèvre et al., 2002) and that KT5720 (PKA inhibitor) completely prevents the follicular fluid-induced AR in human spermatozoa, suggest a role for PKA in this process (De Jonge et al., 1993). In addition, the release of calcium that occurs during AR is stimulated by cAMP and blocked by H89 (PKA inhibitor) in isolated bovine sperm acrosomes (Spungin and Breitbart, 1996). PKC also appears to be involved since PKC inhibitors cause a partial (stauroporine and bisindolylmaleimide) to total (calphostin C) decrease in human sperm AR (De Jonge et al., 1993; Bonaccorsi et al., 1998). Furthermore, bovine sperm AR induced either by A23187 or by PKC activators (phorbol 12-myristate 13-acetate and 1-oelyo-2-acetyl-sn-glycerol), is prevented by stauroporine and H7 (Breitbart et al., 1992; Bonaccorsi et al., 1998).

The inhibition of AR triggered either by mannose-bovine serum albumin or by an antibody raised against the sperm zona pellucida receptor kinase by wortmannin (Fisher et al., 1998) suggests also a role for phosphoinositide 3-kinase (PI3K) in AR. Akt, a downstream effector of PI3K is present in human spermatozoa (Aquila et al., 2004; Nauc et al., 2004) and is involved in capacitation (Nauc et al., 2004), but its Akt during AR has not yet been investigated.

Protein tyrosine kinases (PTK) are of paramount importance in AR and proteins of 80 and 105 kDa are the main substrates of these enzymes (Leyton and Saling, 1989; Tesarik et al., 1996; Baldi et al., 2000). PTK inhibitors (tyrphostin A47, genistein, and lavendustin A) block human sperm AR stimulated by progesterone, (Luconi et al., 1995; Meizel and Turner, 1996; Kirkman-Brown et al., 2002) and LPC and A23187-induced AR are associated with higher levels of Tyr phosphorylation that is prevented by PTK inhibitors (de Lamirande et al., 1998; de Lamirande and Gagnon, 2002). Additionally, triggering of AR with calcium in modelled human spermatozoa (permobilized with streptolysin-O) causes an increase in Tyr phosphorylation that is prevented by PTK inhibitors (genistein, tyrphostin A47, and tyrphostin A51) (Tomes et al., 2004). The role of kinases other than PTK in the modulation of Tyr phosphorylation during AR is not yet established.

Few studies report on the participation of the ERK pathway in AR. Incubation of capacitated human spermatozoa with progesterone causes a redistribution of ERK 1 and 2 proteins from the postacrosomal region to the equatorial segment of the sperm head (Luconi et al., 1998). PD98059 (inhibitor of ERK activation by MEK) prevents human sperm AR induced by A23187 and zona pellucida (Du Plessis et al., 2001) but not by progesterone (Luconi et al., 1998). LPC-induced AR and protein Tyr phosphorylation are also blocked by PD98059 (de Lamirande and Gagnon, 2002). There is presently no data on the involvement of the ERK pathway elements upstream of MEK or on the double phosphorylation of the Thr-Glu-Tyr motif during human sperm AR.

Although published data support the role of kinases in AR, data on the phosphorylation of Tyr residues and the Thr-Glu-Tyr motif during AR of human spermatozoa and regulation of these events are partial or often absent. Most of the studies report on the participation of one kinase and, due to the different experimental protocols used (inducers, inhibitors, etc.), integration of the results is sometimes difficult. Therefore, the first aim of this study was to investigate the role of kinases, PKC, PKA, PTK, PI3K, Akt and of the ERK pathway, in LPC-induced AR. The time course of Tyr and Thr-Glu-Tyr phosphorylation during AR was studied and the regulation of the phosphorylation events by the above mentioned kinases was investigated in an attempt to better understand the signalling pathways involved in AR.

Materials and methods

Materials

Percoll was obtained from Amersham Pharmacia Biotech (Baie d’Urfé, QC, Canada). Lysophosphatidylcholine (LPC), fluorescein isothiocyanate-conjugated Pisum sativum agglutinin and H89 were purchased from Sigma Chemical Co. (St. Louis, MO). CPG78850 was a generous gift from Novartis Pharma Inc. (Basel, Switzerland). PD98059, U126, U124, ZM336372, wortmannin, KT5720, Akt inhibitor, G66976, chelerythrine, herbimycin A, calphostin C, PP2, PP3, tyrphostin A47, tyrphostin A1, sulindac sulphide, β-glycerylphosphate, okadaic acid, and Rp-cAMP-S were purchased from Calbiochem (San Diego, CA). Upstate Biotechnology (Lake Placid, NY) was the supplier of anti-phospho-Tyr (P-Tyr) (clone 4G10) antibody. The polyclonal antibody raised against the phosphorylated Thr-Glu-Tyr motif (P-Thr-Glu-Tyr-P) was bought from Cell Signalling Technology (Beverly, MA). 3-Cyclohexylamino-1-propane sulphonic acid was purchased from Fisher Scientific (Nepean, ON, Canada). Nitrocseulose (0.2 µm pore size; Osmonics Inc., Westborough, MA), donkey anti-rabbit IgG and goat anti-mouse IgG, both conjugated to horse-radish peroxidase (Cedarlane Laboratories Ltd, Hornby, ON, Canada), an enhanced chemiluminescence kit (Lumi-Light; Roche Molecular Biochemicals, Laval, QC, Canada) and radiographic films (Fuji, Minami-Ashigara, Japan) were used for immunodetection of blotted proteins. All other chemicals were of at least reagent grade.

Fetal cord blood was collected at the birthing centre of the Royal Victoria Hospital (Montreal, QC, Canada). Informed consent was obtained from the patients and the ethics board of the Royal Victoria Hospital approved the present study. Fetal cord blood samples were centrifuged (1000 g, 10 min, 4°C) and supernatants were pooled and frozen at −20°C until use. Ultrafiltrate of fetal cord serum (FCSu) was prepared from three pools of 14–24 different samples using YM3 membranes with an exclusion limit of 3 kDa (Amicon, Oakville, ON, Canada).

Chemicals to be tested with spermatozoa were dissolved in distilled water or dimethylsulphoxide which in the incubation media never exceeded 1% (v/v), a condition that does not affect sperm capacitation or AR.

Sperm preparation, treatments and induction of AR

Semen samples from healthy volunteers were washed on four-layer (95–65–40–20%) Percoll gradients buffered in HEPES-balanced saline (115 mM NaCl, 4 mM KCl, 0.5 mM MgCl2, 14 mM fructose and 25 mM HEPES, pH 8.0). Samples were centrifuged for 30 min at 1500 g and sperm cells at the 65–95% Percoll interface and in the 95% Percoll layer were pooled and diluted to 250 × 106 cells/ml in the 95% Percoll solution. Only samples in which progressive motility was greater than 70% were used. Spermatozoa were further diluted in Biggers–Whitten–Whittingham medium (BBW, pH 8.0) (Biggers et al., 1971) devoid of bicarbonate and bovine serum albumin (BSA) and containing 1 mM CaCl2. Spermatozoa were resuspended at 40 × 106 cells/ml in BBW medium without (control) or with FCSu (10%, v/v) as a capacitation inducer and incubated for 3.5 h at 37°C. FCSu induces capacitation and the related hyperactivated motility and protein Tyr phosphorylation (Leclerc et al., 1996, 1997; de Lamirande et al., 1997, 1998). Furthermore, capacitation induced either with FCSu or BSA proceed with similar kinetics and mechanisms and is associated with a cAMP/PKA-dependent Tyr phosphorylation of fibrous sheath proteins (Leclerc et al., 1997), the generation of reactive oxygen species (Herrero et al., 2000), activation of PKA (Lefèvre et al., 2002; O’Flaherty et al., 2004), and modification of the sulphydryl content of Triton-soluble proteins (de Lamirande and Gagnon, 2003). The bicarbonate provided by FCSu (about 2 mM) is sufficient for capacitation since addition of 25 mM bicarbonate to the incubation medium does not further increase in the percentage of capacitation (Leclerc et al., 1996). LPC (2.5 µM) was then added and sperm incubated for an additional 30 min to induce the AR as previously justified (de Lamirande et al., 1997, 1998). ZP3, from oocytes zona pellucida, is the natural stimulant for AR but is not commercially available. LPC was chosen as AR inducer because it is a membrane disturbing lipid previously justified (de Lamirande et al., 1998). Furthermore, bovine sperm AR induced either with FCSu or BSA proceed with similar kinetics and mechanisms and is associated with a cAMP/PKA-dependent Tyr phosphorylation of fibrous sheath proteins (Leclerc et al., 1997), the generation of reactive oxygen species (Herrero et al., 2000), activation of PKA (Lefèvre et al., 2002; O’Flaherty et al., 2004), and modification of the sulphydryl content of Triton-soluble proteins (de Lamirande and Gagnon, 2003). The bicarbonate provided by FCSu (about 2 mM) is sufficient for capacitation since addition of 25 mM bicarbonate to the incubation medium does not further increase in the percentage of capacitation (Leclerc et al., 1996). LPC (2.5 µM) was then added and sperm incubated for an additional 30 min to induce the AR as previously justified (de Lamirande et al., 1997, 1998). ZP3, from oocytes zona pellucida, is the natural stimulant for AR but is not commercially available. LPC was chosen as AR inducer because it is a membrane disturbing lipid found in the amnion liquid of the female genital tract (Grippo et al., 1994) and is as efficient as A23187 to trigger AR and the related protein Tyr phosphorylation (de Lamirande et al., 1997, 1998). Furthermore, LPC is
soluble in aqueous media and, under our experimental conditions, does not affect sperm viability or motility (de Lamirande et al., 1997). Substances such as LPC or A23187, trigger AR in a subpopulation of spermatozoa that were previously incubated under capacitating conditions. Throughout the text ‘acrosome reacting spermatozoa’ or ‘spermatozoa undergoing AR’ will refer to these samples in which a subpopulation of spermatozoa undergo AR after stimulation with LPC. Spermatozoa were washed after treatment with LPC and fixed with ethanol. Inhibitors of kinases were added to spermatozoa 15 min before the addition of LPC. Sperm AR was evaluated after staining with fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (Cross et al., 1986). For each sample, at least 250 spermatozoa were counted.

None of the chemicals used caused a decrease in sperm motility over a 3.5 h incubation period at 37°C. In preliminary experiments, the effect of every treatment on sperm viability was also evaluated by the hypoosmotic

Figure 1. Time course of the phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif in human spermatozoa during LPC-induced AR. (A) Percoll-washed spermatozoa resuspended in BWW medium were incubated for 3.5 h without (−) or with (+) FCSu (10%, v/v) as capacitation inducer. Aliquots were taken (Time 0), LCP (2.5 μM) was added and aliquots were withdrawn 5, 10, 15, and 30 min later. Proteins of 0.1 × 10^6 (for P-Tyr) and 0.6 × 10^6 (for P-Thr-Glu-Tyr-P motif) spermatozoa were loaded in each well, electrophoresed, electrotransferred and immunoblotted using the anti-P-Tyr and anti-P-Thr-Glu-Tyr-P antibodies. The level of phosphorylation was estimated by densitometry analysis as described in Materials and methods. The density value of bands obtained in control spermatozoa (BWW medium alone) after 3.5 h of incubation, before the addition of LPC (Time 0) was used to normalize the other values. Immunoblotting results from an experimental representative of four in which semen samples from different donors were used. Densitometry results are mean ± SEM of 4 values. * value different from that obtained in control spermatozoa after the same incubation period. # value different from that obtained before the addition of LPC. (B) The specificity of the anti-P-Thr-Glu-Tyr-P antibody was verified by preadsorption with the blocking peptide, P-Thr-Glu-Tyr-P, and with a mixture of P-Thr and P-Tyr (5 mM each) as described in Materials and methods.
swelling test after the induction of AR (Aitken et al., 1993). The percentages of dead/acrosome reacted spermatozoa was always very low (2–3%) and was not affected by the treatments (inhibitors, LPC, FCSu).

Differences in the percentages of AR were tested by analysis of variance (two-tails, paired values). Differences between the effects of various treatments were then determined by the protected least-significant difference test and considered statistically significant with $P < 0.05$.

### SDS-PAGE, immunoblotting and densitometry

A time course study for protein phosphorylation (P-Tyr and P-Thr-Glu-Tyr-P) was first performed to determine the best incubation time for the subsequent experiments. Spermatozoa were incubated in the absence or presence of FCSu for 3.5 h. An aliquot of spermatozoa was withdrawn to determine the phosphorylation associated with capacitation (Time 0 in Figure 1). LPC was then added, aliquots were taken 5, 10, 15 and 30 min later. Sperm samples were solubilized with electrophoresis sample buffer containing sodium vanadate (100 mM), β-glycerophosphate (20 mM), sodium fluoride (5 mM), and okadaic acid (10 mM), boiled and centrifuged. Sperm proteins were electroblotteded on 10% polyacrylamide gels and electrotransferred (10 mM Cyclohexylamino-1-propanol sodium sulfonic acid buffer, pH 11.0 containing 10% methanol) to nitrocellulose membranes. The membranes were incubated with a solution of skimmed milk (5%, w/v) in Tris (20 mM, pH 7.8)-buffered saline containing Tween 20 (0.1%, v/v) to nitrocellulose membranes. The membranes were incubated with a solution of skimmed milk (5%, w/v) in Tris (20 mM, pH 7.8)-buffered saline containing Tween 20 (0.1%, v/v) and the anti-P-Thr-Glu-Tyr antibody was diluted 1:1000 in TTBS supplemented with sodium azide (0.1%, w/v) and the anti-P-Thr antibody was diluted 1:10000 in TTBS supplemented with sodium azide (0.1%, w/v) for 45 min at 20°C and washed again with TTBS. Positive immunoreactive bands were detected using the Lumi-Light chemiluminescence kit. Membranes were then rinsed in distilled water and silver stained to verify that an equal amount of protein was loaded in each well.

Specificity of the two antibodies has been demonstrated (Leclerc et al., 1996; Thundathil et al., 2002). However, as the use of the anti-P-Thr-Glu-Tyr-P antibody is relatively new, tests for specificity were repeated. The antibody incubated with its blocking peptide (P-Thr-Glu-Tyr-P; gift from Cell Signalling Technology, Beverly, MA) at a molar ratio of 10:1 (peptide:antibody) did not recognize any protein bands confirming the specificity of the antibody for the P-Thr-Glu-Tyr-P motif (Figure 1B). Preadsorption of the antibody with a mixture of P-Tyr and P-Thr (5 mM each) did not modify the signal obtained (Figure 1B) confirming that the anti-P-Thr-Glu-Tyr-P antibody does not bind to P-Tyr or P-Thr.

Time course results showed that maximal levels of phosphorylation occurred 15 min after addition of LPC. Therefore, the effects of inhibitors of different signalling pathways (Table I) on the phosphorylation of Tyr and Thr-Glu-Tyr were investigated with this incubation time.

The level of phosphorylation was estimated by densitometry of bands using the Un-Scan-It gel (version 5.1) software from Silk Scientific Co. (Orem, UT). Because the intensities of the two protein bands 80 and 105 kDa were often very different, the analysis, when needed, was performed on films of different exposure times to allow the best definition of the bands. In all cases, the contribution of the background was subtracted and care was taken that the measured densities are proportional to the amounts of proteins and that bands analysed are not saturated. For the time course study, intensities were normalized to the values obtained with control spermatozoa (BWW medium alone) after 3.5 h incubation to determine the relative increases in intensities due to addition of LPC to control and capacitated (FCSu; undergoing AR) spermatozoa. In studies performed with inhibitors of kinases, intensities were normalized to the value obtained with spermatozoa undergoing AR to determine the level of inhibition due to these chemicals. Differences between samples and treatments were evaluated by variance analysis (two-tails, paired values) followed by the protected least-significant difference test. A difference was considered to be statistically significant with $P < 0.05$.

### Results

#### Time course study of phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif during LPC-induced AR

Time course for the phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif during human sperm AR was first performed to determine the best incubation time for the subsequent experiments on the regulation of phosphorylation. Spermatozoa were first incubated in the absence (control cells) or in the presence of FCSu (capacitating cells) for 3.5 h. Levels of spontaneous AR (no addition of LPC) were always low and similar for control and capacitated spermatozoa (3–4% data not shown). LPC was then added. The levels of P-Tyr and P-Thr-Glu-Tyr-P were estimated prior to (Time 0 min; basal level for control and capacitated cells) and at various time periods

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**Table I. Inhibitors used in the study**

<table>
<thead>
<tr>
<th>Pathway or kinase</th>
<th>Inhibitor</th>
<th>Target action</th>
<th>IC50 (µM)</th>
<th>Concentration used (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC</td>
<td>chelerythrine</td>
<td>inhibits PKC translocation</td>
<td>0.66</td>
<td>3</td>
<td>O’Toole et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>calphostin C</td>
<td>competes with diacylglycerol</td>
<td>0.05</td>
<td>0.15</td>
<td>Bonaccorsi et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Go6976</td>
<td>Ca²⁺-dependent PKC α1 and B1</td>
<td>0.002–0.03</td>
<td>0.006</td>
<td>Makranz et al. (2004)</td>
</tr>
<tr>
<td>PKA</td>
<td>IB9</td>
<td>ATP-site inhibitor</td>
<td>0.048</td>
<td>10</td>
<td>Leclere et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>Rp-cAMP-S</td>
<td>competes with cAMP</td>
<td>0.006</td>
<td>0.1</td>
<td>de Jonge et al. (1993)</td>
</tr>
<tr>
<td>PTK</td>
<td>tyrophostin A47</td>
<td>receptor type PTK</td>
<td>0.005</td>
<td>0.01</td>
<td>Thundathil et al. (2002); Tomes et al. (2004)</td>
</tr>
<tr>
<td>PP2</td>
<td>non-receptor type PTK</td>
<td>8–12</td>
<td>10</td>
<td>Leclere et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>herbimycin A</td>
<td>competes for Grb2-SH2</td>
<td>0.043</td>
<td>30</td>
<td>Gay et al. (1999); de Lamirande and Gagnon (2002)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Wortmannin</td>
<td>catalytic subunit</td>
<td>0.058–0.072</td>
<td>0.3</td>
<td>de Lamirande and Gagnon (2002)</td>
</tr>
<tr>
<td>Akt</td>
<td>Akt inhibitor</td>
<td>phosphatidylinositol ester analog</td>
<td>5</td>
<td>10</td>
<td>Fisher et al. (1998); Nauc et al. (2004)</td>
</tr>
</tbody>
</table>

References are representative for use of these chemicals on spermatozoa except for the following: *Go6976 tested on macrophages; †Akt inhibitor tested on spermatozoa.
after LPC addition by immunoblotting sperm proteins with specific antibodies (Figure 1).

The antibodies recognized two protein bands of 80 and 105 kDa, similar to those observed during capacitation (Leclerc et al., 1996; Thundathil et al., 2002, 2003) and AR induced by LPC or A23187 (Aitken et al., 1995; de Lamirande et al., 1998). The levels of P-Tyr and P-Thr-Glu-Tyr-P in p80 and p105 increased after the addition of LPC and was higher in acrosome reacting than in control spermatozoa. The phosphorylation of Tyr progressively increased in spermatozoa undergoing AR reaching a plateau at 15 min. The time course of Thr-Glu-Tyr phosphorylation was different. No significant increase was noted at 5 and 10 min but the maximal level was also obtained at 15 min. Because of these results, a 15 min incubation period after LPC addition was chosen for subsequent experiments on the regulation of Tyr and Thr-Glu-Tyr phosphorylation. Results do not rule out the possibility that other proteins may be phosphorylated during AR but the amount of these proteins and/or their level of phosphorylation would then be much lower than those of p80 and p105 since no bands were detected with longer exposure time (data not shown).

**Effect of PKC inhibitors on AR and associated phosphorylations**

In many studies reporting on the role of PKC during AR (Breitbart et al., 1992), inhibitors such as staurosporin and H7 were used even though these chemicals also strongly inhibit other kinases such as PKA (Burnatowska-Hledin et al., 2000). The three inhibitors, chelerythrine, calphostin C and Gö6976 (Table I), used in the present study prevented LPC-induced AR but none, when tested alone, had any effect on the phosphorylation of Tyr and Thr-Glu-Tyr-related to LPC-induced AR (Figure 2). However, phosphorylation levels of p105 were decreased in spermatozoa treated with a mixture of chelerythrine and calphostin C. Combination of the three PKC inhibitors did not cause any additional effect as compared to that of chelerythrine and calphostin C (n = 3; data not shown).

**Effect of PKA inhibitors on AR and associated phosphorylations**

Three PKA inhibitors, H89, KT5720, and Rp-cAMP-S, that act by different mechanisms (Table I) were used. LPC-induced AR was partially prevented by H89, totally by KT5720, and unaffected by Rp-cAMP-S (Figure 3). On the other hand, these inhibitors, used

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**Figure 2.** PKC inhibitors: effect on LPC-induced AR and on the associated phosphorylation of Tyr and of Thr-Glu-Tyr. Percoll-washed spermatozoa resuspended in BWW medium were incubated for 3.25 h without (grey bars) or with (white bar) FCSu (10%, v/v) as capacitation inducer. Chelerythrine (3 μM, Che), calphostin C (150 nM, Calph), or Gö6976 (30 nM, Gö) were added and incubated for 15 min prior to the addition of LPC (2.5 μM). The AR status was evaluated as described in Materials and methods and results are presented in the upper panel. Values are mean ± SEM of eight values obtained with sperm samples from different donors. *: value different from that obtained in control spermatozoa (BWW medium for 3.5 h and then LPC). Proteins of 0.1 £ 10^6 and 0.6 £ 10^6 spermatozoa, respectively for P-Tyr and the P-Thr-Glu-Tyr-P motif, were loaded in each well and immunoblotted as described in Materials and methods. Results of an immunoblotting experiment, repeated five times with similar results and different donors, are shown. The level of phosphorylation was estimated by densitometry analysis as described in Materials and methods. Density of bands was normalized to 100 using the value obtained with spermatozoa undergoing AR (capacitated and then treated with LPC). Results presented in the histogrammes are mean ± SEM of five values. #: inhibition as compared to the value obtained with spermatozoa undergoing AR.
alone or in combination, did not affect the levels of P-Tyr and P-Thr-Glu-Tyr-P of p80 and p105 (Figure 3).

**Effect of PTK inhibitors on AR and associated phosphorylations**

The PTK inhibitors, PP2, herbimycin A, and tyrphostin A47 (Table I), blocked LPC-induced AR (Figure 4). Inactive analogues of tyrphostin A47 and of PP2, tyrphostin A1 and PP3, respectively, had no effect. Treatment with PP2 or herbimycin A did not modify the level of P-Tyr in p105 but the combination of these two substances affected that of p80 (Figure 4). Tyrphostin A47 had an all-or-none effect. In five sperm samples there was a complete inhibition of Tyr phosphorylation related to AR but in the five others no effect was noted (Figure 4). Tyrphostin A1 did not affect the level of P-Tyr of p105 and p80 in any case (data not shown).

The combination of PP2 and herbimycin A, but not each compound individually tested, prevented the increase of P-Thr-Glu-Tyr-P in p80 and p105 (Figure 4). As observed for P-Tyr, tyrphostin A47 totally inhibited the phosphorylation of Thr-Glu-Tyr in 5 out of the 10 sperm samples (Figure 4). Again, tyrphostin A1 did not affect the level of P-Thr-Glu-Tyr-P of p105 and p80 in any case (data not shown).

The effect of tyrphostin A47 appeared to be donor specific and not related to day-to-day experimental conditions. When spermatozoa from two or three donors were tested on the same day, the effect (all-or-none) of tyrphostin A47 on protein phosphorylation was not the same for all donors. However, sperm samples in which Tyr phosphorylation was prevented by tyrphostin A47 were also those in which the phosphorylation of Thr-Glu-Tyr was blocked.

**Effect of wortmannin and of the Akt inhibitor on AR and associated phosphorylations**

An inhibitor of PI3K, wortmannin and Akt (Table I) totally blocked LPC-induced AR (Figure 5). Both substances prevented the Tyr phosphorylation of p80 while the Akt inhibitor had an effect on p80 and p105. The double phosphorylation of the Thr-Glu-Tyr motif was blocked by both inhibitors in p105 but not in p80 (Figure 5).

**Effect of ERK pathway inhibitors on AR and associated phosphorylations**

In previous studies, PD98059 prevented AR induced by LPC (de Lamirande and Gagnon, 2002) but not by A23187 or zona pellucida (Du Plessis et al., 2002). Because MEK can be activated by other kinases than Raf (ex: PKC), inhibitors of elements of the cascade, upstream of MEK, were tested. CGP78850, sulindac sulphide, ZM336372, PD98059, U126, but not U124 (Table I) totally

Figure 3. PKA inhibitors: effect on LPC-induced AR and on the associated phosphorylation of Tyr and of Thr-Glu-Tyr. Sperm treatments and evaluation of acrosomal status, immunoblotting and analysis of the results are described in the caption of Figure 2 and Materials and methods. H89 (10μM), Rp-cAMP-S (200μM, Rp), KT5720 (100nM, KT) were added to the sperm samples after 3.25 h of incubation and LPC 15 min later. Values for LPC-AR are mean ± SEM of six values, obtained with sperm samples from different donors. #: value different from that obtained in control spermatozoa. H89 partially prevented LPC-AR, i.e. the value is higher than that of control spermatozoa but lower than that of FCSu-treated spermatozoa. Results from an immunoblotting experiment, repeated five times with similar results and different donors, are shown. Results of relative intensity are mean ± SEM of five values. #: inhibition as compared to the value obtained with spermatozoa undergoing AR (capacitated and then treated with LPC; normalization standard).
prevented the LPC-induced AR in capacitated spermatozoa (Figure 6). The role of ERK pathway on the phosphorylation of Tyr and Thr-Glu-Tyr was evaluated with CGP78850 and U126, which block the first and the last step of the ERK cascade, respectively. U126 was chosen as MEK inhibitor rather than PD98059 because an inactive analogue, U124, is available. CGP78850 prevented the increase of P-Thr-Glu-Tyr-P for p80 and p105 and that of P-Tyr for p80. U126, but not U124, totally prevented the increase of P-Tyr and P-Thr-Glu-Tyr-P in both p80 and p105 (Figure 6).

**Discussion**

The data presented here indicate that PKC, PKA, PTK, PI3K, Akt and the ERK pathway participate in LPC-induced AR and that most of these transduction elements are involved in the increases of P-Tyr and of P-Thr-Glu-Tyr that occur during AR and affect two protein bands of 80 and 105 kDa. However, these phosphorylations are regulated by different mechanisms.

Most of the published articles supporting the role of kinases in AR deal with the effect of only one kinase and data on the associated phosphorylations are partial or often absent. To better understand the signalling pathways involved in AR, we investigated, in the same series of experiments, the role of kinases in LPC-induced AR and in the regulation of associated Tyr and Thr-Glu-Tyr phosphorylations.

Time course studies first indicated a rise in the phosphorylation of p80 and p105 in spermatozoa undergoing AR (Figure 1). This finding is new for the Thr-Glu-Tyr motif and is consistent with the reported phosphorylation of Tyr during AR induced by LPC (de Lamirande et al., 1998, de Lamirande and Gagnon, 2002) and A23187 (Aitken et al., 1995). Phosphorylation peaked 15 min after addition of LPC (Figure 1), which is expected since AR is completed within 15–30 min (Yanagimachi, 1994).

Sperm capacitation (Leclerc et al., 1996; Thundathil et al., 2002) and AR (Aitken et al., 1995; de Lamirande et al., 1998; present article) affect the levels of P-Tyr and P-Thr-Glu-Tyr-P of p80 and p105. However, regulation of these phosphorylation events (Tyr versus Thr-Glu-Tyr, p105 versus p80, capacitation versus AR) suggests that target proteins during capacitation and AR are different or that identical proteins are phosphorylated at different sites. Tyr and Thr-Glu-Tyr phosphorylations are differently modulated during capacitation (Leclerc et al., 1996, 1997; Thundathil et al., 2002) and the same is true for AR. For example, the level of P-Thr-Glu-Tyr-P on p105, but not that of P-Tyr, is affected by herbimycin A + PP2 (Figure 4), wortmannin (Figure 5) and CGP78850 (Figure 6). Also, wortmannin affected the level of P-Tyr in p80 not the level of P-Thr-Glu-Tyr in p105. Tyr phosphorylation depends on cAMP/PKA and non-receptor type PTK during capacitation (de Lamirande et al., 1997; Leclerc et al., 1997; Baldi et al., 2000; Visconti et al., 2002) but not during AR (Figure 3). Therefore, the increased phosphorylation of p80 and p105 noted in sperm AR does not appear to be due to the participation of a common regulatory pathway leading to the phosphorylation of the same proteins. A more plausible explanation

![Figure 4. PTK inhibitors: effect on LPC-induced AR and on the associated phosphorylation of Tyr and of Thr-Glu-Tyr. Sperm treatments and evaluation of acrosomal status, immunoblotting and analysis of the results are described in the caption of Figure 2 and Materials and methods. Tyrphostin A47 (100 μM, A47), Tyrphostin A1 (100 μM, A1), PP2 (10 nM), PP3 (10 nM), herbimycin A (10 μM, Herb) were added to the samples 15 min prior to the addition of LPC. Values for LPC-AR are mean ± SEM of 10 values obtained with sperm samples from different donors. *: value different from that obtained in control spermatozoa. Results of a representative immunoblotting experiment, repeated 10 times with similar results and different donors, are shown. Tyrphostin A47 was tested on spermatozoa from 10 different samples. In five samples there was a complete inhibition of P-Tyr and P-Thr-Glu-Tyr-P (upper blots, (A)), but in the other five samples, tyrphostin A47 did not affect the phosphorylation levels [lower blots, (B)]. Results of relative intensity are mean ± SEM of five (tyrphostin A47-treated spermatozoa) or ten values (other conditions). #: inhibition as compared to the value obtained with spermatozoa undergoing AR (capacitated spermatozoa and then treated with LPC; normalization standard).](https://academic.oup.com/molehr/article-abstract/11/3/211/1097814)
could be that proteins carrying Tyr and Thr-Glu-Tyr and subjected to phosphorylation during capacitation and AR are different, or the phosphorylation sites on these proteins are different. Herrero et al. (2001) noted that the higher level of P-Tyr of a 85 kDa protein band during capacitation was due to an increased P-Tyr level in many proteins (isoelectric points 5.8–6.4). Such a phenomenon is also possible for AR, and protein bands of 80 and 105 kDa could contain many proteins with different phosphorylation levels and types.

Antigens recognized by the anti-P-Tyr and anti-P-Thr-Glu-Tyr-P antibodies are localized at the principal piece of the sperm flagellum (Leclerc et al., 1997; Thundathil et al., 2003). The increased phosphorylation of p80 and p105 related to capacitation was proposed to be related to the acquisition of hyperactivated motility (Leclerc et al., 1996, 1997; Thundathil et al., 2003). The same explanation is plausible for spermatozoa undergoing AR since only hyperactivated cells can penetrate the viscous environment of the zona pellucida (Stauss et al., 1995).

Results obtained with chelerythrine, calphostin C and Gö6976 (Figure 2) not only confirm the reported (De Jonge et al., 1993; Bonaccorsi et al., 1998) involvement of PKC in human sperm AR but also show the complex role of PKC in related phosphorylations. Calphostin C and chelerythrine, when combined, prevented the phosphorylation of Tyr and Thr-Glu-Tyr in p105 (Figure 2). These data might suggest that either two types of PKC or two steps of PKC activation are needed and when one is inhibited, the other one takes over. Chelerythrine affects the translocation of PKC from the cytosol to plasma membrane, whereas calphostin C hinders the interaction between the catalytic and the regulatory domain by competing at the binding site of diacylglycerol and phorbol esters (Table I). It could be hypothesized that the Tyr and Thr-Glu-Tyr phosphorylation on p105 and related to LPC-induced AR is prevented by blocking the translocation and thus the activation of PKC. Calcium-dependent PKC α1 and B1 did not seem to modulate p80 and p105 phosphorylation since Gö6976, alone or in combination with the two other inhibitors was ineffective (Figure 2). Nevertheless, the three PKC inhibitors blocked AR, indicating that PKC acts on targets other than p80 and p105.

AR induced by A23187 is associated with increases in cAMP levels and PKA activity in human spermatozoa (Lefèvre et al., 2002). Also, inhibitors of PKA and agents that disrupt the PKA A-kinase anchoring proteins (AKAP) interaction block the AR induced by follicular fluid and progesterone (De Jonge et al., 1993; Harrison et al., 2000). However, the role of PKA in Tyr phosphorylation during AR is not clearly established since most data available have been obtained under different conditions (species, AR inducers, etc.) and always only one inhibitor (Baldi et al., 2000; Flesh and Gadella, 2000). H89 and KT5720 prevented LPC-induced AR, whereas Rp-cAMP-S did not (Figure 3). This result could be due to the different modes of action of these chemicals: H89 and KT5720 interfere with the kinase activity, whereas Rp-cAMP-S acts one step earlier and prevents dissociation of the holoenzyme (Table I). Our result with

Figure 5. Wortmannin and Akt inhibitor: effect on LPC-induced AR and on the associated phosphorylation of Tyr and of Thr-Glu-Tyr. Sperm treatments and evaluation of acrosomal status, immunoblotting and analysis of the results are described in the caption of Figure 2 and Materials and methods. Wortmannin (10 nM, Wort) and Akt inhibitor (10 μM, Akt inh) were added to the samples 15 min prior to the addition of LPC. Values for LPC-AR are mean ± SEM of five values obtained with sperm samples from different donors. *: value different from that obtained in control spermatozoa. Results of an immunoblotting experiment, repeated five times with similar results and different donors, are shown. Results of relative intensity are mean ± SEM of at least five values. #: inhibition as compared to the value obtained with spermatozoa undergoing AR (capacitated and then treated with LPC; normalization standard).
Rp-cAMP-S differs from that of Harrison et al. (2000) who reported that Rp-bromo-cAMP prevents AR triggered by progesterone; however, the cAMP analogue was used at 500 μM and also decreased the quality of sperm motility. Our results confirm that PKA is involved in AR but not in protein Tyr phosphorylation (Figure 3) as it was during capacitation (Leclerc et al., 1996; de Lamirande et al., 1997; Baldi et al., 2000; Visconti et al., 2002). PKA substrates and/or downstream effectors during AR might be acrosomal proteins since PKA activation is likely to play a role before and during the release of intracellular calcium stores (Spungin and Breitbart, 1996; Harrison et al., 2000).

The activation of a sperm receptor type PTK by ZP3 (Leyton and Saling, 1989) and progesterone (Tesarik et al., 1996) is fundamental to AR induction and genistein, a general PTK inhibitor competitive for ATP, prevents progesterone-induced AR in human spermatozoa (Kirkman-Brown et al., 2002). Herbimycin A, PP2 and tyrphostin A47 prevented LPC-induced AR (Figure 4), indicating that both receptor type and non-receptor type PTK are required. The mixture of PP2 and herbimycin A blocked the increase of P-Thr-Glu-Tyr-P (p80 and p105) but not that of P-Tyr (p105) (Figure 4), suggesting that non-receptor type PTKs likely act upstream of the MEK or MEK-like kinase responsible for the phosphorylation of Thr-Glu-Tyr rather than on Tyr phosphorylation of p105. These data also hint that several PTK with different sensitivities to inhibitors act on the same target and that when one is inhibited, the other takes over.

Tyrphostin A47 had an all-or-none effect on P-Tyr and P-Thr-Glu-Tyr-P of p80 and p105 (Figure 4). However, the effect of PTK inhibitors depend on the phenomenon studied (capacitation or AR) and the experimental conditions used. During human sperm capacitation, herbimycin A but not genistein or tyrphostin A47 block Tyr phosphorylation (Leclerc et al., 1997) but the increase in P-Thr-Glu-Tyr-P is prevented by tyrphostin A47 but not by PP2 (Thundathil et al., 2002). However, genistein blocks the increase in P-Tyr related to A23187- and progesterone-induced AR (Kirkman-Brown et al., 2002) and tyrphostin A47 inhibits that observed during the calcium-induced AR in streptolysin-O-permeabilized cells (Tomes et al., 2004). Therefore, different PTKs appear to be involved in the phosphorylation events associated with AR and capacitation and the effect of PTK inhibitors on these phosphorylation events during AR depend on multiple variables (stimulant, treatment of cells, etc.).

Wortmannin prevented LPC-induced AR (Figure 5), in agreement with results by Fisher et al. (1998) who used an antibody raised against the sperm zona pellucida receptor kinase or mannose-BSA to trigger AR. Only wortmannin was used in the present study since LY294002, another PI3K inhibitor, promotes calcium influx, capacitation (Nauc et al., 2004) and protein Tyr phosphorylation through a cAMP/PKA-dependent mechanism that is unrelated to inhibition of PI3K (Nauc et al., 2004; Luconi et al., 2004). Possibly because of these non-specific effects, LY294002 does not prevent zona

Figure 6. ERK pathway: effect on LPC-induced AR and on the associated phosphorylation of Tyr and of Thr-Glu-Tyr. Sperm treatments and evaluation of acrosomal status, immunoblotting and analysis of the results are described in the caption to Figure 2 and Materials and methods. CGP78850 (30 μM, CGP), sulindac sulphide (100 μM, SS), ZM336372 (3 μM, ZM), PD98059 (50 μM, PD), U126 (300 nM), U124 (300 nM) were added to the samples 15 min prior to the addition of LPC. Values for LPC-AR are mean ± SEM of six values obtained with sperm samples from different donors. *: value different from that obtained in control spermatozoa. Results of an immunoblotting experiment, repeated six times with similar results and different donors, are shown. Results of relative intensity are mean ± SEM of six values. #: inhibition as compared to the value obtained with spermatozoa undergoing AR (capacitated and then treated with LPC; normalization standard).
cida-induced AR (Du Plessis et al., 2004). Our data confirm that P3K is involved in AR and further indicate its role in phosphorylation since wortmannin blocked the increase in P-Tyr (p80) and P-Thr-Glu-Tyr-P (p105) related to LPC-induced AR (Figure 5). Akt, a downstream effector of P3K, is present in human sperm membranes, flagella and head and is involved in capacitation (Nauc et al., 2004). The present data show that Akt plays also a role in LPC-induced AR and associated phosphorylation of Tyr and Thr-Glu-Tyr (Figure 5). It could be hypothesized that P3K and Akt modulate AR and related phosphorylations through the activation of nitric oxide synthase, an effector of Akt, and the generation of nitric oxide. Nitric oxide is involved in sperm capacitation, related phosphorylation events and AR (Herrero et al., 1999, 2000; Thundathil et al., 2003). Therefore, the axis P3K, Akt, nitric oxide synthase, nitric oxide could modulate the phosphorylation of Tyr and of Thr-Glu-Tyr during AR.

The MEK inhibitor PD98059, prevented AR induced by LPC (de Lamirande and Gagnon, 2002) but not by A23187 and zona pellucida (du Plessis et al., 2002). Figure 6 shows the importance of the whole ERK cascade in LPC-induced AR and phosphorylation of Tyr and Thr-Glu-Tyr (Figure 6). This result confirms our previous data on the inhibition by PD98059 of LPC-induced AR and related P-Tyr (de Lamirande and Gagnon, 2002) but diverge from those of Luconi et al. (1998). This discrepancy could be explained by the use of inducers (progesterone and A23187 versus LPC) that act by different mechanisms. CGP78850 had no effect on P-Tyr levels (p105) and this could be due to lower sensitivity of spermatozoa undergoing AR. A closely related analogue of CGP78850, CGP85793, prevented sperm capacitation and increase in P-Tyr (de Lamirande and Gagnon, 2002), again emphasizing the difference in the regulation between this process and AR.

In conclusion, data obtained with a variety of inhibitors tested in the same series of experiments indicate that PKC, PKA, PTK, P3K, Akt and the ERK pathway are involved in human sperm AR induced by LPC. Our data also show, for the first time, that the phosphorylation of Tyr and of Thr-Glu-Tyr on 80 and 105 kDa protein bands occur rapidly after the onset of AR and are regulated in several different ways. The mechanisms leading to these phosphorylations share similarities since they were both prevented by inhibitors of PKC, P3K and Akt. However, they also diverge since MEK is needed for Tyr phosphorylation, whereas the whole ERK pathway and non-receptor type PTK are required for the phosphorylation of Thr-Glu-Tyr. Additionally, the fact that AR is blocked under conditions that do not affect the two phosphorylations studied indicates that other phosphorylations on Ser, Thr, Tyr are required for AR.

References

Phosphorylation and acrosome reaction

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