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Effect of protein kinase C on guinea pig sperm acrosome reaction induced by progesterone[1]

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KEY WORDS protein kinase C; acrosome reaction; progesterone; phorbol 12,13-dibutyrate; diacylglycerol; calcium; spermatozoa

ABSTRACT

AIM: To investigate the effect of protein kinase C (PKC) on the guinea pig sperm acrosome reaction (AR) induced by progesterone (P_4). **METHODS**: Guinea pig spermatozoa were preincubated in MCM-Ca^{2+} DF medium at 38.5 ℃ under 5 % CO_2/air for 6-6.5 h for capacitation, and were then washed through a three-step gradient Percoll, resuspended in MCM-Ca^{2+} DF and adjusted to 5×10^9 cells/L. The AR was assessed by means of phase-contrast microscopy (expressed as percent of AR) 15 min after different reagents were added. **RESULTS**: In the presence of Ca^{2+} 2 mmol/L, phorbol 12,13-dibutyrate (PDB) or the permanent synthetic diacylglycerol, 1-oleoyl-2-acetyl-sn-glycerol (OAG) initiated the AR of spermatozoa in a concentration-related manner, whereas staurosporine (STA) exhibited an inhibition of the AR. PDB and OAG significantly enhanced P_4-induced AR that was greater than that treated with either agonist separately. STA markedly inhibited the P_4-induced AR. The AR induced by PDB was completely antagonized by inclusion of egtazic acid. Also, both egtazic acid and nifedipine antagonized the AR induced by P_4 and PDB + P_4. In addition, neomycin also prevented the P_4-induced AR. This effect could be partly reversed by PDB. **CONCLUSION**: Activation or inhibition of PKC significantly plays an important role in the signal transduction pathway for the physiological AR.

INTRODUCTION

At the time of fertilization, mammalian spermatozoa undergo exocytosis of the acrosomal granule in response to oocyte-derived agonists. This essential exocytosis process, acrosome reaction (AR), results in a release or exposure of enzymes necessary for penetration of the egg vestments and to fuse with the oocyte's plasma membrane[1]. Progesterone (P_4) is known to be synthesized and secreted by the cumulus cells surrounding the oocyte[2]. Also, P_4 has been described to exert several sperm functions, including motility, capacitation, and the AR, etc[3]. The agonist activates a specific receptor, $GABA_A$-like receptor or $GABA_A/Cl^-$ receptor, that results in the triggering of AR[4,5]. Yet, the mechanism of the AR induced by P_4 is not completely understood. Early studies have shown that the stimulation of P_4 resulted in the activation of a protein tyrosine kinase and the phosphorylation of a 95 kDa protein[6]. The target for this signal transduction pathway is not known. One of the possibilities is that tyrosine kinase could activate phosphoinositidase C and subsequently lead to the hydrolysis of the polyphosphoinositide (PPI)[7] and stimulation of protein phosphorylation[8]. As protein kinase C (PKC) is found in ram, bull and human sperm acrosome, and P_4 can stimulate phosphoinositide breakdown in guinea pig sperm[9], PKC may be involved in a specific PLC pathway in guinea-pig acrosomal exocytosis induced by P_4. The present study was therefore undertaken to examine whether PKC is involved in guinea pig sperm AR induced by P_4.

MATERIALS AND METHODS

Reagents Sodium pyruvate, bovine serum albumin (BSA, Fraction V), *DL*-sodium lactate, HEPES, phorbol 12,13-dibutyrate (PDB), staurosporine (STA), progesterone (P_4), egtazic acid, neomycin, nifedipine and 1-oleoyl-2-acetyl-sn-glycerol (OAG), the membrane-permeable DAG were purchased from Sigma Chem-

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ical Co, St Louis, MO, USA. Percoll was purchased from Pharmacia LKB (Uppsalá, Sweden). All other reagents were of analytical grade.

Medium The culture medium used in all experiments was Ca^{2+}-deficient minimal capacitation medium (MCM-Ca^{2+} DF), containing NaCl 11.76 mmol/L, KCl 2.7 mmol/L, $MgCl_2 \cdot 6H_2O$ 0.49 mmol/L, $NaHCO_3$ 25 mmol/L, *D*-glucose 2.28 mmol/L, sodium lactate 10 mmol/L, sodium pyruvate 1 mmol/L, HEPES 10 mmol/L, BSA 4 g/L and Ca^{2+} 23 μmol/L. The medium was adjusted to pH 7.9 and sterilized through a millipore filter (0.2 μm) before use, which can induce guinea pig sperm capacitation *in vitro* but does not support the acrosome reaction as the AR needs millimolar levels of Ca^{2+}.

Sperm treatment Spermatozoa were freshly obtained from retired breeder guinea pig vasa deferens and cauda epididymides. The sperm suspension was adjusted to 5×10^{13} cells/L and then incubated at 38.5 ℃ under 5 % CO_2 in air for 6 – 6.5 h (capacitation was evidenced by a rapid synchronized AR 15 min following the addition of Ca^{2+} 2 mmol/L). The suspensions were then centrifuged at 690 × *g* for 18 min through a three-step gradient of Percoll (30 % – 55 % – 75 %). The sediment was resuspended in 10 volumes of fresh MCM and centrifuged at 440 × *g* for 8 min. Sperm was resuspended in MCM and adjusted to 5×10^9 cells/L. Finally, the samples were treated with the activator and inhibitor of PKC, P_4, nifedipine, egtazic acid, and neomycin according to the experiments (Ca^{2+} 2 mmol/L existed in all experiments, unless indicated). After 15 min, the AR was assessed by means of a phase-contrast microscopy.

Statistics Data were expressed as $\bar{x} \pm s$ and compared using *t* test.

RESULTS

Effects of PDB, OAG, and STA on the sperm AR To examine the effects of different concentrations of PDB, OAG (both specific activators of PKC activity)[10] and STA (specific inhibitor of PKC activity)[10-11] on the AR, spermatozoa were preincubated for capacitation and were then either stimulated with OAG 5 – 50 μmol/L and PDB 2.5 – 20 μmol/L separately or were inhibited with STA 0.125 – 2.0 μmol/L in the presence of Ca^{2+} 2 mmol/L. As shown in Tab 1, the effects of PDB and OAG were biphasic, with a concentration-dependent increase in the proportion of spermatozoa undergoing AR at PDB concentrations in the range of 2.5 – 15 μmol/L or OAG concentrations in the range of 5 – 10 μmol/L. Further increase in PDB or OAG concentration resulted in a reduced stimulation. However, STA showed an inhibition of AR at 1.0 μmol/L without affecting sperm motility and acrosome integrity (Tab 1).

Tab 1. Effects of OAG, PDB, and STA on acrosome reaction in guinea pig spermatozoa. $n = 3$ **experiments.** $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* **control.**

Treatment/μmol·L^{-1}		Motility/%	Acrosome reaction/%
Control (Ca^{2+} 2 mmol/L)		86.3 ± 2.5	12.1 ± 2.1
PDB	2.5	86.7 ± 2.9^a	13 ± 4^a
	5	87.7 ± 2.5^a	19 ± 6^b
	10	86.7 ± 1.5^a	31.0 ± 2.7^c
	15	86.7 ± 2.1^a	39.3 ± 3.1^c
	20	86.0 ± 1.7^a	28 ± 4^c
OAG	5	86.7 ± 2.9^a	24 ± 10^c
	10	86.6 ± 2.8^a	32 ± 12^c
	25	86.7 ± 2.7^a	21 ± 4^c
	50	83 ± 6^a	13.0 ± 1.7^a
STA	0.125	80 ± 5^a	9 ± 3^a
	0.25	81 ± 4^a	9 ± 4^a
	0.5	81 ± 8^a	9 ± 4^a
	1.0	80 ± 4^a	5.3 ± 2.5^c
	2.0	73.3 ± 2.9^c	6 ± 3^b

Effects of PDB, OAG, and STA on P_4-induced AR To determine whether PKC is involved in the AR of capacitated sperms stimulated by P_4, spermatozoa were preincubated for capacitation as mentioned above, and then exposed to the reagents for 15 min. The results showed that when spermatozoa were treated with P_4 5 μmol/L, PDB 10 μmol/L, or OAG 5 μmol/L, nearly 30 % of spermatozoa underwent acrosome reaction (Tab 1,2). Exposure of spermatozoa to both activators (P_4 + PDB or P_4 + OAG) resulted in significantly higher AR (~ 50 %) than that after treatment with either agonist separately. However, exposure of spermatozoa to P_4 5 μmol/L and STA 1 μmol/L caused a decrease in AR (~ 13 %) which was remarkably lower than that with P_4 alone (Tab 2). STA partly inhibited the AR induced by P_4 + OAG and P_4 + PDB (Tab 2).

Effect of Ca^{2+} on PKC activation To evaluate whether the activation of PKC depends on Ca^{2+} entry, spermatozoa were exposed to egtazic acid 1 mmol/L or Ca^{2+}-deficient medium and then challenged with either

Tab 2. Effects of PDB, OAG, and STA on P_4-induced AR. $n = 4$ experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* P_4. $^iP < 0.01$ *vs* P_4 + OAG. $^kP < 0.05$ *vs* P_4 + PDB.

Treatment	Acrosome reaction/%
Control (Ca^{2+} alone)	9.7 ± 2.9
P_4	33.3 ± 1.7^{ci}
P_4 + PDB	49 ± 3^{cf}
P_4 + OAG	52 ± 3^{cf}
STA + P_4	13 ± 5^{fi}
STA + P_4 + OAG	32 ± 7^{ci}
STA + P_4 + PDB	43.5 ± 2.4^{ck}

Ca^{2+}: 2 mmol/L; P_4: 5 μmol/L; PDB: 10 μmol/L; OAG: 5 μmol/L; STA: 1 μmol/L.

PDB or OAG. Results indicated that the stimulation of AR with PDB alone was significant in the presence of Ca^{2+} 2 mmol/L. However, the AR was completely abolished when sperm were exposed to egtazic acid and then challenged with PDB. Similarly, the occurrence of AR with PDB or OAG was prevented when sperm were suspended in Ca^{2+}-deficient MCM. To investigate whether the activation of PKC during P_4-induced AR was Ca^{2+} dependent, spermatozoa were exposed to egtazic acid 1 mmol/L and P_4 5 μmol/L, and then treated with PDB 10 μmol/L. Results showed that egtazic acid completely antagonized the AR induced by P_4 5 μmol/L and PDB 10 μmol/L. Also, nifedipine blocked the AR induced by P_4 and PDB (Tab 3), indicating that the activation of PKC during the P_4-induced AR depends upon Ca^{2+}.

Effect of neomycin on P_4-induced AR When spermatozoa were treated with neomycin 10 mmol/L, the AR induced by P_4 was prevented. This effect could partly be reversed by inclusion of PDB 10 μmol/L.

DISCUSSION

The present study shows that ≤15 μmol/L concentration of PDB significantly stimulated P_4-induced AR in guinea pig without affecting the sperm motility. The PKC inhibitor, DAG markedly reduced the P_4-induced AR at a concentration which did not affect sperm motility. PDB and DAG(QAG) as well as P_4 are reported to stimulate phosphatidylinositol 4,5-biphosphate hydrolysis and the AR in guinea pig and human sperm[9,10]. These data support our proposal that PKC plays an important

Tab 3. Effect of Ca^{2+} on PKC activation. $n = 4$ experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* PDB. $^iP < 0.01$ *vs* P_4. $^lP < 0.01$ *vs* P_4 + PDB.

Treatment	Acrosome reaction/%
Control (Ca^{2+} alone)	10.8 ± 1.5
PDB	35 ± 5^c
P_4	34 ± 6^c
P_4 + PDB	49 ± 3^{cfi}
Egtazic acid + PDB	2.3 ± 1.3^{cf}
Egtazic acid + P_4	2.8 ± 1.9^{ci}
Egtazic acid + P_4 + PDB	3.5 ± 2.4^{cil}
Nifedipine + P_4	10.5 ± 1.3^{il}
Nifedipine + P_4 + PDB	25.0 ± 2.6^{cil}
PDB (deficient of Ca^{2+})	2.4 ± 1.3^c
OAG (deficient of Ca^{2+})	1.5 ± 0.6^c

Ca^{2+}: 2 mmol/L; egtazic acid: 1 mmol/L; PDB: 10 μmol/L (when combined with P_4) or 15 μmol/L; P_4: 5 μmol/L; Nifedipine: 100 nmol/L; OAG: 10 μmol/L.

Tab 4. Effect of neomycin on P_4-induced AR. $n = 4$ experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* P_4. $^lP < 0.01$ *vs* neomycin + P_4. $^lP < 0.01$ *vs* P_4 + PDB.

Treatment	Acrosome reaction/%
Control (Ca^{2+} alone)	9.7 ± 2.9
P_4	34 ± 6^{ci}
P_4 + PDB	49 ± 3^{cf}
Neomycin + P_4	11.8 ± 2.1^f
Neomycin + P_4 + PDB	16.8 ± 1.0^{cil}

Ca^{2+}: 2 mmol/L; P_4: 5 μmol/L; PDB: 10 μmol/L; Neomycin: 10 mmol/L.

role in signal transduction pathway underlying the P_4-induced AR. Our data propose that sperm PKC is the site of DAG action and that phospholipase A_2 activation occurs after PKC mobilization takes place. Foresta *et al* (1995)[12] reported that STA inhibited Ca^{2+} influx activated by P_4 in human spermatozoa. This study indicates that the inhibitory action of STA on P_4-induced AR may be due to both inactivation of PKC and inhibition of P_4-induced Ca^{2+} influx.

Our results in the present study show that using egtazic acid (a Ca^{2+} chelator) completely antagonized the PKC activation by PDB or PDB + P_4. Nifedipine (a selective L-type Ca^{2+}-channel blocker) blocked the PKC activation induced by P_4 or P_4 and PDB, suggesting that the activation of PKC take places downstream of Ca^{2+} entry. Adding PDB or OAG alone does not result in stimulation

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of AR in the Ca^{2+}-deficient medium. It strongly suggests that PKC involved in P_4-induced AR was a Ca^{2+}-dependent event. It is known that the PKC subspecies (PKCs) are classified into conventional PKCs (cPKC), novel PKCs (nPKC), and atypical PKCs (aPKC)[13]. cPKC is activated by Ca^{2+}, DAG, and phosphatidylserine (PS). nPKC is DAG- and PS-activated enzymes but Ca^{2+}-independent, and aPKC is DAG- and Ca^{2+}-independent, activated by PS and other activators which have not yet been identified. We may postulate that cPKC is involved in the progesterone-induced AR as it is Ca^{2+}-dependent.

In somatic cells, DAG generated by the hydrolysis of PPI activates and mediates the PKC effect. In our experiments, OAG (an analogue of DAG) can stimulate P_4-induced AR, while STA inhibits the AR induced by P_4 and partly inhibits the AR induced by P_4 + OAG and P_4 + PDB. It suggests that PKC is activated and mediated by DAG.

Neomycin is an aminoglycoside antibiotic that is known to bind strongly and selectively to PPI and has been used as a relatively specific inhibitor of PPI metabolism. We examined the effect of neomycin on the P_4-stimulated acrosomal exocytosis. P_4-induced AR was prevented by inclusion of neomycin 10 mmol/L. Recently, we demonstrated that neomycin prevented the hydrolysis of PPI and the occurrence of AR induced by P_4[9], whereas PDB only partly reversed this effect. One possible explanation for this is that neomycin may inhibit a PKC activity necessary for the AR in spermatozoa, by blocking tyrosin phosphorylation of proteins which is related to the sperm exocytosis[14] and thus results in the abolition of acrosomal exocytosis induced by PDB.

To sum up, two possible roles for PKC in mediating sperm AR can be suggested. One is to activate a selective L-type Ca^{2+} channels which is mediated by DAG that is generated by PIP_2 hydrolysis, another is to activated PLA_2.

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蛋白激酶 C 在孕酮激发的豚鼠精子顶体反应中的作用[1]

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关键词 蛋白激酶 C；顶体反应；孕酮；佛波醇 12，13-二丁酸盐；甘油二酯；钙；精子

目的：研究蛋白激酶 C(PKC)在孕酮激发豚鼠精子顶体反应中的作用. **方法**：豚鼠精子在 Ca^{2+} 缺乏 MCM 培养液中，38.5 ℃ 5 % CO_2/空气获能培养(6－6.5) h 然后经 Percoll 密度梯度离心，再混悬于 MCM 中，调数至 5×10^9 个/升. 加入 PKC 激活剂或抑制剂以及相关试剂 15 分钟后，用相差显微镜评价 AR(以 AR% 表示). **结果**：在钙离子存在下，PDB 或 OAG 可激发豚鼠精子 AR，并可显著提高孕酮激发豚鼠精子 AR 的作用. 然而，STA 不仅可抑制 AR，也可阻止孕酮激发豚鼠精子 AR. 同时，依他酸和硝苯地平均可阻断 P_4 以及 P_4 和 PDB 激发豚鼠精子 AR. 此外，新霉素可抑制 P_4 激发豚鼠精子 AR，而且 PDB 可部分逆转这种作用. **结论**：激活或抑制 PKC 可明显增加或降低 P_4 激发的豚鼠精子 AR，表明 PKC 在生理性 AR 的信号转导通路中起重要作用.

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