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Epidermal growth factor induces acrosomal exocytosis in bovine sperm

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Abstract

At the time of fertilization mammalian spermatozoa undergo a Ca^{2+}-dependent exocytotic event, which is known as the acrosome reaction (AR). We describe here that EGF-receptor (EGFR) is localized in the head of bull spermatozoa and that epidermal growth factor (EGF) can induce the occurrence of the AR in its typical dose-dependent manner. Previously we showed that protein kinase C (PKC) is involved in the cascade leading to AR in bull spermatozoa. Here, we show that PKC is involved in the mechanism in which EGF exerts its effect on AR. These findings together with our results which show inhibition of AR by tyrosine-phosphorylation inhibitors, indicate that ejaculated bull sperm contain a typical 170-kDa EGFR which is active in the mechanism leading to AR.

Key words: Sperm; Acrosomal exocytosis; EGF; EGFR; PKC

1. Introduction

The acrosome reaction is a prerequisite for successful fertilization in mammals, defined as a Ca^{2+}-dependent exocytotic event in sperm, in which membrane fusion occurs between the outer acrosomal membrane and the overlying plasma membrane [1,2]. In order for this process to occur, the mammalian sperm in the female reproductive tract undergoes the capacitation process [3,4] in which several modifications of sperm membranes occur including increased lateral mobility of membrane proteins and alterations in membrane fluidity [5,6]. Several lines of research on the mechanism of the acrosomal reaction have suggested the involvement of receptor-mediated signal transduction in this process [7]. The presence of guanine nucleotide binding protein, G_o, has been shown in sea-urchin sperm with a possible role in receptor-effector coupling [8] and egg peptides like react and speract stimulate protein phosphorylation in sea-urchin spermatozoa [9]. In mammalian, G_i-like proteins have been identified in mouse [10] and bovine [11] sperm, and have been implicated as participants in the process of acrosome reaction.

We have shown recently that protein kinase C (PKC), which is a key regulatory enzyme in signal transduction mechanism [12] is present in bovine sperm and involved in the cascade leading to acrosome reaction [13].

In a recent paper, it was reported that mature sperm cells from various mammalian species (human, mouse, rabbit and rat) have functionally epidermal growth factor (EGF) receptors which are localized in the acrosomal region of the spermatozoa [14]. The signal-transduction system utilized by EGF via the EGF receptor involves the generation of second messengers, diacylglycerol and inositol triphosphate [15–17] which stimulates protein kinase C [18] and the release of Ca^{2+} from intracellular stores [19–21] respectively. The mechanism by which these signals are generated appears to involve the stimulation of phospholipase C_γ, possibly by phosphorylation through the EGF receptor kinase [22,23]. In view of these findings, the present study was conducted to investigate the localization of EGF-receptor and its role in the mechanism of bovine sperm acrosome reaction. We show here for the first time that EGF can induce the occurrence of acrosome reaction in mammalian spermatozoa.

2. Experimental

2.1. Sperm preparations

Frozen bull sperm cells, were thawed and diluted at 39°C in medium comprising 150 mM NaCl, 10 mM histidine pH 7.4, were washed by three centrifugations at $780 \times g$ for 10 min. Samples with a minimum of 70% motile spermatozoa were used for experimentation.

2.2. Western blotting

Washed sperm cells (2×10^8 cells/0.2 ml) were lyzed in buffer containing 4% sodium dodecyl sulfate (SDS), 125 mM Tris pH 7.5, 1 mM sodium orthovanodate, 1 mM benzamidine and 1 mM phenylmethylsulfonylfluoride added just before use. The suspension was incubated for 10 min at 25°C and centrifuged at $12,930 \times g$ for 5 min at 4°C. The

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; AR, acrosome reaction; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; STP, staurosporin; dbcAMP, dibutyryl cyclic adenosine mono-phosphate; BAEE, benzoyl arginine ethyl ester; TALP, modified Tyrode's medium.

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supernatant was taken out, and 200 μl of it was added to 30 μl of mixture containing 0.5% Bromophenol blue, 5% glycerol and 2% β-mercaptoethanol. This mixture was boiled for 5 min and the proteins were electrophorized on 7.5% SDS-polyacrylamide gels (SDS-PAGE) according to Laemmli [24]. The proteins were transferred to nitrocellulose paper using buffer comprising of: 25 mM Tris, 192 mM glycine and 20% methanol. For Western blotting, the nitrocellulose paper was blocked with 2% bovine serum albumin in phosphate-buffered saline pH 7.2 (PBS) which contains 0.1% Tween-20 and 0.02% sodium azide for 1 h at room temperature. The paper was then incubated with primary antibody against EGF-receptor (RK2 or Cter diluted 1:10000) (from Dr. J. Yarden, Weizmann Inst.) for 2 h at room temperature, then washed twice with PBS containing 0.1% Tween-20 and incubated for 1 h, room temperature with horseradish peroxidase conjugated donkey anti-rabbit secondary antibody diluted 1:10000 with PBS, 0.1% Tween-20. The paper then washed twice and visualized using the ECL (Amersham) technique.

2.3. Immunocytochemistry studies

Sperm (3×10^6 cells) were collected on glass slides in a cytocentrifuge (1000 rpm, 5 min). The cells were fixed and permeabilized with cold acetone (10 min). For immuno-cytological staining of EGF-receptors in the cells, we used the Zymed streptavidine–biotin–peroxidase (Histostain - SP Kit) system. In this technique a positive reaction is characterized by a reddish brown reaction product. Endogenous peroxidase activity was blocked by a 45 s treatment with periodic acid solution (Zymed) and endogenous avidin and biotin were blocked using the avidin-biotin blocking solution (Zymed), followed by blocking nonspecific antibody binding with 10% nonimmune goat serum, incubated with either pre-immune serum or polyclonal anti-EGFR (PK2) antibody overnight at 4°C in a moist chamber. After washing, the biotinylated secondary antibody was added, and the slides were incubated 30 min, 37°C, washed, and streptavidine–peroxidase and substrate-chromogen were added for 15 min at room temperature. The slides were then washed with water and counter-stained with haematoxylin and examined under the microscope.

2.4. Determination of acrosomal exocytosis

Washed cells (10^8 cells/ml) were capacitated for 4 h at 39°C in modified Tyrode's medium (TALP medium [25]) containing 20 μg of heparin/ml [26]. EGF, (from mouse, Sigma) PMA, $CaCl_2$ or A23187 were then added for another 20 min of incubation. At the end of the incubation, the cells were spun down by centrifugation ($7{,}500 \times g$, 20 min) and the occurrence of the acrosome reaction was determined by measuring the activity of the released acrosin in the supernatant as described by us previously in ram [27] and bull [13,28] spermatozoa. Briefly, the supernatant was adjusted to pH 3.0 with HCl, and acrosin activity was determined by the esterolytic assay using benzoylarginine ethyl ester (BAEE) as substrate and recording the increase in A_{259} with time. The molar absorption coefficient was taken as 1150. The occurrence of the acrosome reaction was confirmed by observing thin sections of spermatozoa in the transmission electron microscope.

3. Results

The detection of EGF-R by western blot analysis with the anti-EGF-R, RK2, (Fig. 1) reveals a significant band at 170 kDa, which is typical to EGF-R from many types of cells. The RK2, a polyclonal antibody generated in A-431 cells, is highly specific for the cytoplasmic domain of the EGF receptor. The localization of the EGF-R in the cells, was detected by immunostaining using the biotin–streptavidin–peroxidase technique. The monoclonal antibody used here was generated against the EGFR of A-431 cells and it showed high immunospecificity for EGF receptors as determined by ELISA. This antibody binds to a carbohydrate residue on the external portion of the receptor molecule. The results in Fig. 2a show that

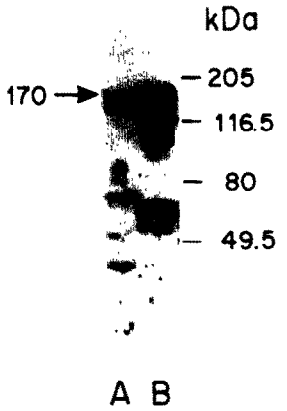

Fig. 1. Western blot analysis for EGFR. Washed sperm cells (2×10^8) were lyzed, the proteins were electrophorized by SDS-PAGE and the proteins were transferred to nitrocellulose paper (see Section 2). The EGFR was localized by using the anti EGFR RK2, which was later detected by horseradish peroxidase conjugated secondary antibody. This experiment was repeated three times and a representative test is given. (A) Sperm cells. (B) A431 cells as positive control for EGFR.

the EGF-R is localized mainly in the apical region of the head, and along the tail. The specificity of the immunostaining was demonstrated by using a non-immune serum (Fig. 2b) in which no staining can be seen. The immunostaining shows the same results whether capacitated or noncapacitated cells were tested. In addition we have seen much less staining in acrosome reacted cells in which the plasma membrane from the apical region of the head disappeared (Fig. 2c).

The occurrence of acrosome reaction in bovine sperm was determined by measuring the exocytosis of the trypsin like acrosin from the cells and the data were confirmed by following the changes in sperm morphology in the electron microscope. Addition of EGF to capacitated sperm cells, resulted in dose dependent increase in acrosin release (Fig. 3) with a maximal effect observed at 1 ng EGF/ml and down-regulation at higher concentrations. The specificity of the EGF effect was demonstrated by pretreatment of the EGF with EGF-antibodies which completely blocked the EGF effect on acrosome reaction. In the presence of 1 ng EGF/ml 30% of the cells are acrosome reacted whereas with anti-EGF treated EGF only 13% acrosome reacted cells were found. Since the spontaneous AR in these experiments was 14%, we conclude that the EGF-dependent AR is completely inhibited by pretreatment of EGF with EGF antibodies. No effect of EGF, above the control, was seen in non-capacitated cells.

In order to establish whether EGF induces the acrosome reaction via its typical tyrosine kinase activation, we tried to block this effect by adding specific tyrosine kinase inhibitors to the cells before the addition of EGF. It is seen in Table 1, that certain tyrophostin which was designed to be a more selective inhibitor of the EGF receptor-associated tyrosine kinase, causes 89% inhibi-

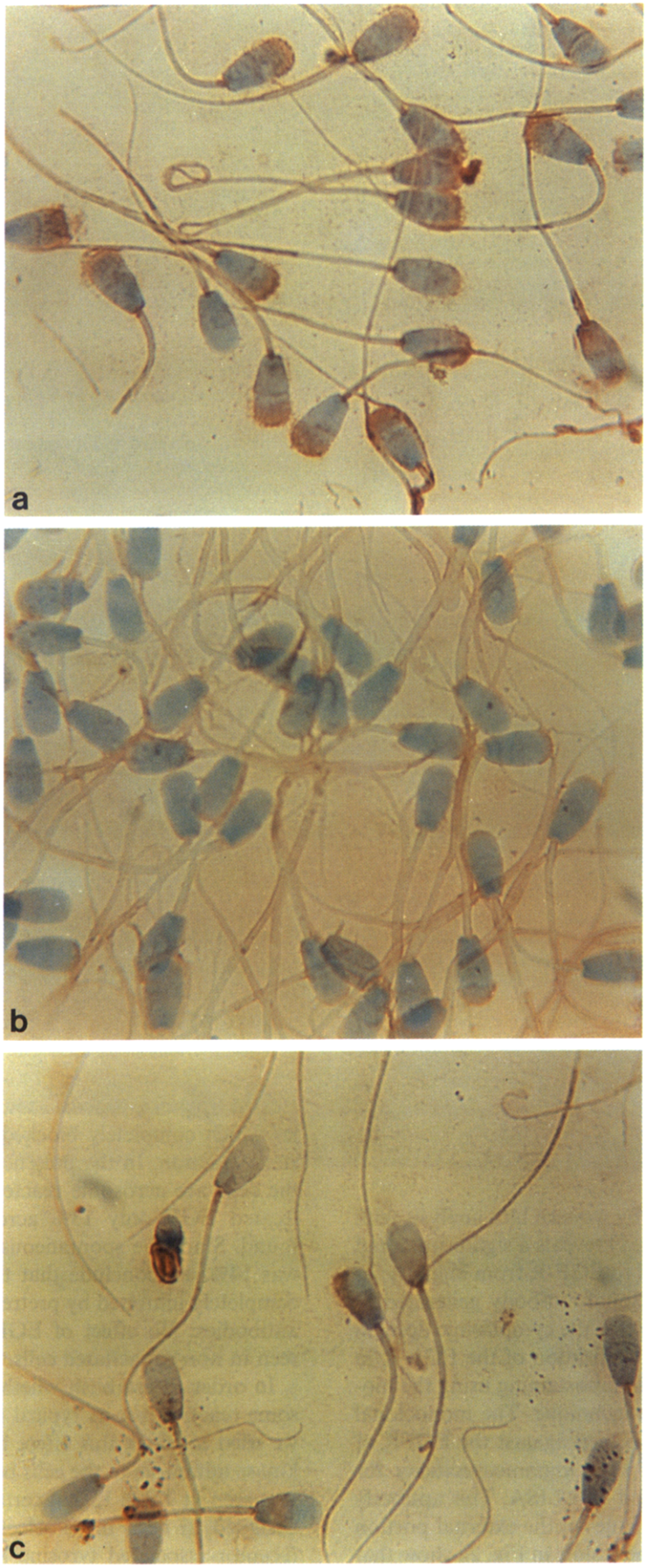
a
b
c

Fig. 2. Localization of EGFR in the sperm cell. Noncapacitated or acrosome reacted washed spermatozoa (3×10^6) were collected on glass slides, fixed, permeabilized, incubated with anti EGFR RK2 or with non-immune serum and stained by using biotinylated secondary antibody and streptoavidine-peroxidase (see section 2). The localization of the EGFR is recognized by the red color at the top edge of the sperm head. (a) Noncapacitated cells. (b) Control of noncapacitated cell incubated with non-immune serum instead of anti-EGFR. (c) Acrosome reacted cells.
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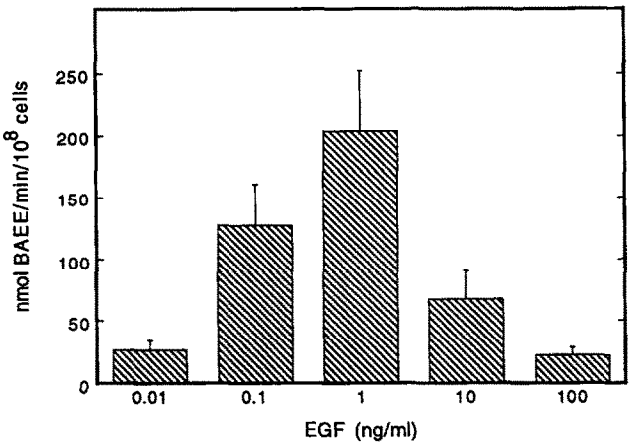

Fig. 3. Effect of increasing EGF concentration on Ca^{2+}-dependent acrosomal exocytosis. Ejaculated bull sperm (10^8 cells/ml) were capacitated for 4 h in TALP containing heparin followed by 20 min of incubation in the presence of 2 mM $CaCl_2$, and increased concentrations of EGF. The activity of the released acrosin from the cells was determined (see section 2) and was given as nmol BAEE/min/10^8 cells. Acrosin activity in absence of EGF or in the presence of anti-EGF (diluted 1:10) treated EGF (1 mg/ml) was 25 ± 8 and 30 ± 10 nmol BAEE/min/10^8 cells, respectively. The anti-EGF antibody (Sigma) was developed in Rabbit against mouse EGF. Acrosin activity in absence of calcium (130 nmol BAEE/min/10^8 cells) was subtracted from each point. Each point represents the mean ± S.E.M. of duplicate determinations from three experiments.

tion of AR induced by EGF, 47% or 50% inhibition of AR induced by PMA or A23187 and only 9% of AR induced by dbcAMP. Another tyrosine kinase inhibitor lavendustin causes also high inhibition (73%) of AR induced by EGF and much lower inhibition by the other AR inducers.

The stimulatory effect of EGF (or PMA) on AR is completely inhibited by staurosporin which is a kinase inhibitor with higher affinity for protein kinase C. When AR was induced by activating the protein kinase A with the addition of dbcAMP, there is no inhibition by staurosporin (see Table 1).

4. Discussion

The presence of signal-transduction components in mammalian spermatozoa, suggest the involvement of receptor-mediated signal transduction mechanisms in sperm physiology. In our recent paper [13] we have shown that PKC, which is a key enzyme in signal transduction mechanism, is present in bovine sperm and involved in the cascade leading to acrosome reaction. It is known in many cells that the signal-transduction system utilized by EGF via the EGFR involves the stimulation of PKC activity [18] via a mechanism by which the EGFR phosphorylates and activates phospholipase-$C_{\gamma 1}$ activity [22,23]. In the present study, we describe for the first time the presence of functionally active EGFR that can transmit EGF-dependent signal resulting in sperm acrosome reaction.

The presence of EGFR is supported by the data showing the typical 170 kDa band labeled by a specific anti-EGFR (Fig. 1). In a recent publication [14] it was shown that sperm cells from mouse, rabbit, human and rat contain an EGFR, but it was claimed that this receptor do not seem to play a major role in fertilization. In their study they showed the effect of the EGF on sperm penetration into zona free hamster egg. The need for the

Table 1
Effect of several inducers and inhibitors on Ca^{2+}-dependent acrosomal exocytosis

Inducer	A23187 (10 μM)		PMA (1.6 nM)		EGF (1 ng/ml)		dbcAMP (0.1 mM)	
Inhibitor	AR	%I	AR	%I	AR	%I	AR	%I
None	233 ± 33	–	116 ± 17	–	155 ± 25	–	138 ± 38	–
STP (1 nM)	110 ± 12	53	9 ± 3	92	16 ± 6	91	140 ± 25	0
Tyrophostin (1 μM)	117 ± 24	50	61 ± 9	47	16 ± 8	89	126 ± 49	9
Lavendastin A (5 μM)	103 ± 21	56	102 ± 35	12	41 ± 12	73	130 ± 17	2

Ejaculated bull sperm (10^8 sperm/ml) were capacitated for 4 h in TALP medium containing heparin followed by 10 min incubation with the inhibitor and then 2 mM $CaCl_2$ and the inducers were added for another 20 min of incubation. The activity of the released acrosin from the cells was determined (see Section 2) and was given as nmol BAEE/min/10^8 cells (AR in the Table) after subtracting the acrosin activity in the absence of Ca^{2+} (with 2 mM EGTA). %I is percent of inhibition. The percent of acrosome reacted cells in the presence of 10 μM A23187, 1.6 nM PMA or 1 ng/ml EGF are 47 ± 17%, 24 ± 4% and 28 ± 7%, respectively, as revealed by observation of thin sections of bull sperm cells in the transmission electron microscope. In the presence of calcium but without the inducer, 38 ± 10 nmol BAEE/min/10^8 cells above the base line was released. The inhibitor Tyrphostin (Cell Biology Products No. 3159SA) used is designed to be selective for inhibition of EFG receptor-associated kinase. This complete experiment was repeated three times and the numbers represent the mean ± S.E.M. of duplicates from the three experiments.

occurrence of sperm AR for this penetration is still controversial, therefore their data cannot exclude the possibility that EGF can affect the occurrence of AR and may have a role in fertilization.

The stimulation of the AR by low concentrations of EGF, its inhibition by specific EGFR-associated tyrosine kinase blockers and the localization of EGFR in the sperm head further support the notion that EGFR is involved in the regulation of the AR. In human sperm, the EGFR is localized in the head as well [29]. The stimulatory effect of EGF on AR is completely blocked by the PKC inhibitor staurosporin (Table 1). The specificity of staurosporin for PKC is determined from the fact that there is no inhibition whatsoever on AR induced by dbcAMP which exerts its effect via activation of cAMP dependent protein kinase A (PKA). In addition, we can see in Table 1 that the effect of the PKC activator, PMA, on AR is completely blocked by staurosporin, while the effect of Ca^{2+} in the presence of the Ca ionophore A23187, is only 53% inhibited by staurosporin. We assume that under the latter conditions, both PKC and PKA are activated to induce the AR. Thus, we conclude that EGFR induced the occurrence of AR via a mechanism in which PKC activation is involved.

The inhibition of the AR by tyrophostin or lavendistin suggest the involvement of tyrosine phosphorylation in AR. When AR is induced by EGF, there is high inhibition (89%, 73%) by these inhibitors due to the fact that the tyrosine phosphorylation of the EGFR itself is blocked and the receptor cannot exert its effect. The inhibition of AR by these inhibitors is much lower (~50%) when the inducers of AR are A23187 or PMA. Under these condition, although the EGFR phosphorylation is bypassed, there are other proteins which undergo tyrosine phosphorylation along the cascade leading to AR. This point is now under investigation in our lab.

We have shown that both capacitated and noncapacitated spermatozoa contain the EGFR, but only capacitated cells undergo the acrosome reaction. We also showed that all of the spermatozoa have EGFR but only 30% of the cells become acrosome reacted by EGF. We suggest that in order to stimulate sperm acrosome reaction by EGF the presence of EGFR in the spermatozoa is necessary but not enough. It is not clear what steps in the cascade leading to acrosome reaction are activated during the time of capacitation, and there is no known way to tell how many of the spermatozoa are indeed capacitated after the incubation. Thus, it is possible that only 30% of the cells become capacitated, and the presence of EGFR in the cells is not part of the capacitation process.

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