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DIFFERENTIAL MODULATION BY PROTEIN KINASE C OF PROGESTERONE-ACTIVATED RESPONSES IN HUMAN SPERM

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Summary. Progesterone exerts important effects on human spermatozoa by rapid non genomic mechanisms of action. It has been demonstrated that processes triggered by this steroid are dependent on the activation of calcium influx through the plasma membrane. Beside calcium, progesterone also induces a rapid plasma membrane depolarization that is dependent on an influx of sodium through a putative progesterone-activated channel located on the plasma membrane. In this study we show that protein-kinase C inhibition inhibits calcium influx activated by progesterone, while leaving the depolarizing effect of this steroid unchanged. These results may be explained by the existence of two progesterone receptors on human sperm plasma membrane, one responsible for calcium influx and modulated by protein-kinase C and the other selectively permeable to sodium that is not under protein-kinase C control. Alternatively, protein-kinase C inhibition might change ion selectively of a single progesterone-activated channel, thus decreasing calcium permeability, while leaving sodium permeability unchanged.

In the recent years there has been an accumulation of studies demonstrating that steroid hormones, beside the classical genomic mechanism of action, can influence cellular functions by rapid non-genomic mechanisms, possibly by binding to external receptors present on cell plasma membrane. The nature of these receptors remains unknown but does not appear to correspond to classical cytosolic receptor for steroids (1,2). Recently it has been demonstrated that progesterone affects important functions also in human spermatozoa by rapid non-genomic mechanism of action (3-5). The hypothesis that progesterone receptors may be present on the sperm surface has been confirmed by studies demonstrating that progesterone conjugated with molecules incapable of crossing the plasma membrane is able to exert effects similar to those of unconjugated progesterone (6,7).

Previous studies from our laboratory have shown that progesterone induces an influx of Ca^{2+} and Na^{+} from the extracellular medium inducing an increase in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ and plasma membrane depolarization in non capacitated human sperm (8). These membrane events are

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Abbreviations used: Ca^{2+}, calcium; Na^{+}, sodium; $[Ca^{2+}]_i$, cytoplasmic calcium concentration; PKC, protein-kinase C.

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strictly correlated with the induction of sperm acrosome reaction although the influx of Ca^{2+} appears to play the principal role (8). Very little is known about the intracellular events that regulate progesterone responses in human sperm. To explore this aspect, the effects of this steroid on sperm $[Ca^{2+}]_i$, plasma membrane potential and acrosome reaction were monitored after sperm treatment with agents known to modulate protein-kinase C (PKC).

The results of the present study show that PKC activity is necessary for $[Ca^{2+}]_i$ rise and acrosome reaction induced by progesterone. PKC activity on the contrary does not influence the depolarizing effects of this steroid. These findings provide indirect evidence for the existence of two different progesterone activated channels (or two different receptor subtypes) in human sperm.

MATERIALS AND METHODS

Sperm collection

Sperm samples were obtained from fertile sperm donors after 3 days of sexual abstinence. Semen samples were allowed to liquefy at room temperature for 30 minutes and then analyzed for semen volume, pH, sperm concentration, motility, viability and morphology. Sperm characteristics were all in the normal range (9). Experiments were performed with spermatozoa isolated with the swim-up technique as previously described (10). After isolation sperm were centrifuged for 10 min. at 800 x g and resuspended in BWW medium (11) at a concentration of $20x10^6$/ml.

Sperm incubation

Sperm suspension were incubated in each specific medium for 2 h in a humidified atmosphere (5% CO_2) at 37 °C in the presence or absence of progesterone 1 μg/ml. In experiments involving inhibition of PKC with staurosporine and 1-(5-isoquino- linesulfoniyl)-2-methylpiperazine, HCl (H-7), sperm aliquots were pre-incubated for different times (0-90 minutes) in the presence of increasing concentrations of each inhibitor before progesterone addition. In experiments evaluating the effects of PKC down-regulation, sperm aliquots were incubated with 100 nM phorbol 12-myristate 13-acetate (PMA) in a humidified atmosphere (5% CO_2) at 37 °C for 6 hours, before progesterone addition. In each experiment sperm aliquots were collected every 30 minutes after progesterone addition for evaluation of motility, viability and acrosome reaction. Acrosome reaction was evaluated according to Talbot and Chacon (12).

Measurement of sperm $[Ca^{2+}]_i$ and plasma membrane potential

Sperm $[Ca^{2+}]_i$ was measured utilizing the fluorescent dye fura-2/AM: sperm suspensions were incubated in BWW for 30 min. at 37 °C in the presence of fura-2/AM (2 μM). $[Ca^{2+}]_i$ was evaluated as previously described (10). Sperm plasma membrane potential changes were monitored with the potential-sensitive dye bis-oxonol at the wavelenght pair 540/580 nm as previously described (8).

Chemicals

Progesterone, staurosporine, H-7, PMA and gramicidin D were purchased from Sigma (St. Louis, MO, USA). Ionomycin was obtained from Calbiochem (La Jolla, CA, USA). Fura-2/AM and bis-oxonol were obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were of analytical grade.

Statistical analysis

Data were analyzed with Stat View II (Abacus Concepts, Berlely, CA) statistical package. Differences between means have been assessed utilizing the Student's t test and $p < 0.05$ was taken to be statistically significant.

RESULTS

Progesterone (1.0 μg/ml) induced a rapid increase of $[Ca^{2+}]_i$ in human non-capacitated sperms followed by a long lasting plateau dependent on an influx of Ca^{2+} from the extracellular medium as demonstrated by the prompt return of $[Ca^{2+}]_i$ to basal levels after extracellular Ca^{2+} chelation with EGTA (fig. 1A). Incubation with staurosporine, a well known PKC inhibitor (13), reduced Ca^{2+} response to progesterone (fig. 1B). The effects of staurosporine were dose- and time-dependent (fig. 2A and 2B).To test further that inhibition of PKC specifically influenced Ca^{2+} response to progesterone, we investigated the effects of another PKC inhibitor, H-7 (14). Pre-treatment of sperm with this agent affected the progesterone-induced Ca^{2+} influx similarly to staurosporine. Similar effects were obtained after prolonged sperm incubation with PMA (100 nM), an experimental protocol which depletes PKC activity (15): under these conditions progesterone failed to induce a $[Ca^{2+}]_i$ rise (data not shown). Thus it is likely that inhibition of progesterone-induced $[Ca^{2+}]_i$ rise by staurosporine and H-7 is due to inactivation of PKC activity. Neither staurosporine nor H-7 treatment had any effect on sperm viability, motility, basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ increase induced by the Ca^{2+} ionophore ionomycin (fig. 1C).

In a previous study we demonstrated that the progesterone-activated channel on sperm plasma membrane, beside Ca^{2+}, was also permeable to Na^+, thus promoting plasma membrane depolarization. Figure 3 shows that pre-incubation with staurosporine did not cause any modification of sperm plasma membrane depolarization in response to this steroid. Treatment of sperm with H-7 and prolonged exposure to PMA gave similar results (not shown).

It is well known that progesterone-induced Ca^{2+} influx is a potent stimulus for the acrosome reaction in non capacitated and capacitated human sperms, independently of the presence of Na^+ in the extracellular medium. Table I reports the effects of sperm treatment with the PKC inhibitors staurosporine and H-7 on progesterone induced acrosome reaction. PKC inhibition by these agents strongly inhibited the ability of the steroid to trigger acrosomal exocytosis confirming that Ca^{2+} influx

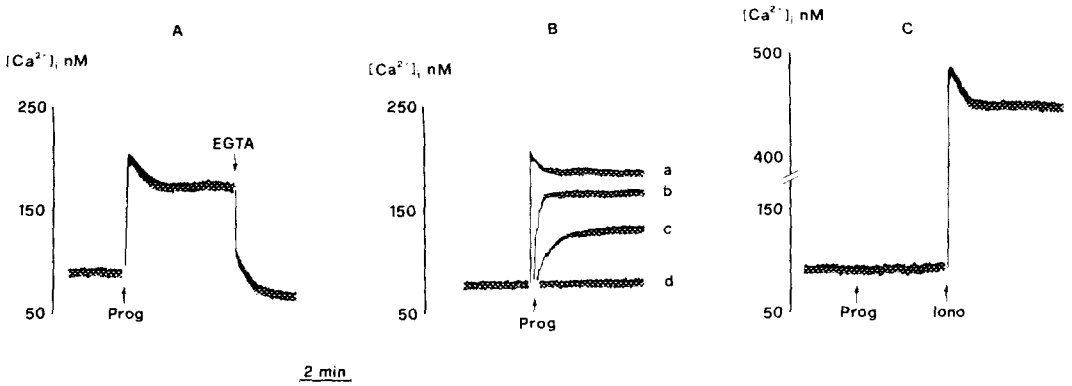

Figure 1. Effects of staurosporine on $[Ca^{2+}]_i$ increases induced by progesterone and ionomycin. Fura-2-loaded sperms were incubated in the absence (A) or in the presence (B) of different concentrations of staurosporine (none, trace a; 1 nM, trace b; 10 nM, trace c; 100 nM trace d) for 60 minutes at 37 °C and then stimulated with progesterone (Prog, 1.0 μg/ml). C, staurosporine (100 nM for 60 minutes) pre-treatment does not abolish the rise in $[Ca^{2+}]_i$ induced by the Ca^{2+} ionophore ionomycin (iono, 1 μM). Representative results from three different experiments are shown.

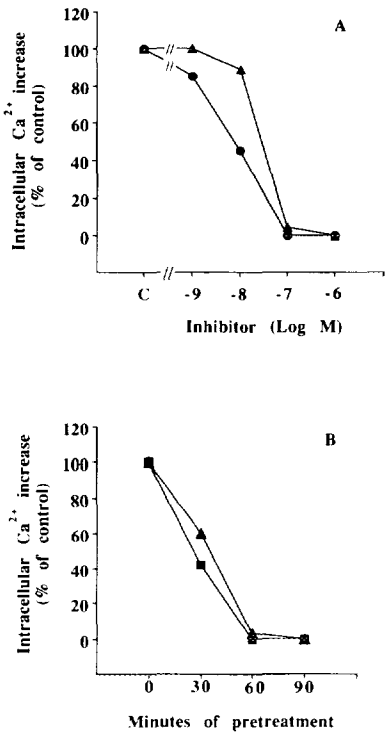

Figure 2. Time and dose dependence of the inhibition by staurosporine and H-7 of progesterone evoked $[Ca^{2+}]_i$ increases in human sperms. A, fura-2-loaded sperms were incubated with increasing concentrations of staurosporine (closed circles) and H-7 (closed triangles) for 60 minutes at 37 °C and then stimulated with progesterone (1.0 μg/ml). B, fura-2-loaded sperms were incubated with 100 nM staurosporine (closed squares) or 100 nM H-7 (closed triangles) for different times at 37 °C and then stimulated with progesterone (1.0 μg/ml). Increases in $[Ca^{2+}]_i$ are expressed as percentage of the value observed without inhibitors treatment (100%). Results are means of three separate experiments.

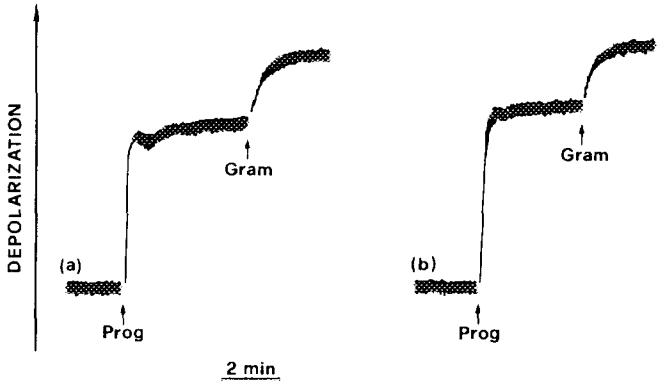

Figure 3. Effects of staurosporine on plasma membrane depolarization induced by progesterone in human sperms. Cells were incubated in the absence (trace a) and in the presence (trace b) of 100 nM staurosporine for 60 minutes at 37 °C and then stimulated with progesterone. Plasma membrane potential changes were monotired with the fluorescent dye bis-oxonol (200 nM). Additions were: progesterone (Prog) 1.0 μg/ml, gramicidin D (Gram) 500 nM.

Table I. Effects of protein-kinase C inhibitors on sperm acrosome reaction induced by progesterone. Sperms were pre-incubated in the absence and in the presence of protein-kinase C inhibitors for 60 minutes and then stimulated with progesterone (1.0 g/ml). Acrosome reaction was evaluated after 120 minutes of incubation with the steroid. * $p < 0.01$ vs both control and samples treated with protein-kinase C inhibitors. Data are means ± S.D. of triplicate measurements from a single experiment representative of three others that gave similar results.

	Acrosome Reaction (%)	
	0	120'
Pre-treatment		
None	4.5 ± 1.2	20.1 ± 2.9 *
Staurosporine (100 nM)	4.1 ± 1.0	4.4 ± 1.1
H-7 (100 nM)	4.0 ± 0.9	4.7 ± 1.2

is a necessary and sufficient condition for progesterone-induced sperm activation, as reported previously (8).

DISCUSSION

The present report shows that staurosporine and H-7, two well known PKC inhibitors (13,14), powerfully inhibited Ca^{2+} influx induced by progesterone, thus suggesting a modulatory role for this kinase in progesterone receptor activation in human sperms. Albeit staurosporine has also been reported to inhibit other kinases, at the doses used in our experiments it appears to be selective for PKC (13). A role for PKC is further supported by the demonstration that prolonged incubation with PMA, a condition that depletes cellular PKC activity (15), also blocked Ca^{2+} influx induced by progesterone. The reduction of progesterone-evoked Ca^{2+} influx by inhibition of PKC activity was correlated with the inhibition of acrosome reaction, confirming that progesterone-induced acrosome reaction requires an influx of Ca^{2+} from the extracellular medium, as previously described (8).

We have recently reported that progesterone also induced in human sperms an influx of Na^{+} that is responsible for plasma membrane depolarization (8). In contrast to Ca^{2+} fluxes, plasma membrane depolarization was not modified by PKC inhibitors. The molecular basis of the different modulation by PKC inhibitors of progesterone-triggered Ca^{2+} influx and plasma membrane depolarization is at present unknown. However, this finding clearly dissociates the two events, ruling out the possibility that plasma membrane depolarization is the consequence of the progesterone-triggered Ca^{2+} influx, as it would be expected for example in the case of stimulation of a Ca^{2+} activated outward Cl^{-} channel. Rather, membrane depolarization appears to be sustained by ion fluxes distinct from Ca^{2+} influx, in agreement with our previous suggestion (8).

Phosphorylation is a wide-spread mechanism for ion channel modulation (16-19). Abolition of Ca^{2+} influx by PKC inhibitors suggests that the phosphorylation state of the progesterone receptor/channel modulates Ca^{2+} influx: high permeability in the phosphorylated state, low permeability in the de-phosphorylated state.

The other novel and interesting observation that rises from the present study is that inhibition of PKC blocked the progesterone-induced Ca^{2+} influx but not that of Na^{+}. These findings rise the question if progesterone in human spermatozoa directly activates two separate channels (two different receptor subtypes coupled to ion channels) or a single channel the state of phosphorylation of which modulates its Ca^{2+} but not Na^{+} permeability. The possible presence for the existence of two types of progesterone-activated channels on sperm plasma membrane has been recently rised (20).

In conclusion, we provide new insights into the mechanisms underlying progesterone-induced ionic responses in human spermatozoa and provide further evidence that human sperm may possess two different progesterone receptor subtypes.

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