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Breakthroughs in Andrology

Chloride Efflux during the Progesterone-Initiated Human Sperm Acrosome Reaction Is Inhibited by Lavendustin A, a Tyrosine Kinase Inhibitor

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ABSTRACT: Previous studies showed that progesterone (P) can initiate the mammalian sperm acrosome reaction (AR) *in vitro* and that a sperm $GABA_A$-like receptor/Cl^- channel is involved in an essential Cl^- efflux mediated by P during the AR. Here, we show that lavendustin A, a potent, specific inhibitor of tyrosine kinase activity, strongly inhibits the P-initiated human AR and the essential P-mediated Cl^- efflux. Lavendustin B, a weak tyrosine kinase inhibitor, had no significant effect. These results suggest that, as part of AR initiation, P mediates tyrosine phosphorylation of the sperm $GABA_A$-like receptor/Cl^- channel.

Key words: $GABA_A$ receptor/Cl^- channel.

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The acrosome reaction (AR), a modified exocytotic event in the sperm head, is essential to mammalian fertilization (Yanagimachi, 1994). Progesterone (P), a putative *in vivo* initiator of the AR, apparently initiates the mammalian AR *in vitro* by acting through plasma membrane receptors that mediate Ca^{2+} influx (see Sabeur et al, 1996 for references) and Cl^- efflux (Turner and Meizel, 1995; Sabeur et al, 1996). The results of a number of studies have strongly suggested that one of the receptors involved in the P-initiated AR is a $GABA_A$ receptor-like/Cl^- channel (Wistròm and Meizel, 1993; Roldan et al, 1994; Shi and Roldan, 1995; Turner and Meizel, 1995). Neuronal $GABA_A$ receptor/Cl^- channels are substrates for tyrosine phosphorylation, and their function is inhibited by tyrosine kinase inhibitors (Moss et al, 1995; Valenzula et al, 1995). Although there have been no published studies of the effect of tyrosine kinase inhibitors on P-mediated Cl^- efflux in sperm, tyrosine kinase inhibitors strongly inhibit the P-initiated human sperm AR (Tesarik et al, 1993; Luconi et al, 1995). Here, we study the effects of a highly selective and potent tyrosine kinase inhibitor lavendustin A and its negative control, a weak inhibitor of tyrosine kinases, lavendustin B (Onada et al, 1989), on the P-mediated Cl^- efflux and the P-initiated AR in human sperm.

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Materials and Methods

Materials

The following materials were purchased: progesterone (4-pregnen-3,20-dione) from Sigma Chemical Co. (St. Louis, MO); Con A-FITC from EY Laboratories Inc. (San Mateo, CA); FITC-Guard from Testog Inc. (Chicago, IL); lavendustin A and lavendustin B from LC Laboratories, (Woburn, MA); Teflon Masked 10 well microscope slides from Cell Line (Newfield, NJ). The 6-methoxy-N-ethylquinolonium (MEQ) was a gift from Dr. J. Biwersi (University of California School of Medicine, San Francisco). All other chemicals were reagent grade and were purchased from Sigma Chemical Co., Fisher Scientific Co. (Pittsburgh, PA), or Mallinckrodt Inc. (Paris, KY). Polypropylene 15-ml centrifuge tubes (Falcon Blue Max) were purchased from Becton Dickinson Labware, Lincoln Park, NJ. For all experiments, deionized water was further purified using a NANO-Pure system (Barnstead/Thermolyne, Dubuque, IA).

Methods

Human semen was obtained from healthy donors by masturbation. A sperm population of >95% motility was obtained by discontinuous Percoll gradient centrifugation and further washing (Thomas and Meizel, 1988). Sperm were then diluted to 3 × 10^6/ml in a modified Tyrodes solution containing 25 mM bicarbonate and 26 mg/ml BSA (plus lactate, pyruvate, glucose,

streptomycin, and penicillin G) and then incubated in 500-μl aliquots (in 15-ml Blue Max polypropylene centrifuge tubes) at 37°C in a humidified 5% CO_2/95% air atmosphere for 24 hours (Turner and Meizel, 1995). This incubation was needed to allow the sperm to undergo cellular changes, collectively known as capacitation, required before sperm can respond to AR initiators (Bedford, 1970).

The percentage of motile sperm and the quality of the motility were determined before and after capacitation, diH-MEQ loading (see below), and chloride measurements. These determinations were made by observing (×125 magnification) a 3.5-μl aliquot of sperm suspension sealed in a 7-mm-diameter well formed by a hardened Teflon-masked microscope slide covered with a coverslip (modified from Thomas and Meizel, 1988).

The 6-methoxy-N-ethyl-1,2-dihydroquinoline (diH-MEQ) was prepared from MEQ as previously described (Turner and Meizel, 1995). Capacitated sperm were pooled as 2-ml aliquots in 15-ml polypropylene tubes and loaded with 50 μM diH-MEQ for 60 minutes at 37°C under a 5% CO_2/95% air atmosphere (Turner and Meizel, 1995). The diH-MEQ undergoes intracellular oxidation to cell impermeant MEQ (Biwersi and Verkman, 1991). Under such loading conditions, MEQ is mainly localized in the human sperm cytosol with noncytosolic compartmentalization ranging from 8% (Turner and Meizel, 1995) to 28% (Turner and Meizel, unpublished results).

After loading with MEQ, sperm were centrifuged through a 40% Percoll gradient (Turner and Meizel, 1995) made with the modified Tyrodes medium but containing 3 mg/ml BSA. This step removed extracellular diH-MEQ. These sperm pellets (containing 70–80% motile sperm) were pooled, washed with the modified Tyrodes medium with 3 mg/ml BSA, and diluted with that medium to 3–4 × 10^6 sperm/ml in preparation for Cl^- efflux studies (Turner and Meizel, 1995). It should be noted that our previous report (Turner and Meizel, 1995) incorrectly listed the diluted concentration of MEQ-loaded sperm as 10 × 10^6/ml. After 5 minutes under a 5% CO_2/95% air atmosphere, tubes containing MEQ-loaded sperm were tightly capped, protected against light by aluminum foil, and maintained at 37°C in a water bath until used.

For each Cl^- assay, a 700-μl aliquot of MEQ-loaded sperm was placed in a quartz microcuvette inside a sample chamber maintained at 37°C, equipped with a magnetic stirring devices, and continuously purged with 5% CO_2/95% air. This sperm suspension was preincubated for 5 minutes with lavendustin A (10 or 100 nM), lavendustin B (10 or 100 nM), or the solvent (0.05% DMSO in H_2O) prior to data collection and the addition of P (3.18 μM) or its solvent (0.05% DMSO in H_2O). Immediately following Cl^- efflux studies, 200-μl aliquots of the remaining MEQ-loaded sperm were preincubated for 5 minutes with lavendustin A (10 or 100 nM), lavendustin B (10 or 100 nM), or the solvent (0.05% DMSO in H_2O). Those pretreated samples were then incubated for an additional 2 minutes with P (3.18 μM) or its solvent (0.05% DMSO in H_2O), fixed with 4% formaldehyde (pH 7.5) in PBS, and assayed for AR the following day using a modified FITC-labeled ConA lectin method (all as previously described by Meizel and Turner, 1993).

An ANOVA model was used to test data for significance. Percentage data were arcsine transformed prior to analysis of variance. One-tailed Dunnet and two-tailed Bonferronni *post hoc* tests were used to determine significance. Values of $P \leq 0.05$ were considered significantly different.

Table 1. *Effect of lavendustin A, a tyrosine kinase inhibitor, on progesterone-mediated Cl^- efflux and progesterone-initiated acrosome reactions in human sperm*

Treatment*	% Acrosome reaction†	% Inhibition of progesterone-stimulated Cl^- efflux†‡
Solvent control	14 ± 4.0[a](5)	
Progesterone (P)	34 ± 5.6[b](5)	0[c] (5)
Lavendustin A 10 nM + P	19 ± 3.5[a](5)	66.0 ± 11.0[d](5)
Lavendustin A 100 nM + P	17 ± 1.4[a](5)	72.5 ± 13.1[d](4)
Lavendustin B 10 nM + P	40 ± 1.0[b](4)	14.5 ± 8.5[c] (4)
Lavendustin B 100 nM + P	39 ± 1.3[b](4)	6.3 ± 6.3[c] (3)

* MEQ-loaded, capacitated human sperm were preincubated for 5 minutes with either 10 nM or 100 nM lavendustin A, lavendustin B (a negative control), or solvent control (0.05% DMSO) and fixed for acrosome reaction assay 2 minutes after the addition of progesterone (P, 3.18 μM) or solvent control (0.05% DMSO). Sperm motility (60–80%) was the same for all treatments within any one experiment.

† Mean ± SEM (number of separate experiments). Values with different letter superscripts are significantly different ($P \leq 0.05$).

‡ Solvent control was subtracted from each treatment prior to calculating % inhibition.

Results

The collision of Cl^- with MEQ quenches the probe's fluorescence (Biwersi and Verkman, 1991). Thus, a decrease in sperm cytosolic [Cl^-] would increase the fluorescence emission of MEQ. In our previous study of human sperm (Turner and Meizel, 1995), we detected a P-mediated increase in MEQ fluorescence units and concluded that the increase represented a Cl^- efflux because it did not occur in the presence of picrotoxin, a blocker of plasma membrane $GABA_A$ receptor/Cl^- channels. The present results demonstrate that 10 nM and 100 nM lavendustin A strongly inhibited both the P-mediated Cl^- efflux (66% and 72% inhibition, respectively; Table 1; Fig. 1) and the P-initiated AR (75% and 84% inhibition, respectively; Table 1). In contrast, 10 nM and 100 nM lavendustin B did not inhibit the AR or significantly inhibit Cl^- efflux (Table 1). Neither lavendustin A nor lavendustin B affected sperm motility (Table 1).

Discussion

The Ca^{2+} influx (see Sabeur et al, 1996 for references) and Cl^- efflux (Turner and Meizel, 1995; Sabeur et al, 1996) are required for the P-initiated AR of human sperm. Here, we have shown that lavendustin A, but not lavendustin B, inhibited the P-initiated human AR and the

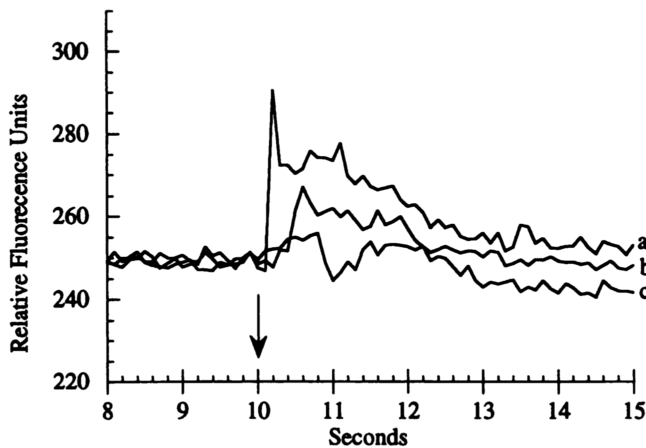

FIG. 1. Inhibition of progesterone (P)-stimulated chloride efflux by lavendustin A. Sperm were preincubated for 5 minutes with 10 nM lavendustin A or solvent control prior to the addition of 3.18 μM P or solvent control. The results shown are of a typical experiment: addition of P (a), lavendustin A + P (b), and solvent control (c). MEQ fluorescence emission (relative fluorescence units) was determined as described in "Materials and Methods." The sperm concentration was 10 × 10^6 sperm/ml in modified Tyrodes medium containing 3 mg/ml BSA. Sperm motility was 60–80%. The percentage of motility was the same for all samples within an experiment. These data are representative of five separate experiments. An increase in fluorescence indicates an efflux of cytosolic chloride. The arrow indicates the addition time of P or the solvent control.

P-mediated Cl^- efflux essential to the AR. Lavendustin A is a potent and specific inhibitor of tyrosine kinase activity that does not inhibit protein kinase A or protein kinase C and requires a 1,000-fold higher concentration to inhibit phosphatidylinositol kinase than to inhibit tyrosine kinase (Onada et al, 1989). The reported IC_{50} of lavendustin A for epidermal growth factor receptor-associated tyrosine kinase activity in an A431 cell membrane fraction is 11 nM and that of lavendustin B, similar in structure to lavendustin A but with one less hydroxyl, is 1.3 μM (Onada et al, 1989). Thus, lavendustin B is an excellent negative control for lavendustin A.

Tyrosine kinase inhibitors do not affect the single, rapid (seconds) P-mediated transient peak in $[Ca^{2+}]_i$ detected in spectrofluorometric studies of human sperm populations (Bonaccorsi et al, 1995; Mendoza et al, 1995). This single transient peak has been shown to be associated with the AR because its inhibition also results in AR inhibition (Pillai and Meizel, 1991; Meizel and Turner, 1993). However, a single cell study of P-treated human sperm detected two Ca^{2+} transients, the second (2–10 minutes after P addition) being associated with the AR (Tesarik et al, 1996) and inhibited by tyrosine kinase inhibitors. There has been one report that tyrosine kinase inhibitors reduce a plateau phase of the P-mediated increase in human sperm $[Ca^{2+}]_i$ (Bonaccorsi et al, 1995), but there is no evidence demonstrating that this plateau phase is important to AR initiation.

Progesterone mediates an efflux of human sperm Cl^- during initiation of the AR, apparently *via* a $GABA_A$-like receptor/Cl^- channel, and blocking that efflux inhibits the AR (Wistrom and Meizel, 1993; Turner and Meizel, 1995; Sabeur et al, 1996). The present studies, demonstrating decreases in P-mediated Cl^- efflux and the P-initiated AR caused by a potent and specific tyrosine kinase inhibitor, provide an explanation for those previous reports of inhibition of the P-initiated human AR by tyrosine kinase inhibitors (Tesarik et al, 1993; Luconi et al, 1995). Moreover because neuronal $GABA_A$ receptor/Cl^- channel function is enhanced by tyrosine kinase-mediated phosphorylation (Moss et al, 1995; Valenzula et al, 1995), the present results suggest that tyrosine phosphorylation may also enhance the $GABA_A$-like receptor/Cl^- channel involved in the P-initiated human sperm AR (Wistrom and Meizel, 1993; Turner and Meizel, 1995).

Neuronal $GABA_A$ receptor/Cl^- channels are pentameric heteroligomers consisting of various combinations of α, β, γ, δ, and ρ subunit types and/or their isoforms ranging from 48 kDa to 64 kDa (Stephenson, 1995). Tyrosine phosphorylation of β and γ subunit types is associated with enhancement of $GABA_A$ receptor/Cl^- channel function, and tyrosine kinase inhibitors inhibit such function (Moss et al, 1995; Valenzula et al, 1995). In a western immunoblot study of human sperm, an antibody against the bovine cerebral cortex $GABA_A$ α subunit detected a 50-kDa protein typical of neuronal $GABA_A$ receptor/Cl^- channel subunit molecular mass but also a 75-kDa protein (Wistrom and Meizel, 1993). Recently, western immunoblot study of human sperm using an antibody against the P-binding site of the intracellular progesterone receptor detected a 50–52-kDa band present on the plasma membrane (Sabeur et al, 1996). Studies of the P-initiated human AR have detected increased phosphorylation, sensitive to tyrosine kinase inhibitors, in proteins of 94–97 kDa (Tesarik et al, 1993; Bonaccorsi et al, 1995; Luconi et al, 1995) and 75 kDa (Bonaccorsi et al, 1995; Luconi et al, 1995). However, it is possible that a sperm $GABA_A$-like receptor/Cl^- channel subunit of less than 75 kDa was tyrosine phosphorylated but at levels too low to be detected in those particular studies. Future work will be necessary to determine which subunit(s) of the human sperm $GABA_A$-like receptor/Cl^- channel is (are) the target(s) for tyrosine phosphorylation during the P-initiated AR.

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