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Progesterone Stimulates GABA Uptake in Human Spermatozoa

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Progesterone can initiate the mammalian sperm acrosome reaction *in vitro,* and studies on the effect of progesterone and several other steroids have demonstrated an increased calcium influx following binding to the sperm membrane. We have previously presented data indicating the presence of a GABA transport protein in human spermatozoa. In this study, we have examined the uptake of radiolabelled GABA into human spermatozoa in the presence of progesterone, other steroids known to elevate intracellular calcium, and steroids known to be ineffective as stimulators of calcium influx. The results demonstrate a twofold increase in GABA uptake following preincubation with progesterone or steroids known to stimulate calcium influx. Steroids with minor effects on calcium influx were less effective as stimulators of GABA uptake.

In order to undergo the exocytotic event called the acrosome reaction (AR), mammalian spermatozoa must pass through *capacitation,* a process involving membrane remodulation and changes in intracellular ionic composition[1]. Progesterone (P) exerts non-genomic effects on human spermatozoa (for review see [2]) and following the original observations that P and 17α-OH-P may induce the acrosome reaction (AR) of human spermatozoa[3], several studies have confirmed that binding of P to the sperm membrane initiates several rapidly occurring intracellular events. An intracellular rise in calcium can be detected immediately following the exposure to P. Due to the diversity of intracellular signalling systems activated upon the binding of P to the responsive cells, the presence of two different receptor subtypes for P in the sperm plasma membrane has been proposed[4].

The demonstration of a rapid interaction between γ-aminobutyric acid $(GABA)_A$ receptors and neurosteroids[5] have initiated several studies suggesting a possible role for GABA in the initiation of the AR. It has been proposed that the sperm progesterone receptor located in the sperm membrane resembles a GABA receptor of the A type[6], and a recent study has demonstrated a progesterone initiated efflux of Cl^- that was inhibited by the $GABA_A$ receptor/chloride channel blockers picrotoxin and bicuculline[7]. Studies on steroid specificity regarding potency of elevating $[Ca^{++}]_i$ do, however, indicate differences between P receptors in human sperm and the properties of the $GABA_A$ specific steroid binding site[8, 9]. In mouse spermatozoa, an increase in acrosomal exocytosis has been reported following exposure of the sperm cell to GABA and muscimol, the latter being a selective $GABA_A$ receptor agonist[10]. Our recent data on the GABA binding properties of human spermatozoa suggest a GABA specific binding site on human spermatozoa that resembles GABA transport proteins (GABA-Tp)[11]. Experimental data from these studies support the presence of a putative GABA-Tp in human spermatozoa that co-transports GABA, Na^+ and Cl^- into the intracellular space of the sperm cell[12]. The present study aimed at investigating the possible interaction between progesterone and

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Abbreviations: AR, acrosome reaction; P, progesterone; GABA, γ-aminobutyric acid; GABA-Tp, GABA transport proteins; Nip, nipecotic acid; SEM, standard error of the mean; $[Ca^{++}]_i$, intracellular free calcium concentration.

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the putative GABA-Tp in human spermatozoa. Other steroid compounds, with previously reported variable ability to elevate $[Ca^{++}]_i$ [8], were also tested with regard to their effect on intracellular GABA accumulation. The data suggest a specific role for P in regulating GABA transport in human sperm cells. Steroids unable to elevate $[Ca^{++}]_i$ were less effective than P in stimulating GABA transport.

MATERIALS AND METHODS

Chemicals. Earle's balanced salt solution (Earle's medium), bovine serum albumin (BSA), GABA, nipecotic acid (Nip) and progesterone (P) were all purchased from SIGMA , St Lois, MO, USA. [^{3}H]GABA was purchased from Dupont (U.K.) Ltd. Hertfordshire, UK. Tris-citrate buffer and dimethyl sulphoxide (DMSO) was purchased from Merck, Darmstadt, GBR. 17β-Methoxy-2α-methyl androst-4-en-3one (U-45345), 17β-Methoxy-2α-carbonitrile-3-oxo androstan-4-ene (U-45342), 6β-Fluoro progesterone (U-8432), 17α-Acetoxy-progesterone (U-5533), 9(11)-Dehydro-2α,12α-dimethyl-testosterone (U-9191) and 17,21-Dimethyl-19-norpregna-4,9-diene-3,20-dione (promegestone, U-56217) were a gift from The Upjohn Company, Kalamazoo, MI, USA

Swim-up preparations of human spermatozoa. Semen samples were obtained from healthy donors after 3 days of abstinence. The ejaculates were allowed to liquefy for 30 min prior to further processing. All semen samples had normal progressive movement, sperm concentration and morphology[13]. A highly motile fraction of spermatozoa was recovered after a 45 min swim-up in Earle's medium supplemented with 1% BSA, all subsequent experiments were performed at 37°C where nothing else is stated. Swim-up fractions from several individuals were pooled and carefully mixed prior to each experiment. The final concentration was between 5 and 20 $\times 10^6$ spermatozoa/ml. The protocol was approved by the local Ethics Committee.

Accumulation of [^{3}H]GABA in human spermatozoa. Samples were left for sperm capacitation for 2.5h prior to addition of Nip/steroids, in samples treated with Nip, the inhibitor was added 5min prior to the steroid. After incubation for 30 min, GABA was added. Incubations were performed in vials (final volume 1 ml/vial) where the swim-up solution was mixed with Nip (1mM in final solution, dissolved in Earle's medium) and/or steroids (dissolved in DMSO, maximum 1% in final solution) at given concentrations. Equal volumes of Earle's medium were added to the controls. [^{3}H]GABA was thereafter added to the samples to a final concentration of 10 nM. Unlabelled GABA was added to parallels examined for AR or motility. After a final 60 min incubation, samples with radiolabelled GABA were centrifuged at 39000g and the pellets washed three times in tris-citrate buffert prior to scintillation count (Wallac 1409 Liquid Scintillation Counter, Finland). Incubations and scintillation counts were carried out in triplicate[12].

Motility. Following the incubations, samples treated with unlabelled GABA from each treatment group were examined regarding motility parameters. A Hamilton-Thorne Motility Analyzer (HTM-S, Ver 7.2, Hamilton Thorn Research, Inc., Danvers, Massachusetts, USA) was used. A minimum of 200 motile cells were analyzed per analyzed sample. The parameter setting was as previously described[12].

Acrosome reaction. Samples examined for AR were prepared and analyzed according to Cross et al[14]. At least 200 cells were examined per sample, results were excpressed as live, reacted sperm in percent of the total sperm number. The supravital stain Hoechst 33258 was used to distinguish dead from live sperm.

Statistics. Results (means $\pm$ SEM) were compared using ANOVA, and for post hoc testing Dunnet's test was used. Results expressed in percentages (AR and GABA uptake) were transformed [arcsin $\sqrt{(\times/100)}$] prior to statistical analysis.

RESULTS

Progesterone (10μM final concentration) induced a more than twofold increase in intracellular [^{3}H]GABA accumulation from 100%$\pm$0 (control) to 202%$\pm$12 when added to the capacitation medium prior to the addition of GABA (Fig 1). When Nip (1 mM final concentration) was added to the capacitating medium prior to GABA, uptake was reduced to 9% $\pm$1 of the control value. Adding Nip prior to P also led to an inhibition of GABA uptake; 15%$\pm$1 compared to the control. No significant changes between controls and treated samples were observed regarding AR (Table I) or motility parameters (data not shown). Post treatment, all samples had motility $>$ 77% (Group means; control 84%$\pm$4, Nip 87%$\pm$3, Nip+P 89%$\pm$2, P 88%$\pm$3).

As P was added to the capacitating spermatozoa at rising concentrations, GABA uptake was increased in a dose dependent manner (fig. 2). Progesterone at 10^{-10}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M (final concentration) increased intracellularly accumulated GABA from 100% (control) to 125%$\pm$16, 160%$\pm$23, 159%$\pm$11, 168%$\pm$14 and 169%$\pm$23 respectively. At 10^{-4} M, P inhibited GABA uptake (65%$\pm$9 vs control).

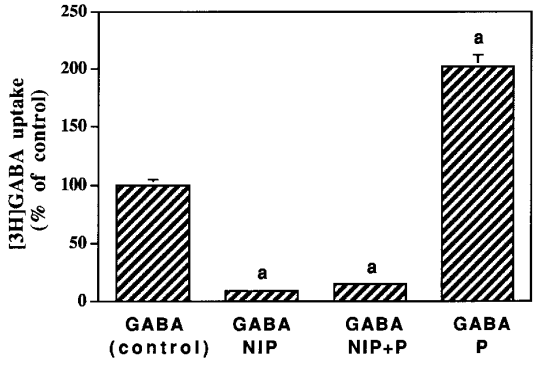

FIG. 1. Effects of progesterone (P) and nipecotic acid (Nip) on [^{3}H]GABA uptake in human spermatozoa. Swim-up preparations of human spermatozoa were capacitated for 2.5 h followed by addition of nipecotic acid (1mM in final solution) and/or P (10μM in final solution), equal volumes of Earle's medium were added to the control. Following a 30 min incubation, [^{3}H]GABA (10 nM in final solution) was added to all samples. Scintillation count was performed 60 min after addition of [^{3}H]GABA. 100% represents [^{3}H]GABA uptake in Earle's medium with no drugs added (control). Each bar represents the mean ± SEM of 5 experiments. **a** = Different from GABA (no drugs added), $p < 0.01$.

In addition to P, six other steroids were tested with regard to their effect on GABA uptake; 17β-Methoxy-2α-methyl androst-4-en-3one, 17β-Methoxy-2α-carbonitrile-3-oxo androstan-4-ene and 6β-Fluoro progesterone have previously been shown to be more effective than P at elevating $[Ca^{++}]_i$ while 17α-Acetoxy-progesterone, 9(11)-Dehydro-2α,12α-dimethyl-testosterone and 17,21-Dimethyl-19-norpregna-4,9-diene-3,20-dione have been reported to be poor stimulators of calcium influx[8]. The three calcium influx stimulating steroids had an effect on GABA uptake not significantly different from P while the three poor stimulators of calcium influx were less effective than P (fig 3 a and b). Four of the six tested steroids did increase GABA uptake above the base level (untreated control). Motility was unaffected following the addition of all tested steroids, and all samples had motility > 82% following treatment (data not shown).

TABLE I
Effects of P and Nip on Sperm Acrosome Reaction
in Presence of 10 nM GABA

Pre-treatment	Acrosome reaction (%)	p[a]
–	7.3 ± 0.6	–
Nip	6.2 ± 1.2	ns
Nip + Prog	7.5 ± 1.7	ns
Prog	11.6 ± 1.7	ns

Sperms were capacitated and pre-treated with Nip and/or P as described in the protocol for figure 1. Pilot experiments revealed no differences between samples treated with 10nM GABA vs. solvent control (Earle's medium). Acrosome reaction was evaluated after 60 min incubation with GABA. Data are means ± SEM of five experiments, 200 cells were evaluated from each sample. $p < 0.05$ was considered significant, ns; not significant vs. control (no pre-treatment, 10nM GABA).

[a] Dunnet's test.

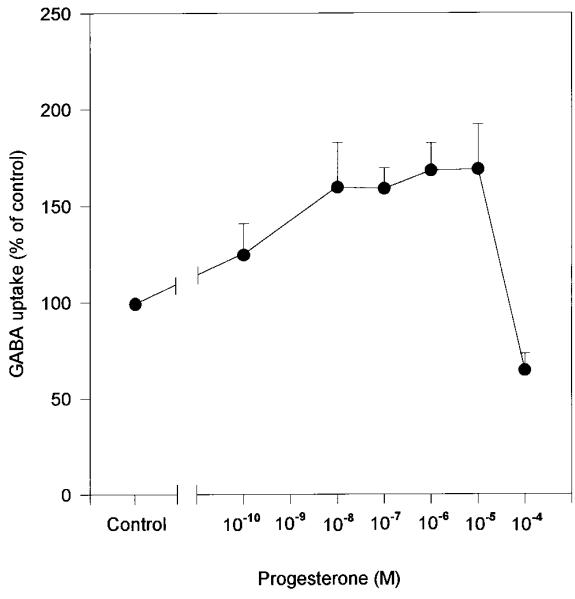

FIG. 2. Effects of increased concentration of progesterone on [^{3}H]GABA uptake in human spermatozoa. Progesterone was added to the swim-up preparations at concentrations ranging from 10^{-10} M to 10^{-4} M. Same protocol as for figure 1. Each bar represents the mean ± SEM of 5 experiments.

DISCUSSION

We have found that progesterone induces a marked increase of GABA uptake into human sperm cells and that this uptake is inhibited by the inhibitor of GABA-Tp; nipecotic acid. The stimulating effect mediated by P could be observed in a concentration interval ranging from 100pM to 10μM. These results indicate a specific role for P in the regulation of GABA uptake in human sperm. A P induced increase in GABA uptake has not previously been described, and the P binding site mediating this effect is not known. Previously, P binding sites on human sperm have been suggested to be related to $GABA_A$ like receptors[6, 7] and it has been proposed that such a receptor is involved in the initiation of the human sperm AR[6]. Since inhibitors of $GABA_{A/B}$ receptors do not inhibit GABA uptake into human sperm[11], the currently observed effect of P on GABA uptake is likely to be mediated separately from the P effect related to the $GABA_A$ like receptor. The suggested GABA-Tp present in human spermatozoa[12] has properties similar to GABA-Tp isolated from mammalian brain[15,16]. Most neurotransmitter transporters contain putative sites for phosphorylation, and regulation of the transport activity by protein kinase systems have been suggested[17]. Two such kinases have been reported to be present in human spermatozoa[18-20] and a recent study has indicated that protein kinase C mediates the P induced rise in intracellular calcium[4], further studies are required to investigate whether these kinases also influence GABA transport into the sperm cell.

The effect on GABA uptake caused by the steroid compounds tested (fig. 3a and 3b) indicate that GABA uptake and calcium influx are coupled. This may suggest a common receptor initiating the two events. Such a receptor may stimulate GABA uptake by activation of intracellular second messengers, involving increased $[Ca^{++}]_i$. Alternatively, stimulation of GABA uptake and increased calcium influx may represent independent events, both initiated by activation of the P receptor. A third possibility is that P may stimulate GABA uptake into synaptic like microvesicles (SLMVs) intracellularly, thereby changing the

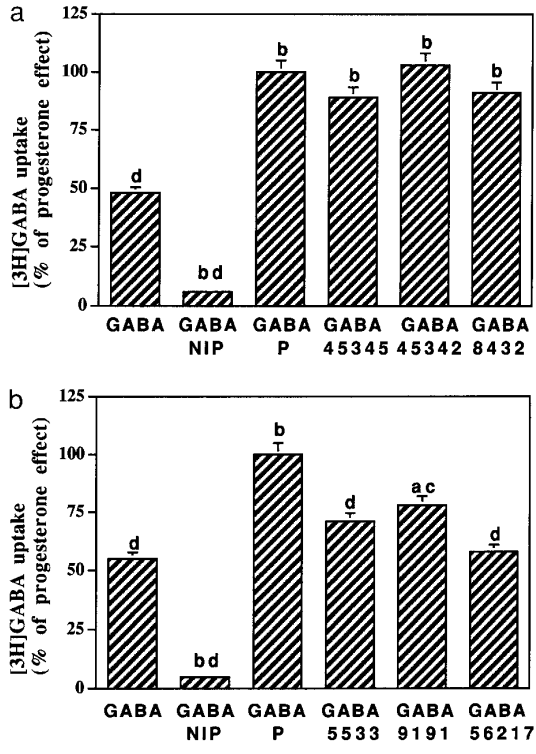

FIG. 3. (**a and b**) Effects of six different steroids on [^{3}H]GABA uptake in human spermatozoa compared to response to progesterone, [^{3}H]GABA uptake stimulated by progesterone was set at 100%, samples treated with nipecotic acid served as negative controls. Same protocol as for figure 1. Each bar represents mean ± SEM of 5 experiments. P = progesterone. **a** = Different from GABA (no drugs added), $p<0.05$; **b** = different from GABA (no drugs added), $p<0.01$; **c** = different from GABA +10μM P, $p<0.05$; **d** = different from GABA + 10μM P, $p<0.01$. (a) U-45345, 17β-Methoxy-2α-methyl androst-4-en-3one; U-45342, 17β-Methoxy-2α-carbonitrile-3-oxo androstan-4-ene; U-8432, 6β-Fluoro progesterone. (b) U-5533, 17α-Acetoxy-progesterone; U-9191, 9(11)-Dehydro-2α,12α-dimethyl-testosterone; U-56217, 17,21-Dimethyl-19-norpregna-4,9-diene-3,20-dione.

equilibrium across the cell membrane leading to increased transport across the plasma membrane. SLMVs accumulating GABA have previously been described in pancreatic β cells and in chromaffine granules[21, 22].

In conclusion, the results from this study suggest a role for progesterone in stimulating GABA uptake in human sperm. The receptor mediating such an effect may have similarities with the plasma membrane receptor that elicits increased calcium fluxes across the sperm cell membrane.

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