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Stimulating Effect of Pyroglutamylglutamylproline-amide, a Prostatic, TRH-Related Tripeptide, on Mouse Sperm Capacitation and Fertilizing Ability In Vitro

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ABSTRACT Pyroglutamylglutamylproline-amide, a prostatic tripeptide with structural similarities to thyrotrophin-releasing hormone (TRH), has been found in the seminal plasma of several mammalian species, suggestive of a biological function relating to spermatozoa. Using chlortetracycline (CTC) fluorescence analysis and in vitro fertilization, we have obtained evidence that the tripeptide stimulates mouse sperm capacitation and fertilizing ability in vitro. The tripeptide at concentrations from 5–500 nM was added to sperm suspensions and cells were assessed with CTC after 40 min, insufficient time for complete capacitation by a majority of spermatozoa under standard conditions of incubation. Concentrations of 25 nM and higher significantly promoted capacitation, as evidenced by a decrease in the proportion of acrosome-intact F pattern spermatozoa, characteristic of uncapacitated cells, and an increase in the proportion of acrosome-intact B pattern spermatozoa, characteristic of capacitated cells. However, there was no significant stimulation of acrosomal exocytosis. These results suggested that peptide-treated cells would be more fertile than their untreated counterparts. This was confirmed using in vitro fertilization, where the presence of 100 nM peptide during sperm preincubation and gamete coincubation significantly stimulated fertilizing ability (peptide, 56.5% of oocytes fertilized; controls, 26.5%). Comparison of the prostatic tripeptide and TRH effects on capacitation revealed that TRH at a concentration of 250 nM was as effective as the prostatic tripeptide in promoting the F ◊ B transition but was less effective or ineffective at lower concentrations. In vitro fertilization assessment of the two peptides, at 100 nM, revealed that only the prostatic tripeptide significantly stimulated fertility. Again, this was consistent with the CTC analyses. Because the prostatic tripeptide can stimulate sperm function in vitro, it is possible that it plays a similar role in vivo and promotes fertilizing ability of ejaculated spermatozoa. We therefore propose that this tripeptide be referred to as fertilization promoting peptide (FPP).

Key Words: Chlortetracycline, In vitro fertilization, Fertilization promoting peptide

INTRODUCTION

A tripeptide, structurally similar to thyrotrophin-releasing hormone (TRH), was isolated and characterized from the rabbit prostate complex (Cockle et al., 1989a,c) and later from human semen (Cockle et al., 1989b). The prostatic peptide, pyroglutamylglutamylprolineamide (pGlu-Glu-ProNH_2), differs at position 2 from TRH, a tripeptide with the structure of pyroglutamylhistidylprolineamide (pGlu-His-ProNH_2). This similarity results in considerable cross-reactivity with TRH antisera, which are usually tolerant of substitutions at position 2. Indeed, TRH immunoreactivity was demonstrated in both the central nervous system and peripheral tissues (reviewed by Hokfelt et al., 1989) before the existence of peptides distinct from TRH had been proved. In the early 1980s, Pekary and colleagues (1980, 1983) reported high levels of TRH immunoreactivity in mammalian prostate tissue and semen that appeared to be chromatographically distinct from authentic TRH. Complete structural characterization was achieved a few years later by Cockle et al. (1989a).

High concentrations of the tripeptide are found in human semen (46.3 ± 16.7 nM, mean ± s.e.m.; Cockle et al., 1989b), similar to the range of TRH concentrations (5–10 nM) demonstrated to give maximal physiological response in an in vitro thyrotroph assay (Martin and Tashjian, 1977). This suggests, therefore, that the prostatic peptide may have a biological function. The most obvious target cell type would be spermatozoa, which would be exposed to the peptide at ejaculation. At present, the peptide has been detected in rabbit and human semen and prostate glands (Cockle et al., 1989a,b; Morrell et al., 1991), as well as in the rat prostate gland (Bilek et al., 1992). It was undetectable in the semen of the bull and ram (Cockle, 1989b) and the prostate gland of the pig (Ashworth et al., 1992).

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In this study we have used a well-characterized mouse sperm in vitro capacitation and fertilization system (e.g., Fraser, 1987; Fraser and McDermott, 1992) to investigate the possibility that the prostatic tripeptide may be able to interact with mammalian spermatozoa to modulate capacitation and fertilizing ability. Initially, we used chlortetracycline (CTC) fluorescence analysis to determine whether the peptide could affect the rate at which sperm cells capacitate and achieve fertilizing potential. The possibility that certain treatments might promote fertilizing ability was explored directly by carrying out in vitro fertilization experiments. A preliminary report of this work has been published recently (Green et al., 1993). Because our data demonstrate that the prostatic peptide consistently stimulates capacitation and fertilization in vitro, we propose that it be named fertilization promoting peptide, or FPP. This acronym is used in presenting and discussing our results.

MATERIALS AND METHODS

Medium and Reagents

The standard medium used in all experiments was a modified Tyrode's (Fraser et al., 1993) containing BSA (crystalline, Sigma, Poole, UK) at 4 mg/ml, which has been shown to support epididymal mouse sperm capacitation and fertilization in vitro.

Pyroglutamylglutamylprolineamide (FPP) was prepared by liquid-phase synthesis starting with prolineamide free base (Sigma). N-tert-butoxy-carbonyl-L-glutamic acid γ-benzyl ester (Sigma) was coupled to prolineamide using equimolar ratios of dicyclohexylcarbodiimide in dimethylformamide. The N-terminal protecting group (butoxy-carbonyl) was removed with trifluoroacetic acid (TFA) and after conversion to the chloride salt the dipeptide was dried in vacuo. Pyroglutamic acid (Sigma) was coupled to the free N-terminus of the dipeptide by the procedure described above. Finally, the benzyl protecting group was removed by bubbling hydrogen bromide through a solution of the peptide in TFA containing 1% (v/v) anisole. The tripeptide was purified by gel filtration and ion-exchange chromatography followed by high-performance liquid chromatography. The identity of the peptide was confirmed by amino-acid analysis followed by fast-atom bombardment mass spectrometry. Ten nmoles of peptide were placed into small plastic tubes, lyophilized, and stored at −20°C. To prepare a stock solution, the contents of one tube were dissolved in 1.0 ml of BSA-free Tyrode's and mixed well by vortexing. This peptide stock (10 μM) was dispensed into smaller aliquots, frozen, and used within 1 month. For use, the stock was thawed and diluted in Tyrode's to produce substocks as required; all were used at a 1/20 dilution. TRH was purchased from Cambridge Research Biochemicals (Gadbrook Park, Northwich, Cheshire, UK). As with the prostatic tripeptide, 10 nmoles of TRH were placed into individual tubes, lyophilized, and stored at −20°C. Stock solutions were prepared, stored, and used as described for the prostatic peptide.

Sperm Suspension Preparation

The contents of the epididymides of one or more mature (>8 wk) TO male mice (A. Tuck and Sons, Battlesbridge, UK) were released into medium, using the ratio of two epididymides per ml. This produces a sperm concentration of 2–3 × 10^7 cells/ml. For experiments using CTC analysis, suspensions were maintained on a warming tray (~37°C) for 5 min to allow cells to disperse and then filtered through short columns of Sephadex G-25 (Medium grade; Pharmacia, Uppsala, Sweden) to remove nonmotile cells (see Fraser et al., 1993). Appropriate volumes of the filtrate were transferred to 30 mm plastic culture dishes (Sterilin, Teddington, UK) under autoclaved liquid paraffin (Boots, Nottingham, UK) and either peptide solution or medium was added. Dishes were incubated at 37°C in an atmosphere of 5% CO_2–5% O_2–90% N_2, the length depending on individual experimental protocols.

Chlortetracycline Assessment

The capacitation state was assessed using chlortetracycline (CTC) staining of cells in suspension to permit accurate timings (described by Fraser and McDermott, 1992). The CTC solution (final pH 7.8) containing 750 μM CTC (Sigma) in a buffer of 130 mM NaCl, 20 mM Tris and 5 mM cysteine (added at the time of solution preparation) was prepared daily and kept wrapped in foil to exclude light. Prior to use, the CTC was kept in the refrigerator but then warmed up to room temperature to avoid cold shock to the sperm cells. Sperm suspension (45 μl) was added to a 0.5 ml foil-wrapped centrifuge tube containing 45 μl CTC solution at room temperature. After careful mixing, 8 μl of 12.5% w/v paraformaldehyde in 0.5 M Tris HCl (pH 7.4) was added and mixed in well. Slides were prepared by placing 10 μl of the suspension on a clean slide. One drop of 0.22 M 1,4-diazabicyclo [2.2.2] octane (Sigma) dissolved in glycerol: PBS (9:1) was mixed in carefully to retard fading of fluorescence. After addition of a coverslip, the slide was firmly compressed between tissues to remove excess fluid. Slides were sealed with colourless nail varnish and stored, wrapped in foil, in the cold. All slides were prepared under yellow sodium illumination. Assessment was carried out on either the same or the following day using an Olympus BHS microscope equipped with phase-contrast and epifluorescence optics; violet light was used to assess cells. The Hg excitation beam was passed through a 405 nm band pass filter and CTC fluorescence emission was observed through a DM 455 dichroic mirror.

In each sample, 100 cells were classified as expressing one of the three main CTC staining patterns: F, with bright fluorescene on the head, which is characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free band in the postacrosomal region, which is characteristic of capacitated, acrosome-intact cells; AR, with dull or absent head fluorescence, which

is characteristic of capacitated, acrosome-reacted cells. Bright fluorescence on the midpiece is seen at all stages. The presence or absence of the acrosomal cap on each cell was verified under phase-contrast illumination (Fraser, 1987). It should be noted that the F ◊ B transition is not a single-step process but rather a gradual one with a time-dependent loss of fluorescence in the postacrosomal region. Therefore, it is very important to set the correct cutoff point for distinguishing B pattern from F pattern cells. As long as there is fluorescence in the postacrosomal region, compared with both the acrosome and the midpiece, we consider this to be an F pattern. Only when a dark band can be seen do we call this a B pattern cell. Additionally we have noted that some cells that appear to have the B pattern with fluorescence analysis are lacking a distinct acrosomal cap when viewed with phase contrast. Since the acrosomal cap is missing, we categorize these as AR cells. This stage may well correspond to the "S" pattern described by Lee and Storey (1985) and probably represents an intermediate stage of acrosomal exocytosis.

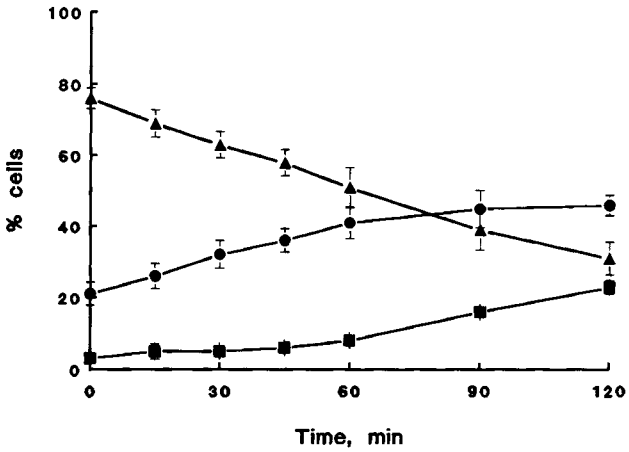

Fig. 1. Time-dependent changes in CTC fluorescence patterns in mouse sperm suspensions incubated in standard Tyrode's medium for a total of 120 min. Data are presented as mean % ± s.e.m. (n = 4). triangles, F pattern; circles, B; squares, AR.

In Vitro Fertilization

Mature TO female mice were induce to superovulate by injecting 7.5 i.u. eCG (Folligon, Intervet, Cambridge, UK) and, 48–54 hr later, 5 i.u. hCG (Chorulon, Intervet), both i.p. Fourteen hr after hCG, cumulus clots were released into standard medium under liquid paraffin. Sperm suspensions were prepared as described above, incubated, and then diluted 1/10 to give a final concentration of ~2 × 10^6 cells/ml; 400 μl droplets were added to paraffin-containing culture dishes. Approximately equal numbers of oocytes were added to all droplets. Gametes were co-incubated for 65 min; oocytes were then transferred to fresh droplets of medium and, at 75 min, fixed with buffered formalin (4% formaldehyde in phosphate-buffered saline). Oocytes were stained with 0.75% aceto-orcein, mounted, and assessed for fertilization. They were considered to be fertilized if they had resumed the second meiotic division and contained a decondensing sperm head (Fraser, 1983).

Statistical Analysis

Data were analyzed using Cochran's modification of the χ^2 test (Snedecor and Cochran, 1980). This was chosen because it is a very stringent test that compares responses *within* replicates; a significant difference requires that responses be consistent and of a reasonable magnitude in all replicates, especially when the number of replicates is relatively low. Each treatment sample was compared with the appropriate control sample.

RESULTS

Series I: What Are the Kinetics of Changes in CTC Patterns in Control Suspensions?

Capacitation is the time-dependent acquisition of fertilizing potential. Because the CTC patterns appear to reflect changes in sperm function (e.g., Ward and Storey, 1984; Fraser and McDermott, 1992), we examined the distribution of cells among the three main CTC categories over 120 min, a time sufficient for these mouse sperm to become highly fertile (Fraser, 1987). Suspensions were prepared and filtered prior to beginning the assessments which were done at 0, 15, 30, 45, 60, 90, and 120 min postfiltration (four replicates, n = 4).

Results (Fig. 1) indicate a steady decrease in the proportion of F pattern cells during the 120 min. A continuing rise in B pattern cells was observed over the first 60 min, after which the values began to plateau. This coincided with the onset of a detectable increase in the proportion of AR pattern cells after 60 min of incubation.

Series II: What is the Effect of FPP on Capacitation and Arosomal Exocytosis?

To determine whether the prostatic tripeptide that we have called fertilization promoting peptide (FPP) can modulate mouse sperm capacitation, FPP at 5–500 nM was added to filtered sperm suspensions. Such suspensions are inherently heterogenous in their composition. Cells were incubated for 40 min (insufficient time for the majority of cells to complete capacitation), then stained with CTC for analysis. Control (FPP-free) samples were prepared at 40 min and also at 120 min, the time routinely used to achieve complete capacitation and high rates of fertilization in vitro in this system (e.g., Fraser, 1987). In order to evaluate such a wide range of concentrations, two overlapping series of experiments were carried out, the first evaluating 0, 5, 10, 25, and 50 nM FPP (n = 3) and the second evaluating 0, 50, 100, 250, and 500 nm FPP (n = 4).

In the lower concentration range (Fig. 2), 25 and 50 nM FPP significantly ($P < 0.05$) stimulated capacitation as evidenced by fewer F pattern and more B pattern cells compared with the control group assessed at

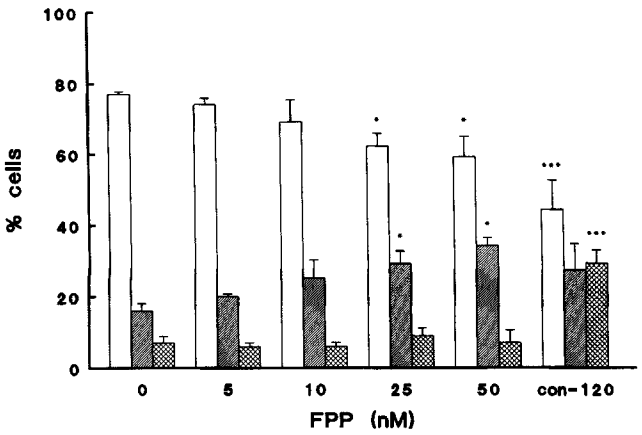

Fig. 2. CTC fluorescence patterns in mouse sperm suspensions incubated for 40 min in the presence of 0–50 nM FPP; control suspensions (0 FPP) were also assessed at 120 min (con-120). Data are presented as mean % ± s.e.m. (n = 3). Open bars, F; diagonally hatched bars, B; cross-hatched bars, AR. $*P < 0.05$, $***P < 0.01$ compared with control suspensions at 40 min.

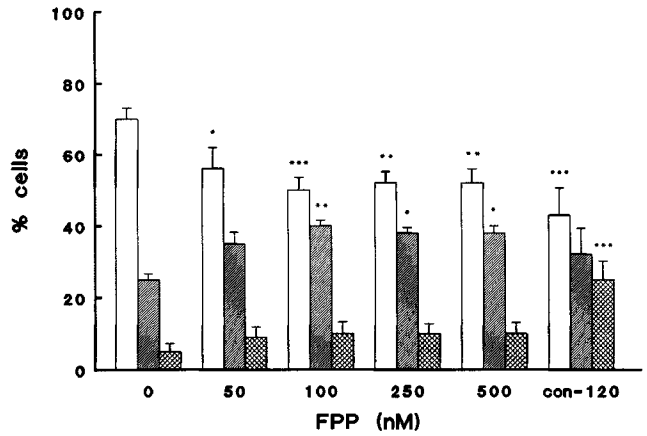

Fig. 3. CTC fluorescence patterns in mouse sperm suspensions incubated for 40 min in the presence of 0–500 nM FPP; control suspensions (0 FPP) were also assessed at 120 min (con-120). Data are presented as mean % ± s.e.m. (n = 4). Open bars, F; diagonally hatched bars, B; cross-hatched bars, AR. $*P < 0.05$, $**P < 0.025$, $***P < 0.01$ compared with control suspensions at 40 min.

the same time point. There was no detectable effect of FPP on the proportion of AR pattern cells. Ten nM FPP appeared to be on the borderline for effectiveness, with some replicates indicating an increase in capacitated cells; overall, it was inconsistent and hence the changes were not significant. Control samples incubated for 120 min showed an even more marked change in distribution of the F, B, and AR pattern cells than did the 40-min-treated FPP suspensions.

In the higher concentration range (Fig. 3), all concentrations were effective in significantly ($P < 0.05$–$P < 0.01$) stimulating capacitation as evidenced by a decrease in the proportion of F pattern cells. A significant decrease in the F pattern, characteristic of uncapacitated cells, usually but not always, produces a significant increase in B pattern cells. This is because capacitated cells are partitioned between the two patterns of B and AR. As can be seen in Figure 3, FPP treatment resulted in both a noticeable rise in B cells and a slight rise in AR cells. Although the changes in the latter category were not statistically significant, they account for the lack of significant change in the B pattern cells in the 50 nM FPP treatment group. Concentrations of 100–500 nM were more effective than 50 nM in promoting the F ◊ B transition. Only control 120 min samples had significantly more ($P < 0.01$) AR pattern cells.

Series III: Does FPP Stimulate Sperm Fertilizing Ability?

Given that CTC assessment indicated a stimulation of capacitation by FPP, we predicted that treated cells would be more fertile than untreated cells. Because the CTC patterns did not change to the same extent seen in fully capacitated suspensions (e.g., Figs. 2, 3), we further predicted that the FPP-treated suspensions would probably not be fully fertile. These predictions were tested using in vitro fertilization.

Sperm suspensions were prepared and, after 5 min, divided into two; one received FPP at 100 nM, the other just medium. Suspensions were preincubated for 40 min and then diluted; one aliquot from the FPP suspension was diluted into FPP-containing medium, another into standard medium; this reduced the final concentration to 10 nM. The control suspension was diluted into standard medium only. Unfertilized oocytes were added and treated as described above (n = 4). There were no obvious differences in the proportion of motile cells in any of the suspensions.

Results (Table 1) indicated that the continuous presence of FPP significantly ($P < 0.025$) and consistently stimulated fertilizing ability. Overall, the proportion of fertilized oocytes was doubled in the presence of FPP (with peptide, 56.5%, without peptide, 26.5%). When the FPP concentration was reduced to ~10 nM, by dilution of treated suspensions into standard medium only, the stimulatory effect of FPP disappeared. This result was puzzling, given that FPP treatment stimulated the F ◊ B transition. The possibility that reducing the FPP concentration caused a B ◊ F reversion, a change that could result in a loss of fertilizing ability, was explored in Series IV.

In two additional experiments, the effect of 500 nM FPP was investigated. There was no evidence for a concentration-dependent response; as with 100 nM FPP, the presence of 500 nM FPP promoted an approximate doubling in the proportion of fertilized oocytes (data not tabulated).

Series IV: Does Diluting FPP-treated Suspensions Cause Cells to Revert to the Uncapacitated Pattern?

To investigate further the loss of fertility observed when FPP-treated suspensions were diluted into FPP-free medium, CTC evaluation of suspensions treated in

TABLE 1. Effect of FPP on Mouse Sperm Fertilizing Ability In Vitro (n = 4)

[FPP]	Preincubation, min	Oocytes fertilized/ total oocytes	(range, %)
0	40	41/155, 26.5%	(15–47)
100 nM	40	104/184, 56.5%**	(34–74)
100 nM ◊ 10 nM	40	35/123, 28.5%	(9–68)

**$P < 0.025$ compared with controls (0 FPP).

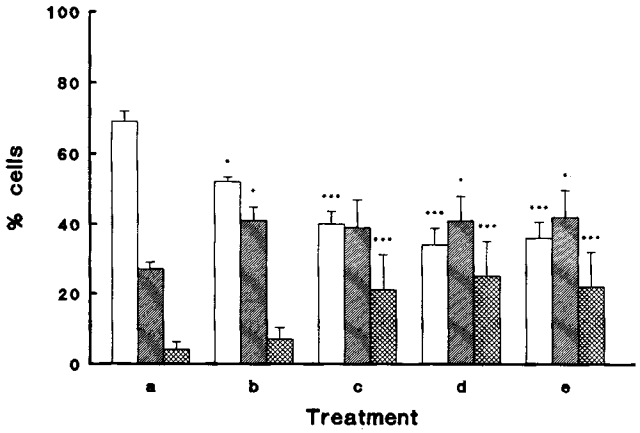

Fig. 4. CTC fluorescence patterns in mouse sperm suspensions incubated in the absence or presence of 100 nM FPP for 40 min, then diluted 1/10 in medium containing either 0 or 100 nM FPP and incubated a further 60 min. The treatment groups are: (a) 0 FPP, 40 min; (b) 100 nM FPP, 40 min; (c) 0 FPP, 100 min; (d) 100 nM FPP, 100 min; (e) 100 nM FPP, 40 min ◊ 10 nM FPP, 60 min. Data are presented as mean % ± s.e.m. (n = 4). Open bars, F; diagonally hatched bars, B; cross-hatched bars, AR. *$P < 0.05$, ***$P < 0.01$ compared with 0 FPP at 40 min (treatment a).

a similar manner was carried out. Suspensions were prepared and filtered as before; one aliquot received 100 nM FPP, the other, none. After 40 min, samples were taken and stained with CTC. Aliquots of the remaining suspensions were diluted 1/10, control suspensions into medium only and FPP-treated suspensions into both FPP-containing and FPP-free medium. These treatments mimicked the preparation of suspensions for in vitro fertilization. After a further 60 min incubation (similar to the sperm:oocyte co-incubation time), cells were stained with CTC and fixed. To concentrate the cells prior to analysis, suspensions were gently centrifuged (~750 × g) to pellet the cells; these were resuspended in a small volume of the CTC-containing supernatant (n = 4).

Results (Fig. 4) indicated that a 1/10 dilution of 100 nM FPP-treated suspensions, reducing the concentration to 10 nM FPP, did not cause the CTC patterns to revert from B ◊ F. After 100 min total incubation, there was no obvious difference between the suspensions continuously incubated in 100 nM FPP and those diluted to 10 nM FPP after 40 min.

Series V: Is TRH as Effective as FPP in Accelerating Capacitation?

Because of the structural similarities between FPP and TRH, we compared the abilities of the two tripeptides to affect capacitation, again using CTC analysis. We evaluated FPP and TRH at concentrations varying from 5–250 nM. In order to assess the two peptides on the same suspensions over this wide range of concentrations, we carried out three series of experiments: 5 and 10 nM (n = 5), 10 and 50 nM (n = 4), and 100 and 250 nM (n = 4). As in Series III, suspensions were prepared, filtered, and then divided into aliquots for treatment. After addition of peptides or medium only (control), suspensions were incubated for 40 min and then stained with CTC; control suspensions were incubated for a further 80 min and then assessed again.

The results have been summarized in tabular form (Table 2) so that responses to the whole range of concentrations can be seen easily. Information in the table indicates changes in the proportion of F pattern cells in individual treatments, compared with untreated controls. Neither peptide had a significant effect at 5 nM. As in Series III, 10 nM FPP had no significant effect in either of the sets of experiments evaluating this concentration, but TRH did ($P < 0.05$) in the second set. Despite this, TRH at 50 nM in the same set had no significant effect, whereas 50 nM FPP produced a significant change ($P < 0.05$) in the F ◊ B transition. At 100 nM, both peptides were effective, with FPP stimulating a greater change ($P < 0.01$) than TRH ($P < 0.05$), whereas at 250 nM, both peptides were equally effective ($P < 0.01$). As in Series III, the effects of the peptides were seen in proportions of F and B pattern cells. There was no significant effect on the proportion of AR pattern cells, except for TRH at 250 nM, where a slight stimulation was noted.

TABLE 2. Summary of Experiments Investigating Stimulation of Capacitation by FPP and TRH†

	Concentration, nM				
Peptide	5	10[a]	50	100	250
FPP	NS	NS/NS	*	***	***
TRH	NS	NS/*	NS	*	***

†n = 13 in total spread over three series of experiments; responses noted indicate changes in the proportion of F pattern cells in individual treatments, compared with untreated controls.
[a]10 nM peptide was assessed in two series; responses for each are noted.
*$P < 0.05$, ***$P < 0.01$ compared with untreated controls.
NS = no significant effect compared with untreated controls.

TABLE 3. Effect of FPP and TRH on Mouse Sperm Fertilizing Ability In Vitro (n = 3)

Peptide	Preincubation, min	Oocytes fertilized/ total oocytes		(range, %)
0	40	22/114,	19.3%	(3–61)
100 nM FPP	40	56/122,	45.9%**	(29–93)
100 nM ◊ 10 nM FPP	40	33/81,	40.7%	(19–72)
100 nM TRH	40	58/145,	40.0%	(30–61)
100 nM ◊ 10 nM TRH	40	42/102,	41.2%	(16–71)

**$P < 0.025$ compared with controls (0 peptide).

Series VI: Do Both FPP and TRH Stimulate Fertilizing Ability In Vitro?

Because both peptides proved able to stimulate capacitation, as determined by CTC analysis, we investigated their ability to enhance fertilizing ability. We chose to compare 100 nM FPP and TRH since FPP appeared to be more effective at this concentration than TRH (see Table 2). The experimental protocol was as described in Series III; after the sperm preincubation, peptide-treated suspensions were diluted both into peptide-containing and peptide-free medium. Thus in each replicate there were five treatments: control (no peptide), 100 nM FPP, 100 ◊ 10 nM FPP, 100 nM TRH, and 100 ◊ 10 nM TRH (n = 3).

Results (Table 3) indicated that 100 nM FPP significantly ($P < 0.025$) stimulated fertilizing ability, with 45.9% of oocytes fertilized as compared with 19.3% in the control group. Whereas the overall proportion of oocytes fertilized (40.0%) in the presence of TRH was noticeably higher than in its absence, the response was not statistically significant. As in Series III, reducing the concentration of FPP to 10 nM by dilution reduced the positive effect of the peptide, although the effect of dilution was not as great in this series; 40.7% of oocytes were fertilized. The reduced concentration of TRH had a similar, but also not significant, effect.

DISCUSSION

In this study we have investigated the possibility that pyroglutamylglutamylprolineamide, a TRH-related tripeptide found in the prostate gland and seminal plasma of several mammalian species, might play a role in modulating sperm functional ability. Our results indicate that the peptide significantly stimulates capacitation in mouse sperm suspensions, as evidenced by more rapid transition from the acrosome-intact F pattern of CTC staining, characteristic of uncapacitated cells, to the acrosome-intact B pattern, characteristic of capacitated cells (e.g., Figs. 2, 3). These changes suggested, correctly, that peptide-treated suspensions would be more fertile than untreated suspensions. Since these results provide the first evidence of a possible physiologically relevant function for the tripeptide, we propose that it be known as fertilization promoting peptide (FPP).

FPP had a significantly stimulatory effect on capacitation, as evidenced by a significant decrease in the proportion of F pattern cells, at 25 nM and higher, i.e. concentrations within the range found in human seminal plasma (Cockle et al., 1989b). Concentrations of 100–500 nM were the most effective, with little evidence for concentration-dependency in the responses noted within this range. Although the peptide significantly stimulated the F ◊ B transition, the changes were not as great as seen in control suspensions incubated for 120 min, sufficient time for completion of capacitation and acquisition of maximal fertility (e.g., Fraser, 1987). These results suggested that FPP-treated suspensions would not have reached maximum fertility within the 40 min incubation used. This proved to be the case. Cells preincubated and assessed in 100 nM FPP were significantly more fertile in vitro than their untreated counterparts (Tables 1, 3). However, the proportions of oocytes fertilized (56.5% and 45.9% respectively) were below the 90–100% usually obtained with fully capacitated suspensions (e.g., Fraser et al., 1993). These observations are consistent with the CTC data.

When the concentration of FPP was reduced to ~10 nM by dilution, the significant stimulation of fertilizing ability was abolished (Tables 1, 3). This was perplexing, since the CTC data obtained from cells treated the same way clearly showed more B pattern cells following FPP treatment for 40 min. Subsequent investigation indicated that the dilution step did not cause the CTC patterns to revert from B ◊ F, suggesting that the reduction in fertility must reflect other changes, possibly in motility. Although no decrease in the proportion of motile cells was observed, perhaps 10 nM FPP is insufficient to sustain hyperactivated motility, which is required for penetration of the murine zona pellucida (Fraser, 1981). Another but less likely possibility is that FPP may facilitate the acrosome reaction, even though it does not stimulate it.

Because FPP and TRH are structurally very similar, we compared the two tripeptides for effects on capacitation and fertility. When cells were incubated in 5–250 nM peptide, CTC analysis revealed that the peptides were equally effective in significantly stimulating capacitation when present at 250 nM, but FPP was more effective than TRH at the lower concentrations. When the two were assessed (at 100 nM) for effects on fertilization in vitro, only FPP caused a statistically significant stimulation of fertility (Table 3), results consistent with the differences observed with CTC (Table 2). Thus the prostatic tripeptide has greater specificity of action. Since this study is the first demonstration of a physiological response to FPP, at present we have no informa-

tion on the peptide's mechanism of action. Given the similarities between TRH and FPP, it is possible that the latter, like TRH, acts via a receptor. However, studies on the mouse TRH receptor have shown that neutral peptides, e.g., TRH, can bind with greater affinity and exert a greater physiological response than charged peptides (Perlman et al., 1992); thus negatively charged peptides like FPP would bind poorly. Our results indicating that both FPP and TRH can evoke the same biological responses in mouse spermatozoa, with FPP being the more effective at lower concentrations, suggest that if a receptor is involved, it is distinct from that for TRH.

The response to FPP was very specific, with the peptide promoting capacitation per se (F ◊ B transition) but not acrosomal exocytosis (B ◊ AR). We were able to detect this FPP-induced effect only because the CTC technique permits the acrosome-intact cells to be further divided into two subgroups on the basis of functional differences. Had we used a more conventional technique, one that simply identifies acrosome-intact and acrosome-reacted cells, we would have concluded, incorrectly, that the peptide was ineffective. Biologically, this response suggests that FPP acts via a specific pathway involved only in capacitation; the resulting acrosome-intact cells would then be capable of responding to the oocyte-associated agonists that trigger acrosomal exocytosis in the fertilizing spermatozoa. In vivo, few mammalian spermatozoa reach the site of fertilization. Strategically, having sperm cells capacitated but acrosome-intact, i.e., ready to interact with oocytes, would maximize the probability of successful fertilization. Given that FPP is found in a setting where it could interact with spermatozoa and that it can specifically stimulate capacitation in vitro, we suggest that FPP may also promote the acquisition of fertilizing ability in vivo.

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