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DEGRADATION OF BRADYKININ IN SEMEN OF RAM AND BOAR

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Abstract—The pattern of bradykinin (BK; Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9])-inactivating peptidases in semen of boar and ram was investigated. The degradation of BK in semen was completely abolished by the metalloprotease inhibitors EDTA and *o*-phenanthroline. Inhibitors of angiotensin-converting enzyme (ACE; EC 3.4.15.1) and phosphoramidon, an inhibitor of neutral metalloendopeptidase (NEP; EC 3.4.24.11), were only partially effective in preventing BK degradation in semen. An additive effect was seen with simultaneous inhibition of both enzymes, resulting in complete abolition of BK degradation. HPLC analysis demonstrated that exogeneous BK in semen is cleaved at Gly[4]-Phe[5], Phe[5]-Ser[6] and Pro[7]-Phe[8]. These results indicate that NEP and ACE are the main peptidases responsible for rapid BK inactivation in semen. The involvement of other peptidases known to be responsible for BK cleavage in other tissues and body fluids, namely carboxypeptidase N (EC 3.4.12.7), post proline cleaving enzyme (EC 3.4.21.26) and aminopeptidase P (EC 3.4.11.9) was excluded. NEP and ACE were shown to be localized mainly in seminal plasma and to a lesser extent on sperm cells.

The nonapeptide bradykinin (BK‡; Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is well known to be a local tissue hormone mediating many vascular and cellular effects. Its target cells are smooth muscle cells, fibroblasts, afferent nerve endings and nerve cells within the CNS, chromaffin adrenal cells, and epithelioid cells of the trachea. It is involved in many physiological and pathological processes, e.g. inflammation, blood pressure, smooth muscle contraction and pain. Its effects are mediated by at least two different receptor subtypes, B_1 and B_2 (for review see Refs 1 and 2).

BK has been shown recently to play a role in mammalian fertilization. It stimulates spermatozoan motility in rams, bulls and men [3–5]. Additionally, it stimulates sperm metabolism with an increase in fructose and oxygen consumption in men [5]. A BK-specific receptor of the B_2-subtype on human spermatozoa was suggested [6]. Moreover, all components of a kallikrein–kinin system including kallikrein, kininogen and kinin inactivating activities have been identified in human semen [7]. All these components are regarded to be stimulants of spermatozoan activity. BK seems to be inactivated by peptidolytic cleavage in all tissues and body fluids investigated so far, including kidney brush border [8], lung [9], blood [10], neuronal perikarya [11] and human neutrophils [12]. In the blood, one of the BK-inactivating enzymes is carboxypeptidase N, also called kininase I (CPN, EC 3.4.12.7), which liberates the C-terminal Arg[9] of BK. The other is angiotensin-converting enzyme, also described as kininase II (ACE; EC 3.4.15.1), releasing sequentially C-terminal Phe[8]-Arg[9] and Ser[6]-Pro[7] [10]. In lung and kidney, the neutral metalloendopeptidase (NEP; EC 3.4.24.11) plays an important role, in addition to ACE, in kinin inactivation [13]. Furthermore, the post proline cleaving enzyme (PPCE; EC 3.4.21.26) takes part in BK degradation in lung and kidney [9, 13]. An aminopeptidase which is specific for cleavage of peptides with proline in the penultimate position from the amino terminus, called aminopeptidase P (APP, EC 3.4.11.9), was demonstrated to inactivate BK in lung, kidney and erythrocytes [13–15]. In human neutrophils BK is mainly inactivated by NEP [12]. A calcium-activated thiolpeptidase was shown to cleave the Phe[5]-Ser[6] bond of the BK molecule in rat isolated neuronal perikarya [11].

In semen of men and farm animals high activities of the two potential kininases ACE and NEP could be demonstrated [16–18]. Moreover, in men there is a strong correlation between semen ACE activities and sperm count as well as sperm motility [7]. Recently, we have obtained similar results with semen of ram (unpublished). Due to the pronounced effects of BK on spermatozoan activity we investigated the pattern of peptidases which inactivate this nonapeptide in semen. In this study, we report that degradation of BK in semen of boar and ram is due to the action of both NEP and ACE. We exclude the influence of any other peptidases, namely CPN, PPCE and aminopeptidase P.

MATERIALS AND METHODS

BK, desArg[9]-BK, desArg[1]-BK, BK[1–5], *o*-phenanthroline, pCMB and Hip-His-Leu were products of Serva (Heidelberg, F.R.G.). Captopril was from

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‡ Abbreviations: ACE, angiotensin-converting enzyme; APP, aminopeptidase P; BK, bradykinin; CPN, carboxypeptidase N; NEP, neutral metalloendopeptidase; pCMB, *p*-chloromercuribenzoate; PPCE, post proline cleaving enzyme.

Squibb (Princeton, NJ, U.S.A.) and phosphoramidon from Sigma (St Louis MO, U.S.A.). Lisinopril was a gift from Dr Stemmler, Merck, Sharp and Dohme (West-Point, PA, U.S.A.), Gly-Pro-Pro-pNA was a kind gift from Dr H.-U. Demuth, MLU (Halle, F.R.G.). Phe-Arg, Phe-Ser-Pro-Phe-Arg and Dansyl-D-Ala-Gly-p(NO_2)Phe-Gly were synthesized by Dr J. Eichler and Dr K. Forner, Research Institute for Molecular Pharmacology (Berlin, F.R.G.).

Semen samples of ram and boar were available from the Institute of Artificial Insemination (Schönow, F.R.G.), and were sufficient to the following limits by microscopical classification: boar, >50% forward-moving spermatozoa (*V*%) and $>0.5 \times 10^9$ counts of spermatozoa/mL; ram, >50% forward-moving spermatozoa (*V*%) and $>1.5 \times 10^9$ counts of spermatozoa/mL.

BK digestion. The incubation mixture (vol. 250 μL) contained 0.05 M Tris–HCl pH 7.4 with 0.03 M NaCl at 37°, 94 μM BK and 20 μg (ram) up to 200 μg (boar) of protein in semen probes and 1 ng protein in probes of purified NEP or ACE. After 0.5–24 hr incubation the reaction was terminated by heating to 96° for 5 min and samples were centrifuged before analysis. For inhibitory experiments a preincubation of the purified enzyme or semen samples with inhibitor was carried out for 15 min before adding BK.

HPLC analysis. HPLC analysis in most cases was performed with an equipment consisting of a LC-9A pump, a SIL-9A automatic sample injector and a SPD-6AV variable wavelength detector with a C-R3A Chomatopac, all from Shimadzu (Kyoto, Japan). The separation was carried out isocratically with 26.5% acetonitrile and 73.5% 0.01 M NaH_2PO_4, 0.15 M $NaClO_4$, pH 2.2 at a flow rate of 1 mL/min. The peptide samples were dissolved in the mobile phase, injected onto a Nucleosil 100 C18 column, 250 × 4 mm, 5 μm particle size (Macherey-Nagel, Düren, F.R.G.) and detected at 215 nm.

The degradation products were identified by addition of peptide standards or by amino acid analysis after fractionation of HPLC peaks. For peak analysis the products were fractionated by a linear gradient of 14–41% acetonitrile in the presence of 0.1% trifluoroacetic acid at a flow rate of 1 mL/min.

For separation of desArg^9-BK from BK HPLC was performed with equipment from Gynkotech (Munich, F.R.G.). Samples were injected onto a Nucleosil 100 C18 column, 250 × 4.6 mm, 5 μm particle size and separation was carried out with a 25–40% acetonitrile gradient in 0.075 M $NaClO_4$, pH 2.2 within 22 min.

Amino acid analysis. The fractions of the HPLC peaks were transferred into hydrolysis tubes and dried by vacuum. The tubes were placed in a gas-phase hydrolysis vessel (Ciba Corning, U.K.). A blank sample to control the level of background and an amino acid mixture were included in each batch of hydrolysis. About 500 μL of 6 N HCL was placed in the bottom of the vessel. For hydrolysis the vessel was purged with nitrogen and then evacuated down to a vacuum of less than 0.1 millibar. The gas-phase hydrolysis was carried out at 110° for 24 hr. After hydrolysis the tubes were dried by vacuum. The amino acids were derivatized with 4-(dimethylamino)azobenzene-4-sulfonylchloride. Separation and identification were performed as described by Knecht and Chang [19].

Enzyme preparation. ACE was isolated from pig lung as described elsewhere [20]. NEP was purified by a modification of the method of Malfroy and Schwartz [21] from pig kidney. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed a single protein band (M_r 88,100) as was shown with Coomassie staining. The purified NEP did not contain any detectable ACE activity.

Determination of enzyme activities. Assay for NEP was performed fluorogenically with Dansyl-D-Ala-Gly-*p*(NO_2)Phe-Gly as substrate as described previously [22]. ACE assay was carried out fluorometrically as already described with Hip-His-Leu as a substrate [22]. Assays of PPCE were performed with the chromogenic substrate Gly-Pro-Pro-*p*-nitroanilide as described by Steinmetzer *et al.* [23] and Lasch *et al.* [24]. Arising *p*-nitroaniline was estimated spectrophotometrically at 405 nm.

RESULTS

Effect of peptidase inhibitors on BK digestion

We initially tested the capability of some well-known specific peptidase inhibitors to protect the BK molecule from degradation in ejaculates of boar and ram. Figure 1 shows the time course of BK degradation in semen of ram. Complete inhibition of BK degradation in semen is achieved if *o*-phenanthroline or EDTA is used. Both inhibitors inhibit metalloproteases. The fact that *p*-chloromercuribenzoate (pCMB) did not alter BK degradation rules out the influence of SH-sensitive peptidases on BK degradation in semen. This applies to PPCE, which takes part in BK degradation in the lung and kidney [9, 25], and the multicatalytic proteinase complex (EC 3.4.24.5), described to be involved in BK degradation in the lung [9]. Specific inhibitors of ACE, captopril, BPP 9α and lisinopril, as well as specific inhibitors of NEP like thiorphan and phosphoramidon inhibited BK degradation only partially. Nearly complete protection of the molecule was achieved by simultaneous inhibition of NEP and ACE by use of phosphoramidon and captopril or phosphoramidon and lisinopril in one experiment. Together these results suggest that initial cleavage of BK in semen of boar and ram is due to the action of both ACE and NEP. We therefore determined the cleavage products which arise from BK, incubated with purified ACE and NEP, and compared them directly with the digestion products of BK in semen.

Digestion of BK by NEP

Purified NEP from pig kidney cleaved the BK molecule at Gly^4-Phe^5 and at Pro^7-Phe^8. The three final products could be confirmed by product analysis as described above, as well as by co-elution with peptide standards, to be Arg-Pro-Pro-Gly (BK 1–4), Phe-Ser-Pro and Phe-Arg. Furthermore, two intermediate products were liberated and were confirmed to be Arg-Pro-Pro-Gly-Phe-Ser-Pro (BK 1–7) and Phe-Ser-Pro-Phe-Arg (BK 5–9) by the

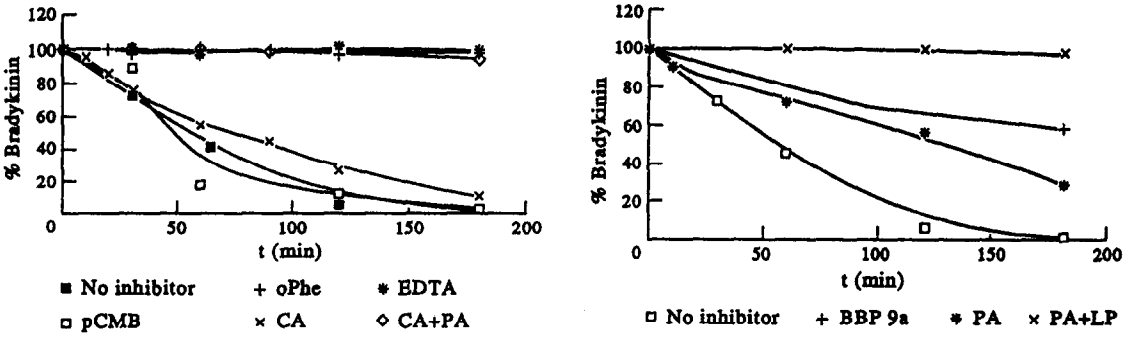

Fig. 1. Inhibition of BK degradation by metalloprotease inhibitors EDTA and *o*-phenathroline (oPhe) (each 1 mM), the inhibitor of SH-sensitive proteases, pCMB (1 mM), ACE-specific inhibitors, BPP 9a, captopril (CA) and lisinopril (LP) (1 μM each), and the NEP-specific inhibitor phosphoramidon (PA) (1 μM). The percentage of intact BK corresponds to the percentage of the area of the appropriate HPLC peak; 100% corresponds to the initial BK concentration (94 μM). Incubations were carried out in 0.05 M Tris–HCl, pH 7.4, at 37° and terminated after time intervals between 10 and 180 min. The graph is split into two for clarity.

methods described above. No further products arose, indicating that our preparation was free of contaminating peptidases. In kinetic studies, the K_m of BK for NEP was 95.8 ± 22.4 μM with a k_{cat} of 465 ± 8.01 min^{-1}. The specificity constant (k_{cat}/K_m) was 4.86 $\mu M^{-1} min^{-1}$ (N = 9, BK concentrations ranging from 9.4 to 376 μM; $r_{sq} = 0.9689$). BK cleavage was fully inhibited by 1 μM phosphoramidon.

Digestion of BK by ACE

Purified ACE from pig lung cleaved the BK molecule at Phe^5-Ser^6 and at Pro^7-Phe^8. The three end products again were confirmed by product analysis and co-elution with standards to be Ser-Pro, Phe-Arg and Arg-Pro-Pro-Gly-Phe (BK 1–5). An intermediate product could be shown to be Arg-Pro-Pro-Gly-Phe-Ser-Pro. Contaminating peptidase activities in our ACE preparation were not detectable. Kinetic studies showed a deviation from Michaelis–Menten kinetics, indicating substrate inhibition (Fig. 2). Therefore, kinetic constants were obtained by fitting the v/S values to Eqn 1 regarding substrate inhibition:

$$v = \frac{V_{max}}{1 + K_m/S_0 + S_0/K_i}. \quad (1)$$

The K_m of BK for ACE was then 98.17 ± 23.3 μM with a k_{cat} of 76.6 ± 11.28 min^{-1}; K_i was 297.3 ± 82.6 μM. (k_{cat} was estimated from the optimum of our v/S characteristic which was only 0.46 × V_{max}.) BK concentration was in the range 5–500 μM (N = 7, $r_{sq} = 0.996$).

Digestion of BK in semen

The next step was to show the products arising during the incubation of BK with semen samples of ram and boar under the same experimental conditions. The results are shown in Fig. 3 (ram). Similar results were obtained for semen of boar. Product confirmation in these analyses was carried out by coelution with standard peptides and with

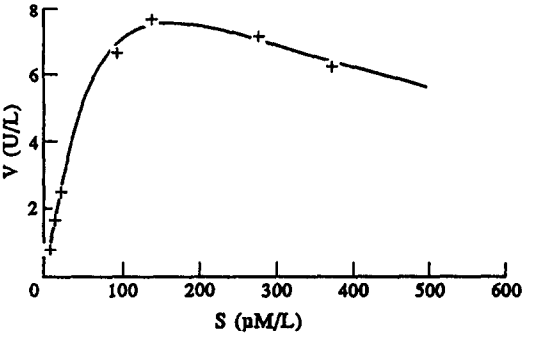

Fig. 2. v/S characteristics of BK hydrolysis by ACE show deviation from Michaelis–Menten kinetics. v is the rate of BK degradation as estimated from HPLC whereby quantitation was achieved by comparison with authentic standards. S is the BK concentration. Results are mean values of three experiments on each substrate concentration. Experiments were carried out at 37° in 0.05 M Tris–HCl, pH 7.4, with 0.2 M NaCl.

BK digestion products released by purified NEP and ACE. Coincubation of BK with semen of both boar and ram gave rise to relatively stable products. These peptide fragments are BK (1–5) and Phe-Arg and include a mixture of products leaving the HPLC column with short elution times (less than 3 min). Comparison with the HPLC elution profiles of digestion products from experiments with purified NEP and ACE indicated that this mixture should include Arg-Pro-Pro-Gly and Ser-Pro. The tripeptide Phe-Ser-Pro is readily degraded in semen probes, whereas there is arising another peak which has not been identified until now. It could be a result of further aminopeptidolytic degradation of one of the hydrolysis products. The presence of aminopeptidases in semen had already been shown in earlier experiments [16]. Intermediate products of

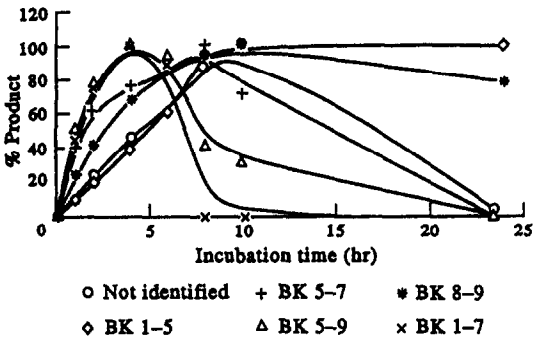

Fig. 3. Products arising from BK degradation by semen of ram. Semen samples containing 20 μg protein (ejaculates diluted 1:500 v/v) were incubated with 94 μM BK in 0.05 M Tris–HCl, pH 7.4, for 0–24 hr. One-hundred per cent product corresponds to the maximum peak area of the respective peptide during the incubation time.

BK degradation in semen are Arg-Pro-Pro-Gly-Phe-Ser-Pro and Phe-Ser-Pro-Phe-Arg.

To determine the initial sites of cleavage we used very dilute semen samples. The use of less dilute semen leads to further degradation which we have not investigated. The products of BK degradation in semen which we have identified indicate that initial cleavage occurs at the sites shown below (arrowed):

$$Arg^1\text{-}Pro^2\text{-}Pro^3\text{-}Gly^4\text{-}Phe^5\text{-}Ser^6\text{-}Pro^7\text{-}Phe^8\text{-}Arg^9.$$
$$\uparrow \quad \uparrow \quad \uparrow$$

These results imply that initial cleavages are due to the combined action of ACE and NEP. The half-life of exogeneous BK in undiluted ram semen can be calculated from our results (Fig. 1) to be about 20 sec and in undiluted boar semen about 2.4 min.

In order to investigate the possible involvement of additional peptidases we carried out HPLC under alternative conditions. The isocratic HPLC separation which was used in the above described experiments did not allow separation of BK from desArg^9-BK, the product of a possible attack of CPN (kininase I). Therefore, we varied HPLC conditions using a gradient as described in Materials and Methods.

Role of carboxypeptidase in BK digestion in semen

Figure 4 shows the decline in exogenous BK in HPLC fractions with lengthening times of incubation with ram semen. The control shows separation of BK and desArg^{9}BK using standard peptides. Carboxypeptidase activity can be excluded by failure to detect its specific cleavage product desArg^{9}BK in these experiments. This product was also not detected when ACE and NEP were inhibited by adding phosphoramidon and lisinopril (1 μM each) to the incubation mixture in order to examine the possibility that desArg^9 is too rapidly degraded to be detected (not shown here). Together these results indicate that carboxypeptidases similar to CPN (EC 3.4.12.7) are not involved in BK degradation in ram semen. Analogous results were obtained with semen of boar (not shown here). In addition, we did not find the product of an attack of aminopeptidase P, desArg^1-BK. This could be further confirmed in an attempt to investigate the ability of semen to cleave the PPCE-specific chromogenic substrate Gly-Pro-Pro-*p*-nitroanilide.

Estimation of enzyme activity of PPCE

Degradation of Gly-Pro-Pro-pNA could be attributed to PPCE cleaving the Pro-pNA linkage and APP cleaving the Gly-Pro linkage, and dipeptidylaminopeptidase IV (EC 3.4.14.4) subsequently cleaving the remaining Pro-Pro-pNA (Fig. 5). The presence of dipeptidylaminopeptidase IV is well documented in semen [16] and could be confirmed in our semen samples. No *p*-nitroaniline was detected in semen samples of boar and ram within 4 hr of incubation with this substrate. In contrast, a control incubation with kidney microvillar membrane yielded an enzyme activity of 0.287 ± 0.07 U/L (N = 5). In addition to the failure of pCMB to inhibit BK degradation this excludes the presence of PPCE in semen. It also provides additional evidence that there is no APP activity in semen. It can therefore be concluded that hydrolysis of BK in semen of boar and ram is solely due to the action of NEP and ACE. To investigate their possible function we sought to localize these peptidase activities.

Localization of NEP and ACE within semen

To investigate the distribution of NEP and ACE in boar ejaculates we centrifuged semen samples at 22,000 *g* for 30 min to separate spermatozoa from seminal plasma. Enzyme activities were estimated with fluorogenic substrates as described in Materials and Methods. We found that 92% of NEP was localized in seminal plasma, as was 95% of ACE. Further high speed centrifugation at 100,000 *g* for 120 min yields all the ACE in the supernatant while 92% of seminal plasma NEP is sedimented.

DISCUSSION

The present study shows that BK degradation in semen of ram and boar is due to the combined action of ACE and NEP. Direct comparison of BK degradation in semen with that by purified NEP revealed that the Gly^4-Phe^5 and the Pro^7-Phe^8 bonds are cleaved by NEP. BK cleavage by ACE occurs at the Phe^5-Ser^6 and Pro^7-Phe^8 bonds. Our inhibition experiments show that both enzymes are equally involved in BK degradation in semen. We obtained K_m values of BK which are in the same range for NEP and ACE ($K_{m\,ACE} = 98.17$ μM; $K_{m\,NEP} = 95.8$ μM). This is not in accord with the results of other authors. Though our K_m of BK for NEP is comparable with the data of Matsas *et al.* [26], the K_m for ACE is usually described in the literature as being in the range 0.4–1 μM [8]. Our HPLC method did not allow us to consider BK concentrations in this range. In view of the deviation from Michaelis–Menten kinetics above 100 μM BK it becomes obvious that our results on the amounts of ACE and NEP involved in BK degradation in semen should be established with physiological BK concentrations.

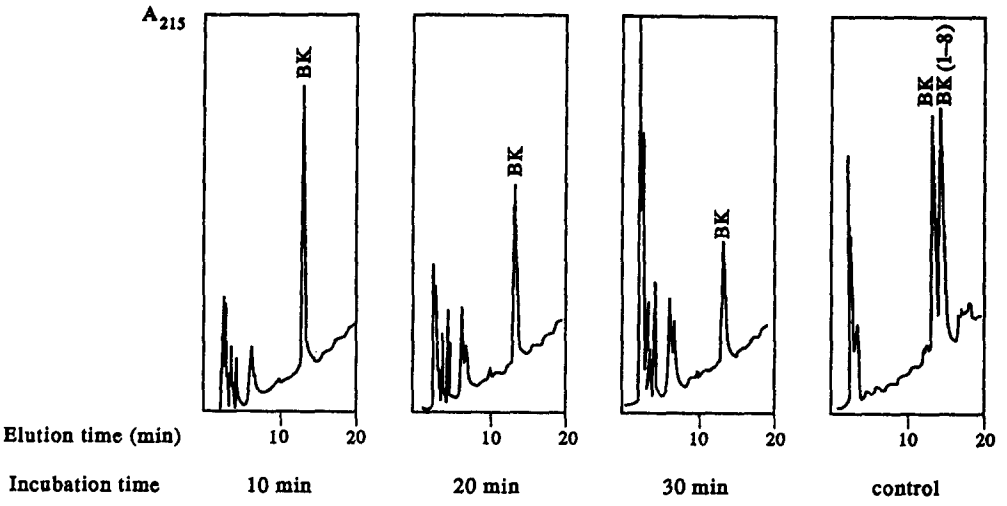

Fig. 4. BK (1–8) does not arise during incubation of BK in semen of ram. Semen samples containing 200 μg protein (ejaculates diluted 1:50 v/v) were incubated with 94 μM BK in 0.05 M Tris–HCl, pH 7.4, for 10, 20 and 30 min. Control shows elution profile of a mixture of BK and BK (1–8), each 94 μM in 0.05 M Tris–HCl, pH 7.4.

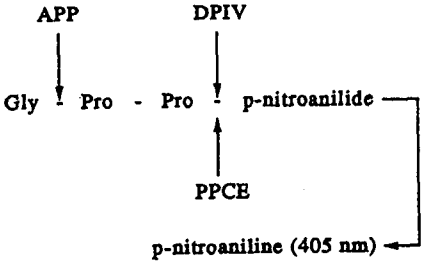

Fig. 5. Alternative hydrolysis of Gly-Pro-Pro-pNA by APP and DPIV or by PPCE.

These are in the nanomolar range [7]. We have excluded the involvement of CPN-like enzymes PPCE and APP in BK degradation in semen of boar and ram. In contrast to our results, Skidgel [27] recently described a carboxypeptidase in human seminal plasma which was able to cleave BK. This enzyme was accompanied in semen by an inhibitor which could be separated from seminal plasma by gel chromatography [27]. Our experiments cannot exclude with certainty the existence of a corresponding enzyme in semen of farm animals.

The activities of BK-inactivating peptidases ACE and NEP in semen of boar and ram are strikingly high when compared with reported specific activities of both peptidases in other tissues and body fluids [28]. They are mainly found in the seminal plasma rather than sperm. Similar results were reported for human semen [18]. Differential behaviour on high speed centrifugation indicates that NEP is apparently membrane associated, whereas ACE is in a soluble form.

What might be the role of these peptidases within seminal plasma? The main function of seminal plasma during fertilization is to prevent premature hyperactivation of spermatozoa. To achieve this, it must contain inhibitors of sperm capacitation as well as of sperm motility (see Ref. 29 for review). Since BK stimulates the activity of spermatozoa, its degradation by high levels of ACE and NEP could prevent premature sperm activation. Peptidases could potentially control the level not only of BK but of other neuropeptides which have been suggested to stimulate spermatozoan activity. This seems to be confirmed by previously reported strong positive correlations between semen ACE activities and sperm motility as well as sperm count [7].

In summary, the inactivation of BK in semen by the action of NEP and ACE could be an important regulatory step in fertilization. ACE and NEP could also be capable of inactivating other neuropeptides involved in sperm function. Modulation of peptidase activities could potentially have important consequences for reproductive processes.

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