

Evidence for the activation of PAR-2 by the sperm protease, acrosin: expression of the receptor on oocytes

Rosealee Smith^a, Alison Jenkins^a, Afrodite Loubakos^a, Philip Thompson^b,
Vanitha Ramakrishnan^c, Jim Tomlinson^c, Usha Deshpande^c, David A. Johnson^d, Roy Jones^e,
Eleanor J. Mackie^f, Robert N. Pike^{a,*}

^aDepartment of Biochemistry and Molecular Biology, Monash University, Clayton, Vic. 3800, Australia

^bDepartment of Medicine, Monash University, Boxhill, Vic. 3128, Australia

^cCOR Therapeutics Inc., South San Francisco, CA 94080, USA

^dDepartment of Biochemistry and Molecular Biology, J.H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA

^eDepartment of Development and Signalling, The Babraham Institute, Cambridge CB2 4AT, UK

^fSchool of Veterinary Science, University of Melbourne, Parkville, Vic. 3010, Australia

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Abstract Proteinase-activated receptor-2 (PAR-2) is a member of a family of G-protein-coupled, seven-transmembrane domain receptors that are activated by proteolytic cleavage. The receptor is expressed in a number of different tissues and potential physiological activators identified thus far include trypsin and mast cell tryptase. Acrosin, a trypsin-like serine proteinase found in spermatozoa of all mammals, was found to cleave a model peptide fluorescent quenched substrate representing the cleavage site of PAR-2. This substrate was cleaved with kinetics similar to those of the known PAR-2 activators, trypsin and mast cell tryptase. Acrosin was also shown to induce significant intracellular calcium responses in Chinese hamster ovary cells stably expressing intact human PAR-2, most probably due to activation of the receptor. Immunohistochemical studies using PAR-2 specific antibodies indicated that the receptor is expressed by mouse oocytes, which suggests that acrosin may play additional role(s) in the fertilization process via the activation of PAR-2 on oocytes. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Protease-activated receptor-2; Acrosin; Fluorescent quenched substrate; Intracellular calcium; Oocyte; Immunocytochemistry

1. Introduction

Proteinase-activated receptor-2 (PAR-2) is the second member of the growing family of proteolytically activated receptors, which couple to G-proteins to produce intracellular responses [1–6]. Proteolytic cleavage of the extracellular domain

of PAR-2 reveals a new amino-terminus which acts as a ‘tethered ligand’ to cause receptor activation. PAR-2 is expressed in a number of tissues, including the gastrointestinal and respiratory tracts, pancreas, kidney, liver, ovary, bone and skin, and is found in epithelial and endothelial cell lines, smooth muscle, T cell lines, neutrophils, osteoblasts and certain tumor cell lines [1–15]. Trypsin appears to activate PAR-2 under physiological conditions in the gastrointestinal and respiratory tracts [16,17], while tryptase may be important in pathological conditions involving mast cell infiltration and/or degranulation [18]. A synthetic receptor agonist peptide (RAP) corresponding to the tethered ligand is also able to activate the receptor [19]. However, activators in many of the tissues where PAR-2 is found have still not been identified and most known proteinases apart from trypsin and tryptase do not activate the receptor, hence knowledge of the physiological and pathological roles of receptor activation is limited.

Trypsin, tryptase and the sperm protease, acrosin, were identified as potential physiological PAR-2 activators by Fox et al. [20] using a four amino acid chloromethyl ketone inhibitor. Due to the fact that proteolytic specificity depends on the amino acid sequence on both sides of the cleavage site, we have developed a fluorescent quenched substrate (FQS) containing both the amino acids prior to and after the cleavage site of PAR-2 (residues 33–41 of human PAR-2). The peptide substrate is based on the principle whereby an amino-benzoic (Abz) acid fluorescent group on the substrate is quenched by a dinitrophenol (Dnp) group attached to the opposite end of the peptide. Cleavage of the peptide then removes the quenching group, allowing the cleaved substrate to fluoresce. Potential activators of PAR-2 must be trypsin-like enzymes capable of cleaving at the Arg³⁶–Ser³⁷ bond within the PAR-2 extracellular domain (Ser³³–Lys–Gly–Arg–Ser–Leu–Ile–Gly⁴⁰).

Acrosin, a trypsin-like serine proteinase found in large quantities in the acrosomal body of spermatozoa of all mammals [21,22], has been suggested to have several functions during fertilization, most notably to facilitate sperm penetration through the zona pellucida, as a secondary binding protein and in dispersal of the acrosomal contents [23–25]. Acro-

*Corresponding author. Fax: (61)-3-99054699.
E-mail: rob.pike@med.monash.edu.au

Abbreviations: PAR-2, proteinase-activated receptor-2; Abz, amino-benzoic acid; Dnp, dinitrophenol; CHO cells, Chinese hamster ovary cells; BSA, bovine serum albumin; APC, activated protein C; [Ca²⁺]_i, intracellular calcium concentration; Fura-2/AM, Fura-2-acetoxymethyl ester; FQS, fluorescent quenched substrate; AEBSEF, 4-(2-aminoethyl)-benzenesulfonyl fluoride

sin has also been found on the inner acrosomal membrane of spermatozoa in the perivitelline space where it may have a role in membrane fusion [26].

In this report we demonstrate the utility and specificity of our FQS to assay acrosin and other proteases for their potential to activate PAR-2. Evidence is provided that acrosin causes PAR-2-mediated intracellular Ca^{2+} mobilization in Chinese hamster ovary (CHO) cells expressing PAR-2 and that mouse oocytes express PAR-2.

2. Materials and methods

2.1. Materials

Tissue culture media and media supplements were purchased from Trace Biosciences (Sydney, Australia). Non-enzymic dissociation solution and bovine serum albumin (BSA) were purchased from Sigma (Sydney, Australia). The antibiotic G-418 sulfate was obtained from Gibco-Life Technologies (Melbourne, Australia) and Fura-2-acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals used were of the highest grade commercially available.

The synthetic peptide, Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys(Dnp)-Asp-OH was synthesized as previously described [27]. Acrosin from boar sperm was purified as described [28]; recombinant human mast cell skin tryptase I was purified as previously described [29] and human thrombin and factor Xa were obtained from Wei Wen Dai (Department of Biochemistry and Molecular Biology, Monash University). The synthetic human PAR-2 agonist peptide, H-SLIGKV-NH₂ (RAP) was synthesized by Auspep Pty. Ltd. (Parkville, Australia). Human plasmin, urokinase, activated protein C (APC), kallikrein and bovine trypsin were purchased from Sigma (Sydney, Australia). Two different anti-mouse PAR-2 antibodies (PAR-2A and PAR-2B), which have been extensively characterized as being specific for PAR-2, were used for immunohistochemistry. Both antibodies were prepared in rabbits immunized with synthetic peptides corresponding to the tethered ligand region of mouse PAR-2. PAR-2A [30] was kindly provided by COR Therapeutics and PAR-2B [15] by the late Prof. Stuart Stone.

2.2. Generation of CHO transfectants expressing PAR-2

Briefly, CHO cells were co-transfected using calcium phosphate with pSV2Neo and either pBJ vector [31] or pBJ vector containing the human PAR-2 full-length coding sequence. Cells were selected with the antibiotic G-418 sulfate for neomycin resistance and individual colonies were isolated by repeated rounds of limited dilution. Single cell clones expressing high levels of PAR-2 were selected by flow cytometry using S14.8.2, a monoclonal antibody specific to the N-terminus of human PAR-2. PAR-2 clones were then screened for high levels of activity using arachidonate release [32] in response to trypsin as a measure of activity.

2.3. Enzyme assays and measurements of kinetic constants

All experiments were performed at 37°C in appropriate assay buffers. Assays using acrosin, plasmin, urokinase, APC, factor Xa and kallikrein were performed in 50 mM Tris-HCl (pH 7.4), 1 mM CaCl_2 , 0.02% (w/v) NaN_3 . Specific assay buffers were used for the following enzymes: trypsin – 0.1 M HEPES (pH 7.4), 0.1 M NaCl, 10 mM CaCl_2 , 0.1% (w/v) PEG (8000), 0.02% (w/v) NaN_3 ; thrombin – 50 mM Tris-HCl (pH 7.4), 10 mM NaCl, 0.2% (w/v) PEG (8000), 0.02% (w/v) NaN_3 ; and tryptase – 0.1 M HEPES (pH 7.5), 10% glycerol (v/v), 10 μM heparin, 0.05% (v/v) brij-35, 0.02% (w/v) NaN_3 . The substrate solution (0.5 ml) was equilibrated to 37°C for 10 min and the enzyme solution added. Enzyme activity was monitored by continuously measuring the fluorescence ($\lambda_{\text{ex}} = 330 \text{ nm}$; $\lambda_{\text{em}} = 440 \text{ nm}$) using a Molecular Dynamics BioLumin 960 Microassay Reader (Pharmacia Biotech, Australia) [33].

Lyophilized PAR-2 peptides were resuspended in dimethylformamide and the concentration of stock solutions determined spectrophotometrically, where the absorption coefficient was assumed to be $10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 360 nm [33]. Fluorescence of peptide products after exhaustive cleavage by trypsin was found to be proportional to concentration. Accordingly, an increase in fluorescence corresponds to an increase in concentration of cleaved peptide. Therefore, from an anal-

ysis of initial velocities obtained at different substrate concentrations, the kinetic parameters, K_m and k_{cat} were determined.

2.4. Cells and culture conditions

The transfected CHO cell line stably expressing PAR-2 and the non-transfected cell line were cultivated in Dulbecco's modified Eagle medium: Hams F-12 (1:1; pH 7.4) containing L-glutamine (200 mM), 10% (v/v) fetal bovine serum, penicillin/streptomycin (5000 U/ml), NaHCO_3 (1.2 g/l) and the selective agent, antibiotic G-418 sulfate (0.565 g/l). They were grown as monolayer cultures (in flasks up to 175 cm^2) under a 5% CO_2 atmosphere of 100% humidity at 37°C.

2.5. Measurement of intracellular calcium ($[\text{Ca}^{2+}]_i$) responses in CHO cells

Cells grown to 80% confluence were washed with phosphate-buffered saline (PBS) and removed from the flasks using non-enzymatic dissociation solution and prepared for $[\text{Ca}^{2+}]_i$ measurements essentially according to published methods [34]. Cells were resuspended at a concentration of 10×10^6 cells/ml in extracellular medium (EM; 121 mM NaCl, 5.4 mM KCl, 0.8 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.8 mM CaCl_2 , 5.5 mM glucose, 25 mM HEPES (pH 7.4)) containing 0.2% (w/v) BSA.

The CHO cells were loaded with the fluorescent indicator Fura-2/AM at room temperature as previously described [34]. Cells were centrifuged prior to fluorescence measurements and resuspended in EM (without BSA) at a concentration of 2×10^6 cells/ml for fluorescence measurements in stirred cuvettes. The ratio of the Fura-2/AM fluorescence at 510 nm after excitation at 340 and 380 nm was monitored using a Perkin-Elmer LS-50B spectrofluorimeter. After initial baseline recording, proteinases (trypsin and acrosin) and receptor agonist, RAP, were added to the cells and the ratio of the fluorescence at the two excitation wavelengths, which is proportional to the $[\text{Ca}^{2+}]_i$, was measured.

2.6. Immunohistochemistry

Ovaries removed from 16 week old mice were prepared for immunohistochemistry and stained according to methods employed by Abraham et al. [15]. Briefly, the ovaries were fixed in paraformaldehyde (4% (w/v) in PBS), cryoprotected in sucrose (25% (w/v) in PBS) and embedded in Tissue-Tek OCT compound. Serial cryosections (10 μm) were treated with 0.3% (w/v) hydrogen peroxide in methanol to quench endogenous peroxidase activity. PAR-2A (1:1000), PAR-2B (2.5 $\mu\text{g/ml}$) or normal rabbit serum (1:1000) were diluted in PBS containing 0.1% (w/v) BSA, applied to sections and then detected using the avidin-biotin-peroxidase complex system (Immunopure, ABC Peroxidase Staining kit, Pierce, Rockford, USA) and 3,3'-diaminobenzidine peroxidase substrate (Sigma, Sydney, Australia), according to the manufacturers' instructions. The sections were counterstained with hematoxylin, dehydrated, mounted and examined with a light microscope.

3. Results and discussion

The PAR-2 fluorescent quenched peptide substrate, Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys(Dnp)-Asp-OH, was firstly evaluated for its accuracy as a predictor of receptor cleavage. The Abz group only fluoresces when the quenching group Lys(Dnp) is released upon proteolytic cleavage. Therefore, activators of PAR-2 would be predicted to hydrolyze the

Table 1
Kinetic parameters of the cleavage of a synthetic peptide corresponding to the activation sequence of PAR-2

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M s}^{-1}$)
Trypsin	13.5	372.0	27.6
Tryptase	29.1	426.0	14.6
Acrosin	22.0	320.0	14.6

Thrombin, plasmin, urokinase, APC, kallikrein and factor Xa were unable to cleave the substrate at concentrations up to 100 nM. The standard error of the mean for each derived kinetic constant was always below 10%.

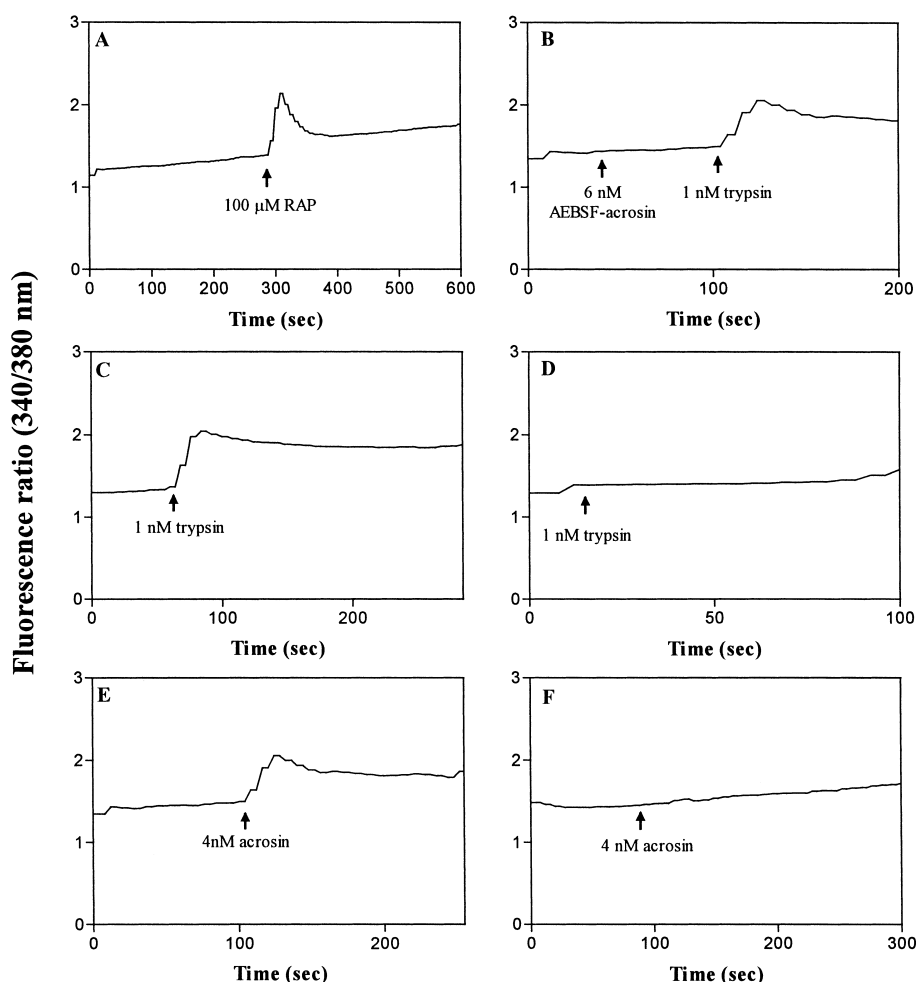


Fig. 1. The $[Ca^{2+}]_i$ response of PAR-2-transfected (A, B, C and E) and non-transfected CHO cells (D and F) in response to RAP, AEBSF-treated acrosin (AEBSF-acrosin), trypsin, or acrosin.

synthetic peptide, while non-activators would be incapable of doing so. In order to test this system, the kinetics of cleavage of the peptide with known activators of PAR-2 (trypsin, trypsinase), as well as known non-activators (thrombin, plasmin, urokinase, APC, kallikrein) were investigated. It was found that trypsin could cleave the substrate at a concentration as low as 0.1 nM, and trypsinase as low as 10 nM, while thrombin and the other known non-activators were unable to cleave the substrate at significantly higher levels (up to 100 nM). Trypsin and trypsinase efficiently cleaved the substrate as exhibited by the high k_{cat}/K_m values (Table 1), consistent with the knowledge that these two proteases are known activators of PAR-2 [1–18]. Additionally, heat-inactivated trypsin and trypsinase were unable to cleave the substrate, demonstrating that the cleavage effect shown by the proteases is due to enzymatic activity. The cleavage of the PAR-2 substrate by trypsin and trypsinase, but not the non-activators, supports the hypothesis that the peptide may be considered as an effective model for determining the potential of a given proteinase to activate the receptor.

Having established the FQS as a suitable model for the PAR-2 cleavage site, we went on to investigate the ability of two proteinases (factor Xa and acrosin), identified by Fox et al. [20] as putative activators of PAR-2, to cleave the FQS.

Firstly, the ability of factor Xa to cleave PAR-2 has been the subject of contradictory data. With regard to data derived from interactions with peptides or peptide inhibitors containing the sequence of the activation site of PAR-2, it has been suggested that factor Xa may be a PAR-2 activator, although the interaction was not as strong as for known activators such as trypsin or trypsinase [20]. Molino et al. [18] showed that factor Xa cleaved a synthetic peptide representing the activation site of the receptor weakly in comparison to trypsin or trypsinase. The studies we carried out with the FQS similarly indicate that the enzyme does not activate PAR-2, which is consistent with previous studies demonstrating that factor Xa does not activate the receptor [35]. A recent study has now shown that factor Xa can activate PAR-2 in vivo [36], however. Thus, while experiments with synthetic peptides representing the activation site of PAR-2 are instructive with regard to the kinetics of cleavage of the sequence by a particular protease, the results with factor Xa suggest that they are interpreted with caution and need to be confirmed by studies with the whole receptor in a cellular setting.

Acrosin was also investigated for its ability to cleave the PAR-2 model substrate. It is known that, like trypsin, acrosin cleaves after arginine or lysine residues, with preference for arginine over lysine [21]. Considering this and previous inhi-

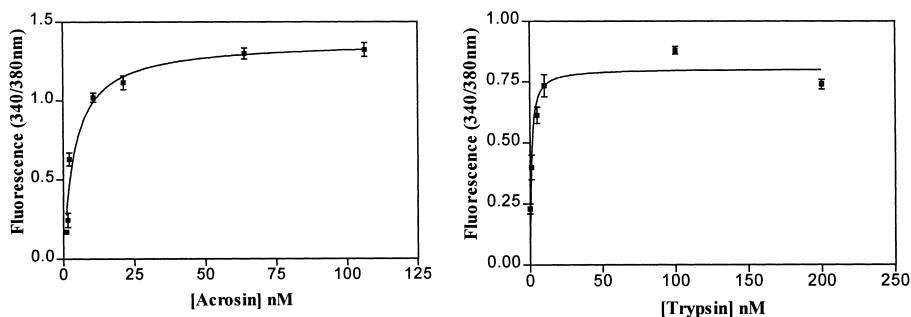


Fig. 2. The $[Ca^{2+}]_i$ response of PAR-2-transfected CHO cells to different concentrations of acrosin (A) and trypsin (B). The $[Ca^{2+}]_i$ values for each enzyme concentration represent the mean $\pm$ S.E.M. derived from three traces similar to those shown in Fig. 1; dose response curves for the two enzymes were also conducted on non-transfected cells, and the low levels of background $[Ca^{2+}]_i$ values were subtracted from the corresponding values for PAR-2-transfected cells to obtain the curves displayed here.

bition studies [20], the PAR-2 FQS was used to examine the potential of acrosin to cleave PAR-2. Acrosin at concentrations as low as 0.1 nM was found to cleave the substrate with similar kinetics to trypsin and tryptase (Table 1), suggesting that the enzyme is indeed a potential physiological PAR-2 activator.

Based on acrosin's ability to hydrolyze the PAR-2 peptide, we sought evidence that the proteinase may be able to cleave within the intact receptor itself. This possibility was addressed by monitoring the increase in intracellular calcium levels of CHO cells stably expressing the intact human form of PAR-2. Acrosin was applied to both the PAR-2-transfected and the non-transfected cells and tested for a $[Ca^{2+}]_i$ response. Trypsin, a well characterized activator of PAR-2, and the RAP were also tested for a response on the cells. The transfected cells showed an increase in $[Ca^{2+}]_i$ in response to RAP treatment (Fig. 1A), whereas a response was not seen in the non-transfected cells (data not shown). Both trypsin and acrosin (Fig. 1C,E), at concentrations as low as 1 nM, were shown to induce significant intracellular calcium responses in the PAR-2-transfected CHO cells, whereas no response was observed in the non-transfected cells at the same enzyme concentrations (Fig. 1D,F). At concentrations higher than 4 nM acrosin or 1 nM trypsin, a response, although significantly smaller, was observed in the non-transfected cells. This response was characterized by a small and slowly developing elevation in calcium concentration (also observed by Nystedt et al. [37] for CHO cells), in contrast to the more rapid responses for the transfected cells. These residual responses in the non-transfected cells may be attributed to the presence of endogenous PAR-2 on the surface of the cells. Additionally, acrosin inactivated by the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) or by boiling was unable to elicit a response in the transfected cells, with the cells showing a normal response when subsequently challenged by trypsin (Fig. 1B).

After adjusting for the small background responses seen in the non-transfected cells, acrosin was found to induce a dose-dependent increase in $[Ca^{2+}]_i$ in the transfected CHO cells. The enzyme concentration inducing half the maximal response (EC_{50}) was 4.1 ± 0.34 nM (Fig. 2A), which is comparable to the EC_{50} of trypsin on the transfected cells of 0.98 ± 0.09 nM (Fig. 2B). These results indicate that acrosin is able to activate PAR-2 on cell surfaces.

As proteinase-activated receptors are activated by irreversible cleavage of the extracellular domain, the receptors under-

go rapid desensitization after a short period of activation by an enzyme agonist. Thus, it has been shown that a single application of trypsin causes marked desensitization of $[Ca^{2+}]_i$ responses (Fig. 3A). Considering this, studies were

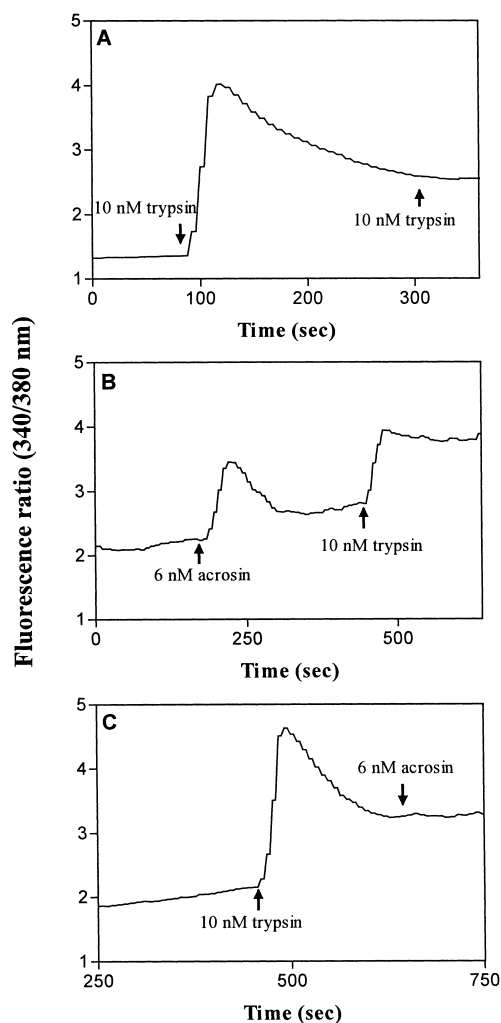


Fig. 3. The effects of desensitization on $[Ca^{2+}]_i$ in PAR-2-transfected CHO cells treated with trypsin and acrosin. The effects of adding (A) 10 nM trypsin followed by another dose of 10 nM trypsin; (B) 6 nM acrosin followed by 10 nM trypsin; and (C) 10 nM trypsin followed by 6 nM acrosin; were tested in CHO cells transfected with human PAR-2.

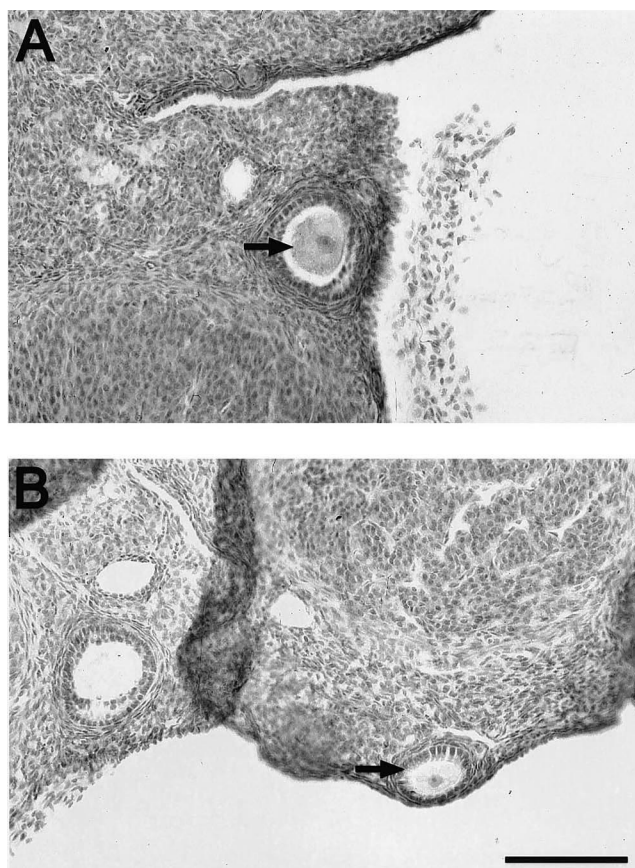


Fig. 4. Immunohistochemical localization of PAR-2 in mouse oocytes. A: Section of mouse ovary stained with PAR-2B antibody. Specific staining is visible throughout the cytoplasm and plasma membrane of an oocyte (arrow) in a primary follicle. Similar staining has also been seen with the PAR-2A antibody. B: Adjacent section of mouse ovary incubated with normal rabbit serum. Bar = 25 μ m.

carried out using the $[Ca^{2+}]_i$ mobilization assay in order to determine if acrosin and trypsin were activating the same receptor. Exposure of the transfected cells to a maximal concentration of trypsin (10 nM – see Fig. 2) desensitized the $[Ca^{2+}]_i$ responses to a subsequent challenge by acrosin (Fig. 3B). When the PAR-2-transfected cells were activated firstly with a sub-maximal dose of acrosin, a secondary response to trypsin was significantly reduced (Fig. 3C). These results strongly suggest that trypsin and acrosin activate a common receptor on the transfected cells, namely PAR-2.

The results obtained imply that acrosin is an activator of PAR-2. In this respect, it is interesting to note that PAR-2 immunoreactivity has previously been detected in the female reproductive tract, with PAR-2 being expressed in the follicle epithelium, follicle smooth muscle and myofibroblasts of the human ovary [7]. Since expression of PAR-2 has not been reported on oocytes, we sought to address this question via immunohistochemical staining of mouse oocytes with antibodies specific for PAR-2. In Fig. 4, oocytes located within the mouse ovary are shown to be specifically stained by antibodies to PAR-2. PAR-2 immunoreactivity was observed in both the cytoplasm and plasma membrane of oocytes in primary, secondary and tertiary follicles.

The potential role of acrosin in the recognition, binding and

penetration of the zona pellucida by sperm has been well documented [24,25]. Here we show that acrosin can induce significant intracellular calcium responses on PAR-2-bearing cells, and that the receptor is expressed by oocytes. These observations suggest that acrosin is likely to activate PAR-2 on oocytes. Such activation processes may play a role in fertilization by enhancing the fusogenicity of the oolemma for the sperm's plasma membrane, or initiating cortical granule exocytosis and the block to polyspermy, or the activation of calcium spiking within the egg cytoplasm which is required for subsequent development [38–40].

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