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Research Article

Endogenous trypsin receptors in *Xenopus* oocytes: linkage to internal calcium stores

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Abstract. The effects of the protease trypsin, externally applied to full-grown oocytes of *Xenopus laevis*, were studied using electrophysiology and fluorometry. The following results were obtained: trypsin in concentrations of 0.1 μg/ml to 1 mg/ml liberated Ca^{2+} from internal stores and evoked large transient currents of up to 5 μA in bath solutions containing 1 mM or no Ca^{2+}. The response desensitized for 50 minutes and recovered at longer times. Transient currents could also be elicited by tryptic impurities in commercially available collagenase used for defolliculation of oocytes. Application of chymotrypsin (0.01 or 1 mg/ml) or of thrombin (3.4 ng/ml or 0.34 mg/ml) neither evoked currents nor desensitized trypsin responses. Incubation with 1 μg/ml Pertussis toxin for 20 to 25 hours prevented the Ca^{2+} release from internal stores and the activation of transient currents by trypsin. We propose that endogenous receptors in the oolemma, specific for trypsin, are linked to internal Ca^{2+} stores via Pertussis toxin-sensitive G proteins. Thus, receptor activation by external trypsin raises internal Ca^{2+} and thereby opens Ca^{2+}-activated Cl^- channels in the oolemma.

Key words. Trypsin; PAR-2 membrane receptor; desensitization; chloride current; G protein; Ca^{2+} fluorescence; *Xenopus* oocyte.

The proteolytic enzyme trypsin can modify specific membrane transport processes. For example, intracellular application of trypsin removes the inactivation of voltage-dependent Na^+ channels [1], decreases the Ca^{2+} sensitivity of L-type Ca^{2+} channels [2] and prevents the spontaneous decline in the activity of ATP-sensitive K^+ channels [3]. In addition to these intracellular effects of trypsin, a novel extracellular action of this enzyme was reported recently for oocytes of the clawed toad *Xenopus laevis* [4]. In these cells, external application of trypsin was shown to induce transient Ca^{2+}-activated Cl^- currents, while intracellular trypsin injection was ineffective. The findings were interpreted by the presence of endogenous trypsin receptors in the oocyte membrane, a release of Ca^{2+} from internal stores after receptor activation, and a subsequent activation of Ca^{2+}-activated Cl^- channels in the oolemma [4].

The aim of this study was to characterize the postulated trypsin receptor in the oolemma of *Xenopus laevis* in more detail, in particular (i) its desensitization properties, (ii) the interaction with other proteolytic enzymes and (iii) the mode of linkage to intracellular Ca^{2+} stores. In addition to these electrophysiological experiments we tried to obtain direct evidence for mobilization of internal Ca^{2+} by external trypsin using

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fluorometry. Some of the results have been published in abstract form [5].

Materials and methods

Oocytes. Fully grown, prophase I-arrested oocytes of *Xenopus laevis* were defolliculated manually with fine forceps or enzymatically using collagenase (Sigma type IA, 2 mg/ml of Barth's solution, at 20 °C, for 3 to 5 hours). Our modified Barth's solution contained (in mM): 100 NaCl, 3 KCl, 1 $CaCl_2$, 0.8 $MgSO_4$, 15 HEPES; 220 mosm, pH 7.6. After collagenase treatment, oocytes were washed in Ca^{2+}-free Barth's solution containing 1 mM EGTA for 5 minutes. For recovery, oocytes were stored in Barth's at 18 to 20°C overnight before use. Each type of experiment was performed on $n = 3$ to 10 oocytes from at least two different batches of oocytes and from two different donor females.

Substances. Trypsin (8 U/mg), trypsin inhibitor from soybean (Type I-S), chymotrypsin (60 U/mg), Pertussis toxin (PTX), cytochalasin D from Sigma and thrombin (97 U/mg) from Serva (Heidelberg, Germany) were applied in the concentrations listed in the text and figure legends. Collagenases were from Sigma (type IA, lots 93H6816 and 122H1112 with tryptic activities of 0.1 to 0.2 U/mg) and from Serva (type 17449, lot 30034C with tryptic activity of 0.1 U/mg).

Electrophysiological measurements. Membrane potentials and membrane currents of the oocytes were measured with a standard two-microelectrode technique using a TURBO TEC 01C from npi electronic (Tamm, Germany); for details see [6]. The electrodes were pulled from borosilicate capillaries with filament and filled with 3 M KCl (potential electrode) or 2 M K^+ citrate plus 60 mM KCl (current electrode). The resistances of potential and current electrodes were between 0.8 and 1.5 MΩ. All electrophysiological measurements were performed at room temperature (18 to 22 °C) under continuous perfusion of the oocytes with the appropriate bath solution in Plexiglas chambers filled to a depth of 3 mm. Membrane resting potentials and membrane resistances were determined approximately 15 minutes after impalement of the electrodes. Oocytes were discarded if the initial membrane resistance was below 400 kΩ in Barth's solution. Membrane currents under voltage clamp conditions as shown in figures 1 to 8 were recorded on a pen recorder from Linseis (Selb, Germany) and represent the total current flowing through the entire oolemma. Outward currents (plotted upwards) correspond to an influx of anions, and inward currents to an efflux of anions. If two measurements were performed on the same oocyte at time intervals longer than one hour or when oocytes were incubated with toxins, the oocytes were washed intensively in Barth's and stored in microtiter wells between the measurements.

Calcium measurements. Changes in intracellular Ca^{2+} concentration were measured by Fura-2 fluorescence at 360 nm (isobestic wavelength) and 380 nm (Ca^{2+} sensitive wavelength). Oocytes were microinjected with 30 to 40 nl of a solution containing 6 mM of Fura-2 pentapotassium salt (MoBiTec, Göttingen, Germany) 10 to 180 minutes before the experiment. No dependence of the results on the time interval between Fura-2 injection and fluorescence measurements could be detected in our experiments. After injection of the dye, the oocytes were transferred into a 4 ml recording Petri dish filled with Barth's or Ca^{2+}-free Barth's

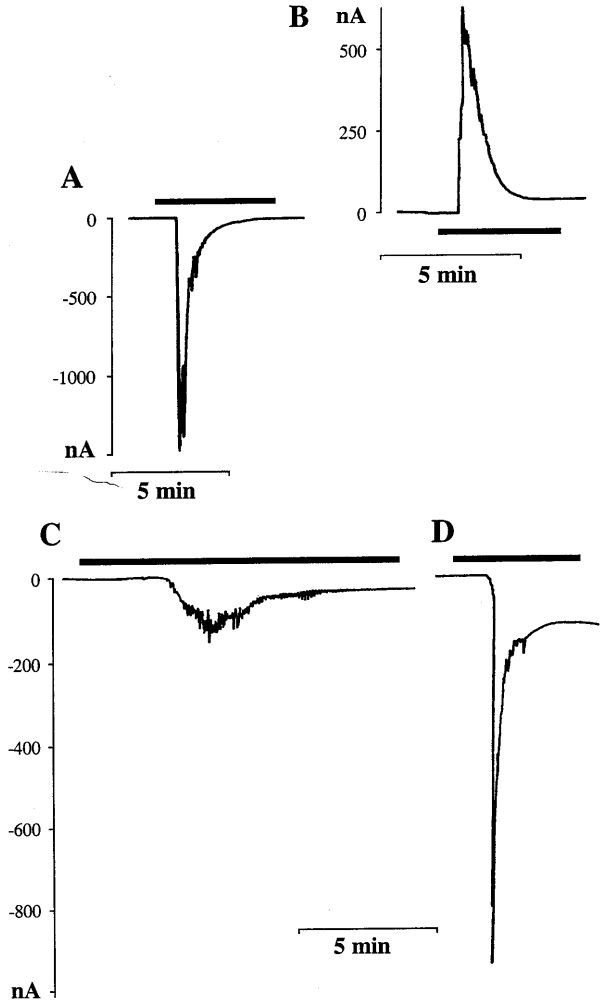

Figure 1. Trypsin responses in Barth's at holding potentials of −70 mV (*A*, *C*, *D*) and 0 mV (*B*). Durations of trypsin application in this and in the following figures are indicated by solid horizontal bars. The current responses were elicited by trypsin concentrations of 10 µg/ml (*A*, *B*, *D*) and 0.1 µg/ml (*C*). Experiments in (*A*, *B*) and (*C*, *D*) were performed on three separate oocytes. Time interval 2 hours between records (*C*) and (*D*).

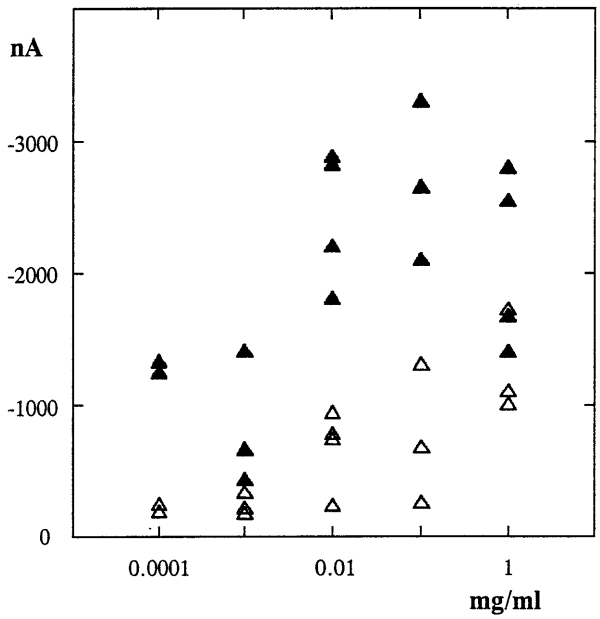

Figure 2. Peak currents of trypsin responses in Barth's. Each symbol represents a measurement on a separate oocyte. Holding potentials were −60 mV (n = 5), −70 mV (n = 24) or −80 mV (n = 3). The experiments were performed in August on oocytes from two donors (▲ and △).

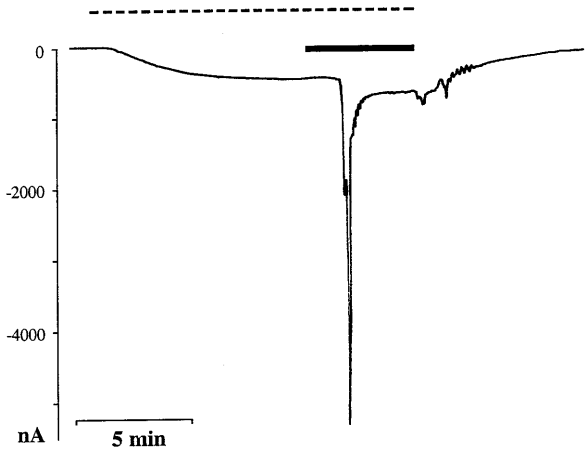

Figure 3. Trypsin response in Ca^{2+}-free Barth's. The oocyte was bathed in Barth's at the beginning and the end of the recording and in Ca^{2+}-free Barth's containing 1 mM EGTA (dashed line). Application of trypsin (10 μg/ml) in Ca^{2+}-free Barth's evoked large transient inward currents. Holding potential was −70 mV.

solution containing 1 mM EGTA, and were fixed with a 0.5 mm thick, ⊃-shaped platinum loop. The chamber with a Fura-2 loaded oocyte was placed on the stage of an upright microscope (Zeiss, Axioskop) and imaged with a ×10, 0.50 objective (Zeiss, Fluar). Pulses of light from a Xenon lamp were generated with a galvanometric scanner (T.I.L.L. Photonics, Munich) which was coupled to the microscope via a light guide. Images were recorded with an integrating slow scan camera (Theta Systems, Munich) and digitized to 12 bits per pixel. Paired images obtained with excitation at 360 and 380 nm were used to calculate pixel ratios (for details see [7]). The ratios are shown as pseudocolours (fig. 9) with blue and red corresponding to low and high intracellular Ca^{2+} concentrations respectively.

Results

Transient currents induced by trypsin. Figure 1 shows transient currents in oocytes elicited by the serine protease trypsin, in the perfusing Barth's solution bathing the oocytes. The currents were inward at a membrane potential of −70 mV (figs 1A, C, D), outward at 0 mV (fig. 1B), rose to a peak value within 30 seconds (figs 1A, B, D) to 2 minutes (fig. 1C) and decayed towards a stationary value after approximately 5 minutes. Comparable transient currents induced by trypsin had been described recently for manually defolliculated *Xenopus* oocytes [4]. Our experiments were mainly conducted on oocytes defolliculated by treatment with collagenase, but manually defolliculated oocytes gave similar results. Our mode of defolliculation did not therefore affect the trypsin response. Almost all (≥95%) defolliculated oocytes responded to trypsin, if the initial membrane resistance exceeded 400 kΩ. After treatment with trypsin (0.1 μg/ml to 1 mg/ml) for up to 12 minutes, the membrane potential V_m reached almost its initial value of approximately −60 mV, whereas the membrane resistance R_m declined to below one half. For example, in twenty experiments with oocytes in Barth's solution, treated with trypsin (10 μg/ml), the following values were obtained: $V_m = -61.1 \pm 2.4$ mV, $R_m = 735 \pm 57$ kΩ (means ± SEM) before application of trypsin and $V_m = -51.9 \pm 3.3$ mV, $R_m = 322 \pm 35$ kΩ approximately 15 minutes after washout of the enzyme. In general, the amplitude of the trypsin-induced transient currents became larger and their onset and offset kinetics faster at increasing concentrations of trypsin in the bath solution (figs 1C, D). Since the charge transfer of the trypsin response is hardly voltage dependent between −40 and −70 mV [4], the peak current values recorded at holding potentials of −60, −70 and −80 mV were pooled. Nevertheless, results from different oocytes were very variable, in particular the peak amplitude of the currents. This is illustrated in figure 2 with a summary of peak current values from experiments performed during one month, on oocytes of two different donors.

The experiments described so far were performed on oocytes in Barth's solution containing 1 mM $CaCl_2$.

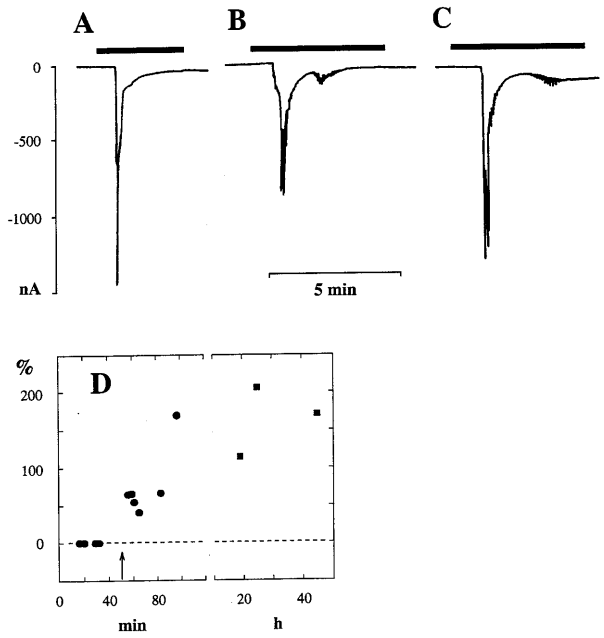

Figure 4. Desensitization and recovery of the trypsin response in Barth's. The records show currents elicited by the first (*A*), second (*B*) and third (*C*) application of 10 μg/ml trypsin to the same oocyte. Currents in (*B*) were recorded 56 minutes after (*A*), currents in (*C*) recorded 18 hours after (*A*). Between the recordings, the oocyte was bathed in Barth's. Holding potential was −50 mV during the recordings of (*A*–*C*). (*D*) Summary of all desensitization and recovery experiments. The maxima of the current responses during the second (●) and third (■) application of trypsin are normalized with respect to the peak current elicited by the first treatment with trypsin. Trypsin concentration was 10 μg/ml in all experiments. The trypsin response was completely desensitized up to 50 minutes after the first application of trypsin (arrow) and recovered at longer periods. Holding potentials were −40 mV (n = 1), −45 mV (n = 2), −50 mV (n = 2), −60 mV (n = 3), −70 mV (n = 2), −80 mV (n = 3).

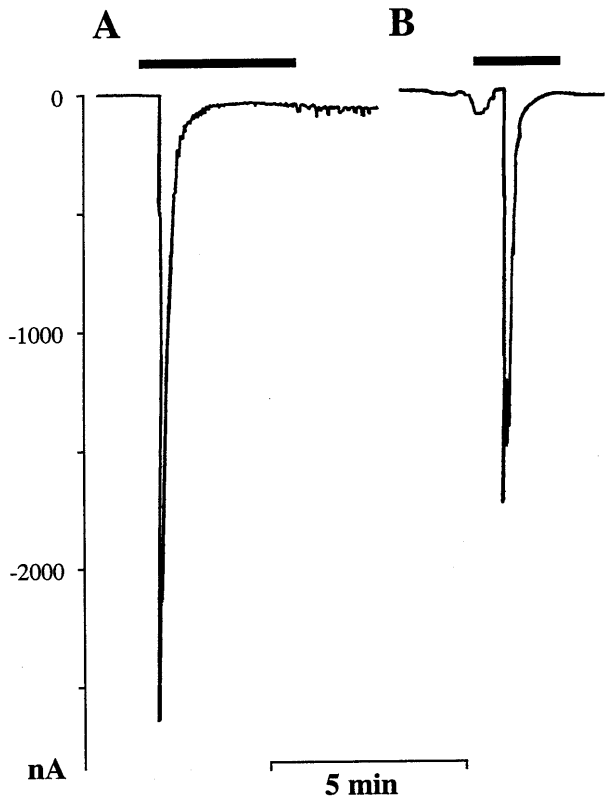

Figure 5. The cytotoxin cytochalasin D does not interfere with the recovery of trypsin receptors. (*A*) A control response was measured by application of trypsin (10 μg/ml Barth's) (bar). Afterwards the oocyte was washed for 1 hour in Barth's, then incubated with cytochalasin D (30 μM, final concentration) for 45 minutes and 87 minutes after the first trypsin application (*B*) again checked for a second response to trypsin (10 μg/ml Barth's) (bar). Holding potential was −70 mV.

However, external Ca^{2+} was not a prerequisite for the trypsin response. Figure 3 shows an experiment in which an oocyte was bathed in Barth's and in Ca^{2+}-free Barth's. After solution exchange, the membrane current declined and reached a stationary value after approximately 7 minutes. A similar reduction of membrane current in external Ca^{+}-free solution was already observed (e.g. [8], fig. 3a). A subsequent application of trypsin in Ca^{2+}-free Barth's elicited large transient currents. The peak current amplitude was −4.9 μA in the experiment shown in figure 3 and -3.4 ± 0.5 μA (mean ± SEM) in a total of five experiments performed with the same protocol.

Desensitization and recovery of the trypsin response. A second application of trypsin did not elicit transient currents, even when the oocyte was washed for 30 minutes with trypsin-free solution [4]. In our experiments we confirmed the desensitization of the trypsin response for time intervals <40 minutes between the first and the second trypsin application. However, partial to full recovery of the response was observed at longer intervals. Figures 4A–C show examples of transient currents evoked 56 minutes and 18 hours after the first application of trypsin to the same oocyte. Figure 4D gives a summary of all similar recovery experiments as a plot of normalized currents at holding potentials between −40 and −80 mV recorded at various times after the first application of trypsin.

To investigate whether the recovery of the trypsin response was dependent on the formation of new membrane receptors, oocytes were incubated in Barth's solution containing the cytotoxin cytochalasin D. This agent is known to inhibit exocytosis of internal membrane vesicles [9] and endocytosis in *Xenopus* oocytes [10]. Despite this treatment (30 μM cytochalasin D for 45 minutes, n = 2; 10 μM cytochalasin D for 20 minutes, n = 3) the trypsin response recovered with similar kinetics (fig. 5).

To explore whether high molecular weight GTP-binding proteins (G proteins) are involved in the trypsin response

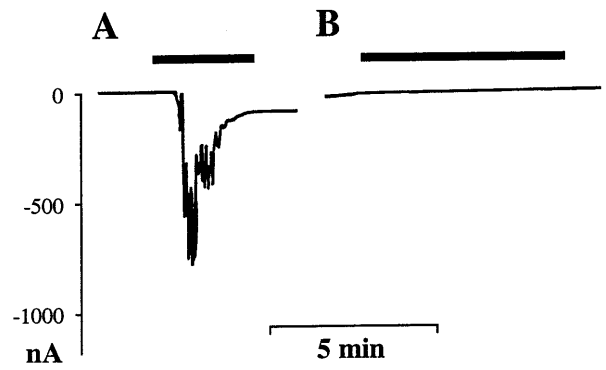

Figure 6. Heterotrimeric G proteins control the trypsin response. (*A*) A control response was measured after application of trypsin (10 μg/ml Barth's) (bar). The oocyte was then incubated with Pertussis toxin (1 μg/ml) for 23 hours and (*B*) subsequently checked for a further response to trypsin (10 μg/ml Barth's) (bar). Holding potential was −80 mV.

and its recovery, currents at −70 mV were first recorded during application of trypsin (10 μg/ml) to oocytes in Barth's. Afterwards, the enzyme was washed out for approximately one hour, and the oocytes were incubated in Barth's with Pertussis toxin (PTX) (1 μg/ml) for 15 to 27 hours. The following treatment with trypsin of 10 μg/ml induced only small transient currents in three oocytes (current amplitudes 6, 23, 25% of their initial values) and no current in three additional cells (fig. 6). Normal trypsin responses could however be elicited after incubation of oocytes in PTX-free solutions for the same period of time as seen in figure 4D.

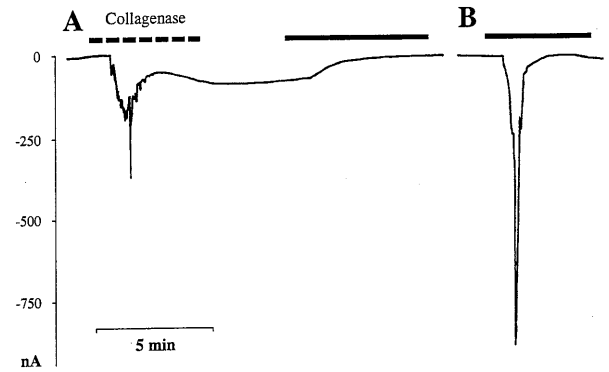

Figure 7. Trypsin-like responses elicited by collagenase. (*A*) Currents induced by collagenase (Serva, 2 mg/ml) dissolved in Barth's. After a pause of 3 minutes, the trypsin response was completely desensitized. (*B*) Recovery of the trypsin response was observed 90 minutes after record (*A*) during a second application of trypsin. All measurements were performed on the same oocyte, trypsin concentration was 10 μg/ml. Between records (*A*) and (*B*), the oocyte was bathed in Barth's. Holding potential was −50 mV during records (*A*) and (*B*).

Desensitization of the trypsin response could be achieved at low trypsin concentrations in the 0.1 μg/ml range (not shown) and even by tryptic impurities in the collagenase batch (fig. 7). Part A of this figure illustrates the effects of collagenase solution (Serva collagenase, 2 mg/ml Barth's, the concentration used for defolliculation of oocytes) which are indistinguishable from the actions induced by trypsin. No trypsin response could be evoked 3 minutes after application of the collagenase solution, whereas the trypsin response recovered after 90 minutes (fig. 7B). Similar effects of collagenase from Sigma or Serva were found in a total of nine experiments. To check whether the proteolytic impurities of the collagenases were of tryptic nature, soybean trypsin inhibitor was used. When oocytes were preincubated in Barth's containing 0.5 to 1 mg/ml (final concentration) of the inhibitor and subsequently treated with the collagenase solution, the currents were strongly reduced or completely inhibited (n = 7). If the inhibitor was applied

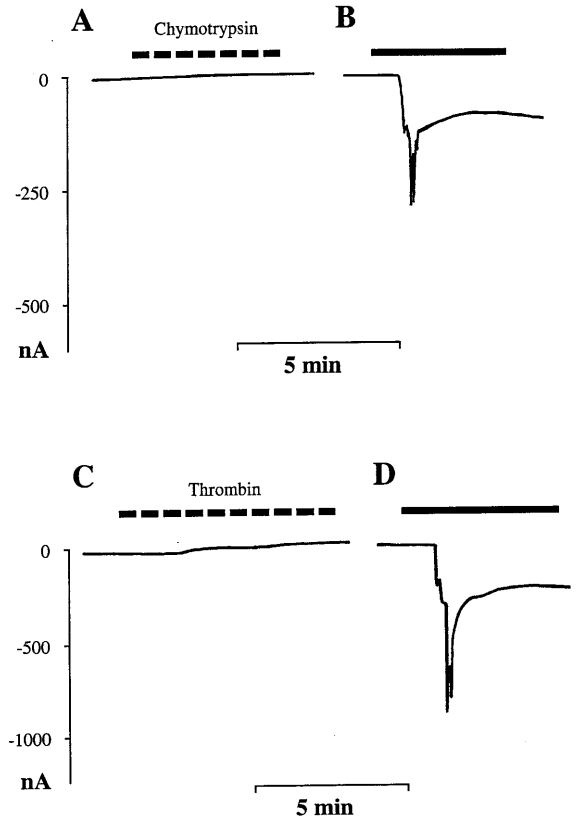

Figure 8. The membrane receptor is specific for trypsin only. (*A*, *B*) Chymotrypsin: (*A*) The oocyte was treated with chymotrypsin (10 μg/ml Barth's) and (*B*) after 5 minutes stimulated with trypsin (10 μg/ml Barth's) (bar). (*C*, *D*) Thrombin: (*C*) The oocyte was treated with thrombin (10 μg/ml) and (*D*) after 1 minute stimulated with trypsin (10 μg/ml Barth's) (bar). Holding potentials were −80 mV for the chymotrypsin and −50 mV for the thrombin experiment.

together with the collagenase, no currents were elicited (n = 5). The trypsin inhibitor itself did not induce Ca^{2+}-activated Cl^- currents in seven experiments (not shown).

The specificity of the trypsin response was tested in experiments with chymotrypsin (0.01 or 1 mg/ml, n = 7) (fig. 8A, B) or thrombin (3.4 ng/ml or 0.34 mg/ml, n = 7) (fig. 8C, D) supplemented to Barth's. Neither of these proteolytic enzymes induced a transient current. Immediately after washout of the enzymes, full-sized trypsin responses could be elicited. Hence, the trypsin response could not be evoked and desensitized by the other proteolytic enzymes tested.

Trypsin liberates Ca^{2+} from internal stores. Fluorescence measurements were employed to study Ca^{2+} signals in oocytes induced by externally applied trypsin. Figure 9 shows examples of Ca^{2+} ratio images obtained before, and at various times after application of trypsin. An increase of Ca^{2+} occurred in oocytes bathed in Barth's (n = 3) (not shown) as well as in oocytes bathed in Ca^{2+}-free Barth's containing 1 mM EGTA (figs 9a–c). Hence, a Ca^{2+} influx into the oocyte was not a prerequisite for the increase of intracellular Ca^{2+}. Due to the limited spatio-temporal resolution of our fluorescence measurement device, we could neither detect local Ca^{2+} transients in the subcortical layer under the oolemma nor resolve their kinetics during the rise of transient currents stimulated by external trypsin. Nevertheless, the spread of Ca^{2+} waves over the entire oocyte, as illustrated in figure 9, occurred in a time range roughly comparable to the electrophysiological trypsin response. Approximately 10 minutes after application of trypsin, the Ca^{2+} fluorescence signal in the oocyte declined again (fig. 9c and nine additional experiments).

After preincubation of oocytes with Pertussis toxin (1 to 3.5 μg/ml Ca^{2+}-free Barth's containing 1 mM EGTA) and subsequent application of trypsin (0.5 to 60 μg/ml Ca^{2+}-free Barth's containing 1 mM EGTA) no Ca^{2+} transients could be detected (figs 9d–f and 13 additional experiments). This inhibitory effect of PTX

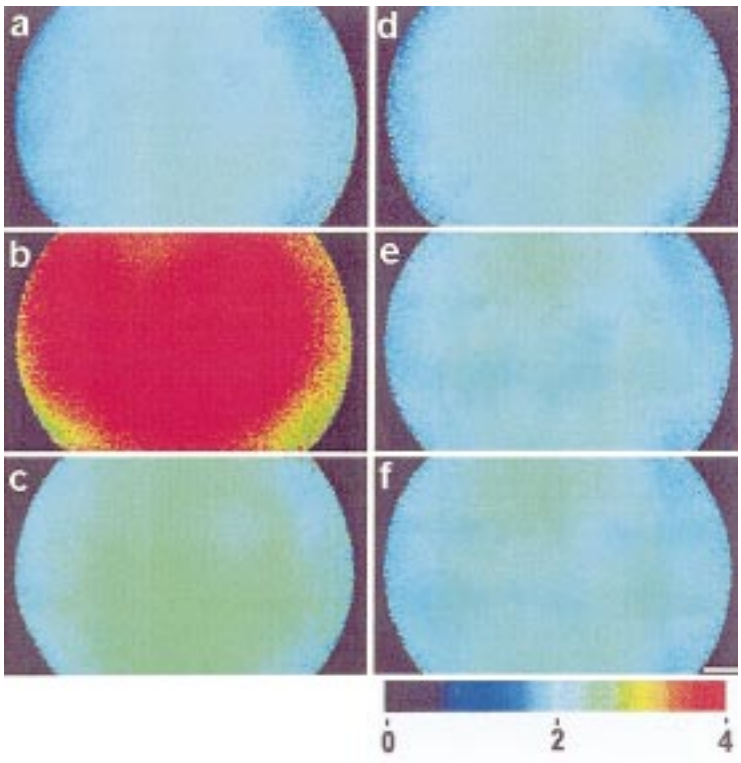

Figure 9. Trypsin-induced Ca^{2+} release in control oocytes (*a–c*) and in oocytes treated with Pertussis toxin PTX (*d–f*). Fura-2 (6 mM) was microinjected (32 nl) 30 to 60 minutes before the experiments, and the oocytes were bathed in Ca^{2+}-free Barth's (containing 1 mM EGTA) at 23 °C (*a–c*): Ca^{2+}-dependent fluorescence was recorded before (*a*), 0.5 minute after (*b*), and 10 minutes after (*c*) addition of trypsin to the chamber (final concentration 60 μg/ml Ca^{2+}-free Barth's containing 1 mM EGTA). (*d–f*): This oocyte was preincubated with 3.5 μg PTX per ml Barth's at 20 °C for 22 hours, bathed in Ca^{2+}-free Barth's containing 1 mM EGTA, and fluorescence was recorded before (*d*), 0.5 minute after (*e*), and 7 minutes after (*f*) addition of trypsin to the chamber (final concentration 60 μg/ml Ca^{2+}-free Barth's containing 1 mM EGTA). All images are ratio images. The ratio colour scale and the 100 μm bar are common to all images (see *f*). Oocytes were mounted with a ⊃-shaped platinum loop in the recording chamber, hence all fluorescence images are truncated at the upper and lower sections of the oocytes.

developed within several hours: reduction of trypsin-induced currents started at 15 hours and reached a maximum at 22 to 25 hours of PTX incubation.

Discussion

The experiments described in this paper confirm and extend a previous investigation of the effects of trypsin on *Xenopus laevis* oocytes [4]. New results obtained in our study are: 1. the Ca^{2+} release and the induction of transient currents by trypsin are Pertussis toxin-sensitive (figs 6, 9d–f). 2. The trypsin response recovers from desensitization after approximately 50 minutes (fig. 4). 3. The recovery cannot be prevented by cytochalasin D (fig. 5). 4. The trypsin response is not desensitized by other proteolytic enzymes (fig. 8). 5. External trypsin increases the Ca^{2+} concentration in oocytes bathed in Barth's or in Ca^{2+} free Barth's (fig. 9).

These results are strong evidence for the presence of specific trypsin receptors in the oolemma that are linked to intracellular Ca^{2+} stores via Pertussis toxin-sensitive G proteins. A similar signalling pathway had been proposed between activation of muscarinic receptors and liberation of internal Ca^{2+} in *Xenopus* oocytes [11]. The increase of internal Ca^{2+} upon receptor activation then transiently opens ion channels in the oolemma and generates the currents seen in our experiments after application of trypsin. The oolemma of *Xenopus* oocytes contains a high number of Ca^{2+}-activated Cl^- channels with biphasic gating kinetics [12–15], and the following observations suggest that these channels mediate the currents induced by trypsin. Intracellular injection of the Ca^{2+} chelator EGTA abolishes the trypsin response [4], the currents elicited by trypsin reverse near the Cl^- reversal potential of -15 mV [4], and an increase of intracellular Ca^{2+} triggers the trypsin response even in the absence of extracellular Ca^{2+} (fig. 3). Despite these agreements between Ca^{2+}-activated Cl^- channels and the ion channels opened by trypsin, there are marked differences between the kinetics of the currents during depolarizing voltage pulses and after application of trypsin. Whereas the currents decay with a time constant near 1 second during a depolarizing voltage step to 0 mV [13], the currents induced by trypsin decline much more slowly at the same potential (fig. 1B).

As shown in figure 4, the trypsin response does not desensitize completely but recovers after approximately 50 minutes. This recovery does not result from recycling of trypsin receptors between oolemma and cytoplasm, because the recovery cannot be prevented by the toxin cytochalasin D, which inhibits endo- and exocytosis (fig. 5). This suggests that desensitization and recovery of the trypsin response are intrinsic processes of the trypsin receptors in the oolemma.

In our experiments, induction, desensitization and recovery of the trypsin response were observed after treatment of oocytes with tryptic impurities in commercially available collagenase at concentrations used for defolliculation (fig. 7). Since the trypsin response required at least one hour for full recovery (fig. 4), oocytes should not be used within this period after enzymatic defolliculation.

Among the various endogenous receptors in the oolemma of *Xenopus laevis* [14], the receptor for lysophosphatidic acid (LPA) and related compounds [16–19] shows the greatest similarities with the trypsin receptor. Activation of both receptors by external agonists leads to long-lasting and transient currents which are observed even in Ca^{2+}-free bath solutions and are abolished by microinjection of Ca^{2+} chelators into theoocyte. Intracellular application of agonists of LPA or trypsin receptors does not evoke transient currents. Furthermore, the receptor responses desensitize with repeated administration of either LPA [18] or trypsin [4], and both responses are mediated through Pertussis toxin-sensitive G proteins [16]. Despite these agreements, the receptors for trypsin and LPA seem to be distinct from each other, because pretreatment with trypsin did not affect a LPA response recorded 30 minutes after washout of trypsin [4]. A high specificity of the receptors for trypsin was also revealed in our experiments showing no after-effects of chymotrypsin or thrombin on the trypsin response (fig. 8) which supports the notion of different trypsin and thrombin receptors [20].

The endogenous trypsin receptor in the oolemma of *Xenopus* oocytes, described in our study, might be related to the recently cloned proteolytically activated receptor PAR-2 [21], because both receptors are coupled to G proteins, and they are activated by trypsin but not by thrombin. The presence of endogenous trypsin receptors in *Xenopus* oocytes explains that trypsin stimulates ^{45}Ca efflux from the cells even without additionally expressed PAR-2 receptors [22]. Endogenous receptors specific for trypsin do not only exist in oocytes, but they have also been found in other cells, for example, vascular endothelial cells [23], human T cell lines [24] and intestinal epithelial cells [25].

The physiological significance of receptors specific for trypsin in the membrane of *Xenopus* oocytes is still unknown. The great variability of the trypsin response among different oocytes and the dependence on the donor (fig. 2) make it unlikely that a high density of trypsin receptors is essential for the homeostasis of oocytes. Instead, trypsin receptors could play a role in the fertilization signal [4], provided the receptors are preserved during oocyte maturation. In cellular biology, trypsin receptors in the oolemma of *Xenopus laevis* offer a unique system to study the linkage between membrane receptors, G proteins, internal Ca^{2+} and ion

channels in a giant cell with electrophysiological measurements and fluorometry.

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