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Trypsin induces Ca^{2+}-activated Cl^- currents in *X. laevis* oocytes

Marcel E. Durieux[a,*], Mina N. Salafranca[a,b], Kevin R. Lynch[b]

Departments of [a]Anesthesiology, [b]Pharmacology and [b]Biochemistry, University of Virginia, Box 238, University of Virginia Health Sciences Center, Charlottesville, VA 22903, USA

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Abstract

The protease trypsin induces Ca^{2+}-activated Cl^- currents when applied in concentrations as low as 0.1 mg/ml to defolliculated, voltage clamped *X. laevis* oocytes. The response is dose-dependent and specific, as other proteases (chymotrypsin, Lys-C and Arg-C), or trypsin pretreated with soybean trypsin inhibitor, did not induce currents. Intracellular trypsin injection did not induce responses. The current does not appear to result from proteolytic activation of the endogenous receptor for lysophosphatidic acid, the only known Ca^{2+}-mobilizing receptor consistently present in oocytes. These results suggest the presence on the oocyte membrane of a specific receptor for trypsin.

Key words: Oocyte; *Xenopus laevis*; Trypsin; Voltage clamp; Membrane receptor; Lysophosphatidic acid

1. Introduction

The oocytes of the African clawed toad *Xenopus laevis* are a tool used commonly for the expression and cloning of membrane receptors of the G-protein-coupled receptor superfamily [1]. One of several advantages of this system is the relative absence of endogenous Ca^{2+} release activating receptors. Although other agonist-induced responses have been described, particularly to muscarinic agonists [2,3], the only responses present consistently in native oocytes are to the phospholipids lysophosphatidic acid (LPA) [4–7] and sphingosine-1-phosphate (S1P) [8], which may well signal through the same receptor. To prevent confounding results when expressing receptors in oocytes, it is important for investigators to be aware of the presence of any endogenous signaling systems in these cells. Here we describe the presence of a novel endogenous oocyte response, a Ca^{2+}-activated Cl^- current induced by the protease trypsin. This response, which is dose-dependent and specific, appears to result from activation of a membrane receptor system.

2. Materials and methods

2.1 Materials

LPA (1-oleyl-2-hydroxy-*sn*-glycero-3-phosphate) was obtained from Avanti Polar Lipids (Alabaster, AL) and was dissolved in a 1% solution of bovine serum albumin (BSA; ICN Biochemicals, Cleveland, OH). The endoproteases Arg-C and Lys-C were obtained from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Oocyte culture and testing

Our technique for *Xenopus laevis* oocyte harvesting and culture has been described previously [4]. Prior to each experiment, oocytes were defolliculated manually. A single oocyte was placed in a recording chamber filled with 3 ml of a solution containing (mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, dextrose 10, HEPES 10 (pH 7.4). Microelectrodes were pulled in one stage from 1.5 mm capillary glass (BBL with fiber, World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C, David Kopf Instruments, Tujunga, CA), and the tips were broken to a diameter of approximately 10 μm. They were filled with 3 M KCl, and tip resistances were usually 1–3 MΩ. The cell was voltage clamped (at −70 mV, unless otherwise noted) using a two-microelectrode voltage clamp amplifier (Oocyte Clamp OC725A, Warner Corp., New Haven, CT), connected to an IBM-compatible personal computer with data acquisition hardware (DAS-8, Keithly Metrabyte, Taunton, MA) and software (OoClamp [9]). Membrane current was sampled at 125 Hz, and recorded for 5 s before, and 55 s after, application of the test compounds. Compounds were delivered in 30 μl aliquots over 1–2 s using a hand-held micropipettor positioned approximately 5 mm from the oocyte. Responses were quantified by integrating the current trace by quadrature and are reported as μC (mean ± S.E.M.). All experiments were performed at room temperature and, unless otherwise noted, at least 5 oocytes were used for each datapoint. For intracellular injections a third micropipette was used, connected to an automated, nitrogen-driven microinjector (PLI-100, Medical Systems Corp., Greenvale, NY). The micropipette was calibrated volumetrically by counting the number of injections needed to expel a known volume of solution. It was then introduced into the cell, and a 50 nl volume was administered. The adequacy of injection was verified by observing the slight increase in cell size on injection.

3. Results

Application of trypsin to defolliculated oocytes voltage clamped at a holding potential of −70 mV induced

*Corresponding author. Fax: (1) (804) 982-0019.
E-mail: med2p@virginia.edu

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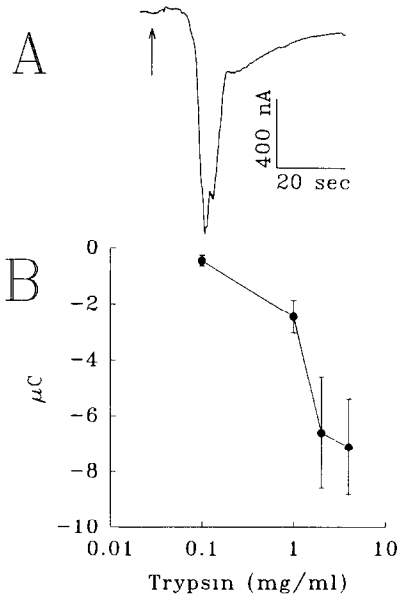

Fig. 1. Trypsin-induced currents in *Xenopus* oocytes. (A) When applied (at arrow) to a defolliculated oocyte voltage clamped at a holding potential of −70 mV, trypsin (2 mg/ml) induces an inward current. Charge transfer is −9.4 μC. (B) Dose-dependency of the oocyte response to trypsin.

transient inward currents (see Fig. 1A). The responses developed after a latency of several seconds, and consisted of a fast inward component, followed by a fluctuating return to baseline over a period of 5–10 s. Typically, 1 mg/ml trypsin induced charge transfers of −2 μC, but responses could be elicited with concentrations as low as 0.1 mg/ml. The response was dose-dependent within the dose range tested (see Fig. 1B). Vehicle alone (BSA 1%) did not induce responses. Even if after trypsin application the oocyte was washed for 30 min with trypsin-free solution, no subsequent trypsin responses could be elicited. Therefore, the trypsin response desensitizes completely.

In shape and kinetics these responses are very similar to Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) induced by activation of endogenous or expressed G-protein-coupled receptors in *Xenopus* oocytes [5]. For this reason we tested the hypothesis that the responses induced by trypsin are $I_{Cl(Ca)}$. Intracellular injection of the Ca^{2+} chelator EGTA (50 nl of a 100 mM solution, resulting in an approximate intracellular concentration of 5 mM) completely abolished the response to subsequent application of trypsin (Fig. 2A). Therefore, an increase in intracellular Ca^{2+} level appears to mediate the response. To establish the nature of the ionic current, we elicited responses to trypsin at various holding potentials, and determined the reversal potential of the current (Fig. 2B). The reversal potential of −15 mV at an extracellular Cl^- concentration of 158 mM indicates that the current is carried mainly by Cl^- ions.

Next, we studied the mechanism of activation of $I_{Cl(Ca)}$ by trypsin. To ensure that the responses were due to trypsin, and not to a contaminant, we treated the trypsin solution (1.6 mg/ml) with soybean trypsin inhibitor before application, and found that oocyte responses were virtually eliminated (−0.4 ± 0.2 μC). Trypsin inhibitor alone did not induce responses (data not shown). To determine whether the action of the protease was intracellular or extracellular, we injected trypsin (50 nl, 10 mg/ml) into oocytes voltage clamped at −70 mV. No responses were observed, yet viability of the oocytes could be confirmed by responses to 1 mg/ml trypsin applied subsequently to the outside of the same oocytes, either after 1 minute (−9.0 ± 2.1 μC; see Fig. 3A) or after 5 min (−7.5 ± 1.6 μC; $n = 4$). These responses were similar to those obtained in oocytes injected with 50 nl water (−11.2 ± 2.7 μC; $n = 3$). Therefore, an extracellular site of action appears likely. To ascertain that the current did not result from nonspecific proteolysis of membrane constituents, we tested several other proteases for their ability to induce currents. Application of chymotrypsin at a concentration of 1 mg/ml resulted in small application artifacts only. Similarly, the proteases Arg-C and

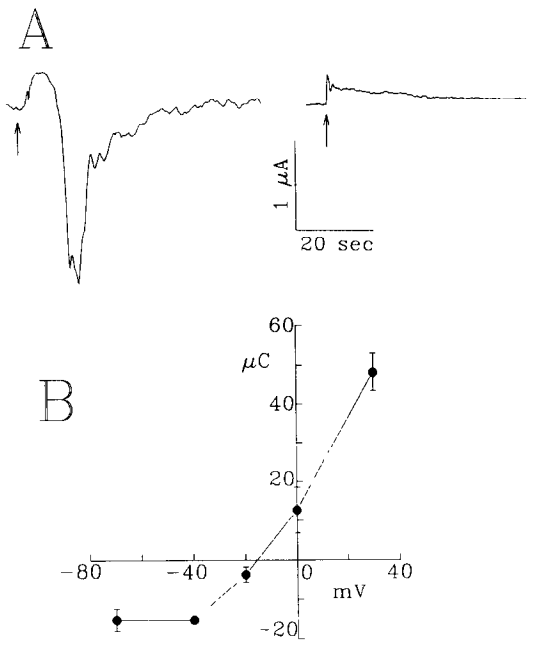

Fig. 2. The trypsin resonse in *Xenopus* oocytes is a $I_{Cl(Ca)}$. (A) Left: control oocyte, injected with 50 nl water, and subsequently exposed (at arrow) to trypsin (1 mg/ml). Charge transfer is −14.3 μC. Right: oocyte, injected with 50 nl EGTA (100 mM), and subsequently exposed (at arrow) to trypsin (1 mg/ml). Except for the application artifact, no response is seen, indicating an increase in intracellular Ca^{2+} concentration mediates the response. Time between injections and applications is approximately 1 minute. (B) Plot of charge transfer versus holding potential. Each points contains data of at least 5 oocytes. A trypsin concentration of 1 mg/ml was used. The reversal potential at −15 mV indicates the current is carried mainly by Cl^- ions.

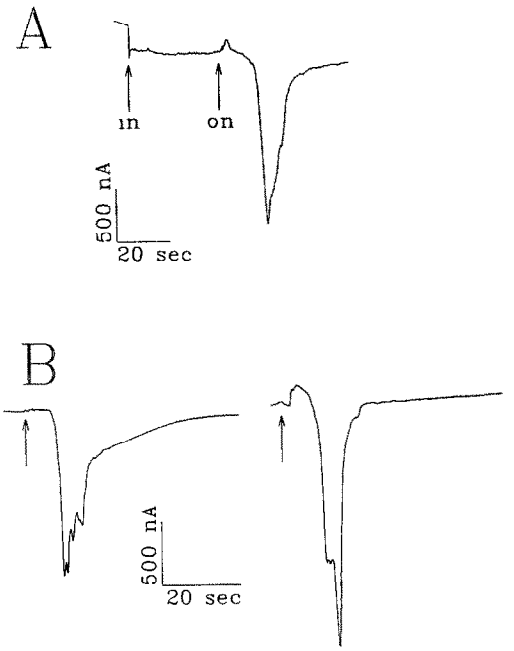

Fig. 3. The trypsin response in *Xenopus* oocytes is mediated through an extracellular system different from the LPA receptor. (A) Trypsin (10 mg/ml, 50 nl) injected into the oocyte ('in') does not elicit responses, whereas trypsin (1 mg/ml) applied subsequently to the outside of the same oocyte ('on') results in a charge transfer of −12.5 μC. Therefore, the site of action is probably extracellular. (B) Left: LPA (10^{-5} M) applied to a control oocyte elicits a charge transfer of -11.1 μC. Right: after treatment of oocyte with trypsin (1 mg/ml) and 30 min wash in trypsin-free solution, LPA (10^{-5} M) still elicits a current (charge transfer −9.2 μC). Therefore, trypsin has not degraded the LPA receptor, making trypsin signaling through this system unlikely.

Lys-C, which cleave a subset of the trypsin cleavage sites, did not induce currents when applied at these concentrations. In addition, responses to trypsin were not reduced by exclusion of Ca^{2+} from, and addition of, 10 mM EGTA to the bath solution, eliminating trypsin-induced Ca^{2+} leakage into the oocyte as an explanation of the $I_{Cl(Ca)}$. Finally, we did not note increases in holding potential after responses to trypsin, indicating the absence of a non-specific increase in membrane leakiness. Thus, the action of trypsin is specific. Another mechanism explaining trypsin-induced $I_{Cl(Ca)}$ would be proteolytic activation of an endogenous G-protein-coupled receptor in the oocyte membrane. The only receptor consistently present in native oocytes is that for LPA [4–7]. When activated by LPA this receptor does not desensitize [4,5]. However, if trypsin activated the LPA receptor by proteolysis one would expect subsequent unresponsiveness to LPA. Therefore, we applied trypsin (1 mg/ml) to defolliculated oocytes, washed the oocytes in trypsin-free solution for 30 min, and then applied LPA (10^{-5} mM). Responses of -8.7 ± 2.4 μC were obtained, indicating that proteolytic digestion of the LPA receptor had not occurred (Fig. 3B).

4. Discussion

We report the presence of an endogenous response to trypsin in defolliculated *X. laevis* oocytes. The response is a $I_{Ca(Cl)}$, appears to have an extracellular site of action, and does not seem to involve activation of the endogenous G-protein-coupled receptor for LPA. Knowledge of this response is relevant for researchers using oocytes for expression of Ca^{2+}-mobilizing receptors.

Based on the data presented, it is not possible to determine the signaling mechanism that links the extracellular action of trypsin to the release of intracellular Ca^{2+}. The specificity of the effect makes general destruction of membrane proteins an unlikely explanation. The presence of response in the absence of extracellular Ca^{2+} eliminates the possibility of trypsin-induced Ca^{2+} leak. The LPA receptor, the only G-protein-coupled receptor known to be present consistently in oocytes, does not appear proteolytically activated. A direct action on the Cl_{Ca} channel cannot be ruled out, but appears unlikely, as: (1) the time delay and response kinetics mimic closely those seen during receptor activation, and (2) a (presumably permanent) change in the channel would be unlikely to result in the transient currents observed. Therefore, the most likely explanation for the trypsin responses appears to be the presence of a specific trypsin receptor in these cells. Based on the response morphology, this receptor might belong to the G-protein-coupled receptor superfamily. In analogy to the thrombin receptor, another G-protein-coupled receptor activated by a protease, this putative trypsin receptor might well be activated through a tethered ligand mechanism [10,11]. This would explain the complete desensitization of the trypsin response. However, the trypsin doses needed to induce currents are higher than those activating other G-protein-coupled receptors, and an alternative explanation of the responses would be trypsin binding, with low affinity, to a receptor in the oocyte membrane for another ligand, as yet unreported. Final determination of the mechanism of action will need additional investigation.

Unresolved at this time is the function of the endogenous trypsin response in oocytes. As $I_{Cl(Ca)}$ in these cells is presumably a fertilization signal [12], the responses to trypsin, LPA or sphingosine-1-phosphate might be involved in signaling that sperm penetration has occurred. Interestingly, in several studies lysophospholipids and trypsin have been shown to have similar effects on components of the reproductive system. Both compounds are able to induce the acrosome reaction and capacitate bovine spermatozoa [13], allowing penetration of the zona pellucida of oocytes. Also, a cyclic nucleotide phosphodiesterase has been isolated from *Xenopus* oocytes that can be activated by trypsin or lysophospholipids, as well as by Ca^{2+}-calmodulin [14]. Several studies have demonstrated the presence of trypsin-related proteases in the ejaculate of mice [15] and

humans [16]. The functional relevance of these findings, as well as the relationship, if any, to our finding of an endogenous oocyte $I_{Cl(Ca)}$ in response to lysophospholipids and trypsin, remains to be evaluated.

References

[1] Kushner, L., Lerma, J., Bennett, M.V.L. and Zukın, R.S. (1989) Methods Neurosci. 1, 3–29.
[2] Kusano, K., Miledi, R. and Stinnakre, J. (1977) Nature 270, 739–741.
[3] Dascal, N. and Landau, E.M. (1980) Life. Sci. 27, 1423–1428.
[4] Durieux, M.E., Salafranca, M.N., Lynch, K.R. and Moorman, J.R. (1992) Am. J. Physiol. 263, C896–C900.
[5] Ferguson, J.E. and Hanley, M.R. (1992) Arch. Biochem. Biophys. 297, 388–392.
[6] Fernhout, B.J.H., Dijcks, F.A., Moolenaar, W.H. and Ruigt, G.S.F. (1992) Eur. J. Pharmacol. 213, 313–315.
[7] Tigyi, G. and Miledi, R. (1992) J. Biol. Chem. 267, 21360–21367.
[8] Durieux, M.E., Carlisle, S.J., Salafranca, M.N. and Lynch, K.R. (1993) Am. J. Physiol. 264, C1360-C1364.
[9] Durieux, M.E. (1993) Comp. Methods Prog. Biomed. (in press).
[10] Vouret-Craviari, V., Obberghen-Schilling, E. van., Rasmussen, U.B., Pavıranı, A., Lecocq, J.P. and Pouyssegur, J. (1992) Mol. Biol. Cell 3, 95–102.
[11] Scarborough, R.M., Naughton, M.A., Teng, W. et al. (1992) J. Biol. Chem. 267, 13146–13149.
[12] Dascal, N. (1987) CRC Crit. Rev. Biochem. 22, 317–387.
[13] Wheeler, M.B. and Seidel, G.E. (1989) Gamete Res 22, 193–204.
[14] Orellana, O., Jedlicki, E., Allende, C.C. and Allende, J.E. (1984) Arch. Biochem. Biophys. 231, 345–354.
[15] Huarte, J., Belin, D., Bosco, D., Sappino, A.P. and Vassallı, J.D. (1987) J. Cell Biol. 1281, 1289.
[16] Huarte, J., Belin, D. and Vassallı, J.D. (1979) Thromb. Res. 16, 463–472.