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Cytotoxicity of Equinatoxin II from the Sea Anemone *Actinia equina* Involves Ion Channel Formation and an Increase in Intracellular Calcium Activity

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Summary. Equinatoxin II is a 20-kDa basic protein isolated from the sea anemone *Actinia equina*. The aim of our work was to investigate the primary molecular basis for the cytotoxic effects of equinatoxin II in two model systems: single bovine lactotrophs and planar lipid bilayers. Previous work has shown that equinatoxin II produces rapid changes in cell morphology, which are dependent on external calcium. It has also been reported that addition of equinatoxin II increases membrane electrical conductance, which suggests that the cytotoxic action of equinatoxin II involves an increase in the permeability of membranes to Ca^{2+}. Extensive changes in cytosolic Ca^{2+} activity are thought to invoke irreversible changes in cell physiology and morphology. In this paper, we show that morphological changes brought about by equinatoxin II in bovine lactotrophs are associated with a rapid rise in cytosolic Ca^{2+} activity, monitored with a fura-2 video imaging apparatus. Moreover, incorporation of equinatoxin II into planar lipid bilayers produces Ca^{2+} permeable ion channels. This suggests that the mode of equinatoxin II cytotoxicity involves the formation of cation (Ca^{2+}) permeable channels in cell membranes.

Key Words fura-2 imaging · planar lipid bilayers · Ca channels · bovine lactotrophs · equinatoxin · sea anemone · *Actinia equina*

Introduction

The cnidocytes of several species of sea anemones (*Actiniidae*) contain short, neurotoxic peptides (2–6 kDa) acting on fast sodium channels and larger (16–20 kDa) cytotoxic and cytolytic polypeptides (Alsen, 1983; Kem, 1988). The latter group consists of structurally related basic toxins which bind to various membrane phospholipids, particularly with sphingomyelin (Bernheimer & Avigad, 1976; Linder, Bernheimer & Kim, 1977; Shin, Michaels & Mayer, 1979; Varanda & Finkelstein, 1980; Turk & Maček, 1986; Kem, 1988; Doyle, Kem & Vilallonga, 1989). The common name "actinoporins" has been proposed for these toxins (Kem, 1988), apparently based on studies of pore formation by *Stichodactyla (syn Stoichactis) helianthus* cytolysin in planar lipid bilayers (Michaels, 1979; Varanda & Finkelstein, 1980).

Most pharmacological and cytotoxicity studies with sea anemone cytolysins have been performed with equinatoxin isolated from *Actinia equina* (Ferlan & Lebez, 1974). Three toxins, equinatoxins I, II and III, were recently isolated (Maček & Lebez, 1988). It was shown that equinatoxin II, which is the most abundant, corresponds to equinatoxin previously described by Ferlan and Lebez (1974). In addition to equinatoxin (i.e. equinatoxin II) pharmacological activities (Sket et al., 1974; Šuput, 1986; Ho et al., 1987; Lee et al., 1988; Šuput, Rubly & Meves, 1988), cytotoxic and cytolytic effects have been clearly demonstrated in various cells. The toxin induces extensive Ca^{2+}- and pH-dependent lysis of erythrocytes (Maček & Lebez, 1981), induces lysis and aggregation of platelets (Teng et al., 1988), tumor cells (Giraldi, Ferlan & Romeo, 1976), and induces cytotoxicity accompanied with morphological alterations and lysis of fibroblasts (Batista, Maček & Sedmak, 1986; Batista et al., 1987).

Pore formation in artificial lipid bilayers by *Stichodactyla helianthus* cytolysins and, on the other hand, cytolytic and cytotoxic effects of equinatoxin II suggest that these effects of sea anemone cytolysins may be, at least in part, ascribed to an intracellular ion imbalance following ion channel formation in cell membranes. An extensive alteration of Ca^{2+} homeostasis is of particular importance as prolonged increases in cytosolic Ca^{2+} activity are thought to invoke irreversible changes in cell physiology and morphology (Choi, 1988). The aim of our

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work was to investigate the primary molecular basis for cytotoxic effects of equinatoxin II; for this, single bovine lactotrophs and planar lipid bilayers were used as model systems.

We found that morphological changes induced by equinatoxin II in bovine lactotrophs were associated with a rapid rise in cytosolic Ca^{2+} activity. Moreover, incorporation of equinatoxin II into planar lipid bilayers produces Ca^{2+} permeable channels. This suggests that the effects of the sea anemone cytolysin, equinatoxin II, are mediated by the formation of cation-permeable channels in cell membranes, where the Ca^{2+} influx is playing an important role.

Materials and Methods

Equinatoxin Purification

Equinatoxin II was purified from the sea anemone *Actinia equina*, and its lethal and hemolytic activity was assayed as described previously (Maček & Lebez, 1981, 1988).

Cell Culture and Solutions

Lactotrophs were prepared from *pars distalis* of pituitary glands of steers obtained from a local abattoir, as previously described (Ingram et al., 1988). Briefly, cells were enzymatically dispersed and enriched on a discontinuous Percoll (Pharmacia, Uppsala) density gradient, allowing isolation of a cell fraction containing 70% lactotrophs. Cells were plated on poly-L-lysine coated glass coverslips. After 2 to 4 days in culture (5% CO_2, 95% humidity), cells were washed in normal recording medium (in mM): 127 NaCl, 5 KCl, 2 $MgCl_2$, 0.5 NaH_2PO_4, 5 $CaCl_2$, 5 $NaHCO_3$, 10 N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), 10 D-glucose, pH = 7.2 (NaOH) and incubated with 1 μM fura-2 acetoxymethylester (Fura-2/AM, Novabiochem, UK; all other compounds from Sigma) at room temperature or at 37°C for 30 min. This resulted in an apparently uniform distribution of fluorescence throughout the cell cytoplasm and is consistent with previous reports (Malgaroli et al., 1987; S.N. Akerman et al., *submitted*).

Cell-coated coverslips were mounted in a 500-μl chamber on the stage of an inverted Nikon-Diaphot microscope. All experiments were carried out at room temperature with a ×100 or ×40 objective.

Equinatoxin II was prepared as a stock solution in the normal bathing medium and applied directly into the chamber by bolus application. Toxin concentration was determined with absorbance measurements at 280 nm using an extinction coefficient of 4.29×10^4 M/cm (Maček & Lebez, 1988).

Fura-2 Imaging

All experiments were performed with a Joyce Loebl Magical imaging system at room temperature. Fura-2 was excited alternately with 340 and 380 nm light by means of a xenon arc lamp and a computer-controlled rotating filter wheel. Emitted light at 510 nm (10 nm bandpass) was collected by an intensified CCD camera (Photonic Science), digitized and stored in the imaging system. Formation of a ratio image (512 × 512, or 256 × 256 pixels) was implemented in a look-up table using the calibration approach described by Grynkiewicz, Poenie and Tsien (1985), with an apparent dissociation constant, K_d, for fura-2 of 135 nM. Recorded data were played back allowing capture of individual ratio frames, production of averaged images and calculation of $[Ca^{2+}]_i$ from the ratio values. Fluorescence intensity ratio values are displayed in figures as the estimated Ca^{2+}-activity rise was beyond the accurate calibration of fura-2. Data were recorded at various acquisition rates (0.18 to 6.25 Hz) and averaged (4 to 32 frames of ratioed images) in order to diminish the noise level and to improve spatial resolution. Continuous traces of average $[Ca^{2+}]_i$ or fluorescence intensity ratios were obtained by defining a region around a cell image, thresholding at an intensity value equivalent to approximately 10 nM, to exclude background noise. This value was chosen after inspection of the image pixel amplitude histogram. The mean fluorescence intensity ratio was calculated over the whole of the defined cell using data of ratio intensity on a pixel-by-pixel basis. Data files were displayed with the use of a spreadsheet (Lotus 123).

Single Channel Recording in Planar Phospholipid Bilayers

Methods of single channel recording were similar to those described by Knowles et al. (1989). Briefly, planar lipid bilayers were painted across a 0.3-mm diameter hole in styrene copolymer with a decane dispersion of 40 mM 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE: Avanti Polar Lipids, Birmingham, AL) to form a neutral bilayer separating 0.2 μm Millipore filtered solutions in the cup ('*cis*') and an outer chamber ('*trans*'). Aqueous solutions were buffered with 5 mM HEPES/N-methyl-D-glucamine, pH 7.5, with salts added as stated. Equinatoxin II was added in aliquots to the *cis* side while stirring.

Experiments were done at room temperature (about 20°C). Current was monitored under voltage-clamp conditions until single-channel activity appeared. A low-noise operational amplifier with frequency compensation was used (Miller, 1982), and data were stored on a video tape after PCM conversion (Sony). Potential differences were recorded *cis* with respect to *trans*, which was held at ground. Current traces were analyzed with a PC-based data acquisition system (CED 1401, UK).

Results

Morphological Effects of Equinatoxin II Application

Equinatoxin II has many clear morphological effects on bovine lactotrophs (Fig. 1). Within 2 min after application of at least 2.3×10^{-7} M equinatoxin II, cells swelled and appeared more granulated with a rougher surface. No changes in morphology were seen after prolonged exposure to lower concentrations of equinatoxin II.

Cytosolic Ca^{2+} Activity

Resting cytosolic Ca^{2+} activity in bovine lactotrophs was usually below 100 nM (Fig. 2*A*), as reported

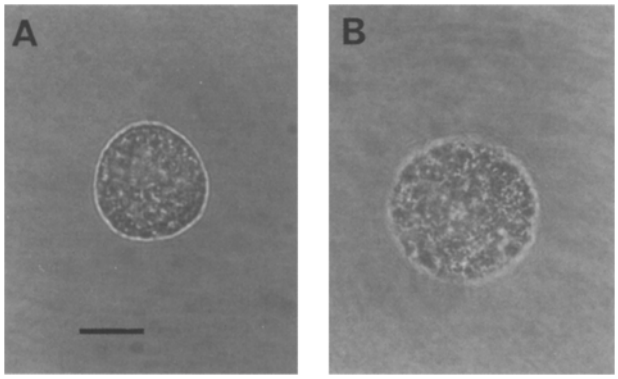

Fig. 1. Morphological features of a bovine lactotroph before (*A*) and 2 min after (*B*) addition of 2.3×10^{-7} M equinatoxin II

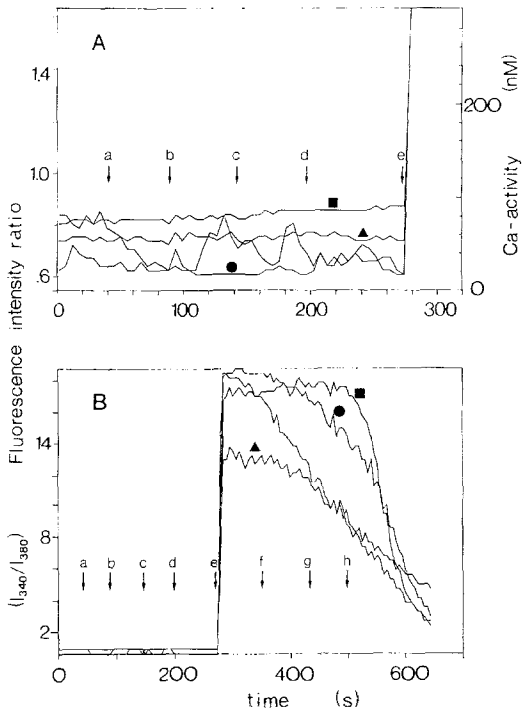

Fig. 2. Dose dependence of intracellular Ca^{2+} activity in bovine lactotrophs to equinatoxin II. (*A*) An increase in resting cytosolic Ca^{2+} was observed only after addition of at least 2.3×10^{-7} M equinatoxin II. Arrows indicate increases in concentration of equinatoxin II to: (*a*) 2.6×10^{-9} M, (*b*) 5.2×10^{-9} M, (*c*) 3.1×10^{-8} M, (*d*) 1.2×10^{-7} M, (*e*) 2.3×10^{-7} M (*f*) 4.4×10^{-7} M, (*g*) 6.5×10^{-7} M, and (*h*) 8.5×10^{-7} M. (*B*) After reaching the threshold concentration, the fluorescence ratio increases rapidly. Acquisition rate was 0.18 Hz. Each curve represents a cell. All cells were in the same objective field (×40). Symbols identify measurements from the same cell

previously (Mason et al., 1988). An increase in cytosolic Ca^{2+} activity was only observed after addition of at least 2.3×10^{-7} M equinatoxin II (the 'threshold' concentration of Fig. 2). Longer exposures at lower concentrations did not affect resting cytosolic Ca^{2+} activity. After reaching the threshold concentration, the fluorescence ratio increased rapidly to a level beyond the accurate calibration for fura-2 ($n = 31$).

There was no correlation between the resting Ca^{2+} activity and the amplitude and duration of the response to equinatoxin II (*compare* Fig. 2*A* and *B*). This may be due to either (i) the intrinsic variability between cells or (ii) differences in dye loading. The limitation of the ratio method is that the intensity of the signal from the 380 nm excitation drops with an increase in Ca^{2+} activity; to avoid division by zero the intensity at 380 nm is set to an arbitrarily low level. Therefore, at very high Ca^{2+} the fluorescence intensity ratio (i.e., the maximal response to equinatoxin II) is proportional to the amount of dye in the cell.

After a variable length of time the signal decayed, presumably due to loss of fura-2 from the cell through the plasma membrane, which has been changed by secondary responses to equinatoxin II. The time-course of fura-2 photobleaching in intact cells had a time-constant of more than 600 sec and would not account for the fluorescence intensity ratio signal decline. The decay in the fluorescence signal was observed on the same time scale as morphological changes.

To try to resolve the kinetics of the increase of fluorescence ratio signal, recordings were made at a higher acquisition rate (6.25 Hz). The resting fluorescence ratio increased to the maximum value within 2 to 3 sec (Fig. 3). The speed and magnitude of this response suggests that this effect is not mediated by a membrane carrier, such as transport ATPases, but instead by an ion channel or pore.

The increase in cytosolic Ca^{2+} activity following equinatoxin II application was due, at least initially, to influx, of Ca^{2+} from the extracellular space. This contention is supported by the following observations: (i) No increase in cytosolic Ca^{2+} activity was seen when cells were bathed in a low Ca^{2+}-containing medium (<100 μM), and (ii) the fluorescence ratio increase started at the periphery of the cell image, with a transient ring of higher Ca^{2+} activity clearly seen near the plasma membrane. This ring became less distinct as cytosolic Ca^{2+} activity increased through the rest of the cell over a few seconds (Fig. 4).

Equinatoxin II Forms Ca^{2+}-Permeable Channels

An addition of 6.5×10^{-7} M equinatoxin II adjacent to a pure phospholipid bilayer resulted in the formation of Ca^{2+}-permeable channels ($n = 6$). Single channel current recordings in a toxin-treated POPE

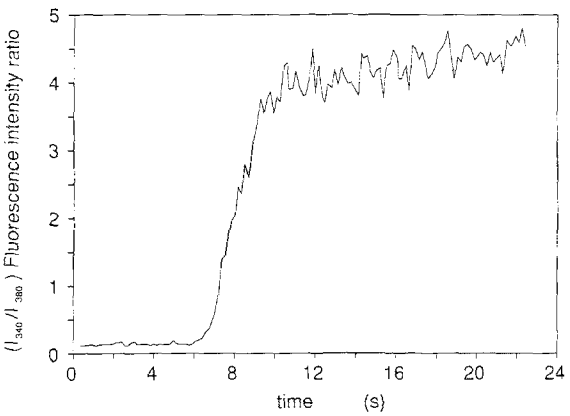

Fig. 3. The effect of equinatoxin II (3.3×10^{-6} M) recorded at a higher acquisition rate (6.25 Hz). Responses of five different cells recorded in the same field of the ×40 objective were averaged. Note different arbitrary units for the fluorescence intensity ratio values, which are due to the use of a different filter wheel

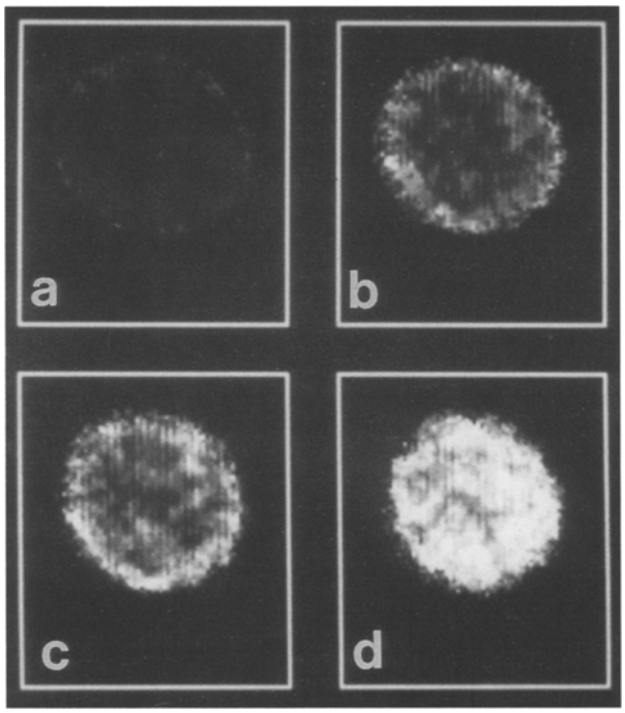

Fig. 4. Fluorescence intensity ratio increase was seen with a transient infilling of the cell volume. Time sequence of averaged (4 frames) fluorescence intensity images from a cell in Fig. 2. (*a*) Spatial cytosolic Ca^{2+} activity distribution at rest, (*b*) 0.9 sec, (*c*) 3.6 sec, and (*d*) 10 sec after the application of equinatoxin II (3.3×10^{-6} M). White indicates higher fluorescence intensity ratio values

bilayer are shown in Fig. 5*A*. At all holding potentials discrete membrane current fluctuations were observed (Fig. 5*B*). Open channel noise is greater than in the closed state, and this can be seen at a faster time scale to be partly due to rapid closures of the open channel to various subconductance state levels. It seems that an apparently open state includes at least one subconductance level (indicated by arrows, Fig. 5).

The main open state of the channel had a unitary conductance of 23 pS in symmetrical 50 mM $CaCl_2$ (Fig. 6), which gives a permeability coefficient (P_{Ca} of 5.3×10^{-17} liter/sec, calculated from the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949) and Ca^{2+} activity of 28.5 mM. In asymmetrical solution (136 : 50 mM $CaCl_2$), the relationship showed rectification, with a reversal potential of −16 mV. This reversal potential is close to the theoretical reversal potential for Ca^{2+} of −11 mV, and suggests a high cation selectivity of equinatoxin II-induced channels. Channel conductance in symmetrical 100 mM KCl was around 100 pS (*not shown*).

Discussion

We have found that the cytotoxic action of equinatoxin II is correlated with a rise in intracellular Ca^{2+} activity, which is effected by formation of Ca^{2+} permeable channels. Above a threshold concentration of equinatoxin II, the time course of morphological changes and increase in cytosolic Ca^{2+} activity are similar. Ca^{2+} entry is very likely to be mediated by ion channels formed *de novo* by the toxin. These results are consistent with previously described effects of *Stoichactis helianthus* cytolysin upon artificial lipid bilayers (Michaels, 1979; Varanda & Finkelstein, 1980). However, our study has clearly demonstrated that the sea anemone cytolytic toxin increases the permeability of the membrane to Ca^{2+}. Given the central role of Ca^{2+} in controlling cellular metabolism (McBurney & Neering, 1987; Miller, 1988; Smith & Augustine, 1988), an irreversible change by a toxin is not surprising. Because of the large electrochemical gradient for Ca^{2+} across the plasma membrane, and the low resting activities of Ca^{2+} in the cytosol, a rapid perturbation of cytosolic Ca^{2+} homeostasis can be mediated by formation of Ca^{2+}-permeable channels. Therefore, we expect that other sea anemone cytolysins, as well as toxins from other groups of organisms, will be found to act by increasing cytosolic Ca^{2+} activities.

Our results are consistent with equinatoxin II effects upon sheep erythrocyte hemolysis (Maček & Lebez, 1981) and rabbit platelet aggregation and lysis (Teng et al., 1988), where an optimal extracellular Ca^{2+} concentration of 1 mM was required. However, neither effect had an absolute requirement for extra-

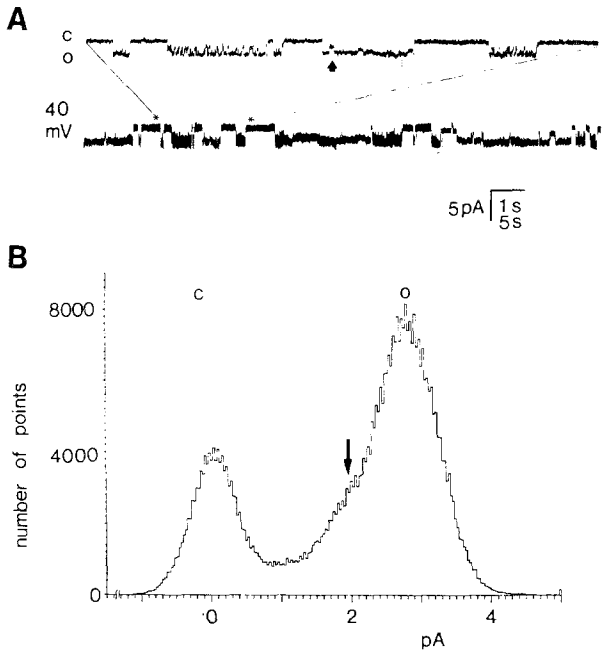

Fig. 5. (*A*) Equinatoxin II (6.5×10^{-7} M) induced single channel activity recorded in a planar lipid bilayer at = 40 mV (POPE) (*O*—open state, *C*—closed state). *Cis* compartment contained 136 mM, and *trans* 50 mM $CaCl_2$. Top trace shows single channel currents displayed at a faster time scale. Note that flickering of channels appears to be due to short closures to at least one subconductance state (indicated by arrow). (*B*) Amplitude histogram of the equinatoxin II-induced single channel currents. Note the hump (indicated by arrow) on the normal distribution of the channel open state, indicating the possible contribution of the putative subconductance state. The current signal (holding potential as in *A*) was filtered at 200 Hz (−3 dB), and digitized at 2 kHz

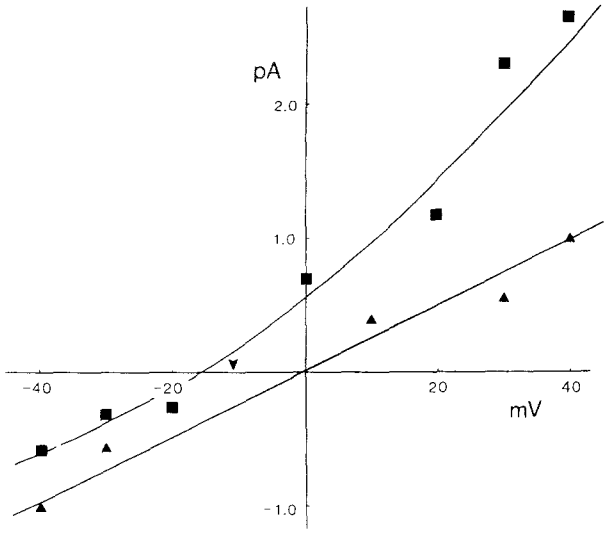

Fig. 6. Current-voltage relations of the equinatoxin II-induced single channel currents in symmetrical (▲, 50 mM *cis:* 50 mM *trans*) and asymmetrical (■, 136 mM *cis:* 50 mM *trans*) $CaCl_2$. The line is of the form $y = 0.023\,x + 0.03$ ($r = 0.99$), and the curve is a Goldman-Hodgkin-Katz relation fitted by eye with a P_{Ca} of 5.3×10^{-17} liter/sec, using Ca^{2+} activity of 68 mM for *cis* and 28.5 mM for *trans* compartments. The reversal potential of −16 mV is close to the theoretical value of −11 mV (*see* arrow) calculated from Nernst equation, using activity coefficients from Robinson and Stokes (1959)

cellular Ca^{2+}. This suggests that channels formed by equinatoxin II are permeable also for other physiologically important ions. This is supported by the finding that channels formed by *Stoichactis helianthus* cytolysin are also permeable for monovalent cations (Michaels, 1979), as are channels formed by equinatoxin II shown in our study and by G. Menestrina (*personal communication*). A gross ion imbalance could contribute to swelling of the lactotroph cell (Fig. 1), either due to the flux of the monovalent cations through the equinatoxin II-formed channels, or through secondary activation of ion channels by the rise in cytosolic Ca^{2+}. In either case, colloid-osmotic lysis of the cells would lead to a loss of the fluorescent dye fura-2 (Fig. 2*B*). Also, increased leakage currents in equinatoxin II-treated single muscle fiber (Šuput, 1986) are consistent with the conclusion that equinatoxin II increases cell membrane permeability for monovalent as well as divalent cations.

The nature of the ion channel formation by the sea anemone cytolytic toxin was described for the first time in detail by Michaels (1979) and Varanda and Finkelstein (1980). They reported slightly different properties of *Stoichactis helianthus* cytolysin ion channels. Michaels (1979) suggested that three toxin molecules aggregate in a lipid bilayer to form a cation-selective channel, whereas an aggregation of four molecules was proposed by Varanda and Finkelstein (1980).

Particularly striking was the dissimilarity in channel kinetics described in the two papers. Our results concerning single channel kinetic characteristics are more consistent with those of Michaels (1979). Channel open times in our experiments are frequently less than one and up to few seconds, closer to the average lifetime of 2 sec reported by Michaels (1979). In contrast, Varanda and Finkelstein (1980) obtained channels which, once observed, remained open. Either the purity of toxin preparations or differences in lipids used might account for these discrepancies. Interestingly, we resolved at least one subconductance level in equinatoxin II-induced ion channels, a feature described for many cation-selective channels (e.g., Hamill & Sakmann, 1981; Barrett, Magleby & Pallotta, 1982; Hunter & Giebisch, 1987; Nagy, 1987), as well as anion-selective channels (e.g., Miller, 1982; Gele-

tyuk & Kazachenko, 1985; Krouse, Schneider & Gage, 1986; Hughes et al., 1987; Smith, Zorec & McBurney, 1989). The role of these subconductance states in equinatoxin II channels is not known. Although the mechanism of channel formation by equinatoxin II is as yet unknown, some sort of cooperativity phenomenon could be envisaged from the all-or-none effect displayed in Figs. 2 and 3. The presence of subconductance states also suggests that the structure of the equinatoxin II channel is complex. Further electrophysiological experiments in combination with molecular genetics approaches would be essential to understand these problems.

Although the formation of ion channels by sea anemone cytolytic toxins is the likely mechanism of their action (Shin et al., 1979; Bernheimer & Rudy, 1986; Kem, 1988) as also demonstrated in our study, some recent results with equinatoxin II indicate that pharmacological activities of sea anemone cytolytic toxins might be more complex, depending on the target membrane, and are distinct from the channel-forming action. It has been shown that equinatoxins I and II block sodium channels in the muscle fiber membrane at concentrations as low as 10–100 pM (Šuput, 1986), and to block potassium channels in frog single myelinated nerve fibers at 10 nM (Šuput et al., 1988). Recently it was shown by means of chemical modification of equinatoxin II that its lethal activity could be discriminated from lipid binding and cytolytic activity (Turk, Maček & Gubenšek, 1989; T. Turk and P. Maček, *submitted*). It will be interesting to discover if the equinatoxin II-induced Ca^{2+} influx shown in this paper is related to the diversity of in vivo pharmacological actions of the toxin reported elsewhere.

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References

Alsen, C. 1983. Biological significance of peptides from *Anemonia sulcata*. *Fed. Proc.* **42:**101–108

Barrett, J.N., Magleby, K.L., Pallotta, B.S. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (London)* **331:**211–230

Batista, U., Jezernik, K., Maček, P., Sedmak, B. 1987. Morphological evidence of cytotoxic and cytolytic activity of equinatoxin II. *Period. Biol.* **89:**347–348

Batista, U., Maček, P., Sedmak, B. 1986. The influence of equinatoxin II on V-79-379 A cell line. *Period. Biol.* **88:**97–98

Bernheimer, A.W., Avigad, L.S. 1976. Properties of a toxin from the sea anemone *Stoichactis helianthus*, including specific binding to sphingomyelin. *Proc. Natl. Acad. Sci. USA* **73:**467–471

Bernheimer, A.W., Rudy, B. 1986. Interactions between membranes and cytolytic peptides. *Biochim. Biophys. Acta* **864:**123–141

Choi, D.W. 1988. Calcium-mediated neurotoxicity: Relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11:**465–469

Doyle, J.W., Kem, W.R., Vilallonga, F.A. 1989. Interfacial activity of an ion channel-generating protein cytolysin from the sea anemone *Stichodactyla helianthus*. *Toxicon* **27:**465–471

Ferlan, I., Lebez, D. 1974. Equinatoxin, a lethal protein from *Actinia equina*-I: Purification and characterization. *Toxicon* **12:**57–61

Geletyuk, V.I., Kazachenko, V.N. 1985. Single Cl^- channels in molluscan neurons: Multiplicity of the conductance states. *J. Membrane Biol.* **86:**9–15

Giraldi, T., Ferlan, I., Romeo, D. 1976. Antitumor activity of equinatoxin. *Chem. Biol. Interact.* **13:**199–203

Goldman, D.E. 1943. Potential, impedance and rectification in membranes. *J. Gen. Physiol.* **27:**37–60

Grynkiewicz, G.M., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450

Hamill, O.P., Sakmann, B. 1981. Multiple conductance states of single acetylcholine receptor channels in embryonic cells. *Nature (London)* **194:**462–464

Ho, C.L., Ko, J.L., Lue, H.M., Lee, C.Y., Ferlan, I. 1987. Effects of equinatoxin on the guinea-pig atrium. *Toxicon* **25:**659–664

Hodgkin, A.L., Katz, B. 1949. The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol. (London)* **108:**37–77

Hughes, D., McBurney, R.N., Smith, S.M., Zorec, R. 1987. Caesium ions activate chloride channels in rat cultured spinal cord neurones. *J. Physiol. (London)* **392:**231–251

Ingram, C.D., Keefe, P.D., Wooding, F.B.P., Bicknell, R.J. 1988. Morphological characterisation of lactotrophs separated from the bovine pituitary by rapid enrichment technique. *Cell Tissue Res.* **252:**655–659

Kem, W.R. 1988. Sea anemone toxins: Structure and action. *In:* Biology of Nematocysts. D. Hessinger and H. Lenhoff, editors. Academic, London

Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G., Ellar, D.J. 1989. A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. *FEBS Lett.* **244:**259–262

Krouse, M.E., Schneider, G.T., Gage, P.W. 1986. A large anion-selective channel has seven conductance levels. *Nature (London)* **319:**58–60

Lee, C.Y., Lin, W.W., Chen, Y.M., Lee, S.Y. 1988. On the causes of acute death produced by animal venoms and toxins. *In:* Progress in Venom and Toxin Research. P. Gopalakrishnakone, and C.K. Tan, editors. pp. 3–14. University of Medicine, National University of Singapore, Singapore

Linder, R., Bernheimer, A.W., Kim, K.-S. 1977. Interaction between sphingomyelin and a cytolysin from the sea anemone *Stoichactis helianthus*. *Biochim. Biophys. Acta* **467:**290–300

Maček, P., Lebez, D. 1981. Kinetics of hemolysis induced by

equinatoxin, a cytolytic toxin from the sea anemone *Actinia equina*. Effect of some ions and pH. *Toxicon* **19**:233–240

Maček, P., Lebez, D. 1988. Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon* **26**:441–451

Malgaroli, A., Milani, D., Meldolesi, J., Pozzan, T. 1987. Fura-2 measurements of cytosolic free Ca^{++} in monolayers and suspensions of various types of animal cells. *J. Cell Biol.* **105**:2145–2155

Mason, W.T., Rawlings, S.R., Cobbett, P., Sikdar, S.K., Zorec, R., Akerman, S.N., Benham, C.D., Berridge, M.J., Cheek, T., Moreton, R.B. 1988. Control of secretion in anterior pituitary cells—linking ion channels, messengers and exocytosis. *J. Exp. Biol.* **139**:287–316

McBurney, R.N., Neering, I.R. 1987. Neuronal calcium homeostasis. *Trends Neurosci.* **10**:164–169

Michaels, D.W. 1979. Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*. I. Formation of transmembrane channels in lipid bilayers. *Biochim. Biophys. Acta* **555**:67–78

Miller, C. 1982. Open-state substructure of single chloride channels from *Torpedo* electroplax. *Phil. Trans. R. Soc. London B* **299**:401–411

Miller, R.J., 1988. Calcium signalling in neurons. *Trends Neurosci.* **11**:415–419

Nagy, K. 1987. Subconductance states of single sodium channels modified by chloramine-T and sea anemone toxin in neuroblastoma cells. *Eur. Biophys. J.* **15**:129–132

Robinson, R.A., Stokes, R.H. 1959. Electrolyte Solutions. Butterworths, London

Shin, M.L., Michaels, D.W., Mayer, M.M. 1979. Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*: II. Effect of membrane lipid composition in a liposome system. *Biochim. Biophys. Acta* **555**:79–88

Sket, D., Drašlar, K., Ferlan, I., Lebez, D. 1974. Equinatoxin, a lethal protein from *Actinia equina*: II. Pathophysiological action. *Toxicon* **12**:63–68

Smith, S.J., Augustine, G.J. 1988. Calcium ions, active zones and synaptic transmitter release. *Trends Neurosci.* **11**:458–464

Smith, S.M., Zorec, R., McBurney, R.N. 1989. Conductance states activated by glycine and GABA in rat cultured spinal neurones. *J. Membrane Biol.* **108**:45–52

Šuput, D. 1986. Effect of equinatoxin on the membrane of skeletal muscle fiber. *Period. Biol.* **88**:210–211

Šuput, D., Rubly, N., Meves, H. 1988. Effects of equinatoxins on single myelinated nerve fibres. *In:* Progress in Venom and Toxin Research. pp. 467–470. P. Gopalakrishnakone and C.K. Tan, editors. National University of Singapore, Singapore

Teng, C.-M., Lee, L.-G., Lee, C.-Y., Ferlan, I. 1988. Platelet aggregation induced by equinatoxin. *Thromb. Res.* **52**:401–411

Turk, T., Maček, P. 1986. Effect of different membrane lipids on the hemolytic activity of equinatoxin II from *Actinia equina*. *Period. Biol.* **88**:216–217

Turk, T., Maček, P., Gubenšek, F. 1989. Chemical modification of equinatoxin II, a lethal and cytolytic toxin from the sea anemone *Actinia equina* L. *Toxicon* **27**:357–384

Varanda, W., Finkelstein, A. 1980. Ion and nonelectrolyte permeability properties of channels formed in planar lipid bilayer membranes by the cytolytic toxin from the sea anemone, *Stoichactis helianthus*. *J. Membrane Biol.* **55**:203–211

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