Trachynilysin, a Neurosecretory Protein Isolated from Stonefish (Synanceia trachynis) Venom, Forms Nonselective Pores in the Membrane of NG108-15 Cells*

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Received for publication, April 9, 2002, and in revised form, August 9, 2002
Published, JBC Papers in Press, August 12, 2002, DOI 10.1074/jbc.M203433200

Trachynilysin, a protein toxin isolated from the venom of the stonefish Synanceia trachynis, has been reported to elicit massive acetylcholine release from motor nerve endings of isolated neuromuscular preparations and to increase both cytosolic Ca2+ and catecholamine release from chromaffin cells. In the present study, we used the patch clamp technique to investigate the effect of trachynilysin on the cytoplasmic membrane of differentiated NG108-15 cells in culture. Trachynilysin increased membrane conductance the most when the negativity of the cell holding membrane potential was reduced. The trachynilysin-induced current was carried by cations and reversed at about −3 mV in standard physiological solutions, which led to strong membrane depolarization and Ca2+ influx. La3+ blocked the trachynilysin current in a dose-, voltage-, and time-dependent manner, and antibodies raised against the toxin antagonized its effect on the cell membrane. The inside-out configuration of the patch clamp technique allowed the recording of single channel activity from which various multiples of 22 pS elementary conductance were resolved. These results indicate that trachynilysin forms pores in the NG108-15 cell membrane, and they advance our understanding of the toxin’s mode of action on motor nerve endings and neurosecretory cells.

Neurotoxins have proved to be valuable tools for understanding molecular mechanisms involved in physiological processes, and their use has been instrumental in the purification and identification of key protein components of excitable membranes and synapses (1–3). Among the neurotoxins that promote neurotransmitter release, the most studied has been a-latrotoxin (α-LTX) for recent reviews, see Refs. 4 and 5), a 120 kDa protein purified from the venom of the black widow spider (Lactrodectus mactans tredecimguttatus), which is known to trigger Ca2+-dependent and -independent release of a variety of neurotransmitters and hormones (6–11). α-LTX forms non-selective cationic pores in planar lipid bilayers (12–14) and in the cytoplasmic membrane of differentiated PC12 (15) and neuroblastoma cells (16). In addition, two families of α-LTX receptors that facilitate pore formation in biological membranes (17, 18) have been identified, i.e. neurexins (19, 20) and latrophilins (21–24), and the pores are involved in the toxin’s massive release of neurotransmitters (4, 5, 7, 9, 11).

Trachynilysin (TLY), a 159 kDa membrane-perturbing (hemolytic) toxic protein isolated from the venom of the stonefish Synanceia trachynis, also greatly increases quantal acetylcholine release from motor nerve endings (25, 26) and catecholamine secretion from large dense-core vesicles of chromaffin cells (27). However, contrary to α-LTX, these two types of release are Ca2+-dependent. Interestingly, neither TLY (25) nor α-LTX (28) affects the number of large dense-core vesicles containing neuropeptides in motor nerve endings despite the depletion of small clear synaptic vesicles. TLY has also been reported to raise the intracellular Ca2+ concentration in cultured mouse hippocampal neurons (29) and adrenal chromaffin cells (27). Simultaneous blockade of L, N, and P/Q voltage-dependent Ca2+ channels caused only a minor reduction of TLY-induced catecholamine secretion and little change in Ca2+ signals (27), and removal of extracellular Ca2+ and addition of EGTA or La3+ completely abolished both secretion and Ca2+ signals. Moreover, depletion of intracellular Ca2+ stores with caffeine inhibited TLY-induced catecholamine secretion by chromaffin cells. These results suggest that transmembrane Ca2+ influx and the resulting mobilization of intracellular Ca2+ stores are required for TLY-induced secretion (27). To determine the mechanism for the Ca2+ influx, we decided to determine whether TLY activates existing ion channels or forms pores in the plasma membrane. Other stonefish toxins; i.e. stonustoxin from Synanceia horrida venom (30) and verrucotoxin from Synanceia verrucosa venom (31), are known to exert their hemolytic activity through pore formation (32).

To the best of our knowledge, voltage clamp studies have not been performed previously with any of the stonefish toxins. In the present studies, the membrane effects and the ability of TLY to form pores were investigated using the patch clamp technique and differentiated NG108-15 hybrid cells (33).

EXPERIMENTAL PROCEDURES

Cell Culture—Undifferentiated neuroblastoma × glioma NG108-15 hybrid cells were grown in monolayer cultures using Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 2 mM glutamine, and 3 μM glycine, as described previously (34). Three days before performing the experiments, the cells were differentiated by adding 0.5 mM dibutyryl cyclic adenosine-monophosphate (Sigma-Aldrich) to the medium and reducing the serum concentration to 1%. The cultures were

*This work was supported in part by the CNRS, by a grant from the Direction des Systèmes de Forces et de la Prospective (to J. M.), and by National Institutes of Health Public Health Service Grant GM-43728 (to A. S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by fellowships from the Ministère de l’Education Nationale de la Recherche et de la Technologie and from the Association Française contre les Myopathies.

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The abbreviations used are: α-LTX, α-latrotoxin; TLY, trachynilysin; EC500, effective concentration (i.e. 50% of maximum).
maintained at 37 °C in a humidified atmosphere containing 95% air, 5% CO₂. Tissue culture reagents were purchased from Invitrogen.

**Trachynilysin—**TLY was purified from stonefish (S. trachynis) venom by sequential anion exchange, fast protein liquid chromatography (FPLC), and size exclusion FPLC (25). Aliquots of the purified toxin (~2 mg of protein/ml) in 10 mM Tris-HCl-buffered saline (pH 7.4) containing 5% glycerol were stored at –60 °C. Before being used in the electrophysiological experiments, TLY was diluted with the standard physiological solution. A fast perfusion system allowed changing the solution around the recorded cell in a fraction of a second. Inside-out patch clamp experiments used TLY diluted with the standard solution filling the patch pipette.

**Anti-TLY Antibodies—**A female New Zealand White rabbit was injected subcutaneously with a water-in-oil emulsion composed of equal parts of Hunter’s TiterMax adjuvant (Sigma) and a solution of purified TLY. Injections were performed on (i) days 0 and 17 with 32 and 158 μg of toxin, respectively, (ii) days 31 and 52 with 317 μg of toxin, and (iii) days 88, 110, and 131 with 1.14 μg of toxin. The rabbit was sedated and exsanguinated on day 139, and the anti-TLY serum was isolated from reconstituted sera (75 mg/ml deionized water) with an lyophilized and stored at 4 °C. Tissue culture reagents were purchased from Invitrogen.

**Immunoblotting—**TLY (0.5 μg of protein/lane) and the protein extract (6 μg/lane) from NG108-15 cells were analyzed under reducing conditions (10% β-mercaptoethanol) by SDS-PAGE with a 12% acrylamide gel. After electrotransfer (4 °C, overnight, 0.2 A) of the separated protein bands onto a nitrocellulose membrane, the membrane was blocked (8 h, 4 °C) with Tris-buffered saline containing 0.1% Tween 20, 5% (v/v) skimmed milk powder, and 1% (v/v) bovine serum albumin, and it was probed (12 h, 4 °C) with the anti-TLY or control IgG preparation diluted (1:500) in the blocking buffer. The rabbit IgG-probed membranes were washed with Tris-buffered saline, and bound antibodies were detected after probing (2 h, 4 °C) with a diluted (1:1000) peroxidase-labeled, anti-rabbit IgG preparation using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences) according to the manufacturer’s instructions.

**Neutralization of TLY Hemolytic Activity—**One hundred and 1000 hemolytic units of TLY, contained in 1 ml volumes of Tris-buffered saline (pH 7.4) supplemented with crystalline bovine albumin (1 mg/ml), were incubated (15 min, 37 °C) with various amounts of anti-TLY IgG and control IgG, and the residual hemolytic activity of the incubated mixtures was determined against rabbit erythrocytes as previously described (36).

**Electrophysiological methods—**Neuroelectric activity was recorded from outside-out patches as previously described (37). The external solution was 135 mM KCl, 5 mM MgCl₂, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES (pH 7.4) (external solution) and 135 mM KCl, 5 mM MgCl₂, 10 mM EGTA, 10 mM HEPES (pH 7.2) (internal solution). The internal solution was filtered through 0.2 μm Millex filters (Millipore, Saint-Quentin en Yvelines, France). The osmotic pressure of the internal and external solutions were 295–305 mosmol and 305–315 mosmol, respectively, as measured with a freezing point osmometer (Knauer, Berlin, Germany). The solutions used to determine the ionic selectivity of the TLY-induced current are shown in Table I. For inside-out patch recordings, the external solution was also used as the internal solution.

**Ionic Selectivity Determination—**To minimize the contribution of voltage-gated K⁺ channels to the macroscopic current, some patch clamp experiments were performed at a holding potential of +60 mV. Under this condition, the transient voltage-dependent currents, including K⁺ currents (38, 39), were inactivated by about 95% 2 min after changing the holding potential. Thus, the different voltage-dependent currents were limited without using any pharmacological agent that could interact with the toxin.

To determine the ionic selectivity of the TLY-induced current, its reversal potential was measured in solutions of various ionic composition using ramp-potentials (150 mV/s) from +60 to −60 mV. Derived from the Goldman-Hodgkin-Katz flux equation (40, 41), Equation 1 (42) gives the reversal potential of a cationic current as a function of the concentration and permeability of each ion species.

$$V = \frac{RT}{F} \ln \left[ \frac{P[K]_o + P[Na]_o + 4P[Ca]_o + 4P[Mg]_o}{P[K]_i + P[Na]_i + 4P[Ca]_i + 4P[Mg]_i} \right] \quad (\text{Eq. 1})$$

In this equation, the terms in square brackets are ion concentrations, \(P_x\) is the permeability of the \(x\) ion species, \(T\) is the absolute temperature, \(R\) and \(F\) are the usual gas and Faraday constants, and \(i\) and \(o\) are the intra- and extracellular compartments, respectively. Ion activities are used rather than concentrations, as in Equation 2 (43). The activity coefficients used for Na⁺, K⁺, Ca²⁺, and Mg²⁺ were 0.75, 0.75, 0.25, and 0.25, respectively.

$$V = \frac{RT}{F} \ln \left[ \frac{0.75 \times P[K]_o + 0.75 \times P[Na]_o + 0.25 \times 4P[Ca]_o + 0.25 \times 4P[Mg]_o}{0.75 \times P[K]_i + 0.75 \times P[Na]_i + 0.25 \times 4P[Ca]_i + 0.25 \times 4P[Mg]_i} \right] \quad (\text{Eq. 2})$$

**Quantification of Macroscopic Current Steps and Low Frequency Fluctuations—**The macroscopic current steps were counted and measured using routines written by G. Ouanounou (this paper) in the Labtalk programming language of Origin software. Current step transitions were detected after computing average currents on a 50 ms time window before and after any given point and calculating the difference. A transition occurred when the difference was higher than twice the variance of the “before” window.

To quantify the current variations that did not occur in steps, each detected step was subtracted from the ensuing samples of the recording, which led to a smoothed trace deprived of step transitions. In other words, the new trace exhibited only low frequency components. Derivation of that signal allowed us to dissociate the changes of membrane conductance into increasing and decreasing components according to the sign of the derivative. Separate integration of the two components of the derivative over the entire analyzed period gave the cumulative currents corresponding to each component.

**Noise Analysis—**Noise analysis was performed using the fast Fourier transform module included in Origin 5 software after filtering off the lower frequencies. The analyzed periods were divided into sections of 1024 samples, and the resulting power spectra were averaged. The mean spectrum, performed on similar base-line sections, was subtracted before fitting to Lorentz Equation 3,

$$S(f) = \frac{S_0}{1 + \frac{f}{f_c}} \quad (\text{Eq. 3})$$

where \(S(f)\) is the power, \(f\) is the frequency, and \(S_0\) is the constant to which the function tends for low frequencies.

The mean channel lifetime (\(\tau\)) was computed from the cut-off frequency (\(f_c\)).

$$\tau = \frac{1}{2\pi f_c} \quad (\text{Eq. 4})$$

The elementary conductance was calculated from the variance (\(\sigma^2\)) and the mean current (\(I_m\)) of the analyzed signal,

...continued. **Pore-forming Activity of Trachynilysin...**
**RESULTS**

**Whole-cell Recording of Membrane Currents during TLY Action**—To determine the effect of TLY on ionic permeability, membrane currents of NG108-15 cells were recorded in the standard external solution using the whole-cell configuration of the patch clamp technique at a holding potential of −60 mV. An inward current developed about 1 min after the addition of 12 nM TLY to the external medium. In the continuous presence of TLY, the induced current rapidly became too large to be recorded accurately. Because of the irreversibility of the TLY effect, the toxin was applied for just 1 min. Under these conditions, the TLY-induced current was limited to a recordable intensity range. A 1 min exposure to 12 nM TLY appeared to be just higher than a threshold duration, since shorter applications of that TLY concentration had no effect other than an increase in electrical noise (data not shown). The threshold duration of toxin exposure could be reached by successive applications, alternating TLY-containing medium with TLY-free medium. This suggests that a minimal number of TLY molecules has to bind to the membrane to produce the membrane effect, and this may partly explain the latency of the effect of the toxin. After 1 min of exposure to TLY, the current increased in a stepwise manner to about −500 to −1000 pA and then fluctuated around this range (Fig. 1A).

Details of the time course of the TLY-induced current at various time scales are shown in Fig. 1B. The TLY-induced activity exhibited macroscopic steps of various sizes that coexisted with current fluctuations without visible steps. Thus, two types of current variations, each with its own frequency domain, compose the TLY-induced current. Despite a large spectrum of step sizes and low frequency variations, visual inspection suggested that the membrane current presented preferential values. To support this view, the first minutes of three recordings were pooled and analyzed as follows. The current was binned in 1 pA-wide classes, and the relative number of samples at any of these levels was computed and plotted versus the bin size (Fig. 1C). The peaks revealed by this time-distribution showed the most frequent intensities of the TLY-induced current. The modes of these peaks, determined by a Gaussian-fitting regression, appeared to be regularly spaced. The mode values were plotted as a function of their rank when sorted in ascending order, and they were fitted with a linear regression equation (inset, Fig. 1C). The slope of the regression line, 27 pA, represents the mean increment between peaks, which suggests that the TLY current is composed of multiples of a common component.

The presence of steps and low frequency fluctuations precluded a deeper analysis of the TLY-induced channel-like activity. However, another type of analysis was performed, as shown later.

**Anti-TLY Antibodies Suppress the Current Induced by TLY**—Before using the anti-TLY IgG preparation in our electrophysiological experiments, its specificity was evaluated by Western blotting and by determining its ability and the ability of the control IgG preparation to neutralize TLY in vitro hemolytic activity. The Western blotting experiments revealed (Fig. 2A) that (i) the anti-TLY IgG bound to TLY α- and β-subunits but not to any of the proteins isolated from NG108-15 cells, and (ii) labeling of the toxin subunits was not observed when the control IgG preparation was used as a probe. The studies examining the specificity of the anti-TLY IgG preparation ability to neutralize TLY in vitro hemolytic activity revealed (Fig. 2B) that incubating 100 and 1000 hemolytic units of TLY with −93 and 698 μg, respectively, of anti-TLY IgG eliminated −90% of the toxin in vitro hemolytic activity. However, incubating 100 hemolytic units of TLY with −1.34 mg of control IgG did not eliminate any hemolytic activity.

Our observation that anti-TLY antibodies neutralized TLY hemolytic activity prompted us to determine whether they could also affect the TLY-induced current. TLY was applied (as described under "Experimental Procedures"), and after the macroscopic induced-current developed, anti-TLY IgG (95 μg/ml, final concentration) was added to the external solution. The TLY-induced current progressively decreased in all 4 cells tested (each from a different culture) until it was completely inhibited within 2–4 min (Fig. 2C). However, control, normal IgG (95 μg/ml, final concentration) did not affect the current induced by TLY or the inhibitory action of a subsequent application of anti-TLY IgG (Fig. 2C). In two of the cells, continuous wash-out of the antibodies allowed about 30% recovery of the

**Fig. 1.** Whole-cell recordings of the membrane current elicited by exposure of NG108-15 cells to TLY. In this figure as in the others 12 nM TLY was applied for 1 min at a holding potential of −60 mV. A, typical example of the onset of TLY-induced current. The horizontal bar above the trace shows the time of toxin application. B, step-like activities appearing at different time bases. The top trace is from the same cell as that in A. C, time distribution of the membrane current induced by TLY. Three recordings were pooled, including the one shown in A, after the base line was subtracted. Currents were binned in 1 pA classes (see "Results"), and the frequency was normalized for the total recording time. The traces in black represent the Gaussian fits of the different peaks. Inset, plot of the modes of the 15 peaks shown in the histogram versus their ascending rank order. The straight line represents the linear regression across all points.

\[
\gamma = \frac{\sigma^2}{I_m(V_m - E_{rev})} \quad \text{(Eq. 5)}
\]

where \(V_m\) is the membrane potential, and \(E_{rev}\) is the reversal potential.
TLY-induced current before the gigaseal was lost (data not shown). In the other two cells, the seal was lost at the beginning of the wash-out period. In addition, exposure of the cells to anti-TLY IgG followed by wash-out (which had no membrane effect) did not prevent the membrane response to subsequent exposure to TLY (data not shown). These results suggest that the anti-TLY IgG bound to the toxin in a reversible manner, and they confirm that TLY binds irreversibly to the cell membrane.

Macroscopic Steps and Low Frequency Current Variations Induced by TLY—Because the membrane current returned to the initial base line in the presence of anti-TLY antibodies, we assumed that all of the TLY-induced increases in conductance were exactly balanced by conductance decreases, and this situation prompted us to assess the proportion of steps and low frequency fluctuations involved in the conductance changes. Therefore, using the algorithm described under “Experimental Procedures,” two recordings (including the one shown in Fig. 2) were decomposed into their step and low frequency components (see Fig. 3, A–B), and the two components were separately analyzed. The number of detected upward events represented only 72% of the downward events. Furthermore, the respective amplitude distributions differed, i.e. the upward events were smaller than the downward events (Fig. 3C). This difference was statistically significant, as demonstrated by the Kolmogorov-Smirnov test performed on the cumulative frequency distribution (p ≤ 0.01). On the whole, the sum of the closing (upward) events corresponded to only 57% of the opening (downward) events. This feature resulted in a global downward deflection of the step component, as shown in Fig. 3B (bottom trace). The analysis did not reveal a common substep; however, the frequency distributions (Fig. 3C, insets) revealed that the most frequent step transitions occurred in the 27 pA class, which may explain the preferential levels of the global TLY-induced current already noticed (Fig. 1C) and characterized by a 27 pA increment.

The low frequency component was analyzed by derivation and by separate integration of the positive and negative parts of the derivative (Fig. 3D). This analysis allowed the separation of periods of increasing and decreasing membrane conductance; i.e. the integral of the positive derivative gives the cumulative current, which corresponded to membrane conductance decreases. Conversely, the integral of the negative derivative corresponded to membrane conductance increases. The membrane conductance increases represented 22% of the conductance decreases, which led to a global upward deflection of the low frequency component of the TLY current, as seen in Fig. 3B (top trace). Thus, during TLY action, 85% of the membrane conductance increases occurred by steps, and 15% occurred by low frequency fluctuations, whereas steps and low frequency fluctuations contributed almost equally (49 and 51%, respectively) to the membrane conductance decreases.

Influence of the Membrane Potential on the Conductance Induced by TLY—All of our previous studies were performed at a membrane potential of −60 mV. Therefore, we decided to examine whether membrane potential influences TLY-induced changes in membrane conductance. At positive potentials, the toxin-induced current was relatively stable and did not exhibit macroscopic steps (only fluctuating at low frequency), in contrast to what was observed at negative potentials (Fig. 4A). Moreover, the TLY membrane conductance increase was greater at positive than at negative holding potentials.

To determine the kinetics of this voltage sensitivity, 60 s step potentials were applied (from the holding potential of +60 mV to the test potential of −60 mV) with different cells previously treated with TLY (Fig. 4A). Transmembrane currents recorded
before toxin application were subtracted from currents recorded in the presence of TLY. When the membrane potential was stepped from +60 to −60 mV, the TLY-induced current reversed and then decreased to about 20% of its initial value in −20 s. The mean current calculated from four recordings (Fig. 4A) was fitted with a single exponential function that had a decay time constant of 5.32 ± 0.05 s (95% confidence interval). Notably, a step from a holding potential of −60 mV to a test potential of +60 mV yielded different kinetics for the membrane conductance changes, i.e. the membrane current increased abruptly about 1 min after the potential change without exhibiting current steps (data not shown).

To determine the current-voltage relationship of the TLY-induced current at the steady state, the potential was changed from +50 mV to −100 mV in −10 mV steps lasting 40 s. The average current during the last 20 s of each step was normalized to the averaged current recorded at +50 mV. As shown by the current-voltage relationship (Fig. 4B), the TLY-induced current reversed at −3 mV in the standard solutions, which suggested that the induced conductance did not have ionic selectivity. The average current was constant from −100 to −40 mV, thus indicating that the membrane conductance increased as the negativity of the potential was reduced. At potentials more positive than the reversal potential, the membrane conductance was almost constant. This corresponds to a so-called “outward rectification.”

Ionic Selectivity of TLY-induced Current—To determine the ion(s) taking part in the TLY-induced current, the reversal potential was measured in solutions of various ionic composition using ramp potentials (from +60 to −60 mV) in a whole-cell configuration. Ramp potentials performed before TLY application were used as controls (Fig. 5, middle trace). The TLY-induced current reversed at about −3 mV (n = 17) in standard solutions (Fig. 5, bottom traces), as was observed previously at the steady state. The reversal potentials of the TLY-induced current recorded in external solutions of various ionic composition are shown in Table I. For each cell, the reversal potential was determined in the external standard solution and in one of the test solutions. Methanesulfonate substitution for Cl had not affect the reversal potential (n = 5),
Fig. 5. Determination of reversal potential of the TLY-induced current. Upper trace, ramp potentials (150 mV/s, +60 to −60 mV) applied to determine the reversal potential. Middle trace, control membrane current recorded before TLY application. Lower traces, recordings of the TLY-induced current from one cell obtained at various times after TLY application (after subtraction of the control current). The current amplitude scale applies to the middle and bottom recordings.

whereas substituting half of the NaCl content with sucrose significantly shifted the reversal potential to a more negative value (n = 3). Thus, neither methanesulfonate nor Cl− contribute to the TLY current. In contrast, TLY caused all of the physiological cations to permeate through the membrane. The relative permeability values, for which calculated reversal potentials using Equation 2 fit best with experimental values, were P_{Ca}/P_{Na} = 1.3, P_{Cm}/P_{Na} = 1.4, and P_{Mg}/P_{Na} = 1.4.

La^{3+} Blocks the Current Induced by TLY—The La^{3+} ion is known to inhibit TLY-induced release of catecholamines from chromaffin cells (27) and the α-LTX-induced current (11, 17) and to perturb the structure of α-LTX pores (11, 44). Therefore, it was of interest to determine whether the current induced by TLY in NG108-15 cells was affected by this cation. At −60 mV, 1 mM La^{3+} added to the external medium completely blocked the current induced by TLY. In the presence of 100 μM La^{3+}, the current returned to the base line, suppressing all low frequency current fluctuations and leaving only fast current transients (Fig. 6, A–B). Lower La^{3+} concentrations partially affected the TLY-induced current. Because of the current fluctuations already described, the La^{3+} effect was quantified by rationing the mean current during La^{3+} application to the control current. A Boltzmann function fit of the data points, obtained with various La^{3+} concentrations, gave an EC_{50} = 512 ± 56 nM (95% confidence limits, Fig. 6C). At all concentrations used, the blockade of the TLY-induced current by La^{3+} was rapidly reversed by washing with the standard medium.

A closer examination of the La^{3+} blockade at 100 μM showed that the transient currents described above exhibited a fast on-current followed by an exponential decay (Fig. 6B). This suggests that the transients correspond to the current steps described above, which are blocked in a time-dependent manner by La^{3+} ions. In addition, the use of the ramp-potential protocol revealed that, in the presence of La^{3+} (100 μM), the current exhibited an outward rectification (data not shown), thus indicating that the La^{3+} effect is voltage-dependent.

Elementary TLY-induced Current—To determine whether the observed macroscopic changes in membrane conductance were due to channel-like activity and whether this activity could explain the time course of the macroscopic TLY-induced current, elementary currents were recorded from membrane patches using the “inside-out” configuration. A drop of buffered TLY solution was deposited at the back of the pipette previously filled with standard external solution. The time necessary for TLY to diffuse to the pipette tip allowed the sealing of the inside-out patch clamp configuration and the confirmation of the absence of endogenous ionic conductances in the membrane patch.

At a pipette potential of −60 mV (i.e., a positive membrane potential), only one channel conductance (22 pS) was detected; i.e., several minutes after inside-out patch achievement, a single channel activity developed (Fig. 7, A–A'), and the number of channels (as well as the opening/closing rate) then increased rapidly. At a pipette potential of +60 mV (i.e., negative membrane potential), several channel conductances were detected. The induction of channel activities began with a 22 pS elementary conductance (Fig. 7, B–B'). After relative stabilization in the open state of 4–8 channels of 22 pS, bursts of 72 and 86 pS channel activity appeared from this current level as base line (Fig. 7, C–D, C'–D'). As time progressed, channel activity became complex and showed elementary conductance multiples of 22 pS and an increased opening/closing rate. However, periods of single channel activity alternated with periods during which the current continuously changed without evident channel activity but with an increased noise level (Fig. 7E). During these periods, the current evolved between two levels corresponding to the open and closed states of 72 and 86 pS channels, without summation with these channel activities.

The frequency spectrum of these periods (provided by fast Fourier transform noise analysis) could be fitted by a Lorentz equation, which indicated that the noise resulted from summation of discrete events (see Ref. 45). These events had a mean open time of about 3.5 ms and an elementary conductance of 22 pS. It should be emphasized that this analysis corresponds to the early stages of TLY activity, because the patch current rapidly became too complex to analyze.

This study shows that the TLY-induced macroscopic current is due at least in part to ion channel-like activity. Interestingly, at the elementary level as at the macroscopic one TLY activity appears to be constituted of various components at negative membrane potentials, whereas only a single component is detectable at positive potentials.

DISCUSSION

The present study demonstrates that brief exposure to TLY induces after a short latent period a potent and irreversible increase in the membrane conductance of NG108-15 cells. Under voltage clamp conditions, the TLY-induced increase in membrane conductance leads to a transmembrane cationic current which, when measured in standard media, reverses at a membrane potential of −3 mV and exhibits an outward rectification. A 1 min exposure to 12 nM TLY appeared to be the time threshold before which time TLY had no apparent effect, and this minimum duration could be reached by successive exposures. This observation suggests that TLY binds irreversibly to the membrane and that a threshold amount of TLY-bound molecules presumably is needed to alter membrane permeability.

Two hypotheses may be proposed to explain the above-mentioned results; either TLY activates one or several types of endogenous ion channels or TLY forms ionic pores by insertion into the cell membrane. Our experimental results revealed that (i) the TLY-induced macroscopic current is made of slow fluctuations and current steps of up to 160 pA at (−60 mV), making it particularly unstable, (ii) TLY elicited channel activity in membrane patches devoid of endogenous channels, and (iii) the observed TLY effect required a threshold amount of bound toxin. These features are inconsistent with the idea that TLY forms pores by activating preexisting channels. Indeed, the
TABLE I

Reversal potential (E_{rev}) of the TLY-induced current in media of various ionic compositions

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>Methane sulfonate</th>
<th>Sucrose</th>
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<td>80</td>
<td>3</td>
<td>-3</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>77</td>
<td>102</td>
<td>0</td>
<td>120</td>
<td>3</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

For each cell, the TLY-induced current E_{rev} was determined in the standard solution and in one of the test solutions, using the protocol for the Fig. 5 data. All reversal potentials were graphically determined.

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The single-channel activity induced by TLY would fluctuate as observed. Also, features similar to those described above have been described for exogenous polypeptides, such as equinatoxin II (46) and α-LTX (12, 15–17), and for endogenous polypeptides such as amylin (47), all of which are known to form pores by insertion into the cell membrane. Thus, our data support the idea that the TLY-elicited pore activity we observed is caused by insertion of TLY into the cell membrane rather than by TLY-induced activation of preexisting silent channels.

Experiments using various holding potentials revealed a large difference in the time course of the TLY-induced current depending on the polarity of the membrane potential. At positive membrane potentials, the macroscopic toxin-induced current fluctuated at low frequency, and inside-out patch recordings exhibited only one elementary conductance of 22 pS. Thus, at positive membrane potentials, the macroscopic TLY current is due to the summation of 22 pS channel-like activities. However, at negative membrane potentials, the macroscopic TLY current was complex and consisted of low and high frequency components. The high frequency component was composed of downward and upward macroscopic current steps, the former being more frequent and exhibiting higher amplitudes than the latter. Thus, step activity leads to a global downward deflection of the membrane current, which is compensated for by an opposite behavior of the low frequency component. This complex behavior was also observed at the channel level, which revealed several conductance values and progressive transitions combined with channel-like activity. Taken together, these observations suggest that the macroscopic downward current steps result from the opening of a large conductance that can close gradually or by small steps. Another possibility is that elementary channels behave independently at positive membrane potentials but that they cooperate at negative potentials, with a higher cooperativity coefficient for opening than for closing. The noise analysis of single channel recordings (Fig. 7, E–E'), which revealed the existence of a discrete event, together with the observation of various conductances (all multiples of the discrete event) are in favor of the second hypothesis.

The influence of the membrane potential on the TLY-induced current at the steady state revealed a strong outward rectification. In contrast, fast ramp currents varied linearly with the membrane potential, which indicates that the elementary conductance did not change with the potential. Notably, current steps still occurred during ramp potentials but only at negative potentials, and they led to abrupt changes in the slope of the current (Fig. 5, bottom). Thus, the outward rectification (Fig. 4B) is due to an increased number of opened TLY-induced conductances overcompensating for the smaller elementary conductance at positive potentials.

La³⁺ was found to block the TLY-induced current. However its EC_{50} was about 5-fold higher for the TLY current than has been reported (48) for voltage-sensitive Ca²⁺ channels in the same cell line. Moreover, the voltage dependence of the La³⁺ effect indicates that it acts via binding to a site located in the pore formed by TLY. In addition, La³⁺ blockade was time- and concentration-dependent. Thus, at 100 μM the kinetics of the blockade of TLY action by La³⁺ were slow enough to allow the TLY-induced conductance opening to produce macroscopic downward current transitions, but they were sufficiently fast to block the TLY pore before its closing and to hide the low frequency fluctuations.

The single-channel activity induced by TLY may result from the formation of TLY channels with intrinsic gates oscillating between the open and closed state or from oligomerization-deoligomerization of the TLY channels. Our results do not allow us to distinguish between these two possibilities. The
complete amino acid sequence of TLY is not yet known, but its reported (25) N-terminal amino acid sequence is similar to that of stonustoxin, for which an amphiphilic α-helix structure was predicted (49) for each subunit. To explain the pore-forming property of stonustoxin, the so-called “carpet-like” model (for review, see Ref. 50) has been proposed (32). This model predicts the existence of a threshold amount of bound toxin for membrane permeation and an instability of the pore structure. Our observations with TLY are in agreement with this model.

The ability of TLY to form cationic pores in the cytoplasmic membrane of NG108-15 cells leads to membrane depolarization, which in turn increases membrane permeation due to outward rectification. Under normal conditions in which the membrane potential is not controlled this mechanism of amplification leads to a rapid and complete depolarization (51) that should induce activation of endogenous voltage-gated channels. However, previous experiments in our laboratory found that low and high voltage-activated Ca\(^{2+}\) currents in NG108-15 cells were not modified by TLY.\(^2\) In addition, the slight participation of voltage-gated L, N, and P/Q Ca\(^{2+}\) channels for TLY-induced catecholamine release from chromaffin cells (27) may result from the depolarizing effect of the toxin. Considering the high Ca\(^{2+}\) permeability of TLY pores and the potent increase in membrane conductance induced by the toxin, the Ca\(^{2+}\) influx through the pores would be expected to be large and to be higher than Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels in NG108-15 cells. Thus, the Ca\(^{2+}\) permeability of the TLY-induced pores is sufficient to account for the external Ca\(^{2+}\) dependence of the TLY-induced release of neurotransmitters from motor nerve terminals and chromaffin cells (25–27). Also, the elicited increase in Ca\(^{2+}\) permeability may be responsible for mobilizing intracellular Ca\(^{2+}\) stores in chromaffin cells (27). Moreover, the latent period between TLY application and its increasing intracellular Ca\(^{2+}\) and stimulating catecholamine release in chromaffin cells is similar to that observed in the present studies of pore formation by TLY. Finally, La\(^{3+}\), which inhibits NG108-15 cell membrane permeation by TLY, also blocks the Ca\(^{2+}\) increase and catecholamine secretion induced in chromaffin cells by TLY (27). Although the Ca\(^{2+}\) permeability of the TLY pores may account for most of the biological effects of TLY, it does not explain why TLY does not trigger the release of large dense core vesicles containing neuropeptides from motor nerve endings. Thus, further studies are needed to explain this seeming paradox.

Acknowledgments—We thank Dr. B. Rouzaire-Dubois for providing the NG108-15 cells used in this study, L. Prado de Carvalho for assistance during preliminary experiments, L. Lane-Guermonprez for help with Western blotting, Dr. J.-M. Dubois and the late Dr. R. Kado for critical discussions and comments on the manuscript, and David McColm for assistance in helping to obtain the S. trachyis venom used as the source of trachynilysin needed in our study.

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Trachynilysin, a Neurosecretory Protein Isolated from Stonefish (Synanceia trachynis) Venom, Forms Nonselective Pores in the Membrane of NG108-15 Cells

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doi: 10.1074/jbc.M203433200 originally published online August 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203433200

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