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α-Latrotoxin Channels in Neuroblastoma Cells

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Abstract: The changes in ionic permeability induced by the application of α-latrotoxin to NG108-15 neuroblastoma x glioma cells were examined using the nystatin perforated-patch technique for whole-cell recording. Complex single channel activity appeared in the plasmalemmas after delays that ranged from 1–20 min in Krebs' solution. The conductance of a channel fluctuated among at least three broad, approximately equispaced bands, the maximum conductance being about 300 pS, and the reversal potential approximately 0 mV. The channels were permeable to Na^+, K^+, Ca^{2+} and Mg^{2+}, poorly permeable to glucosamineH^+ and Cl^-, and were blocked by La^{3+}. The channels stayed fully open in Ca^{2+}-free solutions with 4 mM Mg^{2+}, in solutions with no divalent cations and in solutions with 2 mM Ca^{2+} and 96 mM Mg^{2+}. They opened infrequently if both internal and external Cl^- were replaced by glutamate$^-$. If α-latrotoxin opened similar channels in nerve terminals, the flux of ions through them could account for the massive release of neurotransmitter induced by the toxin.

Key words: Black widow spider — Neurotoxins — Neurotransmitter release — Patch clamp — Ion channels

Introduction

α-Latrotoxin (α-LTx) is the major toxic protein in the venom glands of the black widow spider, *Latrodectus mactans tredecimguttatus,* and it accounts for the principal effects of a homogenate of these glands on the central and peripheral synapses of vertebrates (Frontali et al., 1976; Rosenthal & Meldolesi, 1989). α-LTx acts selectively at nerve terminals where it causes the massive release of a variety of neurotransmitters and, under appropriate conditions, the loss of the small clear synaptic vesicles (Clark et al., 1970; Longenecker et al., 1970; Clark, Hurlbut & Mauro, 1972; Frontali et al., 1976; Tzeng & Siekevitz, 1979*b;* Ceccarelli & Hurlbut, 1980; Matteoli et al., 1988). The receptor for α-LTx, which is confined to the nerve terminals (Tzeng & Siekevitz, 1979*a;* Valtorta et al., 1984), has been purified (Scheer & Meldolesi, 1985) and its predicted amino acid sequence determined (Ushkaryov et al., 1992).

The binding of α-LTx to its receptor is necessary for its action *in situ,* since the toxin affects only those tissues which bear the receptor for it (Rosenthal & Meldolesi, 1989). However, binding is not sufficient for action because transmitters are not released when the toxin binds to its receptor on cells bathed in solutions with La^{3+}, or in solutions without divalent ions (Misler & Hurlbut, 1979; Scheer, 1989; Rosenthal et al., 1990). Since α-LTx forms nonselective cation-specific channels of high conductance through simple planar lipid bilayers (Finkelstein, Rubin & Tzeng, 1976), or through bilayers that contain its receptor (Scheer, Prestipino & Meldolesi, 1986), the toxin-receptor complex at nerve endings might form similar channels through the axolemma, and the flux of cations through them causes the effects of the toxin. To date, however, α-LTx-induced ion channels have been observed only in differentiated PC-12 cells in culture (Wanke et al. 1986). Those channels resemble the channels in the bilayers in that they are nonselective and permanently open, but their unitary

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conductance is only about 15 pS, much lower than the 65–300 pS unitary conductance measured in bilayers. α-LTx stimulates transmitter release from many, but not all (Grasso et al., 1980) preparations, when they are bathed in Ca^{2+}-free solutions (Longenecker et al., 1970; Frontali et al., 1976; Ceccarelli & Hurlbut, 1980; Meldolesi et al., 1983; Fesce et al., 1986), provided that other divalent cations are present at millimolar levels (Misler & Hurlbut, 1979; Misler & Falke, 1987; Rosenthal et al., 1990). Moreover, the concentration of Ca^{2+} inside synaptosomes, as measured by Fura-2 fluorescence, does not rise when transmitter release is stimulated in these Ca^{2+}-free solutions (Meldolesi et al., 1984).

Recently, it was found that the purified receptor for α-LTx binds on columns to purified synaptotagmin, an integral protein in the membrane of synaptic vesicles, and it was suggested that these two proteins normally interact in the nerve terminal, possibly contributing to the docking of a vesicle at an active zone (Petrenko et al., 1991). These observations have prompted the idea that the binding of α-LTx to its receptor might directly trigger the release of a quantum of transmitter from a docked vesicle, through an as yet unidentified mechanism (*for review, see* Petrenko, 1993).

We show here that the undifferentiated neuroblastoma x glioma hybrid clonal cell line NG108-15 bears receptors for α-LTx, and that the toxin forms complex channels of high conductance in the plasmalemmas of these cells at concentrations commensurate with the dissociation constant of the toxin-receptor complex. The gating and permeability properties of these channels are such that the flux of cations through them could account for most of the effects of the toxin at the synapse, if divalent cations other than Ca^{2+} are able to stimulate transmitter release from toxin-treated terminals.

Materials and Methods

Materials

α-LTx was purified from homogenates of venom glands dissected from European black widow spiders according to published procedures (Frontali et al., 1976) and stored at −80°C in small aliquots. For the binding experiments, purified α-LTx was labeled with ^{125}I by acylation with the Bolton-Hunter reagent (N-succimidyl-3-(4-hydroxy 5 [^{125}I] iodophenyl propionate), 1,500 Ci/mmol, Amersham, UK), followed by chromatography on a Sephadex G-50 column (Pharmacia, Sweden). The activity of the purified unlabeled or labeled toxin was tested by measuring its ability to induce release of ^{3}H-dopamine (3.2 Ci/mmol, Amersham, UK) from rat striatal synaptosomes (Meldolesi, 1982).

Neuroblastoma x glioma NG108-15 cells (mock-transfected with the vector pcDNA1) were a gift of Drs Hui-Quan Han and Paul Greengard of the Rockefeller University (New York). The cells were grown in monolayer cultures using DMEM medium supplemented with 10% fetal calf serum, 0.1 mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine (HAT) and were maintained at 37°C in a humidified incubator equilibrated with 95% O_2 and 5% CO_2. The cells were plated either into uncoated 35 mm plastic petri dishes, or into 9 cm plastic petri dishes that held several 15 mm glass coverslips coated with polyornithine, and they were used three to seven days later.

^{125}I-αLTx Binding

Suspensions of NG108-15 cells (approximately 10^7 cells/ml) were exposed to various concentrations of ^{125}I-labeled α-LTx (15 μCi/mmol) in a medium containing: (mM) NaCl, 125; KCl, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2; HEPES-NaOH, pH 7.4, 25; and glucose, 1.1 mg/ml; bovine serum albumin, 10 mg/ml. The incubation was carried out for 20 min at 37°C in Eppendorf tubes. The cells were subsequently chilled in ice and centrifuged for 4 min at 10,000 × *g*. The pellets were rinsed once with 500 μl of incubation medium without toxin and counted in a Beckman Gamma 4000 spectrometer. To determine unspecific binding, we preincubated some samples with an excess of unlabeled toxin.

Electrophysiology

We recorded from isolated rounded cells without long processes. The nystatin perforated-patch technique for whole-cell recording (Horn & Marty, 1988) was used in most of our experiments. The standard bathing solution, which is called Krebs, contained (in mM): NaCl, 145; KCl, 6.3; NaH_2PO_4, 1.2; $MgSO_4$, 1; $CaCl_2$, 2; glucose, 6; NaHEPES, 10; pH 7.4. To test the role of the various ions in carrying the current through the channel opened by α-LTx, we replaced in some experiments most of the NaCl by isosmotic concentrations of glucosamineHCl, KCl, $CaCl_2$, a mixture of $MgCl_2$ and $MgSO_4$ (to keep Cl^- constant), or $MgCl_2$ alone. The stock solution of glucosamineHCl was titrated to pH 7.0 with NaOH, and it contained about 25 mM Na^+. HEPES was omitted from the glucosamineH^+ Krebs. No drugs were used, and all experiments were performed at room temperature, which ranged from about 20 to about 27°C over the course of the year, but varied only about 1°C on any given day. The pipette solution (Falke et al., 1989) contained (in mM): KCl, 66; K_2HSO_4, 33; Na_2EGTA, 0.6; $MgSO_4$, 1; sucrose, 66; NaHEPES, 21 (Na^+, 9); pH 7.4. A bit of this solution was sucked into the tip of a pipette, and the shank was backfilled with the same solution plus nystatin, 250 μg/ml. The stock solution of nystatin in dimethylsulfoxide (DMSO) (50 mg/ml) was prepared fresh each day. The pipettes were pulled from Hilgenberg GMSH borosilicate glass by a BB-CH-PC Mechanex puller and their resistances ranged from 8–12 MΩ The bath electrode was a chlorided Ag wire that had been pushed into a short length of polyethylene tubing filled with 166 mM NaCl plus 2% agar. After a GΩ seal had formed between the cell membrane and the pipette, the holding potential was set to −50 mV and the permeabilization of the patch monitored with the amplifier's transient cancellation circuit, and by eliciting currents with voltage pulses over the range from −100 to +40 mV. Recording was begun when the access resistance to the cell fell below 50 MΩ and the voltage-dependent currents seemed stable. This required 30–90 min in the various cells. All cells showed voltage-dependent outward currents that ranged from 200–1,000 pA at +40 mV, and a few showed inward currents that were less than 200 pA in peak amplitude at 0 mV. The electromotive forces (EMFs) of the resting cells were measured by adjusting the holding potential until the pipette current became zero. Their initial values ranged from about −30 to about −50 mV so that a few pA of inward current flowed at the standard holding potential of −50 mV. The EMFs tended to decline during the experiments, es-

pecially when high concentrations of Ca^{2+} or Mg^{2+} were applied to toxin-treated cells, but the baseline current changed by only a few pA.

In experiments directed to test the α-LTx channel permeability to Cl^- ions, the conventional whole-cell technique was used to record single channel activity (Fenwick, Marty & Neher, 1982). In these instances, we tried to replace cell Cl^- with glutamate$^-$ ions. The pipette solution contained (in mM): Kglutamate, 153; KCl, 4.4; Na_2EDTA, 2; $MgCl_2$, 1.1; NaHEPES, 5 (Na^+ 2); pH, 7.4. Some Cl^- was retained so that the EMF developed between Ag/AgCl recording electrodes could be balanced out.

The pipette current was recorded continuously at a bandwidth of 1 or 2 KHz, and the data were stored on a videotape recorder for later analysis by a computer. Voltage-clamp records were obtained at a bandwidth of 5 KHz. Each experiment was calibrated by applying 25 mV voltage pulses to the EPC-7 model circuit in the whole-cell mode (resistance = 0.5 GΩ). The VCR output was amplified until the calibration pulse was 5 V, digitized by a 12 bit A/D converter and analyzed using pCLAMP 5.5 software (Axon Instruments, Foster City, CA).

Results

α-LTx Binds to NG108-15 Cells and Opens Ion Channels

Undifferentiated neuroblastoma x glioma NG108-15 cells were found to bind iodinated α-LTx in a specific manner. The dependency of the binding on the concentration of α-LTx is shown in Fig. 1. The binding was noticeable already at very low concentrations of the toxin and approached saturation above 10^{-9} M. Nonlinear regression analysis of the data indicated the existence of $36 \pm 13 \cdot 10^3$ binding sites per cell, characterized by high affinity ($K_D = 2.16 \pm 0.34 \cdot 10^{-9}$ M).

The application of nanomolar concentrations of α-LTx to NG108-15 cells in culture was followed by the appearance of bursts of fluctuating inward current that usually appeared 1–20 min after the addition of toxin. The fluctuations increased with time if the toxin was left in the bath, and they gradually decreased after removal of the toxin. These changes in the intensity of the fluctuations were due to changes in the number of channels that were active at any one time in the plasmalemma of any one cell. The maximum intensity varied greatly from cell to cell, and many of our records were obtained from cells with many active channels that passed rather large currents. Figure 2 shows records from a cell which displayed a minimal response to α-LTx: a single burst of fluctuating inward current (*B*) began about 20 min after the toxin had been applied and ended a few min later. This was the briefest and most delayed response ever observed, and might be the result of the activity of a single toxin-induced channel on the cell. *A* and *B* also illustrate the stability of the baseline current (about 3 pA inward). Since it changed by less than 1 pA, no more than one or two 15 pS channels (which would pass <1 pA at −50 mV and be hidden in the baseline noise) could have accumulated in the plasmalemma during the 20 min latent period.

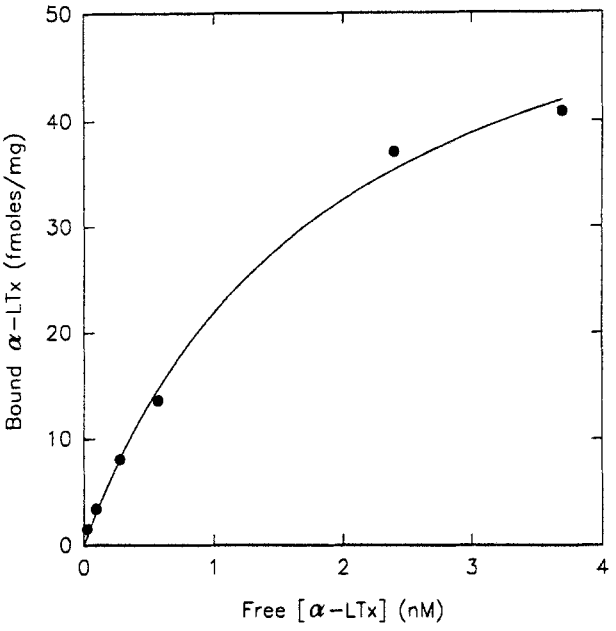

Fig. 1. Binding of α-LTx to NG108-15 neuroblastoma x glioma cells. Specific binding of α-LTx to NG108-15 cells is expressed as a function of the toxin concentration. Mixtures containing NG108-15 cells (10^7/ml) and various concentrations of [^{125}I]α-LTx were incubated for 20 min at 37°C, chilled on ice, and centrifuged. The pellet was rinsed once before counting the radioactivity in a gamma-counter. In the various experiments, unspecific binding ranged between 3 and 14% of the [^{125}I]α-LTx input. The figure illustrates the result of a typical experiment (K_D, $1.88 \cdot 10^{-9}$ M; B_{max}, 59 femtomol/mg protein).

C presents records collected at various times during the burst of current. The current fluctuates among at least three diffuse levels, and transitions occur between any pair of levels. Since the fluctuations were not stationary, and the levels were not clearly resolved, a statistical analysis of the transitions was not carried out. *D* and *E* show that only the fully open state of the channel can be defined unambiguously (arrowhead in *E*), and the rest of this report will describe some of the gating and permeability properties of this state.

Solutions without Ca^{2+} or Divalent Ions Open the Channels

The effects of applying a Ca^{2+}-free Krebs to toxin-treated cells are shown in Fig. 3 (*A* and *B*). In the cell shown in Fig. 3*A*, the mean current increased during the flush, and the fluctuations changed from transient downward deflections arising from a level near 0 pA to transient upward deflections originating from a level near 100 pA. These changes reversed when Krebs was reap-

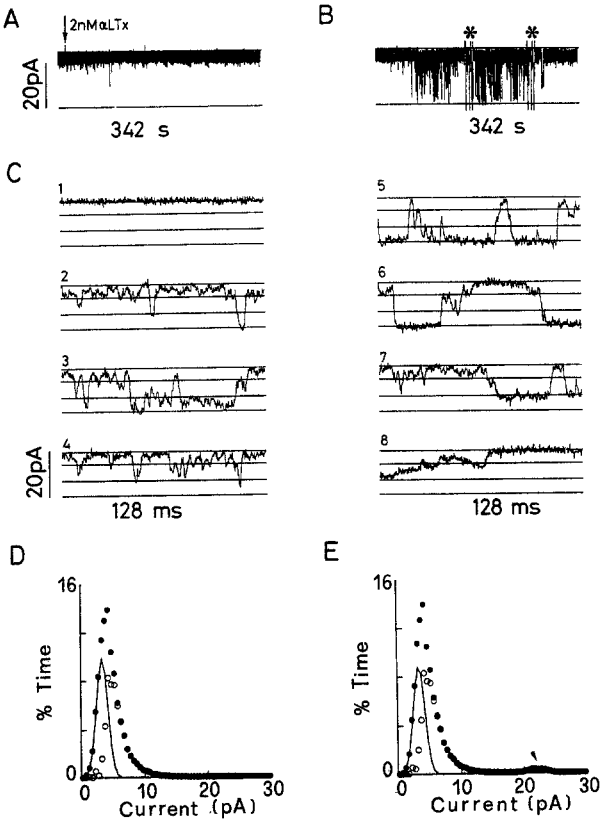

Fig. 2. The minimal response of a cell to α-LTx. (*A*) Control record begun while 2 nM α-LTx was being applied in Krebs (arrow, data read at 500 Hz). (*B*) Record taken about 20 min after toxin was applied, showing the burst of fluctuating current that was recorded from this cell. (*)Times when a set of voltage pulses (*see A*, Fig. 3) was applied. (*C, 1–8*) Selected records collected during the burst (data read at 4 kHz). Traces *1* and *8* show the baseline currents before and after the burst; traces *2* and *7* show current fluctuations at the beginning and the end of the burst; traces *3–6* show current fluctuations at various times during the burst when most of the full openings occurred. The baseline is 2.4 pA, and the other levels are 5.2, 11.5 and 19.3 pA below the baseline. The probability distribution (filled circles) of the current during a 42.6 sec interval at the onset of the burst is shown in *D*, and during the period between the two sets of voltage pulses in *E*. The continuous curves are Gaussian distributions with the same variance as the baseline, but their position and height were adjusted to fit the rising edge of the data. The mean current is 3.3 pA in *D* and 3.4 pA in *E*. The mean ± SD of the baseline current just before the onset of the fluctuations was 2.7 ± 0.9 pA. The open circles are the differences between the adjusted baseline and the data, and they give the distribution of the fluctuations. In all figures, inward current down and holding potential is −50 mV. The upper line is the zero current level, unless indicated otherwise.

plied. The fluctuations then gradually waned, and when the same Ca^{2+}-free solution was reapplied, little happened until the current increased abruptly by 15–20 pA (right panel), as though a single toxin channel had opened. None of these changes in current occurred when the Ca^{2+}-free solution was applied to cells that had not been exposed to α-LTx (*not shown*).

Figure 3*B* shows on a faster time base the changes that occurred in another toxin-treated cell when the Ca^{2+}-free solution was applied. The current began to increase after about 25 sec (center panel), and it seemed to rise in steps. It began to fall about 45 sec after Krebs was reapplied (right panel). No steps are evident in this record.

Similar changes in current occurred when a solution with 1 mM EGTA and no divalent cations was applied to another toxin-treated cell and then removed (Fig. 3*C*). Steps are evident in these records. These changes in current did not occur when divalent cation-free solutions were applied to control cells not treated with α-LTx (*see* Fig. 4, *F* and *G*).

The cells in Fig. 3 seem to exhibit only a few fluctuating channels in Krebs, yet when they are bathed in Ca^{2+}-free, or divalent cation-free solutions, the steady current that finally flows is equivalent to that through many channels.

Current-Voltage Relation

Figure 4 shows the currents produced by a set of voltage pulses applied to a cell in Krebs before (*B*), or after (*C*), it had been treated with α-LTx. The baseline current in the control condition was near 0 pA, and it changed by only a few pA when the membrane potential was changed from −25 to −100 mV. A steady inward current of about 18 pA developed after the toxin was applied, and superimposed on it was a fluctuating current of approximately equal amplitude. Apparently two channels were present: one which was fully open all the time and accounted for the steady component of current, and another which fluctuated among its conductance states and accounted for the fluctuating component of current. Changes in membrane potential changed the magnitude of the steady component and the amplitude and frequency of the fluctuations (*C* and *D*). The current-voltage (*I-V*) relation for the fully open fluctuating channel was linear, its reversal potential was −1 mV, and its conductance was 290 pS (*E*). The continually open channel had a similar *I-V* relation. Its reversal potential was +0.3 mV, and its conductance was 340 pS. Similar *I-V* curves were obtained from eight cells with eight flickering and two steady channels, and their average conductance and reversal potential were 330 ± 37 pS and −2.9 ± 4.5 mV (mean ± SD).

Figure 4 (*F–K*) shows the effects of membrane potential on toxin-induced currents in a different cell bathed either in a Ca^{2+}-free solution with 4 mM Mg^{2+}, or in a solution with no divalent cations. The *I-V* relations were linear, the reversal potentials were near 0 mV and the conductances were equivalent to 4.7–5.3 fully open channels (*K*). Thus, the lack of Ca^{2+} or divalent ions does not affect the *I-V* characteristics of the channels.

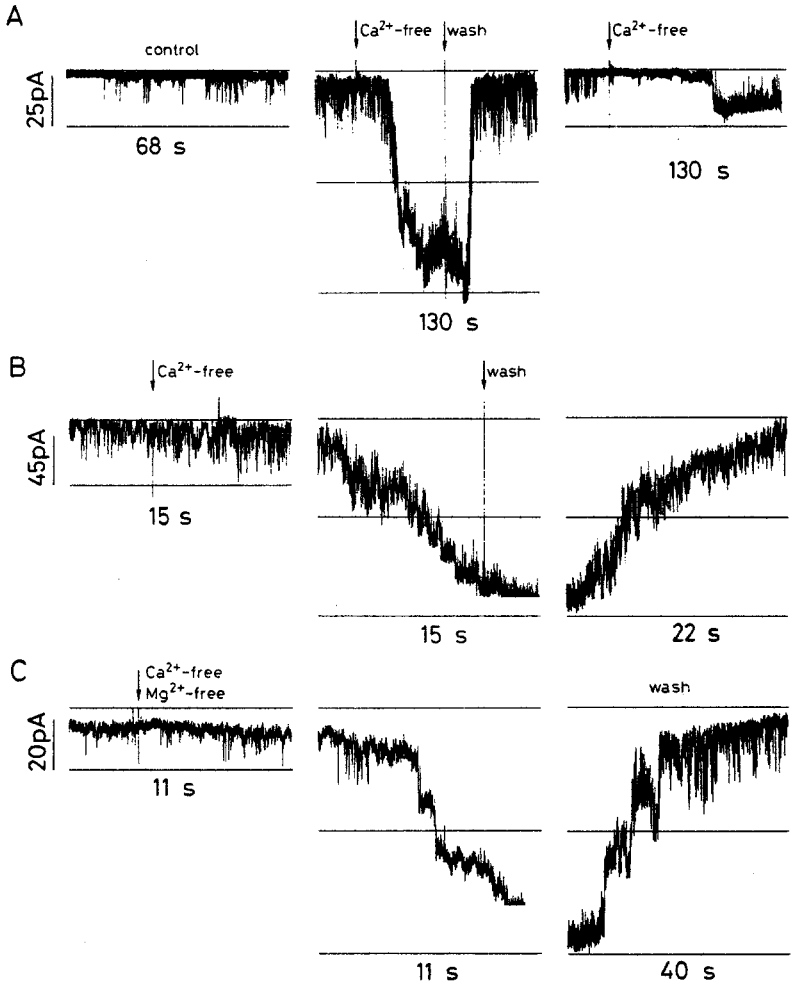

Fig. 3. Effects of Ca^{2+}-free or divalent cation-free solutions on toxin-induced current. Recordings from three different cells exposed to 2 nM α-LTx in Krebs' medium are shown (*A–C*). (*A*) Left panel: Onset of current 6 min after the application of the toxin (data read at 2 kHz). Center panel: changes in current caused by applying Ca^{2+}-free Krebs with 4 mM Mg^{2+} and 1 mM EGTA (first arrow), and then reapplying Krebs (second arrow, data read at 1 kHz). Right panel: changes in current when the same Ca^{2+}-free solution was reapplied about 9 min later (arrow, data read at 1 kHz). (*B*) Left and center panels: the Ca^{2+}-free Krebs with 4 mM Mg^{2+} and 1 mM EGTA was applied at the arrow and, after a delay of about 17 sec, the current increased and went off scale; Krebs was reapplied at the arrow. Right panel: after a delay of 25 sec, the current began to decrease (data read at 4 kHz). (*C*) Left panel: a modified Krebs with no divalent ions and 1 mM EGTA was applied at the arrow. Center panel: the record shows the relatively rapid increase in current which also went off-scale; Krebs was reapplied about 1 min later (*not shown*). Right panel: the current began to decrease about 30 sec after the wash, and it returned to the baseline level (data read at 4 kHz).

Permeability to Ions

Since the reversal potential of the channel indicates that it is relatively nonselective, the inward current through it should be carried either by Na^+ flowing into the cell, or by anions flowing out. Figure 5 shows the results obtained when most of the NaCl of normal Krebs was replaced by isosmotic concentrations of glucosamineHCl, KCl, $CaCl_2$, a mixture of $MgCl_2$and $MgSO_4$ (to keep Cl^- constant), or $MgCl_2$ alone. None of the altered solutions had strong effects on cells that had not been treated with α-LTx (*not shown*). The first and last columns in Fig. 5 illustrate the wide variety in the kinetic behavior of the channels on various cells in Krebs (*A–E*).

GlucosamineH$^+$ (25 mM Na^+) reduces the mean current, the magnitude of the fluctuations and the amplitude of the individual openings (Fig. 5*A*, second and third panel). The latter effect is not fully reversible. These results indicate that the channels are not very permeable to glucosamineH$^+$, and that Na^+ carries most of the inward current in Krebs.

Isotonic K^+ has little effect on the mean current, the magnitude of the fluctuations or the amplitude of the individual openings (Fig. 5*B*). The downward movement of the baseline is presumably due to the reduction in cell EMF caused by K^+. Thus, the channel is about as permeable to K^+ as to Na^+, in keeping with the near zero reversal potential of the channels.

Isotonic Ca^{2+} reduces the mean current, the magnitude of the fluctuations and the amplitude of the individual openings (Fig. 5*C*, second and third panel). However, since current still flows and the amplitudes of the individual openings are reduced only about 50%, the channels are permeable to Ca^{2+}.

The solution with 110 mM Mg^{2+} (constant Cl^-, 2 mM Ca^{2+} and 41 mM SO_4^{2-}) increases the mean current, but reduces the magnitude of the fluctuations and the amplitudes of the individual openings (Fig. 5*D*, second and third panel). The channels are permeable to Mg^{2+}, though the unitary current is reduced by about 60%, and high concentrations of this ion seem to act like low concentrations of Ca^{2+}, causing channels to open. However, since this Mg^{2+}-rich solution contained 41 mM

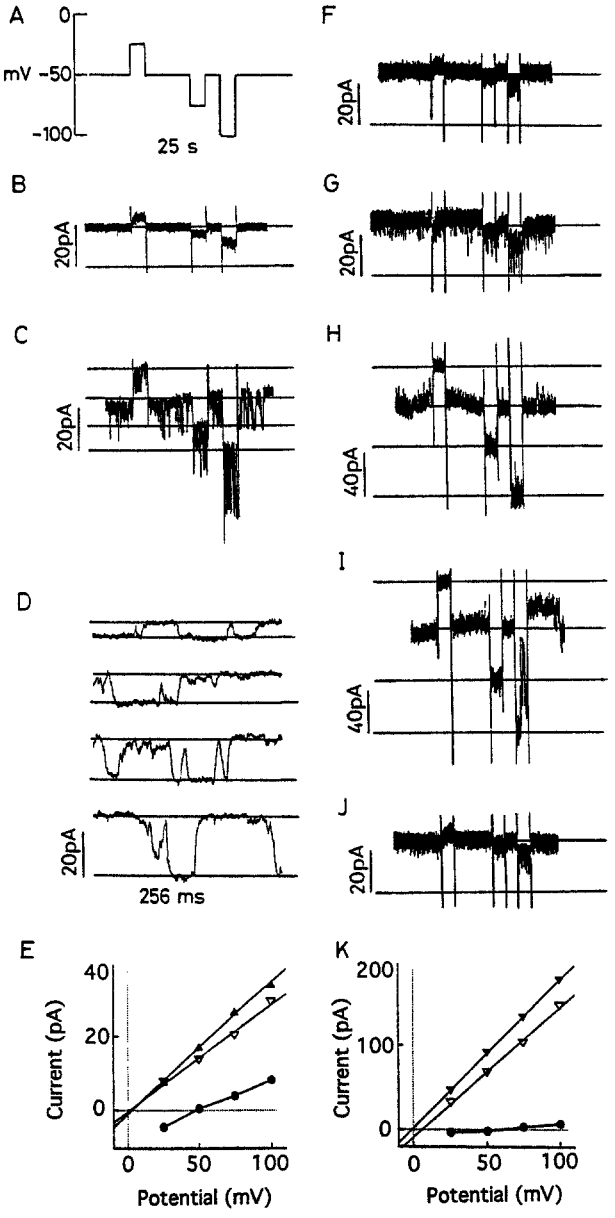

Fig. 4. Effect of membrane potential on toxin-induced currents. Results from two cells treated with 2 nM α-LTx are shown (all data read at 4 kHz). (*A*) Set of voltage pulses: each pulse is 2 sec in duration, and 18 sec elapse between the beginning of the pulse to −25 mV and the end of the pulse to −100 mV. (*B*) Currents recorded from a cell in Krebs before the toxin had been applied. (*C*) Currents recorded after the toxin-induced current had appeared and (*D*) individual full openings (7.2, 14.0, 19.6 and 28.6 pA) of the fluctuating channel at the four levels of membrane potential. The horizontal lines in *C* indicate the mean currents from which the fluctuations arose (3.4, 17.7, 30.6 and 42.6 pA). (*E*) *I-V* relation for the untreated cell (filled circles), the mean of 3–8 fluctuations at each level of potential (open inverted triangles), and the mean current through the continuously open channel (filled triangles). The latter current is the difference between the lines in *C* and the filled circles in *E*. The regression equation (least-squares fit) for the fluctuating current is: $I = -0.3 + 0.288$ V, where I is the magnitude of the inward current (pA) and V is $-1\times$(internal potential) (mV). The equation for the steady current is: $I = 0.1 + 0.335$ V. (*F–K*) Results from a different cell. *F* and *G* show control currents evoked by the set of voltage pulses applied to the cell in Krebs (*F*), or in a modified Krebs with no divalent cations and 1 mM EGTA (*G*), before it was treated with α-LTx. The broadening of the baseline in *G* is due to indigenous channels which became active in this solution. (*H* and *I*) Currents after the cell had been treated with 2 nM α-LTx in Krebs and then, after the channel current had appeared, washed first with a Ca^{2+}-free Krebs with 4 mM Mg^{2+} and 1 mM EGTA (*H*), and then with the solution without divalent ions (*I*). The lines in *H* (27, 65, 104 and 152 pA) and *I* (43, 88, 138 and 187 pA) are drawn through the average currents at the four levels of potential. Some channels in *I* dropped out shortly after the potential was raised to −100 mV, and the line is drawn through the initial value of the current. (*J*) Currents recorded much later in Krebs when the toxin-induced currents had ceased. The cell recovered completely. (*K*) *I-V* relations for the averaged currents before α-LTx was applied (filled circles), and for the toxin-induced currents when the cell was bathed in the Ca^{2+}-free Krebs with 4 mM Mg^{2+} (open triangles) or in the solution without divalent ions (filled triangles). The toxin-induced currents are the differences between the average control currents in *K* and the mean levels in *H* and *I*. The equations for the two regression lines are: $I = 9.1 + 1.54$ V (Ca^{2+}-free) and $I = -1.8 + 1.76$ V (divalent-free), where $V = -1\times$(internal potential). The reversal potentials (internal, referred to the bath) are −6.0 and +1.0 mV, respectively, and the conductances are 1,540 and 1,760 pS, respectively.

SO_4^{2-}, the latter ion might have combined with Ca^{2+} and reduced its concentration enough to cause the openings.

The solution containing 98 mM Mg^{2+}, 2 mM Ca^{2+} and 1 mM SO_4^{2-} (200 mM Cl^-) also increased the mean current and reduced the fluctuations, and these changes were reversible (Fig. 5*E*, second and third panel). When 150 mM glucosamineH^+ was applied, the current quickly fell to almost zero (Fig. 5*E*, last panel), demonstrating that the increase in current was not an artifact. These results confirm that high concentrations of Mg^{2+} open channels even when Ca^{2+} is present at normal levels.

Figure 6 (*A–C*) shows results from an experiment in which α-LTx was applied to a cell bathed in a modified Krebs with 98 mM Ca^{2+}. The fluctuations seem normal, but reduced in amplitude, and the probability distribution shows a clear peak about 7 pA above the baseline. This result confirms that the unitary current through the channels is reduced by about 50% when 98 mM $CaCl_2$ is substituted for 150 mM NaCl.

D and *F* show the effects of a modified Krebs with 98 mM $MgCl_2$ on a cell with a small toxin-induced current. The average current did not increase markedly when Mg^{2+} was applied to this cell, but the fluctuations diminished and the current settled to a relatively steady level about 10 pA below the baseline (*E*). When Krebs was reapplied, the current returned to the original baseline level. *F* shows the probability distributions of the current in the Mg^{2+}-rich solution, and during the final baseline period after Krebs had been reapplied. They are Gaussian and their peaks are separated by 4–6 pA.

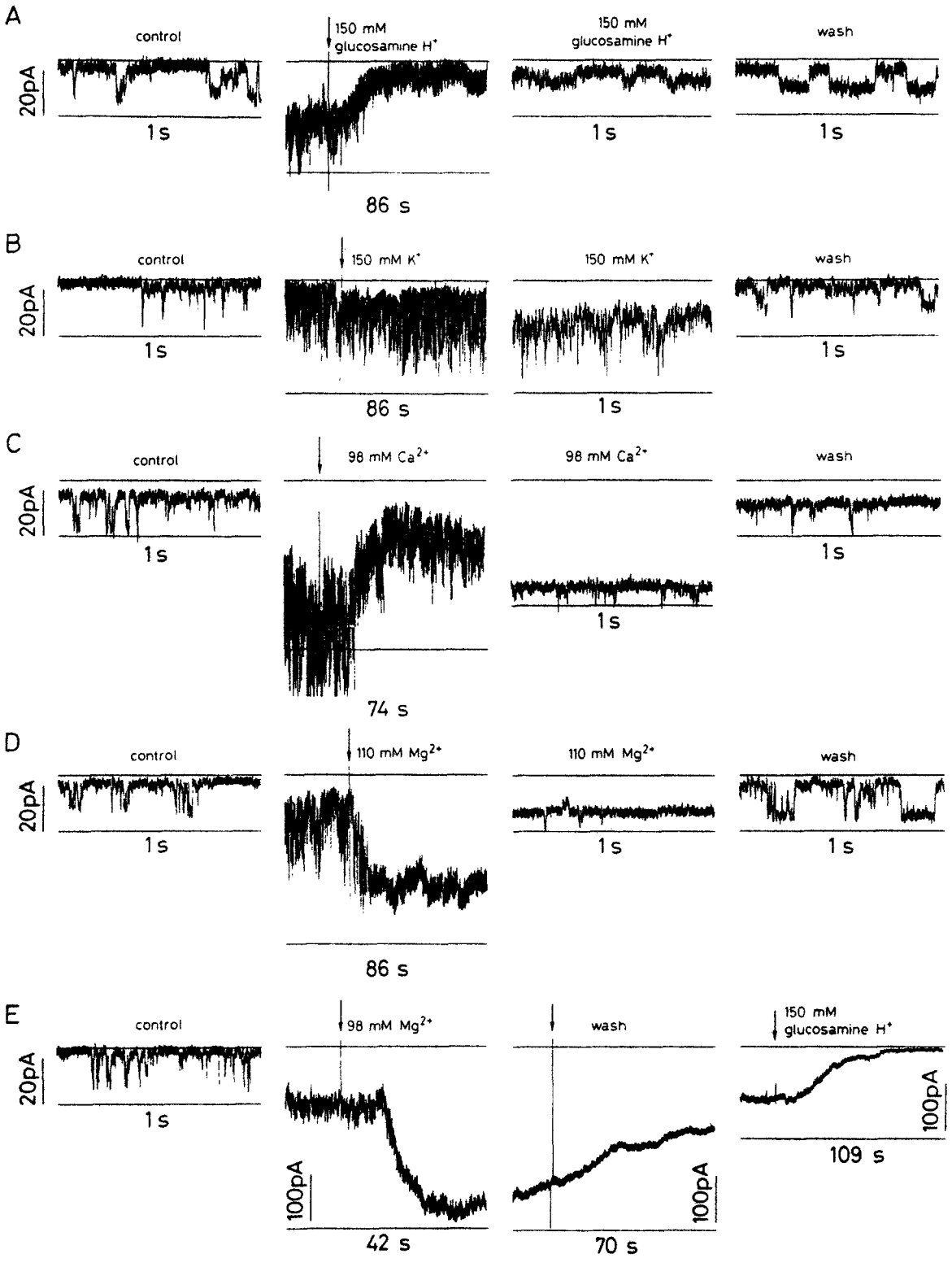

Fig. 5. Effects of Na^+ substitutes on toxin-induced current. Each row of panels shows the effect of a different substitute on a different cell: row *A*, glucosamineH^+ (150 mM, 2 nM α-LTx); row *B*, KCl (150 mM, 2 nM α-LTx); row C, $CaCl_2$ (98 mM, 0.4 nM α-LTx); row *D*, a mixture of $MgCl_2$ (69 mM) and $MgSO_4$ (41 mM) to hold Cl^- constant (2 nM α-LTx). The panels in column 1 show the onset of the current in Krebs, those in column 2 show the changes in current when Na^+ was replaced (arrows) by the substitute ion, those in column 3 show channel openings in the Na^+ substitute, and those in column 4 show openings after recovery in Krebs. Row *E* shows the effects of a modified Krebs with 98 mM $MgCl_2$ (1 mM SO_4^{2-}, 200 mM Cl^-, 2 mM Ca^{2+}). The first panel shows the onset of the current in Krebs (2 nM α-LTx). The current then rose to about 100 pA (second panel), and the Mg^{2+}-rich solution was applied at the arrow. Krebs was reapplied about 2 min later (arrow, third panel), and glucosamineH^+-Krebs was applied after the current had fallen to about 100 pA (arrow, last panel). The current in high Mg^{2+} was too large to see openings of single channels. All data read at 4 kHz, with the exception of column 2, *A–D* read at 2 kHz).

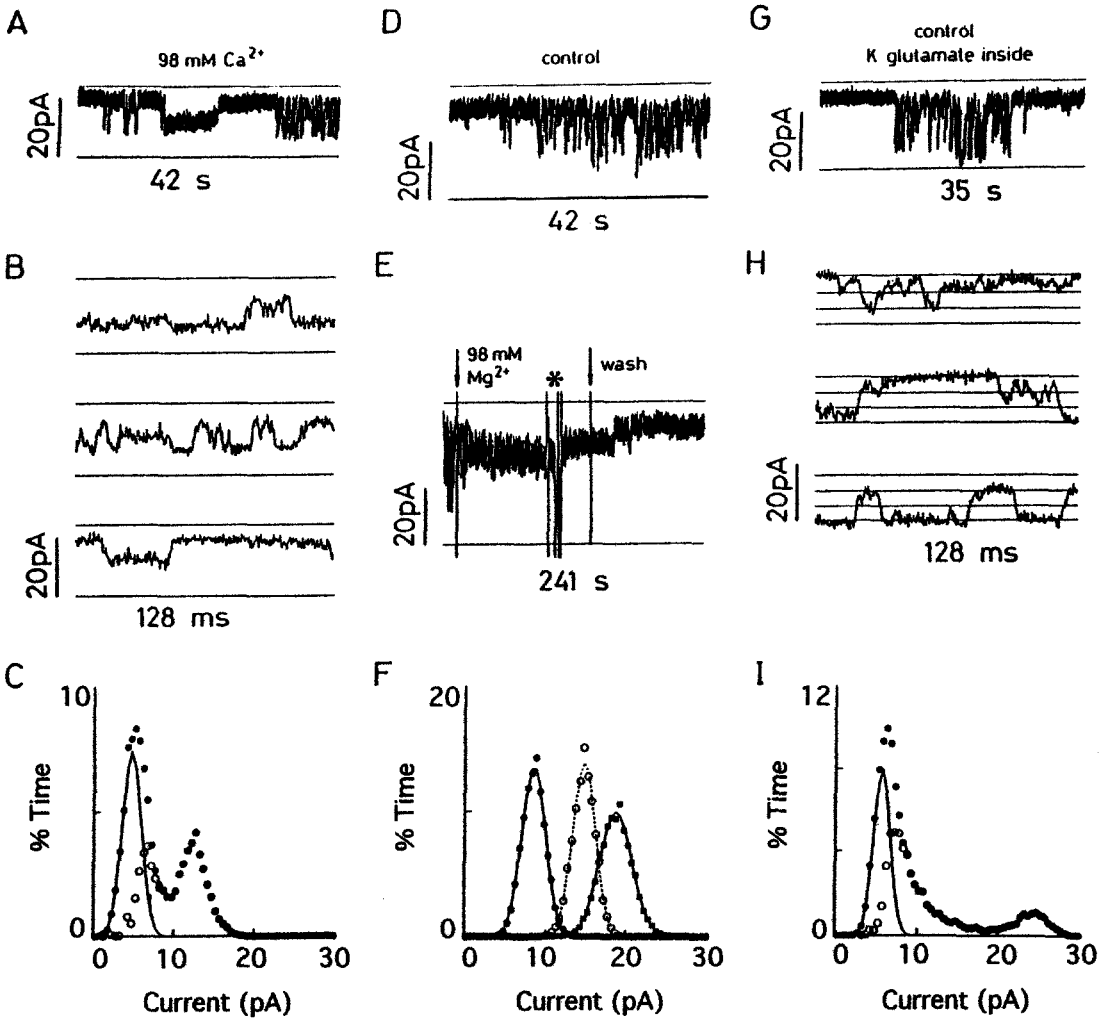

Fig. 6. Effects of Ca^{2+}, Mg^{2+} and glutamate$^-$ on toxin-induced current. (*A–C*) Onset of toxin-induced current in modified Krebs with 98 mM $CaCl_2$. This cell had previously been treated with 0.4 nM α-LTx in Krebs, and the cell was washed thoroughly with the same buffer after the toxin-induced current appeared. The Ca^{2+}-rich Krebs was applied after these currents had stopped, and then 0.8 nM α-LTx was added. *A* shows the onset of current about 5 min later (data read at 4 kHz), *B* shows details of the fluctuations, and *C* shows the probability distribution of the current during the burst. The filled circles are the data, the unbroken line is the probability distribution of the baseline current shifted so as to fit the rising phase of the data. Its mean is 4.75 pA. The open circles are the difference between the data and the adjusted baseline. The baseline current in 98 mM Ca^{2+} just before the onset of current was 4.0 ± 1.2 pA. (*D–F*) Effects of a modified Krebs with 98 mM $MgCl_2$ on a cell with a small toxin-induced current (0.8 nM α-LTx). *D* shows the current in Krebs (data read at 4 kHz), and *E* (data read at 400 Hz) shows the changes that occurred when the Mg^{2+}-rich Krebs was applied (first arrow) and then removed (second arrow). A set of voltage pulses was applied at the asterisk. *F* shows the probability distribution of the current in the Mg^{2+}-rich Krebs just before the set of voltage pulses was applied (filled squares), after the set of pulses but before the reapplication of Krebs (open circles), and during the final baseline in Krebs (filled circles). All data read at 4 kHz. The lines are Gaussian curves fit to the various distributions. Their parameters (mean ± SD in pA) are: 8.5 ± 1.47 (baseline), 14.8 ± 1.44 (Mg^{2+} after pulses) and 18.8 ± 2.00 (Mg^{2+} before pulses). (*G–I*) Currents recorded with a pipette filled with Kglutamate and no nystatin. *G* shows the onset of the current in Krebs about 5 min after 0.4 nM α-LTx was added (data read at 4 kHz), and *H* shows details of the fluctuations. The baseline is 6.4 pA and the three other levels are 5.4, 10.7 and 16.1 pA below this. *I* shows the probability distribution of the current during the burst. Symbols and lines as in *C*. The mean of the fitted baseline is 5.65 pA. The baseline current measured just before the burst was 5.6 ± 1.0 pA.

This result confirms that 98 mM Mg^{2+} opens the channels but carries only about one-third the current carried by 150 mM Na^+.

The channels formed by α-LTx in simple planar bilayers have been reported to be highly selective for cations over anions (Finkelstein et al., 1976). Therefore, it was of interest to test more directly whether the channels in neuroblastoma cells were permeable to Cl^-.

Since inward current should be carried by intracellular Cl^- moving out of the cell, we tried to replace cell Cl^- with glutamate$^-$ (*see* Materials and Methods).

Figure 6 (*G–I*) presents results obtained from a cell with the conventional whole-cell technique. The current fluctuations seem normal, and the probability distribution shows a well-resolved peak about 16 pA above the baseline and a diffuse distribution across the intermediate levels. These results are similar to those shown in Fig. 2, and they suggest that internal Cl^- is not a major current carrier.

When the solution bathing this cell was replaced by Cl^--deficient Krebs with 150 mM Naglutamate, the fluctuations became sporadic and eventually ceased, and they reappeared if Krebs was reapplied (*not shown*). Since the fluctuations that did occur in the glutamate Krebs were of normal amplitude, it appears that the application of Cl^--deficient, or glutamate$^-$-rich, solutions to both sides of the plasmalemma reduces the rate at which the channels fluctuate, and eventually prevents them from opening.

La^{3+} Blocks or Closes Channels

Figure 7 (*A–D*) show the effects of 1 mM La^{3+} applied in a modified Krebs with no PO_4^{3-} or SO_4^{2-}. The fluctuations first increased, and then the current fell to the baseline level (*B*). It did not recover during a 5 min wash with the PO_4^{3-} and SO_4^{2-}-free Krebs, but intense fluctuations reappeared about 20 sec after standard Krebs was applied (*C*). Apparently, the PO_4^{3-} and SO_4^{2-} are needed to remove La^{3+} that is tightly bound to the channels (Scheer, 1989).

Figure 7 (*E–J*) show the results obtained when 1 mM La^{3+} was applied in a solution with 4 mM Mg^{2+} and no Ca^{2+}, PO_4^{3-} or SO_4^{2-}. The current fell almost to zero about 30 sec after La^{3+} was applied, and it rose to a level higher than before when La^{3+} was removed with EGTA (*F*). La^{3+} was reapplied twice more with similar results (*G–J*). It obviously blocks or closes the channels in Ca^{2+}-free solutions, and its effects are reversible.

Discussion

These results show that NG108-15 cells bear receptors for α-LTx and that nonselective, high conductance channels are formed in their plasmalemmas at a toxin concentration commensurate with the dissociation constant of the toxin-receptor complex in these cells. The affinity of the receptors is very high ($K_D = 2 \cdot 10^{-9}$ M) and similar to that reported for the receptors of PC12 cells. However, the density of the receptors is approximately sixfold higher on NG108-15 cells than on PC12 cells (Meldolesi et al., 1983). The conductance of a fully open channel is similar to the conductance of the channel formed by the toxin in simple planar bilayers (Finkelstein et al., 1976) and the channels in the cells are permeable to Ca^{2+} and Mg^{2+} as well as to Na^+ and K^+, as are those in the bilayers. However, in neuroblastoma cells the channels exhibit three properties not described previously in planar lipid bilayers or in PC-12 cells: (i) their conductance usually fluctuates in Krebs' solution; (ii) the fluctuations cease and the channels become permanently open in Ca^{2+}-free solutions, in solutions devoid of divalent cations, or in solutions with normal levels of Ca^{2+} and high levels of Mg^{2+}; and (iii) the channels are blocked by La^{3+}. These properties may be relevant to the pathophysiology of the toxicosis induced by the venom, since each of them can be directly related to a feature of the action of the toxin at nerve terminals.

The formation of nonselective, cation-permeable channels in a nerve ending would depolarize it and, if the depolarization were great enough, block its action potential (Longenecker et al., 1970; Nichols et al., 1982; Mallart & Haimann, 1985; Wanke et al., 1986). Since the channels are permeable to Ca^{2+}, the cytoplasmic concentration of Ca^{2+} should rise and quantal secretion be stimulated (Grasso et al., 1980; Meldolesi et al., 1984). If the channels fluctuate, or the fluctuations occur in bursts, Ca^{2+} might enter the cytoplasm in pulses and quanta be secreted in bursts, as occurs at the neuromuscular junction (Del Castillo & Pumplin, 1975; Fesce et al., 1986; Ceccarelli, Hurlbut & Iezzi, 1988). Agents, such as La^{3+}, that completely block the channels should also block all the other effects of α-LTx, as is observed with rat brain synaptosomes (Scheer, 1989; Rosenthal et al., 1990). The effect of millimolar concentrations of La^{3+} on the action of α-LTx at the frog neuromuscular junction cannot be studied easily, because these concentrations of La^{3+} are powerful stimulants of transmitter release (Heuser & Miledi, 1971; Segal et al., 1985). Since the channels can open in isotonic $CaCl_2$, the ability of high concentrations of Ca^{2+} to suppress the venom-induced quantal secretion at the frog neuromuscular junction (Smith, Clark & Kuster, 1977) cannot be attributed to channel block.

The large increase in current that occurs when cells previously exposed to the toxin in Krebs are bathed in Ca^{2+}- or divalent cation-free solutions suggests that many of the toxin molecules that bind to the plasmalemma in Krebs do not form ion conducting channels. Solutions without Ca^{2+} or divalent cations may convert such bound but inactive molecules into fully open channels, in addition to promoting the opening of already fluctuating channels. This opening of the channels may explain why Ca^{2+}-free solutions with 4 mM Mg^{2+} enhance the deleterious effects of low doses of α-LTx on

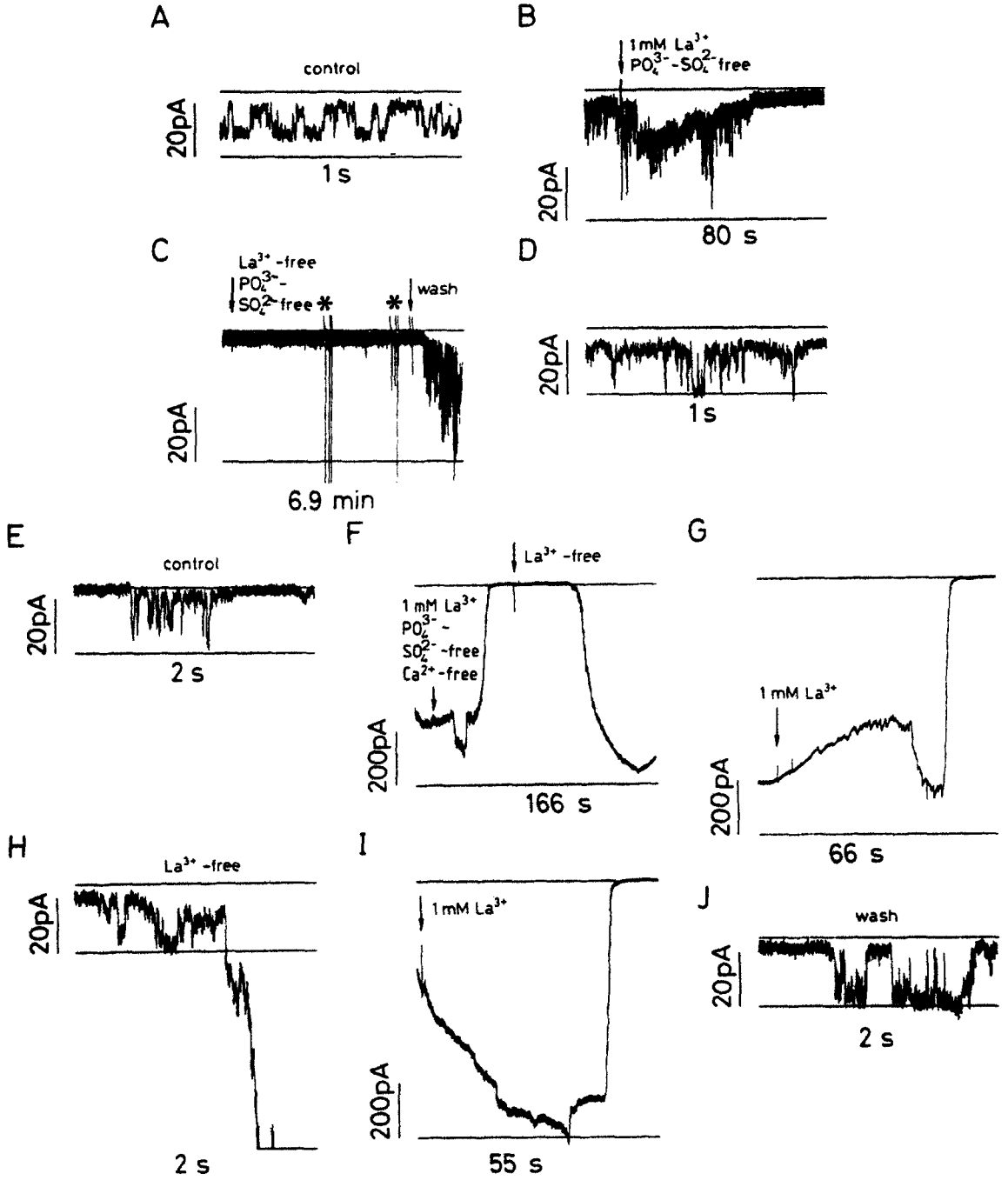

Fig. 7. Effect of La^{3+} on toxin-induced current. (*A–D*) Records from a cell treated with 0.4 nM α-LTx. *A* shows the current in Krebs (data read at 4 kHz). The cell was washed a few min later with a PO_4^{3-} and SO_4^{2-}-free Krebs, and the fluctuations became sporadic. The initial part of *B* shows the fluctuations in the PO_4^{3-} and SO_4^{2-}-free Krebs (data read at 2 kHz). At the time indicated by the arrow, 1 mM La^{3+} was applied and the PO_4^{3-} and SO_4^{2-}-free Krebs was reapplied a few min after the current had fallen to near 0 (first arrow in *C*, data read at 400 Hz). The set of voltage pulses was applied at the asterisks, and standard Krebs was applied at the second arrow. *D* (data read at 4 kHz) shows the currents recorded several min later in Krebs. (*E–J*) Results from another cell which was treated with 1 mM La^{3+} in a modified Krebs with 4 mM Mg^{2+} and no PO_4^{3-}, SO_4^{2-} or Ca^{2+}. *E* shows the onset of current in standard Krebs 1–2 min after 0.4 nM α-LTx had been applied (data read at 4 kHz). The current then increased steadily, the bath was flushed with a modified Krebs containing 2 mM Ca^{2+}, 4 mM Mg^{2+} and no PO_4^{3-}or SO_4^{2-}, and the current finally leveled off at about 500 pA (*F*). A modified Krebs with 1 mM La^{3+}, no Ca^{2+}, 4 mM Mg^{2+} and no PO_4^{3-} or SO_4^{2-} was applied at the first arrow in *F* (data read at 1 kHz), and the La^{3+} was removed at the second arrow by applying a similar solution with no La^{3+} and 1 mM EGTA. The La^{3+} was reapplied about 1 min later (arrow, *G*, data read at 2 kHz). After the current had fallen again almost to zero, La^{3+} was removed (*not shown*). A fluctuating current appeared about 50 sec later (*H*), and it quickly rose in staircase fashion to pass off-scale. La^{3+} was reapplied (arrow, *I*, data read at 2 kHz). After the current had fallen to near zero, a modified Krebs (2 mM Ca^{2+}, 4 mM Mg^{2+} and no PO_4^{3-} or SO_4^{2-}) was applied for 3.3 min (*not shown*), but the current did not recover. The bath was then flushed with standard Krebs, and a fluctuating current appeared about 31 sec later (*J*).

the frog neuromuscular junction, especially its ability to block neuromuscular transmission and vesicle recycling (Ceccarelli & Hurlbut, 1980; Fesce et al., 1986). When low doses of toxin are applied in Krebs, a small number of fluctuating channels should form in a nerve ending, and the average change in its ionic permeability should also be small. Under these conditions, enough Ca^{2+} enters the cytoplasm to stimulate release, but the vesicles apparently recycle normally, the average change in membrane potential is too small to block the action potential and transmission persists (Ceccarelli & Hurlbut, 1980). However, if the bathing solution lacks Ca^{2+} and contains Mg^{2+}, the fluctuating channels would open permanently, inactive channels would be recruited, and the changes in permeability would be much greater. The membrane potential would decrease enough to block the action potential, Mg^{2+} would enter the cell through the open channels and stimulate transmitter release, and a large influx of Na^+ and efflux of K^+ would quickly change the ionic composition of the cytoplasm and thereby block recycling (Larkin et al., 1983).

Although these results are compatible with the idea that the entry of Mg^{2+} or other multivalent cations into the cytoplasm through the toxin-induced channels is the event that causes the secretion from cells bathed in Ca^{2+}-free solutions, they do not explain why a variety of divalent cations can stimulate release from toxin-treated cells, but not from normal cells. These ions might be effective in toxin-treated cells simply because they accumulate to unusually high levels in the cytoplasm where they stimulate release either indirectly by displacing Ca^{2+} from internal storage sites, or directly by substituting for Ca^{2+} in the release process. The latter possibility should not be dismissed, since α-LTx does not raise internal Ca^{2+} when it stimulates secretion from synaptosomes bathed in a Ca^{2+}-free solution (Meldolesi et al., 1984). A further possibility is that the binding of α-LTx to its receptor changes the sensitivity of the release process to Ca^{2+} and/or makes it more sensitive to other divalent cations. Indeed, serotonin may enhance the secretion of transmitters from crayfish motor nerve terminals by increasing the sensitivity of the release process to internal Ca^{2+} (Delaney, Tank & Zucker, 1991).

Based on sequencing data, the α-latrotoxin receptor has been proposed to be a member of the family of neurexins, neuron-specific proteins which bear some homologies to extracellular matrix proteins and which are therefore putatively involved in cell-cell interactions (Uskaryov et al., 1992). These structural characteristics argue against the possibility that the physiological function of the receptor in the nerve terminal is that of being a channel. Therefore, the molecular mechanism by which binding of α-LTx to its receptor induces the opening of cation channels remains to be elucidated. It is possible that the toxin molecule, which is *per se* able to open cationic channels in artificial membranes (Finkelstein et al., 1976), directly participates in the formation of channels in the plasma membrane.

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