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αLATROTOXIN OF THE BLACK WIDOW SPIDER VENOM OPENS A SMALL, NON-CLOSING CATION CHANNEL

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αLatrotoxin, a presynaptically acting polypeptide neurotoxin, induces massive neurotrasmitter release from both synapses of vertebrates and the neurosecretory cells of the line PC12, derived from a rat pheochromocytoma. Single PC12 cells, differentiated by treatment with nerve growth factor, were used to investigate by the patch-clamp technique i) the alterations of the resting cell conditions (membrane potential and resistance) and ii)the microscopic mechanism of the permeability changes that underly the response to αLTx. The toxin was found to open a channel, 15 pS in conductance, that is permeable to various cations (Na^+, K^+ and probably Ca^{2+}) and has little tendency to close. This channel is different from the classical voltage- and receptor-operated channels present in PC12 cells, as well as from the large conductances induced by the toxin in artificial lipid membranes .

αLatrotoxin (αLTx) is a high M_r protein devoid of enzyme activity contained in the venom of the black widow spider. At synapses of vertebrate animals and neurosecretory cells of the line PC12 (derived from a rat pheochromocytoma) αLTx binds with high affinity to a specific surface receptor (1,2) and induces massive neurotransmitter release by exocytosis (2-5) accompanied by plasma membrane depolarization and stimulated ^{45}Ca influx (5-7) that could be due to the activation of a cationic permeability in the plasmalemma. The nature of this channel has not been identified yet, but two sets of observations have been made. First, αLTx-induced ion fluxes are hardly affected by organic blockers of voltage-gated Na^+ and Ca^{2+} channels (tetrodotoxin (TTx) and verapamil (Vp)), and are still evident in cells bathed in Ca^{2+}-free media (5-7); secondly, the toxin alone, when applied to artificial, phospholipid-cholesterol membranes induces the appearance of large conductances, probably due to the insertion of toxin

Abbreviations used. αLTx = αLatrotoxin; TTx = tetrodotoxin; Vp = verapamil; NGF = nerve growth factor.

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molecules across the lipid bilayer (8,9). Whether the last observation has any relevance to the cellular effects induced by αLTx remains to be elucidated.

The present article deals with the ionic events induced by αLTx in PC12 cells differentiated by treatements with nerve growth factor (NGF).By the use of the patch-clamp technique (10), we investigated i)the membrane potential and resistance changes and ii) the single microscopic events responsible for the permeability changes induced by the toxin.

MATERIALS and METHODS

Two cell lines: PC12, a known target of αLTx, and L6, a rat myotube line to be used as control cells, were cultured as described elsewhere (2,11). In order to increase their size and adhesiviness, PC12 cells were differentiated before use by a 9-12 day treatment with NGF (50 ng/ml). Membrane currents and membrane potentials were recorded via the patch-pipette (10) in either the cell-attached (voltage-clamp) or the whole-cell (current-clamp) recording configuration (12). Routinely, in whole-cell experiments a check for the presence of the classical Na^+ and K^+ excitable channels was done before switching to current-clamp. Experiments were performed at 36-37°C on single cells in 35 mm petri culture dishes (Sterilin) and the bath solution was continously exchanged by a conventional perfusion system driven by gravity. During whole-cell experiments the high-K^+ and toxin solution were delivered through a capillary (0.5 mm in diameter) positioned in the vicinity of the cell under study and mounted on a manipulator. The capillary was connected to three distinct reservoirs (saline solutions: standard $\pm$ toxin and high-K^+) operated by electromechanical valves. The dead time for any change was approximatively 1 sec. After analog filtering, data were recorded through PCM modulation (Type 701ES Sony) on a video tape (type 725EG. JVC). A custom-made interface connected the digital data from the PCM (modified for dc signals) to an IEEE card (Tecmar Inc. USA) plugged into a personal computer (M24 Olivetti). The standard saline contained in mM: 140 NaCl, 2.8 KCl_2, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 Hepes, pH 7.2.In some experiments the Ca^{2+} added to the saline was lowered to 10 uM.The high-K^+ solution contained (mM) 110 NaCl and 32.8 KCl. In the cell-attached experiments the pipette contained the standard external solution plus (μM): 25 Vp, 1 atropine, 500 hexamethonium, 1 TTX and 1000 4-aminopyridine. In whole-cell experiments the pipette solution was the following (mM): 140 K-aspartate, 5 $MgCl_2$, 4 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.3 (calculated $[Ca^{2+}]$ 10^{-7} M).

RESULTS

L6 Myotubes Experiments identical to those described below for PC12 cells were carried out on the rat myotubes of the line L6, which are devoid of specific αLTx binding sites (13) In these cells αLTx, at concentrations up to 10^{-8} M, failed to affect either membrane potential or membrane currents.

PC12 cells: effects on membrane potential. During whole-cell (current-clamp) recording the membrane potential (-57 $\pm$ 3 mV; n=16) was monitored for 2-3 minutes waiting for the equilibration of the pipette solution in the cell and then tested for depolarization under a brief

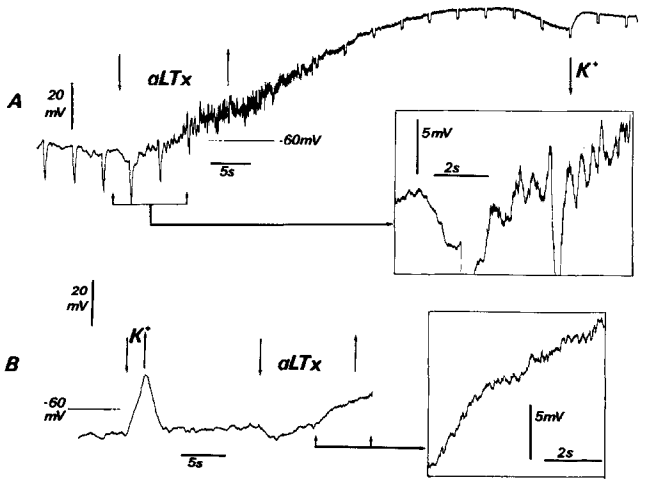

Fig.1. Intracellular membrane potential in PC12 cells.(12 days in culture with NGF) obtained in the whole-cell configuration. Arrows indicate external perfusion with high-K^+ and toxin solutions. A) external solution containing 50 μM Vp and 2μM TTx; hyperpolarizing current pulses of 10 pA were delivered every 4.5 sec; cell resistance was approximatively 3 and 0.4 GOhm before and after toxin perfusion. B) a cell different from that in A) was bathed in standard saline without blockers added. Traces were filtered at 50 Hz. Temperature: 36°C. Insets are enlargements of record A) and B) as indicated by arrows.

extracellular perfusion in the high-K^+ medium (fig.1B).The toxin was then applied for a short period of time. After a delay dependent on the toxin concentration (2 and 15 sec at 2 and 0.3 nM αLTx) depolarization started (Fig.1B). This effect begun with very small positive deflections of the membrane potential (see insets of Fig.1) and within a few tens of seconds reached high values. In order to exclude that the voltage change was by itself responsible for increased inward currents (i.e. that voltage gated channels were activated) experiments with TTx and Vp (Na^+ and Ca^{2+} channel blockers) were performed. Even in the presence of these drugs depolarization by αLTx remained, was sustained and did not reverse (fig1A). During the experiments with αLTx, brief current pulses were delivered to check membrane resistance. Dramatic reductions were always observed (fig.1A). In cells depolarized by αLTx, after toxin was removed, a new perfusion with the high K^+ saline (Nerst equilibrium potential about -37 mV instead of -85 mV in standard saline) induced a supplementary depolarization but a positive potential was never measured (Fig1A). These results indicate that the membrane potential reached by the cell, after the application of αLTx, is due to an equilibrium between the outward K^+ current and the toxin-induced inward current, with a reverse potential around 0 mV. In a low (10 μM) Ca^{2+} medium the effects of αLTx were similar to those obtained at mM $[Ca^{2+}]$, but their time course was slower. Likewise, no major changes were observed when Cl^- in the pipette was replaced by $aspartate^-$. Collectively, the whole-cell

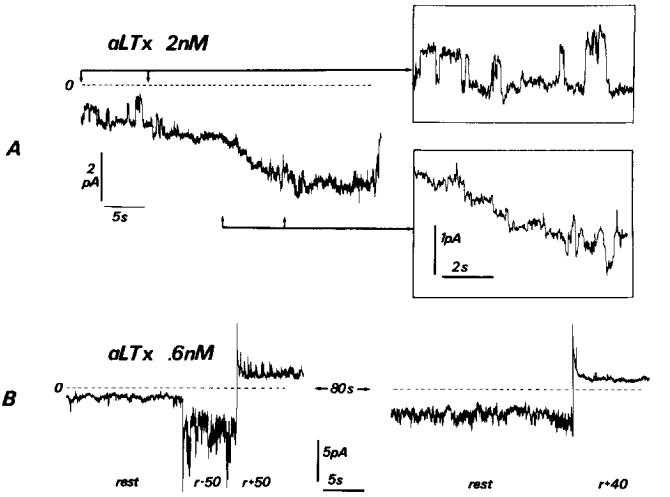

Fig.2. Membrane currents (inward currents downward) in PC12 cells (9 days in culture with NGF) obtained in the cell-attached configuration, with different αLTx concentration. A) record started 20 sec after seal formation at resting potential; notice in the upper inset the open-close kinetics (14 pS, assuming a resting potential of -50 mV) and in the lower inset the step-like increase of the inward current. B) record started 285 sec after seal formation and voltage across the patch was changed as indicated. Notice the outward current at rest+50 mV; the increased current at rest after 80 sec (right part of the record); the lack of fluctuating current (indicating approximately the equilibrium potential of the channel) at rest+40 mV. Traces filtered at 50 Hz Temperature: 37°C.

results indicate that i) cations, and not anions, are responsible for the αLTx-induced currents; and ii) the channel opened by the toxin has no specific selectively for Ca^{2+}, and is permeable to Na^{+} and K^{+}.

PC12 cells: effects on cell patches. Individual events of ion permeation induced by αLTx were characterized by using the cell-attached configuration of the patch-clamp technique, with the toxin present in the pipette together with a mixture of blockers of both voltage- and receptor-operated channels (TTX, Vp, hexamethonium, atropine and 4-aminopyridine). During the approach of the cell by the pipette the positive pressure was removed to avoid diffusion of the toxin to cell regions other than the patch. The first events were inward currents (Fig.2) seen within one minute after the membrane seal, i.e. the time to reach stationary conditions in the layer facing the cell membrane. During the following 2-3 min the current increased progressively in amplitude (Fig 2A). The events recorded were characterized by their extremely variable life time. In some cases openings were short lived (~0.1 sec, upper inset in Fig..2), in others closing did not occur, so that stationary steps of increasing currents appeared, with amplitude very similar to the smallest open-close events (lower inset in Fig.2). The average size of these events was 15 ±5 pS. Only after the occurence of many such events were large deflection seen (not shown), but in no experiments were large currents measured from the very beginning. Extensive control

experiments without toxin were systematically carried out: neither 15 pS, nor large deflection were ever seen. In particular breakdown seal currents were observed in these control cells only after at least 20 minutes from seal formation. In contrast, with the toxin, catastrophic currents were seen in all cases within 4-6 min. In low Ca^{2+} the effect took longer to be seen.

From the experiments of the previous section the mean resting input resistance of PC12 cells was estimated to be 1-4 GOhm. In these cells currents of tens of pA flowing inward in the patch are expected to depolarize the entire cell body (not exposed to toxin) with activation of voltage-gated channels, and appearance of spurious currents in the patch (12). Therefore, the records from high current patches were not considered here. In patches with small αLTx-induced currents (up to 8 pA) membrane potential was hyperpolarized and depolarized from rest in order to test the amplitude and direction of current flow. The fluctuating pattern visible at negative potential (rest-50= -95 mV absolute membrane voltage) was found to reverse its direction at positive potential (rest+50 = +5 mV; fig.2B), implying that the current induced by αLTx flows outward. More than one minute later (Fig.2B) a smaller depolarization (rest+40= -5 mV) caused a practically flat trace. From these measurements it can be concluded that the reverse potential of the αLTx channel is a few mV around zero.

DISCUSSION

Although an effect of αLTx on cation fluxes had been repeatedly suggested in previous studies on both synapses and neurosecretory cells (2-7), the channel opened by the toxin had never been characterized. By the use of the patch clamp technique in two configurations, consistent and complementary information has beeen obtained on a channel previously unrecognized, opened by αLTx in the plasmalemma of PC12 cells. This channel has a small conductance (~15 pS), is permeated by cations (Na^+, K^+ probably Ca^{2+}) but not anions, has little tendency to close and is insensitive to a variety of blockers. These features differentiate the αLTx channel from the known voltage- and receptor-operated channels. Moreover, the channel is substantially different from the large conductance (100-400 pS) that appear in small number in artificial phospholipid-cholesterol bilayers exposed to αLTx and were proposed to mediate the effects of the toxin at its cellular targets (8,9). The persistent opening of even a small number of αLTx channels can account for many effects of the toxin (such as depolarization, increased ^{45}Ca influx, raised cytosolic $[Ca^{2+}]$) previously demonstrated by biochemical and fluorescence techniques at both synapses and neurosecretory cells (5-7). As an example, it can be calculated that at an ending of 5 GOhm of input resistance, the permanent opening of only 5 αLTx channels would

depolarize the cell by about 17 mV, sufficient to cause a pronounced release of neurotransmitter. Many other toxins of natural origin were found previously to affect conductance at the plasmalemma. Some of these toxins are known to affect voltage- and receptor-operated channels (examples: many scorpion toxins for the Na^+ channel; dendrotoxin for the A-channel and another scorpion toxin for the K^+ voltage-dependent channel; αbungarotoxin for the nicotinic receptor)(14-17). Other toxins are endowed with phospholipase activity, and disrupt the permeability barrier of the membrane (14). The mechanism that we describe for αLTx, activation of a discrete, specific channel different from those known up to now, appears novel. Whether this channel is constituted by toxin molecules that insert across the membrane lipid bilayer after binding to the specific αLTx receptor, or by receptor molecules, or by unknown channels coupled to αLTx receptors, remains to be investigated.

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