The mechanism of action of β -bungarotoxin at the presynaptic plasma membrane

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1. The β -bungarotoxin-induced depolarization of the synaptosomal plasma membrane monitored by the efflux of $^{86}\text{Rb}^+$ is potentiated by raising the albumin in the incubation, is Ca^{2+} -dependent and is due neither to inhibition of the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase nor to activation of the voltage-dependent Na^+ channel.
2. Occupancy of the β -bungarotoxin-binding site by dendrotoxin inhibits partially the action of β -bungarotoxin.
3. The efflux of $^{86}\text{Rb}^+$ is paralleled by a release of lactate dehydrogenase from the synaptosome, and the two processes are maximal with 2 nM-toxin.
4. Digitonin induces a release of $^{86}\text{Rb}^+$ and lactate dehydrogenase closely similar to that seen with β -bungarotoxin.
5. It is concluded that the toxicity of β -bungarotoxin for mammalian nerve terminals can be largely accounted for by specific site-directed phospholipase A_2 -induced permeabilization of the plasma membrane.

β -Bungarotoxin, a 23 kDa presynaptic neurotoxin from the venom of the elapid snake *Bungarus multicinctus*, possesses phospholipase A_2 activity (reviewed by Howard & Gundersen, 1980) and binds to a high-affinity site on the synaptosomal plasma membrane (Othman *et al.*, 1982; Rehm & Betz, 1982). β -Bungarotoxin is highly neurotoxic (Othman *et al.*, 1982; Halliwell *et al.*, 1982) and has a variety of effects on isolated synaptosomes consistent with an action at the plasma membrane and an uncoupling of the mitochondria *in situ* (reviewed by Howard & Gundersen, 1980).

At the low or zero albumin concentrations used in most studies the dominant effect is an uncoupling of the mitochondria *in situ* by the fatty acids liberated by the phospholipase A_2 activity at the plasma membrane (Nicholls *et al.*, 1985). Bee-venom phospholipase A_2 uncouples the intrasynaptosomal mitochondria at only slightly higher concentrations (Nicholls *et al.*, 1985). Since β -bungarotoxin is 2000-fold more toxic in relation to its enzymic activity than bee-venom phospholipase A_2 (Othman *et al.*, 1982), it is therefore unlikely that non-esterified fatty acid generation is the primary cause of the toxicity.

Low concentrations of β -bungarotoxin, but not bee-venom phospholipase A_2 , additionally depolarize the plasma membrane (Ng & Howard, 1978; Nicholls *et al.*, 1985). This is not due to fatty acids, since it is potentiated by raising the albumin concentration in the incubation medium (Nicholls *et al.*, 1985). Here we examine the effects of β -bungarotoxin on plasma-membrane integrity and transport when high albumin concentrations are present in the incubation media. In view of the abundance of fatty-acid-binding proteins *in vivo*, these conditions are likely to be more relevant to the toxicity of β -bungarotoxin. Our results indicate that a primary action of the toxin at the plasma membrane is a highly specific, site-directed permeabilization of the synaptosomal plasma membrane.

EXPERIMENTAL

Synaptosomes were prepared from the cerebral cortices of Dunkin–Hartley-strain guinea pigs by Ficoll-gradient centrifugation as described previously (Nicholls, 1978). Synaptosomes were stored as concentrated pellets at 0 °C for not more than 3 h before use. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Isotopic incubations

These were performed as described previously (Nicholls *et al.*, 1985) in stirred open vessels at 30 °C in a medium containing 122 mM-NaCl, 3.1 mM-KCl, 1.2 mM-MgSO₄, 5 mM-NaHCO₃, 0.4 mM-KH₂PO₄, 20 mM-Tes (sodium salt), 10 mM-D-glucose, with the further addition of 16 μM -albumin ('low albumin') or 160 μM -albumin ('high albumin'). The pH was 7.4. Further additions are detailed below.

Isotopic determinations

$\Delta\psi_m$ and $\Delta\psi_p$ were determined simultaneously by a modification (Nicholls *et al.*, 1985) of the method described previously (Scott & Nicholls, 1980). The incubation medium contained additionally 50 μM - $^{86}\text{Rb}^+$ (0.3 $\mu\text{Ci}/\text{ml}$), 1 μM -[^3H]triphenylmethylphosphonium ion (0.13 $\mu\text{Ci}/\text{ml}$) and 5 μM -tetraphenylboron. A synaptosomal pellet (4.5 mg of protein) was resuspended in this medium to give a concentration of 1.5 mg of protein/ml of incubation medium. After allowing 5 min for the plasma membrane to polarize (Scott & Nicholls, 1980), 1.3 mM- Ca^{2+} was added. At defined times further additions were made and 150 μl samples were taken and processed as described previously (Nicholls *et al.*, 1985).

Lactate dehydrogenase release was quantified in the supernatants after centrifugation as described previously (Scott *et al.*, 1980).

Abbreviations used: $\Delta\psi_m$, membrane potential across the inner membrane of synaptosomal mitochondria *in situ*; $\Delta\psi_p$, membrane potential across synaptosomal plasma membrane.

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Fig. 1. Effect of media containing high and low albumin concentrations upon the lowering of $\Delta\psi_m$ and $\Delta\psi_p$ induced by β -bungarotoxin (BBTx)

Synaptosomes were incubated for 32 min in the presence of 1.3 mM- Ca^{2+} , $^{86}\text{Rb}^+$ and triphenylmethylphosphonium ion as detailed in the Experimental section. Where indicated by the arrow, 2.3 nM- β -bungarotoxin was added and potentials were monitored for a further 26 min. (a, b) $\Delta\psi_m$; (c, d) $\Delta\psi_p$. (a, c) Low albumin (16 μM) in medium; (b, d) high albumin (160 μM) in medium.

Toxins

β -Bungarotoxin was purified from the crude venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL, U.S.A.), by fractionation on a CM-Sephadex C-50 column followed by preparative isoelectric focusing as described previously (Spokes & Dolly, 1980). Dendrotoxin was purified from the venom of *Dendroaspis angusticeps* (Miami Serpentarium) as detailed by Harvey & Karlsson (1980). Toxins were stored at -20°C at a concentration of 1 mg of protein/ml and were diluted immediately before use.

Reagents

[^3H]Triphenylmethylphosphonium ion was obtained from New England Nuclear, Dreieich, Federal Republic of Germany. Other radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other reagents were from Sigma Chemical Co., Poole, Dorset, U.K.

RESULTS

Fig. 1 shows that a 10-fold increase in albumin diminishes the effect of β -bungarotoxin upon $\Delta\psi_m$, but potentiates its action at the plasma membrane. The 20 mV lower initial $\Delta\psi_m$ in high albumin is most likely an artefact due to binding of extrasynaptosomal triphenylmethylphosphonium ion by the albumin, leading to an over-estimate of the free external phosphonium cation concentration. Bee-venom phospholipase A_2 at 2.8 nM, which uncouples intrasynaptosomal mitochondria in low-albumin media to the same extent as equimolar β -bungarotoxin (Nicholls *et al.*, 1985), had no effect on $\Delta\psi_p$ in the presence of 160 μM -albumin (results not shown).

A steady depolarization of the plasma membrane could be due to inhibition of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. Sun & Sun (1976) observed such an inhibition with very high toxin concentrations. However, Fig. 2 shows that the rates of depolarization induced by ouabain and β -bungarotoxin in high-albumin medium

Fig. 2. Additivity of β -bungarotoxin (BBTx)-induced and ouabain (Oua)-induced depolarization of the plasma membrane

Synaptosomes were incubated as described in the Experimental section in the presence of 1.3 mM- Ca^{2+} and 160 μM -albumin. Where indicated by the arrow, 2.3 nM- β -bungarotoxin and 100 μM -ouabain were added separately or in combination. $\circ$, Control; $\square$, β -bungarotoxin; $\triangle$, ouabain; $\blacksquare$, β -bungarotoxin plus ouabain.

are strictly additive, confirming the independence of their actions.

An efflux of $^{86}\text{Rb}^+$ would result from an activation of voltage-dependent Na^+ channels. However, since tetrodotoxin does not prevent the depolarization (results not shown), this is unlikely. The phospholipase A_2 activity of β -bungarotoxin is Ca^{2+} -dependent, although a very low activity is seen in Sr^{2+} media (Abe *et al.*, 1977). Fig. 3 shows that Sr^{2+} (together with EGTA to chelate residual Ca^{2+}) leads to a substantial reduction in the depolarization of the plasma membrane. However, since the toxin may still retain detectable phospholipase A_2 activity in the presence of Sr^{2+} (Abe *et al.*, 1977), it is not possible to eliminate the possibility of residual enzymic activity. The binding of β -bungarotoxin to rat synaptosomes is not affected by Ca^{2+} removal (Othman *et al.*, 1982).

Once a linear depolarization is established, the subsequent addition of excess EGTA is without effect (Fig. 3). This could suggest that the Ca^{2+} -dependent action of β -bungarotoxin is not progressive but is completed within a relatively short period.

Dendrotoxin competes for the β -bungarotoxin binding site (Othman *et al.*, 1982). Fig. 4 shows that, although dendrotoxin is itself without detectable effect on $\Delta\psi_p$, it causes a substantial inhibition of the effect of β -bungarotoxin. This suggests that a component at least of the action of β -bungarotoxin requires association with its high-affinity binding site (Othman *et al.*, 1982).

Fig. 3. Bivalent-cation specificity for the plasma-membrane depolarization

Synaptosomes were incubated in the presence of 1.3 mM- Ca^{2+} (closed symbols) or 1.3 mM- Sr^{2+} plus 0.3 mM EGTA (open symbols). Where indicated by the first arrow, 2.3 nM- β -bungarotoxin (BBTx) was added. The second arrow shows the time of addition of 2 mM-EGTA to chelate Ca^{2+} . The pH of the EGTA was adjusted with NaOH to balance the H^+ evolution accompanying the chelation.

Fig. 4. Protection by dendrotoxin (DTx) against β -bungarotoxin (BBTx)-induced plasma-membrane depolarization

Synaptosomes were incubated in the presence of 1.3 mM- Ca^{2+} and 160 μM -albumin. At 32 min, 14 nM-dendrotoxin and 2.3 nM- β -bungarotoxin were added separately or in combination. $\circ$, Control; $\blacktriangle$, dendrotoxin; $\square$, β -bungarotoxin; $\triangle$, dendrotoxin plus β -bungarotoxin.

A general permeabilization of the plasma membrane would result in a loss of $^{86}\text{Rb}^+$ and also a loss of cytosolic markers. Some enhancement of lactate dehydrogenase release has been reported previously (Halliwell *et al.*,

Fig. 5. β -Bungarotoxin (BBTx)-induced release of lactate dehydrogenase (LDH): influence of albumin

Synaptosomes were incubated in the presence of 1.3 mM- Ca^{2+} and 160 μM -albumin (open symbols) or 16 μM -albumin (closed symbols). The release of (a) $^{86}\text{Rb}^+$ and (b) lactate dehydrogenase were monitored after addition of 2.3 nM- β -bungarotoxin.

Fig. 6. Concentration-dependency of β -bungarotoxin (BBTx)-induced $^{86}\text{Rb}^+$ (a) and lactate dehydrogenase (LDH) (b) release.

Synaptosomes were incubated in the presence of 1.3 mM- Ca^{2+} and 160 μM -albumin. The release of $^{86}\text{Rb}^+$ and lactate dehydrogenase were monitored 31 min after addition of toxin.

1982). Fig. 5 shows that β -bungarotoxin causes a time-dependent lactate dehydrogenase release that shows some correlation with that of $^{86}\text{Rb}^+$, since both are potentiated in high albumin. This at first sight appears to indicate a non-specific action of the toxin. However, when plotted as a function of toxin concentration (Fig. 6), the release of both $^{86}\text{Rb}^+$ and lactate dehydrogenase increases up to 2 nM, but is thereafter constant, even when the toxin concentration is increased a further 10-fold. β -Bungarotoxin binds to rat synaptosomes with a K_d of 0.6 nM (Othman *et al.*, 1982); the concentration-dependency for release of $^{86}\text{Rb}^+$ and lactate dehydrogenase is therefore consistent with a dependency for toxin binding, although a more extensive analysis would be required to prove a causal relationship.

In order to establish whether the release of $^{86}\text{Rb}^+$ and lactate dehydrogenase can both be accounted for by a simple permeability change sufficient to allow the slow

Fig. 7. Release of (a) $^{86}\text{Rb}^+$ and lactate dehydrogenase (LDH, b) as a function of digitonin concentration

Synaptosomes were incubated in the presence of digitonin concentrations from 10 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$. Release of $^{86}\text{Rb}^+$ and lactate dehydrogenase was monitored 15 min after addition of the detergent.

egress of cytosolic markers, the toxin action was compared with that of digitonin, which selectively permeabilizes the cholesterol-rich synaptosomal plasma membrane. Fig. 7 shows that increasing digitonin releases both $^{86}\text{Rb}^+$ and lactate dehydrogenase in a ratio comparable with that caused by β -bungarotoxin, although, as would be expected, without reaching a maximum at a given digitonin concentration.

DISCUSSION

Two actions of β -bungarotoxin

In this and a previous paper (Nicholls *et al.*, 1985), two actions of β -bungarotoxin are distinguished. The first is an uncoupling of the mitochondria *in situ* due to fatty acids generated by the toxin's phospholipase A_2 activity at the plasma membrane. This mechanism was originally proposed by Wernicke *et al.* (1975) on the basis of extrapolation from isolated mitochondria. The second action of the toxin results in a general increase in the permeability of the plasma membrane towards ions and cytosolic proteins. This is enhanced by albumin and so cannot be due to the accumulation of non-esterified fatty acid. The effects are consistent with the suggestion of Kelly *et al.* (1979) that the toxin acts by binding to a specific receptor and hydrolysing the phospholipids in its vicinity.

Both effects are potentiated by the binding of β -bungarotoxin to the high-affinity binding site on the plasma membrane. The mitochondrial uncoupling seen in low-albumin media is diminished by dendrotoxin (Nicholls *et al.*, 1985), which competes for β -bungarotoxin binding to the high-affinity site (Othman *et al.*, 1982). The plasma-membrane effect can not only be partially protected against by dendrotoxin (Fig. 4), but also shows a preliminary concentration-dependency (Fig. 6) that is consistent with the affinity of β -bungarotoxin binding to rat cerebrocortical synaptosomes (Othman *et al.*, 1982).

The nature of the plasma-membrane modification

A depolarization of the synaptosomal plasma membrane by β -bungarotoxin has been reported by Ng & Howard (1978) and Sen & Cooper (1978), who used the qualitative criterion of cyanine-dye fluorescence, and a

modest release of plasma-membrane proteins such as lactate dehydrogenase has been reported by a number of workers (Wernicke *et al.*, 1974; Tse *et al.*, 1980; Halliwell *et al.*, 1982; Rehm *et al.*, 1982). However, most studies reported have employed albumin-free media in which the plasma-membrane effect appears to take second place to the mitochondrial uncoupling (Nicholls *et al.*, 1985), and there has been no detailed analysis of the relation between marker-enzyme release, plasma-membrane depolarization, high-affinity binding and toxicity. The present study strongly indicates that these may be intimately related.

The role of albumin

Although albumin would be expected to decrease mitochondrial uncoupling by liberated fatty acids, it is less obvious why it should potentiate plasma-membrane depolarization, or rather permeabilization. One possibility is that the plasma-membrane stability after phospholipase A₂ action might depend on the ability of the lysophospholipid and the fatty acid to remain paired within the bilayer. Selective extraction by albumin of the fatty acids could destabilize the membrane by leaving the lysophosphatide alone in the membrane. The literature is unclear in this respect: in some studies albumin protects membranes against the lytic effects of phospholipase A₂ activity (Nilsson & Dallner, 1977), whereas in others (Gul & Smith, 1974) albumin addition to phospholipase A₂-treated cells induces an immediate structural collapse.

Although very high concentrations of phospholipase A₂ (about 70 nM) have been reported to increase synaptosomal cyanine-dye fluorescence, indicating plasma-membrane depolarization (Sen & Cooper, 1978), nanomolar concentrations of bee-venom phospholipase A₂ that had distinct uncoupling effects on intrasynaptosomal mitochondria had little effect on $\Delta\psi_p$ in our previous study (Nicholls *et al.*, 1985). The conclusion would be that a tightly localized phospholipase A₂ activity would be far more deleterious to the integrity of the plasma membrane than a random attack from a non-binding phospholipase A₂ such as that from bee venom.

Kinetics of β -bungarotoxin action and role of phospholipase A₂ activity

The binding of 5 nM- β -bungarotoxin to synaptosomes is half-maximal in 2.5 min (Othman *et al.* 1982). Since depolarization is linear from the first time point (Figs. 1 and those following), equivalent to a zero-order efflux of $^{86}\text{Rb}^+$, this implies that any permeabilization is complete within about 5 min, since a continued action of the toxin would be expected to lead to an accelerating rate of $^{86}\text{Rb}^+$ and lactate dehydrogenase release. Consistent with this somewhat surprising result is the finding (Fig. 4) that an addition of EGTA 15 min after β -bungarotoxin to inhibit further phospholipase A₂ activity does not affect the depolarization.

Occupancy of the binding site is not sufficient to initiate depolarization, since dendrotoxin is inactive in this respect (Fig. 4); however, the present results do not resolve completely the role of phospholipase A₂ activity in the toxin action. Removal of Ca²⁺ with EGTA and substitution of Sr²⁺ diminishes the central and peripheral toxicity of β -bungarotoxin (Spokes & Dolly, 1980; Halliwell *et al.*, 1982), and in the present study causes a considerable diminution of the rate of plasma-membrane

depolarization (Fig. 3). However, since a low phospholipase A₂ activity is retained in Sr²⁺/EGTA buffers (Strong *et al.*, 1977; Abe *et al.*, 1977; Chang *et al.*, 1977; Spokes & Dolly, 1980; Caratsch *et al.*, 1981), it is not possible to eliminate residual enzymic activity as the cause of the slow plasma-membrane depolarization.

Specificity of β -bungarotoxin action

Lactate dehydrogenase release saturates with respect to both β -bungarotoxin concentration (Fig. 6) and time (Fig. 5), suggesting that not all synaptosomes are responsive to the toxin. Whereas fatty acids released from one synaptosome should be capable of uncoupling any other synaptosome, a membrane-localized lysophosphatide production is unique to an individual synaptosome, and so it is quite feasible that only one subclass of terminal membrane is attacked. In the chick retina (Rehm *et al.*, 1982) cholinergic and γ -aminobutyrate-containing neurons appear selectively sensitive to the toxin. Further identification of toxin-sensitive synaptosomes in the present preparation will require analysis of neurotransmitter contents.

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