Bioenergetic actions of β -bungarotoxin, dendrotoxin and bee-venom phospholipase A₂ on guinea-pig synaptosomes

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1. Low concentrations of β -bungarotoxin or bee-venom phospholipase A₂ cause a progressive Ca²⁺-dependent increase in the proton permeability of the mitochondria within the synaptosomal cytosol, manifested as an increase in oligomycin-insensitive respiration and a partial depolarization of the mitochondrial membrane potential.
2. This uncoupling appears to be a consequence of fatty acids liberated by phospholipase A₂ activity at the plasma membrane, since it can be mimicked by the addition of oleate-albumin complexes, in which case there is no requirement for external Ca²⁺.
3. Dendrotoxin does not affect the mitochondrial proton permeability *in situ*, but protects partially against the uncoupling action of β -bungarotoxin. In contrast, this effect of bee-venom phospholipase A₂ is unaffected by dendrotoxin.
4. β -Bungarotoxin, but not bee-venom phospholipase A₂, induces a slow progressive depolarization of the plasma membrane.
5. The action of β -bungarotoxin at the plasma membrane appears not to be related to fatty acid production, since it is augmented rather than inhibited by raising albumin concentrations in the medium.
6. It is concluded that β -bungarotoxin has at least two actions on intact synaptosomes, both of which may involve interaction at the plasma membrane with a site common to dendrotoxin: first, a mitochondrial uncoupling mediated by fatty acids and, secondly, a depolarization at the plasma membrane.

β -Bungarotoxin acts presynaptically to block neurotransmission at the neuromuscular junction (Oberg & Kelly, 1976; Abe *et al.*, 1977) and at mammalian central synapses (Halliwell & Dolly, 1982). However, its action at the molecular level is elusive. The toxin, which has been purified to homogeneity from the venom of the elapid snake *Bungarus multicinctus* (Spokes & Dolly, 1980), is one of a group which possesses phospholipase A₂ (phosphatide 2-acylhydrolase, EC 3.1.1.4) activity (reviewed in Howard & Gundersen, 1980). The enzymic activity of β -bungarotoxin appears to reside in its 13 kDa A-chain, which has homology with other phospholipase A₂ enzymes (Kondo *et al.*, 1978). Recent investigations of toxin binding to synaptosomes from rat (Othman *et al.*, 1982) and chick (Rehm & Betz, 1982) have shown that β -bungarotoxin binds to a single, saturable, class of

binding sites with K_d values in the range 0.4–0.6 nM. In rat brain these acceptor sites have been shown autoradiographically to reside predominantly in synapse-rich regions of the hippocampus and cerebellum, an observation consistent with the preferential action of the toxin at terminal regions (Halliwell & Dolly, 1982).

Bee-venom phospholipase A₂ has considerably higher enzymic activity than β -bungarotoxin, but does not compete effectively with the latter for high-affinity binding (Othman *et al.*, 1982). It may therefore be employed to elucidate phospholipase-dependent effects of β -bungarotoxin that do not require association with the specific binding site.

Toxin I from the black mamba (*Dendroaspis polylepis polylepis*) and the closely related dendrotoxin from the Eastern green mamba (*Dendroaspis angusticeps*) have a pronounced sequence homology with the 7 kDa B-chain of β -bungarotoxin (Strydom, 1973; Kondo *et al.*, 1978; Harvey & Karlsson, 1982), but in contrast with β -bungarotoxin have no phospholipase activity and facilitate, rather than inhibit, release of transmitter at the

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; SDS, sodium dodecyl sulphate.

neuromuscular junction (Harvey & Karlsson, 1980) and central synapses (Halliwell & Dolly, 1982; Dolly *et al.*, 1984). Dendrotoxin competes with β -bungarotoxin for high-affinity binding to synaptosomes (Othman *et al.*, 1982) and dendrotoxin can antagonize the irreversible inhibition of transmitter release at motor nerve terminals produced by β -bungarotoxin (Harvey & Karlsson, 1982). These single-chain facilitatory toxins could therefore be employed to investigate both the effect of occupancy of the high-affinity binding site and the ability of β -bungarotoxin to modify the bioenergetic functions of the synaptosome when prior addition of dendrotoxin prevents the latter toxin from associating with its binding site.

At the biochemical level β -bungarotoxin uncouples isolated brain mitochondria, apparently as a consequence of the fatty acids produced by its phospholipase A_2 activity (Howard, 1975). The toxin has also been reported to collapse the synaptosomal plasma-membrane potential (judged by the qualitative criterion of Cyanine dye fluorescence (Ng & Howard, 1978; Sen & Cooper, 1978); to inhibit the $(Na^+ + K^+)$ -dependent ATPase (Lin-Shiau & Chen, 1982); to inhibit the uptake and enhance the release of transmitters and metabolites across the synaptosomal plasma membrane (Wernicke *et al.*, 1975; Sen *et al.*, 1976; Dowdall *et al.*, 1979; Tse *et al.*, 1980; Halliwell *et al.*, 1982); and to lower synaptosomal ATP levels, owing to an enhanced energy demand at the plasma membrane (Ng & Howard, 1978). Sen & Cooper (1978) have emphasized the similarity between the actions of high concentrations of β -bungarotoxin and bee-venom phospholipase A_2 , although Ng & Howard (1978) give indications that binding to the pre-synaptic membrane is involved in the potentiation of the action of β -bungarotoxin but not of bee-venom phospholipase A_2 .

In order to understand the effects of β -bungarotoxin *in vitro* it is necessary to employ more discriminating techniques. We have described (Scott & Nicholls, 1980) an isotopic method for quantifying synaptosomal plasma-membrane potential ($\Delta\psi_p$) and mitochondrial membrane potentials ($\Delta\psi_m$) *in situ*, and have previously applied this to an analysis of the presynaptic actions of ionophore A23187 (Åkerman & Nicholls, 1981), α -latrotoxin (Nicholls *et al.*, 1982) and calmodulin antagonists (Snelling & Nicholls, 1984). In the present paper we analyse the time course, concentration-dependency, Ca^{2+} -dependency and mutual competition in the bioenergetic actions of β -bungarotoxin, bee-venom phospholipase A_2 and dendrotoxin on guinea-pig synaptosomes. We conclude that: (a) there is a prominent uncoupling of the mitochondria *in situ* mediated by fatty acids released by the phospholipase action of

β -bungarotoxin at the plasma membrane and that this effect can be antagonized by dendrotoxin; (b) β -bungarotoxin has a distinct effect at the plasma membrane which is not mediated by fatty acids, whereas bee-venom phospholipase A_2 has no significant effect at the plasma membrane at concentrations that partially uncouple the mitochondria *in situ*.

Materials and methods

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 at 0°C as concentrated pellets for not more than 3 h before use. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Isotopic and tetraphenylphosphonium-electrode incubations were performed 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, 20 mM-Tes (sodium salt), 10 mM-D-glucose, together with 16 μ M-albumin to minimize non-specific binding of toxin. 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 of the method described previously (Scott & Nicholls, 1980). The incubation medium contained additionally 50 μ M-⁸⁶Rb⁺ (0.3 μ Ci/ml), 1 μ M-[³H]triphenylmethylphosphonium ion (0.13 μ Ci/ml) and 5 μ M-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 mixture. 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 μ l aliquots were taken and spun through a 1:1 (v/v) mixture of dinonyl phthalate and Dow-Corning 550 silicone oil. Pellets were solubilized in 50 μ l of 5% (w/v) SDS for 2 h at 50°C. A 1 ml portion of scintillant (Scintran Cocktail T; BDH) was added directly to the Eppendorf tube, which was inserted into a 5 ml Minivial and counted for radioactivity in a LKB RackBeta liquid-scintillation counter. In this way losses due to the transfer of the solubilized pellet are avoided. Potentials were determined as described previously, $\Delta\psi_p$ being equated with the ⁸⁶Rb⁺ diffusion potential, and $\Delta\psi_m$ being calculated from the accumulation of triphenylmethylphosphonium ion into the mitochondria *in situ* after correction for accumulation across the plasma membrane (Scott & Nicholls, 1980). A mean value of 2.81 μ l/mg of synaptosomal protein for the sucrose-permeable space in each pellet was determined from parallel experiments and used to

correct the pellet counts for contaminating medium.

Electrode determinations of potential

A tetraphenylphosphonium-ion-selective electrode (Kamo *et al.*, 1979) was constructed as described previously (Nicholls *et al.*, 1982). The electrode showed a Nernstian response over the range 1–5 μM -tetraphenylphosphonium ion. Synaptosomal pellets were resuspended in 3 ml of incubation medium at 1.5 mg of protein/ml and 1.3 mM- Ca^{2+} was added at 5 min. The electrode was inserted into the incubation mixture and 5 μM -tetraphenylphosphonium ion added. The uptake of the cation was recorded continuously and the accumulation, expressed as the ratio of the concentration of the cation in the synaptosome to the residual concentration in the medium, ignoring compartmentation, was calculated.

Oxygen-electrode experiments were performed in Hansa-Tech oxygen-electrode chambers (Hansa-Tech, King's Lynn, Norfolk, U.K.) under conditions identical with those used for the tetraphenylphosphonium-electrode determinations.

Toxins

β -Bungarotoxin was purified from the crude venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL, U.S.A.) as described previously (Spokes & Dolly, 1980). The purification involved fractionation on a CM-Sephadex C-50 column followed by preparative isoelectric focusing. Honeybee (*Apis mellifera*) -venom phospholipase A_2 was prepared as described elsewhere (Othman *et al.*, 1982). 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, Germany. Other isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; tetraphenylphosphonium ion was from Fluorochem, Glossop, Derbyshire, U.K. Other reagents were from Sigma Chemical Co., Poole, Dorset, U.K.

Results

Synaptosomal respiration

The mitochondria within synaptosomes show respiratory control, and respiration may be increased by the addition of protonophores or by the initiation of a rapid ATP utilization with ver-

atridine to allow a dissipative cycling of Na^+ across the plasma membrane (Scott & Nicholls, 1980). Since an oxygen electrode enables the rate-limiting step in any reaction sequence terminating in oxygen uptake to be quantified, experiments may be devised to identify sites of action of toxins which influence energy flow in the synaptosome.

The basic experimental design is shown in Fig. 1. To achieve precisely matched incubation conditions, synaptosomal incubations are divided into two identical oxygen-electrode chambers, to one of which the toxin is added while the other acts as control. In order that conditions should parallel the subsequent potential determinations, Ca^{2+} was not added until 5 min after the start of the incubation, allowing time for the synaptosomes to polarize (Åkerman & Nicholls, 1981). The respiratory inhibition seen when the cation is added is due to inhibition of a partially activated voltage-dependent Na^+ channel (T. S. Sihra & D. Nicholls, unpublished work).

When steady respiratory rates are attained, toxin is added (Figs. 1*b* and 1*d*). In the presence

Fig. 1. β -Bungarotoxin uncoupling of intrasynaptosomal mitochondria: Ca^{2+} -dependency

Synaptosomes (2.2 mg of protein/ml of incubation mixture) were suspended in Ca^{2+} -free incubation medium containing 16 μM -albumin (see the Materials and methods section) and divided equally between two identical oxygen-electrode chambers to provide paired control and experimental traces. After 5 min incubation, 0.5 mM-EGTA (traces *a* and *b*) or 1.3 mM- Ca^{2+} (traces *c* and *d*) was added; 2.2 nM- β -bungarotoxin (BBTx) was added in each experiment. After allowing time for the toxin to act, 100 μM -veratridine (Vt), 10 μM -ouabain (Oua) and 0.25 μg of oligomycin (Oligo) per ml of incubation mixture were added. The rates in parentheses give the final rate of oligomycin-insensitive respiration ($\text{nmol of O}_2 \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$) in each case.

(Fig. 1*d*), but not the absence (Fig. 1*b*), of Ca^{2+} , 2.3 nM- β -bungarotoxin causes a slowly increasing respiration. This could be due to enhanced Na^+ cycling at the plasma membrane, suggested by Abdul-Ghani *et al.* (1981), to an increased ATP demand by undefined plasma-membrane transport processes, as suggested by Ng & Howard (1978), or to an increased mitochondrial proton permeability.

After allowing 5–10 min for the toxin to act, veratridine was added. The respiratory stimulation is due to the enhanced ATP demand to drive the cycling of Na^+ between the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase and the voltage-dependent Na^+ channel. Since β -bungarotoxin does not prevent veratridine from stimulating respiration, this indicates that the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase, the Na^+ channel, mitochondrial substrate supply, the respiratory chain, the ATP synthase and the mitochondrial adenine-nucleotide translocator are all still active in the presence of the toxins.

The additions of ouabain and oligomycin subsequent to veratridine enable the reason for the toxin-induced respiratory increase to be resolved. The rate after ouabain addition represents the basal respiration of the mitochondria, plus that required to maintain synaptosomal ATP requirements other than those ascribable to the Na^+ pump. The final rate after oligomycin addition gives the basal mitochondrial respiration alone, which is in turn determined by the proton permeability of the mitochondrial inner membrane (see Nicholls, 1982).

The accelerated synaptosomal respiration induced by β -bungarotoxin persists after oligomycin addition (Fig. 1*d*); this demonstrates that nanomolar concentrations of the toxin increase the proton permeability of the mitochondrial inner membrane, i.e. cause a partial uncoupling of the mitochondrion. This uncoupling effect requires Ca^{2+} (Figs. 1*a*–1*d*). In the absence of Ca^{2+} the final oligomycin-insensitive respiration is not significantly elevated by incubation with β -bungarotoxin. It is interesting that the ouabain-resistant rate in Figs. 1*a*) and 1*b*) is much lower than the initial respiration before veratridine addition, consistent with the existence of a substantial ATP turnover to maintain Na^+ cycling across the plasma membrane in the Ca^{2+} -depleted synaptosome (T. S. Sihra & D. Nicholls, unpublished work). Fig. 2 shows that bee-venom phospholipase A_2 induces a Ca^{2+} -dependent increase in ouabain-insensitive respiration in the presence of 16 μM -albumin that is very similar to that induced by β -bungarotoxin. The experimental design is identical with that of Fig. 1 and the respiration after addition of both veratridine and ouabain is plotted. As shown below (Fig. 4*e*), this increase

Fig. 2. Respiration of synaptosomes after treatment with bee-venom phospholipase A_2 : influence of veratridine and ouabain

Synaptosomes were incubated in the presence of 16 μM -albumin and 1.3 mM- Ca^{2+} (closed symbols) or 0.5 mM-EGTA (open symbols), exactly as described in the legend to Fig. 1, except that bee-venom phospholipase A_2 (PLA_2) was added in place of β -bungarotoxin. After 8 min exposure to the enzyme, 100 μM -veratridine (Vt) was added and respiration determined ($\bullet$, $\circ$). 100 μM -Ouabain was then added, and the respiratory rate again quantified ($\blacksquare$, $\square$). Results are from a single representative experiment.

persists after oligomycin, and is therefore indicative of an uncoupling of the mitochondria *in situ* very similar to that induced by β -bungarotoxin.

The effect of the toxin and phospholipase on mitochondrial coupling *in situ* can be mimicked by the direct addition of oleate added at a molar ratio of 3:1 with respect to albumin, equivalent to 1.3 μM unbound fatty acid (Spector *et al.*, 1971) (Fig. 3). Unlike β -bungarotoxin or bee-venom phospholipase A_2 , there is no time lag before respiration is stimulated, and no dependency on external Ca^{2+} .

Dendrotoxin, even at 14 nM, causes no uncoupling in the presence or absence of Ca^{2+} (Figs. 4*a* and 4*b*). A slight cycle of stimulated respiration can, however, frequently be seen (e.g. Fig. 4*f*), suggesting that the toxin may be causing some transient alteration in synaptosomal energy demand.

Dendrotoxin partially protects the synaptosome against the uncoupling effect of β -bungarotoxin (Figs. 4*c* and 4*d*). Thus, by the termination of the incubation, β -bungarotoxin respiration is increased by 3.8 nmol of $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ in the absence of dendrotoxin (cf. traces *a* and *c*), whereas in the presence of dendrotoxin the increment is only 2.0 nmol of $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ (cf. traces *b* and *d*). Although this protection is incomplete, it is seen consistently. In

Fig. 3. Oleate induces a Ca^{2+} -independent increase in oligomycin-insensitive respiration

Synaptosomes were incubated exactly as described in the legend to Fig. 1, except that 50 μM -oleate was added where indicated. Incubations contained 16 μM -albumin. Vt, 100 μM -veratridine; Oua, 10 μM -ouabain; Oligo, 0.25 μg of oligomycin/ml.

contrast, protection is not afforded against bee-venom phospholipase A_2 -induced uncoupling, the increments in oligomycin-insensitive respiration being 5.3 and 5.6 $\text{nmol of O} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ respectively in the absence (cf. traces *a* and *e*) and presence (cf. traces *b* and *f*) of dendrotoxin.

Synaptosomal membrane potentials

A partial uncoupling of the intrasynaptosomal mitochondria should manifest itself as a decrease in $\Delta\psi_m$. The tetraphenylphosphonium-selective electrode is sensitive to changes in either synaptosomal $\Delta\psi_m$ or $\Delta\psi_p$ without being able to discriminate directly between the two (Nicholls *et al.*, 1982). Fig. 5 shows the immediate effects of low concentrations of β -bungarotoxin, bee-venom phospholipase A_2 and dendrotoxin on synaptosomal tetraphenylphosphonium-ion accumulation. Values in parentheses give the approximate rate (mV/min) at which either membrane would have to depolarize to give the observed efflux rate. In other experiments (not shown) β -bungarotoxin at a concentration as low as 30 pM induces a significant efflux of the cation, which is a concentration

Fig. 4. Dendrotoxin does not increase oligomycin-insensitive respiration, partially inhibits the uncoupling effect of β -bungarotoxin, but does not protect against bee-venom phospholipase A_2 -induced uncoupling

Synaptosomes were incubated exactly as described in the legend to Fig. 1, except that 14 nM-dendrotoxin (DTx), 2.2 nM- β -bungarotoxin (BBTx) and 2.8 nM-bee-venom phospholipase A_2 (PLA_2) were added where indicated. The broken lines are the extrapolations of the trace before the addition of dendrotoxin to emphasize the slight cycle of respiratory stimulation after the addition of this toxin.

Fig. 5. Depolarization of synaptosomes induced by β -bungarotoxin, dendrotoxin and bee-venom phospholipase A_2 . Synaptosomes were preincubated for 15 min in the tetraphenylphosphonium-electrode chamber in the presence of either 1.3 mM- Ca^{2+} or 1 mM-EGTA to allow tetraphenylphosphonium ion to come to equilibrium across the mitochondrial and plasma membranes. There was no significant difference in the initial tetraphenylphosphonium-ion accumulation ratios in the presence or absence of Ca^{2+} . At the arrows, additions were made of 16 μ M-ouabain (Oua), 40 mM-KCl, 0.48 nM- β -bungarotoxin (BBTx), 1.7 nM-bee-venom phospholipase A_2 (PLA $_2$) or 2.9 nM-dendrotoxin (DTx). Values in parentheses are the rates (mV of membrane potential/min) at which either the plasma or mitochondrial membrane would have to depolarize to produce the observed rate of tetraphenylphosphonium-ion efflux. $TPP_{i/o}$ is the ratio of concentrations of the ion within the synaptosome (with no correction for compartmentation) to that in the incubation mixture.

substantially lower than has previously been reported to affect synaptosomal bioenergetic parameters. Dendrotoxin also causes a slow efflux of tetraphenylphosphonium ion which, however, differs from that induced by β -bungarotoxin and bee-venom phospholipase A_2 in being independent of Ca^{2+} and may be associated with the small respiratory cycle seen in the oxygen electrode (Fig. 4, traces *b*, *d* and *f*). Consistent with the partial antagonism of the uncoupling action of β -bungarotoxin by dendrotoxin (Figs. 4c and *d*) is its ability to decrease the rate at which the former induces tetraphenylphosphonium-ion efflux from the synaptosomes (Fig. 6).

Isotopic determination of $\Delta\psi_m$ and $\Delta\psi_p$ confirms that the initial effect of both β -bungarotoxin and bee-venom phospholipase A_2 is a depolarization of the mitochondrial membrane over a period of 10 min (Fig. 7), as would be predicted from the partial mitochondrial uncoupling revealed by the oxygen electrode. With β -bungarotoxin, but not with bee-venom phospholipase A_2 , there is an additional, slowly developing, plasma-membrane depolarization.

Fig. 6. Dendrotoxin protection against β -bungarotoxin-induced depolarization

Synaptosomes were incubated in the tetraphenylphosphonium-electrode chamber in the presence of Ca^{2+} exactly as described in Fig. 5. Further additions of β -bungarotoxin (BBTx) and dendrotoxin (DTx) were made as indicated. Values in parentheses represent the rate of depolarization (mV/min) as in the legend to Fig. 5. $TPP_{i/o}$ is the accumulation ratio as defined in the legend to Fig. 5.

Whereas the effects of β -bungarotoxin and bee-venom phospholipase A_2 on the mitochondria and $\Delta\psi_m$ can be explained satisfactorily in terms of the generation of free fatty acids, the slow depolariza-

Fig. 7. Time course of β -bungarotoxin and bee-venom phospholipase A_2 effects on $\Delta\psi_m$ and $\Delta\psi_p$ monitored by $^{86}\text{Rb}^+$ and $[^3\text{H}]\text{triphenylmethylphosphonium-ion}$ accumulation

Synaptosomes (1.5 mg of protein/ml of incubation mixture) were preincubated for 25 min. Albumin (16 μM) was present initially and Ca^{2+} was added at 5 min. β -Bungarotoxin (BBTx, *a*) or bee-venom phospholipase A_2 (PLA $_2$, *b*) were added at 25 min. ●, Controls (also shown in *b* as broken lines; ○, 0.4 nM- β -bungarotoxin; □, 2.3 nM- β -bungarotoxin; ■, 2.8 nM-bee-venom phospholipase A_2).

tion of the plasma membrane by β -bungarotoxin suggests that it is having a distinct action that, although Ca^{2+} -dependent, is not related to the phospholipase activity. In Fig. 8 an involvement of free fatty acids in the plasma-membrane depolarization induced by β -bungarotoxin is eliminated by performing incubations in the presence of a ten-times-elevated albumin concentration in order to bind liberated free fatty acids more effectively. The depolarization induced by β -bungarotoxin is increased, and the slight effect of 50 μM -oleate is decreased by high-albumin medium. This is inconsistent with the β -bungarotoxin-induced depolarization at the plasma membrane being a direct action of liberated fatty acids, or an indirect effect (e.g. lowered ATP levels) contingent on mitochondrial uncoupling. The nature of the plasma-membrane depolarization requires further investigation.

Discussion

Such confusion as exists in the literature concerning the molecular actions of β -bungarotoxin and related neurotoxins stems from the difficulties in establishing the relationship between electrophysiological studies and biochemical investigations at the level of the isolated

nerve terminal, from the tendency in both types of study to use high concentrations of toxin, and thirdly from an incomplete ability to monitor the synaptosomal functions which are modified by the toxins.

Although the chick biventer is very sensitive to β -bungarotoxin (Chang & Su, 1982), the mammalian neuromuscular junction requires relatively high concentrations of toxin in order to observe triphasic effects on transmission. These high concentrations have frequently been applied to studies with synaptosomes, despite the report of a high-affinity binding site for β -bungarotoxin with a K_d of less than 1 nM (Othman *et al.*, 1982), and a 50% lethality of β -bungarotoxin injected intraventricularly at a dose of 50 ng/kg body weight (Othman *et al.*, 1982). β -Bungarotoxin at 100–1000 nM has been reported to cause a complete collapse of the plasma-membrane potential (Sen & Cooper, 1978), inhibition of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase (Lin-Shiau & Chen, 1982), inhibition of uptake of transmitters and metabolites (Dowdall *et al.*, 1979; Halliwell *et al.*, 1982) and stimulation of their release (Wernicke *et al.*, 1975; Sen *et al.*, 1976; Dowdall *et al.*, 1979; Tse *et al.*, 1980; Halliwell *et al.*, 1982). Various non-neurotoxic phospholipase A_2 enzymes cause $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase inhibition in the

Fig. 8. Elevated albumin inhibits the action of oleate on $\Delta\psi_p$, but potentiates the action of β -bungarotoxin. Synaptosomes were incubated as described in the legend to Fig. 7. The incubation mixture contained $16\ \mu\text{M}$ -albumin (a) or $160\ \mu\text{M}$ -albumin (b). Further additions were made at 31 min of: $\circ$, $50\ \mu\text{M}$ -oleate; $\blacktriangle$, $2.3\ \text{nM}$ - β -bungarotoxin; $\bullet$ is the control (no addition).

concentration range $30\ \text{nM}$ – $1000\ \text{nM}$ (Lin-Shiau & Chen, 1982) and transient respiratory stimulation followed by inhibition (Noremborg & Lazarewicz, 1982). Although $100\ \text{nM}$ - β -bungarotoxin has been reported to cause no detectable ultra-structural changes to synaptosomes (Sen *et al.*, 1976), $300\ \text{nM}$ -bee-venom phospholipase A_2 caused a massive solubilization of synaptosomal protein (Napias & Heilbronn, 1980).

The problem with the above studies lies in deciding which parameters account for the extreme neurotoxicity of β -bungarotoxin when injected intraventricularly (Othman *et al.*, 1982) and which are non-specific responses to phospholipase A_2 action. The most detailed study of the effects of β -bungarotoxin on synaptosomal bioenergetics, and one of the few that avoids the use of very high toxin concentrations, is that of Ng & Howard (1978). Those authors found that β -bungarotoxin or phospholipase A_2 at concentrations as low as $1\ \text{nM}$ and $4\ \text{nM}$ respectively caused a slow increase in Cyanine-dye fluorescence, which they interpreted as a plasma-membrane depolarization, the assumption being that the dye did not respond to $\Delta\psi_m$. Although our work confirms that both β -bungarotoxin and bee-venom phospho-

lipase A_2 depolarize, the action of bee-venom phospholipase A_2 appears to be localized at the mitochondrial membrane under our conditions. Although β -bungarotoxin and phospholipase A_2 enzymes are both capable of uncoupling isolated brain mitochondria (Howard, 1975), it is likely that both β -bungarotoxin and bee-venom phospholipase A_2 act at the plasma membrane in intact synaptosomes, and that the fatty acids liberated by phospholipase A_2 action at the plasma membrane diffuse across the cytosol and interact with the mitochondrial inner membrane. The ability of added oleate to uncouple the mitochondria *in situ* (Fig. 3) demonstrates that there is no permeability barrier preventing this. The selectivity of β -bungarotoxin for the plasma membrane in media containing high albumin concentrations to bind liberated fatty acids (Fig. 8) is also consistent with a plasma-membrane location for the toxin. Considering the short time courses and low concentrations employed in the present study, it is unlikely that there is significant internalization of the toxin. Thus the first-order kinetics of both toxin binding and dissociation and the trypsin-sensitivity of binding (Othman *et al.*, 1982) both argue for a plasma-membrane localization for the specific toxin-binding site in intact synaptosomes. Our finding that dendrotoxin partially protects against the effect of β -bungarotoxin (but not phospholipase A_2) on the mitochondrial potential is consistent with the view (Oberberg & Kelly, 1976; Abe *et al.*, 1977; Howard & Truog, 1977) that binding of the toxin to a specific presynaptic site potentiates the effect of its phospholipase action at the plasma membrane. It is also in accord with the observation of Harvey & Karlsson (1982) who found that preincubation with $400\ \text{nM}$ -Toxin I inhibits the rate at which $50\ \text{nM}$ - β -bungarotoxin induces blockade of transmission at the neuromuscular junction, and the finding of Ng & Howard (1978) that chemical modification by ethoxyformic anhydride in the presence of L - α -dihexanoyl phosphatidylcholine decreases the ability of β -bungarotoxin (but not phospholipase A_2) to increase the Cyanine-dye fluorescence.

Ng & Howard (1978) suggest that β -bungarotoxin causes an enhanced ATP utilization at the plasma membrane. However, the present work shows that the primary bioenergetic event in medium containing $16\ \mu\text{M}$ -albumin is not an enhanced ATP turnover, but a partial mitochondrial uncoupling, of the type originally proposed by Wernicke *et al.* (1975). There are a number of early reports of the extreme sensitivity of isolated brain mitochondria to uncoupling by β -bungarotoxin (Wagner *et al.*, 1974; Howard, 1975; Kelly *et al.*, 1975). If the respiratory control of intrasynaptosomal mitochondria can be virtually

abolished by 8 min in the presence of 2 nM- β -bungarotoxin in the absence of albumin (Fig. 1f), the mitochondria of synaptosomes incubated for prolonged periods with high toxic concentrations must be highly de-energized. This uncoupling is evidently due to the liberation of fatty acids, and the ability of dendrotoxin to cause a partial protection is in accord with the necessity of the toxin to bind to a receptor on the synaptosomal plasma membrane in order to potentiate its action.

Although binding may account for some of the toxicity of β -bungarotoxin, it is not clear that the 2000-fold discrepancy between phospholipase A₂ activity and lethality of bee-venom phospholipase A₂ and β -bungarotoxin in the rat (Othman *et al.*, 1982) or the mouse (Abe *et al.*, 1977) can be entirely explained by the present findings, since the uncoupling induced by 2 nM- β -bungarotoxin (Fig. 1c) can be mimicked by only 5 nM-bee-venom phospholipase A₂ (Fig. 2). To some extent the neurotoxicity of β -bungarotoxin *in vivo* relative to that of bee-venom phospholipase A₂ will be underestimated by merely presenting them both to a purified synaptosomal preparation. *In vivo*, a foreign phospholipase that is not specifically targeted on to the nerve terminal will be greatly diluted by interacting with the great excess of non-neuronal membrane which is absent from the synaptosomal preparation. However, even after allowing for this, it is likely that the toxicity of β -bungarotoxin may more closely relate to the plasma-membrane depolarization seen in the high-albumin medium (Fig. 8), since what appears to differ between bee-venom phospholipase A₂ and β -bungarotoxin is the ability of the latter to depolarize the plasma membrane at nanomolar concentrations (Figs. 7 and 8). This depolarization is seen in the presence of elevated albumin, and is even enhanced (Fig. 8); thus it cannot be due to fatty acid accumulation. Preliminary investigations (M. Rugolo, J. O. Dolly & D. G. Nicholls, unpublished work) indicate that the plasma-membrane effect may be related to a plasma-membrane permeabilization contingent on binding of β -bungarotoxin to the high-affinity site.

In the present study we could detect little effect of dendrotoxin on the synaptosomal bioenergetics, except for its ability to induce a slow tetraphenylphosphonium efflux (Fig. 5) and to protect against the uncoupling action of β -bungarotoxin (Fig. 4). The dendrotoxin concentrations used here are lower than those reported to stimulate neuromuscular transmission (Harvey & Karlsson, 1980). It is noteworthy that the effect on tetraphenylphosphonium-ion accumulation persisted in the absence of Ca²⁺ (Fig. 5), since the synaptosomal binding of dendrotoxin (Dolly *et al.*, 1984) and the facilitatory effects of an impure dendrotoxin

preparation on the neuromuscular junction also showed an independency from external Ca²⁺ (Harvey & Gage, 1981).

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