Inhibition of β-bungarotoxin action by bee venom phospholipase A₂

BY T. ABE† AND R. MILEDI, F.R.S.

Department of Biophysics, University College, Gower Street, London WC1E 6BT

(Received 10 November 1977)

β-Bungarotoxin (β-BuTX) induces twitching of innervated frog muscle and subsequently blocks transmitter release from motor nerve endings. These actions of β-BuTX are prevented if the nerve–muscle junctions are pretreated with a low concentration of phospholipase A₂ from bee venom.

When the Ca²⁺ in the external fluid is replaced by Sr²⁺, the phospholipase activity of β-BuTX is negligible and, in these conditions, β-BuTX causes a decrease in the amplitude of endplate potentials but does not go on to block transmitter release completely.

INTRODUCTION

β-Bungarotoxin (β-BuTX), a neurotoxin from the venom of Bungarus multicinctus (Chang, Chen & Lee 1973), blocks acetylcholine release from motor nerve terminals, and it has been suggested that the blocking action depends on the phospholipase activity which the toxin normally possesses (Wernicke, Vanker & Howard 1975; Strong, Goerke, Oberg & Kelly 1976; Abe, Limbrick & Miledi 1976; Abe, Alemá & Miledi 1977a, b). We have shown that at the frog neuromuscular junction the amplitude of the endplate potential (e.p.p.), after application of β-BuTX, follows a triphasic course: immediate decrease, then a transient increase and finally a progressive decrease leading to complete blockage of transmitter release (Abe et al. 1976). The last two stages depended on the phospholipase activity of the toxin (Abe et al. 1977a, b). To explore further the mode of action of β-BuTX we have used phospholipase A₂ from bee venom (cf. Shipolini et al. 1971). It was found that this phospholipase strikingly prevents β-BuTX from blocking transmitter release.

METHODS

β-BuTX was purified from the venom of Bungarus multicinctus (Miami Serpentarium Laboratories) as described previously (Abe et al. 1977b). The experiments were carried out on the sartorius nerve–muscle preparation of the frog (Rana temporaria) using conventional techniques (Katz & Miledi 1965). Spontaneous twitching of the muscle induced by the toxin was measured as mentioned previously (Abe et al. 1976). The composition of the normal Ringer

† Present address: Department of Neurochemistry, Brain Research Institute, Niigata, Japan.
solution was, in millimoles per litre: NaCl, 116; KCl, 2.0; CaCl₂, 1.8; sodium phosphate buffer, 1.0 (pH 7.2). For some experiments CaCl₂ was replaced with 6 mM SrCl₂ and the preparation was repeatedly washed in this solution before adding the toxin. Phospholipase A₂, a gift from Dr B. E. C. Banks, was purified from bee (Apis mellifica) venom (Shipolini et al. 1971).

**RESULTS**

**Protection by bee venom phospholipase A₂**

It has been shown previously (Abe et al. 1976) that β-BuTX generates, at some motor nerve endings, impulses which are distributed to the entire motor unit and cause obvious twitching of the muscle. This twitching provides a simple assay of β-BuTX activity and we have used it to see if bee venom phospholipase A₂ alters the effect of β-BuTX. The phospholipase itself did not induce twitching of frog muscle, even at concentrations as high as 20 μg/ml (see also Abe et al. 1977b). Nonetheless, pretreatment of a muscle with the phospholipase inhibited the action of β-BuTX. One hour's preincubation of a muscle with phospholipase A₂ (5 μg/ml) was sufficient to prevent completely the twitching induced by 20 μg/ml of β-BuTX. Since the muscles still contracted to nerve stimulation, even after several hours' exposure to phospholipase and β-BuTX, this fact demonstrates that the phospholipase also prevents the toxin from exerting its usual action of abolishing transmitter release by nerve impulses, and inducing endplate denervation (Abe et al. 1976).

As shown in table 1, even a comparatively short incubation of 15 min in phospholipase was sufficient to protect the motor nerve endings from the subsequent action of β-BuTX. The twitching induced by 5 μg/ml of β-BuTX was largely prevented in the presence of phospholipase, while that induced by 20 μg/ml

<table>
<thead>
<tr>
<th>toxin concentration (μg/ml)</th>
<th>number of twitches in 20 min (experimental/control†)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.097</td>
</tr>
<tr>
<td>10</td>
<td>0.24</td>
</tr>
<tr>
<td>20</td>
<td>0.61</td>
</tr>
</tbody>
</table>

† Ratio of number of twitches by the phospholipase A₂-treated muscle to that by the contralateral muscle. Each value is a mean from 3 pairs of muscles.

(Muscles were incubated with 5 μg/ml of phospholipase for 15 min, and then β-BuTX (5-20 μg/ml) was added. The contralateral muscle from the same frog served as control. Temperature: 20 °C.)
was reduced to 60% of the control value. The fact that this protection is less marked than that observed after 1 h of preincubation suggests that the phospholipase either protects the endings only slowly, or it diffuses into the deep endplates more slowly than β-BuTX.

As already stated, β-BuTX, which possesses phospholipase A₂ activity, causes an immediate decrease in the amplitude of the endplate potentials, followed by a transient increase. In contrast, phospholipase A₂ caused an increase in e.p.p. amplitude, due to increased liberation of transmitter without any prior decrease

![Figure 1](http://rspb.royalsocietypublishing.org/) Effects of β-BuTX on endplate potential amplitude after pretreatment with bee venom phospholipase A₂. Bee venom phospholipase A₂ (5 μg/ml) was added at time 0. Horizontal arrows indicate the period of washing: many rinses with 500 ml of Ringer. At the vertical arrows β-BuTX (5 μg/ml) was added. E.p.p. amplitude is expressed as a percentage of the average before adding the phospholipase A₂. Temperature: 20 °C.

(figure 1). The e.p.p. size increased 2- to 5-fold (mean ± s.d. of seven endplates: 3.8 ± 1.2) in 30 min, and this level was maintained for several hours. The effect of phospholipase on e.p.p. amplitude was partly reversible on removal of the enzyme from the bath, but even after washing the muscle for 1–2 h, the e.p.p. was still larger than control. β-BuTX added after removal of the phospholipase had none of its characteristic effects (cf. figure 1), which shows that the continuous presence of phospholipase in the fluid is not necessary for protection against toxin action. At five endplates the e.p.p. amplitude 30 min after addition of β-BuTX (5 μg/ml) was 98% ± 11% of the value before toxin. In contrast, in non-protected endplates the toxin exerted its characteristic triphasic action and abolished the e.p.p. within 30 min (cf. Abe et al. 1976, 1977a, b).
Action of \( \beta \)-BuTX in Sr\(^{2+}\)-Ringer

It is known that \( \beta \)-BuTX requires Ca\(^{2+}\) for its phospholipase activity, and after substitution of Sr\(^{2+}\) for Ca\(^{2+}\) fails to show significant phospholipase activity (Strong et al. 1976; Abe et al. 1977b). Since Sr\(^{2+}\) can replace Ca\(^{2+}\) in neuromuscular transmission (Miledi 1966; Dodge, Miledi & Rahamimoff 1969) it is possible to test whether \( \beta \)-BuTX has any effect on transmitter release in the absence of its phospholipase activity. To reduce Ca\(^{2+}\) to a very low level, 0.5 mM ethylene-bis (oxoethylenenitrilo)tetraacetic acid (EGTA) was added in some experiments.

The effect of \( \beta \)-BuTX in Sr\(^{2+}\)-Ringer is summarized in table 2, and figure 2 illustrates the time course of \( \beta \)-BuTX on a cluster of a few junctions whose e.p.ps were recorded extracellularly. In all experiments the size of the e.p.p. fell to about

<table>
<thead>
<tr>
<th>EGTA concentration (mM)</th>
<th>number of experiments</th>
<th>amplitude of endplate potentials (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>65, 62, 58</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>60, 48, 49</td>
</tr>
</tbody>
</table>

**Table 2. Effect of \( \beta \)-BuTX (5 \( \mu \)g/ml) on endplate potentials in Ca\(^{2+}\)-free, Sr\(^{2+}\)(6 mM) Ringer**

The average e.p.p. amplitude at various times after addition of toxin is expressed as a percentage of the control value before toxin.

**Figure 2. Effect of \( \beta \)-BuTX on endplate potentials in Ca\(^{2+}\)-free, Sr\(^{2+}\)-Ringer solution.** Ringer solution contained no CaCl\(_2\) and 6 mM SrCl\(_2\). At time 0, \( \beta \)-BuTX (5 \( \mu \)g/ml) was added. E.p.ps of several fibres were recorded with an extracellular electrode. E.p.p. amplitude is expressed as a percentage of the average before toxin. Temperature: 23 °C.
60% of control in 10-20 min and thereafter remained at that level for several hours. Thus, in Sr\(^{2+}\)-Ringer the toxin still has its initial effect of decreasing transmitter release, but the second phase of transient increase and the following complete cessation of release and endplate denervation are missing (cf. Abe et al. 1976, 1977a, b).

**Discussion**

We have previously suggested (Abe et al. 1976, 1977a, b) that the phospholipase A\(_2\) activity of β-BuTX is responsible for inducing the transient increase and subsequent blocking of transmitter release from motor nerve endings. This hypothesis is strengthened by the fact that these two phases of toxin action did not occur in Sr\(^{2+}\)-Ringer, when phospholipase activity is practically abolished. On the other hand, bee venom phospholipase A\(_2\) by itself causes an increase in the amount of transmitter released by nerve impulses and, like β-BuTX, is capable of inducing endplate denervation if applied for a long period (cf. Abe et al. 1976). We do not yet know how the phospholipase increases transmitter release, but several factors could be involved. Phospholipase might alter the action potential of the nerve, increase the amount, or efficacy, of the Ca\(^{2+}\) entering the nerve terminal, or affect transmitter release through the production of lysophosphatidylcholine, which may promote membrane fusion (Howell & Lucy 1969).

Although the phospholipase activity of β-BuTX is clearly an important factor in toxin action, the phospholipase does not mimic the effects of β-BuTX completely. For instance, phospholipase failed to produce the immediate decrease in transmitter release which is caused by β-BuTX. It has been suggested previously (Abe et al. 1976) that this fall in transmitter release reflects the binding of β-BuTX to the presynaptic membrane. The failure of phospholipase to produce this effect may mean that the enzyme does not bind strongly to the nerve terminal membrane. This is also suggested by the finding that the action of phospholipase is partially reversible, while β-BuTX action proceeds even after removal of the toxin from the bathing medium.

Thus it appears that β-BuTX has an inhibitory effect on transmitter release which is not shared by phospholipase. Furthermore, in the absence of Ca\(^{2+}\) the toxin lacks phospholipase activity but still inhibits transmitter release (cf. figure 2). The inhibition seen in Ca\(^{2+}\)-free, Sr\(^{2+}\)-Ringer cannot be attributed to any remaining Ca\(^{2+}\), or phospholipase activity, as it was still observed when EGTA was used. Under these conditions the concentration of residual Ca\(^{2+}\) is much lower than the dissociation constant of the Ca\(^{2+}\)-β-BuTX complex, and any remaining phospholipase activity would be abolished by Sr\(^{2+}\) which inhibits phospholipase activity in the presence of Ca\(^{2+}\) (Abe et al. 1977b).

Our results show that bee venom phospholipase protected the nerve terminals against the action of β-BuTX. Since this protection was still observed when the toxin was applied after removing the phospholipase from the bath, it seems that the protection is not simply due to phospholipase and toxin competing for a
common binding site. One interesting possibility is that the phospholipase leaves behind a change in the membrane which makes it incapable of binding toxin.

In conclusion, it appears that β-BuTX has an inhibitory effect on spontaneous, or evoked, transmitter release, which can be seen in isolation when the toxin is chemically modified (Abe et al. 1977a, b) or when it is applied in Ca-free, Sr-Ringer. This inhibition is presumably due to binding of the toxin to the presynaptic nerve membrane at, or close to, the sites of transmitter release. Subsequently, the toxin’s phospholipase would act on the membrane and cause the transient increase in transmitter release and eventual endplate denervation.

We are grateful to Dr S. Alema for helpful discussion and to the M.R.C. for support. T.A. was a British Council Scholar.

References