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THE EFFECTS OF TETRAPHENYLBORON ON NEUROMUSCULAR TRANSMISSION IN THE FROG

I.G. MARSHALL[1] & R.L. PARSONS

Department of Physiology and Biophysics, College of Medicine, University of Vermont, Burlington, Vermont 05401, U.S.A.

1 The effects of tetraphenylboron (TPB) were studied on the frog sciatic nerve-sartorius muscle preparation.

2 TPB (0.01-1 mM) blocked indirectly elicited twitches of the preparation.

3 TPB (0.01-0.1 mM) produced no depolarization but lowered membrane resistance. TPB increased miniature endplate potential (m.e.p.p.) frequency, the rate of rise of the endplate potential (e.p.p.), and slowed the rate of rise and rate of fall of the muscle action potential.

4 In Mg^{2+} solutions the quantal content of e.p.ps was initially increased by TPB (0.01 mM). This was followed by a decrease of e.p.p. and m.e.p.p. amplitudes, accompanied by a lack of e.p.p. failures.

5 Larger concentrations of TPB (0.1 mM) produced an increase in e.p.p. amplitude followed by the sudden abolition of e.p.ps. This effect was associated with abolition of the nerve terminal spike.

6 TPB (0.1 mM) exhibited no postjunctional blocking action.

7 The results indicate that TPB acts prejunctionally, initially causing an increased release of acetylcholine. Subsequently, transmitter output is reduced by a reduction of quantal size.

Introduction

Tetraphenylboron (TPB), a lipid soluble anion first synthesized by Wittig, Keicher, Rückert & Raff (1949), has been employed in the gravimetric analysis of potassium salts (for review see Flaschka & Barnard, 1960) and of choline esters (Augustinsson & Grahn, 1954), and as a cell dispersing agent (Rappaport & Howze, 1966). It has also been suggested that TPB acts as a cholinoceptor agonist at both parasympathetic neuroeffector junctions (Guideri, Seifter & Seifter, 1968) and the neuromuscular junction (Seifter, Guideri & Seifter, 1968), by interacting with the esteratic site of the acetylcholine receptor (Guideri, Seifter & Seifter, 1972). The claim that cholinoceptor activation could be produced by combination with the esteratic site alone was not substantiated by Marshall (1970) who found that TPB produced no contracture of the chick biventer cervicis muscle. In addition Marshall (1970) postulated that the anti-curare activity of TPB (Seifter *et al.*, 1968) was due to an augmentation of evoked transmitter release rather than to a depolarizing action.

The experiments described have been designed to examine further the actions of TPB on neuromuscular transmission.

Methods

All experiments were performed on the frog (*Rana pipiens*) isolated sciatic nerve-sartorius muscle preparation. Nerve-muscle preparations were dissected in a phosphate-buffered Ringer solution (Parsons, 1969) and were subsequently maintained in a Tris (tris-(hydroxymethyl)aminomethane)-buffered Ringer solution (pH 7.25) at room temperature (17-20° C). The composition of the Tris-Ringer solution was (mM): NaCl 120, KCl 2.5, $CaCl_2$ 1.8, Tris 1. However, when TPB was added directly to the muscle chamber, KCl was omitted from the solution (K^+-deficient Ringer) to avoid precipitation of the water-insoluble potassium salt of TPB. Other modifications of these solutions are indicated in the text.

When necessary, neuromuscular block was produced by reducing the $CaCl_2$ concentration of the Tris-Ringer to 0.9 mM and adding 8 mM $MgCl_2$. Quantal content determinations were

[1] Present address: Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW.

made in this solution by the ratio of e.p.p. to m.e.p.p. amplitudes (del Castillo & Katz, 1954).

Statistical analyses were performed by Student's *t*-test, values of *P* less than 0.05 being regarded as significant. Values quoted in the text are expressed as mean ± standard error of the mean (s.e. mean).

Twitch tension experiments

In these experiments both sartorius nerve-muscle preparations were maintained in K^+-deficient Tris-Ringer solution. The sciatic nerve was stimulated at a frequency of 0.1 Hz by rectangular pulses of 50 μs duration, and of twice the strength required to produce a maximal muscle twitch. Isometric muscle twitches were recorded by Grass FTO3C strain gauges. Nerve-muscle preparations were equilibrated for 30 min in K^+-deficient solution before addition of TPB to one of the preparations. The second preparation served as a control to monitor the effects of prolonged exposure to K^+-deficient solution.

Electrophysiological techniques

After dissection and mounting of the preparation, the entire muscle chamber was secured on the stage of a Bausch & Lomb dynoptic microscope. When transilluminated, it was possible to view the preparation under 300x magnification, using a Leitz long-range objective. By this technique, a nerve twig could be visualized down to the last node of Ranvier and a microelectrode inserted in the corresponding muscle fibre in the immediate vicinity of the chemosensitive post-junctional membrane.

Resting membrane potentials, effective membrane resistances, endplate potentials (e.p.ps), miniature endplate potentials (m.e.p.ps) and muscle action potentials were recorded intracellularly with micropipettes filled with 3 M KCl and having resistances in the range of 6-12 MΩ. Nerve terminal spike activity was recorded extracellularly with micropipettes filled with 1 M NaCl and of low resistance (1-2 MΩ). Both intracellular and extracellular signals were amplified by a solid state neutralized capacitance preamplifier (Industrial Science Associates, Ridgewood, N.Y.) and were displayed simultaneously on Tektronix 565 & 564 oscilloscopes. Oscilloscope tracings were recorded either on Polaroid film or on moving film or paper by a Grass kymograph camera.

Endplate potentials, nerve terminal spikes and junctional action potentials were elicited by stimulation of the sciatic nerve through bipolar platinum electrodes with rectangular pulses of 50 μs duration and of sufficient strength to produce a maximum response. Directly elicited muscle action potentials were produced by stimulating an individual muscle fibre through a second microelectrode inserted in the same fibre approximately 1.3 mm from the recording electrode, with rectangular pulses of 0.01-0.05 ms duration and of sufficient strength to elicit an action potential.

Drug application techniques

TPB was either added to the solution bathing the whole muscle, or perfused directly onto the endplate region of individual muscle fibres (Manthey, 1966; Johnson & Parsons, 1972). In the former case the muscle was bathed in K^+-deficient Tris-Ringer solution, and the responses from many different fibres were measured during exposure to TPB. In the latter case the effect of TPB on the responses of individual fibres was measured over a prolonged period. In this case the muscle was bathed in Tris-Ringer solution although TPB was dissolved in K^+-deficient Tris-Ringer solution. Except for potassium and TPB the perfusion solutions always contained the same constituents as the bathing solution. Microperfusion of individual endplates was achieved by rapidly lowering a micropipette (50-100μm tip diameter) containing the perfusing solution into the bathing medium to a position about 0.1 mm above the fibre surface. The perfusion solution was delivered by hydrostatic pressure from a 15 cm column of solution.

Results

Effects of tetraphenylboron added to the bath

Effects on twitch tension development in indirectly stimulated muscles Relatively large concentrations of TPB (0.1 mM and over) were required to produce measurable effects on twitch tension. TPB 1 mM produced an immediate reduction in twitch amplitude, complete block being obtained in 1-4.5 min (mean 2.5 min; five experiments). The blocking effect of TPB 0.5 mM was slightly slower, taking 3-7 min (mean 5 min; four experiments) to achieve complete inhibition. At both 1 mM and 0.5 mM the blockade of twitch tension could occasionally be relieved by washing, especially if TPB was removed immediately after abolition of twitch tension. However, if the preparation was exposed to TPB (0.5 or 1 mM) for 5-10 min beyond the time of complete block, twitch tension did not recover even with prolonged washing.

The blocking effects of TPB 0.1 mM were slow in onset requiring 12-55 min (mean 34 min; four

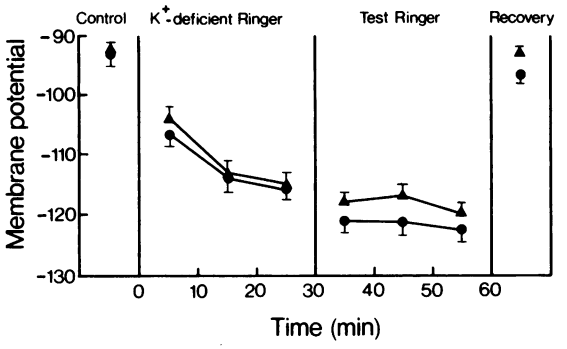

Figure 1 The effects of K^+-deficient solution and subsequent treatment with tetraphenylboron (TPB) 0.1 mM on resting membrane potential. Control values represent the mean (± s.e. mean) of 15-25 potential measurements from individual fibres after approximately 30 min equilibration in normal Tris-Ringer solution. All seven muscles used were then exposed to K^+-deficient solution for 30 minutes. After this period four muscles were exposed to K^+-deficient Ringer plus TPB 0.1 mM (▲), whereas the other three muscles remained in K^+-deficient solution (●). All points represent the mean of measurements made in 6-25 individual fibres. Vertical lines show s.e. mean. Recovery values represent the mean (± s.e. mean) of measurements made in 17 individual fibres 10-30 min after returning the muscles to normal Tris-Ringer solution.

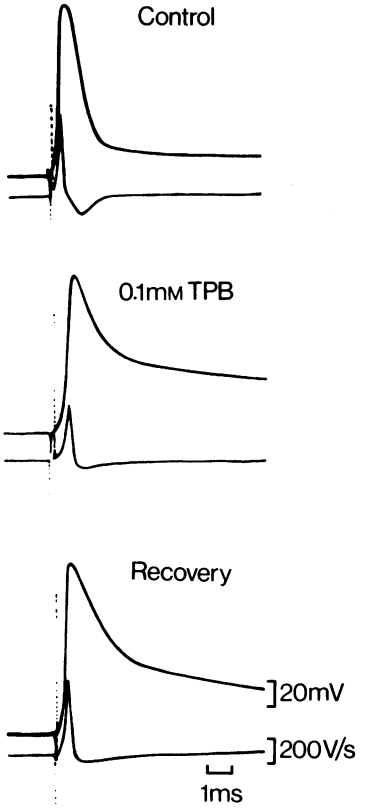

Figure 2 The effects of tetraphenylboron (TPB) 0.1 mM on directly elicited muscle action potentials. The figure shows results obtained in three different fibres in the same muscle. In each case the upper trace is the intracellularly recorded transmembrane potential (Vm), and the lower trace is dVm/dt obtained by electronic differentiation. Traces have been retouched slightly.

experiments) for complete blockade, and were generally preceded by a period of slight (up to 12%) twitch augmentation. The blocking effects of TPB 0.1 mM could not be reversed even by extensive washing.

Concentrations of TPB lower than 0.1 mM produced no change in twitch tension development in preparations exposed to TPB for periods of up to one hour.

Effects on some electrical properties of the muscle fibre Membrane potentials were measured initially in three muscles equilibrated for at least 30 min in the Tris-Ringer solution. The Tris-Ringer solution was then replaced by K^+-deficient Tris-Ringer solution and the membrane potential of different fibres sampled over a period of 60 minutes. In the K^+-deficient solution a rapid hyperpolarization followed by a gradually-developing further hyperpolarization was evident (Figure 1). Such a hyperpolarization has also been observed in K^+-deficient solution by Akiyama & Grundfest (1971). When the muscle was returned to the Tris-Ringer solution the resting potentials fell to control levels. In a comparable series of experiments, in four muscles after 30 min exposure to K^+-deficient solution, the bathing solution was changed to one containing TPB 0.1 mM. The hyperpolarizations observed during 30 min of TPB treatment were not significantly different from those observed during the last 30 min of exposure to K^+-deficient solution (Figure 1).

The influence of TPB 0.1 mM on the input resistance of individual muscle fibres was determined in four preparations. The input resistance measured in muscle fibres equilibrated for at least 15 min in K^+-deficient solution was $0.91 \pm 0.06 \times 10^6\Omega$ ($n = 29$). Subsequent exposure to TPB 0.1 mM for at least 15 min reduced the input resistance significantly ($P < 0.05$) to $0.54 \pm 0.03 \times 10^6\Omega$ ($n = 27$).

TPB altered the configuration of directly stimulated muscle action potentials. Action poten-

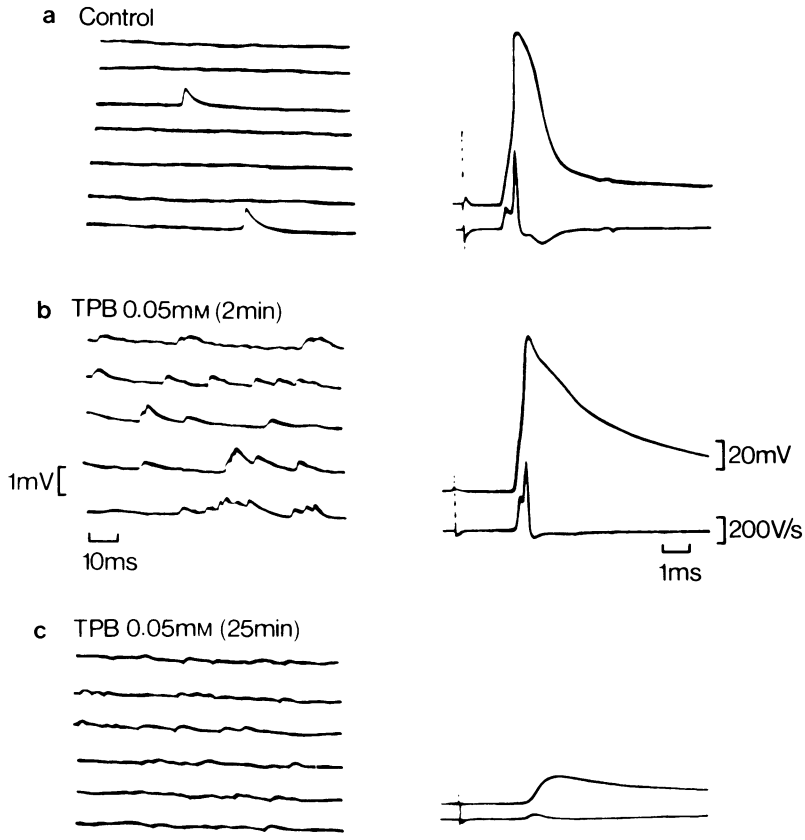

Figure 3 The effects of tetraphenylboron (TPB) 0.05 mM on miniature endplate potentials (m.e.p.ps), endplate potentials (e.p.ps) and indirectly elicited muscle action potentials. The figure shows results obtained in three different fibres of the same muscle. In each fibre representative records of m.e.p.ps (left panels) were obtained prior to recording the effects of nerve stimulation (right panels). In the right panels the upper trace is the transmembrane potential and the lower trace is dVm/dt as in Figure 2. Traces have been retouched slightly. Note that the first action of TPB is to increase dVm/dt of the e.p.p. (b) whereas subsequently the e.p.p. becomes subthreshold (c).

tials were recorded first in muscle fibres equilibrated in K^+-deficient Ringer solution, and then from fibres equilibrated for 10-35 min in the K^+-deficient, TPB 0.1 mM solution. TPB depressed the maximum rate of rise, the maximum rate of fall and the overshoot and greatly prolonged the duration of the action potential (Figure 2). For example, in the muscle preparation illustrated in Figure 2 the maximum rate of rise of the action potential was decreased from 604 ± 6 V/s (mean ± s.e. mean, n =5) to 410 ± 15 V/s, the maximum rate of fall was decreased from 148 ± 2 V/s to 59 ± 1 V/s and the overshoot was reduced from 42 ± 1 mV to 25 ± 1 mV following exposure to TPB 0.1 mM. These changes were only partially reversed after prolonged washing (15-70 min) with TPB-free solution. For example, in the preparation illustrated in Figure 2 the maximum rate of rise of the action potential recovered to 540 ± 19 V/s, the maximal rate of fall to 61 ± 0.5 V/s and the overshoot to 30 ± 1 mV (n = 5). Similar changes were observed in four muscles.

Effects at the endplate region of individual muscle fibres TPB exhibited concentration- and time-dependent effects at individual motor endplates both on the electrical activity following nerve stimulation, and on miniature endplate potential activity. These effects were observed at a lower concentration range (0.01-0.1 mM) than those used in the twitch tension experiments. TPB, in concentrations up to 0.1 mM produced no endplate depolarization.

In the presence of TPB 0.01, 0.05 and 0.1 mM the maximum rate of rise of the e.p.p. was consistently increased, and the time course of the junctional action potential, like that of the directly-elicited action potential, was prolonged (Figure 3). Concomitantly, there was a large increase in m.e.p.p. frequency above control levels (1-2/s), ranging from 40/s (0.01 mM TPB) to over 300/s (0.1 mM TPB). During 60 min exposure to TPB 0.01 mM the changes in e.p.p. and junctional action potential were maintained and were not reversed by 30 min in TPB-free solution.

In the presence of TPB 0.05 or 0.1 mM neuromuscular block of surface fibres occurred, although the pattern of blockade produced by the two concentrations differed.

In the presence of TPB 0.05 mM (three different nerve-muscle preparations) a gradual blockade of neuromuscular transmission developed, as indicated by the appearance of subthreshold e.p.ps after 5, 8 and 17 min exposure respectively (Figure 3). As the time of exposure to TPB 0.05 mM was increased (up to 40 min) the amplitude of the subthreshold e.p.p. continued to decline, in parallel with a decline in the m.e.p.p. amplitude. Frequency of m.e.p.ps also declined from a peak of over 100/s to around 20-60/s. After washing, the m.e.p.p. and e.p.p. amplitudes increased in all three preparations and in one preparation full transmission was restored. Frequency of m.e.p.ps remained elevated (10-100/s) over a 30-60 min recovery period after washing.

In the presence of TPB 0.1 mM complete neuromuscular block occurred precipitously within 5-10 min (three preparations). At this time m.e.p.ps, although reduced in amplitude, were still obvious. Continued exposure to TPB led to further reduction of m.e.p.p. amplitude and frequency. After washing subthreshold e.p.ps were observed in a few fibres of only one preparation. Nevertheless, m.e.p.p. frequency was considerably greater than control, and m.e.p.p. amplitude progressively approached control levels.

Effect on postjunctional sensitivity to carbachol The depolarization of the postjunctional membrane produced by micro-perfusion of carbachol 0.014 mM at individual endplates was measured in fibres equilibrated in K^+-deficient solution for at least 30 min, and in different fibres of the same muscle, after exposure to K^+-deficient TPB 0.1 mM solution for at least 30 minutes. At this time no m.e.p.ps were observed in most TPB-treated fibres. In all cases the depolarization produced by carbachol (0.014 mM) was greater in the TPB-treated fibres, the mean depolarization of 28.5 ± 4.1 mV (mean ± s.e. mean) in the four control fibres being significantly less than that (51.7 ± 4.5 mV) in the four TPB-treated fibres ($P < 0.05$).

Effects of microperfused tetraphenylboron

Effects on endplate potentials To study more quantitatively the relationship between the effects of TPB and time of exposure in individual fibres, the drug was locally microperfused onto individual junctional regions, partially blocked by Mg^{2+} to prevent muscle twitching.

Quantitative determinations were made by comparing the amplitude of e.p.ps and m.e.p.ps during a control perfusion of K^+-deficient Ringer solution with those during perfusion of TPB. With this method, TPB 0.1 mM produced a large increase in e.p.p. amplitude and m.e.p.p. frequency, often accompanied by muscle spiking and contractions, followed rapidly by the precipitous loss of the e.p.p. response. At this time m.e.p.p. frequency remained high. The lower concentration of 0.01 mM TPB produced no sudden fall in amplitude and hence quantal content determinations were performed at this concentration. The increase in mean quantal content ranged from 3.93-14.45 times control in five fibres (Table 1). During control perfusion of K^+-deficient solutions many e.p.p. failures were observed; however, during perfusion with TPB 0.01 mM no e.p.p. failures were evident.

Table 1 Initial effects of tetraphenylboron (TPB, 0.01 mM) on mean quantal content of endplate potentials recorded in Tris-Ringer solution containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (8 mM)

Fibre no.	*Control*			*TPB-treated (5 min)*			
	m.e.p.p. amplitude (mV)	*e.p.p. amplitude (mV)*	*mean quantal content (m)*	*m.e.p.p. amplitude (mV)*	*e.p.p. amplitude (mV)*	*mean quantal content (m)*	*m(TPB) / m(control)*
1	0.39	0.41	1.05	0.35	4.34	12.39	11.80
2	0.53	1.70	3.19	0.47	14.95	32.03	10.04
3	0.60	0.90	1.50	0.62	5.36	8.59	5.72
4	0.64	0.35	0.55	0.46	3.62	7.92	14.45
5	0.43	0.86	1.99	0.38	2.97	7.82	3.93

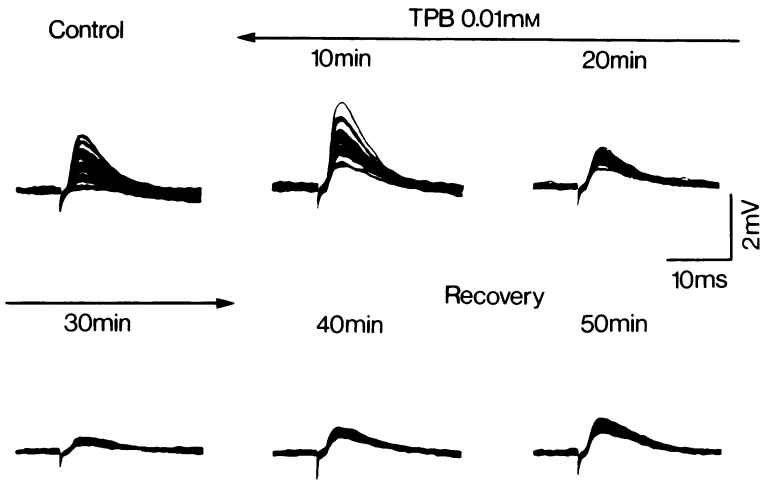

Figure 4 The effects of tetraphenylboron (TPB) 0.01 mM on endplate potentials (e.p.ps) recorded in 0.9 mM $CaCl_2$. 8 mM $MgCl_2$ Tris-Ringer solution. In these experiments all results are from a single fibre monitored for one hour. TPB was applied to the endplate region by microperfusion for 80 min followed by local perfusion with Tris-Ringer solution during the recovery period. Each panel shows 20 superimposed e.p.ps. Note the presence of several failures and wide spread of amplitudes during control measurements. In contrast, after 30 min exposure to TPB no failures are evident.

To study the effects of prolonged exposure to TPB 0.01 mM the membrane potential was maintained at −80 mV in three fibres by passing, if necessary, a d.c. current into the endplate region via a second microelectrode inserted within 50 μm of the recording electrode. Under these conditions TPB (0.01 mM) produced an initial increase in e.p.p. amplitude and m.e.p.p. frequency, followed by a gradual decrease in e.p.p. amplitude that paralleled a fall in m.e.p.p. frequency from its previously high level. No change in the time course of the e.p.p. was noted. The progressive decrease in e.p.p. amplitude was not accompanied by the appearance of failures (Figure 4). The decrease in e.p.p. amplitude was partially reversible by washing. Mean quantal content determinations were performed in two fibres during prolonged microperfusion of TPB (0.01 mM). Initially mean quantal content was increased (3.4 and 2.7 times control respectively), and as e.p.p. amplitude fell after approximately 45 min exposure to TPB, remained greatly above control (4.4 and 2.6 times control respectively). As the final determinations were made at a time when m.e.p.p. amplitude was approaching the noise level of the fibres, these latter determinations are probably underestimates of mean quantal content as only those m.e.p.ps with amplitudes greater than the noise could be measured.

Microperfused TPB produced no reduction of postjunctional sensitivity. In three fibres the depolarization produced by carbachol 0.014 mM (15-18 mV) after 10-15 min local perfusion of TPB (0.1 mM) was as large as that produced by control perfusions of carbachol (14-16 mV).

As TPB had been shown to be capable of augmenting transmission under conditions of low release i.e. low calcium, high magnesium concentrations, it was of interest to determine whether TPB could restore transmission in the absence of calcium. Accordingly muscles were equilibrated in Tris-buffer solutions containing no $CaCl_2$ and 1 mM EGTA ([Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid), and either 2 mM or 4 mM $MgCl_2$. Under these conditions no e.p.ps were elicited by nerve stimulation, and none were observed in the presence of 0.01 or 0.05 mM TPB (three determinations at each concentration) although m.e.p.p. frequency was greatly increased.

Effects on nerve terminal spike activity As the precipitous abolition of e.p.ps observed in the presence of high concentrations was suggestive of a depression of nerve terminal function, extracellular recording of nerve terminal spike (NTS) activity and extracellular e.p.ps were made in the 0.9 mM $CaCl_2$ and 8 mM $MgCl_2$ Tris-Ringer solution. Perfusion of K^+-deficient Ringer solution produced no effect on either NTS or e.p.p. activity. Perfusion of TPB 0.1 mM occasionally produced such a large initial increase in e.p.p. amplitude that action potentials and twitching were observed. However, in most preparations the

initial rise in e.p.p. amplitude was rapidly followed by the simultaneous abolition of NTS and e.p.p. Both the NTS and e.p.ps returned when TPB was removed. No attempt was made to quantitate changes in configuration of the NTS by concentrations of TPB less than 0.1 mM.

Discussion

In the present study no depolarization of the postjunctional membrane was noted when TPB was applied either by addition to the bath or by microperfusion. The observation does not support the view that the negatively charged TPB can activate acetylcholine receptors (Seifter *et al.*, 1968). The results show rather that TPB possesses potent activity on transmitter release, both spontaneous and evoked, and on the ionic conductance properties of the electrically excitable membranes.

The effects of TPB on transmitter release were both concentration- and time-dependent. The initial effect of TPB was to produce an increase both in m.e.p.p. frequency and e.p.p. quantal content. Both of these phenomena may be related to the ability of the lipid-soluble TPB to penetrate the nerve terminal and inhibit calcium uptake by mitochondria, an action it is known to possess in liver cells (Utsumi & Packer, 1967). The possible importance of mitochondria in regulating intraneuronal calcium levels has recently been discussed by Alnaes, Meiri, Rahamimoff & Rahamimoff (1974). Increased intracellular calcium concentration is associated with increases both in m.e.p.p. frequency (Miledi & Thies, 1971) and in e.p.p. quantal content (Crawford & Fettiplace, 1971). It is thought that tetraethylammonium (TEA) increases quantal content by prolonging the presynaptic spike, by a similar mechanism to that by which it prolongs the muscle action potential, namely a decrease in activated potassium conductance (Benoit & Mambrini, 1970). Like TEA, TPB prolongs the muscle action potential, and it is conceivable that concentrations of TPB lower than 0.1 mM may increase the quantal content in part by a prolongation of the nerve terminal spike. Nevertheless, it should be noted that TPB is different from TEA in that the latter drug does not increase m.e.p.p. frequency (Benoit & Mambrini, 1970).

After the initial increase in quantal content, evoked neuromuscular transmission was blocked by two mechanisms. At 0.1 mM, the sudden elimination of the e.p.p. or junctional action potential was shown to be due to an abolition of the nerve terminal spike. As no depolarization was noted in either junctional or non-junctional areas of the muscle fibre, it is unlikely that the nerve terminal block was due to terminal depolarization. However, TPB slowed the rate of rise of muscle action potentials, an effect indicative of a reduced sodium content. Such an action in the fine nerve terminals could lead to nerve terminal block. With prolonged application of lower concentrations (0.01-0.05 mM) of TPB there was a gradual decrease of e.p.p. amplitude, in parallel with a decrease of m.e.p.p. amplitude and frequency, associated with a lack of e.p.p. failures. The lack of failures in the presence of TPB indicates that the probability of transmitter release was above the pre-TPB level. Consequently the decrease in e.p.p. amplitude cannot be attributed to a slowly-developing inhibition of the release process. In addition, as mean quantal content remained well above control levels after continued exposure to TPB the fall in e.p.p. amplitude is probably due either to a fall in quantal size, or to a postjunctional blocking action of TPB.

TPB (0.1 mM) added to the bath increased the depolarization by carbachol (0.014 mM). However, no similar sensitization was observed when TPB was locally applied for only 8-12 min prior to carbachol application. The enhancement produced by TPB added to the bath also occurred in fibres voltage-clamped at −100 mV (Parsons & Spannbauer, unpublished observation), and thus could not be due to either an increase in driving force caused by the slowly developing hyperpolarization (Figure 1) or to an increase in membrane resistance. A similar time-dependent sensitization by 1-fluoro-2,4-dinitrobenzene has recently been described by Edelson & Nastuk (1973).

In view of the lack of postjunctional blocking action of TPB the reduction of e.p.p. amplitude is probably due to a reduction in quantal size, as indicated by the fall in m.e.p.p. amplitude.

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