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THE EFFECTS OF TETRAPHENYLBORON ON SPONTANEOUS TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

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1 The effects of tetraphenylboron (TPB) on spontaneous transmitter release were studied in the frog sartorius muscle preparation.

2 TPB (0.001-1 mM) produced a time-dependent increase in miniature endplate potential (m.e.p.p.) activity that was not sustained. TPB (0.1 mM) produced similar effects on m.e.p.p. frequency in normal Ringer solutions, in the absence of Ca^{2+} or Cl^- and in the presence of excess Ca^{2+} and of tetrodotoxin. The effect of TPB (0.01 mM) was reduced but not abolished in the absence of Ca^{2+}.

3 As m.e.p.p. frequency fell from its peak level in TPB (0.04 mM) m.e.p.p. amplitude was reduced. The reduction of m.e.p.p. amplitude was not prevented by choline (30-300 μM).

4 When m.e.p.p. activity fell below the noise level in the presence of TPB (0.1 mM), lanthanum (0.5 mM) was ineffective in promoting measurable m.e.p.p. activity.

5 The effects of TPB were slowly reversible by washing.

6 The results indicate that TPB acts to reduce the nerve terminal stores of acetylcholine, probably by a combination of rapid release and concomitant inhibition of transmitter storage.

Introduction

Amongst the most striking effects of tetraphenylboron described in the previous paper (Marshall & Parsons, 1975) were a marked increase in spontaneous transmitter release, and a subsequent time-dependent decrease in miniature endplate potential (m.e.p.p.) amplitude and frequency. The present study represents attempts to determine the mechanisms responsible for the changes in m.e.p.ps produced by this negatively charged compound. Some of these results have been presented to the American Society for Pharmacology and Experimental Therapeutics at Montreal (Marshall & Parsons, 1974).

Methods

Conventional intracellular recording techniques, as described in the previous paper (Marshall & Parsons, 1975), were used to record miniature endplate potentials (m.e.p.ps) from the frog sartorius muscle. TPB, dissolved in K^+-deficient Ringer solution (Marshall & Parsons, 1975), was applied to individual neuromuscular junctions by local microperfusion. Muscles were bathed in Tris-buffered Ringer solution (composition (mM) NaCl 120, KCl 2.5, $CaCl_2$ 1.8, Tris 1) except when otherwise stated. Except for TPB and KCl the perfusion solutions always contained the same constituents as the bathing solution. Frequency and amplitude of m.e.p.ps were measured from photographic records of oscilloscope traces.

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).

Results

Effects on m.e.p.p. frequency

Microperfusion of TPB onto individual surface junctional regions produced an increase in m.e.p.p. frequency within a few seconds. After the attainment of peak frequency, there was a gradual fall of frequency. In addition, as the m.e.p.p. frequency decreased, m.e.p.p. amplitude was progressively reduced. Consequently, the accurate measurement

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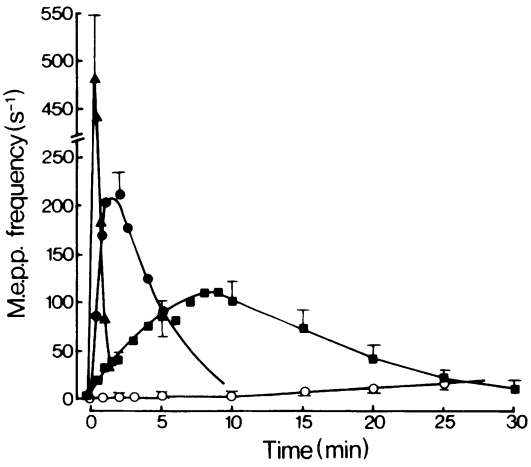

Figure 1 Effects of tetraphenylboron (TPB) on miniature endplate potential (m.e.p.p.) frequency in Tris-Ringer solution. The relationships between time of microperfusion and m.e.p.p. frequency produced by 1 mM (▲), 0.1 mM (●), 0.01 mM (■) and 0.001 mM (○) TPB are shown. Each point represents the mean of 6 determinations. Vertical lines show s.e. mean.

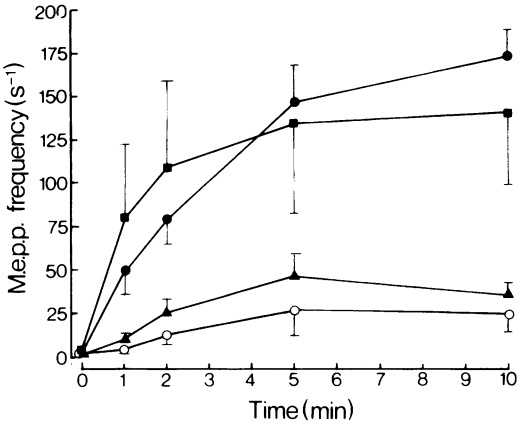

Figure 2 Effects of tetraphenylboron (TPB) (0.01 mM) on miniature endplate potential frequency in normal Tris-Ringer solution (1.8 mM $CaCl_2$, 0 mM $MgCl_2$; ●), high Ca^{2+} Tris-Ringer solution (10 mM $CaCl_2$, 0 mM $MgCl_2$; ■), Ca^{2+}-deficient Tris-Ringer solutions (0 mM $CaCl_2$; 8 mM $MgCl_2$; 1 mM EGTA; ▲; 0 mM $CaCl_2$; 2 mM $MgCl_2$; 1 mM EGTA ○). Each point represents the mean of 5 determinations. Vertical lines show s.e. mean.

of m.e.p.p. frequency was limited as the m.e.p.p. amplitude approached the base-line noise (approx. 0.1 mV).

The effect of TPB was both time- and concentration-dependent (Figure 1). Thus TPB 1 mM produced an explosive increase in m.e.p.p. frequency, followed within 2-3 min by the disappearance of m.e.p.p. activity. In contrast, TPB 0.01 mM produced a slower and less marked increase followed by a loss of measurable activity in approximately 30 minutes. After abolition of m.e.p.p. activity by TPB 0.1 mM, perfusions of either TPB 1 mM or lanthanum 0.5 mM, concentrations that in untreated fibres produced large increases in m.e.p.p. frequency, did not produce any measurable m.e.p.p. activity. It should be noted that at the higher concentrations of TPB the m.e.p.p. frequency was so high that there was summation of individual m.e.p.ps and the accuracy of measurement of frequency from photographic records was limited at frequencies of over 200-300/second. Therefore, the mean values shown on graphs should be regarded as estimates.

Control perfusions of K^+-deficient Tris-Ringer solution produced no significant effects on m.e.p.p. frequency (Table 1).

In order to determine the mechanism of action of TPB on m.e.p.p. frequency, several possible factors modifying the action of TPB were studied.

The effects of TPB 0.1 mM on m.e.p.p.

Table 1 Effects of Ca^{2+} concentration on the increase in m.e.p.p. frequency produced by tetraphenylboron (TPB) 0.1 mM.

Perfusion solution	*Bathing solution*	*M.e.p.p. frequency/s*					
		Control	*1 min*	*2 min*	*5 min*	*10 min*	n
K^+-deficient Tris	Tris	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	1 ± 0.6	6
0.1 mM TPB	Tris	2.2 ± 0.5	203 ± 30	210 ± 26	91 ± 11	10 ± 7	6
0.1 mM TPB	0 mM Ca^{2+} 8 mM Mg^{2+}	4.4 ± 2.6	218 ± 89	198 ± 70	154 ± 30	61 ± 29	6
0.1 mM TPB	0 mM Ca^{2+} 8 mM Mg^{2+} 1 mM EGTA	1.3 ± 0.6	328 ± 13	249 ± 33	54 ± 50	N.M.	3
0.1 mM TPB	10 mM Ca^{2+}	1.1 ± 0.4	236 ± 60	159 ± 31	57 ± 46	12 ± 12	4

N.M. Not measured

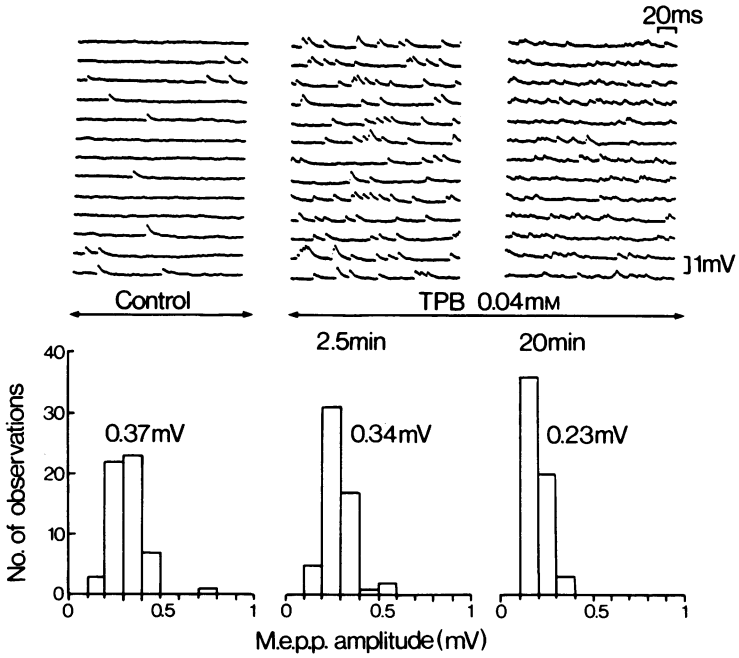

Figure 3 Effects of tetraphenylboron (TPB) 0.04 mM on miniature endplate potential (m.e.p.p.) frequency and amplitude in 10% hypertonic Tris-Ringer solution. The upper panels illustrate the effects of TPB on intracellularly recorded m.e.p.ps. The lower panels are the amplitude distributions corresponding to the records shown; mean m.e.p.p. amplitudes are inscribed. All records are from the same fibre.

frequency in Tris-Ringer solutions were not significantly different ($P > 0.05$) from its effects in Ca^{2+}-deficient Tris-Ringer solutions containing $MgCl_2$ 8 mM and from those in solutions containing $CaCl_2$ 10 mM (Table 1). A marked increase in frequency was also obtained in 3 fibres maintained in Ca^{2+}-deficient solutions containing EGTA 1 mM ([Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; Table 1).

In these experiments the increase in m.e.p.p. frequency produced by TPB 0.1 mM was so great that any small differences in frequency would have been difficult to detect. Thus, additional experiments were conducted with TPB 0.01 mM. At this concentration $CaCl_2$ 10 mM did not significantly increase m.e.p.p. frequency above that recorded in $CaCl_2$ 1.8 mM, whereas in solutions without $CaCl_2$ containing EGTA 1 mM and either 2 mM or 8 mM $MgCl_2$ the increase in m.e.p.p. frequency was significantly reduced, but was not abolished (Figure 2).

TPB (0.1 mM) also produced similar effects on m.e.p.p. frequency to those seen in Tris-Ringer solution, in Cl^--deficient solutions (replacing chloride salts by nitrate salts) and in Tris-Ringer solutions containing tetrodotoxin 1 ng/ml.

Effects on m.e.p.p. amplitude

In the experiments in which changes in m.e.p.p. amplitude were quantified, TPB 0.04 mM was used. At this concentration the change in m.e.p.p. frequency developed slowly, thus allowing accurate measurements of m.e.p.p. amplitude. However, even at this concentration m.e.p.p. activity became indistinguishable from noise within 20-30 minutes. To increase the number of control m.e.p.ps, the resting m.e.p.p. frequency was raised by elevating the tonicity of the Ringer solution 10% by the addition of 25 mM sucrose. In addition, the membrane potential was maintained at −80 mV by passing d.c. current through a second intracellular electrode.

During the initial moments of TPB application, as m.e.p.p. frequency rose, no marked effect on m.e.p.p. amplitude was observed. Measurement of m.e.p.p. amplitude was not attempted at the peak m.e.p.p. frequency in TPB because of summation of m.e.p.ps. As frequency fell it was possible to measure individual m.e.p.ps. At this time TPB produced a large reduction of m.e.p.p. amplitude. The results obtained in one fibre are shown in Figure 3. In many fibres a few m.e.p.ps of large

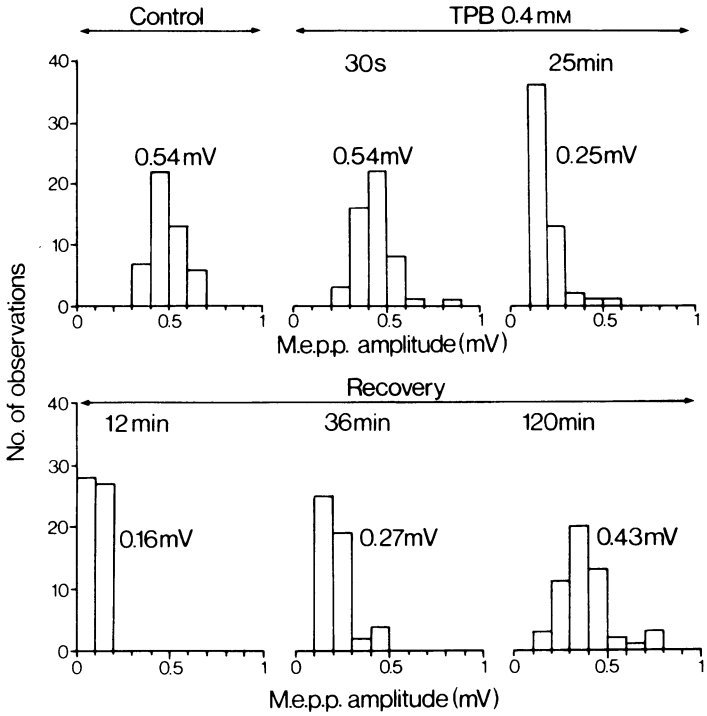

Figure 4 Reversibility of tetraphenylboron (TPB) action on miniature endplate potential (m.e.p.p.) amplitude. The panels represent amplitude distributions, and mean m.e.p.p. amplitudes, measured in 10% hypertonic Tris-Ringer solution. All distributions are from the same fibre. TPB 0.04 mM was microperfused for 60 min at which time no m.e.p.p. activity had been detectable for 10 minutes. Two amplitude distributions taken during the perfusion are shown in the upper panels. Perfusion was then continued with 10% hypertonic Tris-Ringer solution for a further 120 min and m.e.p.p. amplitude distributions made during recovery of amplitude (lower panels).

amplitude (0.5-1.5 mV) were observed at a time when m.e.p.p. activity was almost indistinguishable from noise.

Reversibility of tetraphenylboron effects

In preliminary experiments it was observed that removal of TPB (0.1 mM) from the muscle chamber after abolition of m.e.p.p. activity resulted in the reappearance of m.e.p.ps. Initially the m.e.p.ps were of low amplitude and high frequency and, over a period of 2 h, as amplitude recovered towards control levels, frequency fell towards control. In two fibres perfusions of lanthanum (0.5 mM) during this recovery period produced progressively greater stimulating effects on m.e.p.p. frequency as recovery proceeded.

Amplitude distributions during the recovery of m.e.p.p. amplitude and frequency from the effects of TPB 0.04 mM were constructed in 3 fibres. As recovery was measured in individual fibres, washing was performed by replacing the TPB-containing perfusion pipette by one containing normal Tris-Ringer solution. The example in Figure 4 shows that m.e.p.p. amplitude recovered to 80% of control after 2 h of perfusion with normal Tris-Ringer solution. During the recovery phase, exceptionally large m.e.p.ps (1-1.5 mV) and bursts of m.e.p.ps were occasionally observed in several fibres.

Effect of choline on the action of tetraphenylboron

To determine if the reduction in m.e.p.p. amplitude produced by TPB was a consequence of a hemicholinium-like inhibitory action on neuronal choline uptake, experiments were repeated in which TPB 0.1 mM was perfused onto neuromuscular junctions bathed in choline (30 μM –2 fibres and 300 μM –4 fibres) plus glucose (11 mM). Under these conditions TPB produced a

rise in m.e.p.p. frequency followed by a fall in both m.e.p.p. frequency and amplitude. These effects were indistinguishable from the effects of control perfusions of TPB 0.1 mM.

Discussion

The main effects of TPB on spontaneous transmitter release were an initial increase in m.e.p.p. activity followed by the abolition of such activity. The dramatic increase in spontaneous acetylcholine release was similar to that produced by black widow spider venom (Longenecker, Hurlbut, Mauro & Clark, 1970) and by lanthanum (Blioch, Glagoleva, Liberman & Nenashev, 1968; De Bassio, Schnitzler & Parsons, 1971), being concentration-dependent, and being produced in the absence of extracellular Ca^{2+}. That a large concentration of TPB (0.1 mM) increased m.e.p.p. frequency approximately equally in the presence of and in the absence of Ca^{2+}, indicates that the effect of this concentration is not dependent upon entry into the nerve terminal of extracellular Ca^{2+}. The effect of this large concentration of TPB differed from that of lanthanum in that lanthanum is less effective in the presence of excess Ca^{2+} levels (De Bassio *et al.*, 1971). However, the action of the lower concentration of TPB tested (0.01 mM) was shown to be reduced but not abolished in the absence of extracellular Ca^{2+} indicating that, at low concentrations of TPB, Ca^{2+} entry into the nerve terminals contributes in part to the observed increase in m.e.p.p. frequency. In addition, the action of TPB is independent of chloride ions and unlike that of batrachotoxin (Jansson, Albuquerque & Daly, 1974) and of tityustoxin (Warnick, Albuquerque & Diniz, 1974) is not antagonized by tetrodotoxin.

Currently it is believed that the rate of spontaneous transmitter release is related to intraneuronal Ca^{2+} levels (Miledi & Thies, 1971). Further it is believed that intraneuronal Ca^{2+} levels are regulated to a great extent by mitochondrial sequestration (Alnaes, Meiri, Rahamimoff & Rahamimoff, 1974). Agents such as β-bungarotoxin (Wagner, Mart & Kelly, 1974), ruthenium red, and dicoumarol (Rahamimoff & Alnaes, 1973) and 1-fluoro-2,4-dinitrobenzene (Edelson & Nastuk, 1973) that inhibit mitochondrial Ca^{2+} accumulation or oxidative phosphorylation are known to increase m.e.p.p. frequency. A similar mechanism of action may be responsible for the effects of TPB on m.e.p.p. frequency, as this compound is known to inhibit both mitochondrial Ca^{2+} uptake and oxidative phosphorylation (Utsumi & Packer, 1967). Ready penetration of the highly-lipid soluble TPB into the nerve terminal may account for its rapid onset of action (Liberman & Topaly, 1968), particularly at the higher concentrations used where the action of TPB appeared independent of extracellular Ca^{2+} concentrations.

In TPB, as in black widow spider venom (Longenecker *et al.*, 1970) and lanthanum (De Bassio *et al.*, 1971), the peak m.e.p.p. frequency was not maintained. In the case of TPB this drop in frequency was accompanied by a reduction in m.e.p.p. amplitude which was not due to a post-junctional blocking action (Marshall & Parsons, 1975). In contrast, after a similar period of perfusion of lanthanum 0.5 mM we observed that m.e.p.p. frequency stabilized at 30-100/s, without such a marked fall in amplitude. With black widow spider venom the fall in m.e.p.p. frequency to below control levels, is not associated with a consistent fall in m.e.p.p. amplitude (Longenecker *et al.*, 1970). Thus the fall in m.e.p.p. amplitude produced by TPB does not appear to be simply a consequence of the rapid rate of release. However, the rapid rate of release in combination with an inhibitory action of TPB on either the synthesis or the storage of acetylcholine might be expected to reduce m.e.p.p. amplitude. For example hemicholinium with repetitive nerve stimulation produces a reduction of quantal size (Elmqvist & Quastel, 1965; Jones & Kwanbunbumpen, 1970). However, TPB does not inhibit choline acetyltransferase *in vitro* (Guideri, Seifter & Seifter, 1972) and the lack of antagonistic action of choline suggests that a competitive inhibition of choline uptake is unlikely. An alternative mechanism of action of TPB is an inhibition of vesicular storage of acetylcholine. Recently, it has been proposed that type A botulinum toxin, which reduces m.e.p.p. amplitude possesses such an inhibitory action on acetylcholine packaging (Boroff, del Castillo, Evoy & Steinhardt, 1974). Whittaker (1974) has recently suggested that ATP is involved with vesiculin in the vesicular storage of acetylcholine. We suggest that an inhibition of oxidative phosphorylation by TPB may lead to a deficiency of ATP and thus interfere with acetylcholine storage in the synaptic vesicles.

The high m.e.p.p. frequency that appears after washing of TPB-treated tissues, and the evidence from studies on evoked transmitter release that the probability of release remains enhanced even after prolonged perfusion of TPB (Marshall & Parsons, 1975), suggests that small quantities of acetylcholine, producing effects indistinguishable from the noise, probably continue to be released, despite the apparent abolition of m.e.p.p. activity. That the probability of transmitter release remains high, the quantum size falls towards zero, and

perfusion of lanthanum or a higher concentration of TPB induces no further transmitter release, suggests that TPB depletes 'releasable' acetylcholine stores by a combination of rapid release plus inhibition of storage of transmitter.

The reversibility of the phenomenon indicates that the mechanism of action of TPB is different from that of black widow spider venom, that has been shown to deplete nerve terminals of acetylcholine and vesicles (Longenecker *et al.* 1970; Clark, Mauro, Longenecker & Hurlbut, 1970; Clark, Hurlbut & Mauro, 1972).

Recent preliminary morphological observations indicate that after 30 min exposure to TPB 0.05 mM a partial depletion (approximately 65%) of synaptic vesicles in individual nerve terminals occurs without a concomitant change in vesicle diameter (Marshall, Parsons & Paull, unpublished observation).

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