Br. J. Pharmac. (1987), **90**, 327–334

Effects of diacylglycerol and phorbol ester on acetylcholine release and action at the neuromuscular junction in mice

Richard L.W. Murphy & Margaret E. Smith[1]

Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TJ

1 Miniature endplate potentials and nerve-evoked endplate potentials were studied in a phrenic nerve-diaphragm preparation of the mouse.

2 A phorbol ester, phorbol-12β,13α-dibutyrate, and a diacylglycerol, 1-oleyl-2-acetyl-sn-glycerol, both increased the frequency of miniature endplate potentials. The effect of the phorbol ester on the frequency was still significant when the preparation was incubated in a calcium-deficient solution.

3 The phorbol ester, but not the diacylglycerol, caused a significant increase in the amplitude of the miniature potentials.

4 The phorbol ester also increased the amplitude of the endplate potentials in the presence of (+)-tubocurarine.

5 The mechanisms of action of phorbol ester and diacylglycerol are discussed in relation to the control of acetylcholine release and action at the neuromuscular junction.

Introduction

The hydrolysis of membrane phospholipids, primarily phosphatidylinositol bisphosphate, has been implicated in the responses to stimulation of certain receptors. This hydrolysis takes place via activation of phospholipase C, and results in the formation of inositol 1,4,5-trisphosphate and 1, 2-diacylglycerol. It has been suggested that the former product mobilizes calcium from intracellular stores, and the latter activates protein kinase C (Michell, 1986; for a review, see Berridge, 1984). This kinase has been implicated in signal transduction for a variety of biologically active substances (Nishizuka, 1984). Previous work in this laboratory has shown that phospholipase C activity appears in the bathing medium following nerve stimulation in a phrenic nerve-diaphragm preparation of the rat (Morgan-Harrison *et al.*, 1984). Furthermore, incubation of an isolated phrenic nerve-diaphragm preparation of the mouse with phospholipase C caused an increase in the amplitude of the endplate responses of the muscle (Turner & Smith, 1984). As this effect could be produced by either phosphatidylinositol-specific or phosphatidylcholine-specific enzymes (A.W. Turner & M.E. Smith, unpublished observations), it seemed possible that diacylglycerol was the reaction product responsible for the increase.

It has been found that a phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, promotes exocytosis in 'leaky' chromaffin cells (Knight & Baker, 1983). Phorbol esters have been shown to mimic the action of diacylglycerol on membrane responses (Castagna *et al.*, 1982). It seemed interesting therefore to see whether phorbol esters as well as diacylglycerol influence the release of acetylcholine (ACh) at the neuromuscular junction. Thus the effects of these compounds on presynaptic as well as postsynaptic events at the neuromuscular junction were examined. Both spontaneous release of ACh (miniature endplate potentials, m.e.p.ps) and release evoked by nerve stimulation (endplate potentials, e.p.ps) were investigated in this study.

In some tissues, activation of protein kinase C and mobilization of Ca^{2+} appear to act synergistically (Kaibuchi *et al.*, 1983; Knight & Baker, 1983). As the release of ACh at the neuromuscular junction is influenced by variations in the extracellular calcium concentrations (Fatt & Katz, 1952; Hubbard, 1961), experiments were performed to see whether the effects of phorbol ester at the neuromuscular junction were altered if the preparation was incubated in a calcium-deficient medium.

Since this study was completed, short accounts from two other laboratories (Publicover, 1985; Eusebi *et al.*,

[1] Author for correspondence.

1986) have described an increase in the frequency of m.e.p.ps at the neuromuscular junction of the frog after treatment with 12-O-tetradecanoylphorbol-13-acetate. In the present study we examined the effects of phorbol ester and diacylglycerol on both evoked and spontaneous release of ACh. Their effects on the amplitude of the responses as well as on the resting membrane potential and the effective input resistance, were also assessed. A brief account of part of this work has already been published (Murphy & Smith, 1986).

Methods

Phrenic nerve-diaphragm preparation

All experiments were carried out on diaphragms from mice aged six weeks to six months and were performed at room temperature. The mice were anaesthetized with diethyl ether and decapitated; the thorax was opened and the diaphragm dissected out along with long stumps of the phrenic nerves and transferred to a dish of continuously oxygenated Krebs-Henseleit solution. The latter solution was of the following composition, unless otherwise stated (mM): NaCl 118, KCl 4.7, $CaCl_2$ $2H_2O$ 2.5, $MgSO_4$ $7H_2O$ 2.4, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 5.6 (standard solution). The solution was continuously gassed with a mixture of 95% O_2 plus 5% CO_2 throughout the experiments.

The preparation was divided into hemidiaphragms and one was transferred to a 5 ml organ bath, pinned out on Sylgard resin (Dow-Corning Corporation), and perfused with standard Krebs-Henseleit solution at a flow rate of 4 ml min^{-1}. The stump of the phrenic nerve was placed over a pair of silver bipolar electrodes, and stimulated with square wave voltage pulses of duration 0.06 ms at a frequency of 2 Hz by means of a Digitimer DS2 stimulus isolation unit driven by a Digitimer D 100 pulse programmer. Any hemidiaphragm which failed to respond with a contraction to stimulation of intensity 9 V or less was discarded.

Intracellular recording

The preparation was illuminated from below, and observed through a Nikon SMZ-2 binocular microscope (8–40 × magnification). This allowed the terminal branches of the phrenic nerve to be visualized. Fibres were impaled close to these branches with a capillary glass microelectrode filled with 3 M KCl, of input resistance 15–20 MΩ. The resting membrane potential (RMP) was recorded via a Neurolog NL 102 d.c. preamplifier, and displayed on a digital voltmeter (DMM3, Advance instruments) and on one channel of a Medelec FOR-4.2 four channel oscilloscope. M.e.p.ps and e.p.ps were amplified via a Neurolog NL 106 a.c.-d.c. amplifier, and displayed on another channel. Penetrations were regarded as satisfactory if the RMP was more negative than −60 mV, and the risetime of the m.e.p.ps was 1 ms or less, and that of the e.p.ps was 2 ms or less. At least twenty m.e.p.ps and/or e.p.ps from each endplate were recorded on u.v.-sensitive paper (Kodak type 1895). The amplitudes were corrected for differences in the RMP of the fibres (Katz & Thesleff, 1957; Elmqvist & Quastel, 1965), by means of a simple computer program in BASIC. In some experiments the effective input resistance (Ri) of the fibre was determined by means of a bridge balancing circuit using a 1 nA hyperpolarizing current (although the amplitude of the postsynaptic responses is not necessarily determined by this parameter; Gage & McBurney, 1973).

A series of initial recordings was made and the preparation was then exposed to phorbol-12β,13α-dibutyrate (PDB, Sigma Ltd), 4α-phorbol-12β,13α-dibutyrate (4α-PDB, Scientific Marketing Associates), phorbol (Sigma Ltd) or to 1-oleyl-2-acetyl-sn-glycerol (OAG, Molecular Probes Inc.). The compounds were stored frozen at −15°C as stock solutions. The solvent was dimethylsulphoxide (DMSO, grade I, Sigma). The maximum final concentration of DMSO used was 0.001%. When the effects of the phorbol esters were investigated, the muscle was incubated with the ester for 30 min. The ester was washed out before the test recordings. When the effects of OAG were studied, recordings were made in the presence of the lipid as these compounds are rapidly metabolized (Kaibuchi *et al.*, 1983). As the effect of the phorbol esters had been found to be sensitive to the Ca^{2+} : Mg^{2+} ratio (see below), the concentration of $MgSO_4$ was reduced to 1.2 mM when OAG was used. In other cases where the composition of the Krebs-Henseleit solution was altered (see below), the test compound was dissolved in the appropriately modified solution.

When m.e.p.ps from muscles incubated in a modified solution were examined, an initial series of responses was first recorded in the standard solution. The perfusion medium was then changed to the modified solution and the preparation allowed to equilibrate for 45 min before the next series of recordings. The preparation was then incubated for 30 min in 20 nM PDB (in the modified solution), and further recordings obtained. Finally, the muscle was perfused again with the standard solution, and a final series of responses was recorded.

Endplate potentials

E.p.ps were recorded in a modified Krebs-Henseleit solution containing high Mg_2SO_4, and low $CaCl_2$ (0.5 mM), or in the standard solution containing 1.95 μM (+)-tubocurarine. When the former solution was used, the muscle was first incubated in the standard Krebs-Henseleit solution and a few contrac-

tions were elicited. The solution was then changed to the modified one containing 0.5 mM $CaCl_2$ and the $MgSO_4$ concentration was adjusted until contraction was prevented without complete abolition of evoked release. The $MgSO_4$ concentration which produced this effect was usually 6.6 mM. In early experiments, e.p.ps were evoked at 0.2 Hz and at 2 Hz and in later ones they were evoked at 2 Hz and 20 Hz. As m.e.p.ps could still be observed under these conditions, the average quantal content, i.e. the average number of 'quanta' the size of m.e.p.ps required to generate the observed e.p.p., as well as the amplitude of the e.p.ps could be calculated. The quantal content (m) was calculated by correcting for non-linear summation of voltage with current, using a factor of 0.8 as suggested by McLachlan & Martin (1981), and by the failures method (Del Castillo & Katz, 1954). The calculations were performed using a BBC model B microcomputer, and a simple program written in BASIC.

When recordings were obtained in the presence of (+)-tubocurarine, a few contractions were first evoked in the standard solution, then the antagonist was added to the perfusing solution until contraction was prevented without abolition of the e.p.ps. The preparation was then allowed to equilibrate for at least 45 min before recordings were made. The stimulation protocol was as follows: 20 e.p.ps were recorded at 2 Hz stimulation, then the preparation was allowed to rest for 20 s, and a train of e.p.ps elicited at 20 Hz stimulation. The mean amplitude of the e.p.ps evoked at 2 Hz was calculated. The amplitude of the first e.p.p. in a train was measured and the mean amplitude of the 100th to 109th e.p.p. in the same train was calculated, the latter group giving an estimate of the 'plateau e.p.p.' amplitude. An estimate of the decline of e.p.p. amplitude ('tetanic run-down'; Gibb & Marshall, 1984) was obtained by the formula: decline = 1 − (plateau e.p.p./first e.p.p.), expressed as a percentage.

Results

Miniature endplate potentials

Effect of phorbol ester and diacylglycerol Phrenic nerve-diaphragm preparations were incubated with PDB, in concentrations up to 100 nM, or OAG in concentrations up to 10 μM and the m.e.p.ps were recorded. In control experiments the muscles were incubated with phorbol, which lacks the fatty acyl residues on positions 12 and 13, or with 4α-PDB, a stereoisomer of PDB. Both phorbol and 4α-PDB have been demonstrated to be biologically inactive. None of these substances had any significant effect on the RMP or the Ri of the muscle fibres. The vehicle for these compounds was DMSO, which itself, in the concentrations used, had no effects on any of the parameters measured. Table 1 shows the effect of PDB, OAG, phorbol, and 4α-PDB on the frequency of the m.e.p.ps. Incubation with PDB in concentrations of 20 or 100 nM produced significant increases in the frequency of m.e.p.ps. Incubation with 5 nM or 10 nM PDB had no significant effects. Figure 1 shows typical recordings of m.e.p.ps obtained before and after incubation of a preparation with 20 nM PDB. OAG (250 nM) also caused appreciable increases in the frequency but this substance was not as potent as the phorbol ester. Higher concentrations of OAG (1–10 μM) had no significant effects. Phorbol, in concentrations up to 100 nM, and 4α-PDB (100 nM), had no significant effect.

Table 2 shows the effect of PDB, OAG, phorbol, and 4α-PDB on the amplitude of the m.e.p.ps. A significant increase in the amplitude of the potentials was seen after incubation with PDB in concentrations of 20 or 100 nM. Phorbol (100 nM), 4α-PDB (100 nM), and OAG (250 nM) did not produce any significant change in the amplitude of the responses.

Table 1 Effects of phorbol, 4α-phorbol-12β,13α-dibutyrate (4α-PDB), PDB and 1-oleyl-2-acetyl-sn-glycerol (OAG) on the frequency of m.e.p.ps

Addition	*Before inc.*(Hz)	*After inc.*(Hz)	*% increase*	P
4α-PDB (100 nM)	0.79 ± 0.06 (40,3)	0.80 ± 0.06 (38,3)	1	>0.05
Phorbol (100 nM)	0.53 ± 0.06 (19,2)	0.64 ± 0.03 (20,2)	21	>0.05
PDB (20 nM)	0.69 ± 0.06 (30,3)	1.45 ± 0.18 (38,3)	110	<0.01
PDB (100 nM)	0.43 ± 0.03 (38,3)	1.05 ± 0.06 (38,3)	140	<0.01
OAG (250 nM)	0.69 ± 0.05 (30,3)	0.97 ± 0.11 (30,3)	40	<0.05

The concentration of $MgSO_4$ was reduced to 1.2 mM when OAG was used. The values are expressed as means ± s.e.mean, with the number of fibres, followed by the number of muscles, in parentheses. *P* is the probability, calculated by means of Student's *t* test.

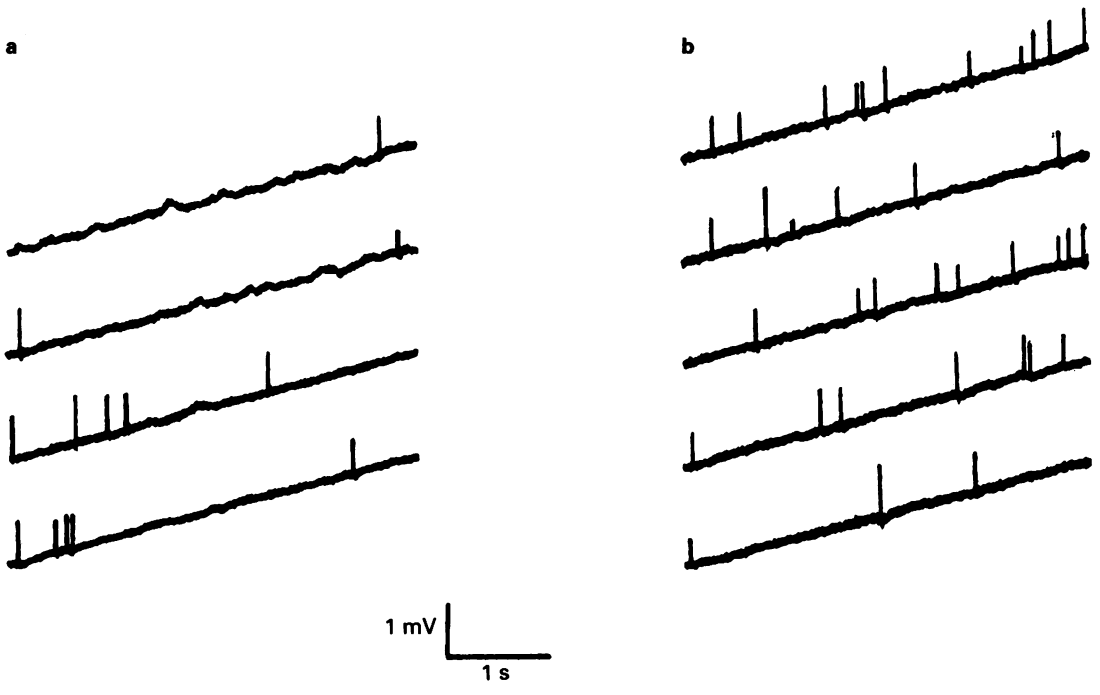

Figure 1 Typical experimental recordings of m.e.p.ps obtained in a mouse hemidiaphragm before (a), and after (b), incubation for 30 min with 20 nM phorbol-12β,13α-dibutyrate in standard Krebs-Henseleit solution.

Table 2 Effects of phorbol, 4α-phorbol-12β,13α-dibutyrate (4α-PDB), PDB and 1-oleyl-2-acetyl-sn-glycerol (OAG) on the amplitude of m.e.p.ps

Addition	*Before inc.* (mV)	*After inc.* (mV)	P
Phorbol (100 nM)	0.74 ± 0.04 (19,2)	0.72 ± 0.04 (20,2)	NS
4α-PDB (100 nM)	1.01 ± 0.04 (40,3)	1.07 ± 0.05 (38,3)	NS
PDB (20 nM)	0.87 ± 0.04 (30,3)	0.98 ± 0.04 (38,3)	<0.05
PDB (100 nM)	1.07 ± 0.03 (38,3)	1.26 ± 0.06 (38,3)]	<0.01
OAG (250 nM)	0.87 ± 0.03 (30,3)	0.88 ± 0.04 (30,3)]	NS

The concentration of $MgSO_4$ was reduced to 1.2 mM when OAG was used. The values are expressed as means ± s.e.mean, with the number of fibres, followed by the number of experiments, in parentheses. *P* is the probability, calculated by means of Student's *t* test. NS, not significant.

Effect of changes in the Ca^+ : Mg^{2+} ratio Muscles were incubated with 20 nM PDB in a Ca^{2+}-deficient solution to see whether the increases in the frequency of m.e.p.ps, described above, could be due to an increase in the permeability of the nerve terminal membrane to Ca^{2+}. The m.e.p.p. frequency measured at the beginning of the experiment in standard solution was 0.86 ± 0.09 Hz ($n = 24$). The frequency fell to 0.19 ± 0.03 Hz ($n = 22$) upon perfusion with the Ca^{2+}-deficient solution. However, subsequent incubation for 30 min with 20 nM PDB caused a significant ($P < 0.05$) rise to 0.32 ± 0.04 Hz ($n = 24$). There was no significant effect on the amplitude of the m.e.p.ps. When the preparation was again perfused with the standard Krebs-Henseleit solution, m.e.p.p. frequency increased to 1.07 ± 0.16 Hz ($n = 24$). Similar results were obtained using a Ca^{2+}-deficient solution containing no EDTA.

Table 3 The effects of phorbol-12β,13α-dibutyrate (PDB, 20 nM) and 1-oleyl-2-acetyl-sn-glycerol (OAG, 250 nM) on the frequency of m.e.p.ps in the presence of various concentrations of Mg^{2+} and Ca^{2+}

Addition	*$MgSO_4$: $CaCl_2$* (mM)	*Initial freq.* (Hz)	*After inc.* (Hz)	*% initial*	P
PDB	2.4 : 2.5	0.69 ± 0.06 (30)	1.45 ± 0.18 (38)	210	<0.01
PDB	2.4 : 0	0.19 ± 0.03 (22)	0.32 ± 0.04 (24)	170	<0.05
PDB	6.6 : 0.5	0.44 ± 0.05 (22)	0.81 ± 0.07 (23)	180	<0.01
OAG	1.2 : 2.5	0.69 ± 0.05 (22)	0.97 ± 0.11 (30)	140	<0.05
OAG	6.6 : 0.5	0.17 ± 0.02 (24)	0.19 ± 0.03 (23)	112	>0.05

The zero Ca^{2+} solution contained 1 mM EDTA. The results are expressed as means ± s.e.mean, with the number of fibres (from three experiments in each case) in parentheses. *P*, the probability, was calculated by means of Student's *t* test.

In order to observe the effects of OAG and PDB on the quantal content of the e.p.ps (see below), muscles were perfused with high Mg^{2+}, low Ca^{2+} solution. The amplitude and frequency of m.e.p.ps were lower in this medium than in the standard solution. Incubation for 30 min with 20 nM PDB had no significant effect on RMP or m.e.p.p. amplitude but an increase (approximately 80%) in the frequency of the m.e.p.ps was again observed. Perfusion of muscles in high Mg^{2+}, low Ca^{2+} solution with 250 nM OAG had no significant effects. The effects on the potency of PDB and OAG of altering the Ca^{2+} : Mg^{2+} ratio are shown in Table 3.

Endplate potentials

(i) Measurements in high Mg^{2+}, low Ca^{2+} solutions In experiments where the effects of PDB were examined, e.p.ps were evoked at 0.2 Hz and 2 Hz, and when OAG was used, they were evoked at 2 Hz and at 20 Hz. It was observed that, within a given series of experiments, the amplitude and quantal content of the e.p.p. were greater at the higher frequency of stimulation. The average quantal content was less than 1, and the average e.p.p. amplitude was smaller than the m.e.p.p. amplitude. PDB had no significant effect on the RMP or Ri of the fibres, or the amplitude of the e.p.p. and the m.e.p.p. There was no significant effect of 20 nM PDB on the quantal content at 2 Hz or 0.2 Hz stimulation, when this value was calculated either according to McLachlan & Martin (1981) or by the failures method. At 2 Hz stimulation m was 0.40 ± 0.06 ($n = 22$) and 0.46 ± 0.06 ($n = 16$) before and after incubation respectively when determined according to McLachlan & Martin (1981). The corresponding values for m determined by the failures method were 0.38 ± 0.07 ($n = 22$) and 0.35 ± 0.05 ($n = 16$) before and after incubation respectively. As noted above the frequency of m.e.p.ps was significantly increased after incubation with PDB. In another series of experiments, perfusion of the muscles with 250 nM OAG under these conditions had no significant effect on any of these parameters.

(ii) Measurements in the presence of D-tubocurarine When recordings were obtained from muscles incubated in the presence of (+)-tubocurarine, the mean amplitude of the e.p.ps evoked at 2 Hz was greater than that of the plateau e.p.ps at 20 Hz, but less than that of the initial e.p.p. in the 20 Hz train. The decline at 20 Hz was in the range 60–70%. Table 4 shows that incubation for 30 min with 20 nM PDB produced a significant (approximately 40%) increase in the mean amplitude of e.p.ps evoked at 2 Hz stimulation. The amplitude of e.p.ps evoked at 20 Hz stimulation was also increased but not significantly. Since the first e.p.p. of a train at 20 Hz was increased more than the plateau e.p.ps, the percentage decline in e.p.p. amplitude was significantly increased. In control experiments, where muscles were incubated for 30 min with 100 nM phorbol, a decrease in the RMP of the fibres from 73.45 ± 0.75 ($n = 42$) to 70.31 ± 0.84 ($n = 48$) was observed ($P < 0.01$). However this substance had no significant effect on the amplitude of the e.p.ps, or on the percentage decline at 20 Hz stimulation. Continuous perfusion of muscles with 250 nM OAG had no significant effects.

Discussion

M.e.p.ps are due to the spontaneous exocytosis from the nerve terminals of the contents of ACh vesicles (see, for example, Ceccarelli & Hurlbut, 1980). Thus the effects of PDB and OAG to increase m.e.p.p. frequency are due to an enhanced release of ACh at the neuromuscular junction via a presynaptic action. After incubation with PDB (20 nM), there was also a significant increase in the amplitude of m.e.p.ps and, in the presence of (+)-tubocurarine, a significant

Table 4 Effects of incubation for 30 min with 20 nM phorbol-12β,13α-dibutyrate (PDB) on e.p.ps in the presence of 1.95 M (+)-tubocurarine

	Initial	*After inc.*	
E.p.p. amp (2 Hz, mV)	0.84 ± 0.06 (41)	1.18 ± 0.11 (42)	**
First e.p.p. (20 Hz)	1.76 ± 0.18 (33)	2.19 ± 0.26 (34)	NS
Plat e.p.p. (20 Hz)	0.60 ± 0.05 (33)	0.67 ± 0.07 (37)	NS
Decline (%)	63 ± 1.8 (33)	68.6 ± 1.3 (34)	*

E.p.p. amp, 2 Hz = mean amplitude of twenty e.p.ps evoked at 2 Hz stimulation. First e.p.p. = amplitude of first e.p.p. in a train evoked at 20 Hz stimulation. Plat. e.p.p. = mean amplitude of 100th to 109th e.p.p. in a train evoked at 20 Hz stimulation. Decline = 1 − (Plat. e.p.p./first e.p.p.) in a given train, expressed as a percentage. Values are expressed as means ± s.e.mean, with the number of fibres in parentheses. The results are combined from five experiments. NS = not significant; $^{**}P < 0.01$; $^{*}P < 0.05$ (Student's t test).

increase in the amplitude of e.p.ps. An increase in the amplitude of the responses, in the absence of changes in the RMP or Ri of the fibres, could be due to a presynaptic effect to increase size of the released quantum. However, it could also be partly due to a postsynaptic effect, since in previous work it was shown that phospholipases of the C type, the action of which could promote the formation of diacylglycerol in the membrane, increased the postsynaptic responses of isolated muscles to ACh and carbachol (Harborne *et al.*, 1978; 1984) and both phospholipase C and PDB increased the binding of a cholinoceptor ligand to isolated sarcolemmal membranes (Turner & Smith, 1985). Thus, although the results of this work have revealed a presynaptic action of PDB, an additional postsynaptic action of this compound cannot be ruled out.

It seems likely, in view of their effects on m.e.p.p. frequency, that PDB and OAG enhance evoked release as well as spontaneous release. The early decline in the amplitude of e.p.ps in the presence of (+)-tubocurarine (Tc) at 20 Hz stimulation may be due to the depletion of a store of synaptic vesicles which are immediately available for release (Elmqvist & Quastel, 1965). However, the decline is probably at least partly due to a presynaptic effect of Tc (see for example, Gibb & Marshall, 1984). The amplitude of the plateau e.p.ps could reflect the rate of mobilization of ACh from a second store. Incubation with PDB produced a greater relative increase in the amplitude of the initial e.p.p. in a 20 Hz train than in the amplitude of the plateau e.p.ps resulting in a greater proportional decline in e.p.p. amplitude. This may be because the synaptic vesicles immediately available for release are closer to the nerve terminal membrane, and are therefore more readily accessible to PDB and OAG.

The frequency of m.e.p.ps recorded in Ca^{2+}-deficient solutions was much lower than that recorded in the standard solution. This had also been observed by Hubbard (1961). Incubation with PDB again produced an appreciable increase in m.e.p.p. frequency. The increase was proportionately smaller than that seen in muscles incubated in standard solution but the relative increases in the two solutions were not significantly different. The quantal content of the e.p.p. was low when recordings were made in high Mg^{2+}, low Ca^{2+} solutions, with a large proportion of nerve stimuli failing to produce an e.p.p. The amplitude and quantal content of the e.p.p. were greater at 2 Hz stimulation than at 0.2 Hz stimulation. The enhancement of evoked release of ACh at high frequencies of stimulation has been observed by other workers (Magleby & Zengel, 1976). Neither PDB nor OAG had a significant effect on the amplitude or quantal content of the e.p.p. It is possible that evoked release is less sensitive to PDB under these conditions than in the presence of Tc, or that it is already maximum under the prevailing ionic conditions. Under these conditions, PDB did not increase m.e.p.p. amplitude.

OAG was less potent than PDB in increasing the amplitude and frequency of the responses. One explanation for this is that diacylglycerol in cell membranes is rapidly phosphorylated to the corresponding phosphatidate (Kaibuchi *et al.*, 1983). Phorbol esters have been demonstrated to mimic the action of diacylglycerol, but are not phosphorylated, and therefore probably cause prolonged activation of protein kinase C (Castagna *et al.*, 1982).

There are several possible explanations for the action of PDB and OAG. One possibility is that the permeability of the nerve terminal to Ca^{2+} is increased. The free calcium ion concentration in a solution containing no added Ca^{2+}, and containing a Ca^{2+} chelator such as EDTA, is less than 100 nM (Kendall & Nahorski, 1984). The intracellular free calcium ion concentration in unstimulated cells is generally in the range 80–100 nM (see for example Vincentini *et al.*, 1985). The inward gradient for calcium ions was

therefore greatly reduced when the muscles were incubated in the Ca^{2+}-deficient solution. The results presented here show that PDB enhanced spontaneous release in these conditions. It is unlikely therefore that the mechanism of action of the phorbol ester is to enhance Ca^{2+} permeability.

Another explanation for the effects of PDB and OAG is that the observed increases are due to the surface-active properties of PDB and OAG. However, the concentrations of OAG and PDB which were effective in this study are lower than those found to cause perturbation in the membrane structure of platelets (Yamanishi *et al.*, 1983). Moreover, incubation with 100 nM 4α-PDB (a stereoisomer of PDB with similar physical and chemical properties) had no significant effect on m.e.p.p. frequency or amplitude (Tables 1 and 2). It seems unlikely therefore that the PDB or OAG increases the ACh responses via membrane perturbation.

The responses of cells to phorbol esters and diacylglycerol have been shown to mimic the effects of endogenous messengers which provoke a 'phosphatidylinositol response' (for a review, see Berridge, 1984). It is therefore possible that the effects of phorbol esters and exogenous OAG mimic those of receptor-mediated production of diacylglycerol. The activation of protein kinase C by phorbol esters and diacylglycerol, leading to stimulation of secretion by platelets, is well documented (Kaibuchi *et al.*, 1983; Yamanishi *et al* ., 1983). Recent evidence has implicated protein kinase C in secretory responses in systems of neural origin (Pozzan *et al.*, 1984; Summers & Creutz, 1985). It has also been found that phorbol (Castagna *et al.*, 1982) and 4α-phorbol esters (Harris *et al.*, 1986) do not activate protein kinase C. The observation that PDB increased m.e.p.p. frequency and amplitude and e.p.p. amplitude at the neuromuscular junction whilst 4α-PDB and phorbol had no significant effect, provides evidence that the mode of action of PDB is via activation of protein kinase C.

It is possible that *in vivo* the production of membrane diacylglycerol is due to the activation of a phospholipase C in the nerve terminal. One candidate for this would be the enzyme shown to be released from nerve-muscle preparations in earlier work (Morgan-Harrison *et al.*, 1984). Alternatively activation of a presynaptic receptor could be responsible for the production of endogenous diacylglycerol in the membrane. Such receptors include muscarinic receptors (Ganguly & Das, 1979), α-adrenoceptors (Kuba, 1970), and substance P receptors (Steinacker, 1977). Adrenocorticotropin peptides have also been shown to increase both phosphatidylinositide metabolism (for a review see Farese, 1983) and spontaneous release of ACh at the neuromuscular junction (Birnberger *et al.*, 1977; Johnston *et al.*, 1983). Furthermore previous work in this laboratory (Haynes & Smith, 1985) has shown that such peptides are present in the terminals of the motor neurones of the mouse at certain times during development. Future work will be directed towards elucidating the membrane mechanisms which mediate the release of ACh at the neuromuscular junction.

This work was supported by the Medical Research Council. We wish to thank Professor C.B. Ferry for making the facilities of the Department of Pharmacy, University of Aston in Birmingham, available for some of these experiments, and to Dr S.S. Kelly for advice. We are grateful to Mr H. Ross for advice on the use of the microcomputer.

References

BERRIDGE, M.J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.*, **220**, 345–360.

BIRNBERGER, K.L., RUEDEL, R. & STRUPPLER, A. (1977). ACTH and neuromuscular transmission: Electrophysiological *in vitro* investigation of the effects of corticotropin and an ACTH fragment on neuromuscular transmission *Ann. Neurol.*, **1**, 270–275.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calicum-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. biol. Chem.*, **257**, 7847–7851.

CECCARELLI, B. & HURLBUT, W.P. (1980). Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.*, **60**, 396–441.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the endplate potential. *J. Physiol.*, **124**, 560–573.

ELMQVIST, D. & QUASTEL, D.M.J. (1965). A quantitative study of end-plate potentials in isolated human muscle. *J. Physiol.*, **178**, 505–529.

EUSEBI, F., MOLINARO, M. & CARATSCH, C.G. (1986). Effects of phorbol ester on spontaneous transmitter release at frog neuromuscular junction. *Pfluegers Arch.*, **406**, 181–183.

FARESE, R.V. (1983). Phosphoinositide metabolism and hormone action. *Endocrine Rev.*, **4**, 78–95.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.*, **117**, 109–128.

GAGE, P.W. & McBURNEY, R.N. (1973). An analysis of the relationship between the current and potential generated by a quantum of acetylcholine in muscle fibres without transverse tubules. *J. membr. Biol.*, **12**, 247–272.

GANGULY, D.K. & DAS, M. (1979). Effects of oxotremorine demonstrate presynaptic muscarinic and dopaminergic receptors on motor nerve terminals. *Nature*, **278**, 645–646.

GIBB, A.J. & MARSHALL, I.G. (1984). Pre- and post-junctional effects of tubocurarine and other nicotinic antagonists during repetitive stimulation in the rat. *J. Physiol.*, **351**, 275–297.

HARBORNE, A.J., SHUTE, J.K. & SMITH, M.E. (1984). Release

of hydrolases and acetylcholine sensitivity in rat skeletal muscle. *J. Physiol.*, **352,** 385–393.

HARBORNE, A.J., SMITH, M.E. & JONES, R. (1978). The effect of hydrolytic enzymes on the acetylcholine sensitivity of the skeletal muscle cell membrane. *Pfluegers Arch.*, **377,** 147–153.

HARRIS, K.M., KONGSAMUT, S. & MILLER, R.J. (1986). Protein kinase C mediated regulation of calcium channels in PC-12 pheochromocytoma cells. *Biochem. biophys. Res. Commun.*, **134,** 1298–1305.

HAYNES, L.W. & SMITH. M.E. (1985). Presence of immunoreactive α-melanotropin and β-endorphin in spinal motoneurones of the dystrophic mouse. *Neurosci. Lett.*, **58,** 13–18.

HUBBARD, J.I. (1961). The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings. *J. Physiol.*, **159,** 507–517.

JOHNSTON, M.F. KRAVITZ, E.A. MEIRI, H. & RAHAMIMOFF, R. (1983). ACTH causes long-lasting potentiation of neurotransmitter release at frog motor nerve endings. *Science*, **220,** 1071–1072.

KAIBUCHI, K., TAKAI, Y., SAWAMURA, M., HOSHIJIMA, M., FUJIKURA, T. & NISHIZUKA, Y. (1983). Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J. biol. Chem.*, **258,** 6701–6704.

KATZ, B. & THESLEFF, S. (1957). On the factors which influence the amplitude of the 'miniature end-plate potential'. *J. Physiol.*, **137,** 267–278.

KENDALL, D.A. & NAHORSKI, S.R. (1984). Inositol phospholipid hydrolysis in rat cerebral cortical slices: II. calcium requirement. *J. Neurochem.*, **42,** 1388–1394.

KNIGHT, D.E. & BAKER, P.F. (1983). The phorbol ester TPA increases the affinity of exocytosis for calcium in 'leaky' adrenal medullary cells. *FEBS Lett.*, **160,** 1, 2, 98–100.

KUBA, K. (1970). Effects of catecholamines on the neuromuscular junction in the rat diaphragm. *J. Physiol.*, **211,** 551–570.

MAGLEBY, K.L. & ZENGEL, J.E. (1976). Augmentation: a process that acts to increase transmitter release at the frog neuromuscular junction. *J. Physiol.*, **257,** 449–470.

McLACHLAN, E.M. & MARTIN, A.R. (1981). Non-linear summation of end-plate potentials in the frog and mouse. *J. Physiol.*, **311,** 307–324.

MICHELL, R.H. (1986). Inositol phosphates: profusion and confusion. *Nature*, **319,** 177–178.

MORGAN-HARRISON, K., SHUTE, J.K. & SMITH, M.E. (1984). Release of phospholipase C following nerve stimulation in an isolated nerve-diaphragm preparation of the rat. *J. Physiol.*, **350,** 56P.

MURPHY, R.L.W. & SMITH, M.E. (1986). Effect of phorbol ester and diacylglycerol on the endplate responses on the mouse diaphragm. *J. Physiol.*, **373,** 64P.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308,** 693–698.

POZZAN, T., GATTI, G., DOZIO, N., VINCENTINI, L.M. & MELDOLESI, J. (1984). Ca^{2+}-dependent and Ca^{2+}-independent release of neurotransmitters from PC12 cells: a role for protein kinase C activation? *J. cell Biol.*, **99,** 628–638.

PUBLICOVER, S.J. (1985). Stimulation of spontaneous transmitter release by the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate, an activator of protein kinase C. *Brain Res.*, **333,** 185–187.

STEINACKER, A. (1977). Calcium-dependent presynaptic action of substance P at the frog neuromuscular junction. *Nature*, **267,** 268–270.

SUMMERS, T.A. & CREUTZ, C.E. (1985). Phosphorylation of a chromaffin granule-binding protein by protein kinase C. *J. biol. Chem.*, **260,** 2437–2443.

TURNER, A.W. & SMITH, M.E. (1984). Effect of phospholipase C on the endplate responses in the mouse diaphragm. *J. Physiol.*, **353,** 93P.

TURNER, A.W. & SMITH, M.E. (1985). Effect of phorbol ester on the binding of α-bungarotoxin to isolated sarcolemma. *Biochem. Soc. Transac.*, **13(5),** 920–921.

VINCENTINI, L.M., DI VIRGILIO, F., AMBROSINI, A., POZZAN, T. & MELDOLESI, J. (1985). Tumor promoter phorbol 12-myristate, 13-acetate inhibits phosphoinositide hydrolysis and cytosolic Ca^{2+} rise induced by the activation of muscarinic receptors in PC12 cells. *Biochem. biophys. Res. Commun.*, **127,** 310–317.

YAMANASHI, J., TAKAI, Y., KAIBUCHI, K., SANO, K., CASTAGNA, M. & NISHIZUKA, Y. (1983). Synergistic functions of phorbol ester and calcium in serotonin release from human platelets. *Biochem. biophys. Res. Commun.*, **112,** 778–786.

(Received May 8, 1986.
Revised August 10, 1986.
Accepted October 27, 1986).