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DUAL EFFECT OF 2,4-DINITROPHENOL ON THE SPONTANEOUS RELEASE OF TRANSMITTER AT THE FROG NEUROMUSCULAR JUNCTION

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Abstract—The uncoupling agent 2,4-Dinitrophenol (DNP) has a dual effect on the spontaneous release of transmitter at the frog neuromuscular junction, causing an initial fall in miniature endplate potential (MEPP) frequency followed by a dramatic rise. The latter effect is probably associated with the release of Ca^{2+} from the mitochondria. The initial fall in MEPP rate is independent of $[Ca^{2+}]_0$ but is largely suppressed by pretreatment with theophylline; it is suggested that DNP also combines with another intracellular Ca^{2+}-store, so reducing Ca^{2+} leakage and causing a fall in the steady-state level of $[Ca^{2+}]_i$. The results confirm that MEPP frequency is largely determined by $[Ca^{2+}]_i$ at the presynaptic terminals.

Excitation at the presynaptic terminals of the neuromuscular junction causes a transient, but marked, increase in calcium permeability (P_{Ca}) of the plasma membrane; Ca^{2+} that enters the cell then produces the synchronized release of quantal packets of transmitter, which is recorded postsynaptically as the endplate potential (EPP). Experimental results also suggest that the intracellular concentration of free calcium ($[Ca^{2+}]_i$) is a major factor governing the frequency of the spontaneous discharge of quantal packets of transmitter, recorded as the miniature endplate potentials (MEPPs) [1, 2]. Thus, the magnitude of the EPP is determined primarily by extracellular Ca^{2+} [3, 4], whilst MEPP frequency is largely governed by intracellular Ca^{2+}.

The steady-state level of $[Ca^{2+}]_i$ in the presynaptic terminals is the resultant of the following factors: (1) P_{Ca} of the plasma membrane (2) the activity of the Ca^{2+} extrusion system in the membrane and (3) exchange with intracellular Ca^{2+}-stores [1, 5, 6]. The mitochondria are one intracellular system that is able to take up Ca^{2+} in cells under certain conditions [7–9] and they have been shown to have an important role in sequestering injected Ca^{2+} in isolated *Chironomus* salivary gland cells [10] and in controlling $[Ca^{2+}]_i$ in rat liver slices [11]. Treatment of either frog or rodent presynaptic terminals with a variety of mitochondrial inhibitors causes a marked increase in MEPP frequency [1, 12–14] and it is most probable that this is because of a release of stored Ca^{2+}. In the present paper we report the results of experiments concerned with the action of the uncoupling agent 2,4-dinitrophenol (DNP) on the presynaptic terminals of the frog neuromuscular junction and show that this agent has a dual action on MEPP frequency in this preparation. These experiments confirm previous findings [5, 6, 15] that there are additional intracellular Ca^{2+}-stores in the nerve terminals that contribute markedly to the buffering of $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Electrophysiological recordings were made with the isolated cutaneous pectoris nerve-muscle preparation of the frog *Rana temporaria*. Frogs were maintained in the laboratory at 5°. All salines in which the preparations were bathed contained NaCl 115 mM, KCl 2.5 mM, Na_2HPO_4 2.15 mM and NaH_2PO_4 0.85 mM at pH 7.1 Calcium concentrations varied such that normal saline contained 1.8 mM $CaCl_2$ but in other salines, however, $[Ca^{2+}]_0$ was maintained constant by the use of Ca^{2+}-EGTA buffers, in which pH was maintained at 7.1 and 0.5 mM EGTA was added, together with the appropriate volume of AnalaR standard volumetric solution of $CaCl_2$. Free Ca^{2+} concentrations were calculated following the method of Portzehl *et al.* [16]. Solutions containing DNP or theophylline were prepared by dissolving these agents in the appropriate saline immediately before use.

The muscle was excised and equilibrated in the appropriate control saline for 45 min at 10°. It was then pinned out in the experimental bath, the microelectrode inserted in the endplate region and the temperature adjusted to the experimental value. Records of MEPPs began after a further 10 min. Electrophysiological recordings were made from the muscle by the use of conventional glass electrodes filled with 3 M KCl; the temperature of the bath was controlled (±1°) by a Peltier device. Potentials were fed through a cathode follower to a Tektronix 502A oscilloscope. MEPPs were recorded on moving film and counted. In any one experiment, the MEPPs were monitored in a single fibre at the intervals shown in the Figs and at least 100 MEPPs were counted. MEPP frequencies were compared using Students '*t*' test.

All inorganic salts used were AnalaR grade. DNP was obtained from BDH, Poole, U.K. Theophylline,

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EGTA were obtained from Sigma Chemical Co., St. Louis, U.S.A.

RESULTS

In control experiments with saline containing 1.8×10^{-3} M Ca^{2+}, no significant variation in MEPP frequency was recorded in any one fibre over a period of 2 hr; small oscillations in MEPP frequency were observed but were always less than 10 per cent of the initial rate. However, considerable variation was found in the basal, control rate of different preparations. For this reason, the results are expressed as a ratio of the control MEPP frequency (F_1/F_0, where F_0 is the control frequency and F_1 the frequency after treatment).

It has been established previously that application of DNP causes a dramatic rise in MEPP frequency at the frog neuromuscular junction [14], a finding that is confirmed in the present study. Figure 1 shows that 10^{-4} M DNP causes a major increase in MEPP frequency some 30 min after application and in some preparations a 100-fold rise in the rate of spontaneous release of transmitter was seen. However, this figure also reveals that DNP consistently produces an initial marked reduction in MEPP frequency which is found during the first 20 min of exposure, a feature of the reponse that has not apparently been reported previously. MEPP frequency usually falls to below 30 per cent of normal values. DNP therefore exerts a dual effect on MEPP frequency.

One possible explanation for this response is that whilst the second, stimulatory phase is likely to be due to the release of Ca^{2+} from mitochondria, the initial inhibition might be because the DNP reduces P_{Ca} and Ca^{2+} inward flux, so allowing $[Ca^{2+}]_i$ to settle to a lower steady-state position. The action of

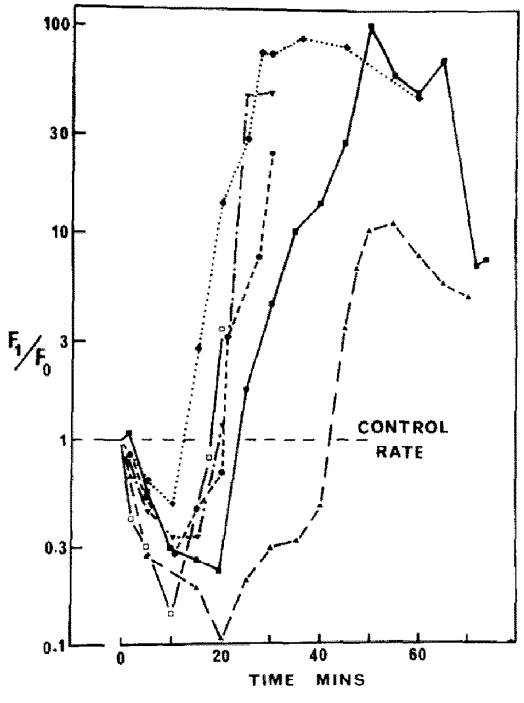

Fig. 1. Effect of DNP on MEPP frequency. Results from six separate experiments shown. Ordinate: frequency of MEPPs (F_1) as a ratio of the control frequency (F_0) before addition of DNP in each experiment. Abscissa: time after addition of 10^{-4} M DNP (min). $[Ca^{2+}]_0 = 1.8 \times 10^{-3}$ M. Temperature = 17°. Essentially similar results were obtained at 25°.

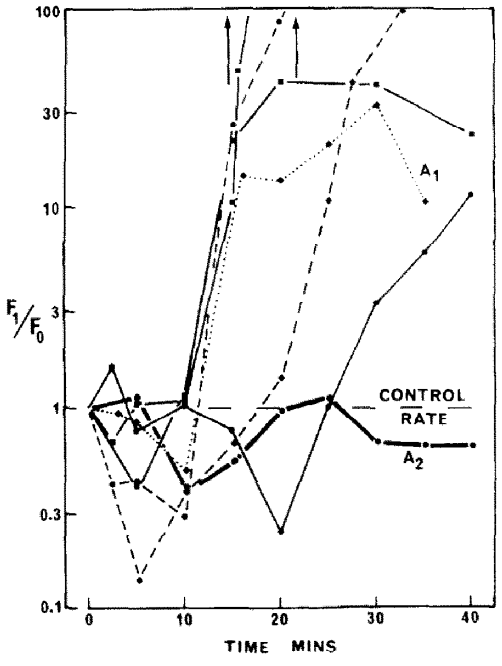

Fig. 2. Effect of DNP on MEPP frequency at low extracellular Ca^{2+}. Details as in Fig. 1, except that $[Ca^{2+}]_0 = 5 \times 10^{-7}$ M, maintained by Ca^{2+}-EGTA buffer system. Seven separate experiments shown; maximum rate in two preparations showed a rate of increase that was greater than 100-fold. Temperature = 17°.

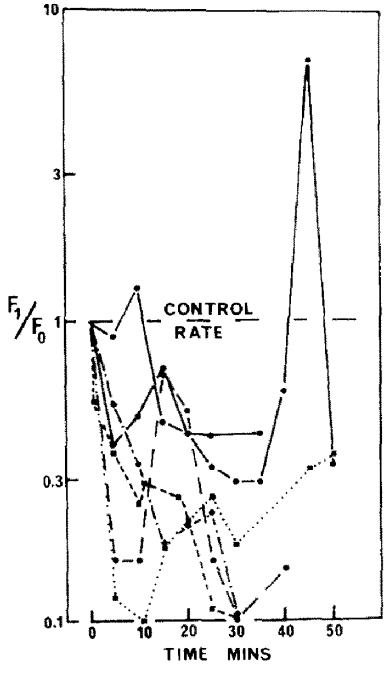

Fig. 3. Effect of DNP on MEPP frequency at low extracellular Ca^{2+}. Details as in Fig. 2. In these preparations the initial fall in MEPP frequency was not followed by the dramatic rise shown in Figs 1 and 2.

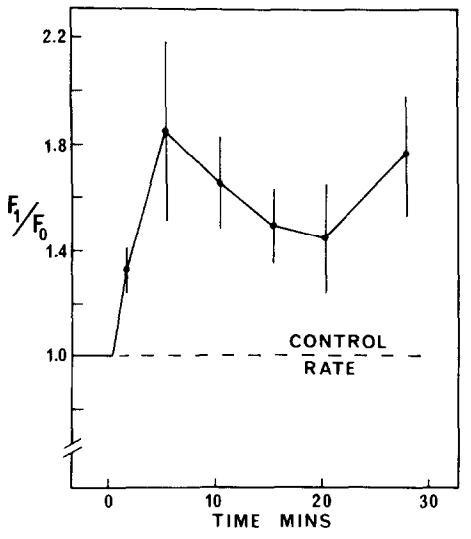

Fig. 4. Effect of theophylline on MEPP frequency. Theophylline = 10^{-3} M; $[Ca^{2+}]_0 = 1.8 \times 10^{-3}$ M; 17°. Points = means of five separate experiments ± S.E.M. Ordinate: frequency of MEPPs (F_1) as a ratio of the control frequency (F_0) before addition of theophylline. Abscissa: time after addition of theophylline.

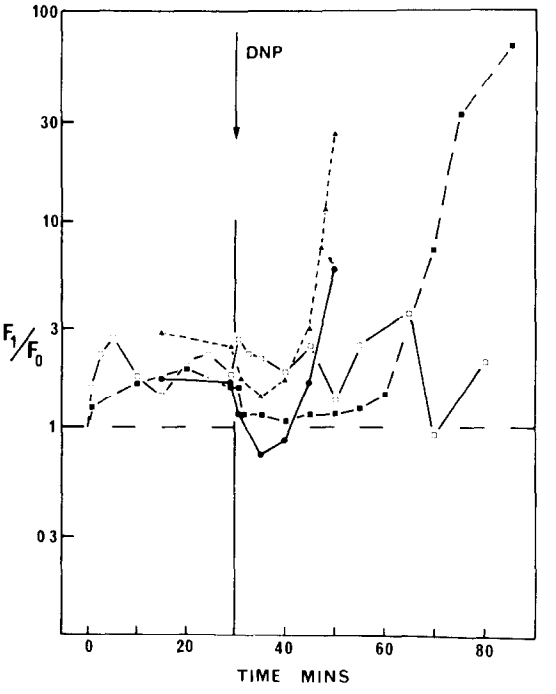

Fig. 5. Interacting effects of theophylline and DNP. The preparation was pretreated with theophylline 10^{-3} M) and DNP (10^{-4} M) was then added at the point shown. Results of four separate experiments. $[Ca^{2+}]_0 = 1.8 \times 10^{-3}$ M. Other details as in Fig. 4.

DNP was therefore tested when the preparation had been equilibrated in a saline containing Ca^{2+} buffered at 5×10^{-7} M, so that $[Ca^{2+}]_0 \simeq [Ca^{2+}]_i$ and Ca^{2+} influx was presumably very low. As a consequence, the steady-state position of $[Ca^{2+}]_i$ is reduced and the absolute MEPP frequency falls to a low rate [6]. Subsequent reductions in F_1/F_0 are, therefore, more difficult to detect but, nevertheless, Figure 2 shows that, under these conditions of low $[Ca^{2+}]_0$ the response to DNP (10^{-4} M) is little affected in many preparations. There is again an initial suppression of the MEPP frequency followed by the usual dramatic increase. However, in other preparations under identical conditions and using the same solutions, a marked fall in MEPP frequency was found, but no subsequent major rise in the rate of spontaneous release was detected. The results of experiments in low $[Ca^{2+}]_0$ of this type are shown in Fig. 3 which confirms that the initial fall in MEPP frequency following DNP treatment is a genuine effect. It is clear, therefore, that the initial fall in MEPP frequency, following DNP treatment, is largely independent of Ca^{2+} influx. Figures 2 and 3 show that, with low $[Ca^{2+}]_0$, there is very different response between different preparations. This difference is illustrated in Figure 2 where the responses labelled A_1 and A_2 are from preparations made from the two cutaneous pectoris muscles of the same frog; in one preparation the later, marked rise in MEPP frequency is seen whereas it is missing in the other.

An alternative explanation for the initial inhibitory effect of DNP would be that it is able to combine with intracellular Ca^{2+} stores, other than the mitochondria, so reducing Ca^{2+}-exchange. Dantrolene sodium has been shown to act in this way, whilst theophylline probably acts in an opposing fashion, by displacing Ca^{2+} from this readily exchangeable store [5]. Theophylline (10^{-3} M) consistently and rapidly causes a significant ($P < 0.01$) increase in MEPP frequency, reaching a maximum effect, an approximate doubling of the rate, in 5 min (see Fig. 4). This action of theophylline has been shown to be independent of external Ca^{2+} and it is unlikely that it operates via a modification of Ca^{2+}-permeability [5]. If the preparation is allowed to equilibrate in 10^{-3} M theophylline for 30 min and 10^{-4} M DNP is then added, there is again an initial suppression of the MEPPs, but the effect is much less marked than when DNP is added without pretreatment with theophylline. The MEPP rate rarely returns to the original control value; the DNP and theophylline appear to be exerting antagonistic effects. The later, stimulatory action of DNP, however, is unaffected by theophylline treatment, confirming the view that the dual action of DNP is the result of two independent effects (Fig. 5).

DISCUSSION

As outlined in the introduction, we believe that $[Ca^{2+}]_i$ is the major factor governing the spontaneous rate of transmitter release [6, 17]. Figure 1 shows that the effect of DNP on the MEPP frequency at the frog neuromuscular junction is a dual one. The results of the experiments with low $[Ca^{2+}]_0$ and with theophylline suggest strongly (1) that both phases of the response to DNP are independent of $[Ca^{2+}]_0$ and are, therefore, probably the result of interactions between DNP and intracellular calcium stores and (2) the initial inhibition is an independent process which is separate from the later, dramatic stimulation. As suggested by previous workers [1, 13, 14] the later, marked rise in MEPP frequency is most

probably the result of mitochondrial uncoupling and the release of stored Ca^{2+}. We believe that the role of the mitochondria, which have a rapid Ca^{2+} uptake mechanism and a slow leak, is likely in this system to be that of a reserve Ca^{2+} buffer mechanism, albeit one that is able to release large quantities of Ca^{2+} into the cytosol when appropriately challenged by pharmacological or chemical agents [8].

The initial fall in MEPP frequency following DNP treatment is still clearly shown when $[Ca^{2+}]_0$ is maintained very low (Figs 2 and 3) and we conclude that DNP does not cause a reduction in Ca^{2+}-influx. Furthermore, under conditions of low $[Ca^{2+}]_0$, the dramatic rise in MEPP frequency was not recorded in some preparations (Fig. 3) and such results suggest that the initial suppression and subsequent rise in spontaneous release are indeed the consequence of two separate phenomena. We suggest that DNP is able to interact with intracellular Ca^{2+} storage sites other than the mitochondria, this interaction promoting a fall in the steady-state position of $[Ca^{2+}]_i$.

We have shown previously [5] that intracellular Ca^{2+}-stores, other than the mitochondria, are important in regulating $[Ca^{2+}]_i$ in the frog presynaptic terminals; dantrolene sodium reduces the leakage from this store whilst theophylline potentiates it (Fig. 4), these two agents having antagonistic effects [5]. This action of theophylline agrees with previous studies that show that caffeine also increases the frequency of MEPPs, even in the absence of external Ca^{2+}, in frog [18] and mammalian motor nerve terminals [19]. These methyl xanthines have two main pharmacological actions: they are phosphodiesterase inhibitors (and hence serve to raise the intracellular levels of cyclic nucleotides) and they can free bound Ca^{2+} from intracellular membranes [20–22], although they probably do not interfere directly with Ca^{2+}-exchange at the mitochondrion [18, 23]. The evidence suggests that cyclic nucleotides do not have an important role in triggering transmitter release either at motor nerve terminals under normal conditions [24, 25] or at the mammalian adrenal [26]. We believe, therefore, that theophylline acts at the presynaptic terminals of the frog neuromuscular junction primarily by facilitating the release of stored Ca^{2+} from intracellular sites. Thus, application of theophylline causes an approximate doubling of MEPP frequency and, if DNP is then added, the initial inhibitory effect of this uncoupling agent is at least partially suppressed (Fig. 5). There are a number of candidates for intracellular Ca^{2+}-binding sites, such as microtubules [27], the endoplasmic reticulum [28] and the inner face of the plasma membrane [5].

It is not clear why, under conditions of low $[Ca^{2+}]_0$, the dramatic release of Ca^{2+} from the mitochondria should be suppressed in certain preparations (Fig. 3). It seems possible that the dual effect of DNP in promoting both a rise and a fall in the steady-state level of $[Ca^{2+}]_i$ is finely balanced and at low $[Ca^{2+}]_0$ its inhibitory action is favoured. Inspection of some of the individual traces in Figs 2 and 3 reveals evidence of this interaction, with an overall reduction in MEPP frequency followed by a modest increase in the rate of spontaneous release.

We conclude (1) that two different intracellular Ca^{2+}-stores (the mitochondria and another unidentified site) are of importance in the control of $[Ca^{2+}]_i$ at the presynaptic terminals and hence in regulating MEPP frequency and (2) that DNP has a dual effect on MEPP frequency and that its action is more complex than hitherto reported. It is able to combine with a readily-exchangeable Ca^{2+} store, reducing Ca^{2+} leakage and causing an initial reduction in MEPP frequency; subsequently it uncouples the mitochondria and the resulting massive Ca^{2+} efflux causes a dramatic increase in the rate of spontaneous transmitter release.

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