British Journal of Pharmacology (1996) 118, 957-960

Dependence on temperature of the effect of dinitrophenol on the release of transmitter quanta at neuromuscular junctions in the mouse diaphragm

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1 The frequencies (F, s^{-1}) of miniature endplate potentials and the quantal content (m) of endplate potentials were measured intracellularly and simultaneously at mouse diaphragm endplates in a bathing solution that contained 0.6 mM Ca^{2+} ions and 5 mM Mg^{2+} ions.

2 Twin pulses at 4 ms intervals gave the quantal contents of the first (m1) and second (m2) responses. The ratio of m2/m1 was taken as an indicator of the temporal facilitation of the release of transmitter.

3 Dinitrophenol (DNP, 10 μM) increased the values of F and m at 36°C. This effect did not depend on extracellular Ca^{2+} ions.

4 The potentiating effect of DNP disappeared at 24°C but the value of m2/m1 remained constant.

5 These results suggest that the effect of DNP is modifiable by temperature which can affect systems that control the intracellular metabolism of Ca^{2+} ions.

Keywords: Dinitrophenol; temperature; temporal facilitation; mouse diaphragm; neuromuscular junction

Introduction

Dinitrophenol (DNP) is a well known uncoupler of electron transport and the synthesis of ATP in mitochondria, where it acts as a protonophore (Loomis & Lippman, 1948). It can reversibly block the excitability of cortical neurones by inducing an increase in potassium conductance (see Erulkar & Fine, 1979), as well as by enhancing the Ca^{2+}-activated current of K^+ ions in rabbit portal vein (Miller *et al.*, 1993). Such a blockade is considered to be due to an increase in the concentration of Ca^{2+} ions within the cytoplasm as a result of the release of Ca^{2+} ions from mitochondria (see Erulkar & Fine, 1979). The release of prostaglandin E_2 that is induced by DNP in rat skeletal muscle can be abolished by organic calcium antagonists, such as nifedipine and verapamil (Majumdar *et al.*, 1994). These results imply that DNP stimulates the influx of Ca^{2+} ions through voltage-dependent channels. The intracellular concentration of Ca^{2+} ions is approximately 10,000 fold lower than its extracellular levels. This gradient is established by the operation of membrane pumps and intracellular storage of Ca^{2+} ions (Murad, 1990). Intracellular Ca^{2+} ions appear to trigger the mechanism that is responsible for the release of transmitter (Katz & Miledi, 1967). Thus, it seems possible that DNP might modify the ability of motor nerve terminals to release transmitter quanta. The present experiments were designed to examine this possibility and to clarify the characteristics of the effect of DNP on the quantal release of transmitter at neuromuscular junctions in the mouse diaphragm.

Methods

Experiments were performed with isolated left phrenic nerve-hemidiaphragm preparations from male *ddY* mice of 8 to 12 weeks of age. The preparation was pinned to a silicone resin, which lined the bottom of a Perspex bath with a capacity of about 30 ml, and it was soaked in Krebs-Ringer solution. The solution was constantly recirculated by means of an 'oxygen lift' system. The bathing solution had the following composition (mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15 and glucose 11. A Ca^{2+}-deficient solution was prepared with addition of 2 mM EGTA (ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid). A bathing solution containing 5 mM Mg^{2+} ions and 0.6 mM Ca^{2+} ions was also prepared for measurements of endplate potentials (e.p.ps). The bathing solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at pH 7.4 and at 36°C, except where otherwise stated. The temperature of the solution in the bath was monitored with a thermister (model MGA-II; Shibaura Electric Co., Tokyo) and held constant by means of an external water jacket and a thermoregulatory device (Thermominder Mini 80; Taiyo, Tokyo) during each experiment.

Intracellular recordings were made with glass microcapillary electrodes, which were filled with 3 M KCl and each of which had a resistance of 4 to 6 MΩ. The electrode was inserted into fibres at the endplate to record potential changes. The signals were led through a high-impedance, unity-gain preamplifier (MEZ-8201; Nihon Kohden, Tokyo) attached to an oscilloscope (VC-11; Nihon Kohden), and stored on an FM instrumentation tape recorder (RMG-5204; Nihon Kohden) when necessary.

Preparations were exposed to the test bathing solution for 20 min before measurements of changes in potential. At each endplate, miniature endplate potentials (m.e.p.ps) were initially recorded for about 1 min, and then the nerve trunk was activated with pulses of 0.1 ms in duration at a supramaximal voltage from an electronic stimulator (SEN-3201; Nihon Kohden) through a suction electrode. Paired pulses with intervals of 4 ms were given 128 or 256 times with intervals of 1.5 s. This trial resulted in a record of e.p.ps of the first and second responses. The measurements were terminated within 3 h of the start of the experiment.

The quantal content (m) was estimated by the method of failures from the following formula:

$$m = \log_e(N/N_0),$$

where N is the number of trials and N_0 is the number of failures (Crawford, 1974).

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Student's t tests were used for statistical analyses and a value of $P<0.05$ was deemed statistically significant.

Drugs used were dinitrophenol (Maruwaka Chemicals, Osaka, Japan) and ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N'*,N'-tetraacetic acid (Sigma, St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade.

Results

Potential changes with amplitudes of 0.1 mV or more and with rise times of 1 ms or less occurred spontaneously at each endplate with noise levels of less than 50 μV, being typical examples of m.e.p.ps. The rate of spontaneous release of transmitter quanta was expressed as the frequency (F, s^{-1}) of m.e.p.ps. In the first trial, the effect of DNP (10 μM) on F was examined (Figure 1). DNP had a stimulatory effect with or without Ca^{2+} ions in the bathing solution. Such a stimulatory effect on F disappeared at 24°C (Figure 2) and thus, it was clearly temperature-dependent. Burst-like potential changes at endplates were sometimes recorded from the preparations that were exposed to DNP, but such changes were not included in the results. At concentrations of 50 μM or higher, DNP frequently caused muscle contraction at 36°C that dislodged the electrode from the endplates and made continuous recording of the potential changes impossible. All of these effects disappeared at 24°C within an exposure period of about 3 h.

The stimulation with twin pulses resulted in two sequential e.p.ps with amplitudes and rise times similar to those of the m.e.p.ps recorded in the same bathing solution. The quantal contents of these e.p.ps, m1 and m2, were estimated by the method of failures. The mean value of m2 was significantly larger than that of m1. Larger values of m2 reflect the increase in the amount of transmitter released in response to the second impulse. This phenomenon is known as temporal facilitation. The ratio of m2/m1 can be taken as an index of such facilitation (Del Castillo & Katz, 1954). Figure 2 shows the effect of DNP on the quantal content (m1) of e.p.ps. DNP the value of m1 at 36°C increased. When the temperature was lowered to 24°C it increased the control level of m1. The stimulatory effect of DNP was blocked at 24°C and thus, it was temperature-dependent. The ratio of m2/m1 did not change in the presence or absence of DNP at either temperature.

Discussion

Dinitrophenol, at 10 μM, increased the rate of asynchronous release of transmitter at 36°C, but this effect was blocked at 24°C. The stimulatory effect was insensitive to the concentration of Ca^{2+} ions in the bathing solution. It is widely accepted that the resting activity for the release of transmitter quanta depends on the internal concentration of Ca^{2+} ions in the nerve terminal (Dudel, 1989; Åkerman & Nicholls, 1983) and on this concentration depends, in turn, the ability of cells to buffer the internal concentration of Ca^{2+} ions at the terminals (Rahamimoff *et al.*, 1978). These observations suggest that, at 36°C, DNP might mobilize Ca^{2+} ions from internal storage sites, such as mitochondria. The effect of DNP might reflect its ability to uncouple oxidative phosphorylation (Loomis & Lippman, 1948). Thus, it is proposed that DNP increases levels of Ca^{2+} ions in the cytoplasm as a result of the release of Ca^{2+} ions from mitochondria (see Erulkar & Fine, 1979).

The evoked release of transmitter is supported mainly by an increase in the influx of Ca^{2+} ions (Katz & Miledi, 1965). The quantal content of e.p.ps increased in the presence of DNP in a temperature-dependent manner. This effect of DNP might be based, in part, on the stimulation of the influx of Ca^{2+} ions through voltage-dependent channels in the presence of external Ca^{2+} ions (Majumdar *et al.*, 1994), as well as on the possible mobilization of Ca^{2+} ions from internal storage sites, as mentioned above.

At 24°C, the spontaneous rate of release of transmitter was very low and it was insensitive to DNP. It is well known that

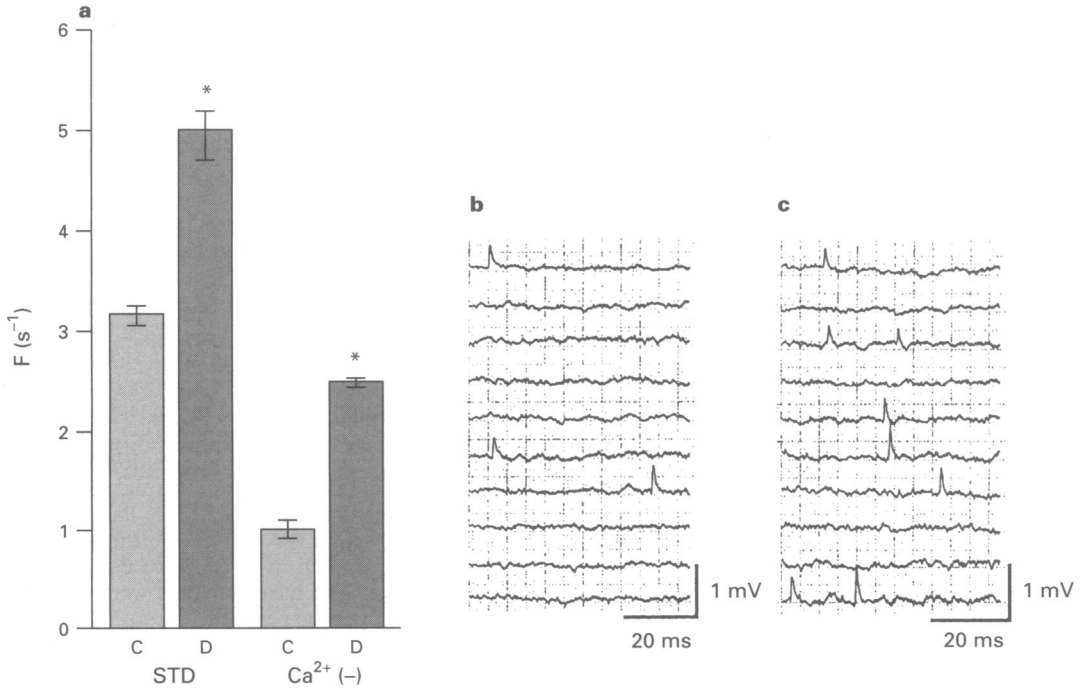

Figure 1 (a) Effects of dinitrophenol on the frequency of m.e.p.ps at neuromuscular junctions of the mouse diaphragm in bathing solutions with or without Ca^{2+} ions at 36°C. Representative results before (b) and after (c) exposure to dinitrophenol (10 μM) on m.e.p.ps. STD, Standard bathing solution; Ca^{2+}(-), STD without Ca^{2+} ions; C, control; D, dinitrophenol (10 μM); F, frequency of m.e.p.ps. n (C in STD) = 256 endplates of 11 preparations; n (D in STD) = 137 endplates of 11 muscles; n[C in Ca^{2+}(-)] = 288 endplates of 16 preparations; n [D in Ca^{2+}(-)] = 280 endplates of 8 muscles.

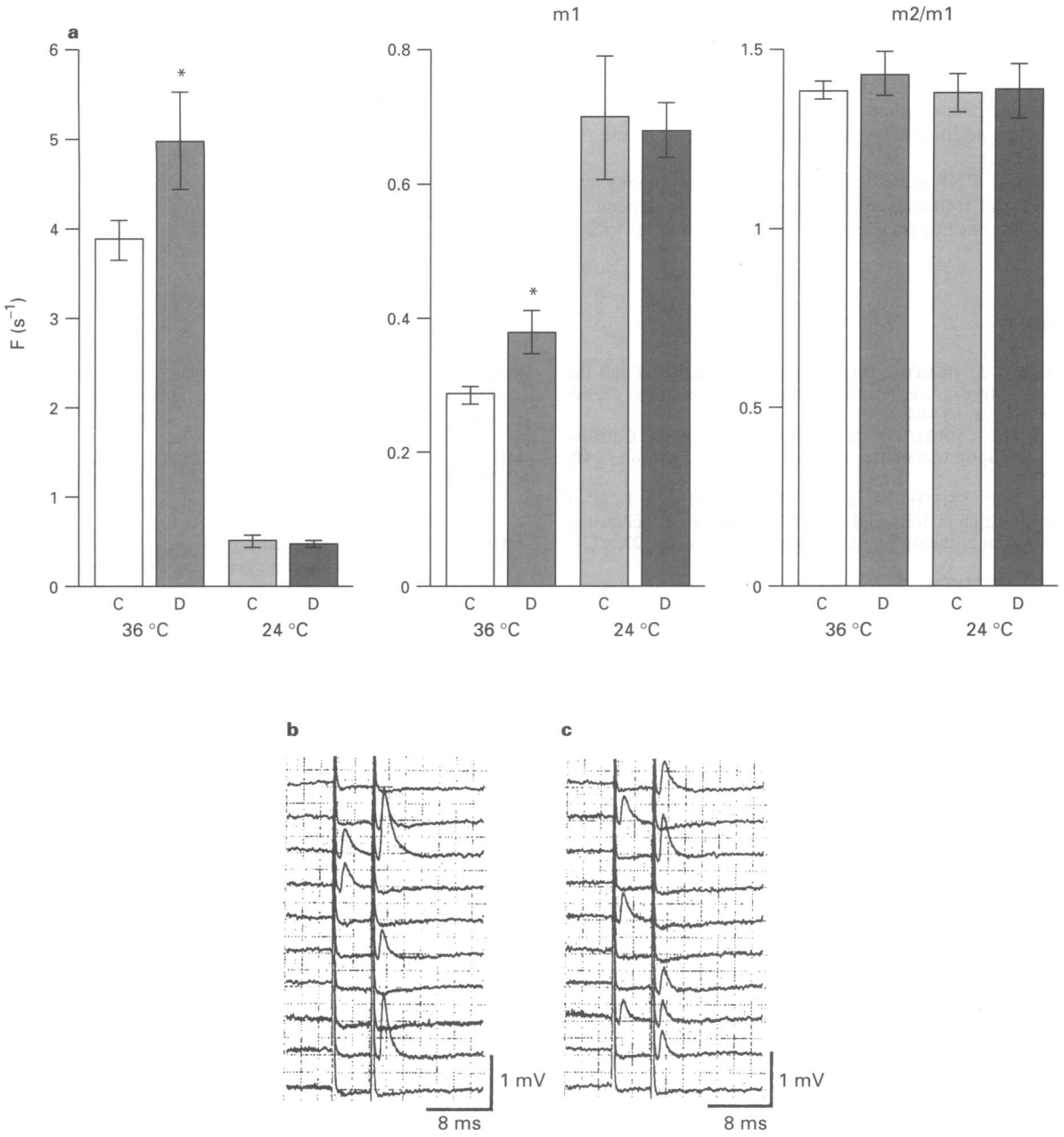

Figure 2 (a) Dependence on temperature of the potentiating effect of dinitrophenol on the release of transmitter quanta at neuromuscular junctions in the mouse diaphragm. Representative results before (b) and after (c) exposure to dinitrophenol (10 μM) on e.p.ps evoked by twin-pulses. F, frequency of m.e.p.ps; m, quantal content of e.p.ps; C, control; D, dinitrophenol (10 μM); $*P<0.05$. Experiments were conducted in a bathing solution that contained 0.6 mM Ca^{2+} ions and 5 mM Mg^{2+} ions.

low temperature reduces the fluidity of membranes. A reduction of membrane fluidity might inhibit the uptake of DNP by the nerve terminals, leading to the reduced activity of this agent. The lipophilicity and monoanionic character of DNP are very important for the effective action of DNP (Winter *et al.*, 1994). A reduction in temperature from 36°C to 24°C increases the evoked release of transmitter, as shown recently (Nishimura *et al.*, 1993a). This effect of temperature, in rat diaphragm muscle, has been proposed to be due to an increase in the probability that quanta are released (Hubbard *et al.*, 1971). At 24°C, the rate of spontaneous release is very low and is independent of changes in millimolar levels of external Ca^{2+} ions (Nishimura *et al.*, 1993b); that is, at 24°C, the evoked release is dependent almost entirely on the external level of Ca^{2+} ions. The increased rate of the evoked release of transmitter must reflect a larger influx of Ca^{2+} ions upon the depolarization of the nerve terminal. The effect of temperature might mask the possible stimulatory effect of DNP on the influx of Ca^{2+} ions, in addition to the reduction of membrane fluidity by low temperature.

The mechanism for the release of transmitter upon paired stimulation is characterized by temporal facilitation. Such synaptic facilitation might reflect the ability of nerve endings to reduce the internal concentration of Ca^{2+} ions, as discussed by Katz & Miledi (1968) and Rahamimoff (1968). The cited authors proposed the 'residual calcium theory' in which intraterminal 'residual Ca^{2+} ions' from the first of a pair of pulses are the basis for twin-pulse facilitation, namely, the increase in quantal release that occurs in response to the second pulse. Two other groups (Charlton *et al.*, 1982; Augustine *et al.*, 1987), using the giant synapse of the squid, demonstrated that an increase in the intracellular concentration of Ca^{2+} is the basis for facilitation after arrival of an action potential at the nerve terminal. The resting level of the in-

tracellular concentration of Ca^{2+} ions must also affect facilitation (Dudel, 1989). According to these observations, facilitation should be modifiable by agents that affect the level and distribution of Ca^{2+} ions inside the cell. However, DNP had no effect on this phenomenon. This result suggests the possible existence of an additional mechanism that is responsible for this phenomenon.

In summary, DNP increased the rates of spontaneous and evoked release of transmitter in a manner that was dependent on temperature but not on the extracellular level of Ca^{2+} ions.

This work was supported by a Grant-in-Aid for Scientific Research (No. 04454118) from the Ministry of Education, Science, Sports and Culture (Japan) to M.N. and by a grant from the Suhara Memorial Foundation to M.N.

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(Received November 7, 1995
Revised February 29, 1996
Accepted March 8, 1996)