*Br. J. Pharmacol.* (1988), **93**, 430–436

# $Zn^{2+}$ stimulates spontaneous transmitter release at mouse neuromuscular junctions

Masakazu Nishimura

Department of Veterinary Pharmacology, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan

**1** Experiments were carried out to examine the effect of $Zn^{2+}$ on the rate of spontaneous release of transmitter at the neuromuscular junction of the mouse diaphragm muscle, in the presence and absence of external $Ca^{2+}$. Miniature endplate potentials (m.e.p.ps) were measured *in vitro*.

**2** $Zn^{2+}$ markedly elevated the frequency of m.e.p.ps without affecting the resting membrane potential of muscle fibres. This effect was time- and concentration-dependent but was independent of the presence of external $Ca^{2+}$. In a $Ca^{2+}$-free bathing solution, $Zn^{2+}$ frequently produced twitching in several fibres. The twitching dislodged the microelectrode. Replacement of the 10 mM NaCl in the $Ca^{2+}$-free solution with equimolar KCl overcame this difficulty. The experiments summarized below were done in the $Ca^{2+}$-free bathing solution which contained 10 mM KCl instead of 10 mM NaCl.

**3** The effect of $Zn^{2+}$ was transient and required a latent period of many minutes. Low temperature (24°C) increased the length of this latent period and reduced the maximum effect of $Zn^{2+}$.

**4** $Zn^{2+}$ increased the frequency of m.e.p.ps in $K^+$-free (replaced with NaCl) solution. The effect appeared with shorter latency in this solution compared to the standard Krebs-Ringer solution.

**5** The effect of $Zn^{2+}$ was partially antagonized by dantrolene sodium or by neomycin. Both agents also reduced the effect of external $Ca^{2+}$ on m.e.p.ps in depolarizing solution.

**6** $Cd^{2+}$ and 2,3-bisphosphoglycerate also elevated the frequency of m.e.p.ps in a manner independent of external $Ca^{2+}$, but the latter compound was much less potent than $Cd^{2+}$.

**7** These experiments provide evidence for a role of intracellularly stored $Ca^{2+}$ in the release of transmitter at the motor nerve terminal. The release of $Ca^{2+}$ from the storage site may be coupled with the metabolism of phosphatidylinositol.

## Introduction

$Zn^{2+}$ has an epileptogenic effect in rats when injected intracerebroventricularly (Donaldson *et al.*, 1971). In the neurones of the guinea-pig olfactory cortex, $Zn^{2+}$ dramatically prolongs excitatory postsynaptic potentials, possibly via some presynaptic action of $Zn^{2+}$ (Smart & Constanti, 1983). It has been suggested that such a hypothetical presynaptic effect may contribute to the epileptogenic effect of intracerebroventricular $Zn^{2+}$. This cation decreases transmitter release depending on extracellular $Ca^{2+}$ at the neuromuscular junctions of amphibian (Benoit & Mambrini, 1970) and chick (Lin-Shiau & Fu, 1980) preparations, and this inhibition is due to competition with $Ca^{2+}$ at the motor nerve terminal (Nishimura, unpublished data).

It has been shown that $Zn^{2+}$ is a potent inhibitor of inositol 1,4,5-triphosphate ($Ins1,4,5P_3$) 5-phosphatase, as are also $Cd^{2+}$, $Ag^{2+}$ and 2,3-bisphosphoglycerate (Storey *et al.*, 1984). This enzyme dephosphorylates $Ins1,4,5P_3$ to generate free inositol (Storey *et al.*, 1984). Thus, inhibition of this enzyme allows the accumulation of $Ins1,4,5P_3$ inside the cell. When $Ins1,4,5P_3$ diffuses into the cytosol $Ca^{2+}$ is released from the endoplasmic reticulum (Berridge, 1984; Berridge & Irvine, 1984). The increase in intracellular $Ca^{2+}$ induces cellular functions such as muscle contraction (Vergara *et al.*, 1985). If this sequence of events occurs when $Zn^{2+}$ is present at the motor nerve terminals, then $Zn^{2+}$ should stimulate the release of transmitter. The present experiments were undertaken to test this possibility and revealed a dramatic, stimulatory effect of $Zn^{2+}$ on the rate of spontaneous release of transmitter, in a manner which was insensitive to external $Ca^{2+}$.

## Methods

Experiments were performed on preparations of isolated, left hemidiaphragm from male *ddy* mice of 11 to 20 weeks old. The preparation was pinned to a silicone resin lining on the bottom of a plastic chamber of about 30 ml capacity, and was soaked in Krebs-Ringer solution. The solution was constantly recirculated by means of an 'oxygen lift' system. The Krebs-Ringer solution was of the following composition (mM): NaCl 136, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15, glucose 11. The $Ca^{2+}$-deficient solution was prepared without addition of EDTA (ethylenediaminetetraacetic acid). The $K^+$-free solution was prepared by replacing 5 mM KCl in the standard Krebs-Ringer solution with equimolar NaCl. All solutions were bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ and maintained at pH 7.3 and 36°C except where otherwise stated. The temperature of the solution in the bath was monitored by a thermistor (Shibaura Electric Co., Model MGA-II) and held constant by means of an external water jacket and a thermoregulatory device (Taiyo, Thermominder Mini 80) during each experiment, at temperatures of 24 and 36°C. To depolarize the presynaptic endings (Liley, 1956), the preparation was equilibrated in Krebs-Ringer solution in which 10 mM NaCl was replaced by equimolar KCl (10 mM $K^+$). The preparation was equilibrated in the 10 mM $K^+$ solution for at least 30 min before addition of any agent.

Drugs used: zinc chloride, cadmium chloride (from Wako), 2,3-bisphosphoglycerate (BPG) (from Sigma), dantrolene sodium (DaNa) (donated by Yamanouchi Pharmaceutical Co., Tokyo) and neomycin sulphate (from Wako). All other chemicals were of analytical grade.

Intracellular recordings were made with glass microelectrodes, filled with 3M KCl, of 6 to 8 MΩ resistance. The electrode was inserted into fibres near endplate regions. The signals were led through a high-impedance, unity-gain preamplifier (Nihon Kohden, MEZ-8201), displayed on an oscilloscope (Nihon Kohden, VC-10) and stored on magnetic tape (Nihon Kohden, RMG-5204).

Recorded miniature endplate potentials (m.e.p.ps) were played back via an analogue to digital converter into a microcomputer (Nihon Kohden, DAB-1100) which was used to calculate the mean amplitude and frequency of m.e.p.ps of 0.1 mV or larger and of 1 ms or less rise times at each endplate, and to plot amplitude and frequency distribution histograms via an X-Y recorder (Nihon Kohden, WX4411). M.e.p.ps were recorded for successive periods of 1 min after exposure to a given solution; from these data the mean frequency of m.e.p.ps ($s^{-1}$) was calculated. Student's *t* tests were used for statistical analysis and a probability of less than 0.05 was deemed statisticaly significant.

## Results

### *Effect of $Zn^{2+}$ on m.e.p.ps*

Figure 1 represents examples of m.e.p.ps and histograms of amplitude for m.e.p.ps from a diaphragm preparation in a standard bathing solution with or without 50 μM $Zn^{2+}$. M.e.p.ps markedly increased in number in the presence of 50 μM $Zn^{2+}$. The increase was usually gradual and took in transitory bursts of high frequency at the maximal effect. In the presence of $Zn^{2+}$, m.e.p.ps did not change their amplitude. M.e.p.ps were measured at an endplate of a diaphragm preparation in a standard solution which contained 100 μM $Zn^{2+}$, over a period of 50 min. The prolonged exposure led to an increase in the frequency of m.e.p.ps (Figure 2). Similar exposure to the standard solution without $Zn^{2+}$ maintained constant rates. The effect of $Zn^{2+}$ required a latent period and occurred transiently. Both the latent period and the duration of the effect varied from fibre to fibre and ranged from 25 to 40 min, and from 15 to 30 min, respectively. Thus, in the presence of $Zn^{2+}$, the maximal effect did not occur simultaneously at all junctions. The peak values were between 68 and 128 $s^{-1}$ in the presence of 100 μM $Zn^{2+}$. A lower concentration of $Zn^{2+}$ (50 μM) produced a similar but less pronounced effect on the frequency of m.e.p.ps. The resting membrane potential of the muscle in the endplate region ($-75.2 \pm 2.1$ mV, mean ± s.e.mean, $n = 26$ endplates of 8 muscles) in the standard solution was not altered by the presence of 100 μM $Zn^{2+}$ ($-73.8 \pm 2.0$ mV, $n = 22$ endplates of 5 muscles) or 50 μM $Zn^{2+}$ ($-74.4 \pm 2.2$ mV, $n = 20$ endplates of 3 muscles).

### *Effect of removal of $Ca^{2+}$*

Removal of $Ca^{2+}$ from the normal solution decreased the membrane potential of the muscle in the endplate region from $75.0 \pm 2.1$ mV ($n = 22$ endplates of 4 muscles in the standard solution) to $70.8 \pm 2.1$ mV ($n = 28$ endplates of 4 muscles in $Ca^{2+}$-free solution). Removal of $Ca^{2+}$ reduced the frequency of m.e.p.ps from $3.62 \pm 0.23$ $s^{-1}$ ($n = 22$ endplates of 4 muscles) to $2.06 \pm 0.20$ $s^{-1}$ ($n = 28$ endplates of 4 muscles). In the $Ca^{2+}$-free solution, recording of m.e.p.ps was possible only intermittently when $Zn^{2+}$ was added, because twitching occurred in several fibres immediately after the addition of $Zn^{2+}$ and this dislodged the glass electrode. When recording was possible, the stimulatory effect of $Zn^{2+}$ was observed with rather short latent periods compared to the effect observed in the standard solution. To overcome this difficulty, 10 mM NaCl in the $Ca^{2+}$-free solution was replaced with 10 mM KCl to give a $Ca^{2+}$-free, 10 mM $K^+$ bathing solution. This solution gave a mean resting potential of $53.2 \pm 0.7$ mV and a mean frequency of

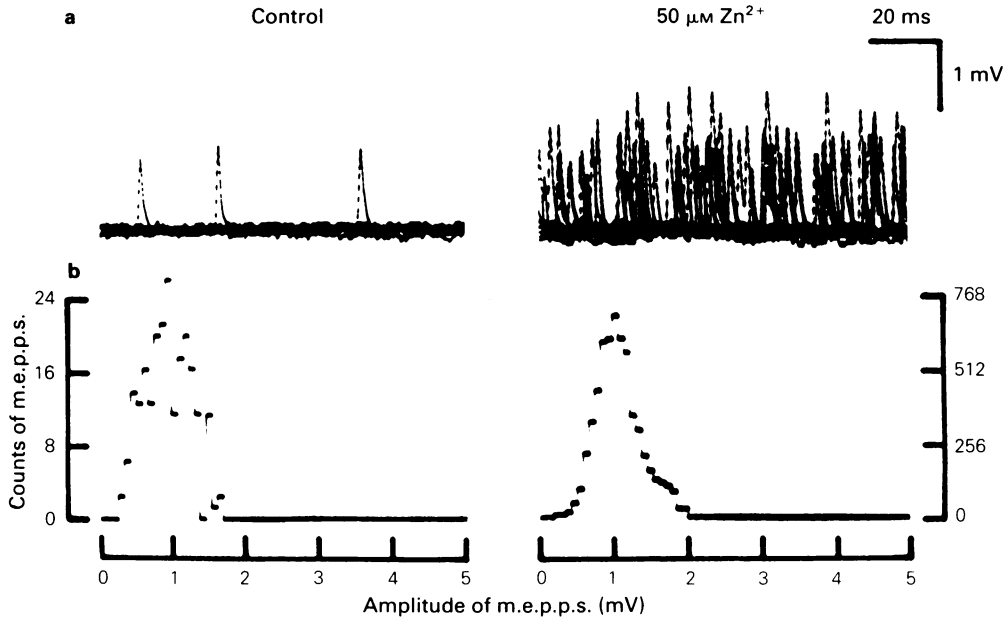


**Figure 1** Examples of m.e.p.ps (a) and histograms of amplitude for m.e.p.ps (b) from a diaphragm muscle of a mouse. All the measurements were recorded from the same fibre. (a) Control m.e.p.ps were recorded 60 min after soaking in a standard Krebs-Ringer solution (superimposed tracing of 10 sweeps); and (right) 40 min after exposure to 50 μM $Zn^{2+}$ in the standard bathing solution (superimposed tracing of 10 sweeps). (b) (left) Control histogram showing a distribution of 178 counts of m.e.p.ps during 1 min (60 min after soaking in the standard bathing solution); (right) histogram showing a distribution of 4757 counts of m.e.p.ps during 1 min (40 min after soaking in the 50 μM $Zn^{2+}$-bathing solution). Resting membrane potentials were between −72 to −75 mV. $Zn^{2+}$ markedly increase the number of m.e.p.ps without altering their amplitude.

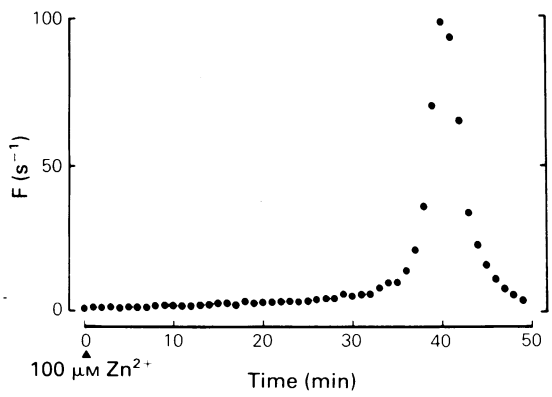


**Figure 2** Time course of an effect of 100 μM $Zn^{2+}$ on the frequency of m.e.p.ps (F, $s^{-1}$) at a mouse neuromuscular junction in Krebs-Ringer solution at 36°C. Ordinate scale m.e.p.p. frequency; abscissa scale, time in min after addition of $Zn^{2+}$. The frequency-elevating effect is transient.

m.e.p.ps of $1.86 \pm 0.07\,s^{-1}$ ($n = 226$ endplates of 56 muscles). The increase in the concentration of KCl suppressed the twitching caused by $Zn^{2+}$. Thus, the subsequent experiments, described below, were performed in this $Ca^{2+}$-free, 10 mM $K^+$ bathing solution.

The presynaptic effect of $Zn^{2+}$ occurred transiently with variable latent periods, as was seen in the standard solution. At most junctions, the latent period for the effect of $Zn^{2+}$ was shortened in the $Ca^{2+}$-free, depolarizing solution; the latent period was 10 to 15 min in the presence of 100 μM $Zn^{2+}$. The effect of $Zn^{2+}$ was detectable over a concentration range from 10 to 100 μM. The latent periods for the effect increased with a decrease in the concentration of $Zn^{2+}$. The maximal effect was dependent on the concentration of $Zn^{2+}$.

*Effect of temperature*

A mean frequency of m.e.p.ps in the $Ca^{2+}$-free, 10 mM $K^+$ bathing solution at 36°C was $1.86 \pm 0.07\,s^{-1}$ ($n = 226$ endplates of 56 muscles). At 24°C, the mean frequency fell to $0.42 \pm 0.16\,s^{-1}$

($n$ = 39 endplates of 6 muscles). $Zn^{2+}$ (50 $\mu$M) was still able to increase the frequency of m.e.p.ps at 24°C (Figure 3). However, the low temperature lengthened the latent period and also increased the duration of the effect of $Zn^{2+}$. The peak value of the frequency of m.e.p.ps in the presence of 50 $\mu$M $Zn^{2+}$ decreased significantly from $46.3 \pm 3.57\,s^{-1}$ ($n = 8$ muscles, 36°C) to $18.8 \pm 4.2\,s^{-1}$ ($n = 6$ muscles, 24°C).

*Blockade of transmitter release*

The stimulatory effect of $Zn^{2+}$ declined with the passage of time as mentioned above. During the decline in the effect of $Zn^{2+}$, 2 mM $Ca^{2+}$ was added to the $Ca^{2+}$-free, 10 mM $K^+$ bathing solution which contained 50 $\mu$M $Zn^{2+}$. The addition of $Ca^{2+}$ did not elevate the frequency of m.e.p.ps. In a second series of experiments, a stimulatory effect of 2 mM $Ca^{2+}$, when added to the $Ca^{2+}$-free, 10 mM $K^+$ bathing solution which contained 50 $\mu$M $Zn^{2+}$ was observed with reduced potency within 10 min after the addition of $Zn^{2+}$. Thus, the decline of the effect of $Zn^{2+}$ with time indicates a blockade of transmitter release or of a significant reduction in the pool of available vesicles.

*Effects of chemical agents or procedures*

Some preparations were rinsed several times for about 30 min in $K^+$-free solution. Then 50 to 100 $\mu$M $Zn^{2+}$ was given to them. $K^+$-free solution alone slightly elevated mean frequency of m.e.p.ps ($4.8 \pm 0.44\,s^{-1}$, $n = 26$ endplates of 6 muscles). In this solution, $Zn^{2+}$ markedly elevated the frequency of m.e.p.ps with rather shorter period of latency than in the standard Krebs-Ringer solution (Figure 4). The effect of 50 $\mu$M $Zn^{2+}$ on the frequency of m.e.p.ps was examined in the presence of 20 $\mu$M dantrolene sodium (DaNa) or 200 $\mu$M neomycin. Figure 5 summarizes the results. Both agents were able to depress the effect of 0.5 mM $Ca^{2+}$ on the frequency of m.e.p.ps in the solution which contained 10 mM $K^+$, whereas they did not affect the frequency of m.e.p.ps in the $Ca^{2+}$-free, depolarizing solution. The treatment with these agents partially inhibited the effect of 50 $\mu$M $Zn^{2+}$.

*Effects of other agonists*

$Cd^{2+}$ and 2,3-bisphosphoglycerate (BPG) were examined for their effect on the frequency of m.e.p.ps in the $Ca^{2+}$-free, 10 mM $K^+$ bathing solution. Both agents were able to raise the frequency of m.e.p.ps in this solution. Their effects required latent periods. The effect of $Cd^{2+}$ was transient. However, no decline of the effect of BPG was observed during the observation period (within 60 min). $Cd^{2+}$ were able to reproduce the effect of $Zn^{2+}$ at similar concentrations (Figure 6). The effect of BPG was much less potent than those of $Zn^{2+}$ and $Cd^{2+}$. The frequency of m.e.p.ps was $6.66 \pm 0.43\,s^{-1}$ ($n = 20$ endplates of 5 muscles) when measured from 50 to 60 min after the addition of 100 $\mu$M BPG. Higher concentrations of BPG (> 200 $\mu$M) decreased the amplitude of m.e.p.ps frequently making it impossible to measure them.

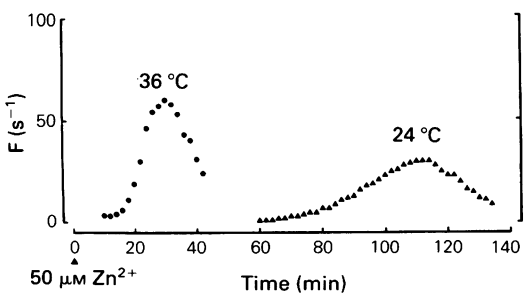


**Figure 3** Effect of 50 $\mu$M $Zn^{2+}$ on m.e.p.p. frequency in $Ca^{2+}$-free, 10 mM $K^+$ bathing solution at 24°C compared to that at 36°C. Ordinate scale, m.e.p.p. frequency (F, $s^{-1}$); abscissa scale, time in min after addition of $Zn^{2+}$, early recordings of the frequency of m.e.p.ps are omitted in the case of the low temperature.

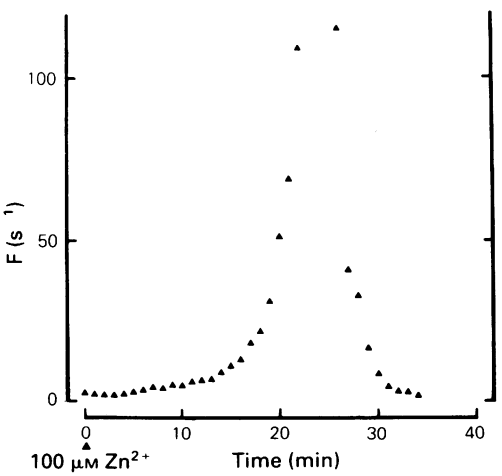


**Figure 4** Effect of 100 $\mu$M $Zn^{2+}$ on the frequency of m.e.p.ps at a mouse neuromuscular junction in $K^+$-free Krebs-Ringer solution. A preparation was pre-soaked for about 30 min in $K^+$-free bathing solution and washed with this solution, then 100 $\mu$M $Zn^{2+}$ was added. Ordinate scale, frequency of m.e.p.p. (F, $s^{-1}$); abscissa scale, time in min after addition of 100 $\mu$M $Zn^{2+}$. The results shown are typical of three similar observations.

## Discussion

The present experiments indicate that $Zn^{2+}$ elevates the frequency of m.e.p.ps at the mouse neuromuscular junction. Factors controlling the frequency of m.e.p.ps are presynaptic in nature (del Castillo & Katz, 1954). The frequency of m.e.p.ps is equivalent to the rate of spontaneous quantal release of transmitter (Katz, 1966). Thus, $Zn^{2+}$ must raise the rate of spontaneous release of transmitter by acting at a presynaptic site. The effect of $Zn^{2+}$ did not depend upon the presence of external $Ca^{2+}$. According to the 'calcium hypothesis' (Katz & Miledi, 1965), the release of transmitter is mediated by $Ca^{2+}$ inside the nerve ending (Katz & Miledi, 1965; Rahamimoff & Yaari, 1973). $Ca^{2+}$ utilized for the release of transmitter can be supplied from intracellular storage sites (Blaustein *et al.*, 1980; Brinley, 1980). The concentration of free $Ca^{2+}$ inside the nerve terminal is very low in a resting state. Thus, the presynaptic effect of $Zn^{2+}$ is probably mediated by increased levels of $Ca^{2+}$ in the nerve terminal, which must be supplied from an intracellular store of $Ca^{2+}$. This hypothesis is supported by the results of experiments presented here, in which the effect of $Zn^{2+}$ was reduced to some extent in the presence of DaNa. This antagonist has been shown to inhibit the release of $Ca^{2+}$ from intracellular storage (Desmedt & Hainaut, 1977) and also to reduce the release of transmitter stimulated by caffeine and theophylline from the motor nerve terminal (Statham & Duncan, 1976). The present experiments also indicate that DaNa reduced the effect of external $Ca^{2+}$ on the rate of spontaneous release of transmitter, from the nerve terminal equilibrated in depolarizing solution.

The stimulatory effect of $Zn^{2+}$ may possibly contribute to its epileptogenic activity in the central nervous system when it is injected intracerebroventricularly in the rat (Donaldson *et al.*, 1971). It has been demonstrated that $Zn^{2+}$ potently inhibits $Na^+$, $K^+$-ATPase activity of rat brain microsomes *in vitro* and that low levels of activity of this enzyme can be measured locally in the rat brain treated intraventricularly with $Zn^{2+}$. The intracerebroventricular $Zn^{2+}$ is epileptogenic, and ouabain, a specific inhibitor of $Na^{2+}$, $K^+$-ATPase, is similarly epileptogenic. At the mouse neuromuscular junction, ouabain facilitates the rate of transmitter release in a $Ca^{2+}$-free, high $K^+$ solution (Nishimura, 1986). This effect of ouabain is much less potent than the effect of $Zn^{2+}$ observed in the present study. The present experiments revealed no blocking effect of $K^+$-free solution on the $Zn^{2+}$-induced increase in the rate of release of transmitter. Thus, although the inhibition of $Na^+$, $K^+$-ATPase may be an important factor, it may not be the only mechanism that can explain the cellular response. There is growing evidence that $Zn^{2+}$ may act by stimulating an

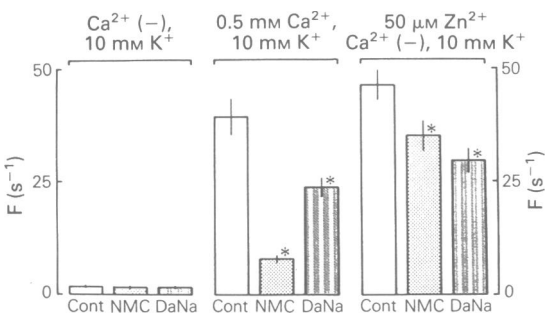


**Figure 5** Effects of 0.5 mM $Ca^{2+}$ and 50 μM $Zn^{2+}$ on the frequency of m.e.p.ps at the mouse neuromuscular junction in $Ca^{2+}$-free, 10 mM $K^+$ bathing solution with or without 200 μM neomycin (NMC) or 20 μM dantrolene sodium (DaNa). Ordinate scales m.e.p.p. frequency (F, $s^{-1}$). Vertical lines show s.e.mean of 12 to 16 experiments. Neomycin and DaNa were added 20 min before the addition of $Ca^{2+}$ or $Zn^{2+}$. M.e.p.ps in a solution without added $Ca^{2+}$ or $Zn^{2+}$ were measured from 30 to 50 min after the addition of the antagonist. When 0.5 mM $Ca^{2+}$ was added to the 10 mM $K^+$ bathing solution m.e.p.ps were measured from several endplates within 20 min, 5 min after the addition of $Ca^{2+}$. Frequency of m.e.p.ps in the presence of $Zn^{2+}$ is plotted as the maximum value measured within 50 min after the addition of $Zn^{2+}$. Both neomycin and DaNa inhibited the effects of external $Ca^{2+}$ and $Zn^{2+}$ on the frequency of m.e.p.ps.

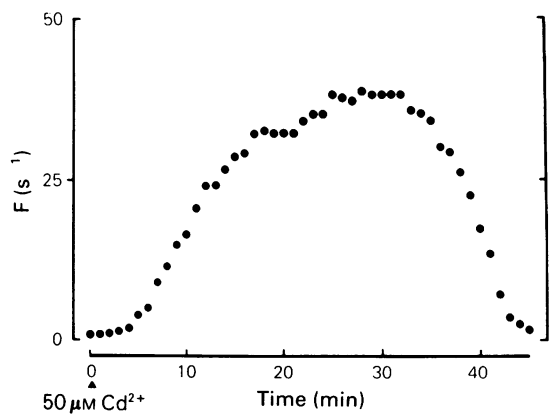


**Figure 6** Time course of the effect of 50 μM $Cd^{2+}$ on the frequency of m.e.p.ps at a mouse neuromuscular junction in a $Ca^{2+}$-free, 10 mM $K^+$ bathing solution at 36°C. Ordinate scale, m.e.p.p. frequency (F, $s^{-1}$), abscissa scale, time in min after addition of $Cd^{2+}$. The effect of $Cd^{2+}$ required a latent period and occurred transiently.

intracellular messenger system (Storey *et al.*, 1984; Vergara *et al.*, 1985).

There are some sites for storage of $Ca^{2+}$ inside the cell (Blaustein *et al.*, 1980; Brinley, 1980). Those sites are predominantly in the endoplasmic reticulum and in the mitochondria. Various factors have been shown to liberate $Ca^{2+}$ from these sites, thereby accelerating cellular functions mediated by $Ca^{2+}$ (Berridge, 1986). In fact, the release of acetylcholine from frog (Crawford, 1975) and mouse (Nishimura *et al.*, 1984) neuromuscular junction and from rat brain synaptosomes (Adam-Vizi & Ligeti, 1984) can be accelerated by several agents in the absence of external $Ca^{2+}$. The effects of these agents are considered to be due to increased levels of $Ca^{2+}$ mobilized intracellularly. For example, the mitochondrion releases $Ca^{2+}$ in the presence of FCCP (carbonylcyanide p-trifluoromethoxyphenyl hydrazone) which is highly toxic to this organelle (Blaustein *et al.*, 1980; Brinley, 1980). The released $Ca^{2+}$ is effective in increasing the release of transmitter (Nishimura *et al.*, 1984). $Zn^{2+}$ has been observed to promote the liberation of $Ca^{2+}$ from the sarcoplasmic reticulum of skeletal muscle (Vergara *et al.*, 1985). If this effect of $Zn^{2+}$ also occurs at the endoplasmic reticulum in the nerve terminal, the presynaptic effect of this cation, as presented here, is explainable. This hypothesis is supported by the evidence in these experiments that the effect of $Zn^{2+}$ is mimicked by $Cd^{2+}$ and BPG which have been shown to promote the release of $Ca^{2+}$ from the endoplasmic reticulum (Streb *et al.*, 1983).

A mechanism has been proposed for the liberation of $Ca^{2+}$ from the endoplasmic reticulum (Streb *et al.*, 1983; Vergara *et al.*, 1985; Berridge, 1986). Electrical activation releases inositiol 1,4,5-triphosphate ($Ins1,4,5P_3$) (Vergara *et al.*, 1985) and $Ins1,4,5P_3$ mediates the release of $Ca^{2+}$ from the endoplasmic reticulum as an intracellular messenger. $Zn^{2+}$, $Cd^{2+}$, $Ag^{2+}$ and BPG have been reported to inhibit $Ins1,4,5P_3$ 5-phosphatase which catalyzes the dephosphorylation of $Ins1,4,5P_3$ (Storey *et al.*, 1984). $Li^+$ inhibits $Ins1,4,5P_3$ breakdown and also increases m.e.p.p. frequency with a time course similar to the effect of $Zn^{2+}$ (Carmody & Gage, 1973). Thus, $Ins1,4,5P_3$ may accumulate inside the nerve terminal in the presence of these agonists. Neomycin inhibits the production of $Ins1,4,5P_3$ by reducing hydrolysis of phosphatidyl inositol 4,5-bisphosphate as do neamine, spermine and poly-L-lysine (Schacht, 1976; Vergara *et al.*, 1985). Neomycin partially attenuated the effect of $Zn^{2+}$ on transmitter release. However, it inhibited to some extent the effect of $Ca^{2+}$ on the rate of spontaneous release of transmitter in a depolarizing solution. It has been postulated that neomycin has a $Mg^{2+}$-like effect, competitively inhibiting the action of $Ca^{2+}$ at the nerve terminal and, thus preventing the release of transmitter (Vital Brazil & Prado-Franceschi, 1969; Fiekers, 1983). External $Ca^{2+}$ in the depolarizing solution must be transported into the nerve terminal across the cell membrane so that it can then act on a $Ca^{2+}$-regulated process (Katz & Miledi, 1965; Rahamimoff & Yaari, 1973). Thus, the possibility that neomycin reduces the transmembranous movement of $Zn^{2+}$ remains to be proven.

In summary, $Zn^{2+}$ stimulates the rate of spontaneous release of transmitter from the motor nerve terminal by a release of $Ca^{2+}$ stored intracellularly. $Ins1,4,5P_3$ may be involved in the mechanism of release of $Ca^{2+}$.

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (No. 60560333).

## References

ADAM-VIZI, V. & LIGETI, E. (1984). Release of acetylcholine from rat brain synaptomomes by various agents in the absence of external calcium ions. *J. Physiol.*, **353**, 505–521.

BENOIT, P.R. & MAMBRINI, J. (1970). Modification of transmitter release by ions which prolong the presynaptic action potential. *J. Physiol.*, **210**, 681–695.

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

BERRIDGE, M.J. (1986). Regulation of ion channels by inositol triphosphate and diacylglycerol. *J. Exp. Biol.*, **124**, 323–335.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.

BLAUSTEIN, M.P., RATZLAFF, R.W. & SCHWEITZER, E.S. (1980). Control of intracellular calcium in presynaptic nerve terminals. *Fed. Proc.*, **39**, 2790–2795.

BRINLEY, JR., F.J. (1980). Regulation of intracellular calcium in squid axons. *Fed. Proc.*, **39**, 2778–2782.

CARMODY, J.J. & GAGE, P.W. (1973). Lithium stimulates secretion of acetylcholine in the absence of extracellular calcium. *Brain Res.*, **50**, 476–479.

CRAWFORD, A.C. (1975). Lithium ions and the release of transmitter at the frog neuromuscular junction. *J. Physiol.*, **246**, 109–142.

DEL CASTILLO, J. & KATZ, B. (1954). Changes in end-plate activity produced by presynaptic polarization. *J. Physiol.*, **124**, 586–604.

DESMEDT, J.E. & HAINAUT, K. (1977). Inhibition of the intracellular release of calcium by dantrolene in barnacle giant muscle fibres. *J. Physiol.*, **265**, 565–585.

DONALDSON, J., ST. PIERRE, T., MINNICH, J. & BARBEAU, A. (1971). Seizures in rats associated with divalent cation inhibition of $Na^+$-$K^+$-ATPase. *Canadian J. Biochem.*, **49**, 1217–1224.

FIEKERS, J.F. (1983). Effects of the aminoglycoside

antibiotics, streptomycin and neomycin, on neuromuscular transmission. I. Presynaptic considerations. *J. Pharmacol. Exp. Ther.*, **225**, 487–495.

KATZ, B. (1966). *Nerve, Muscle, and Synapse*. New York: McGraw-Hill Book Company.

KATZ, B. & MILEDI, R. (1965). The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. Roy. Soc. B.*, **161**, 495–503.

LILEY, A.W. (1956). The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. *J. Physiol.*, **134**, 427–443.

LIN-SHIAU, S.-Y. & FU, W.-M. (1980). Effects of divalent cations on neuromuscular transmission in the chick. *Eur. J. Pharmacol.*, **64**, 259–269.

NISHIMURA, M. (1986). Factors influencing an increase in spontaneous transmitter release by hypoxia at the mouse neuromuscular junction. *J. Physiol.*, **372**, 303–313.

NISHIMURA, M., TSUTSUI, I., YAGASAKI, O. & YANAGIYA, I. (1984). Transmitter release at the mouse neuromuscular junction stimulated by cadmium ions. *Arch. Int. Pharmacodyn. Ther.*, **271**, 106–121.

RAHAMIMOFF, R. & YAARI, Y. (1973). Delayed release of transmitter at the frog neuromuscular junction. *J. Physiol.*, **228**, 241–257.

SCHACHT, J. (1976). Inhibition by neomycin of polyphosphoinositide turnover in subcellular fractions of guinea-pig cerebral cortex in vitro. *J. Neurochem.*, **27**, 1119–1124.

SMART, T.G. & CONSTANTI, A. (1983). Pre- and postsynaptic effects of zinc on in vitro prepyriform neurones. *Neurosci. Lett.*, **40**, 205–211.

STATHAM, H.E. & DUNCAN, C.J. (1976). Dantrolene and the neuromuscular junction: Evidence for intracellular Ca stores. *Eur. J. Pharmacol.*, **39**, 143–152.

STOREY, D.J., SHEARS, S.B., KIRK, C.J. & MICHELL, R.H. (1984). Stepwise enzymatic dephosphorylation of inositol 1,4,5-triphosphate to inositol in liver. *Nature*, **312**, 374–376.

STREB, H., IRVINE, R.F., BERRIDGE, M.J. & SCHULZ, I. (1983). Release of $Ca^{2+}$ from a nonmitochondrial intracellular store in pancreatic aciner cells by inositol-1,4,5-triphosphate. *Nature*, **306**, 67–69.

VERGARA, J., TSIEN, R.Y. & DELAY, M. (1985). Inositol 1,4,5-triphosphate: A possible chemical link in excitation-contraction coupling in muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 6352–6356.

VITAL BRAZIL, O. & PRADO-FRANCESCHI, J. (1969). The nature of neuromuscular block produced by neomycin and gentamycin. *Arch. Int. Pharmacodyn. Ther.*, **179**, 78–85.

*(Received February 20, 1987.*
*Revised September 2, 1987.*
*Accepted September 11, 1987.)*