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Relative potencies of metal ions on transmitter release at mouse motor nerve terminals

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1 The effects of a range of metal ions were systematically studied at the mouse neuromuscular junction in order to investigate the type of calcium channel present at the nerve terminal.

2 Endplate potentials and miniature endplate potentials were recorded from the phrenic nerve diaphragm muscle preparation with glass microelectrodes.

3 Endplate potential amplitudes and quantal contents were reduced by manganese (IC_{50} 220 μM), cadmium (IC_{50} 11 μM), cobalt (IC_{50} 340 μM), and nickel (IC_{50} 420 μM). Miniature endplate potentials were not affected by these ions at concentrations equal to the IC_{50}s. Gadolinium did not reduce endplate potentials up to 100 μM.

4 Comparisons made with known channel types in neuroblastoma cell lines suggest that the calcium channels at the motor nerve terminal are different from those types studied in the cell lines, although most similarity is shown to the high-voltage activated calcium channel types.

Keywords: Calcium channels; metal ions; neuromuscular junction

Introduction

The calcium channels at the nerve terminal of the neuromuscular junction are of key importance in neuromuscular transmission. They open in response to an action potential arriving at the nerve terminal. Calcium entry through these channels initiates a series of events that results in the release of the neurotransmitter, acetylcholine. There is, however, very little known about the detailed nature of these presynaptic voltage-operated calcium channels. This is mainly because the nerve terminal is too small for making either intracellular or patch-clamp recordings. The object of this work was to help clarify the properties of these presynaptic calcium channels indirectly by systematically studying the relative potencies of a range of metal ions on neuromuscular transmission.

Calcium channels have been characterized in other tissues by their electrophysiological and pharmacological properties. The main classification has been into T, L, N and P type channels although others are being proposed, e.g. Q and R (Nowycky *et al.*, 1985; Bean, 1989; Llinas *et al.*, 1989; Zhang *et al.*, 1993; Wheeler *et al.*, 1994). The T type channel has a low threshold of voltage activation; L and N type channels are high-threshold activated. The L type channels are sensitive to dihydropyridines, while N type channels are blocked by ω-conotoxin GVIA. The P type channel is a high-threshold current not blocked by either dihydropyridines or ω-conotoxin, but blocked by a toxin from funnel-web spider venom (Llinas *et al.*, 1989; Cherksey *et al.*, 1991). Evidence is available (Cesario *et al.*, 1989; Dascal, 1990; Cherksey *et al.*, 1991) suggesting the location of N and P type channels as being predominantly neuronal.

It has already been shown that transmission at the mouse neuromuscular junction is unaffected by dihydropyridines (Burges & Wray, 1989) or ω-conotoxin (Sano *et al.*, 1987; Protti *et al.*, 1991), suggesting that the mouse nerve terminal calcium channel is neither L nor N type. Transmission at the frog neuromuscular junction can be blocked by ω-conotoxin suggesting that there is probably some degree of species variation in the nature of the calcium channel present (Sano *et al.*, 1987). Furthermore, funnel web spider venoms (FTX and ω-Aga-IVA) have been shown to block transmission at the mouse neuromuscular junction (Uchitel *et al.*, 1992; Protti & Uchitel, 1993), indicating similarities with the P type channel.

Several inorganic cations are known to be effective in blocking currents through calcium channels for a range of tissues. There are differences in the potencies of these ions between different calcium channel types (Narahashi *et al.*, 1987; Kasai & Neher, 1992). For instance, nickel ion is a potent blocker of T type channels, whilst cadmium ion is a potent blocker at L and N type channels, and gadolinium (under certain conditions, Docherty, 1988; Boland *et al.*, 1991) is selective for N type channels. Although there have been previous studies using metal ions (e.g. Meiri & Rahamimoff, 1972; Toda, 1976; Forshaw, 1977; Lin-Shiau & Fu, 1980; Hamilton & Smith, 1992), systematic comparisons have not been made. In this paper, the blocking potencies of a range of cations (Ni^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Gd^{3+}) on neuromuscular transmission have been systematically measured, and the results compared with blocking potencies already known for calcium channel types in other tissues. Our data provide further information about the properties of these channels at the neuromuscular junction.

Methods

Mouse isolated phrenic nerve diaphragm preparations were perfused (5-10 ml min^{-1}) with oxygenated (95% O_2, 5% CO_2) Krebs solution at room temperature (21-24°C). The composition of the Krebs solution was (mM); NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 2.52. In order to record endplate potentials, tubocurarine (3-5 μM) was added to the solution to prevent muscle contraction. Intracellular voltage recordings were made as previously described (Lang *et al.*, 1983) with glass microelectrodes filled with 3 M KCl, resistance 10-15 MΩ, resting potentials in the range -60 mV to -75 mV. The phrenic nerve was stimulated at 0.5 Hz (pulse width 0.1 ms, supramaximal voltage 5-15 V). All data were stored on magnetic tape and subsequently analysed by computer after digitisation at 5 kHz. The quantal content was calculated from the endplate potential (e.p.p.) amplitude using the variance method, as previously described in Lang *et al.* (1983). At least 90 e.p.p.s (3 min at

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0.5 Hz) were always used for quantal content calculations. Non-linear summation of e.p.p.s was considered negligible because e.p.p. amplitudes were always kept small by tubocurarine. Quantitative comparisons of potencies have been made using e.p.p. amplitudes rather than quantal content, since postsynaptic sensitivity was shown not to be affected by these ions at the concentrations used here (see Results). Furthermore, e.p.p. amplitudes could be more accurately determined than quantal content, which was subject to greater experimental scatter.

The actions of a range of metal ions (cadmium, nickel, manganese, cobalt and gadolinium as chloride salts) were studied by perfusing them onto the preparation during continuous recording of endplate potentials evoked by stimulation of the phrenic nerve. The effect of these metal ions was also studied similarly on spontaneous miniature endplate potentials (m.e.p.ps) in the absence of any tubocurarine or nerve stimulation. No corrections were made to endplate potential or miniature endplate potential amplitudes for variations in resting potentials since the latter were found to be unaffected by metal ions (see Results).

After impalement of each muscle fibre the resting membrane potential was allowed to stabilise for a few minutes. Then control e.p.ps were recorded for 3 min before the test ion was perfused onto the preparation. E.p.ps were continuously recorded during a further 4 min application of the ion. After this application period, the ion was washed off and the e.p.ps continually recorded for a further 3–4 min to follow the recovery of the original e.p.p. amplitude.

Student's paired or unpaired t test was used as appropriate, with the level for significance set at $P<0.05$.

Results

The resting membrane potential (RMP) of the mouse diaphragm muscle fibres was continuously monitored throughout each recording. The membrane potential was not statistically significantly altered by application of any of the ions at any concentration (paired t test). The mean RMP for all recordings was -67.1 ± 0.6 mV before application of ions and -65.9 ± 0.6 mV ($n=107$ endplates) after 4 min application of ions.

Effects of the metal ions on e.p.ps

A range of concentrations of each metal ion was applied to the diaphragms, and e.p.ps were continuously recorded at 0.5 Hz. Cadmium, nickel, manganese and cobalt were all found to reduce the amplitude of the e.p.ps (see Figure 1 for example traces). In each case, the reduction in amplitude occurred rapidly as the ions were washed on, and was readily reversible on washout (see Figure 2 for examples). Gadolinium applied (Figure 2) at concentrations of 5, 10, 100 μM was without effect on e.p.p. amplitudes (higher concentrations could not be tested because of limited solubility).

A range of concentrations of each of the ions was applied to endplates, and concentration-response curves for each ion were generated from these data (see Figure 3). The data are presented as the mean e.p.p. amplitude in the presence of metal ion, as a percentage of the pretreatment e.p.p. amplitude.

From these concentration-response curves, IC_{50}s were obtained for each ion, taking the IC_{50} as the concentration of the ion required to reduce the e.p.p. amplitude to 50% of the control level. The IC_{50}s obtained were: manganese 220 μM, cadmium 11 μM, cobalt 340 μM and nickel 420 μM.

Quantal contents were also determined from the e.p.p. data. Concentration-response curves were obtained for quantal contents for each ion studied (data not shown). There was a similar pattern of reductions in quantal content by the ions to the reduction of e.p.p. amplitudes, indicating a presynaptic action of the ions at these concentrations. As the experimental scatter for e.p.p. amplitudes was less than for quantal content, the IC_{50}s generated from e.p.p. amplitude data were used for relative potency comparisons (see Discussion).

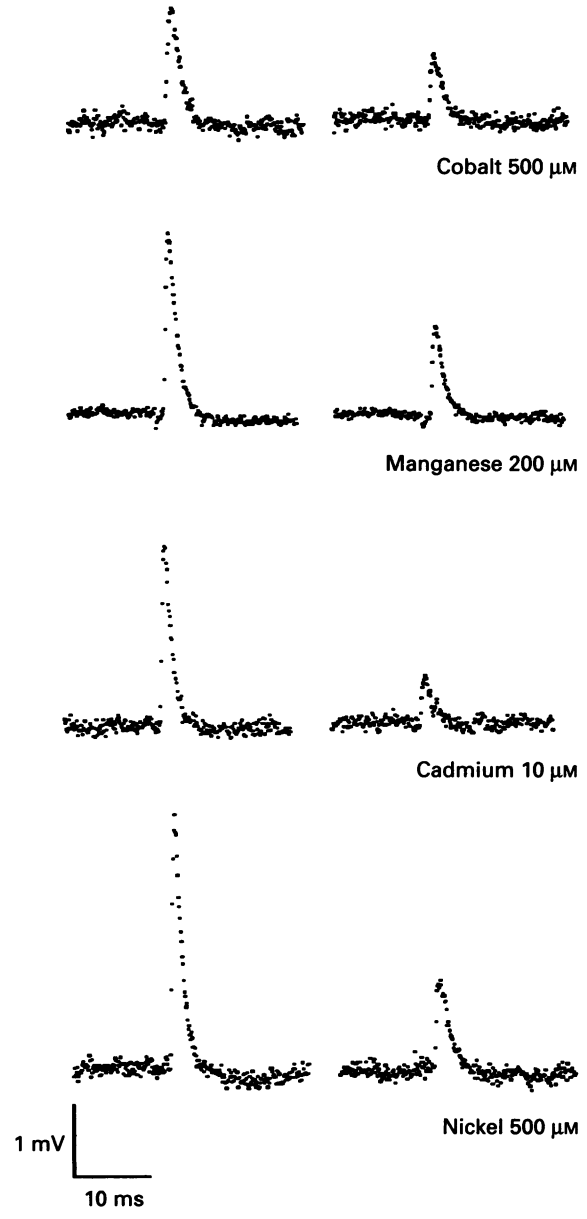

Figure 1 Examples are given of e.p.ps measured from muscle fibres before and during application of metal ions (cobalt, manganese, cadmium and nickel) at concentrations given on the figure. In these examples, the stimulus artifacts were below the level of the noise.

Effect of metal ions on m.e.p.ps

The effect of the metal ions on the size of m.e.p.ps was examined in a similar way to the effect of the ions on e.p.p. amplitudes by recordings made during continuous perfusion with metal ions. Figure 4 shows examples of m.e.p.ps measured prior to application of the ions, and in the same fibres in the presence of the metal ions. The concentrations used in these experiments (given on the figure) correspond very closely to the IC_{50} concentrations for the reduction of e.p.p. amplitude, calculated as described above. The mean m.e.p.p. amplitudes from several endplates are shown in Table 1. There were no significant effects on the mean m.e.p.p. amplitudes with cadmium, nickel or manganese ions at the concentrations used. Cobalt appeared to reduce the mean m.e.p.p. amplitude;

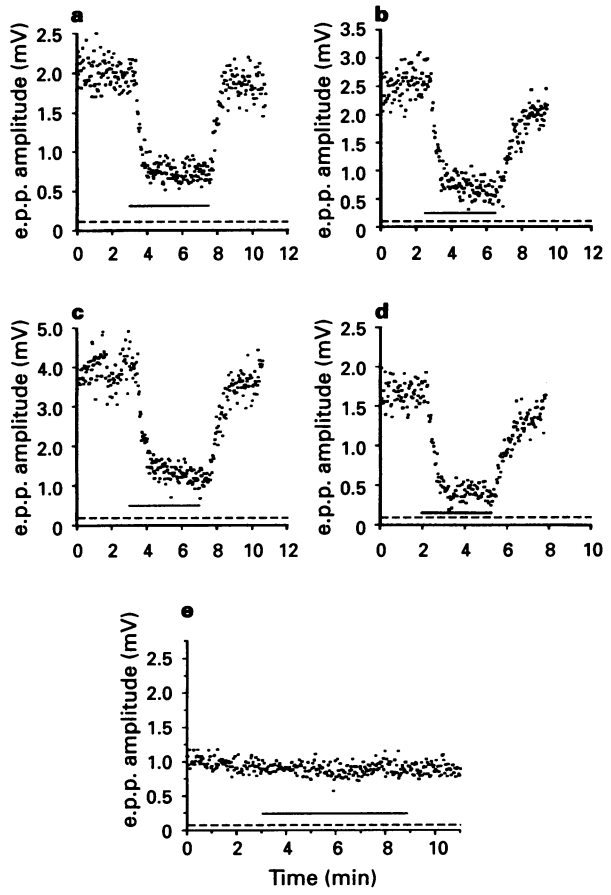

Figure 2 Effects of different metal ions on endplate potential amplitudes at the mouse neuromuscular junction. In each case the data are from a single continuous recording and e.p.p. amplitudes are plotted against time. The horizontal dashed line represents the amplitude of the baseline noise. The bars indicate when the metal ions were perfused onto the preparation: (a) manganese chloride 400 μM; (b) cadmium chloride 10 μM; (c) cobalt chloride 500 μM; (d) nickel chloride 500 μM; (e) gadolinium chloride 10 μM.

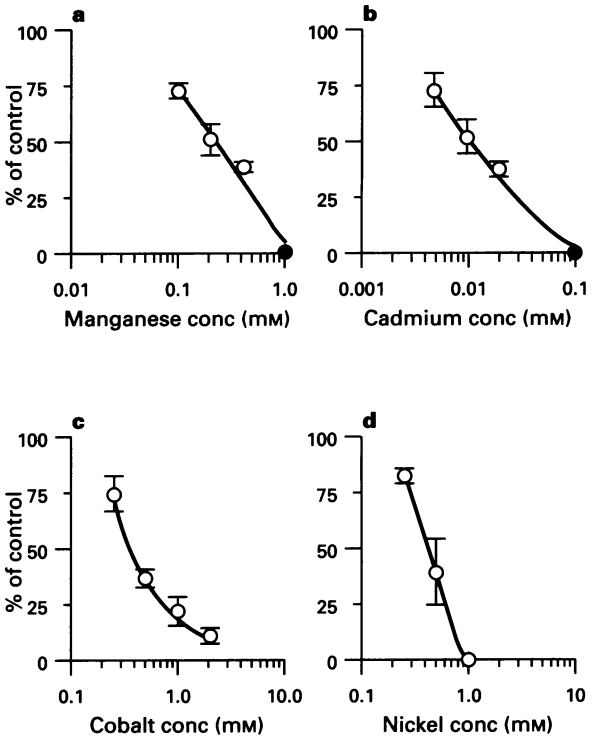

Figure 3 Concentration-response curves for the block of e.p.p. amplitudes by metal ions. In each case e.p.p. amplitude is plotted as a percentage of control against the concentration of metal ions. Points (○) correspond to means (±s.e.mean) of 4–13 endplates. At high concentrations, e.p.ps fell below the level of the baseline noise. (●) (a) Manganese; (b) cadmium; (c) cobalt; (d) nickel.

although this reduction was small, it was statistically significant (paired t test). To investigate this further, recordings were also made at a higher concentration of cobalt (500 μM). No significant effect of cobalt (paired t test) was seen on the mean m.e.p.p. amplitude with this higher concentration (Table 1). These results therefore support the interpretation of lack of postsynaptic action of these ions at the concentrations used.

The effect of the metal ions on the m.e.p.p. frequency was also studied. No significant effects (paired t test) on m.e.p.p. frequency were observed with any of the metal ions tested (Table 1).

Discussion

Our data show that there is no effect of the ions at the concentrations used on m.e.p.p. frequency. Since resting m.e.p.p. frequency is mainly governed by intracellular processes, this suggests a lack of intracellular action of these ions at these concentrations. Some studies have observed an increase in the frequency of m.e.p.p.s with cadmium (Forshaw, 1977; Cooper & Manalis, 1984; Nishimura *et al.*, 1984; Guan *et al.*, 1987) or cobalt (Weakly, 1973), but such effects were seen at high concentrations of the ion concerned or at low calcium concentrations. It is unlikely that this effect plays a significant role at the concentrations used in the studies presented here.

The results described here for lack of effect on m.e.p.p. amplitudes, together with the similar pattern of reductions between quantal content and e.p.p. amplitude, show that the metal ions affect e.p.p. amplitude by a reduction in the number of packets of ACh released with each nerve stimulation, and that the effect is not due to any action on the postsynaptic membrane. As the concentrations of metal ions used do not affect presynaptic sodium or potassium channels (Penner & Dreyer, 1986), the most likely target for the metal ions is the calcium channel in the nerve terminal responsible for transmitter release.

It is not very meaningful to compare absolute values of IC_{50}s between different studies as these values vary with the recording solutions, preparation used, and other experimental conditions. In order to make systematic comparisons, the relative potencies of a range of ions using identical experimental conditions needs to be considered as we have done in the present study. Previous studies on the effects of metal ions on the neuromuscular junction e.p.p. have generally examined only the effects of a single metal ion (e.g. Balnave & Gage, 1973; Weakly, 1973; Forshaw, 1977; Molgo *et al.*, 1991; Satoh *et al.*, 1982) and have not, as in the present study, systematically compared a range of metal ions.

However, there have been two other studies using a series of metal ions, but they have measured the effect of the ions on twitch tension (Lin-Shiau & Fu, 1980) and extracellularly recorded presynaptic ion channel currents (Hamilton & Smith, 1992). In the first of these studies (Lin-Shiau & Fu, 1980), the effects of the ions on direct muscle stimulation-induced twitches were compared with the effects of indirect nerve stimulation-induced muscle twitches. However, without measuring e.p.p. and m.e.p.p. amplitudes, it is not possible to say how much of the observed block of the muscle twitch tension is due to a reduction in the amount of transmitter released. Relative potency values calculated from their data do however correlate well with the relative potencies calculated from the present data for the reduction of e.p.p. amplitudes.

Using a focal extracellular electrode placed in the region of

the nerve terminal, currents can be recorded which are a reflection of ion channel currents in the nerve terminal (McArdle *et al.*, 1981; Brigant & Mallart, 1982; Konishi & Sears, 1984; Mallart, 1985; Hamilton & Smith, 1992). Using this technique, Hamilton & Smith, (1992) studied the effects of three metal ions (cadmium, cobalt and nickel) on a component of this current thought to be due to calcium channels in the nerve terminal. However, it is difficult to determine whether the currents observed by these techniques correspond to the currents through those calcium channels which are responsible for transmitter release. It has been suggested that there may be at least two types of calcium channel in the nerve terminals (Penner & Dreyer, 1986), but whether calcium entry through one or more than one type of channel is responsible for transmitter release is uncertain. On comparing potencies relative to cadmium, the relative potency of cobalt at reducing focally recorded currents is similar to that for reducing e.p.p. amplitude (this study); however, nickel is relatively less potent at reducing focally recorded currents. This suggests that the data of Hamilton & Smith (1992) may indeed be based on channels not concerned with transmitter release.

Relative potencies of metal ions at the neuromuscular junction calcium channel can be used to make comparisons with work done on specific types of calcium channel studied in other preparations. As at the neuromuscular junction, it is rare for a range of concentrations to be applied and for IC_{50} values to be calculated. However, two studies have been made in which a range of metal ions have been applied to the calcium channels in neuroblastoma cell lines NG108 15 and NIE-115 (Narahashi *et al.*, 1987; Kasai & Neher, 1992). From the IC_{50} values quoted in these latter studies, it is possible to calculate relative potencies for L, N (Kasai & Neher, 1992) and T (Narahashi *et al.*, 1987) type channels. These relative potencies have been compared with the relative potencies obtained in the present study on the release of neurotransmitter from the nerve terminal. The relative po-

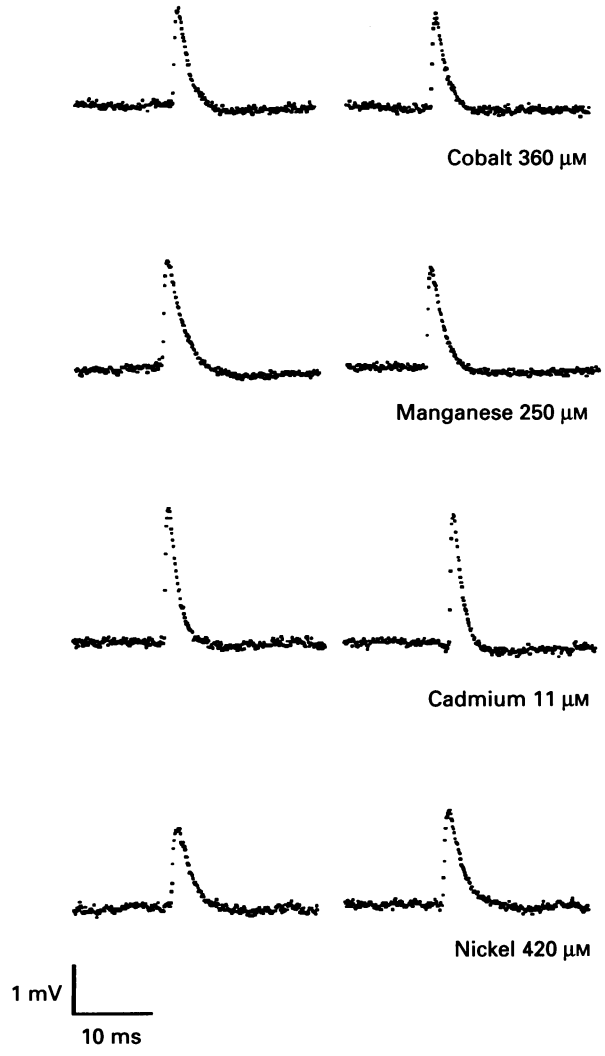

Figure 4 Examples of the lack of effect of metal ions on m.e.p.ps. Examples are given of m.e.p.ps recorded from muscle fibres before and during applications (same fibre in each case respectively) of the metal ions (cobalt, manganese, cadmium and nickel) at concentrations given on the figure.

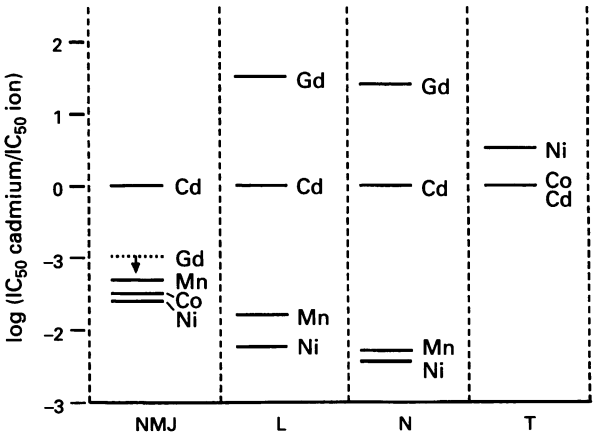

Figure 5 Ion potencies relative to cadmium at the motor nerve terminal and at different channel types in neuroblastoma cells. The ion potencies relative to cadmium (defined as IC_{50} cadmium/IC_{50} ion) are plotted for the present data obtained at the motor nerve terminal (NMJ), L and N calcium channel types from NG108 15 cells (Kasai & Neher, 1992); and for T type channels from N1E-115 cells (Narahashi *et al.*, 1987). The dotted line for gadolinium indicates that the highest concentration tried on the neuromuscular junction had no effect.

Table 1 Effect of metal ions on m.e.p.ps

	Pretreatment		*Presence of ions*		
Ion	*m.e.p.p. amplitude* (mV)	*m.e.p.p. frequency* (s^{-1})	*m.e.p.p. amplitude* (mV)	*m.e.p.p. frequency* (s^{-1})	*Number of endplates*
Nickel (420 μM)	1.5±0.2	1.0±0.2	1.4±0.2	1.3±0.4	6
Cadmium (11 μM)	1.2±0.3	3.4±1.4	1.1±0.3	3.5±1.5	6
Manganese (250 μM)	1.2±0.2	6.3±4.5	1.1±0.1	3.6±2.1	6
Cobalt (360 μM)	1.4±0.2	0.6±0.1	1.2±0.2*	0.4±0.1	6
Cobalt (500 μM)	1.3±0.3	–	1.2±0.2	–	5

For each metal ion tested, the mean m.e.p.p. amplitude and frequency (±s.e.mean) is given before and during application of the ions. *$P<0.05$, Student's paired t test.

tency data, presented graphically in Figure 5, would indicate that the calcium channel in the nerve terminal responsible for transmitter release is not L, N or T-type.

This conclusion assumes that only one type of channel is responsible for transmitter release (Penner & Dreyer, 1986) at the neuromuscular junction. However, there is evidence that more than one type of calcium channel may be involved in transmitter release e.g. in the hippocampus (Wheeler *et al.*, 1994) but it is not known if this is the situation at the neuromuscular junction. The relative lack of effect of nickel at low concentration (250 μM) in the present experiments appears to rule out an appreciable contribution from T type channels even in association with other types of channels. Since the relative potencies of metal ions are not known for P, Q and R type channels, further speculation is inappropriate at this stage.

Gadolinium ions failed to inhibit the transmitter release at concentrations up to the limit of its solubility. However, the potency of gadolinium has been shown to be dependent on the concentration of bicarbonate ions present in the solution (Boland *et al.*, 1991). There were no bicarbonate ions present in the recording solution used to measure the L and N-type currents (Kasai & Neher, 1992). The recording solution used in the present experiments did, however, contain bicarbonate ions and this is likely to explain the lack of effect of gadolinium.

If one therefore discounts the relative potencies for gadolinium, then the relative potency profile for the neuromuscular junction can be seen to bear the greatest resemblance to that for L and N-type channels rather than T-type channels (Figure 5). One could, therefore, speculate that the nerve terminal calcium channel has a higher degree of similarity to these high voltage-activated channels (in the region that the metal ions bind to block the channel) as compared to the low voltage activated T-type channel.

The metal ions are thought to bind to the calcium binding region in the pore of the ion channel hence preventing the passage of calcium ions through the pore (Nachslen, 1984). Thus one could conclude that, for the calcium channel at the neuromuscular junction, the pore region of the channel is different from L, T and N type channels. However, it has the highest resemblance to the high voltage activated L and N type channels.

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