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Two different presynaptic calcium currents in mouse motor nerve terminals

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Summary. 1. Extracellular recordings of potential changes under the perineural sheath of nerve bundles close to some of the nerve terminals were performed using the M. triangularis sterni of the mouse.

2. The nerve signals consisted of a predominant double-peaked negativity which was often preceded by a small positive deflection. While the first negative peak is related to the propagating nerve action potential, the second negative deflection can be attributed to a potassium conductance since it was selectively blocked by tetraethylammonium (TEA) or 3,4-diaminopyridine (3,4-DAP).

3. Combined application of TEA and 3,4-DAP gave rise to a prolonged positive-going wave which was blocked by Cd^{2+}, thus, indicating its underlying cause to be a Ca current.

4. Ionophoretic application of TEA and Cd^{2+} to the endplates affected potassium and calcium components of the subendothelial signals, respectively, thus indicating their presynaptic origin. This finding is supported by the decrease of the amplitude of these components with increasing distance from the endplate region.

5. Maximal effects on K conductance attainable with 3,4-DAP could still be potentiated by TEA, indicating the presence of at least two distinct sets of K channels.

6. The prolonged positive potential induced by TEA and 3,4-DAP consisted of a fast and slow component, both of which can be attributed to Ca conductances with different characteristics.

7. The fast positive signal component is attributed to the voltage-dependent Ca channel, responsible for the initiation of transmitter release. Its amplitude and duration depend on extracellular Ca^{2+}-concentration. The fast component is still present when Ca^{2+} is substituted by Sr^{2+} or Ba^{2+}. It is blocked by Cd^{2+} and Mn^{2+} in the millimolar range, but remains unaffected by organic Ca-antagonists.

8. The slow positive signal component, whose physiological role remains to be elucidated, also depends on extracellular Ca^{2+}-concentration. It is reduced by frequent nerve stimulation. The slow component can be carried by Sr^{2+} or Ba^{2+} and it is blocked by Cd^{2+} and Mn^{2+} in the micromolar range. In contrast to the fast 'Ca potential' the slow one is reduced by verapamil and diltiazem but not by the 1,4-dihydropyridines nitrendipine and nisoldipine. Concentration-response curves can best be described assuming two dissociation constants.

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Key words: Neuromuscular junction – Presynaptic – Calcium channel – Ca-antagonist – Potassium channel

Introduction

Calcium currents and their heterogenous characteristics have been described in a variety of experimental preparations (for reviews, see Hagiwara and Byerly 1981; Tsien 1983). Their crucial role in excitation-secretion coupling has been confirmed (Katz and Miledi 1967, 1968; Katz 1969; Llinas and Nicholson 1975). Direct studies of presynaptic Ca currents can hardly be achieved except at the squid giant synapse (Llinas et al. 1976, 1981a, b) and only few investigations of presynaptic currents have been made at endplates (Hubbard and Schmidt 1963; Katz and Miledi 1965, 1968; Braun and Schmidt 1966).

Recently, Brigant and Mallart (1982) presented results which indicated spatially separated distribution of Na channels (in the heminode region) and K and Ca channels (in the terminal arborizations) in mouse motor nerve endings. This was achieved by focal extracellular recording applied to a very thin muscle preparation (McArdle et al. 1981) which allowed precise positioning of extracellular electrodes along the nerve terminal. However, Konishi and Sears (1984) demonstrated that under certain conditions a small TTX-sensitive inward current is detectable in the terminal region. Focal current recordings at nerve terminals display low signal to noise ratio. Using the technique of subendothelial recording from preterminal motor axons (Katz and Miledi 1968) Gundersen et al. (1982) recorded signals of remarkable amplitude simply by inserting the electrode under the perineural sheath of frog nerve bundles. The same technique has been applied by Mallart (1984) to a mammalian nerve-muscle preparation. These investigations suggested the presence of passive currents of presynaptic origin in the parent axon. However, only indirect evidence has been given so far that this is the case. We applied this technique to the triangularis sterni preparation of the mouse and provide evidence for the presence of passively conducted presynaptic currents in mammalian nerve bundles. Local application of channel blocking agents to the endplate induced changes in the subendothelial signals, thus providing evidence of their presynaptic origin.

We have examined the prolonged presynaptic depolarization which becomes apparent after blocking potassium currents with tetraethylammonium and 3,4-diaminopyridine (Brigant and Mallart 1982). This potential consists

of two components, both of which can be attributed to presynaptic Ca conductances, although differing in frequency dependence and sensitivity to inorganic as well as organic calcium-antagonists.

Methods

Experiments were performed on the M. triangularis sterni of adult mice (McArdle et al. 1981) visualized at a ×400 magnification by a Zeiss microscope equipped with Nomarski interference contrast optics (see Dreyer et al. 1979). The preparation was continuously perfused (3–6 ml/min) with a modified Krebs solution of the following composition (mM): NaCl 115, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 25, Na_2HPO_4 1, glucose 11, gassed with 95% O_2/5% CO_2 (pH 7.3). For investigations with Cd^{2+} and Mn^{2+} a Ringer's solution of the following composition was used (mM): NaCl 145, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, glucose 11 and Hepes 5 (buffered at pH 7.3), gassed with 100% O_2. All experiments were performed at room temperature (21–24° C).

Signals following nerve stimulation through a suction electrode were recorded inside the endothelial tube of nerve bundles (containing 2–4 nerve fibres) using glass microelectrodes filled with 0.5 M NaCl (resistance: 4–10 MΩ). The reference electrode was a chlorided silver wire in the recording chamber. The penetration of the perineural sheath (sheath of Henle) was accompanied by the registration of a negative potential in the range of 2–6 mV. The 'input resistance' was measured by feeding square pulses with one electrode and recording potentials with another one. It was estimated to be about 0.5 MΩ.

This technique allows one to record potential changes between the recording electrode and the reference electrode notionally sited at the open end of the sheath where the synaptic terminals emerge. These potential changes reflect the sum of longitudinal currents flowing along the outside of the axons within the endothelial tube. Signal amplitude usually ranged between 2–12 mV depending on the position of the electrode, the sheath resistance and the quality of the seal between electrode and perineural endothelium. The electrogenesis of the potentials observed is difficult to interpret. However, the use of channel blocking agents allowed us to identify waveform components related to K and Ca currents. The local administration of these drugs to the endplate identified the nerve terminal as the source at which these currents were generated (see Results). It thus appears that the potentials recorded at some distance from the nerve terminals arise from passive currents which result from presynaptic conductance changes. Within the endothelial tube considerable longitudinal gradients of potentials may be generated due to the insulating properties of the perineural sheath.

Any change of the properties of the sheath would tend to alter the magnitude of the recorded waveforms. Since we wanted to use this technique to quantitate drug effects on the presynaptic Ca currents, changes of the sheath properties were monitored using the amplitude of the propagating nerve action potential. In our experiments, unless very high concentrations of Ca-antagonists were used, we observed no changes of the nerve spike indicating the actions of these drugs on the sheath resistance to be negligible. This conclusion is further supported by the finding that the local

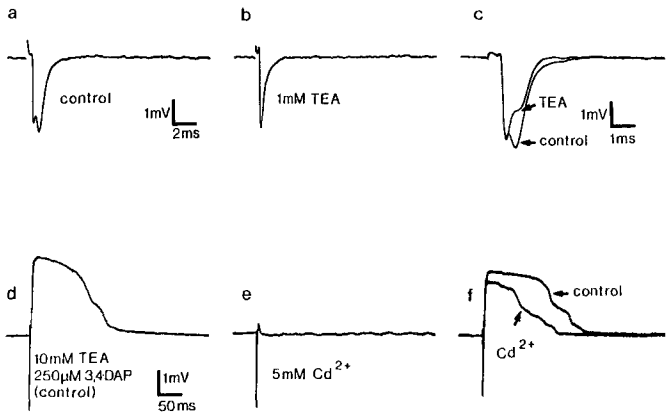

Fig. 1a–f. Effects of TEA and Cd^{2+} on subendothelial nerve signals. **a** Control signal; **b** bath application of 1 mM TEA; **c** ionophoretic application of TEA to an endplate in the vicinity of the recording electrode; **d** positive voltage deflection in the presence of 10 mM TEA and 250 μM 3,4-DAP; **e** block of the positive wave by bath application of 5 mM Cd^{2+}; **f** ionophoretic application of Cd^{2+} to an endplate

application of Cd^{2+} to the perineural sheath at the site of the recording electrode showed no effect on the signal. Microelectrodes for ionophoretic application had resistances of 80–100 MΩ when filled with TEA (1 M) or $CdCl_2$ (1 M).

To avoid the contribution of postsynaptic responses d-tubocurarine (50 μM) was added to the bath solution. Investigations on the 'Ca potentials' were made in the presence of tetraethylammonium chloride (TEA, 1–10 mM) and 3,4-diaminopyridine (3,4-DAP, 50–250 μM) as indicated in the results. Repetitive firing of the nerves which occurred in the presence of K channel blockers was abolished by addition of 100 μM procaine.

Results

Subendothelial signals

Figure 1a shows a nerve signal following supramaximal nerve stimulation. It consisted of a predominant double-peaked negativity which was often preceded by a small positivity. This waveform closely resembles focally recorded signals obtained at the transition between myelinated axon and non-myelinated terminal (Brigant and Mallart 1982) where evidence has been presented that the first negativity is due to a Na influx and the second negative peak corresponds to a passive current generated by the K efflux in the nerve terminals. In close analogy to what has been described by these authors the second negative deflection of the subendothelial signal can selectively be inhibited by 1 mM tetraethylammonium (TEA) (Fig. 1b) suggesting this signal component also to be due to a K conductance. Higher doses of TEA (10 mM) in combination with 250 μM 3,4-diaminopyridine (3,4-DAP) gave rise to a prolonged positive voltage deflection (Fig. 1d) which was effectively blocked by 5 mM Cd^{2+} (Fig. 1e), thus indicating its underlying cause to be a Ca current (see also Gundersen et al. 1982). Since it is known that in mammalian nodes of Ranvier the role of K conductance is negligible (Chiu et al. 1979) and Ca conductance not expected to be present there, we suspected these potentials to be the reflections of currents originating

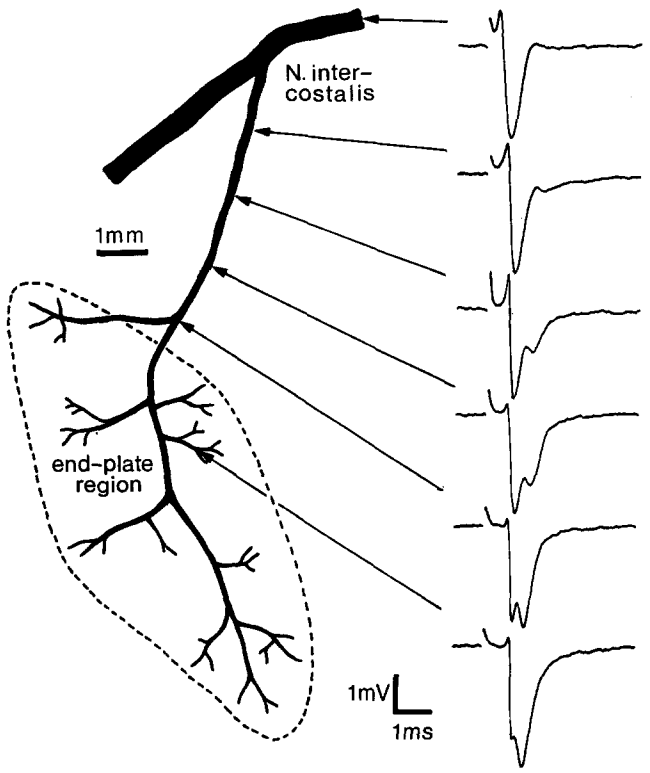

Fig. 2. Wave forms recorded at different points of the nerve as indicated by *arrows*. End-plate region is marked by *dashed line*. Note the reduced amplitude of the second negative peak with increasing distance from the endplate region

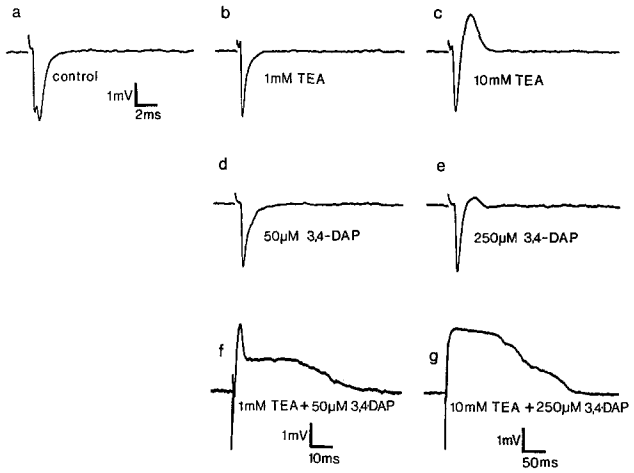

Fig. 3a–g. Effects of TEA and 3,4-DAP on subendothelial signals. **a** Control; **b** 1 mM TEA; **c** 10 mM TEA; **d** 50 μM 3,4-DAP; **e** 250 μM 3,4-DAP; **f** 1 mM TEA + 50 μM 3,4-DAP; **g** 10 mM TEA + 250 μM 3,4-DAP

from the presynaptic terminals. This would also account for the reversed polarity of the potentials related to K and Ca currents compared to focal recordings at the terminal itself. To substantiate this presumption we applied channel blocking agents to the endplate, while recording signals nearby in the nerve bundle.

Figure 1c shows the effect of TEA on the subendothelial signal when ionophoretically applied to the terminal arborizations of a motor axon. Obviously, the reduction of the second negativity is due to the blockade of K channels located in the presynaptic membrane of the neuromuscular junction. However, the component was not completely blocked, although this was the case in focal recordings obtained at the terminal region itself (not illustrated). Thus, the degree of signal reduction corresponds to the contribution of the treated terminal to the recorded signal.

Similar results for the prolonged positive wave could be obtained with the local administration of Cd^{2+} to the endplate after having blocked potassium channels with TEA (10 mM) and 3,4-DAP (250 μM). Figure 1f shows the suppression of the positive-going voltage deflection in the nerve bundle as a probable consequence of Ca channel blockade by Cd^{2+} in the nerve terminal. Control experiments in which TEA or Cd^{2+} were applied to the nerve bundle showed no effect on the signal.

Further evidence for the presence of longitudinal currents in the perineural tube originating from the presynaptic membrane is illustrated for the K component in Fig. 2. The second negative peak of the nerve signal diminished with increasing distance from the endplate regions (marked by dashed line) until only a predominantly negative waveform preceded by a small positivity remained (Fig. 2, top record). This latter signal reflects the propagating action potential since it is completely abolished in the presence of TTX (not shown). It remains unaffected by TEA (10 mM) and 3,4-DAP (250 μM).

These results indicate the presence of passively conducted currents of presynaptic origin in the space enclosed by the perineural sheath of nerve bundles. The major advantages of the subendothelial recording technique compared to focal recordings at the terminal are 1) improved signal to noise ratio, due to the contribution of presynaptic currents of several endplates to the signal; 2) stable recording over long periods allowing quantitative experiments.

Effects of TEA and 3,4-DAP

Figure 3 shows the effects of potassium channel blockers on the subendothelial wave form. While 1 mM TEA or 50 μM 3,4-DAP (Fig. 3b, d) effectively reduced the second negative spike of the control signal (Fig. 3a), higher concentrations gave rise to a positive voltage deflection (Fig. 3c, e). 3,4-DAP displayed maximal effects at 250 μM. In contrast TEA concentrations of 20–30 mM still slightly increased amplitude and duration of the positive wave (not illustrated). These findings strongly suggest the presence of at least two different sets of K channels, one of which is virtually 3,4-DAP-insensitive (Mallart 1984; see also Dubois 1981).

A combination of both agents gave rise to a large positive deflection which seems to consist of two components (Fig. 3f): a fast one of 6 ms duration and a prolonged one of 60 ms duration at a stimulation rate of 1/min. Raising the concentrations of K channel blockers further increased the positive-going wave, causing the two components to overlap (Fig. 3g). The duration of this potential was in the range of 150–350 ms varying with the muscle preparation and the position of the recording electrode.

Frequency dependence

Frequent nerve stimulation exhibited different effects on the two components of the positive wave. To distinguish these actions further, different concentrations of K channel

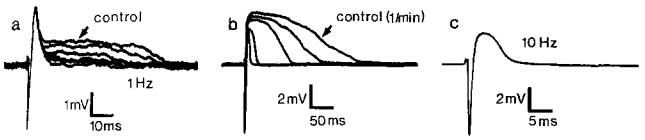

Fig. 4a–c. Effect of frequent nerve stimulation on 'Ca potentials'. **a** Control signal (*arrow*) was obtained with 1 mM TEA + 50 μM 3,4-DAP at a stimulation rate of 1/min. Five nerve stimuli at 1 Hz were subsequently applied. **b** Control signal (*arrow*) was obtained with 10 mM TEA + 250 μM 3,4-DAP at a stimulation rate of 1/min. Reduced positive waves correspond to stimulation frequencies of 0.033 Hz, 0.1 Hz, 1 Hz and 10 Hz respectively. They were taken after 15–30 nerve stimuli, when signals remained stable. **c** Same signal as shown in **b** recorded at 10 Hz in faster time sweep

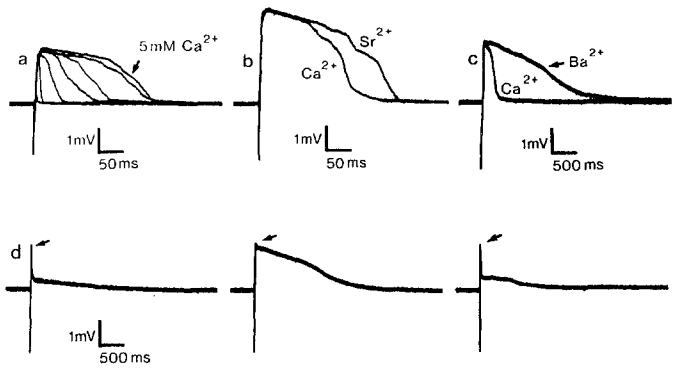

Fig. 5a–d. Effects of Ca^{2+}, Sr^{2+} and Ba^{2+} on positive signal components. **a** Actions of different external Ca^{2+}-concentrations on the subendothelial signal. The *arrow* indicates the signal taken at 5 mM Ca^{2+}, following signals correspond to 2.5, 2, 1.5, 1 and 0.5 mM Ca^{2+}, respectively. No added Ca^{2+} + 2 mM EGTA almost completely reduced positive deflections. **b** Equimolar substitution of 2.5 mM Ca^{2+} through Sr^{2+}; **c** equimolar substitution of 2.5 mM Ca^{2+} through Ba^{2+} (stimulation rate 1 pulse/5 min); **d** same as in **c**, except showing three consecutive signals taken at a stimulation rate of 1/min. Note that the fast component remains unaltered (*arrows*)

blockers were used. The control signal in Fig. 4a corresponds to a stimulation rate of 1/min and displays the fast and the slow component. Subsequent pulses applied at intervals of 1 s rapidly diminished the slow component leaving the fast one unaffected. After a resting period of 1 min the control signal was restored. However, with high frequency stimulation (10 Hz) over a longer period (20 min) the recovery of the slow component took more than 30 min.

Even with higher concentrations of TEA and 3,4-DAP the two components could be separated by increasing the stimulation rate. Figure 4b shows signals obtained at different stimulation frequencies. Amplitude and duration for any given frequency remained stable after some 15–30 pulses. At frequencies higher than 10 Hz no further reduction of the positive deflection could be observed. We will refer to the remaining positivity as the fast component (Fig. 4c) although a small contribution of the slow component cannot be excluded. For convenience experiments were conducted at a stimulation rate of 1/min, although longer pulse intervals in the range of several minutes showed even a more prolonged wave of up to 600 ms duration.

Effects of external Ca^{2+}, Ba^{2+} and Sr^{2+}

To clarify if both components of the prolonged positive wave are due to Ca^{2+} influx, external Ca^{2+} concentrations were varied. Both components of the positive voltage deflection were critically dependent on extracellular calcium concentration (Fig. 5a). With no calcium and 2 mM EGTA added to the bath solution all positivity was virtually absent. Increasing extracellular calcium concentration enhanced both, amplitude and duration of fast and slow signal components. While the fast component saturated at less than 1 mM Ca^{2+}, the duration of the slow component further increased with raising of external calcium, saturating at 5 mM.

Ba^{2+} and Sr^{2+} have been shown to substitute Ca^{2+} as inward current carriers through Ca channels so far characterized (see Hagiwara and Byerly 1981). Hence, equimolar substitution of external Ca^{2+} (2.5 mM) through Ba^{2+} and Sr^{2+} should provide further indications of the nature of the potentials recorded. Sr^{2+} effectively carried the fast and slow signal components. The duration of the positive wave was even slightly prolonged (Fig. 5b). After the exchange of Ca^{2+}-containing solution with Ba^{2+}-containing solution a great prolongation of the positive wave could be observed (Fig. 5c). At a stimulation rate of 1 pulse/5 min the duration of the positive potential increased to several seconds. One possible explanation is that Ba^{2+} blocks additional K channels spared by TEA and 3,4-DAP. This would then cause the prolongation of presynaptic depolarization. However, when stimulating with 1 pulse/min the amplitude of the slow component yielded large variations. Usually it seemed to be determined by the magnitude of the preceding positive wave, i.e. that large positive deflections were usually followed by signals with no or small positive potentials and vice versa. A sequence of three consecutive traces is shown in Fig. 5d. The fast component remained unaltered throughout the experiments. Likewise, when a pulse was applied at the end of the long positive wave, the slow component was completely abolished, whereas the amplitude of the fast component remained stable. These findings further indicate the presence of at least two distinct Ca channels in the presynaptic membrane (see Discussion).

Effects of calcium-antagonists

Amongst other divalent cations Cd^{2+}, Mn^{2+} and Co^{2+} have been found to inhibit Ca currents by competing with Ca^{2+} at the cation binding site without passing the channel.

Figure 6a and d show dose dependent effects of Cd^{2+} on the slow signal component. Cd^{2+} in a concentration of only 10 μM blocks the slow component almost completely. In contrast, inhibition of the fast component required a concentration of 15 mM Cd^{2+} (Fig. 6b). Similar results were obtained with other divalent cations like Co^{2+} and Mn^{2+}, although in somewhat higher concentrations.

Some types of Ca channels exhibit considerable sensitivity to organic compounds such as verapamil, diltiazem, nifedipine, etc. (Kohlhardt et al. 1972; Fleckenstein 1977; Lee and Tsien 1983; for review see Rosenberger and Triggle 1978) while others do not (Nachshen and Blaustein 1979; Gotgilf and Magazanik 1977).

Verapamil selectively reduced the slow component (Fig. 6c) at concentrations in the nanomolar range, although even high concentrations (10 μM) failed to block it completely. These concentrations showed no effect whatsoever on the fast component (Fig. 6f) suggesting another

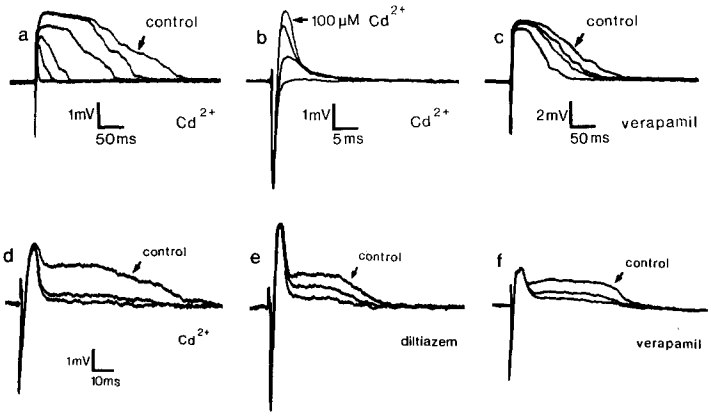

Fig. 6a–f. Effects of organic and inorganic calcium antagonists on the 'Ca potential'. **a** Effect of 10^{-8}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M Cd^{2+} on the control signal (*arrow*); **b** effect of Cd^{2+} (1, 5 and 15 mM) on the fast component after the slow component was diminished by 100 µM Cd^{2+} (*arrow*); **c** effect of 10^{-9}, 10^{-7} and 10^{-5} M verapamil on the control signal (*arrow*); **d** effect of 10^{-6} and 10^{-5} M Cd^{2+} on the slow component; **e** effect of 10^{-6} and 10^{-5} M diltiazem on the slow component; **f** effect of 10^{-7} and 10^{-6} M verapamil on the slow component. In **a–c** the concentrations of TEA and 3,4-DAP were 10 mM and 250 µM, respectively; in **d–f** they were 1 mM and 50 µM, respectively

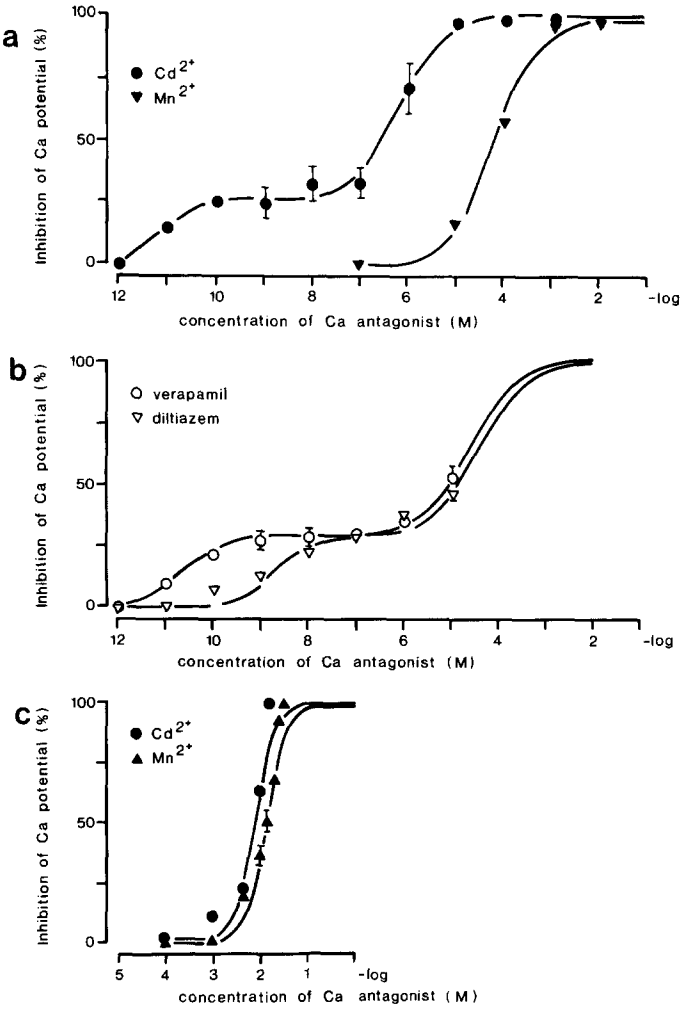

Fig. 7a–c. Concentration-response relationship between calcium antagonists and 'Ca potentials'. **a** Effects of Cd^{2+} (●) and Mn^{2+} (▼) on total 'Ca potential'; **b** effects of verapamil (○) and diltiazem (▽) on total 'Ca potential'. Blocking effects of these antagonists on the nerve spike prevented measurements at concentrations higher than 10 µM; **c** effects of Cd^{2+} (●) and Mn^{2+} (▲) on the 'Ca potential' after more than 95% of the total positive deflection has been blocked by 100 µM Cd^{2+} and 1 mM Mn^{2+}, respectively. Data for these concentrations were arbitrarily set to 100% and correspond fairly exactly to the fast 'Ca component' recorded with 10 Hz stimulation. Data for concentrations higher than 10 mM Cd^{2+} or 20 mM Mn^{2+} were not incorporated into least-squares fits since also a marked depression of the nerve spike could be observed. Values for each point are the mean of three independent experiments (±SEM) and designate the area enclosed by the positive waves and the baseline. Theoretical curves were fitted by the least-squares method according to the Eqs. $Y=[Y_{max1}/1+(K_{D1}/A)]+[Y_{max2}/1+(K_{D2}/A]$ in **a, b** and $Y=[Y_{max3}/1+(K_{D3}/A)^2]$ in **c**, respectively. K_D is the apparent dissociation constant, A is the concentration of Ca-antagonists and Y_{max} is maximal inhibition. The calculated data are listed in Table 1

fundamental difference between the two 'Ca potentials'. Diltiazem, another organic antagonist, showed similar effects on the slow signal component (Fig. 6e). It also did not affect the fast one. However, with 100 µM, both verapamil and diltiazem exhibited depressing effects on the nerve spike, suggesting unspecific blocking of Na channels. Another interesting finding is that the 1,4-dihydropyridines nitrendipine (10 µM) and nisoldipine (10 µM) had no effect on the fast and slow signal components. Blocking action of the inorganic as well as the organic antagonists could at least partially be reversed by raising external calcium.

Figure 7 summarizes our findings showing the concentration-response-curves of some calcium-antagonists. Responses are defined as the area enclosed by the positive waves and the baseline. Inhibition curves for the slow Ca potential (Fig. 7a, b) could best be fitted assuming two apparent dissociation constants (K_D) except for Mn^{2+}, where two constants could not be distinguished. Figure 7c shows a more detailed curve of the action of Cd^{2+} and Mn^{2+} after more than 95% of total positive potential has been blocked by 100 µM Cd^{2+} and 1 mM Mn^{2+}, respectively. At these concentrations the remaining positivity corresponds fairly exactly to the fast component recorded with 10 Hz stimulation and was arbitrarily set to 100%. It should be noted that concentrations exceeding 20 mM Mn^{2+} and 10 mM Cd^{2+} also reduced the nerve spike. Data for these concentrations are supplied in the plot but not incorporated into least-squares fitting. The calculated data from fitted curves are listed in Table 1.

Discussion

Recent investigations on presynaptic currents (Gundersen et al. 1982; Mallart 1984) used a recording technique in which microelectrodes were inserted under the perineural sheath of nerve bundles. From indirect evidences it was suggested that electric signals recorded there reflect currents entering or leaving the presynaptic terminal in response to nerve stimulation. To provide further evidence for the presence of passively conducted, presynaptic currents inside the perineural tube of nerve bundles, we applied known channel blockers to an endplate and observed reduction of nerve

Table 1. Parameters derived from least-squares fits to concentration-response curves[a]

	Slow Ca component				Fast Ca component[b]	
	Max. inhibition		Max. inhibition		Max. inhibition	
	Y_{max1} (%)	K_{D1} (M)	Y_{max2} (%)	K_{D2} (M)	Y_{max3} (%)	K_{D3} (M)
Verapamil	29	$2.9 \cdot 10^{-11}$	71	$1.9 \cdot 10^{-5}$	–	–
Diltiazem	30	$1.8 \cdot 10^{-9}$	70	$3.1 \cdot 10^{-5}$	–	–
Cd^{2+}	26	$9.4 \cdot 10^{-12}$	74	$6.6 \cdot 10^{-7}$	100	$7.8 \cdot 10^{-3}$
Mn^{2+}	–	–	100	$6.8 \cdot 10^{-5}$	100	$1.4 \cdot 10^{-2}$

[a] See Fig. 7
[b] Here the parameters were determined using the co-operative model with the Hill-coeffizient $n = 2$
K_D = apparent dissociation constant

signal components. The present experiments were done to investigate presynaptic calcium conductances in mammalian motor nerve terminals.

Blockade of the repolarizing K-efflux in nerve terminals with TEA and 3,4-DAP unmasks two prolonged positive waves. In our recording situation any positive deflection indicates either a positive outward current at the site of the recording electrode (within the perineural sheath) or a positive inward current generated distantly in the nerve terminals. The first possibility can be ruled out simply because outward currents are not expected to be found in mammalian myelinated axons (Chiu et al. 1979). Not surprisingly, TEA and 3,4-DAP showed no effect whatsoever on the nerve signal recorded far away from the endplate region indicating the absence of a significant K-conductance in the nodal membranes. Presumably, there is an attenuation of the first negative deflection (the external nerve spike at the site of the recording electrode) due to passive currents originating from precedingly and succeedingly activated nodes of Ranvier which would tend to render the tip of the electrode more positive, as witnessed by the initial small positivity and the final positive 'hump' in Fig. 2 (top record). It appears that in the presence of TEA and 3,4-DAP the positive waves recorded inside the perineural sheath close to the nerve endings correspond to positive inward currents in the nerve terminals. Brigant and Mallart (1982) could not detect other inward than Ca^{2+} currents in mouse nerve terminals. This has been contested by Konishi and Sears (1984) who found a TTX-sensitive inward current which appeared to be partly dependent on Ca^{2+} but insensitive to Co^{2+}. If the latter interpretation is correct, then with K channels blocked by TEA and 3,4-DAP the terminal action potential would be of long duration (Brismar 1981) and possibly show up as a positive deflection of the sub-endothelial signal. However, the results presented here provide pharmacological evidence that all presynaptic inward current detectable with this method appears to be due to Ca^{2+}-influx rather than Na^{+}.

Both signal components are strictly dependent on extracellular Ca^{2+}-concentration. 'Saturation' occurs for the fast component at less than 1 mM Ca^{2+}, while the slow component 'saturates' at 5 mM external Ca^{2+}. We certainly failed to eliminate completely K conductance, hence, restricting the amplitude and duration of these potentials. Thus, saturation in our system means maximal response under the given conditions.

The effects of Cd^{2+} and Mn^{2+} on both components further indicate these deflections to be due to Ca conductances. The concentration-response curves clearly indicate at least two different Ca conductances associated to Ca channels with different dissociation constants. While the slow component is blocked by Cd^{2+} and Mn^{2+} in the micromolar range, the fast component is blocked in the millimolar range. Another striking difference between the two positive potentials is revealed by the differential effects of verapamil. While the fast component remains unaffected by verapamil the slow component is considerably reduced at concentrations in the nanomolar range. From the data obtained for Cd^{2+}, verapamil and diltiazem the Ca current associated with the slow signal component can best be described assuming two apparent dissociation constants, suggesting the presence of two binding sites. Another possible explanation is the presence of two subtypes of Ca channels constituing the slow component.

Interestingly, the 1,4-dihydropyridines nitrendipine and nisoldipine had no effect on the slow and fast 'Ca potentials', although considered to be the most potent organic Ca antagonists (see for review Schwartz and Triggle 1984). This further stresses the idea of tissue specific heterogeneity of Ca channels. Ca channels with similar pharmacological properties have recently been found in the somatic membrane of avian dorsal root ganglion neurones (Boll and Lux 1985).

All Ca channels so far studied are permeable to Ba^{2+} and Sr^{2+}. In our experiments Sr^{2+} effectively substituted for Ca^{2+} in eliciting the fast and slow signal components. The duration was even enhanced. This result may not be due to higher mobility of Sr^{2+} through the Ca channel, but instead to an effect on potassium conductance. Sr^{2+} has been reported to be less effective in activating Ca-dependent K channels (Gorman and Hermann 1979).

Ba^{2+}-ions were also able to carry both components of the positive deflection. At a stimulation rate of 1 pulse/5 min the duration of the slow potential was greatly enhanced to values of several seconds. This prolongation may be due to the blockade of Ca-dependent K channels by Ba^{2+} (for reviews see Latorre and Miller 1983; Hermann and Hartung 1983). However, when stimulating with 1 pulse/min the magnitude of the slow component showed large variations. Amplitude and duration of the slow component of consecutive signals largely depended on the magnitude of the preceding Ba^{2+} influx, i.e. that signals with large positive deflections were followed by signals with no or small slow

positive potentials. This effect may be due to intracellular accumulation of Ba^{2+} which may then cause the inactivation of the channels carrying the slow component, as witnessed by the reduction of the slow positive potential. Since the fast component showed no alterations, even if a second pulse was applied at the end of a prolonged wave, one has to assume that Ba^{2+}, whatever the mechanism underlying this phenomenon, inactivates the slow component, leaving the fast one unaffected. A fast decrease of a current carried by Ba^{2+}-ions through Ca channels in the somatic membrane of mollusc neurones has been reported recently (Kostyuk et al. 1985).

Since neither Ba^{2+} (2.5 mM) nor Cd^{2+} (3 mM) had any significant effect on the second negative peak recorded in the absence of TEA and 3,4-DAP (not illustrated) it is suggested that Ca-dependent potassium conductance is negligible under these conditions. However, these channels become important when high amounts of Ca^{2+} enter the synapse, as is the case after reduction of K conductance with TEA and 3,4-DAP. In contrast to Ba^{2+}, TEA or 3,4-DAP alone effectively suppressed the 'K component' of nerve signals (see Fig. 3). While 3,4-DAP (250 μM) displayed maximal effects on K conductance attainable with this blocker, addition of TEA still potentiated this effect. From these experiments it is suggested that at least two distinct sets of potassium channels might be present in the presynaptic nerve terminal. For the same preparation used in this study the presence of two different potassium channels, one of which being Ca-dependent, has been suggested by Mallart (1984) using a different experimental approach.

Another property of the slow 'Ca potential' namely its depression with increasing stimulation frequency remains puzzling. There may be more than one mechanism involved. It is known that the block of K conductance by aminopyridines is relieved by sustained or repetitive depolarization (Ulbricht and Wagner 1976; Meves and Pichon 1977; Kirsch and Narahashi 1978). Since the frequency dependent reduction was also observed with very high concentrations of 3,4-DAP and TEA or in the presence of TEA alone we regard this effect to be small. Similarly, one would not expect periods of 30 min for the recovery of the signal subsequent to a prolonged phase (20 min) of repetitive stimulation. We suggest the main mechanism contributing to the decrease of the slow current to be the Ca entry itself. Increased $ICaI_i$ by repetitive stimulation may have two synergistic effects. On the one hand it could activate Ca-dependent K channels (Hermann and Hartung 1983; Latorre and Miller 1983). On the other hand it could block Ca currents (for review see Eckert et al. 1981).

Our results indicate the presence of two different presynaptic Ca currents in mouse motor endings being responsible for potentials recorded within the perineural tube. While the fast current may be attributed to voltage dependent Ca channels responsible for the nerve-evoked transmitter release the origin of the slow current is somewhat more difficult to interpret. It might result from a local regenerative process (a graded type of 'Ca-spike') associated with a distinct set of voltage dependent Ca channels activated more slowly at more positive potentials. The physiological role of this current remains to be elucidated.

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