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Effects of diltiazem and verapamil on responses to acetylcholine

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1 The calcium channel antagonists diltiazem and verapamil were found to alter the average lifetime of ion channels activated by acetylcholine (ACh).

2 Average channel lifetime was determined from the decay phase of miniature endplate currents at the neuromuscular junction of mouse hemidiaphragms and from direct recording of single channel currents activated by ACh from BC_3H1 mouse tumour cells in culture.

3 Both diltiazem and verapamil reduced average channel lifetime in a dose-dependent manner. For each drug, concentrations as high as 20 μM – 100 μM were required to decrease channel lifetime by 50%.

4 Single channel recording experiments also showed that both diltiazem and verapamil greatly decreased the frequency of opening events at concentrations as low as 2 μM to 5 μM. This finding is consistent with an enhancement of receptor desensitization.

Introduction

Calcium channel antagonists (CCA) such as diltiazem and verapamil are widely used for treatment of supraventricular arrhythmias and ischaemic heart disease (Reves *et al.*, 1982; Paoletti & Govoni, 1987). In general, these drugs act by inhibiting calcium influx through slow calcium channels in cardiac and smooth muscle cells. Diltiazem and verapamil bind to separate sites associated with the calcium channel, although both sites are allosterically linked to the dihydropyridine receptor (Schramm & Towart, 1985).

Numerous reports suggest that CCA also have effects on the neuromuscular system. Diltiazem, verapamil and nifedipine potentiate the actions of neuromuscular blocking agents (Bikhazi *et al.*, 1982; 1983; Williams *et al.*, 1983; Durant *et al.*, 1984; Del Pozo & Baeyens, 1986; Wali, 1987). In neuromuscular preparations, twitches produced by both direct (muscle surface) and indirect (nerve) stimulation are inhibited by verapamil (Bondi, 1978; Publicover & Duncan, 1979; Kraynack *et al.*, 1983a; Wali, 1987). Twitches produced by indirect stimulation are depressed to a greater extent, suggesting a possible inhibition of neuromuscular transmission (Ribeiro *et al.*, 1979; Kraynack *et al.*, 1983b; Lawson *et al.*, 1983). Verapamil also decreases contracture produced by application of acetylcholine (ACh) (Chiarandini & Bentley, 1973; Wali, 1987). These results suggest that CCA inhibit neuromuscular transmission through postsynaptic mechanisms.

The postsynaptic effects of calcium channel antagonists on neuromuscular transmission may possibly be due to alterations in the properties of ACh-activated ion channels. In molluscan neurones, D600 (methoxyverapamil) reduces both sodium and chloride currents activated by ACh and decreases the time constant of current relaxations in response to voltage jumps (Bregestovski & Iljin, 1980; Slater *et al.*, 1983). At the frog neuromuscular junction, D600 reduces the sensitivity of the endplate to ACh and carbachol and increases the decay rate of postsynaptic currents (Bregestovski *et al.*, 1980; Adam & Henderson, 1986).

The experiments described in this paper were therefore designed to study effects of diltiazem and verapamil on the average lifetime of channels activated by ACh. Two methods have been used to determine average channel lifetime: measurement of the decay phase of miniature endplate currents (m.e.p.cs) at the motor endplate, and direct recording of ACh-activated single channel currents using patch clamp techniques. Results show that diltiazem and verapamil depress responses to ACh and decrease average channel lifetime. These findings may help to explain decreases in twitch tension sometimes observed with CCA, and may also help to account for their interaction with other neuromuscular blocking agents.

Methods

For endplate experiments, diaphragms were removed from male Swiss Webster mice (25–45 days old) under ether anaesthesia. Hemidiaphragms were pinned to the bottom of a Sylgard-lined chamber with insect pins. Muscles were continuously superfused with a Ringer solution containing (mM): NaCl 115, KCl 4.5, Na_2HCO_3 25, NaH_2PO_4 1.0, $MgSO_4$ 1.0, $CaCl_2$ 2.0 and glucose 12, pH 7.3, which was bubbled with 95% O_2: 5% CO_2. Diltiazem (5–100 μM, Marion Laboratories) and (±)-verapamil (50–500 μM, Sigma) were added directly to the perfusate.

Miniature endplate currents (m.e.p.cs) were recorded focally from endplate regions of muscle fibres by use of an extracellular electrode. Electrodes were filled with Ringer solution, had resistances of 100–500 kΩ and tip diameters of 5–20 μm. Signals were high pass filtered at 1 Hz and low pass filtered at 3.5 kHz by means of an 8-pole Bessel filter. M.e.p.cs, including a segment of baseline prior to each event, were digitized at 50 μs per point. Records were discarded if they contained overlapping events or artifacts, or if the rise time of the m.e.p.c. was greater than about 300 μs. M.e.p.cs were then averaged and the time constant of decay, τ, was determined by a linearized least squares fit between 15% and 90% of the peak to $I(t) = I(0)\exp(-t/\tau)$, where I is the averaged current at time t. Usually 100–200 m.e.p.cs were averaged for each measurement. M.e.p.cs were collected 20–25 min after drug application. All experiments were performed at controlled room temperature (18–23°C).

For single channel experiments, patch clamp techniques were used to record single channel currents activated by ACh from cell-attached patches of BC_3H1 cells grown in culture. BC_3H1 cells are derived from a mouse cerebrovascular tumour, and express nicotinic receptors for ACh when allowed to differentiate in serum-free media (Munson *et al.*, 1982; Olson *et al.*, 1983). ACh-activated channels from BC_3H1 cells have been studied extensively by Sine & Steinbach (1984, 1986) and Sine & Taylor (1979, 1980, 1981).

Cells were grown in tissue culture flasks (Strauch & Rubenstein, 1984) at 37°C in a 5% CO_2-enriched atmosphere using Dulbecco's modified Eagles's medium containing glucose 1 g l^{-1}, and supplemented with 10% heat-inactivated foetal calf serum, L-glutamine 0.32 mg ml^{-1}, penicillin 100 u ml^{-1} and streptomycin 100 μg ml^{-1}. When the cells had grown to confluence, they were induced to promote synthesis of ACh receptors by incubation in a serum-free N2SF modified hormone-supplemented culture medium containing RPMI 1640, bovine serum albumin 5 μM, insulin 5 μg ml^{-1}, transferrin 100 μg ml^{-1}, progesterone 20 nM, putrescine 100 μM, Na_2SeO_3 30 nM, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid 10 mM pH 7.2. After 3–5 days in serum-free media, the cells were treated with 0.5% trypsin in 0.1% EDTA phosphate-buffered saline to lift them off culture flasks, then subcultured onto microscope cover slips. Cells were used 1–3 days later.

Cells attached to cover slips were transferred to a solution containing (mM): NaCl 100, KCl 4.0, $CaCl_2$ 2.0, $MgCl_2$ 5.0 and HEPES 10.0, pH 7.4. Electrodes contained this solution plus ACh 50 nM–1 μM. Diltiazem and verapamil were added to both the electrode and bathing solutions. Experiments were performed at room temperature, and all data were collected at +75 mV hyperpolarized relative to cell resting potential, which was estimated to be −59 mV. Signals were low pass filtered at 3 kHz by use of an 8-pole Bessel filter, and single channel currents, together with a segment of baseline prior to each event, were digitized at 50 μs per point. Channels were then analysed to determine amplitude and open time. Average channel amplitude was calculated from the mean amplitude of events > 500 μs in duration. Channel opentime distributions were described as the sum of 1 or 2 exponential components estimated by the maximum likelihood method using a non-linear least squares approximation according to the P3R function of BMDP statistical package. A single component was considered adequate if the correlation between the 2 time constants generated by a 2 component fit was greater than 0.5.

Data collection and analysis were performed on an IBM PC/XT or AT equipped with a Tecmar LabMaster A/D board and 576k memory utilizing pCLAMP software package (Axon Instruments, Burlingame, California) and Lotus 123.

Results

M.e.p.cs recorded in the absence and presence of diltiazem and verapamil are shown in Figure 1. Both drugs decreased the amplitude of the currents and increased the rate at which they decayed.

Measurements of current decay were made using averaged m.e.p.cs, as shown in Figure 2. Semilogarithmic plots of the decay phases are linear, demonstrating that m.e.p.c. decay was well described by a single exponential function of time. In the experiments illustrated, diltiazem 100 μM decreased τ to 51% of control, while verapamil 100 μM decreased τ to 57%.

Both diltiazem and verapamil decreased channel lifetime in a dose-dependent manner. Figure 3 illustrates the relationship between τ and concentration for the two drugs. τ, expressed as a fraction of its value in the absence of drug, is plotted as a function of drug concentration on a logarithmic scale. Diltiazem was slightly more potent than verapamil in decreasing τ. At 100 μM, diltiazem reduced τ to 0.49 ± 0.11% of control

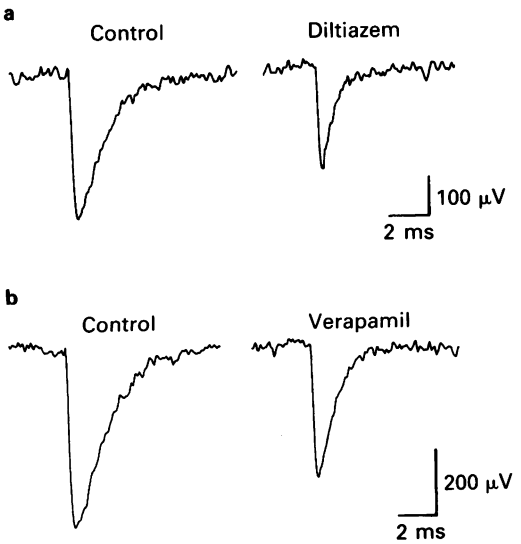

Figure 1 Examples of individual m.e.p.cs recorded in the absence and presence of (a) diltiazem 100 μM and (b) verapamil 100 μM. Each drug reduces current amplitude and increases current decay rate.

(mean of 6 endplates ± s.e.mean), whereas 100 μM verapamil decreased τ to 0.69 ± 0.09% ($n = 6$). Since the decay rate of m.e.p.cs is usually determined by the rate at which open channels close (or convert to some other non-conducting state), the decreases in τ seen with diltiazam and verapamil suggest that these drugs decrease the average time that channels stay open once they have been activated by ACh.

Single channel recording techniques were used to confirm the apparent reduction in channel opentime produced by diltiazem and verapamil. ACh-activated single channel currents were recorded from BC_3H1 cells in culture.

Frequency histograms of channel opentimes often contained an excess of short events beyond that predicted for a simple exponential distribution. For cells exposed to ACh alone, 13 out of 20, or 65% required two exponential components, τ_f and τ_s ($\tau_f < \tau_s$), to describe opentime distributions. The presence of two components suggests that there are at least two open states of the channel. The significance of the faster component is unclear, while the slower component would correspond to average channel lifetime at the endplate as measured by fluctuation analysis (Colquhoun & Sakmann, 1985) or m.e.p.c. decay.

Verapamil did not alter τ_f, which measured 0.30 ± 0.08 ms (mean ± s.e.mean from $n = 3$ cells) at 20 μM compared with 0.36 ± 0.07 ($n = 13$) with ACh alone. At diltiazem concentrations greater than 5 μM, opentime distributions were well described by single

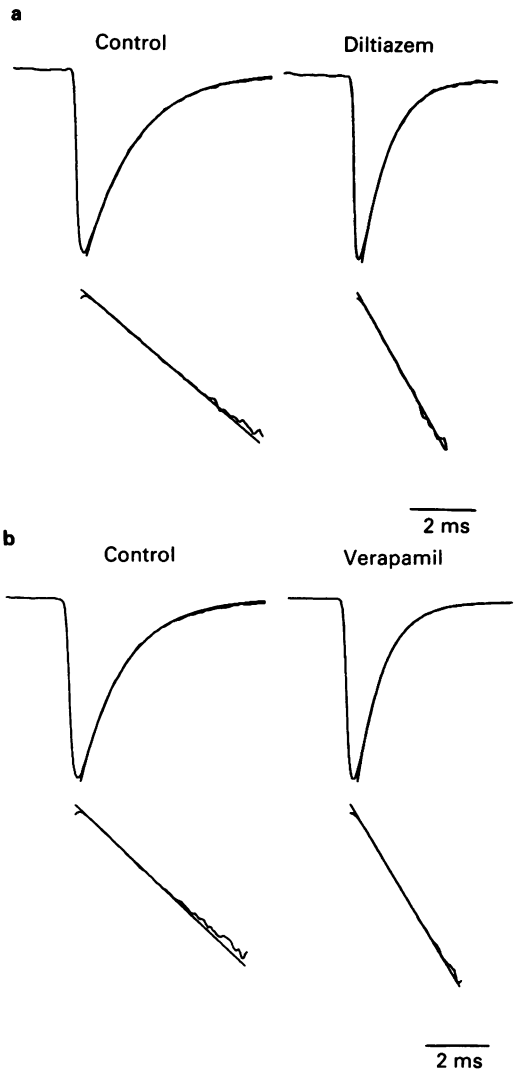

Figure 2 Averaged m.e.p.cs obtained in the absence and presence of (a) diltiazem and (b) verapamil. Semi-logarithmic plots of the decay phases are shown below each current, and calculated exponential curves are superimposed on the data. Currents have been normalized to the same peak height to facilitate comparison of decay phases. In the experiments illustrated, diltiazem 100 μM decreased τ from 1.50 to 0.77 ms, and verapamil 100 μM decreased τ from 1.38 to 0.79 ms.

exponential functions requiring only 1 time constant τ_s ($n = 12$). A faster component, if it existed, might not be detectable due to bandwidth limitations of the recording system.

Both diltiazem and verapamil shortened the time constant of the slower component of opentime his-

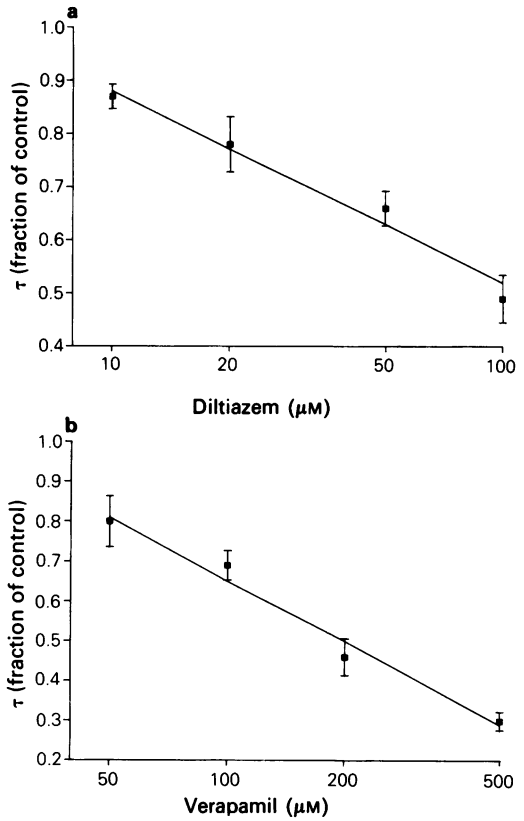

Figure 3 Dose-response curves illustrating decreases in the time constant of m.e.p.c. decay produced by (a) diltiazem and (b) verapamil. Values of τ are expressed as fractions of paired controls. Each value is the mean of 3 to 6 experiments with s.e.mean shown by vertical lines. Lines are least squares fits.

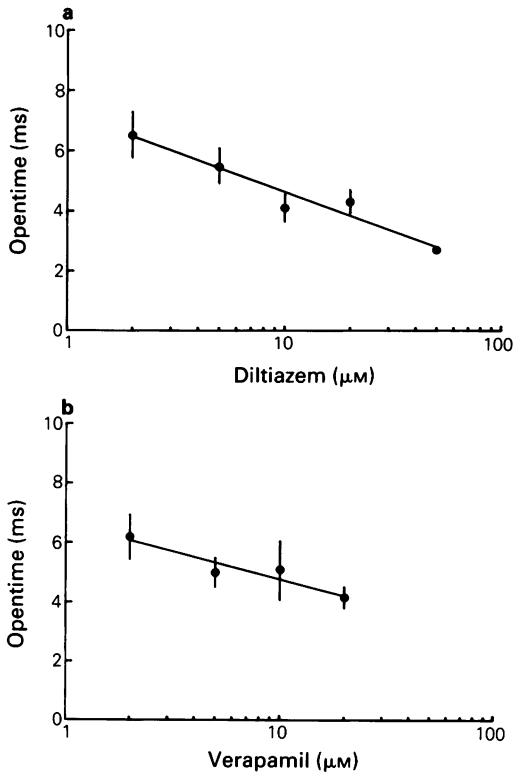

Figure 4 Dose-reponse curves showing decreases in the opentime of acetylcholine (ACh)-activated single channel currents produced by (a) diltiazem and (b) verapamil. The time constant of the slower component of opentime histograms was used as an indication of mean channel opentime. Each value is the mean of 3 to 11 cells, with s.e.mean shown by vertical lines. Lines are least squares fits.

tograms, and did so in a dose-dependent manner (Figure 4). Diltiazem 50 μM reduced τ_s to about 43% of control, from 6.32 ± 0.18 ms ($n = 20$) to 2.71 ± 0.07 ms ($n = 3$). Verapamil 20 μM reduced τ_s to 65% or 4.13 ± 0.33 ms ($n = 3$).

Diltiazem and verapamil were usually present in both the recording electrode and bathing solution. When diltiazem was present only in the electrode, its efficacy was reduced. Diltiazem 50 μM in the bath reduced the time constant to 43% of control compared with 75%, or 4.75 ± 0.46 ms ($n = 5$) when diltiazem was present only in the electrode.

Single channel amplitude was not consistently affected by either diltiazem or verapamil. In the absence of drug, mean channel amplitude at +75 mV hyperpolarized relative to cell resting potential was 2.70 ± 0.03 pA (mean $\pm$ s.e.mean from $n = 24$ cells). Average channel amplitude was 2.52 ± 0.09 pA ($n = 6$) at 20 μM diltiazem and 2.74 ± 0.01 pA ($n = 3$) at 20 μM verapamil.

One obvious effect of diltiazem and verapamil was to decrease the rate at which channels were activated by ACh. Figure 5 illustrates results of experiments in which the frequency of channel openings was measured as a function of drug concentration. At 500 nM ACh, openings averaged 568 per min ($n = 4$), although this number varied greatly due to differences in the number of channels in each patch. In the presence of diltiazem or verapamil, openings dropped dramatically, to about 50 per min at 20 μM, and sometimes disappeared entirely.

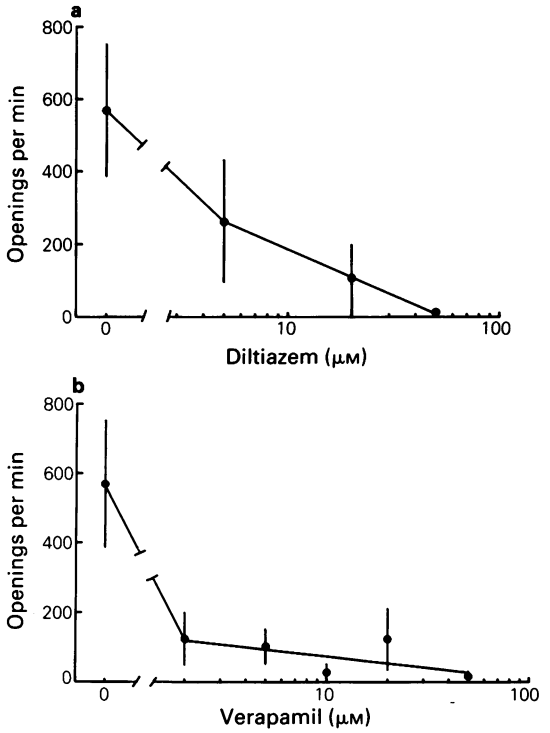

Figure 5 Measurement of frequency of channel openings as a function of drug concentration at acetylcholine 500 nM for (a) diltiazem and (b) verapamil. Drugs were present in both electrode and bathing solution. Channel openings decreased rapidly with increasing concentration. Each point is the mean of 4 to 9 cells with s.e.mean shown by vertical lines. Lines have been drawn by eye.

Discussion

The main finding of these experiments is that diltiazem and verapamil have anti-acetylcholine properties. These drugs appear to affect ACh-activated channels in two distinct ways; they decrease channel lifetime, but also reduce the frequency of channel activation. These effects, however, were observed at concentrations 20–100 times higher than those encountered clinically. In man the therapeutic blood level of diltiazem is 0.1–0.4 μM (50–200 ng ml^{-1}, Marion Laboratories product information on Cardizem), while plasma levels of verapamil are about 0.25–0.8 μM (125–400 ng ml^{-1}, Knoll Pharmaceutical Co. product information on Isoptin). In other *in vitro* experiments, concentrations of 5 μM diltiazem or 1 μM verapamil have been used to block calcium currents in isolated ventricular cells (Lee & Tsien, 1983), while 63 μM diltiazem or 10 μM verapamil are required to produce 50% inhibition of calcium currents in skeletal muscle (Walsh *et al.*, 1986). Thus the concentrations used in these experiments may not be entirely inappropriate.

The lower levels of diltiazem and verapamil expected during clinical use may still produce significant alterations in the function of ion channels activated by ACh. Even a small reduction in channel opentime or a decrease in the rate of channel activation will decrease the safety factor for neuromuscular transmission. Such a decrease will become especially important when transmission is also depressed by other agents. Thus these findings are consistent with numerous reports in the literature that CCA potentiate effects of other neuromuscular blockers.

One possible explanation for the decreased number of single channel opening events observed with diltiazem and verapamil is an enhancement of desensitization (Adam & Henderson, 1986). Single channel recording involves multiple reactivations of a small number of channel proteins in the membrane patch, and thus an enhancement of desensitization would hinder channel reactivation and decrease the frequency of opening events. Although the amplitude of m.e.p.cs was only slightly affected by diltiazem and verapamil, m.e.p.cs are produced by one-time activation of several hundred channels simultaneously, rather than multiple activations of the same channels. Thus m.e.p.c. results do not contradict a possible increase in receptor desensitization.

Unfortunately, the theory that diltiazem and verapamil act by enhancing desensitization could not be tested directly by single channel recording techniques. The low rates of channel activation in the presence of diltiazem and verapamil made it extremely difficult to collect sufficient numbers of opening events to determine the number of states occupied by the channel and to make precise measurements of transition rates between states. Further experiments are needed to ascertain the precise mechanisms by which CCA interact with ion channels activated by ACh.

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