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Effects of dihydropyridine calcium antagonists on rat midbrain dopaminergic neurones

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1 The effects of the dihydropyridine calcium channel antagonists, nifedipine and nimodipine (300 nM–30 μM) were tested *in vitro* on intracellularly recorded dopaminergic neurones in the rat ventral mesencephalon.

2 Bath applied nifedipine and nimodipine inhibited in a concentration-dependent manner the spontaneous firing discharge of the action potentials, whereas, the dihydropyridine calcium channel agonist, Bay K 8644 increased the firing rate.

3 Pacemaker oscillations and bursts of action potentials were produced by loading the cells with caesium. Nifedipine and nimodipine reduced the rate and the duration of the caesium-induced membrane oscillations and decreased the number of action potentials in a burst. During the blockade of potassium currents the dopaminergic neurones often developed a prolonged (100–800 ms) afterdepolarization that was also inhibited by dihydropyridines.

4 The spontaneous discharge of calcium spikes was also inhibited by both dihydropyridine calcium antagonists. The apparent input resistance and the level of membrane potential were not affected by the dihydropyridine calcium antagonists.

5 If the action potential duration was less than 150 ms the shape of the spike was not clearly influenced by both calcium antagonists. However, when the duration of the action potential was longer than 150–200 ms due to the intracellular injection of caesium ions plus the extracellular application of tetraethylammonium (10–50 mM), both nifedipine and nimodipine reversibly shortened the plateau potential.

6 It is suggested that nifedipine and nimodipine depress the rhythmic and bursting activity of the dopaminergic cells and shorten the calcium action potential by blocking dihydropyridine-sensitive high-threshold calcium currents.

Keywords: Nifedipine; nimodipine; Bay K 8644; substantia nigra; ventral tegmental area; intracellular electrophysiological recordings; calcium

Introduction

The regular pacemaker-like electrical activity of dopaminergic cells in slices of the ventral mesencephalon is highly dependent on a set of intrinsic membrane conductances (Silva & Bunney, 1988; Lacey *et al.*, 1989; Grace, 1991), which should make them capable of rhythmic firing even in the absence of synaptic inputs (Hainsworth *et al.*, 1991). Furthermore, it has recently been reported that the rhythmic pattern of firing in these cells could be transformed in a bursting one under particular conditions (e.g. K^+ channels blockade by the extracellular application of apamin, Shepard & Bunney, 1991).

Several subtypes of calcium channels (L, N, T and P) have already been characterized at neuronal level (Tsien *et al.*, 1991). Among them, it has been clearly demonstrated that the L-type channels are sensitive to agonists and antagonists of the dihydropyridine class (Bellmann *et al.*, 1983; Greenberg *et al.*, 1984; Gahwiler & Brown, 1987; Regan *et al.*, 1991; Sayer *et al.*, 1993), whereas T, N and P type channels are almost unaffected by these agents. The role of the different types of calcium channels in regulating the functioning of the dopaminergic cells has not yet been fully investigated. It is interesting to note that the substantia nigra has a high density of nitrendipine (a dihydropyridine antagonist) binding sites which are reduced in patients with Parkinson's disease (Nishino *et al.*, 1986). In addition, dihydropyridine Ca^{2+}-channel antagonists have recently been reported to inhibit the firing rate of mesencephalic dopaminergic cells (Ugedo *et al.*, 1988; Nedergaard *et al.*, 1993). In order to study further the role of the dihydropyridine-sensitive calcium currents on the spontaneous rhythmic and bursting firing rate and on the shape of the action potentials, we applied the organic Ca^{2+} channel antagonists, nifedipine and nimodipine to midbrain dopaminergic cells intracellularly recorded *in vitro* in a rat brain slice preparation.

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Methods

Preparation of the tissue

A detailed description of the methods has been published elsewhere (Lacey *et al.*, 1989; Mercuri *et al.*, 1991). Albino Wistar rats (150–300 g) (Morini, Reggio Emilia) were anaesthetized with ether and killed by a blow to the chest. The brain was removed and horizontal slices (thickness 300 μm) were cut by a vibratome starting from the ventral surface of the midbrain. A single slice containing the substantia nigra and the ventral tegmental area was then transferred to a recording chamber and completely submerged in an artificial cerebrospinal fluid warmed to 35°C. The perfusing solution contained (mM): NaCl 126, KCl 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.4, glucose 11, $NaHCO_3$ 25, gassed with 95% O_2 and 5% CO_2. In low-sodium experiments (20–40% of control) NaCl was replaced by equimolar choline chloride and scopolamine (10 μM) was added to avoid cholinergic effects. Zero calcium solutions were obtained by replacing $CaCl_2$

with $MgCl_2$ (10–30 mM). When $CoCl_2$ was added to the solution NaH_2PO_4 was omitted. The ventral tegmental area (VTA) and the substantia nigra pars compacta (SN) were identified with a dissecting microscope as the regions medial (VTA), rostral and caudal (SN) to the medial terminal nucleus of the accessory optic tract.

Recordings

The recording electrodes (Clark 1.5 mm, thick wall), pulled by a Narishige vertical puller, were filled with 2 M KCl or 2 M CsCl and had a tip resistance of 40–80 MΩ. The signals were obtained by an amplifier (Axoclamp-2A, Axon Instruments) and were displayed on a pen recorder (Gould 2400 S) or saved on a tape recorder (Biologic) for off-line analysis. Data were presented as a mean ± s.e.mean.

Drugs

Drugs were bath-applied via a three-way tap system. Complete exchange of the bath solution occurred in about 1 min. The following drugs were used: dopamine hydrochloride, tetraethylammonium chloride (TEA), tetrodotoxin (TTX), all of which were dissolved in water and were obtained from Sigma. Nifedipine, nimodipine and Bay K 8644 (methyl 1,4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) were obtained from Bayer. They were dissolved in 100% ethanol before dilution in the external solution. The final ethanol concentration in all test and control solutions ranged between 0.1 and 0.3%. These concentrations of ethanol did not produce *per se* any detectable electrophysiological effect on the dopaminergic cells. The lights were dimmed throughout the experiments to prevent inactivation of the dihydropyridines.

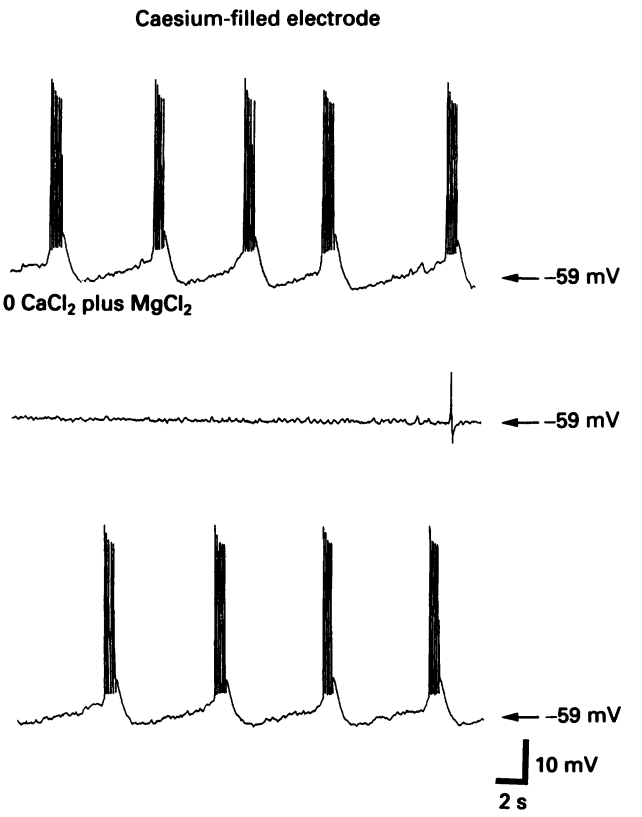

Figure 1 The caesium-induced burst firing is calcium-dependent. Oscillographic traces showing the reversible depressant effect of a solution with nominally no calcium on the bursting activity induced by the intracellular diffusion of caesium ions in a dopaminergic neurone.

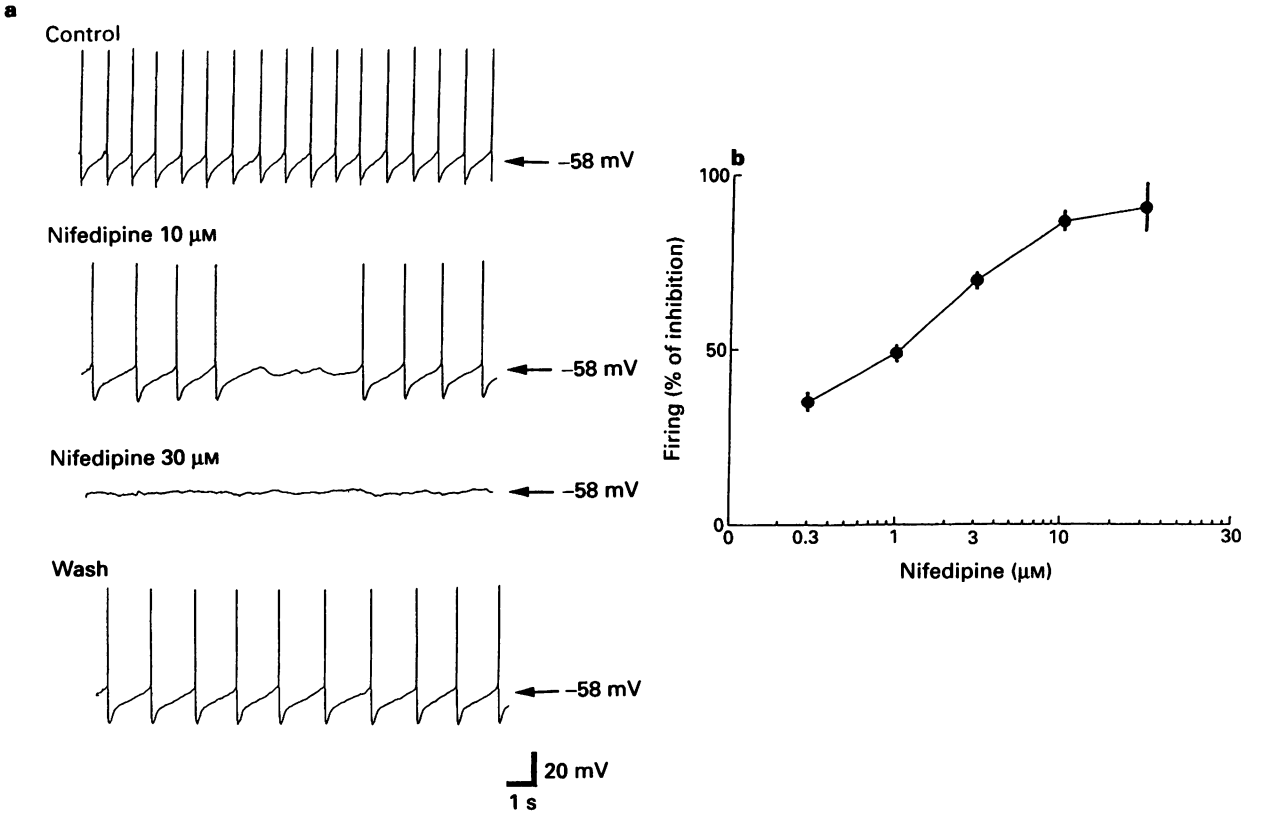

Figure 2 Effects of nifedipine on the spontaneous activity of dopaminergic cells. (a) Oscillographic records showing the concentration-dependent reduction of the spontaneous firing rate caused by nifedipine. (b) Dose-response relation for the inhibitory effects of nifedipine. Each point (4–7 experiments) shows mean effects ± s.e.mean.

Results

The experiments described in this paper are based on a total of 54 dopaminergic cells (30 in the substantia nigra pars compacta and 24 in the ventral tegmental area). The electrophysiological and pharmacological properties of these cells have been widely described (Grace & Onn, 1989; Kita *et al.*, 1986; Lacey *et al.*, 1989; Johnson & North, 1992; Hajos & Greenfield, 1993). All the neurones included in this study fired rhythmically spontaneous action potentials at a frequency of 1.9 ± 0.5 Hz. They showed a pronounced voltage- and time-dependent inward rectification (Ih) and had a hyperpolarizing response to dopamine application. The steady-state input resistance measured by a long hyperpolarizing pulse (1–7 s, 10–15 mV) ranged between 80 and 195 MΩ. Sixteen cells were also impaled with Cs-filled electrodes. They showed a progressive broadening of the action potential (10–80 ms) and eight of them developed a pattern of bursting activity, consisting of pacemaker depolarizations (0.1–0.7 Hz) which gave rise to a plateau-spike and sequence of two to ten action potentials (Figure 3d) (Mercuri *et al.*, 1994). The interspike interval within the burst increased as the burst progressed. In fact, the first, fourth and ninth interspike intervals were 27.9 ms ± 1.4 (30 bursts), 41.6 ms ± 2.5 (24 bursts) and 57.5 ms ± 1.4 (8 bursts) respectively. The average interspike interval between spikes occurring in a burst was 44.5 ± 3.1 ms. The bursting pattern of activity was depressed by 0 mM Ca^{2+} plus 25 mM $MgCl_2$ solutions ($n = 3$) (Figure 1).

Actions of dihydropyridines

Application of nifedipine (300 nM–30 μM) inhibited the spontaneous electrical activity of 32 dopaminergic cells in a concentration-dependent manner (Figure 2). Nifedipine at 1 μM reduced the spontaneous firing by about 50% of control (Figure 2b). This effect had a slow onset (2–4 min) and peaked in 7–12 min. An incomplete recovery was observed upon prolonged washout (more than 2 h). Nifedipine produced neither changes in membrane potential nor in the apparent input resistance (not shown). In cells loaded with caesium the rhythmic spontaneous firing activity was inhibited and the pacemaker oscillations of the membrane potential were depressed and narrowed in the presence of nifedipine (1–30 μM) ($n = 6$) (Figure 3). The narrowing of the pacemaker oscillations was mainly due to a marked inhibition of the depolarizing-after-potential (100–800 ms) that followed the plateau-spike (Figure 3d). The number of action potentials above the depolarizing oscillation was also reduced (Figure 3d).

Bath-applied nimodipine (1–30 μM) also inhibited the spontaneous firing of dopaminergic cells ($n = 11$) (Figure 4) and reduced the number of action potentials when a bursting pattern of activity was generated by the intracellular diffusion of caesium ions ($n = 3$) (not shown). Our experiments did not systematically examine the effects of a wide range of nimodipine concentrations on the dopaminergic cells. However, the effects observed with nimodipine were similar to those seen with the same concentrations of nifedipine. Five dopaminergic cells were also exposed to Bay K 8644 (1 μM); this drug increased the rate of firing of three cells by 20 ± 8% of control and slightly depolarized the membrane (1–3 mV) (Figure 5). In some neurones that were completely inhibited by nifedipine ($n = 4$) the spontaneous firing could be partially restored by superfusing the L-type Ca^{2+} channel agonist.

Actions of dihydropyridine antagonists on the spontaneous activity of calcium-dependent action potentials

The neurones loaded with caesium or treated with TEA (10–30 mM) were able to fire spontaneous calcium potentials even when the cells were treated with TTX (1 μM) or the extracellular concentration of sodium was reduced to 26.2 mM (choline chloride substitution). Under these conditions nifedipine (10–30 μM) ($n = 3$) (Figure 6) and nimodipine (30 μM) ($n = 2$) reduced or blocked the spontaneous activity of the calcium spikes.

Actions of nifedipine and nimodipine on the shape of the spike

In control conditions (KCl-filled electrodes), nifedipine and nimodipine did not change the shape of the action potentials significantly (measured at the midpoint of spike height)

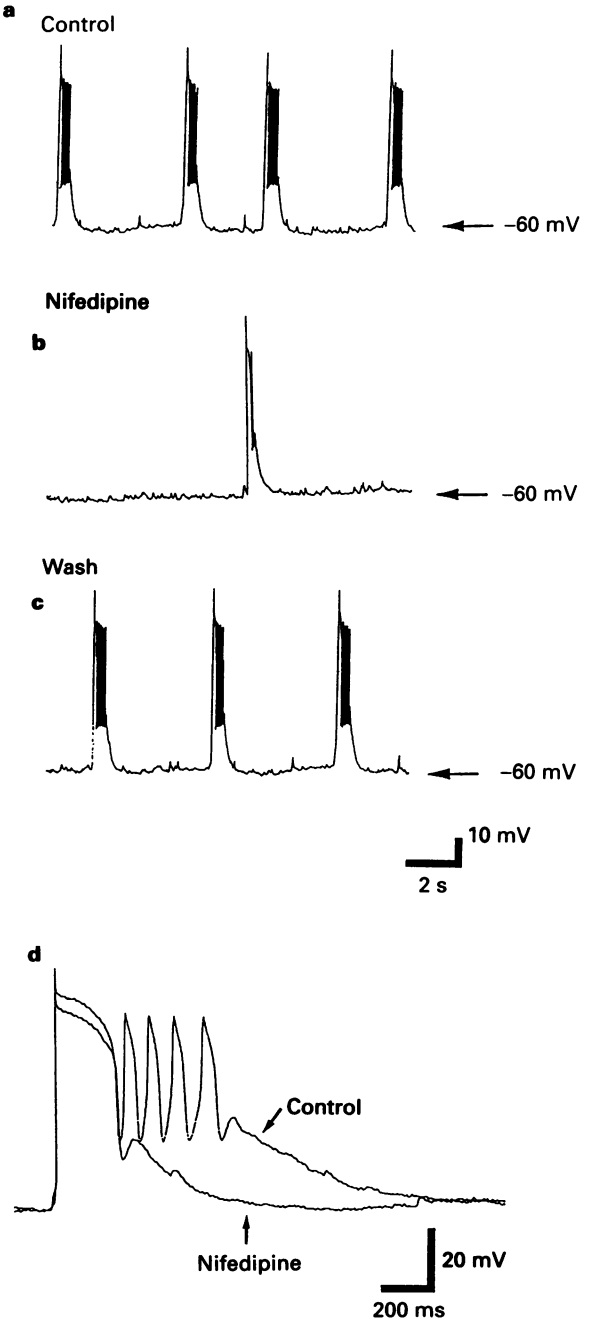

Figure 3 Effects of nifedipine on the spontaneous bursting activity. (a) This cell was impaled with a caesium-filled electrode; later (5 ms) depolarizing membrane oscillations and spontaneous bursts developed. (b) The application of nifedipine (10 μM) decreased the amplitude of depolarization and reduced the number of spikes in the burst. (c) Wash. (d) Superimposed traces to show the depressant effect of nifedipine on the depolarizing envelope which produced burst firing.

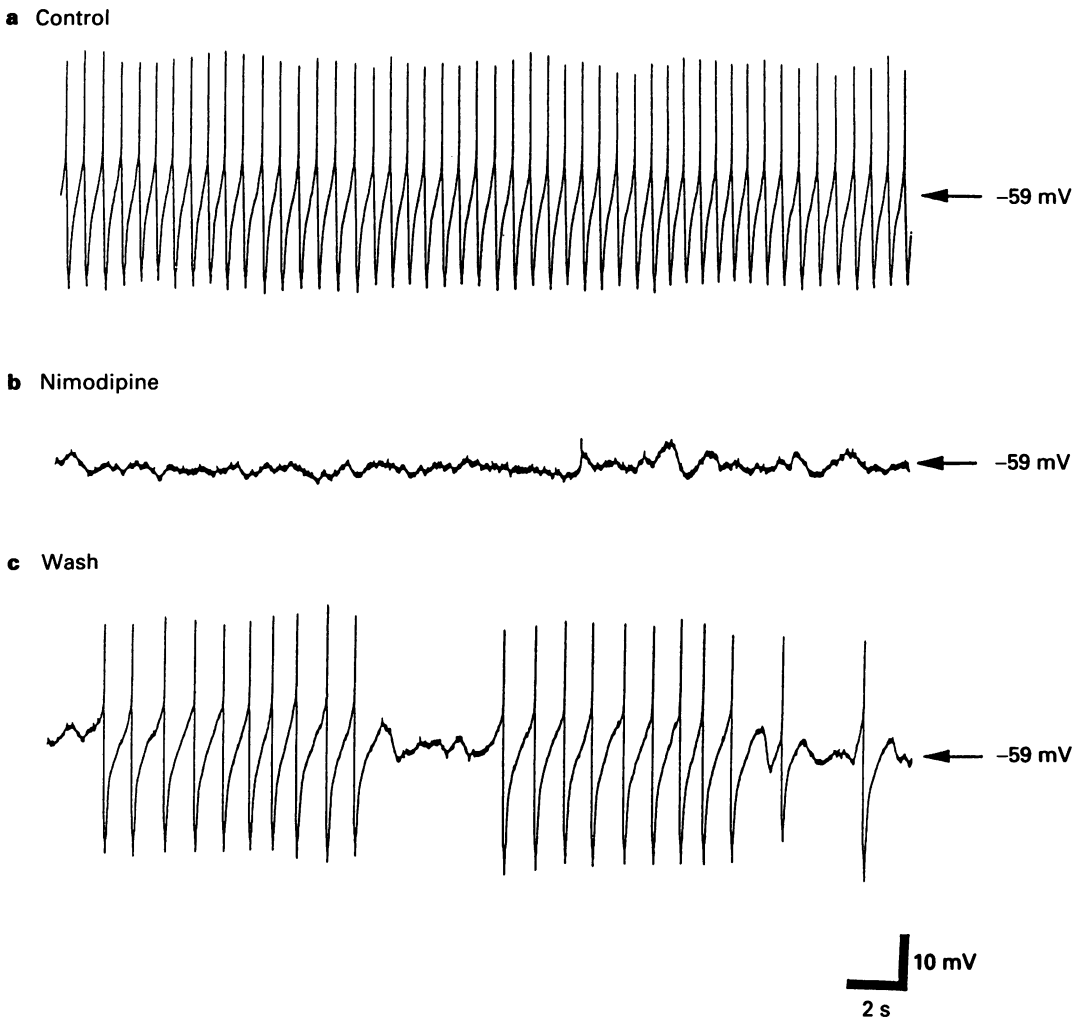

Figure 4 Inhibitory effects of nimodipine on pacemaker firing activity. The spontaneous firing (a) was blocked by nimodipine (10 μM) (b). Note that this is a chart recording. The full amplitude of the action potential is not reproduced because of the low frequency of the pen recorder.

(Figure 7a(i)) ($n = 10$). In addition, the shape of the after-hyperpolarization was not clearly affected by the dihydropyridines (Figure 7a(ii)) ($n = 13$). A lack of effect on the shape of the action potential was also observed even in cells impaled with CsCl-filled electrodes. It has to be pointed out that the maximal duration of the spike obtained with caesium-filled electrodes was around 80 ms. However, when a long-lasting spike (above 150–200 ms) was produced by applying 10–50 mM TEA on the caesium-loaded cells, both calcium antagonists, applied at concentrations (3–30 μM) likely to be maximally effective based on findings in other neurones (Regan *et al.*, 1991; Moyer *et al.*, 1992; Sayer *et al.*, 1993), were able to reduce the duration and the amplitude of the plateau potential. The shortening of the spike caused by the dihydropyridine calcium antagonists was either observed on sodium and calcium-dependent plateau potentials or on calcium plateau spike evoked in the presence of TTX (1 μM) (Figure 7b(i,ii)). Nifedipine (3 μM) reduced the duration and the amplitude of the spike to 66.5 ± 8.4% ($n = 7$) and 84.5 ± 2.7% ($n = 7$) of the respective controls. Nimodipine (3 μM) also shortened the duration of the plateau-spike and depressed its amplitude to 74.2 ± 4.2 ($n = 4$) and 89.5 ± 1.8 ($n = 4$) respectively. A block or a reduction of the amplitude and duration of the spike was also produced by the inorganic calcium channel blocker cobalt (1–3 mM) ($n = 4$) (Figure 7b (iii,iv)). In order to maintain the shape of the long lasting action potential a constant depolarizing pulse was injected intracellularly at a rate of 0.03–0.1 Hz while the neurones were manually clamped at − 70, − 85 mV.

Discussion

The present study suggests that calcium currents sensitive to dihydropyridine antagonists modulate the spontaneous rhythmic and bursting firing in the dopaminergic mesencephalic cells. The rhythmic and bursting activity observed *in vitro* could be compared with the firing properties previously recorded from the dopaminergic cells *in vivo*, which consisted in regular, irregular and burst discharge of action potentials (Grace & Bunney, 1983; 1984). It has to be pointed out that the burst firing evoked in cells loaded with caesium *in vitro* exhibits a shorter interspike interval (about 45 ms) than that observed in bursts occurring *in vivo* (about 70 ms). Although a normal synaptic connectivity between cells is required for the modulation of the discharge patterns typically observed *in vivo* (Shepard & Bunney, 1991), in the light of the ability of the potassium channel blockers (tetraethylammonium, barium, apamin and caesium) to trigger and/or increase bursts either *in vivo* (Grace & Bunney, 1984) or *in vitro* (see also Shepard & Bunney, 1991), it could be hypothesized that an inhibition of potassium-mediated outward currents and an increase of voltage-dependent inward currents play a key role in the bursting behaviour observed in both experimental conditions.

Action of dihydropyridines on firing rate

The rhythmic activity of the dopaminergic neurones is mainly regulated by the interplay of threshold and suprathreshold

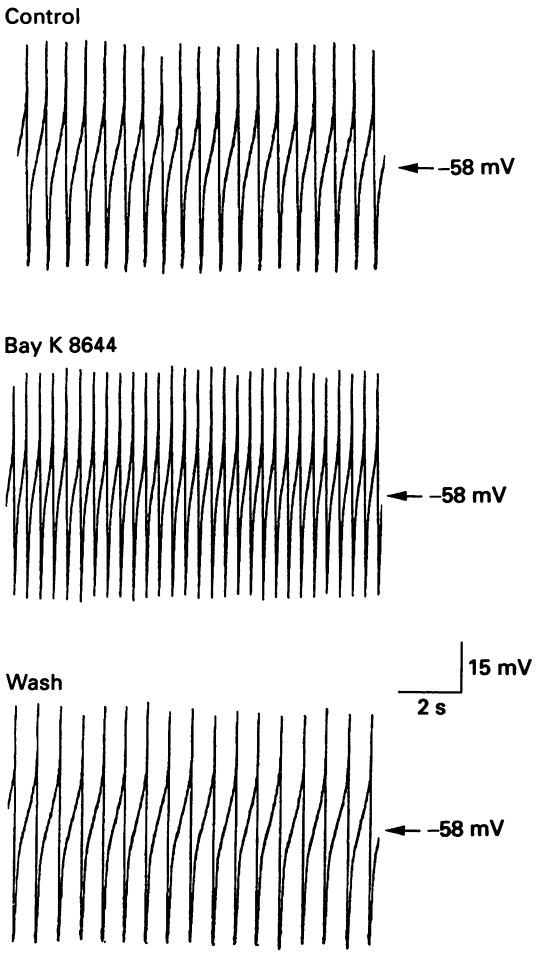

Figure 5 Bay K 8644 increases the spontaneous firing rate. The discharge of the spontaneous action potential in a ventral tegmental area cell is increased in a reversible manner by Bay K 8644 (1 μM). Note that the full amplitude of the spike is not reproduced because of the low frequency of the chart recorder.

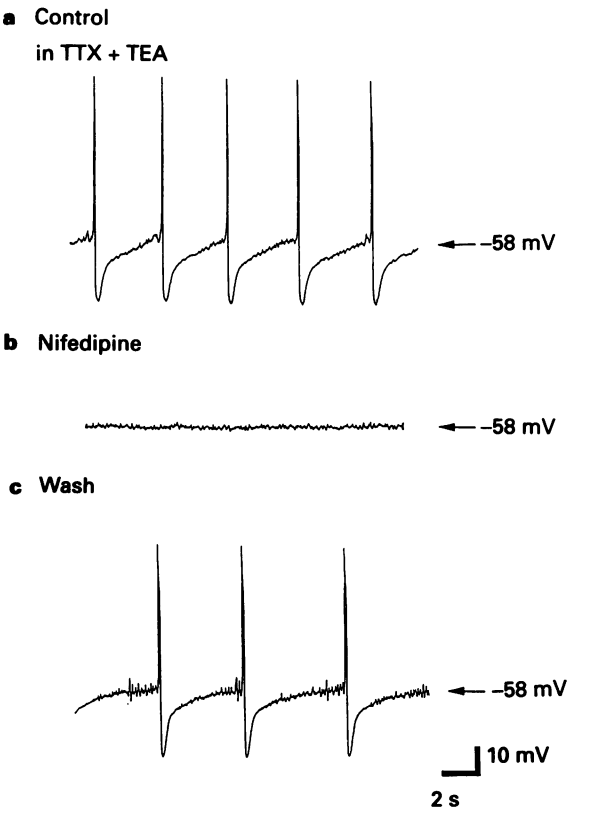

Figure 6 Dihydropyridine calcium channel antagonists depress the spontaneously occurring calcium spikes. Nifedipine (10 μM) inhibited the spontaneous activity of calcium action potentials (b). These spikes occurred in the presence of tetrodotoxin (TTX, 1 μM) and tetraethylammonium (TEA, 30 mM) (a).

currents intrinsic to the membrane (for a review see Connor, 1985). Among these are: (a) the voltage-activated inward current which appears to be carried by Na^+ and Ca^{2+} ions (Grace & Onn, 1989; Grace, 1991), the Na^+-K^+-dependent inwardly rectifying current (Lacey & North, 1987; Silva *et al.*, 1990) and the low- and high-threshold Ca^{2+} currents (Llinas *et al.*, 1984; Kang & Kitai, 1993b); (b) the Ca^{2+}-activated outward potassium current, mainly responsible for the afterhyperpolarization (Grace & Bunney, 1983), and the outwardly rectifying potassium currents (Grace & Onn, 1989; Silva *et al.*, 1990). It has already been demonstrated that calcium currents sustain the spontaneous firing of dopaminergic cells. The treatment of the neurones with inorganic calcium channel blockers and with solutions containing low-calcium depresses the spontaneous firing rate suggesting that calcium inward currents are determinant in generating and maintaining the intrinsic pacemaker (Llinas, 1988; Fujimara & Matsuda, 1989; Grace, 1991; Mercuri *et al.*, 1991; Kang & Kitai, 1993a).

The bursting firing of the dopaminergic cells produced by intracellular caesium loading is also dependent on calcium influx. In fact, it is depressed by lowering the extracellular concentration of calcium. The ability of the dihydropyridine calcium antagonists to affect, in a concentration-dependent manner, not only the rhythmic but also the bursting activity proves that L-type Ca^{2+} channels, sensitive to these agents, contribute to the inward currents underlying the two different patterns of spontaneous cellular discharge. The observation that the dihydropyridine calcium channel agonist, Bay K 8644 (Schramm *et al.*, 1983; Nowycky *et al.*, 1985) increases the spontaneous firing activity also suggests a role of the dihydropyridine-sensitive calcium currents in the regulation of the interval between spikes. In line with the present study, it has already been reported that bath-applied nifedipine decreases the spontaneous activity of guinea-pig dopaminergic neurones in the substantia nigra (Nedergaard *et al.*, 1993) and intravenous application of nimodipine decreases the regular and burst firing discharge of rat dopaminergic neurones in the ventral tegmental area (Ugedo *et al.*, 1988).

To account for the effects of the dihydropyridines on pacemaker firing activity, it could be suggested that a high-threshold dihydropyridine-sensitive Ca^{2+} current, preferentially located in the dendrites (Llinas *et al.*, 1984; Nishino *et al.*, 1986; Grace, 1990), is necessary to trigger either the spikes or the membrane depolarization causing bursting activity. The Na^+/Ca^{2+} spikes generated at the soma might in turn reactivate the dendritic calcium depolarizations, thus contributing to the electrical autorhythmicity of the dopaminergic cells. The importance of the high-threshold calcium currents in sustaining a rhythmic firing is also supported by a computer modelling of cardiac pacemaking in the sinoatrial node (Egan & Noble, 1987). An additional effect of dihydropyridine on low-threshold calcium currents could also account for the slowing of the regenerative rhythmic depolarizations (Nedergaard *et al.*, 1993); however, a recent whole cell patch-clamp study has reported that the calcium current which underlies the pacemaker-like depolarization in dopaminergic cells is not blocked by nifedipine (Kang & Kitai, 1993b).

Effects on the shape of the action potential

An important finding of this study was the shortening of the plateau action potential and of the depolarizing after poten-

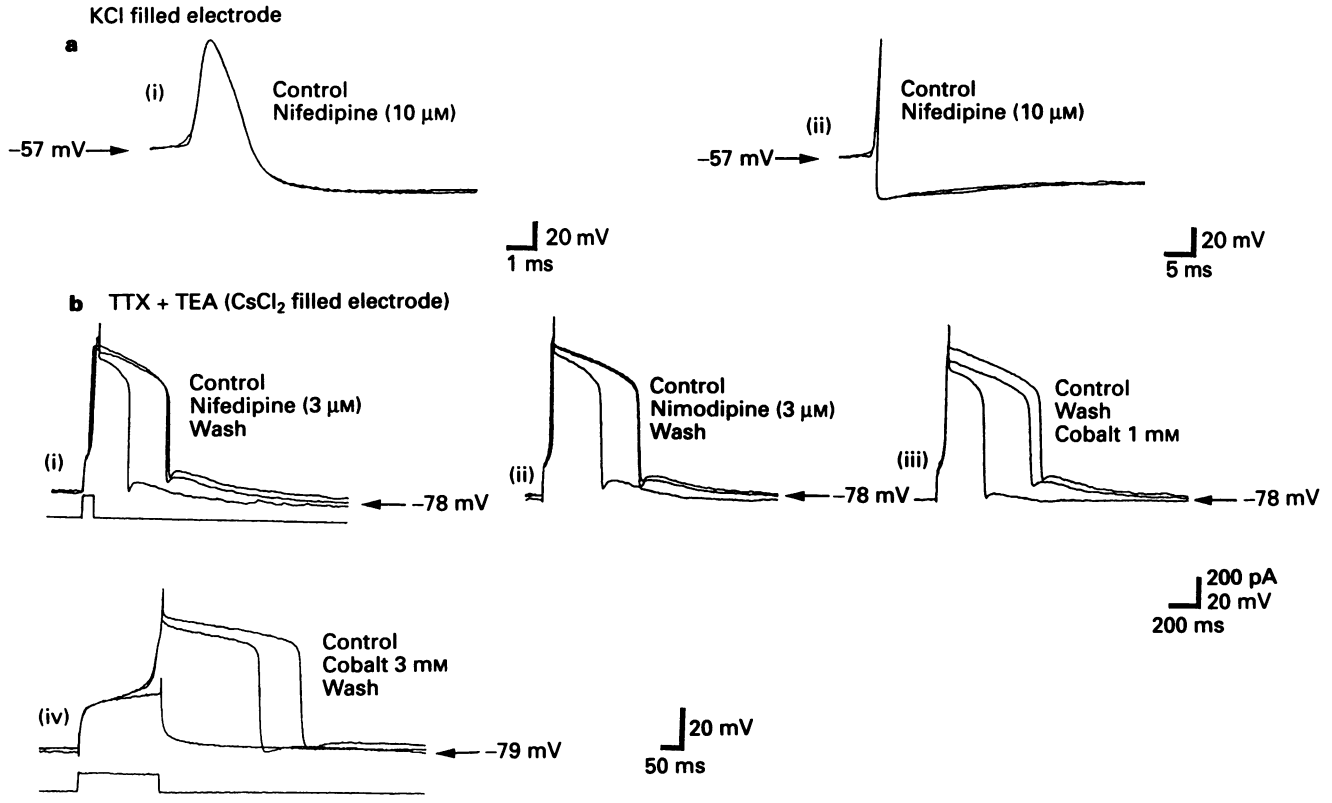

Figure 7 Effects of dihydropyridines on the shape of the action potentials. (a) (i) Nifedipine (10 μM) did not affect the shape of the action potentials recorded from a dopaminergic cell in control conditions (KCl-filled electrodes). (ii) The spike afterhyperpolarization was also unaffected by the dihydropyridine. Note that each trace is an average of four sweeps. (b) (i) Inhibitory effect of nifedipine (3 μM) on the amplitude and duration of the plateau potential in a cell impaled with caesium and treated with tetraethylammonium (TEA, 30 μM) and tetrodotoxin (TTX, 1 μM). The membrane was set to − 78 mV and a small, just threshold, depolarizing pulse was delivered at 0.03 Hz. The pulse elicited a long lasting TTX-insensitive spike that outlasted it. Upper voltage, The three traces in (i), (ii) and (iii) show control, effects of drugs and wash. The lower trace in (a) shows current. (ii) The TTX-insensitive calcium spike was also reversibly inhibited by nimodipine (3 μM). (iii) Depressant effects on the plateau-potential of cobalt (1 mM). (iv) The application of cobalt (3 mM) on this dopaminergic cell completely blocked the calcium spike. Note that (1) all the records in a (i), b (ii) and c (iii) were taken from the same dopaminergic cell; (2) the dihydropyridines reduced the calcium spikes without requiring a further increase of the injected current.

tials by dihydropyridine calcium antagonists. This suggests that a relevant source of calcium influx during a prolonged spike-like and post-spike depolarization is through L-type calcium channels and is consistent with the increased efficacy of the dihydropyridines at depolarized membrane potentials (Bean, 1984; Bean *et al.*, 1986; Jones & Jacobs, 1990). It is also evident that the dihydropyridine-induced shortening of the spike is dependent on the duration of the action potential. The plateau-spike has to be longer than 150 ms to observe the depressant effect of the dihydropyridines.

It has recently been shown that nifedipine blocks the TTX-resistant slow oscillation of the membrane whereas it does not affect the shape of the spike in dopaminergic cells (Nedergaard *et al.*, 1993 but see Fujimara & Matsuda, 1989; Kang & Kitai, 1993a,b). The apparent difference from our study could be explained by the fact that nifedipine was tested on action potentials that were not broad enough to cause the plateau-like depolarization and the post-spike depolarizing potentials which may have dendritic origin. The preferential blocking effects of dihydropyridine antagonists of L-type calcium channels during a maintained depolarization of the membrane could account for this phenomenon (Bean, 1984; Sanguinetti & Kass, 1984; Bean *et al.*, 1986; Hess, 1990; Jones & Jacobs, 1990).

It is interesting to note that Higashi *et al.* (1990) have already shown that organic calcium channel blockers preferentially depress the post-spike prolonged dendritic depolarization in hippocampal neurones.

Relationship between the electrophysiological effects of dihydropyridine and dopamine release

The level of extracellular dopamine is strictly dependent on the rate and the mode of firing of the dopaminergic neurones (Gonon, 1988). Although the contribution of the L-type calcium current in controlling dopamine release is still controversial, in agreement with our electrophysiological data showing an inhibitory effect of dihydropyridine antagonists, it has been reported that L-type Ca^{2+} channels control the influx of calcium (de Erausquin *et al.*, 1992) and the amount of dopamine released from dopaminergic cells in dendrites, and synaptosomes (Woodward & Leslie, 1986; Chaudieu *et al.*, 1992; Brouard *et al.*, 1993).

Therapeutic implications

The reduction of the voltage-dependent calcium influx by dihydropyridine Ca^{2+} antagonists might protect dopaminergic neurones from excessive intracellular loading of calcium ions during an excessive membrane depolarization caused by an ischaemic/anoxic episode (Choi, 1990).

Furthermore, the inhibitory effect of dihydropyridines on the spontaneous firing of the dopaminergic neurones might also be relevant to the control of movements, of drug seeking behaviour and of mental performances. Thus, dihydropyridine calcium antagonists could turn out to be useful

for the treatment of psychiatric and neurological disorders (Scriabine *et al.*, 1989).

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