The action of amlodipine on voltage-operated calcium channels in vascular smooth muscle

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1 Amlodipine, a dihydropyridine derivative largely ionized at physiological pH, inhibited calcium channel currents in single vascular smooth muscle cells isolated from rabbit ear artery in a concentration-dependent manner.

2 Amlodipine inhibited the current-voltage relationship for calcium channel currents across the range of test potentials used. However, the effect of amlodipine was more marked on more depolarized test potentials. Amlodipine also shifted the steady-state inactivation curve for calcium channel currents in a hyperpolarized direction.

3 The potency of amlodipine as determined from the steady-state inhibition of calcium channel current induced by the drug was dependent on the holding potential of the cells. Use of a more depolarized holding potential increased the potency of amlodipine.

4 Onset of amlodipine-induced inhibition was relatively rapid at both -60 mV and -40 mV holding potential. The use of a more depolarized holding potential increased the rate of association of amlodipine. No recovery from amlodipine-induced inhibition was seen over a 20 min period following washout of the drug.

5 In addition to voltage-dependence, the action of amlodipine showed use-dependence, in that the effect of amlodipine was more marked when calcium channel currents were evoked frequently. Increasing the frequency of activation of calcium channel currents did not alter the apparent onset rate of amlodipine-induced inhibition, but increased the degree of inhibition achieved by the drug.

6 The electrophysiological properties of amlodipine, particularly its voltage-dependence are probably important determinants of its action *in vivo*.

Keywords: Vascular smooth muscle; calcium channels; dihydropyridine; voltage clamp; amlodipine

Introduction

Voltage-operated calcium channels represent a major route by which calcium enters vascular smooth muscle cells (Bolton, 1979; Tsien, 1983), and play an important part in excitation-contraction coupling (Van Breemen & Saida, 1989). Dihydropyridines block entry of calcium into the vascular smooth muscle cell by an action on voltage-operated calcium channels (Tsien, 1983). Dihydropyridines are believed to act by binding to a site, or sites, associated with the voltage-operated calcium channel, so causing an alteration in gating characteristics. This change in gating leads to reduced calcium entry, consequently the drugs are known as calcium antagonists or calcium channel blockers.

Amlodipine (2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-2-methoxycarbonyl-6-methyl-1,4-dihydropyridine) is a novel dihydropyridine derivative. Although structurally related to other dihydropyridines such as nifedipine its chemical properties differ from typical dihydropyridines. Unlike the majority of dihydropyridines, amlodipine is relatively insoluble in organic solvents (partition coefficient in octanol/buffer ($K_{p_{oct}}$) = 29, cf. nifedipine $K_{p_{oct}}$ = 145), and exists primarily in an ionized form at physiological pH (pK_a = 9.02) (Mason *et al.*, 1989).

In vivo, the cardiovascular effects of amlodipine are largely due to vasodilatation, this action of amlodipine is relatively slow in onset and of long duration (Burgess & Dodd 1990). Similar vasorelaxant properties have been observed *in vitro*. In rat isolated aortic strips Burges *et al.* (1987) demonstrated that the onset and offset of amlodipine were much slower than nifedipine, and in radioligand binding studies, Rigby *et*

al. (1988) reported that [3H]-amlodipine displayed slow rates of association and dissociation with dihydropyridine binding sites in microsomes derived from dog ventricular myocytes.

Although it is a dihydropyridine, the action of amlodipine may also involve binding to non-dihydropyridine sites on the voltage-operated calcium channel; ligand binding studies indicate that amlodipine interacts not only with a dihydropyridine binding site but also with a site which binds diltiazem (Rigby *et al.*, 1988; Nayler & Gu, 1992) and another that binds phenylalkylamines such as (–)-D888 (Vaghy, 1992).

The object of these studies was to examine the effect of amlodipine on voltage-operated calcium channels in vascular smooth muscle cells.

Methods

Single smooth muscle cells were freshly dispersed from rabbit ear artery by a modification of the procedure of Benham & Bolton (1986). Short segments (1–2 mm) of artery were incubated for 50–60 min in a modified physiological salt solution containing $10 \mu M$ calcium, 2 mg ml^{-1} bovine serum albumin, 1 mg ml^{-1} collagenase (Worthington 130 u mg^{-1}), 0.5 mg ml^{-1} papain (Sigma 15 u mg^{-1}) and 5 mM dithiothreitol respectively. Cells were dispersed by mild agitation in this low-calcium physiological salt solution. After centrifugation the cells were resuspended in normal physiological salt solution (PSS) containing (mM): NaCl 130, KCl 6, CaCl_2 1.7, MgCl_2 1.2, glucose 14 and HEPES 10.7 buffered to pH 7.2 with NaOH. The cells were stored on cover slips at 4°C and used within 6 to 8 h. The experiments were performed using the whole-cell configuration of the patch-clamp technique

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(Hamill *et al.*, 1981) by means of a List EPC-7 patch-clamp amplifier. Patch pipettes had resistances of 3 to 5 MΩ. The internal (pipette) solution contained (mM): NaCl 126, MgCl₂ 1.2, EGTA 2, Mg₂ ATP 2, TEA 10, and HEPES 11 buffered to pH 7.2 with NaOH. The experiments were carried out in 'high-barium solution' containing (mM): BaCl₂ 110 and HEPES 10 buffered to pH 7.2 with TEA-OH to increase the size of the inward current elicited by depolarization and to minimise calcium-dependent inactivation of currents (Aaronson *et al.*, 1988).

An IBM compatible PC provided the voltage-clamp command pulses through a Labmaster A/D interface board using commercially available software (PCLamp 5.5, Axon Instruments, CA, U.S.A.). Data were recorded on-line, or on digital audio tape using a DAT recorder (Biologic, France) and later analysed off-line after analogue-to-digital conversion at between 2.5–10 kHz depending on the voltage protocol used. The currents were digitally filtered at 2 kHz and leak currents were subtracted digitally using average values of steady leakage currents elicited by a 10 mV hyperpolarizing pulse (Aaronson *et al.*, 1988). All recordings were made at room temperature (22–25°C).

Drugs were applied by bath perfusion or by a concentration-jump technique previously described in detail (Hering *et al.*, 1987). This enabled the application of the drugs within less than 10 ms. Data are presented as means values ± s.e. mean of *n* observations. Concentration-response data were fitted to a logistic function:-

$$E = \frac{C}{E_{\max} + \frac{C}{(K_D)^n}}$$

where *E* is effect of drug, *E*_{max} is maximum effect of drug, *C* is the concentration of drug, *K*_D is the apparent affinity and *n* is the pseudo-Hill slope, using nonlinear regression analysis and commercially available software on an IBM PC compatible computer (Inplot 3.1 and 4, GraphPAD Software, CA, U.S.A.).

Steady-state inactivation data similarly were fitted to a Boltzmann function:

$$I = \frac{I_{\max}}{1 + \exp(V - V_h)/k} + C$$

where *I* is the normalized peak current at any potential, *I*_{max} is the maximum peak current evoked by such a step, *V*_h is the holding potential at which inactivating current is half inactivated, *k* is a slope factor and *C* is the fraction of non-inactivating current which is present in these cells (Hughes *et al.*, 1990).

Drugs

Amlodipine (a gift from Pfizer, U.K.) was prepared as a stock solution (10 mM) in ethanol. Subsequent dilutions were made in distilled water.

Results

Effect of amlodipine on inward currents

Amlodipine (10 pM–10 nM) induced a marked concentration-dependent inhibition of calcium channel current in single rabbit ear artery cells held under voltage clamp at –60 mV and –40 mV. Examination of the current-voltage relationships suggested that the inhibitory effects of amlodipine were more marked on more depolarized test potentials although amlodipine did induce inhibition of currents evoked by depolarization near to threshold (Figure 1). The potency of amlodipine as estimated from the concentration-response

relationship was increased about 10 fold at the more depolarized holding potential of –40 mV (Figure 2).

The rate of onset of amlodipine-induced inhibition was examined at two holding potentials, –60 mV and –40 mV. As indicated above, amlodipine is more potent at depolarized potentials so the rate of onset of amlodipine-induced inhibition was compared using concentrations of amlodipine chosen to produce around 30 and 75% inhibition of calcium channel currents (1 and 10 nM at –60 mV, and 100 pM and 1 nM at –40 mV). At –60 mV, the onset of amlodipine-induced inhibition was relatively rapid; mean time constant of onset (*τ*) = 33.3 s (1 nM) and 7.6 s (10 nM) (*n* = 3–4). At –40 mV the mean *τ* of amlodipine-induced inhibition was 25 s (100 pM) and 7.6 s (1 nM) (*n* = 3) (Figure 3). From these kinetic data it was possible to estimate the affinity of amlodipine for the voltage-operated calcium channel from the equation derived from Schwarz and colleagues (1973):

$$K_D = \frac{\tau' \times [\text{Amlodipine}] - \tau \times [\text{Amlodipine}]}{\tau - \tau'}$$

Where *τ* is the mean time constant of onset with the lower concentration of amlodipine, and *τ'* is the onset time constant using the higher concentration of amlodipine.

Substituting the values obtained at –60 mV and –40 mV membrane potentials gave affinities of 286 pM and 29 pM respectively. These results compare favourably with the values of 240 pM and 34 pM for the *IC*₅₀ derived from the concentration-response relationship.

Following washout of amlodipine no recovery from amlodipine-induced inhibition was seen at either holding potential during a 20 min recovery period.

Effect on availability

The above studies indicate that the potency of amlodipine is increased at more depolarized potentials and suggest that amlodipine may bind with higher affinity to an inactivated state of the voltage-operated calcium channel. This was further investigated by examining the effect of amlodipine (1 nM) on the availability of inward current following a 6 s conditioning pulse sufficient to induce steady-state inactivation of calcium channel current in these cells (Aaronson *et al.*, 1988). In the presence of amlodipine current was inhibited at all potentials (Figure 4) and amlodipine also shifted the mid-point of the inactivation curve to more negative potentials (control = –26 ± 3 mV; amlodipine = –47 ± 6 mV; *n* = 4 in each case) without significantly altering the slope factor (*k*) (control *k* = 7.1 ± 0.2; amlodipine *k* = 7.4 ± 1.0; *n* = 4 in each case). From the mean shift in the inactivation curve we calculated the dissociation constant for the inactivated state (*K*_i) according to the approach described by Bean and colleagues (1986) assuming one to one building to resting and inactivated states in a simple two state model. Using this model

$$\Delta V_h = \frac{k \ln (1 + [\text{Amlodipine}]/K_i)}{(1 + [\text{Amlodipine}]/K_i)}$$

where *ΔV*_h is the shift in the mid point of the inactivation curve, *k* is the slope factor, [Amlodipine] is the concentration of amlodipine used, and *K*_i is the *IC*₅₀ of amlodipine measured at a holding potential of –60 mV when there is negligible inactivation. With *ΔV*_h = 21 mV, *k* = 7 mV, *K*_r = 240 pM *K*_i was calculated to be 10 pM.

The effect of frequency of activation

In general, dihydropyridines do not show use-dependence (Terada *et al.*, 1987; Honore *et al.*, 1989; Kuga *et al.*, 1990). However Kass & Arena (1989) reported that amlodipine did display use-dependence in isolated myocardial cells. We examined the effect of increasing the frequency of stimulation on the inhibition produced by amlodipine. Over the range

Figure 1 The effect of amlodipine on calcium channel currents in rabbit ear artery cells. Cells were bathed in 110 mM Ba^{2+} and voltage clamped at -60 mV. (a) Control currents evoked by 200 ms depolarization steps from -50 mV to $+50$ mV in the absence of drug; (b) currents evoked by the same depolarization steps in the presence of amlodipine 1 nM. (c) The effect of amlodipine on the peak current-voltage relationship; (●) control; (○) amlodipine 100 nM; (▲) amlodipine 1 nM; (■) amlodipine 10 nM; (□) amlodipine 100 nM. The voltage protocol and scale bar are indicated.

0.03–1 Hz the magnitude of inhibition induced by 10 nM amlodipine was increased by increasing the frequency of stimulation, although inhibition of current did occur in the absence of activation (Figure 5). The rate of onset of amlodipine-induced inhibition was little altered when currents were elicited every 10 s; τ being 28 ± 4 s ($n = 4$) for 10 nM amlodipine at -60 mV holding potential compared with 33 ± 3 s ($n = 4$) when currents were evoked at 1 Hz. In contrast the steady-state inhibition induced by 10 nM amlodipine when currents were evoked at 0.1 Hz was significantly less ($53 \pm 8\%$, $n = 4$) than when currents were evoked at 1 Hz ($70 \pm 7\%$, $n = 4$).

Discussion

Amlodipine, although a dihydropyridine, is unusual in that it is almost completely ionized at physiological pH; the condi-

tions of this study. As has been previously reported for other dihydropyridines in vascular smooth muscle (Bean *et al.*, 1986; Nelson & Worley, 1989; Kuga *et al.*, 1990), we found that amlodipine-induced inhibition is affected by the resting membrane potential. Depolarization increases the potency of amlodipine. This effect of membrane potential appears to be, at least in part, due to an increase in the association rate constant at depolarized membrane potentials. In these studies we were unable to see any recovery from amlodipine-induced inhibition at any holding potential so it is unclear whether membrane potential can also influence the rate constant for drug-receptor dissociation. However in a previous study (Hering *et al.*, 1993) using another dihydropyridine, (–)-202 791, membrane potential had no effect on offset rate constant.

In addition to inhibiting calcium channel currents, amlodipine also caused the steady-state inactivation curve for calcium channel currents to be shifted to more polarized

Figure 2 Concentration-response relationship for amlodipine-induced inhibition of peak calcium channel currents in cells voltage clamped at -60 mV (■) and -40 mV (□). Calcium channel currents were evoked every second using a 20 ms pulse to $+10$ mV from a holding potential of -60 mV. The average peak current was measured at each concentration of drug in each cell, once stable inhibition was achieved. Results are normalized with respect to the calcium channel current in the absence of any drug. Each point is the mean (with s.e.mean) of 4–5 observations in different cells.

potentials. Similar findings have previously been reported for other dihydropyridines and a common explanation is that the increased affinity at depolarized membrane potentials and the shift in the availability curve reflect a high affinity of the dihydropyridine for the inactivated state or states of the voltage-operated calcium channel (Bean, 1984; Sanguinetti & Kass, 1984). Using the model proposed by Bean *et al.* (1986) we calculated the affinity for the inactivated state of the calcium channel to be around 10 pM. This predicts that at -40 mV (when from the availability curve around 20% of channels will be inactivated compared with $<5\%$ of channels at -60 mV) the apparent affinity constant should fall from 200 pM to around 40 pM. This agrees reasonably well with the calculated IC_{50} for amlodipine at -40 mV of 34 pM. In myocardial cells studied by whole cell voltage clamp, Kass & Arena (1989) reported that amlodipine induced little or no block of calcium channel currents in myocardial cells unless cells were depolarized to or below -50 mV. This contrasts with our findings where block of calcium channel current can be seen in these cells at hyperpolarized membrane potentials when little inactivation is likely to occur (see Figure 4). However, compared with voltage-dependent calcium channels in myocardial cells, calcium channels in the resting state in vascular smooth muscle appear to have higher affinity for dihydropyridines (McCarthy & Cohen, 1989). The basis of this difference between the voltage-dependent calcium channel in cardiac and vascular smooth muscle is unclear, but has been reported previously (see Nelson & Worley, 1989).

The onset of amlodipine-induced inhibition of calcium

Figure 3 Onset of amlodipine-induced inhibition at (a) -60 mV and (b) -40 mV holding potential. Calcium channel currents were evoked at 1 Hz using a 20 ms pulse to $+10$ mV from the holding potential. After a period in the absence of drug to ensure that currents were stable and did not 'run down', amlodipine was added to the chamber by a concentration-jump technique. Currents recorded at 10, 20, 30 and 60 s following addition of drug are shown.

Figure 4 Effect of amlodipine on availability of calcium channel current in a single rabbit ear artery cell. Inactivation was induced by a 6 s prepulse. Data were fitted to a Boltzmann function as described in Methods. V_h indicates the potential at which current was inactivated by 50%, V_{hc} indicates this potential in the absence of drug, V_{haml} the potential in the presence of 1 nM amlodipine. Data are representative of 4 similar studies.

Figure 5 Effect of activation frequency on amlodipine-induced inhibition. Examples taken from single ear artery cells voltage clamped at -60 mV. Amlodipine 10 nM (■) or vehicle (○) was added to the cell at the time indicated by the upper bar. Current was elicited by a 20 ms step to $+10$ mV at the rate indicated in the lower bar. The graph shows peak current plotted against time. The result for amlodipine is representative of 4 similar experiments.

channel currents in single vascular smooth muscle cells is rapid; near IC_{50} concentrations achieve stable inhibition in less than 1 min. Rapid onset of action of amlodipine has also been seen in studies in single myocardial cells (Kass & Arena, 1989). These findings contrast with studies of the onset of amlodipine-induced inhibition of contraction in isolated blood vessels, where amlodipine-induced inhibition is extremely slow (Burgess *et al.*, 1987; Hughes *et al.*, 1991). Amlodipine is a charged compound relatively insoluble in organic solvents (Mason *et al.*, 1989), but several recent studies have shown that amlodipine readily partitions into lipid bilayers containing phospholipid and that insertion of amlodipine into the lipid bilayer occurs rapidly (Mason *et al.*, 1989; Seelig & Ganz, 1991).

The existence of a membrane pathway for the association of dihydropyridines with the calcium channel has been proposed previously (Rhodes *et al.*, 1985; Herbet *et al.*, 1986). In this model, access of the dihydropyridine molecule to the voltage-operated calcium channel is by lateral diffusion within the cell membrane. Such lateral diffusion within an essentially 2-dimensional structure decreases the time taken for drug and channel to interact (Rhodes *et al.*, 1985). Such a scheme may explain both the rapid onset of action of amlodipine seen in these studies as well as the apparent irreversibility of amlodipine-induced inhibition since amlodipine will tend not to diffuse out of the membrane (Mason *et al.*, 1989).

Although such a model can explain the rapid onset and slow offset of amlodipine seen in our studies it does not resolve the difference between the onset rates seen here and those observed in studies of isolated vessels. Bauerle & Seelig (1991) reported that both amlodipine and nimodipine had high mobility in lipid bilayers, so it seems unlikely that lateral diffusion would be restricted in the plasma membrane. We suggest that this difference reflects difficulty in amlodipine gaining access to multicellular preparations. Amlodipine may insert rapidly into the outer leaflet of the plasma membrane, but because of its polar side chain be unable to diffuse across the plasma membrane. Diffusion into the tissue could therefore not occur across cells and would be slowed in intact arteries. It is interesting to note that amlodipine is unable to block voltage-activated calcium channels when it is applied from the intracellular side (Kass & Kwan, 1992) suggesting that the ability of amlodipine to traverse the cell membrane is limited.

The finding that amlodipine displays use-dependence is of interest and similar findings were reported by Kass & Arena in myocardial cells (1989). Use-dependence is a well recognised feature of some calcium antagonists such as verapamil and diltiazem (Ehara & Kaufmann, 1978; Kuga *et al.*, 1990) but not dihydropyridines (Terada *et al.*, 1987; Honore *et al.*, 1989; Kuga *et al.*, 1990). The degree of use-dependence shown by amlodipine in these studies appears to be less than that shown by the phenylalkylamine, D600, in cells derived from the same tissue (Hering *et al.*, 1989). In the case of D600, almost no block of calcium channel current was seen following addition of D600 in the absence of channel activation. In contrast in this study amlodipine did cause block of calcium channel currents even in the absence of activation although frequency-dependent block of current by amlodipine was also clearly seen in these experiments. The molecular basis of use-dependence is uncertain but the most widely accepted explanation is that access of ionized drug to its receptor on the voltage-operated calcium channel is limited when the voltage-operated calcium channel is closed (i.e. resting or inactivated), so blocking and unblocking require open channels (Hille, 1984). It is therefore possible that this property of amlodipine may relate to the largely ionized nature of the drug under study conditions or alternatively reflect actions of amlodipine at non-dihydropyridine binding sites on the voltage-activated calcium channel (Rigby *et al.*, 1988; Nayler & Gu, 1992; Vaghy, 1992).

It is well known that vascular smooth muscle, particularly under vasoconstrictor influences such as those that exist *in vivo*, will be relatively depolarized (Nield, 1983). Moreover, there is evidence that experimental hypertension is associated with increased depolarization of vascular smooth muscle in resistance arteries (Harder & Hermsmeyer, 1983; Cheung, 1984; Stekiel *et al.*, 1986). Such a state of depolarization will make vascular smooth muscle much more susceptible to amlodipine-induced inhibition of voltage-operated calcium channels than other less depolarized tissues such as the heart. Indeed a similar explanation may underlie the apparent tissue selectivity of dihydropyridines towards different vascular beds (Towart *et al.*, 1985). Different regions of the circulation may be relatively more or less depolarized (Harder, 1983) and consequently the apparent potency of dihydropyridines may

differ between different vascular beds or even regions within a single vascular bed.

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