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pH-dependent block of the L-type Ca^{2+} channel current by diltiazem in human mesenteric arterial myocytes

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Abstract

Inhibition of the L-type Ca^{2+} channel current (I_{Ba}) by diltiazem was characterised in human mesenteric arterial myocytes. External (pH_o) and internal (pH_i) pH was varied to alter the proportion of drug in charged and neutral forms. Diltiazem (20 μM) reduced I_{Ba} amplitude by approximately half at pH_o 7.2 and 9.2 at holding potential −60 mV. The I_{Ba} decay was increased by diltiazem at $pH_o = 9.2$ (97% uncharged), but not at 7.2. The IC_{50} for inhibition of I_{Ba} by diltiazem at holding potential −60 mV was decreased from 51 to 20 μM at pH_o 7.2 and 9.2, respectively. At holding potential of −90 mV, but not −60 mV, tonic block increased and use-dependent block decreased as pH_o was raised from 6.2 to 9.2. Diltiazem also caused a hyperpolarizing shift in I_{Ba} availability at alkaline pH_o. The results suggest that raising pH promotes Ca^{2+} channel blockade by increasing the proportion of uncharged diltiazem.

Keywords: Diltiazem; Ca^{2+} channel; Ca^{2+} channel antagonist; (Human); Vascular smooth muscle cell; pH

1. Introduction

The mechanism of inhibition of cardiovascular L-type Ca^{2+} channels by the widely used benzothiazepine diltiazem remains surprisingly obscure in contrast to that for dihydropyridines and phenylalkylamines. Although a number of studies have compared the effect of diltiazem on L-type Ca^{2+} channels to that of other Ca^{2+} channel antagonists in cardiac myocytes (Kanaya et al., 1983; Lee and Tsien, 1983; Uehara and Hume, 1985), snail neurones (Novales-Li et al., 1990) and smooth muscle cells (Terada et al., 1987; Kuga et al., 1990), few reports have been devoted solely to this drug (Tung and Morad, 1983; Kanaya and Katzung, 1984; Xiong et al., 1990). Comparative studies have shown that diltiazem blocks Ca^{2+} channels in a voltage- and use-dependent manner (attributed to a state-dependent inhibition) with features intermediate between those described for dihydropyridines and phenylalkylamines (Lee and Tsien, 1983; Uehara and Hume, 1985; Xiong et al., 1990). However, whether use-dependency is due primarily to an interaction with inactivated or with open channels has not been resolved.

Diltiazem has a pK_a of 7.7, and so exists in both charged and neutral forms at physiological pH. It has not been established, however, whether diltiazem primarily accesses the channel in its charged or neutral forms. This unresolved question has important implications regarding the mechanism of Ca^{2+} channel blockade by benzothiazepines. According to the modulated receptor hypothesis, originally proposed in order to explain the mechanisms of block of sodium channel by local anaesthetics (Hille, 1977b), but subsequently also applied to the interaction of many drugs with the Ca^{2+} channel (e.g., Bean, 1984), a hydrophilic pathway (provided by the open channel) is required for the charged drug to reach its receptor. On the other hand, the neutral molecule is thought to reach the channel via a hydrophobic pathway within the membrane, binding with high affinity when inactivation occurs. Uehara and Hume (1985) found that acidification of the external solution from 7.4 to 6.4, which will increase the amount of the charged form of the drug, altered the blocking effect of diltiazem on Ca^{2+} channels in frog

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atrial myocytes in a manner not entirely consistent with predictions based on the modulated receptor hypothesis. Recently, it has been reported that the permanently charged benzothiazepine derivative, (*cis*)-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-3-methyl-6-(trifluoromethyl)-1-[2-(trimethylammonio)ethyl]-2*H*-1-benzazepin-2-one iodide (SQ32,428), had no effect on current inactivation and did not cause use-dependent inhibition of Ca^{2+} channels in smooth muscle and cardiac cells (Hering et al., 1993; Seydl et al., 1993). Diltiazem itself, however, produced a use-dependent block and shifted the steady-state inactivation for Ca^{2+} channel current, and also increased the rate of Ca^{2+} channel current decay (Uehara and Hume, 1985; Xiong et al., 1990; Hering et al., 1993).

In the present report, we altered pH of external and internal solutions to vary the relative proportions of charged and neutral form of diltiazem in order to define the mechanism of blockade of L-type Ca^{2+} channels by the drug in freshly isolated human mesenteric arterial smooth muscle cells. Our results establish for the first time that the neutral form of diltiazem plays the pre-eminent role in channel blockade. Evidence is also presented that external pH affects the interaction of diltiazem with the channel.

2. Materials and methods

Experiments were carried out on single smooth muscle cells freshly isolated from human mesenteric (omental) arteries obtained during surgery at St. Thomas's Hospital, as approved by the St. Thomas's Ethical Committee. Methods of cell isolation, recording of currents in freshly isolated cells using the conventional whole cell patch clamp technique, and data analysis have been described in detail elsewhere (Smirnov and Aaronson, 1992a). Cells were placed in a small chamber (0.1 ml) which allowed turnover of about 95% of the perfusing solution within 10 s (Smirnov and Aaronson, 1996). All experiments were performed at room temperature.

L-type Ca^{2+} channel currents were studied in the presence of 10 mM Ba^{2+} in the external solution in order to increase the current amplitude and reduce the possible contribution of Ca^{2+}-induced inactivation of Ca^{2+} channels (Smirnov and Aaronson, 1992b). The main external solution contained (mM): 120 NaCl, 1 CsCl, 1.2 $MgCl_2$, 10 $BaCl_2$, 4 tetraethylammonium chloride, 10 glucose, and 10 HEPES. The composition of the pipette solution was (mM): 135 CsCl, 2.5 $MgCl_2$, 2 adenosine 5-triphosphate disodium salt (Na_2ATP), 10 HEPES, 10 EGTA. The pH of the external and pipette solutions was adjusted with NaOH and CsOH, respectively, to 7.2 for the control solution, or 6.2 and 8.2 for the external test solutions as indicated in the text. For pH 9.2, 5 mM HEPES was replaced with equimolar amount of 2-*N*-cyclohexylaminoethanesulfonic acid in both external and pipette solutions.

Diltiazem hydrochloride and other chemicals were obtained from Sigma (Poole, Dorset, UK). Values in the text and figures are presented as means ± S.E.M. Student's *t*-test was used to assess the significance of differences between mean values, with $P < 0.05$ considered to indicate significance, unless otherwise stated.

3. Results

3.1. Effect of pH_o on the concentration-dependency of I_{Ba} block by diltiazem

The concentration-dependency of I_{Ba} block by diltiazem was investigated using 100 ms voltage steps to +10 mV at 0.1 Hz, with the addition of increasing concentrations of the drug to the bath. Similar experiments were performed at two levels of pH_o, 7.2 and 9.2 at both holding potentials of −90 and −60 mV. Fig. 1A illustrates, for example, the inhibition of I_{Ba} at holding potential of −90 and pH_o 7.2 in one cell.

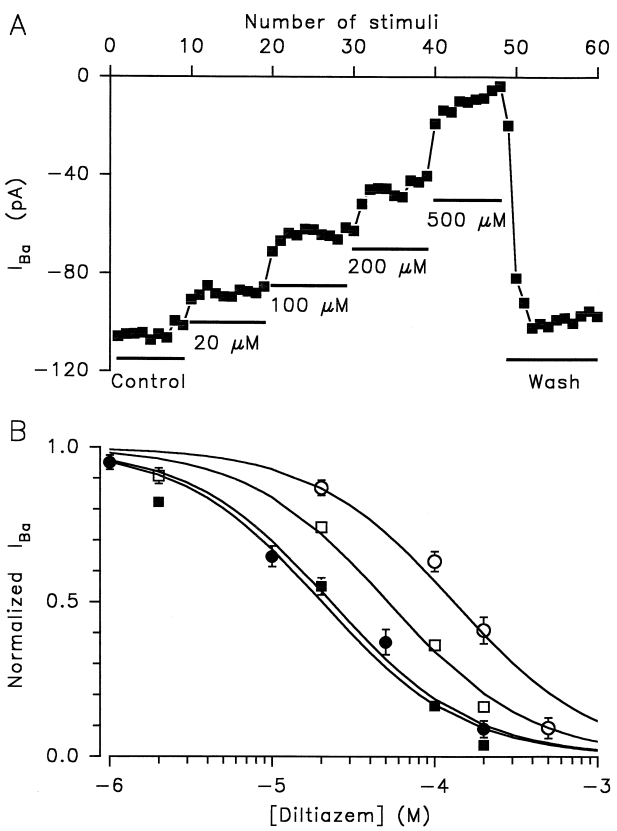

Fig. 1. Concentration-dependency of I_{Ba} blockade by diltiazem. (A) I_{Ba} peak amplitude measured under control conditions and in the presence of various concentrations of diltiazem as indicated in μM. Holding potential of −90 mV, pH_o 7.2. The cell was stimulated at 0.1 Hz with 100 ms depolarizing pulses to +10 mV. (B) shows the concentration-dependencies for block of I_{Ba} obtained in 4–5 cells at pH_o 7.2 (circles) and 9.2 (squares) at holding potentials of −90 (open symbols) and −60 mV (filled symbols). Solid lines are Langmuir curve fits with apparent IC_{50} values of 131.5 (○), 23.1 (●), 51.7 (□) and 20.3 (■) μM.

Fig. 1B illustrates that the inhibition of I_{Ba} by diltiazem at holding potential of −90 mV was approximately 2.6 times more potent at pH_o 9.2 ($IC_{50} = 51$ μM) than at pH_o 7.2 ($IC_{50} = 131$ μM). Conversely, no significant difference was observed when cells were held at −60 mV (IC_{50} were 20 and 23 μM at pH_o 9.2 and 7.2, respectively). At both pH_o, diltiazem was significantly more potent at a holding potential of −60 mV.

3.2. pH_o dependency of the effect of diltiazem on I_{Ba} decay

In order to evaluate the effect of diltiazem on the kinetics of Ba^{2+} currents through Ca^{2+} channels (I_{Ba}), 500 ms voltage steps were applied from a holding potential of −60 mV to test potentials between −20 and +40 mV in the absence and presence of 20 μM diltiazem, both at extracellular pH (pH_o) 7.2 and 9.2. I_{Ba} showed little inactivation at membrane potentials negative of −10 mV. At more positive potentials, however, I_{Ba} inactivated more quickly, and this could be described by single exponential (Fig. 2). The mean time constants (τ_{500}) obtained are listed in Table 1. Although values of τ_{500} at pH_o 9.2 tend to be somewhat higher than those at pH_o 7.2, possibly due to a surface potential-mediated hyperpolarizing shift of activation in the more alkaline solution, this difference was not significant. A low external concentration of diltiazem (5 μM) did not affect significantly the kinetics of I_{Ba} decay. At pH_o 7.2, 20 μM diltiazem tended to accelerate slightly the rate of I_{Ba} decay (Fig. 2A and Table 1), although this effect was also not significant.

At pH_o 9.2, however, the rate of I_{Ba} decay was significantly increased by 20 μM diltiazem (Fig. 2B), resulting in a decrease of τ_{500} by 61–74% over the range of the test potentials examined (Table 1).

3.3. Which form of diltiazem interacts with Ca^{2+} channel?

At pH_o 7.2, the calculated bath concentrations of the charged and neutral forms of diltiazem ($pK_a = 7.7$) were 15.2 and 4.8 μM, respectively, while at pH_o 9.2, the bath concentrations were 0.6 and 19.4 μM, respectively. The greater acceleration of I_{Ba} decay at pH_o 9.2 compared to 7.2 can be seen by comparing Fig. 2A and B. Considering that in both cases the pH of the pipette solution was set at 7.2 and assuming that only the neutral form of diltiazem can diffuse through the cell membrane, it can be calculated that at steady state the internal charged concentration of diltiazem would be 15.2 μM at pH_o 7.2 and 61.3 μM at pH_o 9.2. These results suggested that the greater effect of diltiazem at pH_o 9.2 compared to pH_o 7.2 might be caused either by an increase in the concentration of the neutral form of diltiazem on either side of the membrane, or by the increase in the internal charged diltiazem concentration. In line with these possibilities, application of 80 μM diltiazem when both pH_o and pH_i were set at 7.2, under

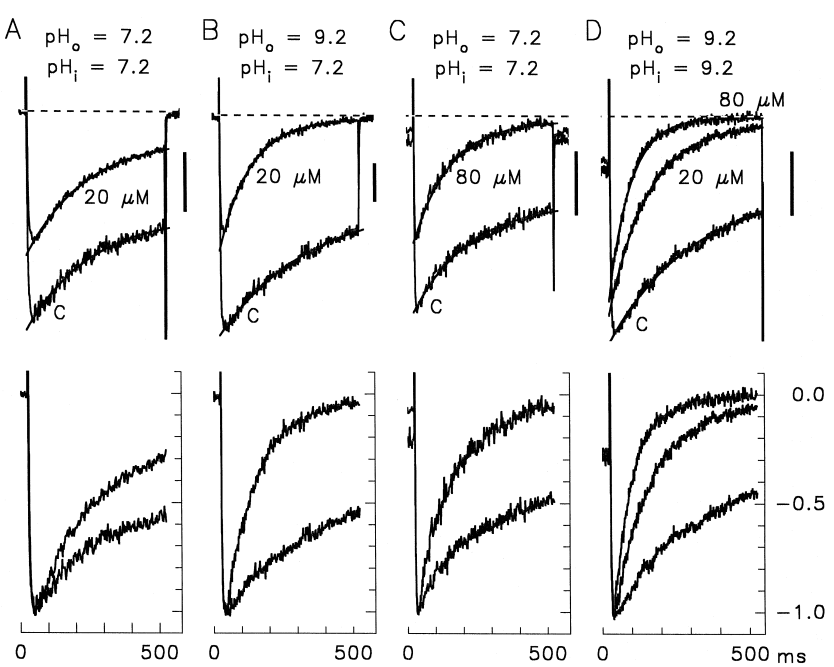

Fig. 2. Effect of diltiazem on the time-course of I_{Ba} decay at different pH levels. (A–D) show current recorded at +10 mV in the absence (marked C) and the presence of diltiazem (μM). Extracellular and intracellular pH levels are indicated at the top of each panel. Holding potential −60 mV. Solid lines are monoexponential fits with the following time constants (τ_{500}): 271 and 236 ms (A), 335 and 114 ms (B) 204 and 134 ms (C), and 279, 128 and 71 ms (D) in the absence and in the presence of the indicated concentrations of the drug, respectively. Vertical calibration bars indicate 50 pA. The bottom row shows the same set of currents, each scaled to the same peak to emphasize changes in the rate of current decay.

Table 1
Effect of pH_o and various diltiazem concentrations on the time constant of I_{Ba} decay

V_m (mV)	Control	$pH_o = 7.2$			$pH_o = 9.2$	
		5 μM	20 μM	80 μM	Control	20 μM
0	380 ± 38 (16)	444 ± 206 (5)	309 ± 41 (7)	218 ± 43 (6)[b]	545 ± 164 (7)	141 ± 10 (6)[b]
10	223 ± 13 (16)	229 ± 30 (5)	186 ± 11 (9)[a]	115 ± 11 (6)[c]	271 ± 26 (7)[d]	97 ± 6 (6)[c]
20	183 ± 13 (16)	170 ± 24 (5)	164 ± 11 (9)	86 ± 9 (6)[c]	206 ± 12 (7)	75 ± 5 (6)[c]
30	180 ± 11 (16)	190 ± 28 (5)	156 ± 12 (9)	77 ± 7 (6)[c]	184 ± 8 (7)	68 ± 4 (6)[c]
40	159 ± 10 (16)	187 ± 27 (5)	150 ± 12 (8)	86 ± 10 (5)[c]	189 ± 15 (7)	74 ± 7 (5)[c]

[a] $P < 0.05$ (one-tailed *t*-test).
[b] $P < 0.05$ (two-tailed *t*-test).
[c] $P < 0.001$ (two-tailed *t*-test).
[d] $P < 0.05$ (one-tailed *t*-test) in comparison to the control at pH_o 7.2
Number of cells studied is indicated in parentheses. pH_i was 7.2 throughout.

which condition the external concentration of neutral diltiazem would be 19.2 μM, and the internal steady-state concentration of charged diltiazem would be 60.8 μM, accelerated I_{Ba} decay to an extent similar to that observed with 20 μM diltiazem at pH_o 9.2, over a range of test potentials (Fig. 2C and Table 1).

To clarify which of the forms of diltiazem, charged or neutral, was more important in promoting the decay of I_{Ba}, the effect of diltiazem on I_{Ba} kinetics was investigated when both the external and internal pH levels were adjusted to 9.2. In this case diltiazem should be almost entirely (97%) in its neutral form on both sides of the cell membrane and, therefore, if the charged form of the drug was necessary for the block, very little effect on I_{Ba} amplitude and kinetics would be expected. Under these conditions, the current in the absence of diltiazem decayed more slowly ($\tau_{500} = 683 \pm 158$ ms, $n = 5$) than at pH_i 7.2 ($P < 0.001$). The decay rate of I_{Ba} was significantly increased by 50% at 20 μM and by 76% at 80 μM of diltiazem with τ_{500} equal to 342 ± 40 ($n = 5$) and 164 ± 25 ms ($n = 4$), respectively (Fig. 2D).

These results suggested that the rate of I_{Ba} decay was markedly accelerated under conditions in which diltiazem was almost entirely in its neutral form, implying that the charged form of the drug was not necessary for this effect. Furthermore, comparison of panels A and D of Fig. 2

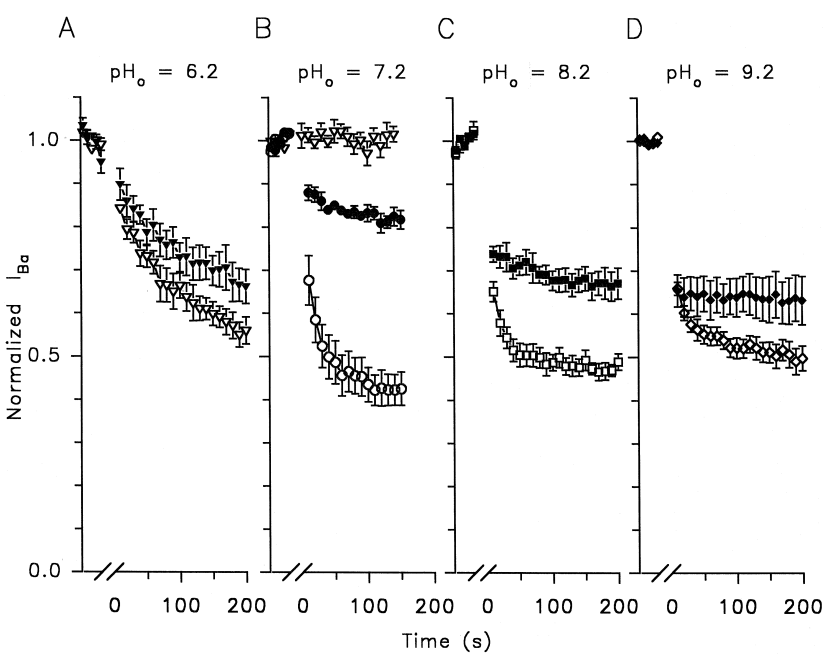

Fig. 3. Summary of tonic and use-dependent inhibition of I_{Ba} by diltiazem (20 μM) at various pH_o. The peak current amplitudes were plotted against the duration of the experiment. The break on the time scale represents the 2 min gap between application of the drug and first stimulus, values to the left of the break represent current amplitude before drug was added. Cells were stimulated at 0.1 Hz from holding potentials of −90 (filled symbols) or −60 (open symbols) mV. The open triangles in panel B show current amplitude in the absence of diltiazem. Each point and bars represent mean ± S.E.M. for 5–7 cells.

shows that given equal external and internal total concentrations of diltiazem, the effect on current decay was larger when the drug was predominantly in its neutral form (i.e., both pH_o and $pH_i = 9.2$), than when it was predominantly in its charged form (i.e., both pH_o and $pH_i = 7.2$).

3.4. Effect of pH_o on tonic and use-dependent I_{Ba} blockade by diltiazem

To compare the effect of diltiazem on tonic and use-dependent inhibition of I_{Ba} at various pH_o, a standard experimental protocol was used. Approximately 2–3 min after rupture of the patch membrane, at which time the run-up of I_{Ba} was completed and its amplitude had stabilised, the cell was clamped at either −60 or −90 mV and stimulated repeatedly by 100 ms voltage steps to +10 mV applied at 0.1 Hz. At this rate of stimulation, the amplitude of I_{Ba} remained unchanged under control conditions (Fig. 3B, open triangles). Five control stimuli were applied in order to assess the control current, and then 20 μM diltiazem was added to the bath for 2 min, during which time the cell was not stimulated. This concentration of diltiazem was chosen because it produced approximately 50% inhibition of I_{Ba} at the control holding potential (−60 mV) and pH_o (7.2). Two minutes after the application of drug, stimulation at 0.1 Hz was restarted.

Fig. 3 summarises the results obtained in groups of 5–7 cells at each pH_o using this protocol with 20 μM diltiazem and holding potentials of −90 and −60 mV at pH_o 6.2, 7.2, 8.2 and 9.2.

Fig. 3A illustrates that the tonic component of inhibition of I_{Ba} was small and independent of the holding potential at pH_o 6.2 when the bulk of diltiazem was charged. However, a progressive use-dependent block, which was not completed during the period of stimulation, developed at both holding potentials of −90 and −60 mV. An increase in pH_o to 7.2 resulted in a dramatic change in the inhibitory effect of diltiazem on I_{Ba} (Fig. 3B). The use-dependent inhibition of I_{Ba} amplitude was substantially reduced at the holding potential of −90 mV. When cells were held at −60 mV the tonic blockade of I_{Ba} was now significantly larger than that at −90 mV, and a sizeable use-dependent block occurred, which was faster than that observed at pH_o 6.2.

Further elevation of pH_o to 8.2 and 9.2 resulted in a progressive increase in the tonic blockade of I_{Ba} at −90 mV, whereas the tonic blockade observed at −60 mV remained virtually unchanged (Fig. 3C and D). The use-dependent block at both holding potentials tended to diminish, and was almost absent at pH_o 9.2 and holding potential of −90 mV.

It is noteworthy that the extent of tonic block at pH_o 7.2 and 9.2 was consistent with that implied by the concentration-dependency of block illustrated in Fig. 1B. In both cases, inhibition of I_{Ba} observed using a holding potential of −60 mV was pH_o-independent, while block obtained with a holding potential of −90 mV was greater at pH_o 9.2 than at pH_o 7.2.

3.5. Modulation of I_{Ba} inactivation by diltiazem and pH_o

Another important feature of the inhibitory effect of diltiazem on Ca^{2+} channel currents is its ability to modulate the voltage-dependent inactivation (also referred to as availability) of the channel. Fig. 4 shows the effect of several concentrations of diltiazem on the availability of I_{Ba}. This was measured by subjecting cells held at −60 mV to a 30 s conditioning potential in the range between −100 and +40 mV, followed by a test pulse to +10 mV. This pulse duration allowed us to measure Ca^{2+} channel availability under virtually steady-state conditions (Smirnov and Aaronson, 1992b). The peak current during the test pulse after each conditioning potential was normalised with respect to that after a conditioning step to −100 mV and this was used as a measure of the fraction of Ca^{2+} channels available for activation at a particular voltage.

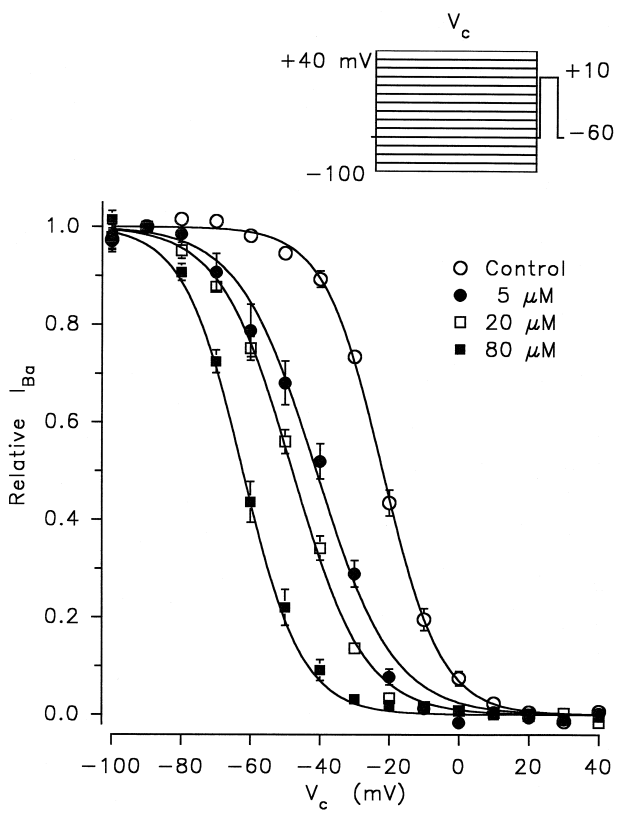

Fig. 4. Effect of three concentrations of diltiazem (as indicated) on the steady-state availability for I_{Ba}. Voltage protocol shown in the inset; V_c indicates the 30 s conditioning potential applied every min. Interpulse interval 30 ms. All current amplitudes were normalized to that measured after the conditioning pulse to −100 mV. Data were fitted with the Boltzmann equation with a half-inactivation potential, V_h, and slope factor k_h, equal to −21.9 and 8.4 mV (Control), −41 and 11.1 mV (5 μM), −47.9 and 10.4 mV (20 μM), and −62.2 and 8.6 mV (80 μM).

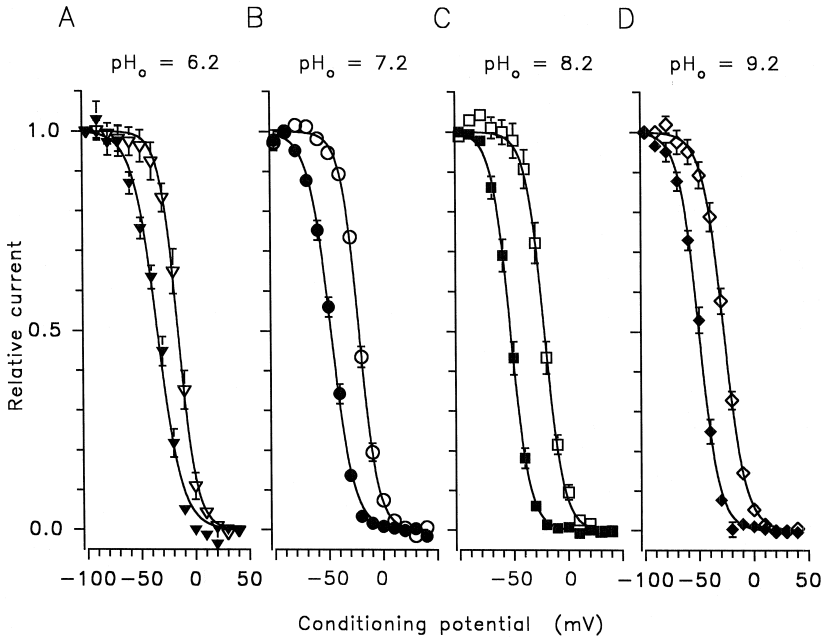

Fig. 5. Modulation of the steady state inactivation of I_{Ba} by diltiazem (20 μM) at several pH$_o$, as indicated. The steady-state availabilities were measured as described in the legend to Fig. 4. Data were fitted with the Boltzmann equation (solid lines) with V_h and k_h equal to −15.5 and 8.5 mV and −34.6 and 11.1 mV (A), −21.9 and 8.4 mV and −47.9 and 10.4 mV (B), −21.6 and 8.6 mV and −52.8 and 8.7 mV (C), −27.2 and 9.9 mV and −50.1 and 9.3 mV (D) in the absence and presence of diltiazem, respectively.

Data were fitted with the Boltzmann function. The steady state availability was progressively shifted in a concentration-dependent manner by diltiazem, with shifts in V_h of 19, 26, and 40 mV, at diltiazem concentrations of 5, 20, and 80 μM, respectively.

In order to investigate whether pH$_o$ modified this response, the availability of I_{Ba} in the absence and presence of 20 μM diltiazem was measured at several pH$_o$ values, as presented in Fig. 5. At pH$_o$ 6.2, the steady-state availability was shifted by 19 mV towards negative membrane voltages in the presence of the drug (Fig. 5A, Table 2). This shift was increased to 26 and 31 mV at pH$_o$ 7.2 and 8.2, respectively (Fig. 5B and C, Table 2). At pH$_o$ 9.2, however, the shift diminished to 22 mV (Fig. 5D, Table 2).

4. Discussion

Increasing the pH$_o$ in the range between 6.2 and 9.2, which increased progressively the proportion of the neutral form of diltiazem, resulted in a number of alterations in the block of I_{Ba} in human mesenteric arterial myocytes by the drug: (1) the tendency of diltiazem to accelerate the decay of I_{Ba} was enhanced, (2) the potency of block was in-

Table 2
Modulation of voltage-dependent characteristics for I_{Ba} by pH$_o$ and 20 μM diltiazem

pH$_o$		Inactivation			Activation	
		Half-inactivation potential V_h (mV)	Slope factor k_h (mV)	ΔV_h (mV) ($V_{h,D}-V_{h,C}$)	Half-activation potential V_m (mV)	Slope factor k_m (mV)
6.2	Control	−15.9 ± 4.7 (6)	8.6 ± 1 (6)		+10.4 ± 3 (10)	6.3 ± 0.6 (10)
	Diltiazem	−34.7 ± 3.2 (5)	11.3 ± 2.3 (5)	18.8	–	–
7.2	Control	−21.8 ± 2.5 (9)	8.4 ± 0.8 (9)		+2.8 ± 4 (26)	6.4 ± 0.3 (26)
	Diltiazem	−47.9 ± 2.8 (7)	10 ± 1.4 (7)	26.1	–	–
8.2	Control	−22.1 ± 5.3 (9)	8.3 ± 2.2 (9)		+2.1 ± 3.3 (8)	7 ± 0.4 (8)
	Diltiazem	−52.9 ± 4 (7)	8.4 ± 0.9 (7)	30.8	–	–
9.2	Control	−27.9 ± 4.2 (11)	10.4 ± 3 (11)		−4.6 ± 2.1 (8)	6.4 ± 0.3 (8)
	Diltiazem	−50.2 ± 3.7 (10)	9.1 ± 1.4 (10)	22.3	–	–

ΔV_h represents the difference between the half-inactivation potential in the presence ($V_{h,D}$) and absence ($V_{h,C}$) of the drug at the same pH$_o$.
Number of cells studied is indicated in parentheses.

creased, (3) the tonic component of the blockade, particularly at the holding potential of −90 mV, was increased, (4) the extent of use dependent block, particularly at holding potential of −90 mV, was decreased, and (5) there was an increased diltiazem-induced hyperpolarizing shift in the steady-state inactivation of I_{Ba}, except at pH_o 9.2.

4.1. Effects of diltiazem on decay of I_{Ba}

The rate of decay of I_{Ba}, which presumably reflects the block of open or inactivated states of the channel (Lee and Tsien, 1983; Bean, 1984), was accelerated in the presence of diltiazem. As shown in Fig. 2, the extent of this acceleration varied with the concentration of diltiazem and with internal and external pH. By manipulating pH, it was possible to vary the proportions of the drug in the charged and neutral forms. This then should also affect the steady-state intracellular concentrations of both forms. In order to determine which form of diltiazem was more important in modulating the decay of I_{Ba}, the ratios of the τ_{500} in the absence and presence of 5–80 μM drug at different external and internal pHs were plotted against the log of the calculated steady state-concentrations of external/internal neutral diltiazem (Fig. 6A), internal charged diltiazem (Fig. 6B), and external charged diltiazem (Fig. 6C). There was no visible relationship between the acceleration of current decay and either external or internal charged diltiazem. Conversely, the acceleration of current decay at various external and internal pH was clearly related to the concentration of neutral diltiazem. The apparent correlation between the current decay and increasing concentration of the charged form of diltiazem observed when the external bulk concentration of the drug was increased at constant pH_o (open symbols in Fig. 6B and C) is probably related to the increased concentration of the neutral form which occurred concurrently. These results suggest that diltiazem reaches its binding site on the L-type channel in the neutral form via a hydrophobic pathway and thus modulates I_{Ba} kinetics in human mesenteric arterial myocytes. This conclusion is also supported by radioligand binding studies in vascular smooth muscle, skeletal and cardiac muscle (Hering et al., 1993; Seydl et al., 1993) where much more potent binding to L-type Ca^{2+} channels of the neutral diltiazem derivative of diltiazem (*cis*)-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-3-3-methyl-6-(trifluoromethyl)-1-[2-(dimethylamino)ethyl]-2*H*-1-benzazepin-2-one (SQ32,910) than its permanently charged analogue SQ32,428 has been demonstrated. In addition, it was also found that SQ32,428 did not change the current kinetics, was not frequency-dependent, and did not shift the steady-state inactivation dependency of the Ca^{2+} current in vascular myocytes (Hering et al., 1993). However, at present we cannot exclude entirely the possibility that diltiazem may diffuse to the Ca^{2+} channel binding site in its neutral form and bind only when the channel becomes open. Further experimental evidence is required to assess this possibility.

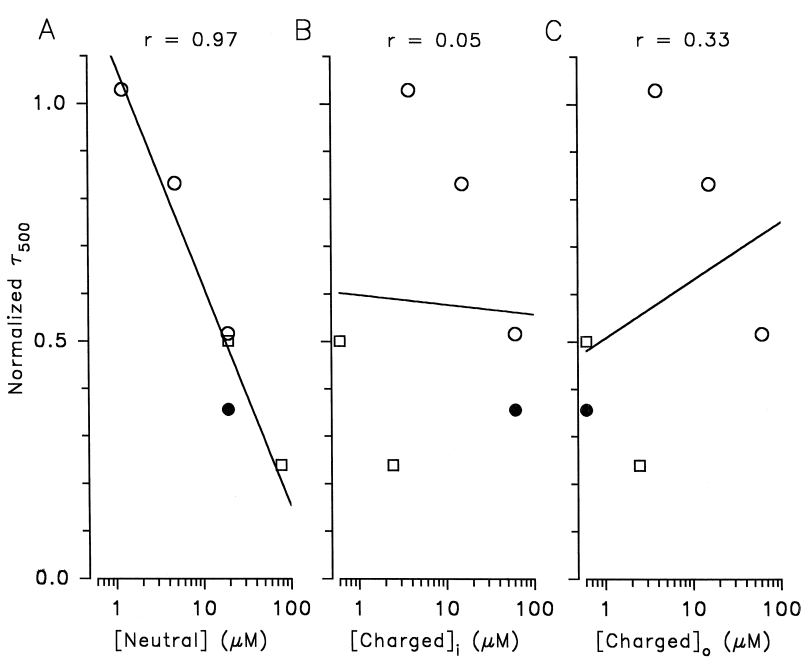

Fig. 6. Relationship between the calculated amounts of charged and neutral forms of diltiazem and changes in decay rate of I_{Ba}. The 'normalized τ_{500}' was calculated as the ratio between τ_{500} measured in the presence of diltiazem and that in the absence of the drug, such that a decreased relative τ_{500} indicates an acceleration of decay (see Table 1 and text for further description). Symbols represent the normalized τ_{500} at pH_o and pH_i equal to 7.2 and 7.2 (○), 9.2 and 7.2 (●), and 9.2 and 9.2 (□), respectively, with external diltiazem concentrations of 5, 20 and 80 μM. Straight lines are regression lines fitted through all points, with regression coefficients shown above each panel.

4.2. The concentration-dependency of I_{Ba} blockade

The assumption that diltiazem acts on Ca^{2+} channel currents in human mesenteric arterial smooth muscle cells in its neutral form could also explain the differences in the potency of the drug at two holding potentials at pH_o 7.2 and 9.2. It has been found that the IC_{50} for inhibition was 51 μM at pH_o 9.2 and 131 μM at pH_o 7.2 (Fig. 1). This 2.6-fold difference in potency of block was close to that predicted if the block of the current was due to the neutral form of diltiazem, since the concentration of the neutral form should be 3.2-fold higher at pH_o 9.2 than at pH_o 7.2. On the assumption that the neutral form was exclusively responsible for suppressing the current, and correcting for the effect of pH_o on the proportions of charged and neutral drug, the IC_{50} values are brought into closer agreement; the IC_{50} at pH_o 7.2 would be 32 μM, while that at pH_o 9.2 would be 50 μM. The lack of effect of pH_o on the concentration-dependency at a holding potential of −60 mV is discussed below.

4.3. Effects of pH_o on tonic block of I_{Ba}

The tonic block by 20 μM diltiazem at the holding potential of −90 mV was increased when the external solution was alkalinised (Fig. 3), suggesting that the resting channel is blocked by the neutral form of the drug. The calculated IC_{50} (38 μM) for the tonic inhibition of I_{Ba} by the neutral form of diltiazem at the holding potential of −90 mV at pH_o between 6.2 and 9.2 is also in good agreement with the values obtained from the concentration-dependent inhibition of the current at pH_o 7.2 and 9.2 (see above). At the holding potential of −60 mV, the tonic inhibition of I_{Ba} increased between pH_o 6.2 and 7.2, as was also found in cardiac cells (Uehara and Hume, 1985). However, that tonic block did not increase further as pH_o was raised above 7.2 (Fig. 3). This is unexpected, since the amount of the tonic block should continue to increase with pH_o in line with the concentration of the neutral form of diltiazem, as found at −90 mV. It is noteworthy, however, that this lack of effect of pH_o between 7.2 and 9.2 on tonic block at the holding potential of −60 mV was entirely consistent with our finding that the potency of blockade of the current was increased by altering pH_o in this range at the holding potential of −90 but not −60 mV (see above).

4.4. Effects of pH_o on I_{Ba} availability

We found that at pH_o 7.2, diltiazem caused a concentration-dependent hyperpolarizing shift in the steady state inactivation of I_{Ba} (Fig. 4). This effect seems to be unlikely due to the effect of diltiazem on I_{Ba} recovery for the following reasons. Our preliminary data showed that I_{Ba} recovery from inactivation measured after a 150 ms membrane depolarization to +10 mV from the holding potential of −60 mV could be described by two exponentials with fast and slow time constants equal of 86 ± 11 ms and 1564 ± 402 ms ($n = 7$), respectively. Although the recovery from inactivation was slower in the presence of 20 μM diltiazem measured at pH_o and pH_i 7.2 (134 ± 26 ms and 3629 ± 1060 ms for fast and slow time constants, correspondingly, $n = 7$, data not shown), it could not explain the shift in availability measured using our experimental protocol. Also, in the presence of the drug Ca^{2+} channels started to inactivate in the voltage range where very little channel activation is taking place (Fig. 4), which additionally supports the idea of interaction of diltiazem predominantly with the inactivated state of the Ca^{2+} channel.

The shift in the steady state inactivation by Ca^{2+} channels antagonists been attributed to a preferential binding of blockers to inactivated channels (Bean et al., 1983; Bean, 1984; Uehara and Hume, 1985). Recently, Hering et al. (1996) have used chimeras of diltiazem-sensitive and -insensitive Ca^{2+} channel α subunits to demonstrate that diltiazem interacts with a transmembrane segment IVS6 of the L-type channel which has an important influence on the rate of channel inactivation.

In order to evaluate whether diltiazem binds to the inactivated state also in the neutral form we used the theoretical approach described previously in detail by Bean (1984). The drug binding to the resting and inactivated state of Ca^{2+} channels could be estimated on the basis of the drug-induced shift of current availability according to the equation:

$$-\Delta V_h = k \ln\left[(1 + D/K_i)/(1 + D/K_r)\right], \quad (1)$$

where k is the slope factor for the control steady state availability, ΔV_h is the difference between the midpoint of the inactivation function in the presence and absence of diltiazem, and K_i and K_r are binding constants for the inactivated and resting state of the channel, respectively. Assuming that $K_r = 50$ μM for neutral diltiazem (see above) and obtaining ΔV_h for $pH_o = 7.2$ from Table 2, calculated values of K_i were 0.14, 0.21 and 0.12 μM for the total diltiazem concentrations of 5, 20 and 80 μM, respectively. These results suggest that diltiazem in its neutral form binds to the inactivated state for Ca^{2+} channels 200–400 times more strongly than to the resting state. This estimated affinity to the inactivated Ca^{2+} channels in human mesenteric arterial myocytes is approximately 10 times higher than that reported for I_{Ba} in rabbit mesenteric arterial myocytes (Xiong et al., 1990) at pH_o 7.2.

The higher affinity of diltiazem for the inactivated than for the resting state of the Ca^{2+} channel could be responsible for the apparent increase in the potency of the drug at the holding potential of −60 mV at pH_o 9.2 in comparison to that at 7.2 (Fig. 1). In order to assess this, we calculated the apparent dissociation constant K_{app} for drugs

acting on the resting and inactivated channels with differing affinities K_r and K_i, respectively, using the equation:

$$K_{app} = 1/\left[(h/K_r) + (1-h)/K_i\right], \quad (2)$$

where h is the fraction of channels in the resting state at that potential in the absence of drug (Bean, 1984). K_r values of 32 μM at $pH_o = 7.2$ and 50 μM at $pH_o = 9.2$ for the neutral form of diltiazem were estimated from the block of the channel at holding potential of −90 mV as discussed above. Values of h at a holding potential of −60 mV, obtained from the Boltzmann fit of availabilities (Fig. 5), were 0.98 and 0.95 at pH_o and K_i values, calculated using Eq. (1), were equal to 0.21 and 1.79 μM for pH_o 7.2 and 9.2, respectively. The calculated K_{app} were 8 and 21 μM of the neutral form of diltiazem, implying the total drug concentration of 25 and 22 μM at pH_o 7.2 and 9.2, respectively. These values agreed very closely with the observed IC_{50}s at a holding potential of −60 mV (23 and 20 μM at pH_o 7.2 and 9.2, respectively, Fig. 1). The results of the inactivation studies could therefore be used with the concentration dependency observed at a holding potential of −90 mV to predict accurately the concentration dependency found at holding potential of −60 mV. This internal consistency of the results supports our suggestion that the neutral form of diltiazem is of predominant importance in blocking the channel, and provides indirect confirmation that the apparent affinity of the drug for the inactivated channel is diminished at high pH_o.

However, when the effect of the whole range of pH_o on the diltiazem-mediated shift in I_{Ba} availability was examined (see Fig. 5 and Table 2), calculated K_i values were 0.08, 0.21, 0.29 and 1.79 μM for pH_o 6.2, 7.2, 8.2 and 9.2, respectively. There was therefore a progressive trend towards a decrease in the calculated potency of drug binding to inactivated channels as pH_o increased which may indicate that protons somehow modulate the binding of diltiazem to the inactivated state of Ca^{2+} channel in human mesenteric arterial myocytes. A similar suggestion was proposed by Uehara and Hume (1985), who found that the recovery of the Ca^{2+} channel from block by diltiazem, which they proposed was largely due to an interaction of the drug with the inactivated state of the channel, was slowed when the pH_o was reduced from 7.4 to 6.4. Uehara and Hume (1985) suggested that the effect of pH_o on the interaction of diltiazem with the Ca^{2+} channel might be due to the existence of both pH_o-dependent and -independent binding sites for diltiazem. Conversely, Schwarz et al. (1977) and Hille (1977a,b) suggested that neutral local anaesthetic molecules bind to a receptor site associated with the Na^+ channel, and may then be protonated by H^+ entering the external mouth of the channel. However, to resolve these possibilities further experimental work is required.

To summarise the points discussed above, we propose that diltiazem binds to the resting and inactivated Ca^{2+} channel mainly in its neutral form. The contribution of open channel block by the charged form of the drug is comparatively small under physiological pH_o presumably due to its low affinity to a binding site within the Ca^{2+} channel (Hering et al., 1993; Seydl et al., 1993). The effects of pH_o on block can mainly be explained by alterations in the proportions of the charged and neutral forms of diltiazem. Our results also suggest, however, that elevations of pH_o may have the additional effect of somewhat reducing the affinity of the inactivated channel for diltiazem.

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