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Specific inhibition of stretch-induced increase in L-type calcium channel currents by herbimycin A in canine basilar arterial myocytes

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1 The effects of protein-tyrosine kinase (PTK) and protein-tyrosine phosphatase (PTP) inhibitors on voltage-activated barium currents (I_{Ba}) through L-type calcium channels increased by hypotonic solution were investigated in canine basilar arterial myocytes by the whole-cell patch-clamp technique.

2 I_{Ba} was elicited by depolarizing step from a holding potential of −80 to +10 mV and identified by using an L-type calcium channel agonist, Bay K 8644 (100 nM), and an L-type calcium channel blocker, nicardipine (1 μM).

3 Hypotonic superfusate induced cell swelling and acted as a stretch stimulus, which reversibly increased peak I_{Ba} amplitude at +10 mV. I_{Ba} was also decreased by nicardipine (1 μM) under the hypotonic condition.

4 PTK inhibitors such as herbimycin A (30 nM), genistein (10 μM), and lavendustin A (10 μM) decreased I_{Ba} enhanced by hypotonic solution. Genistein also decreased I_{Ba} in a concentration-dependent manner under the isotonic condition. The inactive genistein analogue daidzein (10 μM) had no effect on I_{Ba} under either the isotonic or hypotonic condition. By contrast, herbimycin A did not decrease I_{Ba} under the isotonic condition. Sodium orthovanadate (10 μM), a PTP inhibitor, increased I_{Ba} under both conditions.

5 The present results suggest that cell swelling by hypotonic solution increases the L-type calcium channel currents in canine basilar artery and that herbimycin-sensitive PTK activity is primarily involved in the enhancement of calcium channel currents.

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Keywords: Canine basilar artery; herbimycin A; L-type calcium channel; mechanical stimulation; patch clamp; swelling; tyrosine phosphorylation

Abbreviations: DMSO, dimethyl sulphoxide; Gen, genistein; HMA, herbimycin A; Hypo, hypotonic; I_{Ba}, voltage-activated barium current; Iso, isotonic; Nic, nicardipine; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; TRIZMA, tris(hydroxymethyl)amino-methane; SOV, sodium orthovanadate; V_h, holding potential

Introduction

It has been reported that some voltage-activated channels showed mechano-sensitivity in various kinds of muscle cells and tissues. For instance, L-type calcium channel currents in rabbit cardiac myocytes (Matsuda *et al*., 1996) and delayed rectifier potassium channel currents in guinea-pig ventricular myocytes (Sasaki *et al*., 1992) were increased by mechanical stimulation, such as osmotic cell swelling or cell inflation *via* the patch pipette. Furthermore, it has been revealed that L-type calcium channels in rat basilar artery (Langton, 1993) and large-conductance calcium-activated potassium channels in rabbit pulmonary artery (Kirber *et al*., 1992) were also sensitive to mechanical stimulation. Recently, Xu *et al*. (1996) suggested that osmotic cell swelling increased L-type calcium channel currents in gastric smooth muscle.

It has also been demonstrated that the activity of L-type calcium channels can be regulated by different types of kinases, such as protein kinase A (PKA) (Sperelakis *et al*., 1994) and protein kinase C (PKC) (Hartzell & Rinderknecht, 1996). These two kinases phosphorylate serine and threonine residues on the α- and β-subunits of these channel proteins (Nastainczyk *et al*., 1987; Hell *et al*., 1993). Recently, a great deal of interest has appeared in the literature regarding another class of kinase, the protein tyrosine kinase (PTK) (Pfeiffer *et al*., 1995; Cataldi *et al*., 1996; Strauss *et al*., 1997). However, in vascular smooth muscle cells, only a few publications about the influence of PTK on ion channels have been published, in contrast to the number of publications about the interaction of PKA or PKC with ion channels (Wijetunge *et al*., 1992; Wijetunge & Hughes, 1995; Nelson *et al*., 1997).

In the present study, to elucidate the involvement of PTK activity in stretch-induced increase in L-type calcium channel currents, we investigated the effects of the PTK inhibitors such as herbimycin A (Uehara *et al*., 1989; Uehara, 1997), genistein (Akiyama *et al*., 1987), and lavendustin A (Onoda *et al*., 1989), or sodium orthovanadate, an inhibitor of protein tyrosine phosphatase (Swarup *et al*., 1982), on voltage-activated barium current (I_{Ba}) through L-type calcium channels enhanced by mechanical stimulation in canine basilar arterial

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myocytes using whole-cell voltage-clamp method. Hypotonic superfusion was employed, as it provides an alternative way to apply mechanical stress to a cell (Matsuda *et al*., 1996; Xu *et al*., 1996; 1997). Our results suggest that herbimycin A-sensitive PTK activity is specifically involved in the enhancement of L-type calcium channel currents by mechanical stimulation of the canine basilar artery.

Methods

Preparation of single cells

Single smooth muscle cells were isolated from canine basilar artery by the modified methods described elsewhere (Hagiwara *et al*., 1992; Matsuda *et al*., 1996; Xu *et al*., 1996). In brief, mongrel dogs of either sex and weighing 10–18 kg were exsanguinated after anaesthetization with an intravenous injection of sodium pentobarbitone (30 mg kg^{-1}). The basilar artery was isolated and cleaned of connective tissue in HEPES-buffered Krebs–Henseleit solution. The artery was cut into small segments (1 mm × 2 mm), and these segments were incubated for 60 min at 37°C in calcium-free Hank's solution. Then, they were digested for 15–25 min at 37°C in calcium-free Hank's solution containing 1.5 mg ml^{-1} collagenase (type IA, Sigma), 0.1 mg ml^{-1} protease, 0.2 mg ml^{-1} ATP, 2 mg ml^{-1} trypsin inhibitor, and 2 mg ml^{-1} BSA. After digestion, the supernatant was discarded, and the softened muscle segments were transferred again into calcium-free Hank's solution and incubated for 15 min at 37°C. Single cells were dispersed by gentle agitation with a wide-pore glass pipette. Isolated canine basilar myocytes were kept in the Kraft-Brühe (K-B) solution (Isenberg & Klöckner, 1982) at 4°C until used. All experiments were carried out at room temperature within 6 h including harvesting cells and performing actual experiments.

Electrical recordings

Isolated cells were transferred to a small chamber on the stage of an inverted microscope (IMT-2, Olympus, Tokyo, Japan). The chamber was perfused with the isotonic external solution (0.5 ml min^{-1}). The final pipette tip resistance was 2–5 MΩ when filled with the standard internal solution, and these pipettes were used to make a gigaohm seal of 1–2 GΩ. Standard patch-clamp techniques were used (Hamill *et al*., 1981). An Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.) in conjunction with a TL-1 DMA interface (Axon Instruments, Foster City, CA, U.S.A.) was used to record membrane currents and command pulses were applied by using a computer (Business VEISA 486/33, Advanced Logic Research, Singapore) and pClamp software version 6.0.3 (Axon Instruments, Foster City, CA, U.S.A.). The currents were filtered at 1 kHz. The sampling interval was 0.5 ms from a holding potential of −80 mV, and the cells were depolarized to various potentials at a frequency of 0.2 Hz to minimize calcium channel rundown. The compensation of capacitative transients and leakage currents was performed by the patch-clamp amplifier settings. The data were displayed on a computer monitor (Flex VIEW 2X, Advanced Logic Research, Singapore) and a digital oscilloscope (COR-5521, Kikusui Electronics, Tokyo, Japan).

To examine the effect of mechanical stimulation on membrane current in the whole-cell configuration, we adopted hypotonic cell swelling (Matsuda *et al*., 1996; Xu *et al*., 1996).

Cell volume measurement

Changes in cell form were displayed on a computer (Power Macintosh 7600/120, Apple Computer Inc, Cupertino, CA, U.S.A.) through a CCD camera (Television Camera CTC-2100, Ikegami Tsushinki, Tokyo, Japan) mounted on an inverted microscope, and the extent of change in cell volume was estimated roughly by fitting to an equation (Fay & Delise, 1973) as follows:

$$V = 1/6\ \pi abL \quad (1)$$

where V is the cell volume (μm^3), L is the longest axis (μm), and a and b are the remaining two axes (μm). The area (A) of the two-dimensional projection of the ellipsoid is given by $A = \pi/4$ aL. If one assumes that a/b is identical in two ellipsoids, which is equivalent to assuming identical eccentricity of their cross-sectional profiles, then the ratio of volumes of the two ellipsoids is given by:

$$V_1/V_2 = (A_1)^2/(A_2)^2 \times L_2/L_1 \quad (2)$$

Solutions and drugs

HEPES-buffered Krebs–Henseleit solution containing (in mM): NaCl 142.88, KCl 5.88, $MgSO_4$ 1.18, $CaCl_2$ 2.55, glucose 11.1, and HEPES 5 was adjusted to pH 7.4 with NaOH. Calcium-free Hank's solution containing (mM): NaCl 125, KCl 5.4, KH_2PO_4 0.44, $NaHCO_3$ 15.5, Na_2HPO_4 0.34, glucose 10, and sucrose 2.9 was adjusted to pH with HCl. K-B solution containing (mM): KOH 110, glutamic acid 70, taurine 10, KCl 25, KH_2PO_4 10, HEPES 5, EGTA 0.5, and glucose 11 was adjusted to pH 7.4 with HCl. The isotonic external solution (321.6 mosmol l^{-1}) containing (mM): $BaCl_2$ 50, HEPES 5, and mannitol 150 was adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane (TRIZMA). When the effect of hypotonic cell swelling on the voltage-activated barium currents (I_{Ba}) was examined, mannitol was removed from the isotonic external solution (Matsuda *et al*., 1996). Thus, the hypotonic external solution (155.5 mosmol l^{-1}) containing (mM): $BaCl_2$ 50, and HEPES 5 was adjusted to pH 7.4 with TRIZMA. The standard caesium-rich internal solution containing (mM): CsCl 140, HEPES 5, ATP 4, and EGTA 4 was adjusted to pH 7.4 with NaOH. All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, U.S.A.), Wako (Osaka, Japan), and Dojindo (Kumamoto, Japan). Herbimycin A, genistein, daidzein, sodium orthovanadate, and nicardipine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Bay K 8644 was procured from Wako (Osaka, Japan). Lavendustin A was purchased from Calbiochem (La Jolla, CA, U.S.A.). These drugs, except sodium orthovanadate, were dissolved in dimethyl sulphoxide (DMSO) and diluted over 1000 times into the bath solution, the final concentration of DMSO being kept under 0.1%. We confirmed that 0.1% DMSO had no effect on any of the membrane currents, as reported by Ogata *et al*. (1997).

Data and statistical analysis

In current-voltage plots, peak current amplitudes were plotted against the step potentials of the electrical stimulation. The data were expressed as mean ± s.e.mean. Maximum current amplitudes were estimated from peak currents induced by voltage step from −80 mV to +10 mV. Statistical significance was estimated by paired or unpaired Student's *t*-test, or by Tukey's test after analysis of variance (ANOVA). *P* values of less than 0.05 were considered to be statistically significant.

Results

Effect of osmolarity change on voltage-activated barium currents (I_{Ba})

Membrane potential was clamped by the whole-cell patch-clamp method. Whole-cell currents carried by barium ions were recorded in canine basilar arterial myocytes (Figure 1). Inward currents were elicited by depolarizing pulses to +10 mV from a holding potential of −80 mV under isotonic conditions (Figure 1A). The current-voltage (I-V) relationship indicated that the maximum current was obtained at +10 mV, the threshold potential for activation was about −40 mV, and the reversal potential was about +50 mV. These properties suggest the presence of an L-type calcium channel current (Figure 1B). The peak inward current in whole-cell recording was increased by the L-type calcium channel agonist Bay K 8644 (100 nM) to 176.9±9.6% ($P<0.05$, $n=5$) of the control current. The inward current was inhibited by the L-type calcium channel blocker nicardipine (1 μM) to 26.7±8.5% ($P<0.01$, $n=18$) of the control (113.5±19.5 pA, $n=18$). This residual current was designated as nicardipine-insensitive component. The increase in current produced by Bay K 8644 was reversed on washing with drug-free solution (106.1±8.4% of the control, $n=5$). Likewise, the inhibitory effect was recovered by washout of nicardipine (63.2±6.0% of the control, $n=5$). These results suggest that the inward current passing through the L-type calcium channel appears to be predominant in the whole-cell recording in canine basilar arterial myocytes.

To elucidate the effect of hypotonic cell swelling on I_{Ba} carried through the L-type calcium channels, we added nicardipine (1 μM) to the hypotonic external solution. Figure 1A shows traces of I_{Ba} at +10 mV (holding potential, $V_h=-80$ mV) during exposure to isotonic (control) and hypotonic solutions, and to hypotonic solution containing nicardipine. When the isotonic external solution (321.6 mosmol l^{-1}) was replaced by the hypotonic external solution (155.5 mosmol l^{-1}), the peak I_{Ba} was increased to 184.3±11.5% ($P<0.01$, $n=45$) of the control (123.8±18.4 pA, $n=45$) within 10 min; and the increased peak I_{Ba} was recovered to 115.6±13.2% ($n=5$) of the control by changing the external solution from hypotonic to isotonic. In addition, the peak I_{Ba} enhanced by the hypotonic solution was inhibited by nicardipine to 14.5±8.7% ($P<0.01$, $n=6$) of I_{Ba} in hypotonic solution (230.3±28.4 pA, $n=6$), and the current was recovered to 62.2±10.9% ($n=4$) after washout with hypotonic solution. As shown in Figures 1A,B, the nicardipine-insensitive component of I_{Ba} was not changed in the isotonic and hypotonic solutions containing nicardipine (isotonic: 30.3±9.6 pA, $n=18$; hypotonic: 35.1±8.6 pA, $n=4$; $P>0.05$). These results suggest that I_{Ba} increased by hypotonic solution mostly passed through the L-type calcium channels. Figure 1B shows the I-V relationship of I_{Ba} in isotonic and hypotonic solutions, and isotonic and hypotonic solutions containing nicardipine. Hypotonic superfusate increased I_{Ba}, whereas the hypotonic superfusate in the presence of nicardipine decreased I_{Ba} enhanced by hypotonic solution at each test potential. However, the potential at which the peak inward current was elicited and the threshold and reversal potentials were not changed under hypotonic conditions in the absence or presence of nicardipine (Figure 1B).

Obvious changes in cell volume were observed under the microscope, and were assessed roughly by fitting the extent of these changes to the equation described by Fay & Delise (1973, see Methods). Cell volume under the isotonic condition was 2034.2±28.9 μm^3 ($n=4$), whereas the corresponding value was

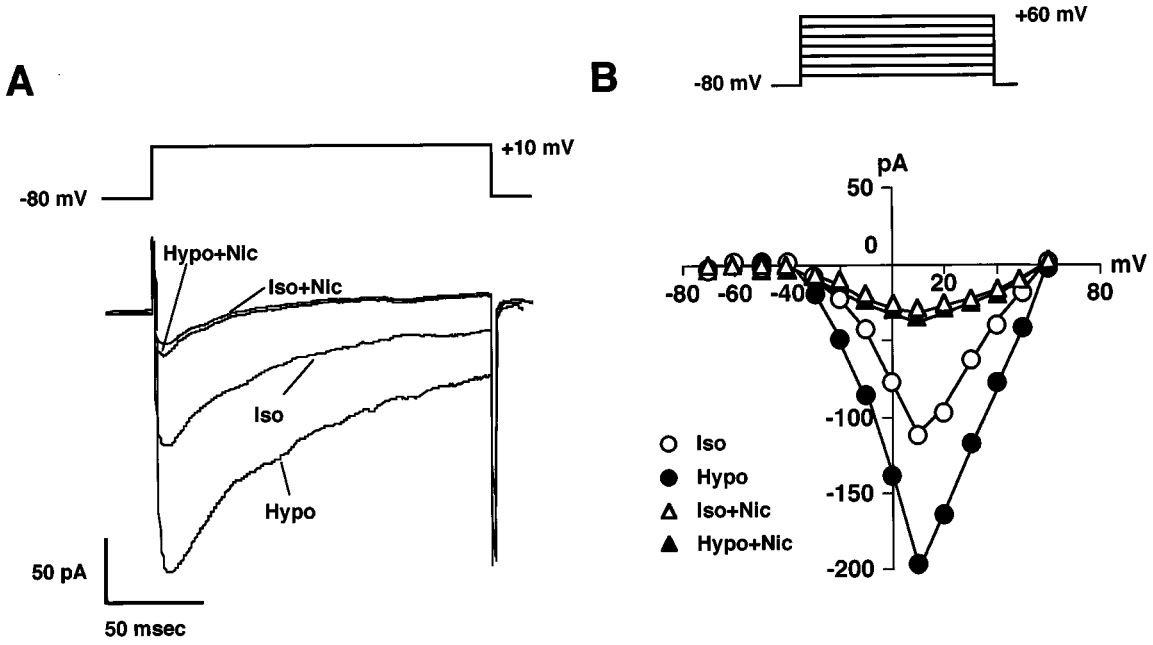

Figure 1 Effect of osmolarity change on I_{Ba}. (A) Effect of nicardipine (Nic, 1 μM) on I_{Ba} enhanced in canine basilar arterial myocytes. The external solution was changed from the isotonic solution (Iso) to the hypotonic external solution (Hypo). Then, the hypotonic solution was replaced by the isotonic solution containing nicardipine (Iso + Nic). Thereafter, the isotonic solution was changed to the hypotonic solution containing nicardipine (Hypo + Nic). I_{Ba} was elicited by depolarizing pulses to +10 mV from a holding potential (V_h) of −80 mV. (B) I-V relationships of I_{Ba} for the same cell shown in (A) in the isotonic external solution (Iso), the hypotonic external solution (Hypo), the isotonic solution containing nicardipine (Iso + Nic) and the hypotonic external solution containing nicardipine (Hypo + Nic). I_{Ba} was activated by incremental 10 mV depolarizing steps from a V_h of −80 mV to +60 mV. Barium ion (50 mM) was used as the charge carrier. All experiments were carried out at room temperature of 23°C.

3811.6±396.3 μm^3 ($P<0.01$, $n=4$) under the hypotonic condition. The extent of changes in both I_{Ba} and cell volume was dependent on the osmolarity of superfusate, and the change in I_{Ba} was linearly related to that in cell volume ($r=0.98$, $P<0.01$) (Figure 2). Pretreatment with nicardipine (1 μM) for 5 min had no apparent effect on increase in cell volume by hypotonic solution. Thus, the increase in cell volume by hypotonic solution was associated with the enhancement of I_{Ba} passed through the L-type calcium channels.

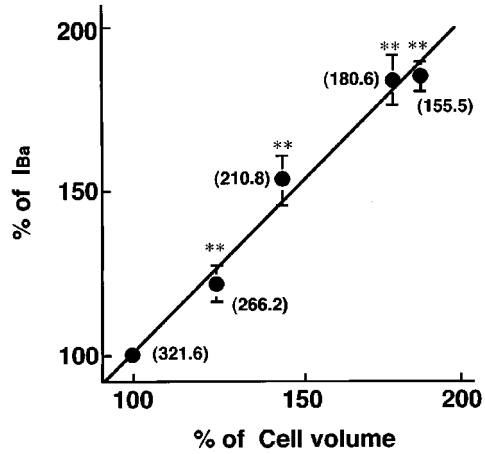

Figure 2 Relationship between cell volume and I_{Ba} changes induced by hypotonic solution. I_{Ba} was elicited by depolarizing pulses to +10 mV from a holding potential (V_h) of −80 mV. Cell volume and peak value of I_{Ba} under isotonic condition (321.6 mosmol l^{-1}) are taken as 100%. Regression analysis indicated that the relationship between cell volume and I_{Ba} approximated linear function ($y=1.02x+1.58$; $r=0.98$, $P<0.01$). The numerical value in a parenthesis represents osmolarity (in mosmol l^{-1}). Each point represents the mean±s.e.mean of four experiments. $^{**}P<0.01$ compared with corresponding control values.

Effects of herbimycin A on I_{Ba} under isotonic and hypotonic conditions

To assess whether L-type calcium channels were regulated by tyrosine phosphorylation, we investigated the effects of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) inhibitors on I_{Ba} under isotonic and hypotonic conditions. The results are summarized in Table 1. Herbimycin A (30 nM), which acts on PTK by directly binding to its reactive SH groups (Uehara *et al*., 1989; Uehara, 1997), had no apparent effect on the peak I_{Ba} under the isotonic condition (Figure 3A and Table 1). As shown in Figure 3B, herbimycin A caused no change in the I-V relations. Herbimycin A (30 nM) had no apparent effect on the nicardipine-insensitive component of I_{Ba} (31.3±9.1 pA, $n=4$) in the isotonic solution.

On the contrary, the extracellularly-applied herbimycin A (30 nM) caused a significant change in I_{Ba} under the hypotonic condition. Figure 4A shows traces of I_{Ba} at +10 mV ($V_h=-80$ mV) during exposure to isotonic (control) and

Table 1 Effects of protein-tyrosine kinase and phosphatase inhibitors on L-type calcium channel currents under isotonic and hypotonic conditions

Inhibitor	*% of the barium current* *Isotonic*	*Hypotonic*
Vehicle	95.8±6.8	97.2±2.0
Herbimycin A (30 nM)	94.6±4.7	72.4±1.1**
Genistein (10 μM)	71.8±4.1**	56.7±6.0**
Daidzein (10 μM)	105.9±5.3	92.5±4.7
Lavendustin A (10 μM)	72.4±1.1**	73.9±4.6**
Orthovanadate 10 (μM)	121.0±5.3**	120.7±3.6**

Each value represents the mean±s.e.mean of 4–8 separate experiments. Vehicle shows the isotonic and hypotonic external solutions containing 0.1% DMSO. The barium current was elicited by depolarizing pulse to +10 mV from a V_h of −80 mV. The barium currents in the isotonic and hypotonic solutions were taken as 100% under isotonic and hypotonic conditions, respectively. $^{**}P<0.01$.

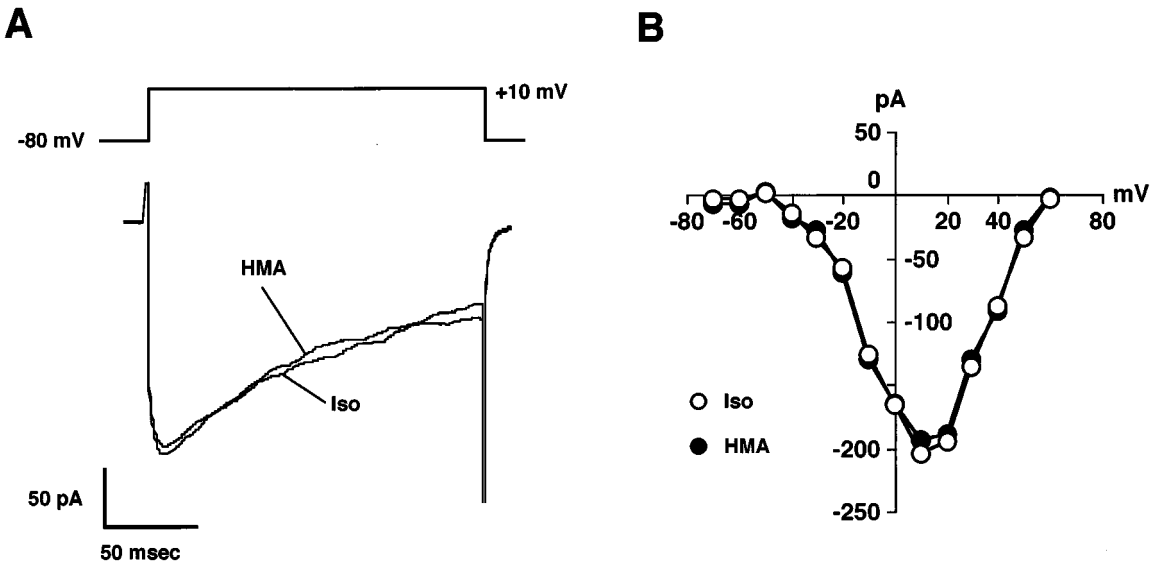

Figure 3 Effect of herbimycin A on I_{Ba} in canine basilar arterial myocytes induced by the isotonic external solution (Iso). (A) Representative tracings of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −80 mV in the absence or presence of herbimycin A (HMA, 30 nM). The peak inward current in this cell was not changed from 200 pA after application of herbimycin A. (B) Current-voltage (I-V) relationships of I_{Ba} for the same cell shown in (A) in the absence or presence of herbimycin A. I_{Ba} was activated by incremental 10 mV depolarizing steps from a V_h of −80 mV to +60 mV.

hypotonic solutions, and to hypotonic solution containing herbimycin A. The peak I_{Ba} enhanced by hypotonic solution was decreased by herbimycin A (Table 1), and the current was recovered to 80.2±2.2% ($n=4$) after washout with hypotonic solution. The recovery from the effect of herbimycin A was rather incomplete. As shown in Figure 4B, herbimycin A caused no shift of the I–V relations, though herbimycin A decreased I_{Ba} at each test potential. In canine basilar cells, herbimycin A exerted a concentration-dependent (1–30 nM) inhibition on I_{Ba} under the hypotonic condition (Figure 4C).

To alternatively confirm that the nicardipine-sensitive component of I_{Ba} is primarily affected by cell-swelling, we excluded the nicardipine-insensitive component(s) of I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −40 mV (Figure 5A). When the isotonic external solution was replaced by the hypotonic external solution, the I_{Ba} was increased to 195.2±13.1% ($n=5$) of the control (Figure 5B). Nicardipine (1 μM) abolished I_{Ba} under both isotonic and hypotonic conditions. However, herbimycin A (30 nM) effectively decreased the I_{Ba} enhanced by hypotonic solution without affecting I_{Ba} in isotonic solution.

Effects of genistein and lavendustin A on I_{Ba} under isotonic and hypotonic conditions

Genistein (10 μM), which inhibits PTK by competing with ATP for the binding on the enzyme (Akiyama *et al*., 1987; Cataldi *et al*., 1996), affected I_{Ba} under the isotonic condition. Figure 6A shows traces of I_{Ba} at +10 mV ($V_h=-80$ mV) during exposure to isotonic solutions in the absence (control) or presence of genistein (10 μM). The extracellularly-applied genistein significantly decreased the peak I_{Ba} (Table 1). The

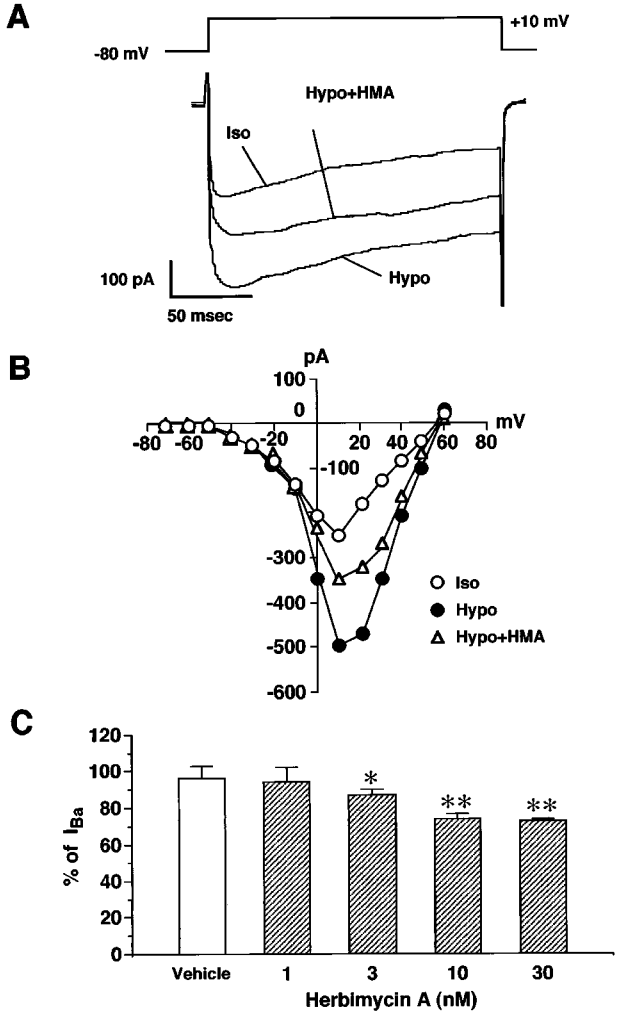

Figure 4 Effect of herbimycin A on I_{Ba} in canine basilar arterial myocytes enhanced by the hypotonic external solution. (A) Representative tracings of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −80 mV in the absence or presence of herbimycin A (HMA, 30 nM). (B) I–V relationships of I_{Ba} for the same cell shown in (A) in the isotonic external solution (Iso), the hypotonic external solution (Hypo), and the hypotonic external solution containing herbimycin A. I_{Ba} was activated by incremental 10 mV depolarizing steps from a V_h of −80 mV to +60 mV. (C) Concentration dependence of the inhibitory effect of herbimycin A on I_{Ba} under the hypotonic condition is illustrated. I_{Ba} elicited by the hypotonic external solution in the absence of herbimycin A is taken as 100%. Each column represents the mean±s.e.mean of 4–6 experiments. $^*P<0.05$ and $^{**}P<0.01$ compared with corresponding control values.

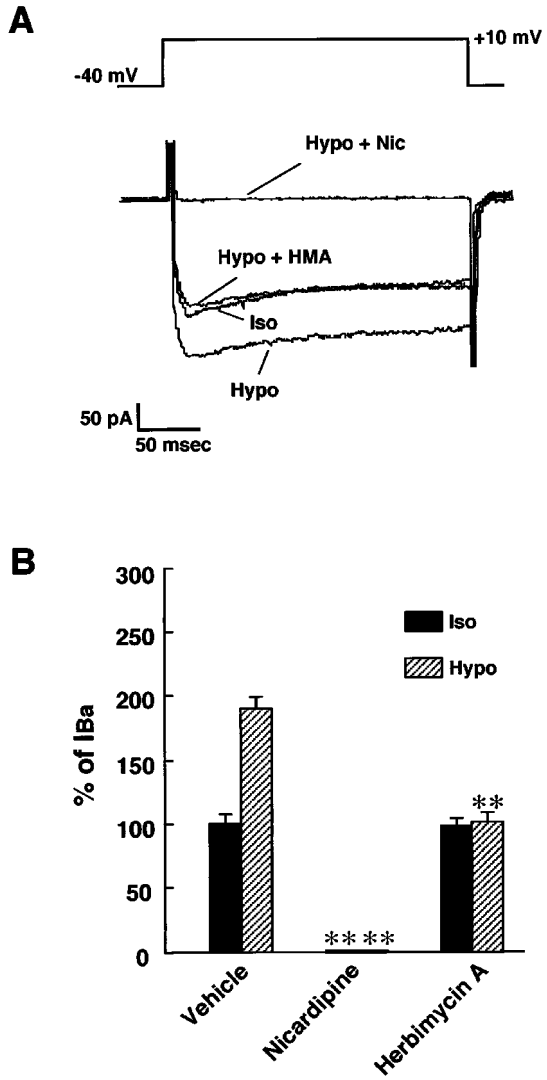

Figure 5 Effects of herbimycin A and nicardipine on I_{Ba} passing though L-type calcium channels. (A) Representative tracings of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −40 mV under isotonic condition (Iso) and those under hypotonic condition (Hypo) in the absence and presence of herbimycin A (HMA, 30 nM) or nicardipine (Nic, 1 μM). (B) Effects of herbimycin A and nicardipine on I_{Ba} passing though L-type calcium channels under isotonic and hypotonic conditions. I_{Ba} in the isotonic external solution in the absence of drugs is taken as 100%. Each column represents the mean±s.e.mean of 4–6 experiments. $^{**}P<0.01$ compared with corresponding control values.

current was almost recovered to the control level after washout (99.0±5.1% of the control, $n=7$). Although genistein decreased I_{Ba} at each test potential, it caused no shift in the I–V relations (Figure 6B). Genistein exerted a concentration-dependent (1–100 μM) reduction of I_{Ba} in the isotonic solution (Figure 6C). Genistein (10 μM) had no apparent effect on the nicardipine-insensitive component of I_{Ba} (data not shown). On the other hand, the inactive genistein analogue daidzein (10 μM) did not exert any influence on I_{Ba}. Under the isotonic condition, daidzein caused no significant changes in the peak I_{Ba} (Table 1). Furthermore, lavendustin A (10 μM), which binds to the PTK at a site distinct from the ATP binding site (Onoda *et al*., 1989; Xiong *et al*., 1995; Cataldi *et al*., 1996), also decreased the peak I_{Ba} (Table 1).

The extracellularly-applied genistein (10 μM) also significantly decreased I_{Ba} under the hypotonic condition. Figure 7A shows the changes in the peak inward current in response to hypotonic solution and genistein. The peak I_{Ba} was immediately increased by hypotonic solution and reached its maximum value within 10 min. When genistein was added to hypotonic solution, the peak I_{Ba} decreased to near the same level before swelling. The recovery of the peak I_{Ba} was observed after washout of genistein with hypotonic solution. Subsequent application of the isotonic external solution returned both cell volume and the peak I_{Ba} to the basal (control) level before application of the hypotonic solution (Figure 7A). Figure 7B shows traces of I_{Ba} at the points (a–c) labelled in Figure 7A. Genistein decreased the peak I_{Ba} elicited by the hypotonic solution (Table 1), and the current was recovered to 86.6±5.2% ($n=7$) after washout of it with hypotonic solution. As shown in Figure 7C, genistein decreased I_{Ba} enhanced by

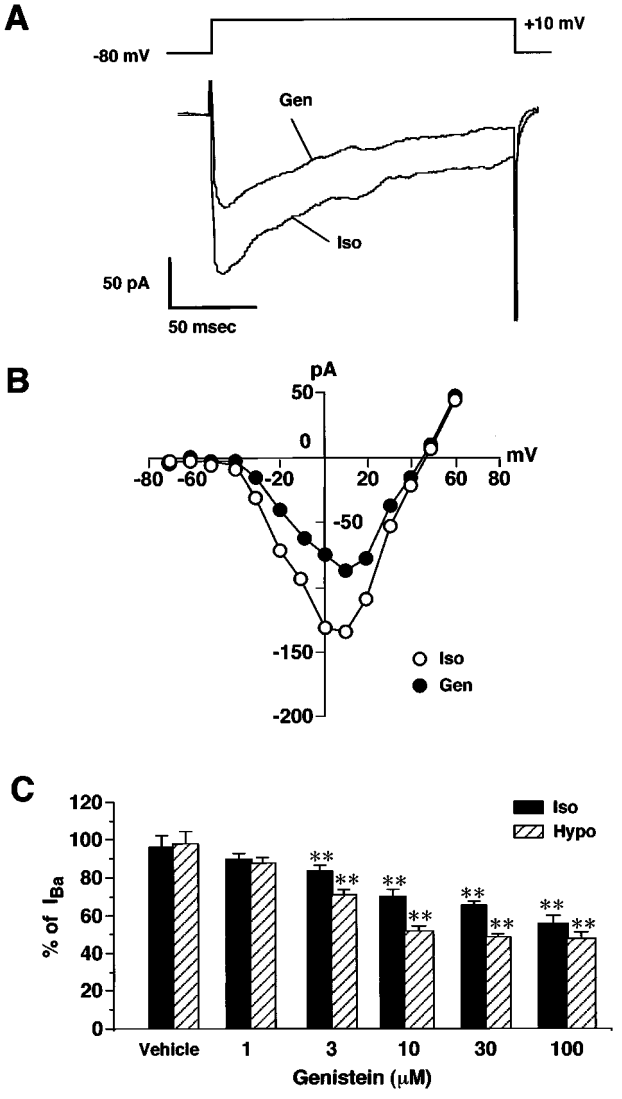

Figure 6 Effect of genistein on I_{Ba} in canine basilar arterial myocytes induced by the isotonic external solution (Iso). (A) Representative tracings of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −80 mV in the absence or presence of genistein (Gen, 10 μM). (B) Current-voltage (I–V) relationships of I_{Ba} for the same cell shown in (A) in the absence or presence of genistein. I_{Ba} was activated by incremental 10 mV depolarizing steps from a V_h of −80 mV to +60 mV. (C) Concentration dependence of the inhibitory effect of genistein on I_{Ba}. I_{Ba} elicited by the isotonic or the hypotonic external solutions in the absence of genistein is taken as 100%. Each column represents the mean±s.e.mean of 6–8 experiments. $^{**}P<0.01$ compared with corresponding control values.

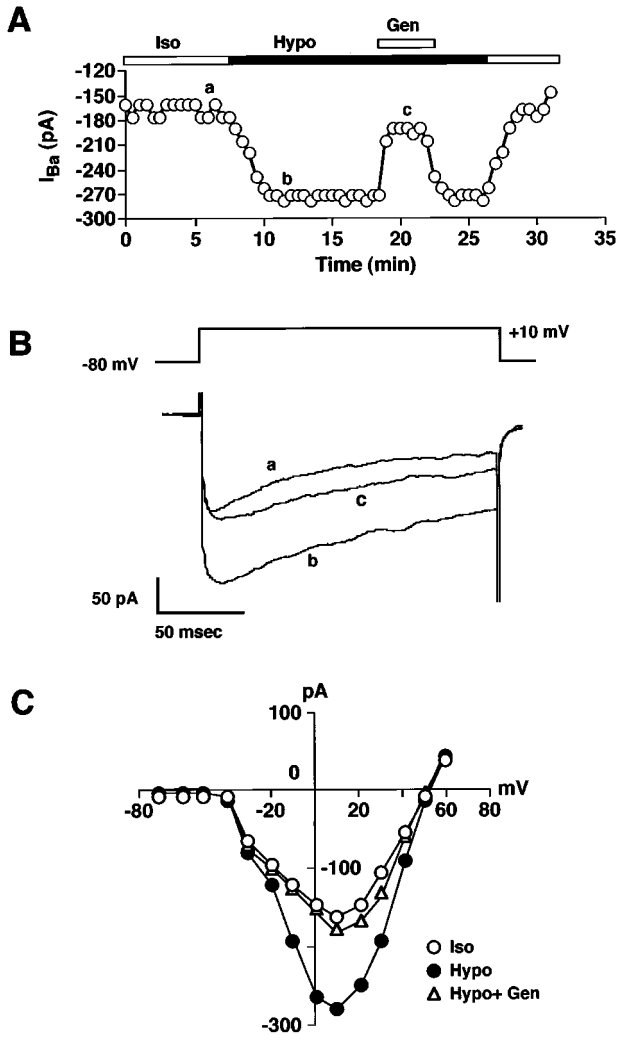

Figure 7 Effect of genistein on I_{Ba} in canine basilar arterial myocytes enhanced by the hypotonic external solution. (A) Time course of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −80 mV every 30 s under isotonic (Iso) (a) and hypotonic (Hypo) conditions in the absence (b) or presence (c) of genistein (Gen, 10 μM). (B) Representative tracings of I_{Ba} from the three time-points labelled in (A). (C) I–V relationships of I_{Ba} for the same cell shown in (A) and (B) in the isotonic external solution, the hypotonic external solution, and the hypotonic external solution containing genistein. I_{Ba} was activated by incremental 10 mV depolarizing steps from a V_h of −80 mV to +60 mV.

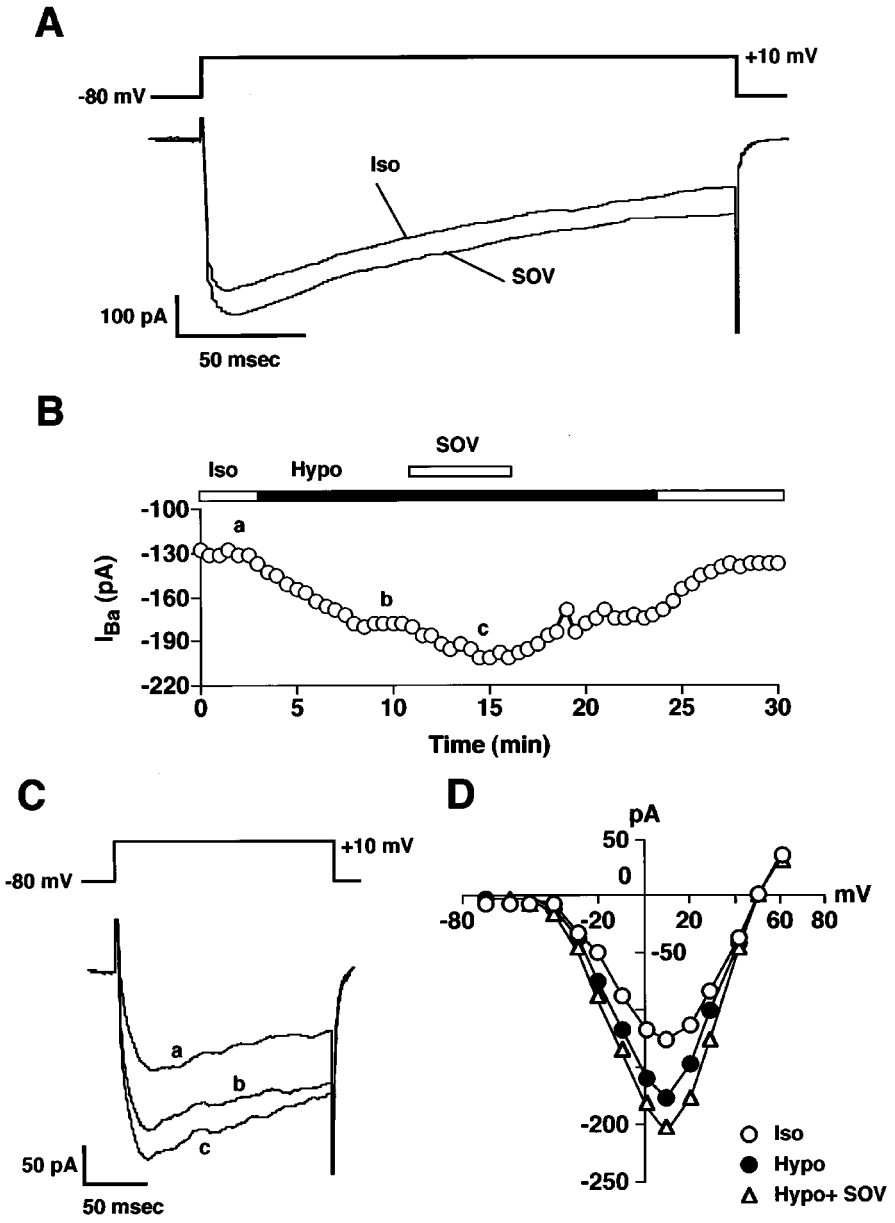

Figure 8 Effect of sodium orthovanadate on I_{Ba} in canine basilar arterial myocytes under isotonic (Iso) (A) and hypotonic (Hypo) (B–D) conditions. (A) Representative tracings of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −80 mV in the absence or presence of sodium orthovanadate (SOV, 10 μM). (B) Time course of the peak I_{Ba} elicited by depolarizing pulses to +10 mV from a V_h of −80 mV every 30 s under isotonic (a) and hypotonic conditions in the absence (b) or presence (c) of sodium orthovanadate (10 μM). (C) Representative tracings of I_{Ba} from the three time-points labelled in (B). (D) I–V relationships of I_{Ba} for the same cell shown in (B) and (C) in the isotonic external solution, the hypotonic external solution, and the hypotonic external solution containing sodium orthovanadate. I_{Ba} was activated by incremental 10 mV depolarizing steps from a V_h of −80 mV to +60 mV.

hypotonic solution at each test potential. However, genistein caused no shift in the I–V relations. Genistein also exerted a concentration-dependent (1–100 μM) reduction of I_{Ba} in the hypotonic solution (Figure 6C). The IC_{40} (concentration producing 40% inhibition) of genistein in the isotonic (56.2±8.6 μM, $n=8$) was significantly larger than that in the hypotonic solutions (5.3±2.8 μM, $P<0.01$, $n=6$). By contrast, daidzein (10 μM) had no effect on I_{Ba}. In hypotonic solution containing daidzein, the peak I_{Ba} slightly decreased (Table 1). Furthermore, lavendustin A (10 μM) also significantly decreased the peak I_{Ba} in the hypotonic solution (Table 1), and the current was recovered to 87.7±4.0% ($n=5$) after washout of the hypotonic solution.

Effects of sodium orthovanadate on I_{Ba} under isotonic and hypotonic conditions

The extracellularly-applied sodium orthovanadate, a well-known PTP inhibitor (Swarup *et al*., 1982), led to a reversible increase in I_{Ba} under both isotonic and hypotonic conditions. As shown in Figure 8A, the peak I_{Ba} was increased by sodium orthovanadate (10 μM) under the isotonic condition (Table 1), and recovered to 104.1±3.8% ($n=8$) after the washout. Figure 8B shows the changes in the peak inward current in response to hypotonic solution and sodium orthovanadate. Figure 8C shows traces of I_{Ba} at the points (a–c) labelled in Figure 8B in isotonic and hypotonic solutions, and in hypotonic solution containing sodium orthovanadate. When sodium orthovana-

date was superfused after hypotonic cell swelling, the peak I_{Ba} was increased in the hypotonic solution (Table 1), and was almost completely recovered to $100.6 \pm 1.5\%$ ($n=5$) after the washout with hypotonic solution. Sodium orthovanadate further increased the I_{Ba} that had been enhanced by hypotonic solution at each test potential. However, sodium orthovanadate caused no shift in the I-V relations (Figure 8D).

Discussion

The results of the present study, obtained by means of the whole-cell patch-clamp technique, demonstrated that herbimycin A, a PTK inhibitor, specifically decreased voltage-gated barium currents (I_{Ba}) enhanced by the hypotonic external solution in canine basilar arterial myocytes, whereas genistein, another type of PTK inhibitor, decreased I_{Ba} under both isotonic and hypotonic conditions. Furthermore, we found that sodium orthovanadate, a PTP inhibitor, increased I_{Ba} in these cells under both isotonic and hypotonic conditions.

In the present study, using Bay K 8644 and nicardipine, which are L-type calcium channel agonist and blocker, respectively, we firstly confirmed that I_{Ba}, elicited by depolarizing pulses from a V_h of -80 mV, passed through mainly the L-type calcium channels in isolated canine basilar myocytes. However, a nicardipine-insensitive component of I_{Ba} remained, indicating that I_{Ba} passed though partially the non L-type calcium channels. Furthermore, we found that I_{Ba} was enhanced by osmotic cell swelling in hypotonic solution, and that hypotonic superfusion increased I_{Ba} at each test potential (Figure 1). The extent of changes in both I_{Ba} and cell volume was dependent on the osmolarity of superfusate, and the change in I_{Ba} was correlated well with that in cell volume (Figure 2). The increased I_{Ba} was completely inhibited by nicardipine (Figure 1) though the drug has no effect on osmotic cell swelling. I_{Ba} elicited by depolarizing pulses from a V_h of -40 mV was also enhanced by the osmotic cell swelling, and this enhanced I_{Ba} was completely abolished by nicardipine (Figure 5). Therefore, our results indicate that I_{Ba} enhanced by hypotonic solution mostly passes through the L-type calcium channels. Similar results were obtained in guinea-pig gastric myocytes (Xu *et al*., 1996).

Recently, there have been a number of reports about the effect of tyrosine phosphorylation on the L-type calcium channel activity. Strauss *et al*. (1997) reported that the activity of L-type calcium channels was regulated by PTK and protein kinase C (PKC) in cultured rat and human retinal pigment epithelial cells. Cataldi *et al*. (1996) also reported that PTK activation caused an increase in L-type calcium channel currents, whereas PTP activation decreased these currents, in GH_3 cells. In vascular smooth muscle cells, L-type calcium channel currents (Wijetunge & Hughes, 1995; 1996; 1998) and intracellular calcium concentration (Nelson *et al*., 1997) are increased by tyrosine phosphorylation. In the present study, I_{Ba} was decreased by genistein (Figures 6 and 7, Table 1) and lavendustin A (Table 1), and was further increased by sodium orthovanadate (Figure 8, Table 1) under both isotonic and hypotonic conditions. However, the inactive genistein analogue daidzein did not affect I_{Ba} under either condition (Table 1). Genistein had no effect on the nicardipine-insensitive component of I_{Ba}. These results suggest that tyrosine phosphorylation, which depends on the balance between PTK and PTP activities, regulates the activity of L-type calcium channels in the canine basilar artery.

On the contrary, herbimycin A decreased I_{Ba} only under the hypotonic condition (Figures 3, 4 and 5, Table 1). The concentration-dependent and specific inhibitory action of herbimycin A on I_{Ba} increased by hypotonic solution indicates that herbimycin A-sensitive PTK activity may be exclusively involved in the mechanism underlying the enhancement of L-type calcium channel currents under the hypotonic condition.

As to the effect of herbimycin A, a cytoplasmic non-receptor tyrosine kinase of 60,000 dalton, known as c-Src, is widely distributed in smooth muscle cells (Di Salvo *et al*., 1989) and is a common target of herbimycin A (Uehara *et al*., 1989; Uehara, 1997). It has been reported that intracellular application of human c-Src increased L-type calcium channel currents (Wijetunge & Hughes, 1995) and intracellular application of peptide A, an inhibitor of c-Src, inhibited the effects of c-Src and also reduced L-type calcium channel currents in rabbit ear arterial cells (Wijetunge & Hughes, 1995; 1996). The PTK activity of adhesion molecules such as c-Src was suggested to play an important role in regulating voltage-operated calcium channel in vascular smooth muscle cells. Furthermore, c-Src is found in association with the cytoskeleton (Felice *et al*., 1990) and concentrated around cellular attachment sites to the extracellular matrix, such as focal adhesions (Richardson & Parsons, 1995). C-Src is active in its dephosphorylated state, and its activation seems to be modulated by mechanical stretch (Lehoux & Tedgui, 1998). Xu *et al*. (1997) proposed that in gastric smooth muscle, the actin cytoskeleton might mediate I_{Ba} increased by hypotonic solution and that the role of this cytoskeleton in channel currents is active only after mechanical stimulation such as hypotonic cell swelling. In our experiments, herbimycin A decreased only I_{Ba} enhanced by hypotonic stimulation in canine basilar arterial myocytes (Figure 3, 4 and 5, Table 1). Therefore, we consider that adhesion molecules and cytoskeletal proteins may play an important role in regulating L-type calcium channel activated by mechanical stimulation such as hypotonic cell swelling. This is, to our knowledge, the first study showing the specific effect of herbimycin A on I_{Ba} enhanced by hypotonic solution in canine basilar arterial cells.

It has been reported that genistein, besides inhibiting PTKs, can also block other protein kinases such as protein kinase A (PKA) and PKC (Akiyama *et al*., 1987), both of which are known to regulate L-type calcium channels (Sperelakis *et al*., 1994; Hartzell & Rinderknecht, 1996). Genistein also had direct inhibitory and activating actions on the delayed-rectifier potassium channel (Washizuka *et al*., 1997) and the cystic fibrosis transmembrane conductance regulator chloride channel (Hwang *et al*., 1997; Zhou *et al*., 1998) in guinea-pig ventricular myocytes, respectively. Although it is possible that genistein inhibits the L-type calcium channel activity by a PTK-independent mechanism, the IC_{50} value for genistein inhibition of L-type calcium channels (about 10 μM) was much lower than that for PKA and PKC blockade (Akiyama *et al*., 1987). Furthermore, the specificity of genistein action on L-type calcium channels *via* PTKs was confirmed further by the inability of daidzein (Table 1). In addition, extracellularly-applied staurosporine (1 nM), a serine/threonine protein kinase inhibitor, did not significantly change the peak I_{Ba} under the hypotonic condition (our unpublished observations). Herbimycin A and lavendustin A, two other type of PTK inhibitors devoid of PKA or PKC inhibitory action (Uehara *et al*., 1989; Onoda *et al*., 1989) and structurally unrelated to genistein, effectively inhibited the calcium channel activity in canine basilar arterial myocytes. Consequently, our results strongly suggest that PTK activity is primarily involved in the regulation of L-type calcium channels in canine basilar arterial cells.

In summary, our results suggest that cell swelling by hypotonic solution increases the L-type calcium channel currents in canine basilar arterial myocytes and that

herbimycin-sensitive PTK activity is primarily involved in the enhancement of calcium channel currents under the hypotonic condition.

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