

## Characteristics of Ca Currents in Rabbit Basilar Arterial Smooth Muscle Cells

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= Abstract = In order to determine the exact nature of Ca channels involved in various cerebrovascular contractile behaviour including vasospasm, we performed experiments to identify and characterize the types of Ca channels in rabbit basilar arterial smooth muscle cells by using kinetic and pharmacologic tools. Single smooth muscle cells were enzymatically isolated from rabbit basilar artery. Single cells were voltage-clamped, and membrane currents were recorded using the whole-cell configuration of patch clamp technique. The measured cell capacitance ( $C_m$ ) was  $19.2 \pm 0.65$  pF ( $n = 21$ ) and input resistance ( $R_{input}$ ) was  $2.04 \pm 0.12$  G $\Omega$  ( $n = 12$ ). These passive membrane properties are similar to other cerebrovascular smooth muscle cells. Inward  $Ca^{2+}$ -channel current was recorded. Replacement of external  $Ca^{2+}$  (2 mM) with  $Ba^{2+}$  (10 mM) increased the amplitude of the current and did not shift the I-V relationship for  $I_{Ba}$  in comparison with that for  $I_{Ca}$ . Changing the holding potential from -80 to -40 mV decreased the current amplitude but did not shift the voltage dependence. No detectable low-threshold, rapid inactivating inward current was observed. Steady-state activation and inactivation curves for  $I_{Ca}$  ( $V_{1/2(act)}$  -4.4 mV;  $V_{1/2(inact)}$  -22.3 mV) and  $I_{Ba}$  ( $V_{1/2(act)}$  -7.5 mV;  $V_{1/2(inact)}$  -20.5 mV) were determined. The theoretical 'window current's amplitude was calculated for  $I_{Ca}$  and  $I_{Ba}$ . Calcium channel current was almost completely inhibited by 1  $\mu$ M nifedipine and enhanced by Bay K 8644, suggesting this is carried by 'L-type' but not by 'T-type' calcium channel. Bay K 8644 significantly shifted activation curve to the negative potential. Both 8-br-cAMP (0.1 ~ 1 mM) and 8-br-cGMP (0.1 ~ 1 mM), a membrane permeable cyclic nucleotides, decreased the current amplitude. From the above results, it is suggested that only 'L-type' Ca-current ( $I_{Ca-L}$ ) exists in rabbit basilar arterial smooth muscle cells.

Key Words : L-type Ca currents, basilar artery, patch clamp, window current.

### INTRODUCTION

Arterial tone, which underlies the maintenance

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of peripheral resistance in the circulation, is a major contributor to the control of blood pressure. The contractile force of arterial smooth muscle is regulated by the intracellular concentration of  $Ca^{2+}$ . This is thought to occur by way of the dependence of myosin light chain phosphorylation on a kinase activated by the calcium-calmodulin complex. The phosphorylation leads to actin-myosin interaction and so to force development (Somlyo, 1985; Hai and Mur-

phy, 1989; Nelson et al., 1990).

Excitation-contraction coupling (E-C coupling) in vascular smooth muscle involves a rise in the intracellular  $\text{Ca}^{2+}$  concentration that is in part dependent upon an influx of  $\text{Ca}^{2+}$  through voltage-gated calcium channels. In a variety of tissues, two or three populations of voltage-activated calcium channels have been analyzed on the basis of whole-cell and single channel calcium current recording (Nowycky *et al.*, 1985; Bean, 1985). It is now generally accepted that most vascular smooth muscle cells (SMCs) possess two types of voltage-activated calcium channels, usually termed T-type and L-type, respectively (Bean *et al.*, 1986; Friedmann *et al.*, 1986; Benham *et al.*, 1987; Aaronson *et al.*, 1988; Loirand *et al.*, 1989; Ganitkevich and Isenberg, 1990).

Contraction and relaxation of cerebrovascular smooth muscle cells regulate cerebral blood flow and pressure and it is well known that the electrical and pharmacological properties of the cerebral arteries are different from other peripheral arteries (Faraci and Heistad, 1990). There exists a high degree of electromechanical coupling in cerebral arteries and the level of membrane potential largely determines the active state of cerebrovascular smooth muscle (Harder *et al.*, 1984). Calcium channels of arterial smooth muscle are believed to play an important role in cerebral circulatory control in both normal and pathological conditions (Faraci and Heistad, 1990). Cerebral arteries have been shown to be more sensitive than peripheral arteries to dihydropyridine  $\text{Ca}^{2+}$ -channel antagonist and agonist (Asano *et al.*, 1993), and dihydropyridine  $\text{Ca}$ -channel antagonist have proved useful in the management of disorders such as hypertension, stroke, migraine, and cerebral vasospasm (Worley *et al.*, 1991).

There is a little information available on the electrophysiological properties of cerebrovascular SMCs despite the physiological and clinical importance of this vascular bed for the control of cerebral blood flow. To determine the exact nature of  $\text{Ca}$  channel involved in various cerebrovascular contractile behavior including vasospasm, identification and characterization of the  $\text{Ca}$  channel types present in this tissue must be

done first. Thus present study was undertaken to identify and characterize the type of  $\text{Ca}$  current of rabbit basilar arterial SMCs by using kinetic and pharmacological tools.

## MATERIALS AND METHODS

### 1. Cell Isolation

Single smooth muscle cells were enzymatically isolated from rabbit basilar artery. Rabbits (New Zealand white rabbit, 1.5~2.0 kg) were anesthetized with sodium pentobarbital (40 mg/kg, i.v.) and exsanguinated. The brain was then rapidly removed and placed in phosphate-buffered cold Tyrodes solution containing (mM): NaCl 147, KCl 4,  $\text{MgCl}_2$  2,  $\text{CaCl}_2$  2,  $\text{NaH}_2\text{PO}_4$  0.42,  $\text{Na}_2\text{HPO}_4$  1.81, glucose 5.5, pH 7.35. Then the segment of basilar artery was gently dissected from the surface of the brain stem and placed in a  $\text{Ca}^{2+}$ -free phosphate-buffered Tyrode's solution ( $\text{CaCl}_2$  was omitted from the above compositions). Tunica adventitia, surrounding connective tissue and side branches were removed gently under a stereomicroscope. The artery was longitudinally dissected and rinsed to remove the residual blood in vessel. The artery was first moved to a nominally  $\text{Ca}$ -free Tyrode's solution containing (mM): NaCl 138.5, KCl 6, glucose 10,  $\text{MgCl}_2$  0.5, HEPES 5 (pH 7.35 with Tris), cut into small pieces and then the segments were stored at 4 °C for 15 minutes. Collagenase (1.5-1.8 mg; Wako pure chemicals, Osaka, Japan), bovine serum albumin (2 mg; essentially fatty acid free, Sigma, St. Louis, USA) and dithioerythritol (1~1.5 mg; Sigma) was dissolved into 1 ml of  $\text{Ca}$ -free Tyrode's solution. Then the arterial segments were incubated in this enzyme cocktail at 35 °C for 13-15 minutes. After collagenase treatment, segments were transferred to modified Kraftbrühe solution (KB medium; Isenberg and Klockner, 1982) containing (mM): L-glutamic acid (free acid) 50, KCl 40, taurine 20,  $\text{KH}_2\text{PO}_4$  20,  $\text{MgCl}_2$  3, glucose 10, HEPES 10, EGTA 0.5 (pH 7.35 with KOH), and single cells were dispersed by gentle agitation with glass pipette. Isolated single cells were stored at 4 °C until use.

## 2. Electrophysiological Recordings

An aliquot of single basilar arterial smooth muscle cells in suspension were added to the recording chamber (0.5 ml) mounted on an inverted microscope (Olympus, IMT-2, Japan). Solutions were superfused through the chamber by gravity at a rate of 2~3 ml/min. Experiments were performed at room temperature. Single cells were voltage clamped, and membrane currents were measured using the whole-cell configurations of patch clamp technique (Hamill *et al.*, 1981). Patch pipettes were made from borosilicate glass capillaries (inner diameter, 1.5 mm), pulled on a two-step vertical puller (Narishige, PP-83, Japan), and fire-polished with a microforge (Narishige, MF-83); the pipettes had a resistances of 3~5 M $\Omega$ . To measure whole-cell currents after gigaseal formation, access to the cell interior was obtained by rupturing the membrane at the tip of the pipette with additional negative pressure. Series resistance was not compensated. Membrane currents were recorded using patch-clamp amplifier (Axon instruments, Axopatch 1-D, USA).

Membrane currents were monitored on a digital oscilloscope (Philips, PM 3350, Japan), and data were digitized on-line with an analog-to-digital interface (Axon instruments, Labmaster TL-1 DMA interface). Data that were digitized on-line were stored on an IBM-AT compatible computer. Whole-cell currents were filtered at 5.0 kHz and digitized. All data analysis was performed with pCLAMP 5.5.1 software (Axon Instruments). Leakage current subtraction was performed on data, if necessary.

## 3. Solutions

### 1) Bath solutions

#### (1) Normal Tyrode's solution (mM)

NaCl 138.5, KCl 6, CaCl<sub>2</sub> 1.8, glucose 10, MgCl<sub>2</sub> 0.5, HEPES 5 (pH 7.35 with Tris)

#### (2) Barium solution for calcium-channel current recording (mM)

NaCl 125, KCl 5, BaCl<sub>2</sub> 10, glucose 10, MgCl<sub>2</sub> 0.5, HEPES 10 (pH 7.35 with NaOH)

### 2) Pipette solutions for Ca-current recording

CsCl 135, MgCl<sub>2</sub> 4, HEPES 10, Na<sub>2</sub>ATP 2, EGTA 10 (pH 7.35 with CsOH) 135, MgCl<sub>2</sub> 4, HEPES 10, Na<sub>2</sub>ATP 2, EGTA 10 (pH 7.35 with CsOH)

## 4. Drugs

N, N, N', N'-tetraacetic acid (EGTA), nifedipine, sodium nitroprusside (SNP), 8-bromo-cAMP, 8-bromo-cGMP were all obtained from Sigma Chemicals.

## 5. Statistics

Results are expressed as mean  $\pm$  SEM. Statistical significance was evaluated using Student's *t* test for unpaired observations. Differences were considered significant at *p* observations. Differences were considered significant at *p* < 0.05.

## RESULTS

### 1. Passive Membrane Properties

The passive membrane properties of enzymatically isolated cells from the rabbit basilar artery are shown in Table 1. Smooth muscle cells isolated were relaxed spindle-shaped and the length of cells varied but not more than 150  $\mu$ m long, 10  $\mu$ m wide. Under current clamp the isolated cells have a resting membrane potential of  $-29 \pm 1.2$  mV (*n* = 16) when the pipette was filled by the solution containing K-aspartate with 10 mM EGTA. At the same conditions by using 10~20 mV hyperpolarizing pulses (step pulse or ramp) at holding potential of -60 mV, the membrane input resistance and cell capacitance were measured. The measured cell capacitances (*C<sub>m</sub>*) was  $19.2 \pm 0.7$  pF (*n* = 21) and input resistances (*R<sub>input</sub>*) was  $2.04 \pm 0.12$  G $\Omega$  (*n* = 12). Assuming a value of 1  $\mu$ F/cm<sup>2</sup> for specific membrane capacitance, the estimated mean cell surface area corresponded to  $1924 \pm 69$   $\mu$ m<sup>2</sup>. The geometric surface area, assuming a cylinder (120  $\mu$ m long, 6  $\mu$ m wide), was 1130  $\mu$ m<sup>2</sup>. Part of this difference arises from extensive caveolae in smooth muscle cells (Abe and Tomita, 1968). Multiplying the surface area by the input resistance gives a

**Table 1.** Passive membrane properties of smooth muscle cells from the rabbit basilar artery

Membrane potential ( $V_m$ , mV)	$-29 \pm 1.2^{\text{e}}$
Cell capacitance ( $C_m$ , pF)	$19.2 \pm 0.65$
Input resistance ( $R_{\text{input}}$ , $G \Omega$ )	$2.04 \pm 0.12$
Electrical cell surface area ( $A_m$ , $\mu\text{m}^2$ )	$1924 \pm 69^{\text{e}}$
Specific membrane resistance ( $R_m$ , $k\Omega\text{cm}^2$ )	39.2
Space constant ( $\lambda$ , cm)	0.485

<sup>e</sup> Pipette was filled by the solution containing K-aspartate with 10mM EGTA

<sup>e</sup> Assumed specific membrane capacitance of  $1.0 \text{ F/cm}^2$

specific membrane resistance ( $R_m$ ) is about  $39.2 \text{ k}\Omega\text{cm}^2$ . Using following equation,  $\lambda = (d/4R_m/R_i)^{1/2}$ , a space constant ( $\lambda$ ) of 0.485 cm was determined on the basis of the measured cell diameter ( $d$ ) of  $6 \mu\text{m}$ , a calculated specific membrane resistance ( $R_m$ ) of  $39.2 \text{ k}\Omega\text{cm}^2$ , and assuming a specific internal resistivity ( $R_i$ ) of  $250 \Omega\text{cm}$  (Abe and Tomita, 1968). Such a value of the space constant predicts that the entire membrane of a single rabbit basilar arterial cell would be virtually isopotential both in the resting state and during activation of ionic channels.

## 2. Calcium Current ( $I_{\text{Ca}}$ )

### 1) Run-down of the $\text{Ca}^{2+}$ -current

After establishing whole-cell clamp, the  $\text{Ca}^{2+}$ -channel current began to decrease with time, which has been called as 'run-down'. In a preliminary experiment, run-down was assessed by repetitively measuring current during test pulses of 100 ms to +10 mV, at intervals of 20 s (data not shown). In most cases ( $n=5$ ), run-down was biphasic, with an initial period of rapid run-down, followed by a slower phase. Time course of the run-down was well fitted by double exponentials. But the degree of run-down was somewhat variable from cell to cell. The time required for the current to decrease to half its maximum value was  $12.5 \pm 2.5 \text{ min}$  ( $n=4$ ).

### 2) The properties of ionic permeation

Calcium current ( $I_{\text{Ca}}$ ) was recorded in the physiological  $[\text{Ca}^{2+}]_o$  of 2.0 mM (Fig 1A, C). With holding potential of -80 mV, depolarization beyond -30 mV elicited an inward current, peak-

ed at +10 mV and reversed between +50 mV and +60 mV. The current amplitude was relatively small and peak amplitude varied between 30 and 90 pA (mean  $-58.0 \pm 13.4 \text{ pA}$ ,  $n=7$ ).

Replacement of 2.0 mM  $\text{Ca}^{2+}$  with 10 mM  $\text{Ba}^{2+}$  increased the current amplitudes about 3.9-folds (mean  $-229.7 \pm 42.6 \text{ pA}$ ,  $n=15$ ). The  $I_{\text{Ba}}$  exhibited slower inactivation time course than the  $I_{\text{Ca}}$  and did not completely inactivated during 400 ms step pulses. With holding potential of -80 mV, depolarization beyond -40 mV elicited an inward current, peaked at +10 mV and reversed between +60 mV and +70 mV (Fig 1B, C). Thus the I-V relation was not changed by replacement of  $\text{Ca}^{2+}$  with  $\text{Ba}^{2+}$ .

Current-voltage relations of both currents never showed inflexions or 'hump' in the negative potential range such as might result from activation of a separate population of T-type Ca-channels.

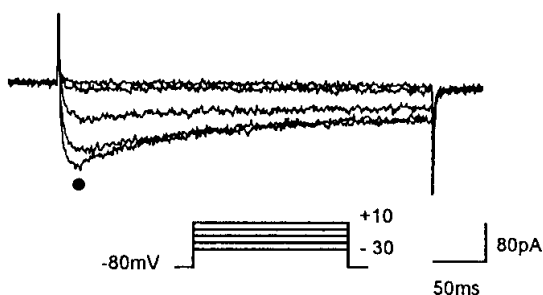
### 3) Effect of holding potentials

Several investigators have been able to separate two types of calcium currents (T- and L-type) in vascular smooth muscle by changing the holding potentials (Yatani *et al.*, 1987; Benham *et al.*, 1987; Bean, 1989; Smirnov and Aaronson, 1992). In those studies, a holding potential of -40 or -30 mV was sufficient to completely inactivate T-type calcium current. Therefore, the effect of changing the holding potential from -80 to -40 mV was also investigated, since the less negative holding potential would be expected to lead to relatively more inactivation of T-type channels, and so to less transient current on depolarization (Fig 2). The represen-

A.  $\text{Ca}^{2+}$  2mM



B.  $\text{Ba}^{2+}$  10mM



C.

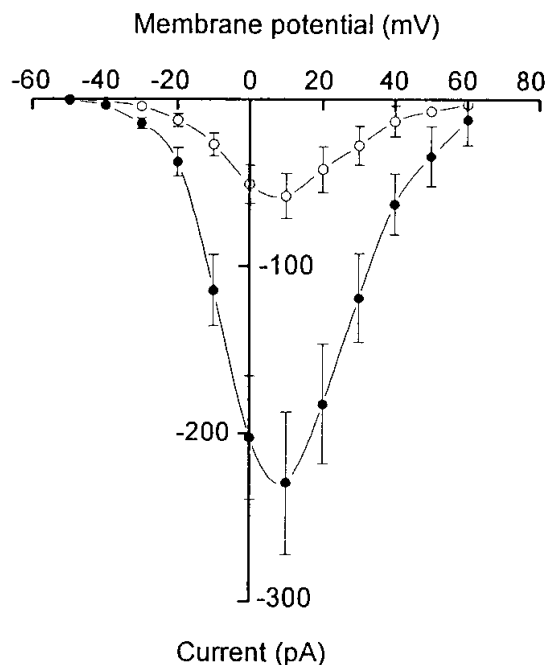


Fig. 1. The ionic permeation properties of  $I_{\text{Ca}}$ . Inward Ca-channel currents were recorded in the presence of external 2 mM  $\text{Ca}^{2+}$  (panel A; open circles in panel C, mean  $\pm$  SEM,  $n = 7$ ) or 10 mM  $\text{Ba}^{2+}$  (panel B; filled circles in panel C, mean  $\pm$  SEM,  $n = 15$ ). The inward current was increased about 3.9 folds by replacement of external  $\text{Ca}^{2+}$  with  $\text{Ba}^{2+}$ . I-V relationship was not changed and peaked at about +10 mV (panel C).

tative current trace of  $I_{\text{Ba}}$  was illustrated in Fig 2A ( $V_h = -80$  mV) and 2B ( $V_h = -40$  mV). Although  $I_{\text{Ca}}$  (squares) and  $I_{\text{Ba}}$  (circles) (Fig 2C) amplitudes were decreased with holding potential of -40 mV (filled squares or circles), current-voltage relations of both current are similar in shape: the thresholds of current activation were between -30 and -20 mV, peaked at +10 mV in both cases. Digital subtraction of the currents activated from holding potential of -40 mV from the currents activated from holding potential of -80 mV did not reveal a rapidly inactivating component at any of the test potentials.

4) Steady-state voltage dependence of  $I_{\text{Ca}}$

(1) Steady-state activation

Steady-state activation ( $d_{\infty \text{act}}$ ) curves were

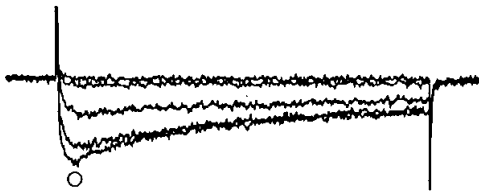
determined from the I-V relationship (Klockner and Isenberg, 1985) by using Eq. 1 (Fig 3; upper panel, circles):

$$I_{\text{Ca}} = g_{\text{Ca}} \times (V - E_{\text{rev}}) \quad (\text{Eq. 1})$$

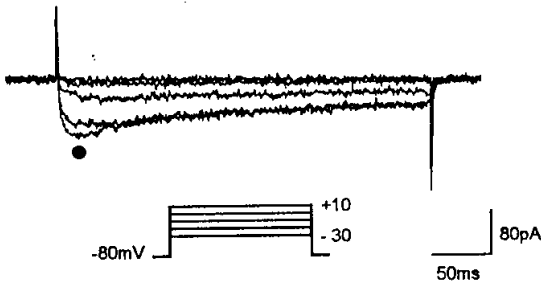
where  $I_{\text{Ca}}$  is the peak amplitudes of the whole-cell current,  $g_{\text{Ca}}$  is the conductance,  $V$  is the test potential and  $E_{\text{rev}}$  is the reversal potential of the Ca current. Apparent reversal potentials, between +60 and +70 mV, obtained from the reversal point of the I-V curves were used because of the precise concentration of  $[\text{Ca}^{2+}]_i$  and  $[\text{Ba}^{2+}]_i$  was not known. Within a limited range, the obtained values of the reversal potential had a negligible effect on the parameters calculated.

The continuous line through the data was

A.  $V_h = -80\text{mV}$



B.  $V_h = -40\text{mV}$



C.

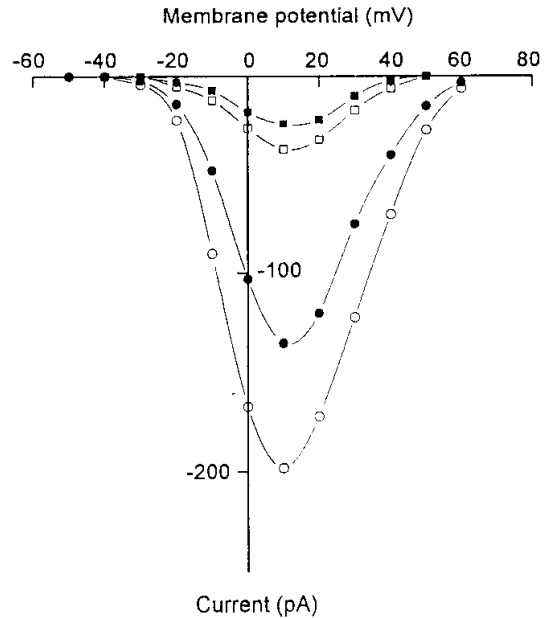


Fig. 2. The effect of holding potentials on calcium channel current.  $I_{Ba}$  was recorded from holding potential of  $-80\text{ mV}$  (panel A) and  $-40\text{ mV}$  (panel B) at the test potentials indicated. Changing the holding potential from  $-80\text{ mV}$  to  $-40\text{ mV}$  decreased the current amplitude but did not shift the voltage dependence (panel C). No detectable low-threshold, rapidly inactivating current (T-type) was observed. Circles,  $I_{Ba}$ ; Squares,  $I_{Ca}$ ; Open circles or squares,  $V_h = -80\text{ mV}$ ; Filled circles or squares,  $V_h = -40\text{ mV}$

drawn using a Boltzmann equation (Eq. 2)

$$d_{\infty \text{act}} = 1 / (1 + \exp((V_t - V_{1/2}) / k_1)) \quad (\text{Eq. 2})$$

where  $d_{\infty \text{act}}$  is the steady-state activation gating variables,  $V_t$  is the test potential,  $V_{1/2}$  is the half activation potential and  $k_1$  is the slope factor of the curve.  $V_{1/2}$  are  $-4.42\text{ mV}$  and  $-7.48\text{ mV}$ , and  $k_1$  are  $-6.47\text{ mV}$  and  $-5.72\text{ mV}$  for  $I_{Ca}$  (solid line) and  $I_{Ba}$  (dotted line), respectively.

(2) Steady-state inactivation

A modified double-pulse protocol was used to measure the steady-state inactivation ( $f_{\infty \text{inact}}$ ) of  $I_{Ca}$  and  $I_{Ba}$  as a function of membrane potentials (Fig. 3; upper panel, squares). Pre-pulse potential ranging from  $-80$  to  $+60\text{ mV}$  were applied for a duration of  $2\text{ s}$ . Following a  $10\text{ ms}$  inter-

pulse interval at a potential of  $-80\text{ mV}$ , the membrane potential was raised to a test potential of  $+10\text{ mV}$  for  $400\text{ ms}$ . This current was then normalized to the largest current ( $I/I_{\text{max}}$ ) and plotted for each prepulse potential. Plotted data are well fitted by a Boltzmann equation (Eq. 3),

$$f_{\infty \text{inact}} = 1 / (1 + \exp((V_t - V_{1/2}) / k_2)) \quad (\text{Eq. 3})$$

with a half inactivation voltage ( $V_{1/2}$ ) of  $-22.3\text{ mV}$  and  $-20.5\text{ mV}$ , and slope factor ( $k_2$ ) of  $+6.44\text{ mV}$  and  $+6.40\text{ mV}$  for  $I_{Ca}$  (solid line) and  $I_{Ba}$  (dotted line), respectively.

At pre-pulse potentials positive to  $+20\text{ mV}$ , the steady-state inactivation curves showed tendency to reactivate, thus made a U-shape (data in Fig. 3 were trimmed at  $+20\text{ mV}$ ). Such a U-

shaped inactivation curve is an usual phenomenon for calcium current and has been interpreted to indicate evidence for a Ca-dependent inactivation mechanism (Klockner and Isen-berg, 1985; Ohya *et al.*, 1988). When barium ion is used as a charge carrier, however, the U-shaped curve became less dominant but not completely abolished (data not shown). Therefore this simple interpretation, Ca-dependent inactivation, will have to be retested and further examination is needed.

### 5) Window current of $I_{Ca}$

It is apparent from Fig 3 that when the  $d_{\infty act}$  and  $f_{\infty inact}$  curves superimposed, there is a 'window' of current from -40 to 0 mV where channels are available a 'window' of current from -40 to 0 mV where channels are available for activation but never fully inactivated. To evaluate the potential dependence of such a window current, it was estimated that the theoretical sustained current through the Ca-channels in the normal  $Ca^{2+}$  (2 mM) and  $Ba^{2+}$  (10 mM) solutions, taking into account both the activation and inactivation processes. The expression used was as follows:

$$\text{Window } I_{Ca} = \text{peak } I_{Ca} \times d_{\infty act} \times f_{\infty inact} \quad (\text{Eq. 4})$$

Where  $d_{\infty act}$  is the function of the steady-state activation curve, and  $f_{\infty inact}$  is the function of the steady-state inactivation curve.

The calculated window currents are presented in Fig 3 (lower panel). Although the window current carried by 10 mM  $Ba^{2+}$  is much larger than that carried by 2.0 mM  $Ca^{2+}$ , the potential dependency of current amplitude is similar for both situations. It is also apparent that a sustained inward current, although the amplitudes are very small, increased near exponentially within the physiological membrane potential range (-60 to -40 mV). This predicted window current amplitudes are somewhat similar to the values that are measured at the end of the 2 s depolarizing pulse to +10 mV, which are sustained sustained non-inactivating current during long-pulse duration: 2 to 3 pA for  $I_{Ca}$ , and 15 to 20 pA for  $I_{Ba}$ .

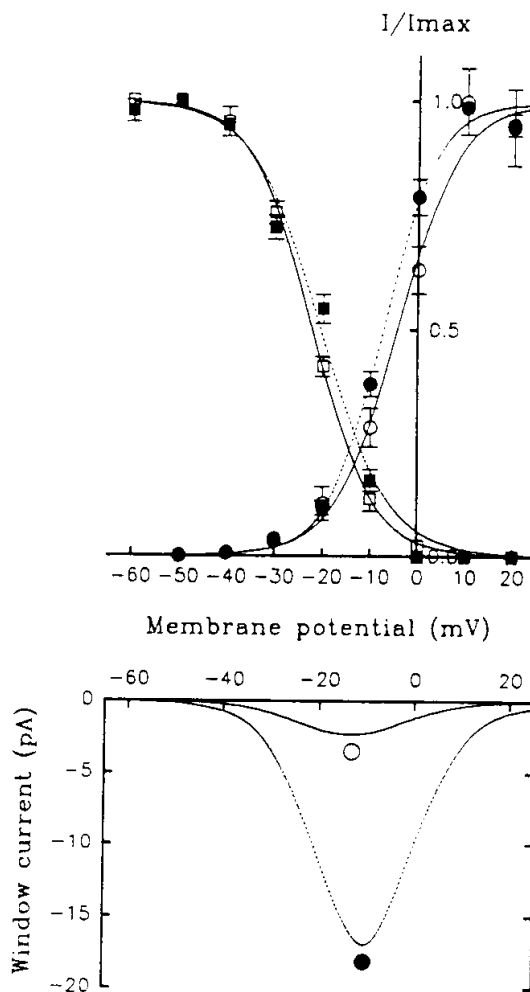


Fig. 3. Steady-state voltage dependence of Ca-current ( $I_{Ca}$ ). Upper panel: Steady-state activation and inactivation curves for  $I_{Ca}$  (open circles or squares; mean  $\pm$  SEM,  $n = 5$ ) and  $I_{Ba}$  (filled circles or squares; mean  $\pm$  SEM,  $n = 6$ ) were drawn. For  $I_{Ca}$ :  $V_{1/2(act)} = -4.4$  mV,  $V_{1/2(inact)} = -22.3$  mV. For  $I_{Ba}$ :  $V_{1/2(act)} = -7.5$  mV,  $V_{1/2(inact)} = -20.5$  mV. Lower panel: 'Window current' amplitude was calculated from the peak Ca-current, activation and inactivation curves for  $I_{Ca}$  (solid line) and  $I_{Ba}$  (dotted line) using Eq. 4.

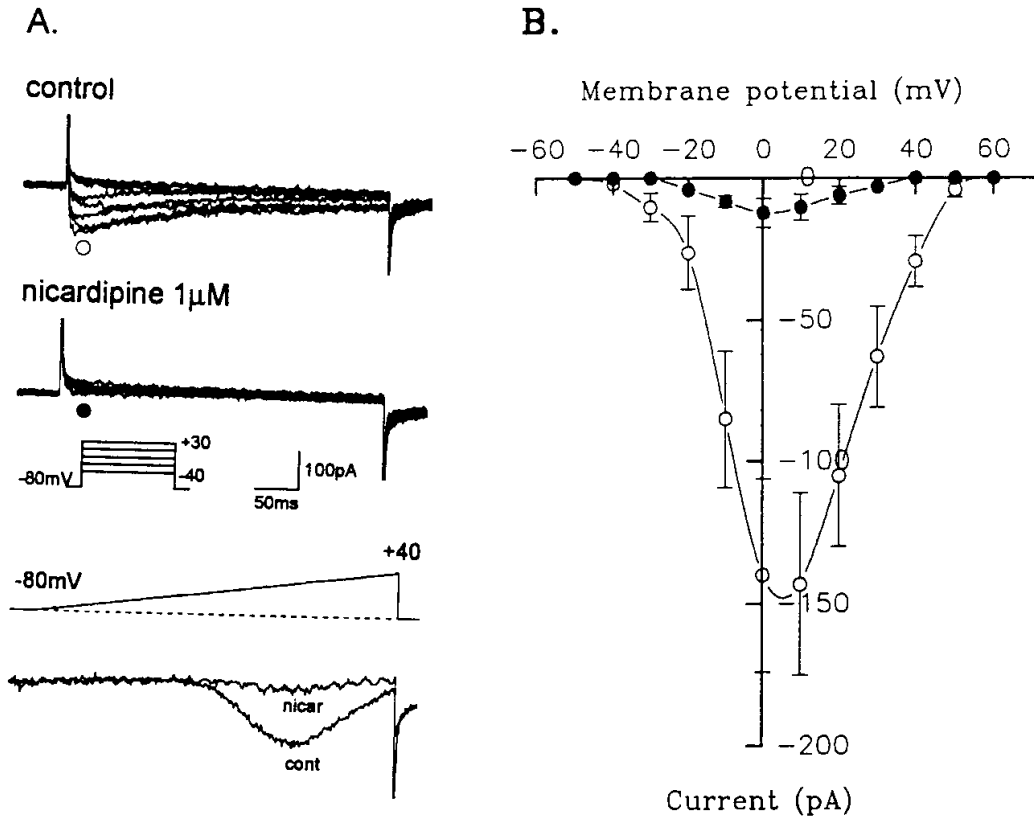


Fig. 4. Effect of nicardipine on Ca-current. Panel A: Nicardipine (1  $\mu$ M), a dihydropyridine L-type Ca-channel blocker, almost completely blocked the  $I_{Ba}$ . Calcium channel current was elicited by step-pulses (upper traces) or ramp depolarization (lower trace) Panel B: Current amplitude was markedly decreased by nicardipine without marked change of current-voltage (I-V) relationship (mean  $\pm$  SEM, n = 5).

#### 6) Pharmacology of $I_{Ca}$

The effect of dihydropyridine Ca-channel blocker, nicardipine, was tested. Representative traces and mean I-V relations are shown in Fig 4. One micromolar nicardipine markedly reduced the  $I_{Ba}$  at all test potentials. I-V relations before and after the treatment of nicardipine was not changed.

The effect of dihydropyridine Ca-channel agonist, Bay K 8644, was also tested in barium solution. Fig 5 shows representative traces in one cell. Bay K 8644 (100 nM) largely increased the current amplitude and the peak of the I-V

curve for Bay K 8644, in contrast to nicardipine, was shifted 10 mV in the hyperpolarizing direction (peaked at 0 mV, Fig. 5B). The activation curve was parallel shifted to the negative direction ( $V_{1/2}$  of -4.2 mV and -17.4 mV for control and Bay K 8644, respectively). Agents that shift the activation curve to more negative potentials would increase open probability of the channel (Nelson *et al.*, 1990). Thus the effect of Bay K 8644 is mainly due to an increasing open probability of the Ca-channel (Fig. 5C). Inactivation curve was also slightly shifted to the negative direction (Fig. 5D).



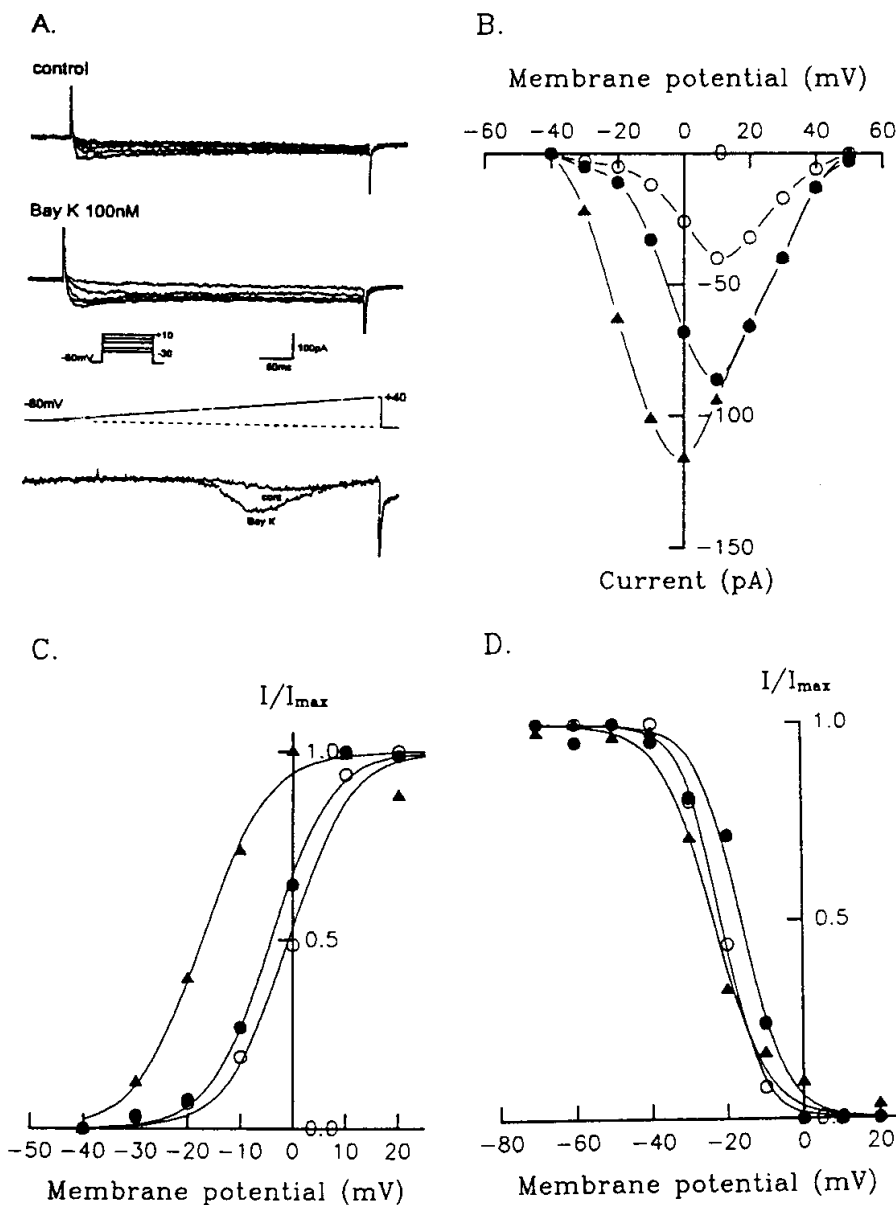
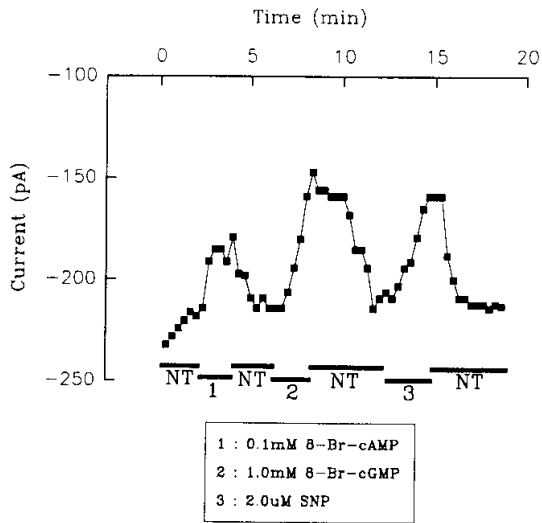


Fig. 5. Effect of Bay K 8644 on Ca-current. Panel A: Bay K 8644 (100 nM), a dihydropyridine L-type calcium channel agonist, enhanced the barium current amplitude. Panel B: Bay K 8644 shifted the I-V relationship to the negative direction and I-V relationship peaked at 0 mV. Currents were recorded in same cell; 2 mM [Ca<sup>2+</sup>]<sub>o</sub> without Bay K (open circles), 10 mM [Ba<sup>2+</sup>]<sub>o</sub> without Bay K 8644 (filled circles), 10 mM [Ba<sup>2+</sup>]<sub>o</sub> with 100 nM of Bay K (filled triangles). Panel C: Steady-state activation curve was also left-shifted. Half activation potential ( $V_{1/2(act)}$ ) was changed from -4.2 mV to -17.5 mV by Bay K 8644. Panel D: Steady-state inactivation curve was also slightly changed. Half inactivation potential ( $V_{1/2(inact)}$ ) was changed from -16.4 mV to -23.7 mV



**Fig. 6.** Effect of cyclic nucleotides on Ca-current ( $I_{Ca}$ ). Membrane permeable cyclic nucleotides, 8-bromo cAMP and 8-bromo cGMP, decreased the  $I_{Ca}$ , in a reversible manner. Step pulses to +10 mV with holding potential of -80 mV were repeated every 20 s and the peak current's amplitudes were plotted against time. Sodium nitroprusside (2  $\mu$ M), a known activator of guanylate cyclase, also decreased the current amplitude in a similar manner.

In other experiments, modulatory effect of several drugs on  $I_{Ca}$  was tested (data not shown). Effect of isoproterenol (0.2~10  $\mu$ M) was somewhat variable from cell to cell, in 3 of 10 cells  $I_{Ca}$  was decreased, in 4 of 10 cells the current was increased by isoproterenol and in remainder cells showed no effect. Serotonin (1  $\mu$ M) increased the current and I-V relations was shifted to the negative potential (10 mV), thus the effect was similar to Ca-channel agonist Bay K 8644. Sodium nitroprusside (2  $\mu$ M) decreased the current about 40% and the action was rapid and reversible. Sodium nitroprusside did not shift the I-V relations.

These effect of the drugs suggest that intracel-

lular second-messenger systems, especially cyclic nucleotides, can modulate the channel current. Therefore direct effect of the cyclic nucleotides on the Ca-channel current was tested. Membrane-permeable cyclic nucleotides, 8-bromo-cAMP and 8-bromo-cGMP, were applied to the cells. 8-Bromo-cAMP (0.1 to 1 mM) and 8-bromo-cGMP (0.1 to 1 mM) reduced the Ca-channel current (Fig. 6). Reducing effect of the 8-bromo-cGMP was more potent than that of the 8-bromo-cAMP. Two micromolar sodium nitroprusside, which is known as an activator of guanylate cyclase, mimicked the effect of 8-bromo-cGMP. Before the prominent run-down occurred the inhibitory effect of these two nucleotides was potent.

## DISCUSSION

### Passive membrane properties

Cell-length (< 150  $\mu$ m long, 10  $\mu$ m wide) and membrane capacitance ( $19.2 \pm 0.7$  pF) measured in this experiments were similar to that of the rat basilar (Stockbridge *et al.*, 1992; Langton and Standen, 1993) and rabbit basilar (Worley *et al.*, 1991), guinea-pig basilar (West *et al.*, 1992), and cat cerebral (Bonnet *et al.*, 1991) arterial smooth muscle cells. Many investigators reported that the membrane potentials obtained from enzymatically dispersed vascular smooth muscle cells were ranged from -25 to -65 mV, and exhibit marked variation among vascular beds and species: -32 mV for rabbit coronary artery (Matsuda *et al.*, 1990), -48 mV for rabbit portal vein (Hume and Leblanc, 1989), -52 mV for canine renal artery (Gebland and Hume, 1992), -36 mV for human cystic artery (Akbarali *et al.*, 1992). In the present experiment, observed membrane potential was  $-29 \pm 1.2$  mV. These values are somewhat lower than measurements in tissue strips as mentioned above. This discrepancy, however, is not fully understood yet. One possible explanation is due to the alterations in resting conductance associated with cell disaggregation (Fleischmann *et al.*, 1993), or the lack of an inward-rectifying potassium current which is the major determinant of the negative resting potential in cardiac cells (Matsuda *et al.*, 1990).

Smooth muscle cells in arteries measured by intracellular recordings in vitro, have stable membrane potentials between -60 and -75 mV (Hirst and Edward, 1989; Brayden, 1990). Membrane potentials measured in vivo are in the range of -40 to -55 mV (Nelson *et al.*, 1990).

### Calcium current

The existence of two types (T- and L-type) of voltage-dependent calcium currents has been described in a wide variety of vascular smooth muscle cells (Arronson *et al.*, 1988; Bean, 1989; McDonald *et al.*, 1994). These currents are distinguished by their differences in activation threshold, inactivation potential dependence, kinetics of inactivation, single channel recording of conductance, and pharmacological sensitivities.

It is well known that the L-type calcium channel is more permeable to  $Ba^{2+}$  and  $Sr^{2+}$  than  $Ca^{2+}$  whereas the T-type current has an equal permeability to both  $Ca^{2+}$  and  $Ba^{2+}$ . In the present studies, in 2 mM  $[Ca^{2+}]_o$  and 10 mM  $[Ba^{2+}]_o$ ,  $I_{Ba}$  amplitude was 3.9-fold larger than  $I_{Ca}$ . The separation of vascular smooth muscle T- and L-type calcium currents can be achieved by eliciting test pulses from two different holding potentials (McDonald *et al.*, 1994). In the present studies, the subtracted current traces from two different holding potentials (-80 and -40 mV) did not reveal a fast transient component. Furthermore, in both  $Ca^{2+}$  and  $Ba^{2+}$  solutions, the I-V relationship did not exhibit a 'hump' along the negative slope region due to the sum of two current components. These results indicate that rabbit basilar artery smooth muscle cells have an only one type of calcium current (L-type).

However, it was also reported that two types of dihydropyridine-sensitive Ca-channels were present in rabbit basilar artery. In addition to the most common conductance level (~24 pS) observed for dihydropyridine-sensitive Ca-channel (L-type Ca-channel), small conductance (~12 pS) Ca-channel which was also DHP-sensitive was recorded in rabbit basilar artery by using cell-attached patch-clamp (Oike *et al.*, 1990; Worley *et al.*, 1991). These two Ca-channels have a similar voltage dependency and pharmacological characteristics, thus it was difficult

to discriminate these two channels by using whole-cell recordings (Oike *et al.*, 1990). It remains to be seen whether the two different conductance level channels subserve different physiological functions.

The sensitivity of L-type Ca-current and the relative insensitivity of T-type Ca-current to dihydropyridine Ca-channel agonists and antagonists have been well described (Bean *et al.*, 1986; Bean, 1989). Consistent these previous reports in smooth muscle cells, the dihydropyridine Ca-channel antagonist nicardipine almost completely abolished the current in rabbit basilar artery. Dihydropyridine Ca-channel agonist Bay K 8644 increased the current and shifted the I-V relations and activation curve to more hyperpolarized potentials. In physiological states, membrane depolarization beyond -40 mV would not be the most effective method to increase  $P_{open}$ , since inactivation and decrease in driving force would tend to offset increase in activation. However agent that shifts the activation curve to more negative potentials (eq. Bay K 8644, serotonin, epinephrine) would increase  $P_{open}$  (Nelson *et al.*, 1990).

Since arterial smooth muscles are usually non-excitable, and do not always depolarize markedly during E-C coupling, it is clear that modulation of  $Ca^{2+}$  influx near the in vivo resting membrane potential may be of great physiological importance. Smooth muscle cells in arteries and arterioles, in vitro, have stable membrane potentials between -60 and -75 mV (Hirst and Edward, 1989). Membrane potentials measured in vivo (Nelson *et al.*, 1990) are in the range of -40 to -55 mV. The membrane potential of arterial smooth muscle cells in vivo (-40 to -55 mV) therefore falls in the range in which the current through Ca-channels is strongly voltage dependent. This Ca-channel current could thus have physiological relevance to tone maintenance. When cerebral arteries are subjected in vitro to physiological transmural pressure, they depolarized to between -40 and -55 mV and develop tone (Harder, 1984; Brayden and Wellman, 1989). It should be stress that  $P_{open}$  of the channel is a continuous function of membrane potential, with no threshold; in other words, a Ca-channel al-

ways has a finite  $P_{open}$ . Thus apparent "threshold" for a whole cell Ca-currents are simply detection thresholds. In rabbit basilar artery SMCs, small window current was predicted even at a membrane potential of -50 mV, which is clearly seen in the presence of 10 mM  $Ba^{2+}$  (see Fig. 3). Although this window current amplitudes seem to be finite, this amount would be enough to raise intracellular calcium concentration at physiological states.  $Ca^{2+}$  influx through even a small number of Ca-channels is sufficient to lead to significant changes in intracellular free  $Ca^{2+}$  concentration, because of the small volume of arterial smooth muscle cells (~1 pl). Thus  $Ca^{2+}$  entry through Ca-channels over the physiological range of membrane potentials could supply smooth muscle cells with sufficient  $Ca^{2+}$  to maintain steady contraction (Nelson *et al.*, 1990; McDonald *et al.*, 1994). The significance of the window current for E-C coupling is suggested by the fact that it is predicted to change by an approximately e-fold factor with a 10 mV shift of the membrane potential from -60 mV in either direction (Nelson *et al.*, 1990).

Regulation of function through the modulation of voltage-dependent Ca channels by neurotransmitters and neuropeptides appears to be a widespread phenomenon. Only recently have direct demonstrations of modulation of arterial smooth muscle Ca-channels been provided. In cardiac myocytes, it has been well established that  $\beta$ -adrenergic stimulation increases the amplitudes of the L-type Ca-channel current by activating adenylate cyclase to produce cAMP, which in turn stimulates cAMP-dependent phosphorylation of the channel or a channel subunit (Trautwein and Hescheler, 1990). In contrast, there is still considerable controversy concerning the effect of  $\beta$ -adrenergic stimulation and cAMP on calcium current in vascular smooth muscle cells. Although tissue diversity may be partially responsible for some of the reported differences in  $\beta$ -adrenergic modulation, some of these differences may be related to experimental conditions (Ishikawa *et al.*, 1993). In this study from rabbit basilar artery, both cAMP and cGMP decreased the Ca-current. The effect of cGMP was mimicked by sodium nitroprusside that can raise

the intracellular concentration of cGMP. Therefore in rabbit basilar artery it is suggested that cyclic nucleotide-mediated 2nd messenger systems can modulate the calcium channels.

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