Mechanisms of the Inhibitory Action of Semotiadil Fumarate, a Novel Ca Antagonist, on the Voltage-Dependent Ca Current in Smooth Muscle Cells of the Rabbit Portal Vein

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ABSTRACT—Effects of semotiadil on the voltage-dependent Ca current (I_{ca}) were investigated in dispersed smooth muscle cells of the rabbit portal vein. At a holding potential of −100 mV, semotiadil (≥0.1 μM; dissolved in dimethylsulphoxide, DMSO) inhibited the I_{ca} in a concentration-dependent manner (IC_{50}=2.0 μM, Hill’s coefficient = 1.0). At a holding potential of −80 mV or −60 mV, the concentration-inhibition curve observed in the presence of semotiadil was shifted to the left compared with that observed at −100 mV; and semotiadil shifted the voltage-dependent inactivation curve to the left. The curve for the decay of I_{ca} was fitted with two time constants. Semotiadil (<1 μM) reduced the slow but not the fast time constant. The curve for the recovery from I_{ca} inactivation also consisted of two time constants, and semotiadil (1 μM) prolonged the slow recovery. Semotiadil dissolved in deionized water more potently inhibited I_{ca} than semotiadil dissolved in DMSO. At pH 10.0, semotiadil did not modify the voltage-dependent inactivation curve. However, recovery from the inactivation was much faster at pH 10.0 than at pH 7.3. These results indicate that the voltage-dependent inhibition of I_{ca} by semotiadil may be due to binding of the ionized drug during the inactivated state and also inhibition of the transition from the inactivated to the resting state. Long-lasting inhibition of I_{ca} after removal of semotiadil may be due to tight binding of semotiadil on the channel through a hydrophobic site.

Keywords: Portal vein, Ca current, Ca antagonist, Vasodilatation

Semotiadil fumarate, (+)-3,4-dihydro-2-[5-methoxy-2-[3-[N-methyl-N-[2-[3,4-methylenedioxy) phenoxy]ethyl]amino]propoxy]phenyl]-4-methyl-3-oxo-2H-1,4-benzothiazine hydrogen fumarate (SD3211) is a non-dihydropyridine (DHP) type Ca antagonist that binds to a different site of the Ca channel from other Ca antagonists (1, 2). This drug had a long-lasting inhibitory action on the Ca-induced contraction in depolarized smooth muscle tissues of the pig coronary artery, and this inhibitory action was antagonized by an increase in extracellular concentrations of Ca, while this drug had no effect on the A23187-induced contraction. Thus, Nishimura et al. (1) estimated that this drug acts on the voltage-dependent Ca channel of smooth muscle cells. It is well known that both DHP and non-DHP types of Ca antagonists inhibit voltage-dependent Ca channels in a voltage-dependent manner. However, mechanisms of voltage-dependent inhibition induced by these Ca antagonists might differ. For example, DHP derivatives are thought to bind to the channel mainly in an inactivated state and are known to have both agonistic and antagonistic actions on the Ca channels in smooth muscle cells (3, 4). On the other hand, non-DHP type Ca antagonists, such as diltiazem, verapamil and flunarizine, are known to block Ca channels in the open state as well as in the resting and inactivated states, and their frequency- and voltage-dependent block of Ca channels is thought to be due to their open and inactivated channel blocking actions. Hescheler et al. (5) reported that in cardiac cells, an ionized form of D600 blocks the open Ca channel from the inside of the cell. However, Kass et al. (6), using amlodipine, an ionized DHP-derivative, postulated that the ionized form of amlodipine acts from the outside of the membrane, because intracellular perfusion of the drug did not inhibit the Ca current. In smooth muscle cells of the rabbit ileum and portal vein, Ca antagonists including D600 are believed to block Ca channels from the outside of the membrane (7, 8). These results may indicate that different Ca an-
agonists act on different kinds of Ca channels from different pathways and in different manners.

Recently, Kass et al. (6) reported that the neutral form of amlodipine produces a voltage-dependent tonic block and the ionized form produces an use-dependent block of Ca channels in the guinea pig ventricular cell. Furthermore, they reported that recovery from the inactivation is incomplete in the presence of the ionized form, but complete in the presence of the neutral form (holding potential, $-40 \text{ mV}$), suggesting that different mechanisms of inhibition on the channel may occur with different ionization states of the drug. These results coincide well with those reported by Uehara and Hume (9). On the other hand, Xiong et al. (10) reported that, in the rabbit mesenteric artery, changes in pH have no effect on the inhibitory actions on Ba current observed in the presence of a diltiazem derivative. Semotiadil has a dissociation constant (pKa) of 9.03, and most of the drug molecules are distributed as the ionized form at pH 7.3. Therefore, investigations of the actions of semotiadil in relation to the ionized forms of the drug may be informative to understand the inhibitory action of Ca antagonists on the Ca current in vascular smooth muscle cells. Until now, the action of semotiadil, a benzothiazine type Ca antagonist, on $I_{\text{Ca}}$ has not been investigated in detail. I intended to elucidate the mechanisms of the inhibitory action of semotiadil on $I_{\text{Ca}}$ in smooth muscle cells of the rabbit portal vein.

**MATERIALS AND METHODS**

Male albino rabbits (Nippon White, 1.8–2.0 kg) were anesthetized with sodium pentobarbitone (40 mg/kg, i.v.; Pitman-Moore Co., Indianapolis, IN, USA). The portal vein was dissected out and the surrounding connective tissue was carefully removed. The tissue was then cut into small pieces (0.5 x 2 mm) with fine scissors in fresh physiological salt solution (PSS).

**Preparation of single smooth muscle cells**

Dispersion of the smooth muscle cells was similar to the method described by Momose and Gomi (11) and Inoue et al. (12). In brief, small pieces of the portal vein (0.5 x 1–2 mm) were incubated in nominal Ca-free PSS with 0.1% collagengase (Wako Pure Chem., Osaka), 0.1% trypsin inhibitor (type IIa; Sigma Chem., St. Louis, MO, USA) and 0.3% bovine serum albumin (essentially fatty acid free; Sigma Chem.) for 30 min. The tissue was gently agitated with a blunt-tipped glass pipette until the solution became cloudy, and then the debris was removed with fine nylon mesh (200 x 200 pm) and centrifuged at 1000 rpm for 1 min. The pellet was then re-suspended in a stock solution (0.5 mM Mg – 0.5 mM Ca solution) containing 0.1% trypsin inhibitor and 0.3% bovine serum albumin. The cell suspension was stored in ice-cold water.

**Electrical recording**

Recordings of the membrane currents were similar to those described by Hamill et al. (13). One drop of the cell suspension was added to a small chamber (0.4 ml in volume) placed on the stage of a differential interference inverted microscope (TMD-Diaphoto; Nikon Co., Tokyo). Patch electrodes (2–3 MΩ) were prepared with an electrode puller and heat polisher (PP-83 and MF-83; Narishige Sci. Inst. Lab., Tokyo) and were manipulated by a three-dimensional oil-driven micromanipulator (MO-102; Narishige Sci. Inst. Lab.) or electric-driven micromanipulator (Leitz Wetzler GmbH, Wetzler, FRG). A high resistance seal (> 50 GΩ) was obtained by application of negative pressure (10–30 cmH₂O), and the patch membrane was then ruptured by further application of negative pressure (30–50 cmH₂O) as described by Hamill et al. (13).

For recording an isolated Ca current, the pipette was filled with a high CsCl solution containing 4 mM EGTA (ethyleneglycol-bis (b-aminoethylether)-N,N,N,N'-tetra-acetic acid) and the bath was perfused with PSS. Ca current was monitored on a high-gain digital oscilloscope and a conventional thermo-writing pen recorder (VC-10 and RJG-4124; Nihon Kohden, Tokyo) through a patch clamp amplifier (CEZ-2200; Nihon Kohden and Axopatch-1D; Axon Instrum., Foster, CA, USA), and the data were stored on a video-cassette tape through a PCM data recording system (DC-3 kHz; F-51, National Co., Tokyo and PCM-501E; Sony Co., Tokyo). Capacitative current was minimized using capacitative and Rs compensation. Subtraction of capacitative and leak currents was made by the “P/n” method (14) for measurement of the amplitude of the isolated Ca inward current. A short rectangular square pulse was applied through an electronic stimulator (SEN-7203; Nihon Kohden) to evoke Ca current. The current was analyzed with “pCLAMP” software (Axon Instrum.) through an analogue-to-digital converter (TL-1; Axon Instrum.) and a personal computer (AMSC-ATC, Alps Elect. Co., Nagoya). A hard copy of the current trace was obtained by a laser printer (L880S; Kyocera Co., Tokyo).

The solution was continuously perfused through the chamber at a flow rate of 3 ml/min, and the over-flow was siphoned off by means of a water pump. Exchange of the solution in the experimental chamber took 0.5 min. In several experiments similar to those shown in Fig. 4B, semotiadil was rapidly applied (within hundreds of msec) using principally the same device described by Yellen (15). All experiments were performed at room temperature.
**Solutions**

Solutions used in the present experiments were PSS and high CsCl solution. The ionic composition of PSS was: 135 mM NaCl, 6 mM KCl, 2.5 mM CaCl₂ and 12 mM glucose. The ionic composition of high CsCl solution was: 110 mM CsCl, 20 mM TEACl, 5 mM MgCl₂, 12 mM glucose, 5 mM Na₂ATP (adenosine triphosphate disodium) and 4 mM EGTA. The pH of the solutions was kept at 7.25 ± 0.05 by addition of 10 mM N-(2-hydroxyethyl)piperazine-N-2-ethansulfonic acid (HEPES; Dojin Kagaku, Kumamoto), titrated with Tris(hydroxymethyl)-aminomethane (Tris; Sigma Chem.).

For cell dispersion and cell storage, nominally Ca-free and 0.5 mM Mg–0.5 mM Ca solutions, respectively, were used, with the following ionic compositions: (nominally Ca-free solution) 138 mM NaCl, 6 mM KCl, 12 mM glucose and 5 mM HEPES; and (0.5 mM Mg–0.5 mM Ca solution) 136 mM NaCl, 6 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 12 mM glucose and 5 mM HEPES.

**Drugs**

Drugs used in the present experiments were semotiadil fumarate (pKa=9.03; Santen Pharmac. Co., Osaka). Semotiadil (100 mM) was dissolved in dimethylsulphoxide (DMSO) and diluted in PSS to the desired final concentration. In some experiments, the drug (0.01–10 μM) was directly dissolved in PSS. The pH values in the presence of drug were readjusted just before application. The final concentration of DMSO was less than 0.01%, and this concentration did not affect I_{Ca}.
Statistics

Statistical significance was estimated by Student's t-test, and P-values less than 0.01 were considered to indicate a statistically significant difference.

RESULTS

Effects of semotiadil on $I_{\text{Ca}}$ in the smooth muscle cells of rabbit portal vein

Figure 1Aa shows the current-voltage relationship in the presence or absence of semotiadil obtained at the holding potential of $-100 \text{ mV}$ (100 msec pulse duration). Semotiadil (1 $\mu$M) reduced the amplitude of $I_{\text{Ca}}$ and showed a voltage-dependent inhibition of the current at the holding potential of $-100 \text{ mV}$ (Fig. 1, A and B). When $I_{\text{Ca}}$ recorded before and during application of semotiadil were superimposed after matching their peak heights, the two currents overlapped completely at any given depolarizing pulse less positive than $+10 \text{ mV}$ (Fig. 1Ac). Figure 1C shows the relationships between the relative amplitude of $I_{\text{Ca}}$ and concentrations of semotiadil at three different holding potentials. For these experiments, $I_{\text{Ca}}$ was evoked by a depolarizing pulse of 0 mV applied every 30 sec, and the amplitude of $I_{\text{Ca}}$ before application of semotiadil was normalized as 1.0. At the holding potential of $-100 \text{ mV}$, semotiadil (> 30 nM) inhibited $I_{\text{Ca}}$ in a concentration-dependent manner, and 100 $\mu$M semotiadil completely inhibited $I_{\text{Ca}}$ ($IC_{50}=2.0 \mu\text{M}$). This concentration-response relationship observed at the holding potential of $-100 \text{ mV}$ was shifted to the left by elevation of the holding potential to $-80 \text{ mV}$ or $-60 \text{ mV}$ ($IC_{50}=0.90 \mu\text{M}$ at the holding potential of $-80 \text{ mV}$ and 84 nM at $-60 \text{ mV}$).

$I_{\text{Ca}}$ evoked by a long depolarizing pulse (> 15 sec) to 0 mV from the holding potential of $-100 \text{ mV}$ in PSS declined with two different time constants (fast component, 62.2±8.4 msec; slow component, 2.41±0.28 sec, n = 18), and the $I_{\text{Ca}}$ ceased within 15 sec (Fig. 2A). Application of semotiadil (1 $\mu$M) inhibited the amplitude of $I_{\text{Ca}}$ (Fig. 2Ba), and it sped the decay of the slow component of current inactivation. However, semotiadil (< 1 $\mu$M) did not change the decay of the fast component (Fig. 2Bb). Table 1 summarizes the effects of semotiadil on the amplitude (a) and the time constant (r) of both fast and slow components of $I_{\text{Ca}}$ and its effects on the relative inhibition of $I_{\text{Ca}}$ (semotiadil/control). In the control, the amplitude of the fast component relative to the slow component was not modified by changes in the amplitude of the membrane depolarization (test pulse), and the time constants of both fast and slow components were reduced in a voltage-dependent manner (from $-20 \text{ mV}$ to 20 mV). Application of semotiadil (up to 1 $\mu$M) only reduced the time constant of the slow component but not that of the fast component. However, higher concentrations of semotiadil (>3 $\mu$M) reduced the time constants of both components.

Semotiadil produced a slow onset of $I_{\text{Ca}}$ inhibition and voltage-dependent inhibition

Figure 3A shows the time course of development of the current inhibition by 1 $\mu$M semotiadil. The maximum inhibition of $I_{\text{Ca}}$ induced by semotiadil (1 $\mu$M) was obtained at 10 min after application, and the time to reach the half inhibition of $I_{\text{Ca}}$ by semotiadil was about 2 min. As shown in Fig. 3Aa, when a depolarizing pulse was applied after an interval of 4 min in the presence of semotiadil (1 $\mu$M), the amplitude of $I_{\text{Ca}}$ induced by the first resumed stimulation was slightly smaller than that observed before application of semotiadil but consistently larger than that.
Table 1. Effects of semotiadil on the fast and slow components of the amplitude and time constant of decay of $I_{Ca}$ and on relative inhibition of $I_{Ca}$

| Membrane potential (mV) | Control | 0.1 µM | Semotiadil 1 µM | 3 µM |
|------------------------|---------|--------|----------------|------|
| $\tau_{fast}$ (msec)   |         |        |                |      |
| -20                    | 106.2±13.2 | 106.1±19.8 | 104.0±20.2 | 79.1±11.0* |
| -10                    | 85.1±8.4   | 85.7±9.0  | 80.3±3.1  | 54.4±7.7** |
| 0                      | 62.2±8.4   | 64.6±7.3  | 58.3±7.3  | 48.6±10.1* |
| 10                     | 64.0±10.1  | 60.7±5.6  | 56.0±14.7 | 45.9±5.8*  |
| 20                     | 64.6±3.4   | 59.2±11.0 | 51.1±13.2 | 44.1±3.9** |
| $\tau_{slow}$ (sec)    |         |        |                |      |
| -20                    | 3.23±0.61  | 2.05±0.43* | 1.05±0.34** | 0.386±0.051** |
| -10                    | 2.55±0.54  | 1.80±0.35* | 0.698±0.21** | 0.330±0.070** |
| 0                      | 2.41±0.28  | 1.58±0.10** | 0.671±0.090** | 0.272±0.10** |
| 10                     | 1.90±0.35  | 1.21±0.34* | 0.446±0.27** | 0.219±0.10** |
| 20                     | 1.89±0.53  | 0.488±0.080* | 0.387±0.090* | 0.301±0.11* |
| $a_{fast}$             |         |        |                |      |
| -20                    | 0.73±0.04  | 0.76±0.05  | 0.71±0.06  | 0.71±0.06  |
| -10                    | 0.82±0.04  | 0.80±0.03  | 0.79±0.04  | 0.77±0.02  |
| 0                      | 0.88±0.05  | 0.83±0.04  | 0.81±0.06  | 0.82±0.03  |
| 10                     | 0.91±0.02  | 0.85±0.04  | 0.82±0.08  | 0.80±0.05* |
| 20                     | 0.90±0.02  | 0.82±0.02* | 0.77±0.05* | 0.77±0.05* |
| $a_{slow}$             |         |        |                |      |
| -20                    | 0.20±0.04  | 0.19±0.04  | 0.24±0.06  | 0.28±0.07** |
| -10                    | 0.03±0.03  | 0.17±0.02  | 0.16±0.05  | 0.20±0.03* |
| 0                      | 0.11±0.03  | 0.13±0.04  | 0.16±0.04  | 0.18±0.04* |
| 10                     | 0.075±0.02 | 0.15±0.04  | 0.19±0.08  | 0.22±0.05** |
| 20                     | 0.11±0.03  | 0.21±0.04  | 0.21±0.05  | 0.27±0.07* |
| Semotiadil/Control     |         |        |                |      |
| -20                    | 0.87±0.08  | 0.71±0.11  | 0.66±0.12** |
| -10                    | 0.88±0.04  | 0.68±0.07  | 0.46±0.03** |
| 0                      | 0.90±0.02  | 0.63±0.03  | 0.46±0.05* |
| 10                     | 0.82±0.03  | 0.58±0.08  | 0.47±0.02* |
| 20                     | 0.78±0.03  | 0.59±0.06  | 0.36±0.07** |

Each parameter was calculated using the following equation: $I_{Ca(t)} = a_f \cdot \exp(-t/\tau_f) + a_s \cdot \exp(-t/\tau_s)$, where $I_{Ca(t)}$ is the relative amplitude of $I_{Ca}$ recorded at time $t$ after application of a depolarizing pulse; and $a_f$, $a_s$, $\tau_f$ and $\tau_s$ are the relative amplitudes and time constants for the fast and slow components in the presence of 0.1 µM, 1 µM and 3 µM semotiadil, respectively. * and ** indicate P values less than 0.01 and 0.001, respectively. Each column represents the mean of 3–18 observations with S.D. The ratio semotiadil/control is the relative inhibition of the peak amplitude of $I_{Ca}$ in the presence of 0.1 µM, 1 µM and 3 µM semotiadil.

recorded at 4 min with continuous application of pulses (Fig. 3Aa, filled squares and filled circles). This suggests that continuous, repetitive stimulation (every 30 sec) accelerated the current inhibition. Removal of semotiadil partly restored the amplitude of $I_{Ca}$. As shown in Fig. 3Aa, the amplitude of an irreversible inhibition of $I_{Ca}$ observed after removal of semotiadil was not dependent upon the number of stimulations. The amplitude of $I_{Ca}$ evoked after 14 min by a single stimulation (open triangle) was slightly smaller than that observed in the absence of the drug and almost the same as that by the first resumed stimulation at 4 min. To investigate whether or not irreversible inhibition following application of semotiadil is voltage-dependent, semotiadil (1 µM) was applied at different membrane potentials (-120 mV, -100 mV, -60 mV and -20 mV) for 4 min. Recovery of the current amplitude evoked by stimulation every 30 sec (depolarization pulse to 0 mV and pulse duration of 100 msec) was measured after removal of the drug at the holding potential of -100 mV (Fig. 3B). When semotiadil (1 µM) was applied at the holding potential of -100 mV, the amplitude of $I_{Ca}$ was inhibited to 0.88±0.053 times the control (n=4; measured before application of semotiadil at the same holding potential). When semotiadil was applied at the membrane potential of -20 mV, the amplitude of $I_{Ca}$ evoked by the first stimulation was inhibited to 0.17±0.012 (n=3) times the control. However, it was restored to 0.74±0.087 (n=3) times the control.
control within 2 min. These results indicate that the voltage-dependent current inhibition induced by semotiadil was easily restored when the membrane was held at \(-100\) mV or more negative potential.

**Effects of semotiadil on the voltage-dependent inactivation**

Voltage-dependent inactivation of the \(I_{Ca}\) was examined before and after application of semotiadil using the experimental protocol shown in Fig. 4. In the absence of semotiadil (control), inactivation of the current occurred at \(-40\) mV or more positive depolarizing pulses (Fig. 4A, open circles). Ten minutes after application of semotiadil (1 \(\mu M\)), the voltage-dependent inactivation curve was shifted to the left, and the amplitude of the remaining \(I_{Ca}\), observed by application of depolarization pulses over 0 mV, was suppressed (Fig. 4A, filled circles). Increase in the duration of conditioning pulse (30 sec) shifted the inactivation curve to the left and reduced the amplitude of the remaining \(I_{Ca}\). The results show that the conditioning pulse to inhibit \(I_{Ca}\) to half \((V_{half})\) was \(-50.6\) mV with a slope factor of 6.0 mV (Fig. 4A, triangles). Further increase in the duration of the conditioning pulse did not change either parameter. In the presence of semotiadil (1 \(\mu M\)), the steady state inactivation curve was estimated from the current inhibition obtained at different holding potentials \((-40\) mV, \(-60\) mV, \(-80\) mV, \(-100\) mV and \(-120\) mV), and inactivation of the current occurred at the holding potential of \(-80\) mV with a \(V_{half}\) of \(-70.6\) mV (Fig. 4A, filled triangles).

To investigate the effects of semotiadil (1 \(\mu M\)) on the voltage-dependent inactivation of the fast component of \(I_{Ca}\), various amplitudes of short conditioning pulse (300 msec duration) and test pulse (0 mV and 100 msec pulse) separated by 20 msec were applied. In the absence of semotiadil, at the end of the conditioning pulse to \(-20\) mV, 0 mV and +20 mV, the amplitude of the fast component was markedly inhibited to 0.04 times, 0.01 times and 0.01 times the control, respectively (calculations were made from the equation introduced in Table 1), while the amplitude of the slow component was decreased to 0.91 times, 0.88 times and 0.85 times the control, respectively (see Table 1). On the other hand, in the presence of semotiadil (1 \(\mu M\)), a part of the slow component was also inactivated within the 300 msec conditioning pulse (0.75 times at \(-20\) mV, 0.64 times at 0 mV and 0.46 times at +20 mV; Table 1). Therefore, to observe the effects of semotiadil on the fast component, the value of the slow component was subtracted from the total \(I_{Ca}\) and normalized in the presence or absence of semotiadil (Fig. 4B). The obtained \(V_{half}\) was \(-33.2\) mV (in the absence of semotiadil) and \(-34.9\) mV (semotiadil 1 \(\mu M\)). These results indicate that semotiadil (1 \(\mu M\)) has almost
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**Fig. 4.** Effects of semotiadil (1 μM) on the voltage-dependent inactivation of $I_{Ca}$. (A) Conditioning pulses of various amplitudes were applied (up to 40 mV, 5 sec duration) before application of the test pulse (to 0 mV, 100 msec duration). The holding potential was kept at −100 mV, and the membrane potential was returned to −100 mV. To minimize the contamination of capacitative surge into $I_{Ca}$, the interval between the conditioning and test pulses was kept at 20 msec. The peak amplitude of $I_{Ca}$ evoked by each test pulse was measured before and after application of semotiadil. The amplitude of $I_{Ca}$ in the absence of semotiadil without application of any conditioning pulse was normalized as 1.0, and the results are expressed relative to this (open circles, in the absence of semotiadil). Filled circles are expressed as the normalized value relative to 1.0, measured without application of the conditioning pulse in the presence of semotiadil. Each symbol indicates the mean of 4–6 observations. Lines were drawn by fitting the data to the following equation using the least squares method, $I'_{Ca}(V) = (I'_{Ca(\text{max})} - C)/(1 + \exp [(V - V_{half})/k])$, where $I'_{Ca(V)}$ and $I'_{Ca(\text{max})}$ are relative amplitudes of $I_{Ca}$ obtained at a conditioning pulse to $V$ mV and that without a conditioning pulse, respectively; $C$ is the sum of the relative amplitude of the non-inactivating component of $I_{Ca}$ obtained after application of the 5 sec conditioning pulse and the reactivated component of $I_{Ca}$ measured after 20 msec interval; $V$ is the amplitude of the conditioning pulse; $V_{half}$ is the amplitude of the conditioning pulse necessary to inhibit to half $I_{Ca}$ evoked by the test pulse; and $k$ is the slope factor for the Boltzmann curve. The following values were used for curve fitting: (control, open circles), $I'_{Ca(\text{max})} = 1$, $V_{half} = -33.1$ mV, $k = 6.0$ mV, $C = 0.19$; (1 μM semotiadil with 5 sec conditioning pulse, filled circles; relative inhibition of $I_{Ca}$ in the presence of 1 μM semotiadil, 0.61 ±0.069), $I'_{Ca(\text{max})} = 1$, $V_{half} = -45.8$ mV, $k = 6.0$ mV, $C = 0.09$; Steady state inactivation curves were plotted. Each value was obtained by changing the holding potential (30 sec) instead of application of a 5 sec conditioning pulse and the reactivated component of $I_{Ca}$ measured after 20 msec interval; $V$ is the amplitude of the conditioning pulse; $V_{half}$ is the amplitude of the conditioning pulse necessary to inhibit to half $I_{Ca}$ evoked by the test pulse; and $k$ is the slope factor for the Boltzmann curve. The following values were used for curve fitting: (control, open circles), $I'_{Ca(\text{max})} = 1$, $V_{half} = -33.1$ mV, $k = 6.0$ mV, $C = 0.19$; (1 μM semotiadil with 5 sec conditioning pulse, filled circles; relative inhibition of $I_{Ca}$ in the presence of 1 μM semotiadil, 0.61 ±0.069), $I'_{Ca(\text{max})} = 1$, $V_{half} = -45.8$ mV, $k = 6.0$ mV, $C = 0.09$; Steady state inactivation curves were plotted. Each value was obtained by changing the holding potential (30 sec) instead of application of a 5 sec conditioning pulse. (control of steady state inactivation, open triangles), $I'_{Ca(\text{max})} = 1$, $V_{half} = -50.6$ mV, $k = 6.0$ mV, $C = 0.00$. (steady state inactivation by 1 μM semotiadil, filled triangles), $I'_{Ca(\text{max})} = 1$, $V_{half} = -70.6$ mV, $k = 6.0$ mV, $C = 0.00$. (B) Voltage-dependent inactivation curves obtained by application of a 300 msec conditioning pulse. To measure the voltage-dependent inactivation of the fast component, the contribution of the relative amplitude of the slow component was subtracted from the total $I_{Ca}$. For the control, it was assumed that 20% of the total amplitude of $I_{Ca}$ was the slow component, and this current was not inactivated within 300 msec ($t_{slow} > 1.8$ sec at conditioning pulse less positive than +20 mV) as estimated from Fig. 2. Therefore, a constant value of 0.2 times the control value (total amplitude) was subtracted. For semotiadil, the relative amplitude of the slow component measured by stimulation with 300 msec pulse duration was calculated from the time constant and relative amplitude of the slow component shown in Table 1. Finally, the relative amplitude of $I_{Ca}$ without application of a conditioning pulse was re-normalized as 1.0. Lines are fitted with the following values for the parameters: (control, open circles), $V_{half} = -33.2$ mV, $k = 6.0$ mV, $C = 0.28$; (1 μM semotiadil, filled circles; relative inhibition of $I_{Ca}$ in the presence of 1 μM semotiadil, 0.61 ±0.015), $V_{half} = -34.9$ mV, $k = 6.0$ mV, $C = 0.12$. 

**A**

- **5 sec**
  - -100 mV
  - 0 mV
  - 20 msec

**B**

- **300 msec**
  - -100 mV
  - 0 mV
  - 20 msec
no effect on the voltage-dependent inactivation of the fast component.

Effects of semotiadil on the recovery of I$_{ca}$ from the inactivation

To study the effects of semotiadil (1 μM) on the recovery process of I$_{ca}$, a pair of pulses separated by various intervals was applied. The amplitude of I$_{ca}$ evoked by a pre-pulse was normalized as 1.0, and the amplitudes of I$_{ca}$ evoked by the test pulse were plotted against the interval. In the absence of semotiadil, the amplitude of I$_{ca}$ was restored with time constants of 100 msec and 4.26 sec, when the I$_{ca}$ was evoked by a test pulse (0 mV, 100 msec duration) preceded by a depolarizing pulse (pre-pulse; 5 sec duration) with various intervals (Fig. 5A, open circles). With a 500 msec interval, 0.7 times the control I$_{ca}$ amplitude was restored and with a 10 sec interval, the amplitude of I$_{ca}$ was completely restored. In the presence of semotiadil (1 μM, filled circles), the time constant of the slow component was prolonged (11.4 sec), but that of the fast component remained unchanged (100 msec). With an interval of 30 sec, the current amplitude was not completely restored (0.88±0.11 times the control evoked by a pre-pulse, n=5) and with a 500 msec interval, 0.16±0.06 times (n=5) the control amplitude was restored. On removal of semotiadil, the slow time constant of the current did not recover. However, the amplitude of I$_{ca}$ was completely restored with an interval of 30 sec (τ$_r$=7.6 sec, filled squares). In the presence of a high concentration of semotiadil (10 μM), both time constants for the recovery were markedly prolonged (τ$_r$=2.9 sec, τ$_r$=43 sec).

Similar experiments were also performed with the use of a short pre-pulse (300 msec duration, Fig. 5B). In the absence of semotiadil, the fast component of I$_{ca}$ evoked by a depolarizing pulse to 0 mV from the holding potential of -100 mV was completely inactivated within 300 msec, but more than 95% of the slow component remained. Therefore, the recovery time course observed by a 300 msec pre-pulse mainly reflects the recovery of the fast component. In the absence of semotiadil, values of the time constants for the fast and slow components of recovery were almost the same as those observed in Fig. 5A (300 msec pre-pulse τ$_f$=100 msec and τ$_r$=4.25 sec); and with a 500 msec interval, more than 0.72 times the control amplitude was restored. Semotiadil (1 μM) reduced the fraction of the fast recovery to 0.32 and prolonged the time constant of the slow component (5.94 sec). These results were the same as those obtained with the 5 sec pre-pulse (Fig. 5A). These results suggest that semotiadil inhibited the recovery process, especially the slow component, from the inactivation of I$_{ca}$. At the holding potential of -80 mV, the time constant of the slow component, but not that of the fast component, was in-
creased (the fast component, 100 msec and the slow component, 33.0 sec). On further shift of the holding potential to the depolarized level of 60 mV, the time constant of the slow component was prolonged (78.6 sec) without affecting the fast component. Semotiadil (1 AM) did not change the time constant of the fast component (100 msec at 80 mV and 60 mV), but markedly prolonged the time constant of the slow component (68.5 sec at -80 mV and 275 sec at -60 mV).

Fig. 6. Effects of pH of the test solution on the relationships between inhibition of ICa and concentration of semotiadil. Semotiadil was dissolved in PSS. (A) The membrane was kept at the holding potential of 100 mV and depolarizing pulses to 0 mV (100 msec duration) were applied. Each symbol indicates the mean value of 3-6 observations which were obtained at pH values of 7.3 (triangles), 9.0 (squares) and 10.0 (circles), respectively. Lines were fitted with the equation shown in Fig. 1C and the following values were used for curve fitting: (pH=7.3) \( K_D = 10^{-6.3} \) M, \( n = 0.9 \); (pH=9.0) \( K_D = 10^{-6.2} \) M, \( n = 0.9 \); (pH=10.0) \( K_D = 10^{-6.0} \) M, \( n = 0.9 \). (B) The membrane was kept at -80 mV, and the pulse to 0 mV was applied. The following values were used for curve fitting: (pH=7.3) \( K_D = 10^{-7.7} \) M, \( n = 0.9 \); (pH=9.0) \( K_D = 10^{-7.1} \) M, \( n = 0.9 \); (pH=10.0) \( K_D = 10^{-6.8} \) M, \( n = 0.9 \).

Effects of pH on ICa and actions of semotiadil
Semotiadil is a strong hydrophobic drug (octanol/water coefficient > 1000 at pH 7.0) and has a pKa value of 9.03. Therefore, in the present experiments, semotiadil
was usually dissolved in DMSO to increase the solubility in water and then diluted in aqueous solution. However, semotiadil at less than 100 μM could be dissolved in the solution directly. In the present experiments, to compare the effects of semotiadil on the I_{Ca} at different values of pH, semotiadil was dissolved in the PSS directly. As shown in Fig. 6A (pH = 7.3, open triangles), semotiadil (dissolved directly in PSS) had an inhibitory action on I_{Ca} that was about 10 times stronger than when the drug was dissolved in DMSO (cf Fig. 1). Changes in the pH did not shift the concentration-inhibition curves for semotiadil (Fig. 6A), when I_{Ca} was evoked by a depolarizing pulse to 0 mV from the holding potential of -100 mV. On the other hand, when a depolarizing pulse to 0 mV was applied from the holding potential of -80 mV, the concentration-inhibition curves observed at pH 9.0 and 10.0 were shifted to the right compared with that at pH 7.3 in a pH-dependent manner.

Figure 7A shows the effects of semotiadil on the voltage-dependent inactivation curve obtained at pH 10.0. The same experimental protocol was used as in Fig. 4A. Alkalization of the solution shifted the voltage-dependent inactivation curve to the left (V_{1/2} = -33.1 mV at the pH 7.3 and -55.5 mV at the pH 10.0). The V_{1/2} value obtained in the presence of 1 μM semotiadil was -58.6 mV, indicating that the voltage-dependent inactivation observed at pH 10.0 was not modified by 1 μM semotiadil. To study the effects of semotiadil (1 μM) on the recovery process of I_{Ca} at pH 10.0, a pair of pulses was applied with changes in the interval similar to those used in the experiments shown in Fig. 5A. In the absence of semotiadil (open circles), there were two recovery time constants for current recovery (fast time constant 179 msec, slow time constant 4.31 sec). In the presence of semotiadil (1 μM, filled circles), the time constant of the slow component was prolonged (7.69 sec), but that of the fast component remained unchanged (180 msec, Fig. 7B). Recovery from the inactivation in the presence of semotiadil observed at pH 10.0 was faster than that observed at pH 7.3 (7.69 sec vs. 11.4 sec).

DISCUSSION

The present experiments showed that when a long depolarizing pulse (>15 sec) was applied to the membrane, the I_{Ca} was inhibited with two time constants (fast and slow components). It is known that in many smooth muscle cells, two types of Ca channels, namely L- and T-type Ca channels, exist (guinea pig coronary artery (16), rabbit basilar artery (4), rabbit ear artery (17), guinea pig portal vein (18)). Most of the reports showed that T-type Ca channels were inactivated rapidly and was resistant to any given Ca antagonists. In the present experiments, the fast component of I_{Ca} recorded from the rabbit portal vein was inhibited by semotiadil to the same extent as the slow component. I also observed (unpublished observations) that nicardipine, diltiazem and Cd inhibited the current amplitudes of both fast and slow components to the same extent, and the amplitude ratio of the fast component against the total amplitude of I_{Ca} was not dependent on the amplitude of the depolarizing pulse. Using the cell-attached configuration of the patch-clamp procedure, only one class of unitary current with a single channel conductance of 27 pS was recorded (the pipette was filled with 90 mM BaCl$_2$ solution and the bath was perfused with 140 mM KCl solution). Therefore, it is postulated that the existence of two components of current inactivation may indicate the presence of two inactivated states, but not two subtypes of Ca channel. According to the receptor modulated hypothesis (19), each Ca channel is in either the resting, open or inactivated state. As recovery of the channel from the inactivated state also showed two components, the voltage-dependent Ca channel in the rabbit portal vein may have two inactivated states.

In the rabbit portal vein, when the membrane was kept at a holding potential more negative than -80 mV, a depolarizing pulse to 0 mV produced the same amplitude of I_{Ca}. This indicated that all Ca channels were kept in the resting state when the membrane was held at -80 mV or more negative potential. Although semotiadil (1 μM) inhibited the amplitude of I_{Ca} at any conditioning potential and shifted the steady-state inactivation curve to the left, the amplitude of I_{Ca}, evoked by a depolarizing pulse from holding potentials of -100 mV or -120 mV, showed the same value. Thus, at the holding potential of -100 mV or more negative potential, semotiadil did not inhibit I_{Ca} in a voltage dependent manner (resting state block) (Fig. 3B).

When the holding potential was elevated to -80 mV or -60 mV from -100 mV, the concentration-inhibition curve of I_{Ca} shifted to the left and these results were coincident with the shift of the voltage-dependent inactivation curve observed on application of semotiadil. Therefore, the inhibitory action of semotiadil seems to occur more potently at the inactivated state rather than the resting state.

The dissociation constant for drug binding to the channel at the inactivated state could be estimated from the shift of the voltage dependent inactivation curve and the concentration-inhibition relation curve obtained at the resting state using the following equation (9, 20, 21): 

\[ -\Delta V_{1/2} = k \times \ln \left( \frac{1+([N]/K_a)}{1+([N]/K_r)} \right) \]

where \( \Delta V_{1/2} \) is the amplitude of the shift of the voltage dependent inactivation curve, k is a slope factor for the inactivation curve, [N] is the concentration of drug applied, and K_a and K_r are the dissociation constants of semotiadil for the
inactivated and resting states of Ca channels, respectively. The estimated $K_i$ value was 87 nM, when $V_{\text{half}}$ was obtained from the 5 sec conditioning pulse (when $K_i$ was estimated from the steady state inactivation curve, as 24 nM). As $K_i$ was estimated to be 2.0 $\mu$M from the concentration-inhibition curve at the holding potential of $-100$ mV, semotiadil may bind to the inactivated state with nearly 82 times higher potency than to the resting state.

With application of a 300 msec conditioning or pre-pulse, the fast component of $I_{Ca}$ was completely inactivated and more than 80% of the current inactivated was restored within 500 msec (fast recovery). In the case of the 5 sec pre-pulse application, 80% of the current amplitude was restored more slowly. These results suggested that semotiadil (1 $\mu$M) accelerates the transition from the fast to slow inactivated state and reduces the transition rate of semotiadil to the fast inactivated state.

It has been reported that semotiadil produces a long-lasting inhibition of the contraction in a depolarized rabbit aorta (1) and of the blood pressure in conscious dogs (22, 23). In the present experiments, semotiadil (>0.1 $\mu$M) produced a long-lasting inhibition even after removal of the drug. Semotiadil markedly inhibited the slow component of the recovery phase, and the current recovery was incomplete at the holding potential of $-60$ mV. However, as shown in Fig. 3B, long-lasting inhibition of $I_{Ca}$ after removal of semotiadil was not dependent on the holding potential level during drug application, but depended on drug concentrations. Furthermore, as recovery of $I_{Ca}$ from the inactivation was not completely restored by removal of semotiadil (Fig. 5A), long-lasting inhibition of $I_{Ca}$ by semotiadil may be due to tight binding to the channel.

As semotiadil has a pKa value of 9.03, nearly 98% of the semotiadil is ionized at pH 7.3 under physiological conditions. According to Kass et al. (6) and Uehara and Hume (9), the ionized Ca antagonist produced a use-dependent inhibition, while the neutral drug produced a tonic inhibition of $I_{Ca}$. In the present experiments, however, when the holding potential was kept at $-100$ mV (the channel was in the resting state), the concentration-inhibition curve remained the same with changes in the pH (Fig. 6A), suggesting that both ionized and neutral forms of semotiadil had inhibitory actions on $I_{Ca}$. In the present experiments, when the drug was dissolved directly in PSS, DMSO (<0.01%) itself had no effect on the amplitude of $I_{Ca}$, and this agent did not prevent the restoration of the current amplitude when DMSO (0.01%) was added before or after application of semotiadil. Furthermore, semotiadil dissolved in propyleneglycol also produced a weak inhibitory action (N. Teramoto, unpublished observation). These results indicated that hydrophobic sites of semotiadil may be important for producing the blocking action. As semotiadil is a hydrophobic compound (octanol/water coefficient = 560 at pH = 6.0 and >1000 at pH = 7.0; T. Kawashima and G. Kawatsu, personal communication) and the drug dissolved in DMSO produced an inhibitory action in the same concentrations at pH 7.3 and 10.0 when the channel was in the resting state, this drug may act on the Ca channel in the resting state through a hydrophobic site of the channel and the ionized drug may act on the Ca channel mainly at the inactivated state (at pH = 7.3). Since there is one binding site for semotiadil on the Ca channel as estimated from the concentration-inhibition curve at three different pH conditions, it is plausible to postulate that semotiadil anchors in the membrane near the Ca channel due to its hydrophobic property and inhibits the resting channel. In addition, the ionized drug further acts on the open and inactivated channels. Wu et al. (24) reported that with Bay K-8644 dis-
solved in different kinds of solvents (DMSO, ethanol, polyethylene glycol), Bay K-8644/DMSO inhibited the T channel current in NIE-115 cells, whereas Bay K-8644/ethanol or Bay K-8644/polyethylene glycol failed to do so. They also reported that the inhibition of T channel currents and more potent actions on L channel currents by DHPs are apparently due to a direct interaction of DMSO and DHP molecules, i.e., DMSO may change DHP molecules, physically or chemically. These results may suggest that different solvents in this experiment may exert an effect on the ring distortion of semotiadil. Further studies might bring me some interesting clues about the ring distortion and rotation of semotiadil by using infrared spectroscopy and other techniques.

In conclusion, although high concentrations of semotiadil (>3 µM) act as a nonspecific blocker of the Ca channel in the resting, open and inactivated states, low concentrations of drug (≤1 µM) selectively bind to the inactivated state and inhibit the Ca channel, and this agent also slowed the transition rate from inactivated to resting state. This inhibitory mechanism was markedly modified by the level of the holding potential and pH. In general, the resting membrane potentials of various smooth muscle cells are between −70 mV and −50 mV (25) and most semotiadil molecules were ionized at physiological pH. Therefore, semotiadil, in an ionized form, tightly binds to the membrane and blocks the inactivated Ca channel of the smooth muscle cells by preventing the slow component of the recovery process.

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