Modulation by Mibefradil of the Histamine-Induced Ca\textsuperscript{2+} Entry in Human Aortic Endothelial Cells

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ABSTRACT—The effect of mibefradil, known as a T- and L-type Ca\textsuperscript{2+} channel antagonist, on the histamine-induced Cl\textsuperscript{-} current and Ca\textsuperscript{2+} entry was investigated in human aortic endothelial cells by the fluorescence measurement of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) combined with the patch clamp method. Mibefradil (10 μM) inhibited both the Cl\textsuperscript{-} current and Ca\textsuperscript{2+} entry in a concentration-dependent manner with an IC\textsubscript{50} value of 4.8 and 2.6 μM for the Cl\textsuperscript{-} current and [Ca\textsuperscript{2+}], respectively. These values were comparable to those reported for the inhibition of the T-type Ca\textsuperscript{2+} channel and other Cl\textsuperscript{-} channels. The suppression of Ca\textsuperscript{2+} entry is not caused by the inhibition of the Cl\textsuperscript{-} current and the resulting depolarization since the inhibition was still observed under the voltage clamp condition. These results suggest that mibefradil is a potent blocker not only for the agonist-induced Cl\textsuperscript{-} current but also Ca\textsuperscript{2+} entry channels in vascular endothelial cells.

Keywords: Aortic endothelial cell, Ca\textsuperscript{2+} concentration, Cl\textsuperscript{-} channel, Fura-2, Mibefradil

Previous studies have shown that in vascular endothelial cells, stimulation of H\textsubscript{1}-receptor with histamine can activate both a Cl\textsuperscript{-} current and Ca\textsuperscript{2+} entry from the extracellular space (1, 2). Although cascade reactions including inositol 1,4,5-triphosphate are involved, it is not clear which second messengers are responsible for the activation of these channels (1, 3). The Cl\textsuperscript{-} current is not a Ca\textsuperscript{2+}-activated one, because high concentrations of BAPTA in the patch electrode failed to abolish the current (1, 2). As for Ca\textsuperscript{2+} entry, agonist-induced nonselective cation channels can be possible candidates (3, 4), but capacitative Ca\textsuperscript{2+} entry (CCE) channels (5) might also be involved in the histamine-induced Ca\textsuperscript{2+} entry in human aortic endothelial cell (HAEC) since application of a store-depleting inhibitor of endoplasmic reticulum Ca\textsuperscript{2+}-ATPase pumps, such as thapsigargin, can also activate Ca\textsuperscript{2+} entry (3, 6, 7). Interestingly, during histamine application, the sustained [Ca\textsuperscript{2+}], elevation was suppressed by reducing external Cl\textsuperscript{-} or by Cl\textsuperscript{-} channel blockers, N-phenylantranilic acid (NPA) and niflumic acid in HAEC (7, 8). Similar results have been reported in rat mucosal-type mast cells where Cl\textsuperscript{-} channel blockers suppressed extracellular Ca\textsuperscript{2+}-dependent secretion of serotonin (9). These findings indicate that Cl\textsuperscript{-} channels may act as a regulator for Ca\textsuperscript{2+} entry channels or that Cl\textsuperscript{-} channels may modulate Ca\textsuperscript{2+} entry by altering the membrane potential (1, 10). For example, Ono et al. (1) have shown that the membrane potential of HAEC is largely determined by Cl\textsuperscript{-} during histamine application. It is thus possible that inhibition of Cl\textsuperscript{-} channels causes membrane depolarization, thereby decreasing [Ca\textsuperscript{2+}], during histamine application.

On the other hand, it is well known that a number of Cl\textsuperscript{-} channel blockers, including NPA and niflumic acid, are not necessarily selective for Cl\textsuperscript{-} channels but also inhibit Ca\textsuperscript{2+} entry channels (9, 11, 12). In this respect, the availability of a specific experimental tool to inhibit selectively the Cl\textsuperscript{-} channels would be very useful to assess the functional relationship between Cl\textsuperscript{-} channels and Ca\textsuperscript{2+} entry pathway in vascular endothelial cells. Nilius et al. (13) have reported that mibefradil, a Ca\textsuperscript{2+} channel antagonist that acts on both L- and T-type Ca\textsuperscript{2+} channels with tenfold selectivity for T-type channels (14, 15), is a more potent and selective Cl\textsuperscript{-} channel antagonist than NPA, niflumic acid and 9-anthracene carboxylic acid (9-AC). In the present study, therefore, we have made an attempt to examine whether mibefradil can selectively inhibit histamine-induced Cl\textsuperscript{-} current in HAEC. The results suggest that mibefradil is a potent blocker not only for the Cl\textsuperscript{-} current but also the agonist-induced Ca\textsuperscript{2+} entry.

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MATERIALS AND METHODS

Culture of endothelial cells

HAEC at passage 3 were purchased from Clonetics (San Diego, CA, USA). The cells were grown in the culture medium (endothelial basal medium, Clonetics) supplemented with 2% or 5% fetal bovine serum, bovine brain extract protein contents (3 mg/ml), human recombinant epidermal growth factor (10 μg/ml), 1 mg/ml hydrocortisone, 50 μg/ml gentamicin and 50 ng/ml amphotericin B in 5% CO₂-containing air at 37°C. The culture medium was exchanged every 48 h until a subconfluent growth stage was obtained. The cells were detached by exposure to 0.025% trypsin in a Ca²⁺- and Mg²⁺-free solution containing 0.01% EDTA for approximately 180 s, diluted in the culture medium, and then reseeded on coverslips (9 × 9 mm) coated with fibronectin (Biomedical Technologies, Stoughton, MA, USA) with a cell density of approximately 2,500 cells/cm². We kept the cells in culture for 1 − 4 days before use. No differences were found in the experimental results obtained by using these types of culture media.

The protocols for animal experimentation described in this paper were previously approved by the Animal Committee, Akita University; the “Guidelines for Animal Experimentation” of the University were completely adhered to in all subsequent animal experiments.

Solution and drugs

The HEPES-buffered saline (HBS) used as the standard bath solution contained 136.9 mM NaCl, 5.4 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 11.1 mM glucose and 5.0 mM HEPES. The pH was adjusted to 7.4 with NaOH.

The pipette solution for the nystatin perforated patch electrode contained 120 mM potassium gluconate, 30 mM KCl, and 10 mM HEPES, and pH was adjusted to 7.2 with KOH. Nystatin (Sigma Chemical, St. Louis, MO, USA) was dissolved in methanol as a 10 mg/ml stock solution and added to external solutions to obtain a final concentration of 200 − 400 μg/ml. Fura 2-AM (Dojindo Laboratories, Kumamoto) was dissolved in dimethyl sulfoxide as 1 mM stock solution and diluted in 1 ml HBS with 2 μl 10% cremophor EL (Sigma Chemical). The final concentration of the Ca²⁺ indicators was 5 μM. Mibefradil was kindly provided by Hoffman-La Roche, Ltd. (Basel, Switzerland), and it was applied at concentrations between 1 and 30 μM from an aqueous 30 mM stock solution.

The bath solution was warmed by the use of the water jacket around the perfusion tube. The temperature of the chamber was kept at around 36°C. The flow rate was constantly maintained at 2 − 3 ml/min during the experiments.

Measurement of [Ca²⁺].

The method used to measure [Ca²⁺] was essentially similar to that described previously (1, 7, 8). After loading fura-2 by incubation with HBS containing 5 μM fura-2AM for 30 − 60 min at 37°C, cells were washed several times with HBS to remove extracellular fura-2AM. Fluorescence measurement of [Ca²⁺], in single cells was performed using a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic Co., Tokyo). Excitation wavelength was alternated between 340 and 380 nm at 400 Hz, and emission fluorescence was measured at 500 nm by a photomultiplier. The emission intensities at 340 and 380 nm excitation together with the current and voltage signals were sampled every 1 s onto the hard disk of a computer (Macintosh Iici) running SuperScope software (version 1.1; GW Instruments Inc., Somerville, MA, USA). Autofluorescence, measured at the end of each experiment on cell-free parts of the coverslips, was subtracted from the raw signals at each excitation wavelength before calculating the fluorescence ratio, F340/F380. In this study, [Ca²⁺] was expressed as the fluorescence ratio.

Voltage clamp and recording technique

Single endothelial cells on coverslips were voltage clamped using the nystatin perforated patch clamp technique (1, 16, 17). The tip diameter of the pipette was about 2 − 5 μm and the resistance ranged from 3 to 5 MΩ when filled with the internal solution. To measure the I-V relation, ramp pulses (dV/dt = ±0.2 V/s) were applied from a holding potential of −10, −50 or −80 mV; and the I-V relations were measured during the hyperpolarizing portion of the ramp pulses. The current and voltage signals were digitized online at 500 Hz using pCLAMP software (version 6.0.3; Axon Instruments, Burlingame, CA, USA). The liquid junction potential between the pipette solution and HBS was electrically compensated.

The theoretical concentration-response curve is drawn by the least squares fit with the Hill’s equation,

\[ I = I_0 \left(1 + \frac{[\text{mibefradil}]}{IC_{50}}\right)^n \]

where \( I \), \([\text{mibefradil}]\), \( IC_{50} \), and \( n \) indicate the relative amplitude of either Cl⁻ current (Fig. 2) or the F340/F380 ratio (Fig. 4), the mibefradil concentration, the half-inhibitory concentration, and Hill’s coefficient, respectively. The results are expressed as means ± S.E.M.

RESULTS

Inhibition by mibefradil of the histamine-induced Cl⁻ current

We first examined whether mibefradil affected the histamine-induced Cl⁻ current in HAEC. After confirming the membrane perforation under the nystatin-perforated patch clamp condition, ramp pulses (dV/dt = ±0.2 V/s) were applied every 20 s from a holding potential of −50 mV (Fig. 1). Upon application of histamine, the holding current
was shifted toward the inward direction accompanied by an increase of the current deflection in response to ramp pulses. After obtaining the maximal effect, the current decreased gradually to a relatively steady level within a couple of minutes. Mibefradil (1, 10 μM) was then applied, causing a decrease of the current (Fig. 1, A and B: c, d, e). The reversal potential of the histamine-activated current measured before and during mibefradil application was $-29.4 \pm 2.2$ mV ($n = 5$) and $-30.4 \pm 1.6$ mV ($n = 5$), respectively. These values are near to the value (approximately $-40$ mV) expected for a Cl\(^{-}\) electrode from the Nernst equation. Therefore, the histamine-activated current could be the Cl\(^{-}\) current (1). The concentration-dependent suppression of the Cl\(^{-}\) current was obtained by measuring the Cl\(^{-}\) current amplitude at +50 mV and normalized in reference to the value before mibefradil application (Fig. 2). The half maximal inhibition was obtained at 4.8 μM. This value is comparable to those reported for the inhibition of the volume-activated or Ca\(^{2+}\)-activated Cl\(^{-}\) current by mibe-
fradil (13). When the concentration of mibefradil was further increased up to 30 μM, the membrane became leaky and the giga-ohm seal was disrupted.

**Effects of mibefradil on the histamine-induced Ca\(^{2+}\) entry**

As previously reported (8), histamine (10 μM) produced a biphasic elevation of [Ca\(^{2+}\)]\(_i\) in HAEC (Fig. 3). The initial transient increase is due to release of Ca\(^{2+}\) from intracellular store sites (7, 18, 19), and the following sustained elevation is caused by Ca\(^{2+}\) entry from the extracellular space (8).

Effects of mibefradil on Ca\(^{2+}\) entry were examined when [Ca\(^{2+}\)]\(_i\) reached a sustained level. Out of 25 cells examined, mibefradil decreased the histamine-induced increase in [Ca\(^{2+}\)]\(_i\) in 19 cells (Fig. 3A), while it slightly enhanced [Ca\(^{2+}\)]\(_i\) in 3 cells (Fig. 3B) and had no effect in other 3 cells. Quantitative evaluation was made by measuring the sustained [Ca\(^{2+}\)]\(_i\) elevation in reference to the value before mibefradil application. On average of all 25 cells examined, 10 μM mibefradil decreased the sustained [Ca\(^{2+}\)]\(_i\) to $0.31 \pm 0.08$ (mean ± S.E.M., $n = 25$). At 30 μM mibefradil, the sustained [Ca\(^{2+}\)]\(_i\) was further decreased to $0.18 \pm 0.07$ (mean ± S.E.M., $n = 7$).

The concentration-response relationship obtained from 31 cells is summarized in Fig. 4. The suppressing effects of mibefradil became apparent at approximately 1 μM and maximal at around 30 μM. The half-maximum inhibition was obtained at 2.6 μM.

**Simultaneous measurements of the effect of mibefradil on histamine-induced increase in [Ca\(^{2+}\)]\(_i\) and the Cl\(^{-}\) current**

The inhibition of histamine-induced Ca\(^{2+}\) entry might be derived secondarily from the inhibition of the Cl\(^{-}\) current. Namely, the inhibition of the Cl\(^{-}\) current might have induced membrane depolarization, which in turn reduced the driving force of Ca\(^{2+}\) entry and thereby decreased [Ca\(^{2+}\)]\(_i\). In the experiment shown in Fig. 5, the effects of mibefradil

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**Fig. 1.** Inhibition by mibefradil of the histamine-induced Cl\(^{-}\) current. A: After confirming membrane perforation using the nysta-
tin-perforated patch clamp technique, ramp pulses were applied every 20 s from a holding potential of −50 mV. The vertical deflection indicates the current response to ramp pulses. The dotted line is the zero current level. B: Superimposed I-V curves were obtained at the times indicated by corresponding letters in A (a – f).

**Fig. 2.** Concentration-response relationship of mibefradil on the histamine-induced Cl\(^{-}\) current. The amplitude of the histamine-
induced Cl\(^{-}\) current was measured at +50 mV and normalized in reference to the value before mibefradil application. Open circles show the averaged value, and standard errors are shown by vertical bars. Number of measurements at each concentration is indicated in parentheses. The continuous curve is drawn by the least squares fit with the Hill’s equation, $I = I_m / (1 + ([\text{mibefradil}] / \text{IC}_{50})^h)$, where $I$, [mibefradil], $I_m$, and $n$ indicate the relative amplitude of the Cl\(^{-}\) current, the mibefradil concentration, the half-inhibitory concentra-
tion and Hill’s coefficient, respectively. The $\text{IC}_{50}$ value is 4.8 μM and $n$ is 1.0.
on \([\text{Ca}^{2+}]_i\), were examined under the voltage-clamp condition where possible change of the membrane potential could be avoided. During the sustained phase of \([\text{Ca}^{2+}]_i\) elevation, depolarization of the membrane potential from \(-80 \text{ mV}\) to \(-10 \text{ mV}\) markedly decreased \([\text{Ca}^{2+}]_i\) (19 ± 6%, \(n = 6\)), and repolarization recovered \([\text{Ca}^{2+}]_i\). The finding is consistent with a previous study that the sustained \([\text{Ca}^{2+}]_i\) elevation is largely due to \(\text{Ca}^{2+}\) entry through the plasma membrane (1, 8). In the presence of histamine, mibefradil (10 \(\mu\text{M}\)) decreased both \([\text{Ca}^{2+}]_i\) (51 ± 6%, \(n = 6\)) and the histamine-induced \(\text{Cl}^-\) current. Subsequent depolarization of the membrane potential further decreased \([\text{Ca}^{2+}]_i\) in a reversible manner. These results indicate that mibefradil decreases \([\text{Ca}^{2+}]_i\) independently from the secondary change of the membrane potential caused by the \(\text{Cl}^-\) current suppression.

DISCUSSION

It is well established that \([\text{Ca}^{2+}]_i\) increases in response to several vasodilator substances, such as bradykinin, thrombin and ATP, in vascular endothelial cells (20). These substances induce a biphasic increase in \([\text{Ca}^{2+}]_i\), in a way similar to the histamine-induced \([\text{Ca}^{2+}]_i\) increase (1, 8). The present study has demonstrated that mibefradil inhibited
the sustained increase in [Ca\(^{2+}\)]\(_i\) induced by histamine. The inhibition occurred even under the voltage-clamp condition, indicating that mibefradil blocks Ca\(^{2+}\) entry independently from the change of the membrane potential. This is in good agreement with a previous finding that mibefradil decreased [Ca\(^{2+}\)]\(_i\) in calf pulmonary artery endothelial cells where the changes in membrane potential were prevented by high K\(^+\) solution (13). When considered together with the facts that mibefradil inhibits various types of voltage-dependent Ca\(^{2+}\) channels (14, 21, 22) and Cl\(^-\) channels (13), the present results may indicate that mibefradil directly blocks the Ca\(^{2+}\) entry pathway in vascular endothelial cells.

Another possible explanation, though it was not demonstrated in the present study, is that the Cl\(^-\) channel may act as a regulator for Ca\(^{2+}\) entry in HAEC. Regulatory role of Cl\(^-\) channel or related structures in ionic transport has been suggested previously in other tissues; for example, cystic fibrosis transmembrane conductance regulator (CFTR) that functions as a Cl\(^-\) channel controls other membrane or membrane-associated proteins such as epithelial Na\(^+\) channels, voltage-gated K\(^+\) channels, and water channels (23). Wei et al. (24) have shown that TRP4, a member of the family of six transmembrane helix channels that are possible candidates for CCE in vascular endothelial cells (3, 25), is necessary for CFTR activation in mouse aortic endothelial cells.

In the present study, the IC\(_{50}\) values for the inhibition of the Cl\(^-\) current and [Ca\(^{2+}\)]\(_i\) were 4.8 \(\mu\)M and 2.6 \(\mu\)M, respectively. These values are comparable to those reported so far for the block of T- (K\(_T\) = 2.7 \(\mu\)M), L- (K\(_L\) = 18.6 \(\mu\)M) and N-type Ca\(^{2+}\) channels (K\(_N\) = 1.4 \(\mu\)M) (21, 22), and Ca\(^{2+}\)-activated (K\(_A\) = 4.7 \(\mu\)M) or volume-activated Cl\(^-\) current (K\(_V\) = 5.4 \(\mu\)M) in vascular endothelial cells (9). On the other hand, it has been reported that the agonist-induced Ca\(^{2+}\) entry in vascular endothelial cells are also blocked by various substances such as several micromolar Gd\(^{3+}\) or La\(^{3+}\) (3), niflumic acid (2) and SK&F 96365 (2). It may be concluded that mibefradil is one of the potent blockers for the histamine-induced Ca\(^{2+}\) entry in HAEC.

It should be noted that mibefradil unexpectedly increased [Ca\(^{2+}\)]\(_i\) in some unclamped cells. Such response might be explained by assuming that the inhibitory action of mibefradil was more potent for the Cl\(^-\) current than for Ca\(^{2+}\) entry. The resting membrane potential of HAEC has been reported to show bimodal distribution with peaks at \(-17.8\) mV and \(-67.5\) mV, and activation of the Cl\(^-\) current induced either depolarization or hyperpolarization depending on the membrane potential before histamine application (1). We thus speculate that some cells would have been depolarized and others hyperpolarized after block of the Cl\(^-\) current, and these changes would have lead to decrease or increase of the driving force for Ca\(^{2+}\) entry.

The physiological role of Cl\(^-\) channels has been reported in the regulation of cellular excitability, transepithelial transport, cell volume regulation, and acidification of intracellular organelles in many cells (3, 26). In fact, mibefradil, used as a Cl\(^-\) channel blocker, suppressed the proliferation of calf pulmonary artery endothelial cells (13) and rat microvascular endothelial cells (27). The authors (27) suggested that the block of Cl\(^-\) channels impaired the cell volume change, which should normally occur during cell proliferation. It has also been reported that intracellular Ca\(^{2+}\) is important in cell proliferation and growth. For example, inhibition of T-type Ca\(^{2+}\) channels in smooth muscle cells prevented the anomalous infiltration/growth of damaged tissues associated with the development of arteriosclerosis (28). In endothelial cells, voltage-dependent Ca\(^{2+}\) channels are not involved in the regulation of Ca\(^{2+}\) entry into the cells (3), and therefore the Ca\(^{2+}\) entry pathway appears to act as a major source of Ca\(^{2+}\) entry. In support of this view, vascular endothelial growth factor (VEGF) induces store depletion and triggers activation of CCE channels, resulting in Ca\(^{2+}\) influx that might be important for controlling angiogenesis (3). It might be, therefore, speculated that in addition to block of the Cl\(^-\) current, inhibition of Ca\(^{2+}\) entry contributes to the antiproliferative action of mibefradil in vascular endothelial cells.

Mibefradil is a potent peripheral and coronary vasodilator, which stabilizes sinoatrial nodal activity and reduces ischemic afterload without negative inotropy (29). Its vascular selectivity has been attributed to both its T-type Ca\(^{2+}\) channel selectivity and its highly voltage-dependent blocking activity for L-type Ca\(^{2+}\) channels (21). However, effects of mibefradil on endothelial cells have been given little attention in previous studies. It should be emphasized that mibefradil inhibits both endothelial Cl\(^-\) channels and Ca\(^{2+}\) entry with potency comparable to that for inhibiting voltage-dependent Ca\(^{2+}\) channels. In vascular endothelial cells, changes in [Ca\(^{2+}\)]\(_i\) play a key role in the regulation of the synthesis and release of different vasoactive factors (3, 30). It is thus conceivable that the vasodilatory effect of mibefradil might be, at least in part, derived from the suppression of endothelial Ca\(^{2+}\) entry. Namely, a decrease in [Ca\(^{2+}\)]\(_i\) in endothelial cells may suppress the synthesis and release of vasoconstricting factors, thereby causing the vasodilatory effect. For revealing the clinical relevance of mibefradil, further studies are clearly necessary to know whether or not inhibition of Cl\(^-\) current and/or Ca\(^{2+}\) entry in vascular endothelial cells contributes to vasodilator effects of mibefradil.

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