Histamine Induces K\textsuperscript{+}, Ca\textsuperscript{2+}, and Cl\textsuperscript{-} Currents in Human Vascular Endothelial Cells

Role of Ionic Currents in Stimulation of Nitric Oxide Biosynthesis

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Abstract The nature of the membrane currents mediating agonist-induced Ca\textsuperscript{2+} entry and enhanced nitric oxide (NO) production in endothelial cells is still unclear. Using both perforated-patch and conventional whole-cell clamp technique, we have studied the membrane response associated with histamine stimulation of human vascular endothelial cells. In perforated-patch experiments, the initial histamine (10 \mu mol/L)-induced current reversed close to the K\textsuperscript{+} equilibrium potential and was blocked by tetrabutylammonium ions (TBA, 10 mmol/L). In addition, a TBA-insensitive current that developed slowly in the presence of histamine was recorded. This delayed histamine-induced current reversed close to neutral potential and was inhibited by SK&F 96365 (25 \mu mol/L), a putative blocker of receptor-operated Ca\textsuperscript{2+} channels. Similar histamine effects were observed in conventional whole-cell experiments using pipette solutions with low Ca\textsuperscript{2+}-buffering capacity. Strong buffering of intracellular free Ca\textsuperscript{2+} suppressed the initial, but not the delayed, current response. The delayed component of histamine-induced current was substantially inhibited by the Ca\textsuperscript{2+} channel blocker N-phenyl-
thranilic acid (NPA, 100 \mu mol/L), and an eightfold change in the Cl\textsuperscript{-} gradient shifted the reversal potential of this current by 30 mV. In Cl\textsuperscript{-}-free solutions, histamine induced an SK&F 96365-sensitive NPA-resistant current, which, according to reversal potential measurements in 20 mmol/L extracellular Ca\textsuperscript{2+}, corresponded to a cation conductance with 13- to 25-fold selectivity for Ca\textsuperscript{2+} over K\textsuperscript{+}. Both SK&F 96365 and TBA strongly suppressed histamine-induced rises in intracellular free Ca\textsuperscript{2+} and cellular cGMP levels, whereas NPA did not. Our results provide the first demonstration that three distinct ionic conductances contribute to the histamine-induced membrane response of endothelial cells. It is suggested that histamine induces a Cl\textsuperscript{-} conductance that is apparently not involved in Ca\textsuperscript{2+} homeostasis and regulation of NO biosynthesis, while, in parallel, joint activation of a rapidly induced K\textsuperscript{+} permeability and a slowly developing cation permeability mediate Ca\textsuperscript{2+} entry and stimulation of endothelial NO production. (Circ Res. 1994;75:304-314.)

Key Words • endothelial cells • patch clamp • Ca\textsuperscript{2+} entry • endothelium-derived relaxing factor/nitric oxide

Agonist-induced Ca\textsuperscript{2+} entry is a major determinant of vascular endothelial functions. In particular, the formation of endothelium-derived relaxing factor (EDRF, or nitric oxide [NO]) is known to be in large part regulated via Ca\textsuperscript{2+} entry.\textsuperscript{1,2} The nature of the Ca\textsuperscript{2+} entry mechanism(s) activated by receptor agonists such as bradykinin, ATP, and histamine in endothelial cells is still speculative. Activating agonists appear to enhance the plasma membrane permeability for both K\textsuperscript{+} and Ca\textsuperscript{2+} in endothelial cells.\textsuperscript{3,4} Agonist-induced increases in endothelial K\textsuperscript{+} conductance, which most likely support Ca\textsuperscript{2+} entry by increasing the electrochemical gradient,\textsuperscript{5,7} have been extensively characterized.\textsuperscript{6,8-10} However, the pathways and mechanisms underlying the agonist-induced increase in Ca\textsuperscript{2+} conductance, and thus the regulation of endothelial function, are as yet unclear. Nonselective cation channels have been identified in the plasma membrane of endothelial cells, and an involvement of these cation channels in agonist-induced Ca\textsuperscript{2+} entry has been suggested.\textsuperscript{11-15} The reported permeability properties of these nonselective cation channels differ considerably, and there is divergent evidence regarding the mechanism of channel activation.\textsuperscript{12,13,15} Direct ligand gating\textsuperscript{15} and second-messenger–mediated modulation of channels\textsuperscript{12,13} have been suggested. Besides these two principal mechanisms of channel modulation, considerable evidence has recently accumulated for a linkage between agonist- or drug-induced depletion of intracellular Ca\textsuperscript{2+} stores in the endothelium and the activation of a Ca\textsuperscript{2+} entry pathway.\textsuperscript{16-18}

Characterization of agonist-induced Ca\textsuperscript{2+} currents in endothelial cells is impeded by the fact that most endothelial preparations lack detectable agonist-induced inward currents or that such currents are minute compared with the large, agonist-evoked K\textsuperscript{+} current.\textsuperscript{6,8,19} However, histamine stimulation of endothelial cells from human umbilical vein has been found to result in a sustained membrane current that displays a reversal potential close to 0 mV, ie, a feature consistent with the idea of histamine-activated Ca\textsuperscript{2+} entry through receptor-coupled nonselective cation channels.\textsuperscript{20} However, the tonic conductances contributing to this histamine response, as well as their role in histamine-induced changes of endothelial functions, have not yet been evaluated in detail. The relatively slow time course of current development following receptor stimulation was interpreted in terms of second-messenger–mediated activation of ion channels. We have recently
reported that both the histamine-induced current and NO production in human endothelial cells can be reversibly inhibited by the Ca\textsuperscript{2+} entry blocker SK&F 96365.\textsuperscript{21} In the present study, we have extended the analysis of the histamine-induced endothelial membrane conductances and addressed the question of the functional role of histamine-induced ionic currents. We provide evidence that histamine induces K\textsuperscript{+}, Ca\textsuperscript{2+}, and Cl\textsuperscript{−} currents in human endothelial cells and demonstrate the functional significance of both the K\textsuperscript{+} and the Ca\textsuperscript{2+} conductance for regulation of endothelial NO production.

Materials and Methods

Cell Culture

Human endothelial cells were isolated and cultured as described previously.\textsuperscript{21} Experiments were performed with cells kept in primary culture (1 to 3 days after isolation) and with subcultured cells (passage 1). Purity of endothelial cell preparations was tested by indirect immunofluorescence staining using anti-von Willebrand factor.

Electrophysiology

Endothelial cells plated on glass coverslips were transferred to an experimental chamber (200 \( \mu \text{L} \)) that was connected to a perfusion system. Whole-cell membrane currents were recorded with either the standard patch-clamp technique or the amphotericin B-perforated-patch technique.\textsuperscript{22,23} Patch pipettes (Clark Electromedical Instruments) had a resistance of 1 to 3 MΩ, and series resistance in perforated-patch whole-cell experiments was in the range of 20 to 40 MΩ. To allow optimal exchange between pipette solution and cytoplasm in conventional whole-cell recordings, the experiments were, in general, started after a delay of at least 10 minutes after membrane rupture. All experiments were carried out at room temperature.

Voltage clamp and current amplification were performed with a List EPC/7 patch-clamp amplifier (List). Voltage-step and voltage-ramp protocols were applied by using pCLAMP 5.5 software. Time courses of histamine effects were studied by holding the cells at \(-60\) mV and applying voltage-ramp protocols (0.04 mV/ms every 5 or 10 seconds), which allowed the assessment of transient changes in the current-to-voltage (I-V) relation. Compositions of bath and pipette solutions are listed in the Table. In general, in perforated-patch experiments solution G was used in the pipette, and amphotericin B (240 μg/mL, Sigma) was added to the pipette solution immediately before use. Osmotic pressure of the solutions was adjusted to values between 290 and 315 mOsm, either by variation of the HEPES concentration or by use of the nonelectrolyte mannitol, which has been reported to exert only marginal effects on electrode standard potentials or ion activities.\textsuperscript{24} After transfer of the cells from culture medium to the various external solutions, part of the cells were rounded up and consequently detached from the coverslips. Although the use of mannitol-containing solutions is expected to favor cell shrinkage, a similar degree of cell shrinkage was observed in all external solutions. Since cell shrinkage may affect cell responsiveness, cells that exhibited changes in morphology were excluded from experimentation.

Liquid junction potentials between pairs of solutions were measured directly, with the junction potentials at the tip of the 3 mol/L KCl electrode assumed to be negligible. According to the assumption that junction potentials between pipette solutions and the cell interior are approximately zero, correction for liquid junction potentials was performed by setting the holding potential equal to the negative liquid junction potential during offset adjustment.\textsuperscript{25} With all pairs of solutions, offset drift was <5 mV within 20 minutes. Reversal potentials of histamine-induced currents were derived from the intersection of current responses to voltage ramps in the absence (I-V curve of unstimulated cell) and in the presence of histamine.

Measurement of Intracellular Free Ca\textsuperscript{2+}

Endothelial cells were loaded with fura 2-AM as described previously.\textsuperscript{1,21} In brief, cultured endothelial cells were harvested by enzymatic digestion (0.25% trypsin and 0.02% EDTA) and resuspended in culture medium (Optitmem 1) containing 2 μmol/L fura 2-AM. After a 45-minute incubation at 37°C, the cells were washed twice with HEPES buffer containing (mmol/L) NaCl 145, KCl 5, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2.5, and

Compositions of Bath and Pipette Solutions

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\* The given CaCl\textsubscript{2}, EGTA, and BAPlA concentrations correspond to a free Ca\textsuperscript{2+} concentration of \( \approx 30 \) nmol/L according to Robertson and Potter.\textsuperscript{26} In some experiments, CaCl\textsubscript{2} and BAPlA were omitted in solution F and 0.1 EGTA was added, corresponding to a free Ca\textsuperscript{2+} concentration of \( \approx 10 \) nmol/L, with an assumed Ca\textsuperscript{2+} contamination of 10 μmol/L.

pH of all solutions was adjusted to 7.4 with N-methyl-D-glucamine (NMDG).

The compositions of the various solutions, including their NaCl, KCl, CaCl\textsubscript{2}, MgCl\textsubscript{2}, EGTA, and HEPES concentrations, are listed in the following table. The bathing solution is composed of NaCl 137, KCl 5, CaCl\textsubscript{2} 2.5, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 1, and HEPES 5.5. The internal solution contains NaCl 145, KCl 5, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2.5, and HEPES 5.5. The pH of all solutions was adjusted to 7.4 with N-methyl-D-glucamine (NMDG). The given CaCl\textsubscript{2}, EGTA, and BAPlA concentrations correspond to a free Ca\textsuperscript{2+} concentration of \( \approx 30 \) nmol/L according to Robertson and Potter.\textsuperscript{26} In some experiments, CaCl\textsubscript{2} and BAPlA were omitted in solution F and 0.1 EGTA was added, corresponding to a free Ca\textsuperscript{2+} concentration of \( \approx 10 \) nmol/L, with an assumed Ca\textsuperscript{2+} contamination of 10 μmol/L.
HEPES 10, pH 7.4, and resuspended at a final concentration of 1.25 × 10⁶ cells per milliliter. Fura 2 fluorescence was monitored by the ratio–fluorescence spectroscopy technique with a dual-wavelength spectrofluorometer (Shimadzu RF 5000). The fluorescent Ca²⁺ indicator was excited alternately at 340 and 380 nm, and emission was collected at 500 nm. The signal ratio (340/380) was determined after autofluorescence correction, and free intracellular Ca²⁺ was calculated according to Grynkiewicz et al.²⁶ A dissociation constant of 224 nmol/L was assumed for the fura 2/Ca²⁺ complex at 37°C.²⁷ Calibration factors Rₘₐₓ and Rₘᵟᵢₐₜ (maximum and minimum fluorescence ratio, respectively) and S₀/Sₙ₀ (the ratio of fluorescence values at 380 nm in the absence and presence of saturating Ca²⁺ concentrations) were determined in situ by the addition of 1 µmol/L ionomycin in the presence of 2.5 mmol/L Ca²⁺ (to obtain Rₘₐₓ and S₀) and by subsequent addition of excess (20 mmol/L) EGTA (to obtain Rₘᵟᵢₐₜ and Sₙ₀).

Measurements of cGMP

Endothelial cGMP levels were measured by radioimmunoassay as previously described.¹²² Values are given as mean±SEM from the indicated number of experiments.

Statistics

Averaged data are given as mean±SEM from the indicated number of experiments. Statistical analysis was performed by using Student’s t test for paired or unpaired values. Differences were considered statistically significant at P<.05.

Materials

All tissue culture media were from Gibco-BRL, and ingredients were from Flow Laboratories. SK&F 96365 (1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride) was kindly provided by Dr Janet Merrit from SmithKline Beecham, antibodies for indirect immunofluorescence staining were from Boehringer Mannheim, N-phenylanthranilic acid (NPA) was from Aldrich, and fura 2-AM was from Lambda Fluorescence Technology. All other enzymes, drugs, and chemicals were purchased from Sigma.

Results

In most endothelial cell preparations studied to date, agonist activation was found to be associated with a dramatic increase in the K⁺ conductance of the cells, whereas membrane currents attributable to agonist-activated Ca²⁺ entry were barely detectable.⁶⁸,¹⁹ In contrast, human endothelial cells respond to histamine with a large sustained membrane current, which under physiological ionic conditions clearly moves more positive than the K⁺ equilibrium potential.²⁰,²¹ This histamine-induced current has been interpreted in terms of a nonselective cation conductance, which may provide the basis of stimulated Ca²⁺ entry. However, this hypothesis has not yet been rigorously tested. Therefore, using both the permeabilized and the conventional whole-cell patch-clamp technique, we have studied histamine-induced changes in endothelial membrane conductances in more detail.

Histamine-Induced Membrane Currents Recorded by the Permeabilized-Patch Technique

Fig 1 shows a typical histamine-induced current response recorded by the permeabilized-patch technique. Solution E (low Cl⁻, high K⁺, Table) was used in the pipette, and the cell was bathed in a normal physiological extracellular solution (solution A). The cell was held at −60 mV, and slow depolarizing voltage ramps were applied to determine the I-V relation. In resting cells, the I-V relation depicted clear inward rectification. The zero current potential in unstimulated cells was in the range of −30 to −60 mV. On addition of 10 µmol/L histamine to the bath solution, a biphasic change in membrane currents was observed as illustrated for potentials of −80 and 0 mV in Fig 1 (upper panel). The initial phase of the histamine-induced response was characterized by a rapid increase in the current recorded at 0 mV. Zero current potential increased transiently during this initial phase of histamine stimulation, and the I-V relation derived from voltage-ramp protocols revealed that the histamine-induced current reversed close to the expected K⁺ equilibrium potential. After a significant delay (20 to 60 seconds), a slowly developing inward current was observed at −80 mV. This slowly developing current corresponded to a histamine-induced shift of the reversal potential in the positive direction (Fig 1, lower panel). On washout of histamine, the membrane conductance slowly declined, and an I-V relation fairly consistent with control was obtained within 3 to 4 minutes.

As a first step, to reveal the involvement of a Ca²⁺ conductance in the slowly developing histamine-induced current, we studied the effects of histamine in solutions exclusively containing Ca²⁺ and K⁺ as possible charge-carrying cations; Na⁺, which may carry a substantial amount of current by permeation through either nonselective cation¹⁴ or anion channels,³⁰ was excluded. Fig 2 illustrates typical current responses recorded in high-Ca²⁺ (20 mmol/L) Na⁺-free extracellular solution (solution B). Under these experimental conditions, the resting I-V relation and histamine-induced changes in the I-V relation were similar to those observed in Na⁺-containing normal physiological extracellular solution (Fig 1). Fig 2A shows current responses obtained
from a sequence of voltage steps before (control) and during stimulation of a cell with 10 μmol/L histamine. The cell was held at −60 mV, and test pulses (duration, 400 milliseconds) were delivered every 10 seconds to test potentials in the range of −120 to +40 mV. The corresponding I-V relations are shown in Fig 2B. In the presence of 10 μmol/L histamine, membrane currents, measured 3 minutes after histamine administration, reversed at ≈−30 mV. I-V relations obtained by application of slow voltage-ramp protocols were in agreement with those obtained from peak current values measured in voltage-step protocols (compare panels B and D of Fig 2). In Fig 2D, graphs illustrate the initial current response (left graph, corresponding to point 2 in Fig 2C) and the delayed response (right graph, corresponding to point 3 in Fig 2C). Two distinct histamine-induced membrane conductances developing with different time courses are clearly evident from the I-V relation obtained from voltage-ramp protocols 30 seconds and 2 minutes after application of histamine (time points 2 and 3 in Fig 2). Similar effects of histamine were observed in 32 of 34 cells obtained from 12 different umbilical veins. The sustained histamine-induced current reversed at −32±5 mV, and the histamine-induced inward current amounted to 67±12 pA at −80 mV.

We have previously reported that the sustained current response to histamine is completely suppressed by the imidazole-derivative SK&F 96365.21 It was thus of interest to investigate whether the initial histamine-induced current is also sensitive to SK&F 96365. This was tested by stimulation of cells with histamine (10 μmol/L) in the presence of 25 μmol/L SK&F 96365. A typical experiment is illustrated in Fig 3A. In the presence of SK&F 96365, histamine application still induced an early current, which exhibited an I-V relation similar to that of the initial histamine-induced current observed in the absence of SK&F 96365. How-
ever, the delayed response was suppressed. The reversal potential of this transient histamine-induced current \((-77\pm5 \text{ mV}, n=3)\) is in agreement with the idea of endothelial Ca\(^{2+}\)-dependent K\(^+\) channels being transiently activated by release of Ca\(^{2+}\) from intracellular stores. In an attempt to block this current, we tested tetrabutylammonium chloride (TBA), a compound known to block Ca\(^{2+}\)-activated K\(^+\) channels in endothelial cells.\(^6,8\) Fig 3B illustrates the typical effect of histamine in the presence of 10 mmol/L TBA. The initial component of current was not detectable, but the slowly developing current was still present, now reversing at slightly positive voltages \(+(10\pm4 \text{ mV}, n=4)\). Apparently, two components of histamine-induced membrane currents were dissected by the use of the Ca\(^{2+}\) entry blocker SK&F 96365 and the K\(^+\) channel blocker TBA, suggesting activation of two distinct permeation pathways by histamine.

**Histamine-Induced Membrane Currents Recorded With Conventional Whole-Cell Clamp Technique**

The nature of the histamine-induced current response was further investigated by use of the conventional whole-cell recording technique. The resting I-V relation in K\(^+\)-containing solutions depicted inward rectification at voltages negative to \(-60 \text{ mV}\) and, in most experiments, some outward rectification at positive voltages \(>(+20 \text{ mV})\), which was rarely observed in permeabilized-patch experiments. Histamine again induced membrane currents with high reproducibility, i.e., in 22 of 25 cells. Fig 4A and 4B show, in comparison, the responses recorded with a high-K\(^+\)-low-Cl\(^-\) intracellular (pipette) solution (solution H, Table) containing different concentrations of Ca\(^{2+}\) chelators. When 0.1 mmol/L EGTA was added, the current responses were similar to those observed in permeabilized-patch recordings (compare Fig 4A with Fig 2). However, when higher concentrations of Ca\(^{2+}\) chelators (5 mmol/L EGTA plus 2 mmol/L BAPTA) were included into the pipette solution (with CaCl\(_2\) added to give free Ca\(^{2+}\) concentrations in the 10- to 30-nmol/L range), the initial response, but not the delayed response, to histamine was suppressed (Fig 4B). These results indicate that the initial histamine-induced current is mediated by intracellular Ca\(^{2+}\), whereas the delayed response is not. The mean reversal potential of the histamine-induced current observed with the high-K\(^+\)-low-Cl\(^-\) pipette solution containing EGTA plus BAPTA (solution F, Table) was \(-9\pm5 \text{ mV} (n=5)\). This value may be explained by the opening of ion channels that are permeable for both K\(^+\) and Ca\(^{2+}\). However, this value does not preclude the contribution of a Cl\(^-\) current, which in case of perfect Cl\(^-\) selectivity would be expected to reverse at \(-24 \text{ mV}\) under these conditions.

To test for a possible contribution of Cl\(^-\) to the observed histamine-induced current, we varied the Cl\(^-\) gradient in our experiments and found that the reversal potential of the histamine-induced current was markedly dependent on the Cl\(^-\) gradient. Fig 4C illustrates the current response typically recorded with a high-Cl\(^-\) (144 mmol/L)-containing pipette solution (solution G, Table). The histamine-induced current reversed at \(+21\pm3 \text{ mV} (n=4)\); with 18 mmol/L Cl\(^-\) in the pipette, the current reversed at \(-9\pm5 \text{ mV} (n=5)\). Thus, an \(\sim 8\)-fold (8.3-fold) change in the intracellular Cl\(^-\) concentration, which is expected to shift the reversal potential of a perfectly Cl\(^-\)-selective current by 52 mV, resulted in a 30-mV shift of the reversal potential of the histamine-induced current. This result clearly indicates a contribution of Cl\(^-\) to the delayed histamine-induced membrane current in human endothelial cells.

To further test the hypothesis of a histamine-induced Cl\(^-\) conductance, we tested the Cl\(^-\) channel blocker NPA for its ability to block the histamine response. This compound was chosen since it has been reported to block Cl\(^-\) conductance in human endothelial cells.\(^31\) As illustrated in Fig 5A, 100 \(\mu\)mol/L NPA inhibited the histamine-induced current reversibly. The extent of inhibition was similar to that obtained with 25 \(\mu\)mol/L SK&F 96365 (Fig 5B). Our results indicate that histamine induces a substantial anion conductance that may overlap with the current corresponding to the actual Ca\(^{2+}\) entry pathway.

This idea was tested by studying the effects of histamine in Cl\(^-\)-free solutions. When all Cl\(^-\) was replaced by aspartate (solutions C and J, Table), histamine was still able to induce a membrane response. Time courses of histamine-induced inward currents recorded at \(-60\) and
0 mV in Cl\textsuperscript{−}-free solutions are illustrated in Fig 6. With K\textsuperscript{+} as the major intracellular cation, the reversal potential of the histamine-induced current, i.e., the potential corresponding to the intersection of I-V curves in the absence and presence of histamine, was +37±5 mV (n=4). The histamine-induced current was clearly dependent on extracellular Ca\textsuperscript{2+}, as illustrated in Fig 6A. Perfusion of the cell with a nominally Ca\textsuperscript{2+}-free solution in the continuous presence of histamine reversibly reduced the inward current. The histamine-induced current recorded in Cl\textsuperscript{−}-free solutions again exhibited sensitivity to SK&F 96365 (Fig 6B) but was resistant to NPA (100 μmol/L, n=3, Fig 6C). Fig 6C shows the current response evoked by histamine in symmetrical 140 mmol/L aspartate solutions (solutions D and J, Table). Under these conditions, the current reversed at +26±3 mV; thus, a 26-fold change in extracellular aspartate resulted in an ~10-mV shift of reversal potentials. This observation indicates that a small fraction of the histamine-induced current is carried by aspartate. Nonetheless, the finding that reversal potentials were 26 mV more positive than aspartate equilibrium is consistent with a substantial contribution of Ca\textsuperscript{2+} to the observed current. When applying constant field theory for membrane permeabilities to monovalent and divalent cations,\textsuperscript{32,33} the measured reversal potentials corresponded to permeability ratios of Ca\textsuperscript{2+} to K\textsuperscript{+} (P\textsubscript{Ca}/P\textsubscript{K}) in the range of 13 to 25. In nominally Ca\textsuperscript{2+}-free extracellular solution containing 140 mmol/L aspartate, the reversal potential of the histamine-induced current was shifted to −22±6 mV (n=3). With Cs\textsuperscript{+} as a monovalent cation and methanesulfonate as a substitute for Cl\textsuperscript{−} (solutions E and K, Table), the current induced by histamine reversed at =+60 mV (+58±12 mV), which corresponds to calculated permeability ratios of Ca\textsuperscript{2+} to Cs\textsuperscript{+} (P\textsubscript{Ca}/P\textsubscript{Cs}) in the range of >70. As shown in Fig 6D, reversal potentials were barely affected by changes in the extracellular Cs\textsuperscript{+} methanesulfonate concentration.

Functional Effects of Agents That Block Histamine-Induced Membrane Currents

In parallel with the electrophysiological experiments, we investigated the functional effects of the K\textsuperscript{+} channel blocker TBA, the Cl\textsuperscript{−} channel blocker NPA, and the Ca\textsuperscript{2+} entry blocker SK&F 96365. All three compounds were found to modulate the histamine response in...
electrophysiological experiments. It was thus of interest to test their ability to affect the histamine-induced intracellular Ca\(^{2+}\) signal and, as a functional consequence, the production of NO. Fig. 7 illustrates the effects of these compounds on the histamine-induced intracellular Ca\(^{2+}\) signal. Stimulation of endothelial cells with 100 \(\mu\)mol/L histamine caused an initial transient rise in [Ca\(^{2+}\)], from an average value of 116±6 to 872±57 nmol/L (n=10), which was followed by a sustained [Ca\(^{2+}\)] plateau at 355±40 nmol/L. Preincubation (40 seconds) of the cells with SK&F 96365 markedly modified the intracellular Ca\(^{2+}\) signal (Fig 7A), in that the sustained phase of elevated [Ca\(^{2+}\)] (plateau phase) was significantly reduced (to 140±10 nmol/L, n=6), whereas the initial transient peak was barely affected (847±51 nmol/L). Preincubation with TBA (Fig 7B), on the other hand, resulted in a statistically significant suppression of both the transient peak (to 675±28 nmol/L, n=5) and the sustained plateau phase of the intracellular Ca\(^{2+}\) signal (to 225±32 nmol/L). In contrast, NPA (100 \(\mu\)mol/L) did not affect the histamine-induced intracellular Ca\(^{2+}\) response (n=4), as illustrated in Fig 7C. A histamine-induced intracellular Ca\(^{2+}\) peak of 828±57 nmol/L and an intracellular Ca\(^{2+}\) plateau at 312±34 nmol/L were observed in the presence of 100 \(\mu\)mol/L NPA. Higher concentrations of NPA substantially quenched the fura 2 signal, which impeded reliable analysis of the data. Inhibition of the intracellular Ca\(^{2+}\) signal by TBA and SK&F 96365 was expected to result in suppression of the histamine-induced NO formation. To confirm this functional effect of TBA and SK&F 96365, we measured intracellular cGMP levels, which are known to correspond well with intracellular NO formation.\(^{28}\) A typical experiment is illustrated in Fig 8. Histamine elevated the cellular cGMP levels within 4 minutes up to five times the control values. This effect was largely dependent on the presence of extracellular Ca\(^{2+}\) and significantly blocked by 10 \(\mu\)mol/L TBA or 25 \(\mu\)mol/L SK&F 96365. Consistent with the lack of effect of NPA on intracellular Ca\(^{2+}\) signals, the Cl\(^-\) channel blocker (up to 300 \(\mu\)mol/L) failed to inhibit histamine-induced increases in cellular cGMP.

**Discussion**

Endothelial cells cultured from human umbilical vein are a frequently used model for studies on agonist activation of vascular endothelium. This cell type responds to saturating histamine concentrations in a fashion typical of receptor-mediated activation of electrically nonexcitable tissues, displaying an agonist-induced intracellular Ca\(^{2+}\) signal comprising a transient Ca\(^{2+}\) peak followed by a maintained Ca\(^{2+}\) plateau, which reflects a sustained phase of enhanced Ca\(^{2+}\) entry from extracellular space.\(^{24-36}\) In the present study, we provide evidence that histamine induces three distinct membrane conductances in these cells. The histamine-induced current response comprised transient and sustained responses, each of which exhibited distinctly different I-V relations. Within an initial phase of \(~20\) to 30 seconds, the current induced by histamine displayed a reversal potential close to the expected K\(^+\) equilibrium. After prolonged exposure to histamine, the reversal potential of the current shifted slowly toward positive potentials, indicating delayed activation of a second component of current. This slowly developing histamine-induced current was affected by changes in the Cl\(^-\) gradient, suggesting the contribution of a histamine-induced anion conductance. Experiments in Cl\(^-\)-free solutions clearly demonstrated the ability of histamine
to induce a Ca$^{2+}$ current that also contributes to the delayed membrane response. It is concluded that histamine enhances the K$^+$, Cl$^-$, and Ca$^{2+}$ permeability of the endothelial cell membrane.

**Histamine-Induced K$^+$ Permeability**

The I-V relation observed in resting unstimulated human umbilical vein endothelial cells displayed marked inward rectification, as reported for many other endothelial cell preparations.$^{5,6,8,10-13}$ Interestingly, in conventional whole-cell experiments, the majority of cells also exhibited some outward rectification that was rarely seen in permeabilized-patch experiments. Outward rectification may result from voltage-dependent activation of K$^+$ channels, such as large-conductance Ca$^{2+}$-dependent K$^+$ channels, which have been identified in this cell type,$^{13}$ but other as-yet-unidentified voltage-dependent K$^+$ or anion channels may also be involved. Why this outward rectification is not present in permeabilized-patch recordings is unknown. In permeabilized-patch experiments, histamine elicited a rapid initial current response, exhibiting a reversal potential near −80 mV. This histamine-induced current was conserved in conventional whole-cell clamp experiments when pipette solutions with low Ca$^{2+}$ buffer capacity (up to 0.5 mmol/L EGTA) were used. The rapid response was suppressed by (1) extracellular application of the K$^+$ channel blocker TBA (10 mmol/L) or (2) intracellular application of higher concentrations of Ca$^{2+}$ chelators. These results suggest histamine-induced stimulation of Ca$^{2+}$-activated K$^+$ channels. Enhanced activity of endothelial K$^+$ channels has been observed during agonist stimulation of various endothelial cell preparations, including bovine and pig aortic endothelial cells.$^{6,8,10}$ In an early study on histamine-induced membrane currents in human endothelial cells,$^{20}$ no such agonist-activated K$^+$ currents were observed. However, in these experiments pipette solutions containing 1.1 mmol/L EGTA were used, providing Ca$^{2+}$ buffer capacity that may be sufficiently high to suppress Ca$^{2+}$-dependent activation of K$^+$ channels at the single-channel level, so far only large-conductance Ca$^{2+}$-dependent K$^+$ channels have been identified in endothelial cells of human umbilical vein.$^{13}$ This channel type may contribute at least in part to the observed rapid current response to histamine.

**Histamine-Induced Cl$^-$ Permeability**

The most striking membrane response elicited by histamine in human endothelial cells is a slowly rising, long-lasting current that reverses close to 0 mV.$^{6,20}$ This membrane response is of particular interest, since agonist-induced inward currents at the resting potential of the cells (ie, currents that may correspond to Ca$^{2+}$ entry) have only rarely been observed in most other endothelial cell preparations. Although histamine-induced activation of nonselective cation channels, which are primary candidates for the endothelial Ca$^{2+}$ entry pathway, may explain the observed membrane response, the contribution of other ionic conductances has not yet been investigated. When permeabilized-patch experiments were conducted in the presence of the K$^+$ channel blocker TBA, the reversal potential of the sustained histamine-induced current shifted slightly to positive potentials (compare Figs 2 and 3B), indicating some contribution of K$^+$-selective channels to the sustained response. The reversal potentials observed in the presence of TBA or with high intracellular concentrations of Ca$^{2+}$ chelators were close to the Cl$^-$ equilibrium potential; thus, the hypothesis of a histamine-induced Cl$^-$ conductance was tested. A 30-mV shift in the reversal potential was obtained with an eightfold change in the Cl$^-$ gradient. For a perfectly Cl$^-$-selective conductance, a reversal potential shift of 52 mV would be expected; thus, activation of highly Cl$^-$-selective channels as the sole basis of the histamine-induced slow membrane response may be excluded. Our results, however, clearly suggest a partial contribution of Cl$^-$ to the observed current. Agonist activation of endothelial Cl$^-$ channels has previously been reported,$^{30,37}$ and an NPA-sensitive Cl$^-$ conductance has been identified in human endothelial cells.$^{31}$ Such Cl$^-$ conductance may well be stimulated during histamine activation. Indeed, the Cl$^-$ channel blocker NPA inhibited the slow histamine response, which further confirmed the idea that the slow histamine response involves activation of endothelial Cl$^-$ channels. Our results, however, do not preclude the possibility that part of the current response is due to stimulated Ca$^{2+}$ entry. Interestingly, in Cl$^-$-containing solutions, both the putative Ca$^{2+}$ entry blocker SK&F 96365 (25 μmol/L) and NPA (100 μmol/L) inhibited a substantial part of the histamine response. This finding may be explained by the existence of a single type of slowly developing histamine-induced conductance that is sensitive to both NPA and SK&F 96365. However, further experimentation yielded results demonstrating the existence of a histamine-induced cation conductance that is largely insensitive to NPA. The marked inhibitory effect of SK&F 96365 in Cl$^-$-containing solutions may reflect its ability to block two distinct histamine-induced conductances, ie, a cation- and an anion-conducting pathway. Since in our experiments, intracellular Ca$^{2+}$ was effectively buffered by EGTA+BAPTA-containing pipette solutions, indirect inhibition of the histamine-induced anion conductance via lowering of [Ca$^{2+}$] appears less likely than a direct effect of SK&F 96365. A direct inhibitory effect of SK&F 96365 on endothelial Cl$^-$ channels is in line with recent reports showing that this imidazole derivative interferes rather nonselectively with various membrane transport systems including Ca$^{2+}$-ATPases and voltage-gated ion channels.$^{36,38,39}$

**Histamine-Induced Ca$^{2+}$ Permeability**

To unmask histamine-induced Ca$^{2+}$ currents in human endothelial cells, we attempted to minimize the current flow through histamine-activated Cl$^-$ channels by substituting aspartate or methanesulfonate for Cl$^-$. As possible charge-carrying cations, K$^+$ or Cs$^+$ in the intracellular solution and Ca$^{2+}$ in the extracellular solution were used. Although measurement of reversal potentials at different aspartate gradients suggested that a slight but significant aspartate conductance exists in histamine-stimulated cells, variation of the methanesulfonate gradient was apparently without effect on histamine-induced currents. With K$^+$ as the intracellular cation, the current induced by histamine in symmetrical aspartate solutions reversed at +26 mV. According to constant field theory, this current would correspond to a cation conductance with a 13- to 25-fold selectivity
for Ca\(^{2+}\) over K\(^+\), and the calculated selectivity for Ca\(^{2+}\) over Cs\(^+\) was even higher. The existence of a rather Ca\(^{2+}\)-selective histamine-induced membrane current was further supported by the finding that the histamine-induced current was suppressed when extracellular Ca\(^{2+}\) was reduced to nominally zero. Although reversal potentials clearly shifted in the negative direction during reduction of extracellular Ca\(^{2+}\), the Ca\(^{2+}\)-to-K\(^+\) permeability ratio calculated from reversal potentials in nominally Ca\(^{2+}\)-free solutions, which were assumed to contain ≈50 μmol/L Ca\(^{2+}\), was >10-fold higher than in 20 mmol/L Ca\(^{2+}\)-containing extracellular solutions. This striking difference may be explained by the existence of additional histamine-modulated membrane conductances, such as the aspartate conductance discussed above, and/or by a change in permeability properties of the involved cation channel due to the reduction of extracellular Ca\(^{2+}\). Nonetheless, the reversal potentials measured in Cl\(^-\)-free solutions unequivocally demonstrate the ability of histamine to induce a cation conductance that exhibits moderate selectivity for Ca\(^{2+}\). At the single-channel level, histamine has been found to modulate endothelial nonselective cation channels\(^{4,15}\) in vascular endothelial cells. Both studies describe endothelial cation channels that are activated by histamine and exhibit comparable unitary conductance but considerably different cation permeability ratios (P\(_{Ca}/\)P\(_{Na}\)), ie, 0.2\(^{14}\) and 15.7.\(^{15}\) It is tempting to speculate that the Ca\(^{2+}\) current observed in the present study may reflect the opening of an ion channel similar to the latter more selective cation channel. However, the channel described by Yamamoto et al\(^{15}\) was apparently modulated by histamine via a direct mechanism. Such direct agonist regulation is rather inconsistent with the slow onset of the current response observed in the present study. Thus, the ion channel involved in the histamine-induced inward current reported in the present study may be different from the channels identified so far.

In a very recent report, Nilius et al\(^{40}\) suggest that histamine-induced Ca\(^{2+}\) entry into human endothelial cells involves an ion channel with lower permeability for Ca\(^{2+}\) than for K\(^+\) and Na\(^+\). These authors exclude the possibility of a contribution of Cl\(^-\) to the histamine-induced current on the basis of observing reversal potentials different from those expected for perfect Cl\(^-\) selectivity. However, endothelial Cl\(^-\) channels have been found to exhibit significant permeability for Na\(^+\) (P\(_{Cl}/P_{Na} = 5\)).\(^{30}\) Thus, at a physiological sodium gradient, the current through endothelial Cl\(^-\) channels is expected to reverse to more positive levels than E\(_{Cl}\). Consequently, the observed shift of reversal potentials to more negative values on removal of extracellular Na\(^+\)\(^{40}\) may as well be explained by a current that involves endothelial Cl\(^-\) channels. Moreover, the finding that inhibition of protein kinase C (PKC) evokes a current similar to the one induced by histamine\(^{40}\) may be interpreted in terms of modulation of endothelial Cl\(^-\) channels, since cellular regulation of such channels via PKC has previously been suggested.\(^{30}\) Estimation of a low Ca\(^{2+}\) selectivity by Nilius et al\(^{40}\) may therefore be due to histamine-induced current flow through endothelial Cl\(^-\) channels that has not been eliminated.

Possible Signaling Mechanisms Involved in Histamine Stimulation of Ca\(^{2+}\) Permeability

In some endothelial preparations, inward currents that may reflect Ca\(^{2+}\) entry have previously been reported. However, these membrane currents were observed with rather low incidence, which impeded characterization.\(^{20,41}\) The rather poor reproducibility has been explained by a small inward current component activated by an easily diffusible second messenger, which is quickly diluted in conventional whole-cell experiments.\(^{42}\) Using the permeabilized-patch technique, which preserves intracellular signaling, we have indeed observed a sustained histamine-induced inward current with extremely high reproducibility (>95%). The idea of second-messenger-mediated Ca\(^{2+}\) entry was further strengthened by the finding that the agonist-induced inward current was found to develop quite slowly, ie, a phenomenon also reported for a bradykinin-induced current observed in bovine aortic cells.\(^{43}\) However, in experiments recording histamine-induced membrane currents by use of the conventional whole-cell patch-clamp technique, we found that even when excessive exchange between cytoplasm and pipette solution was allowed, histamine still induced slowly developing membrane currents with almost similar reproducibility. Thus, the current response of human endothelial cells to histamine is not disturbed by cell dialysis. The use of intracellular solutions containing high concentrations of Ca\(^{2+}\) chelators did not prevent histamine-induced inward currents. This result argues against a role of intracellular Ca\(^{2+}\), which has previously been suggested to mediate the slow current response.\(^{20}\) On the other hand, a signaling mechanism that has to be considered is that of Ca\(^{2+}\)-release–mediated Ca\(^{2+}\) entry. Depletion of intracellular Ca\(^{2+}\) stores appears to trigger stimulation of Ca\(^{2+}\) entry, as suggested from electrophysiological as well as functional studies in various non-electrically excitable tissues.\(^{16,18,43-47}\) A close relation between the Ca\(^{2+}\) content of intracellular Ca\(^{2+}\) pools in the endothelial cell and the plasma membrane Ca\(^{2+}\) permeability has been suggested.\(^{16,18}\) Moreover, it has been reported that thapsigargin, which causes depletion of Ca\(^{2+}\) stores via inhibition of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, induces a current very similar to the one induced by histamine.\(^{17}\) The characteristics of the histamine-induced Ca\(^{2+}\) current described here are consistent with the characteristics of store depletion–induced Ca\(^{2+}\) entry, in that the time course of current development is slow, as expected for the complex signaling pathway postulated for depletion-induced Ca\(^{2+}\) entry.\(^{45,46}\)

Role of Histamine-Induced Ionic Currents in Regulation of Endothelial NO Production

The histamine-evoked membrane response was found to be sensitive to the K\(^+\) channel blocker TBA, the Cl\(^-\) channel blocker NPA, and the Ca\(^{2+}\) entry blocker SK&F 96365. Whereas TBA preferentially blocked the rapidly activated K\(^+\) conductance, both NPA and SK&F 96365 inhibited the slowly developing, sustained component of the histamine response. SK&F 96365 inhibited the current response in Cl\(^-\)-containing as well as Cl\(^-\)-free solutions. On the other hand, NPA exerted its inhibitory effect only when Cl\(^-\) was present as the charge carrier. These results indicate that NPA blocks a histamine-induced Cl\(^-\) conductance, whereas SK&F
96365 most likely affects both the anion conductance and the Ca\(^{2+}\) entry pathway. When measuring histamine-induced intracellular Ca\(^{2+}\) signals in the endothelial cells, we found that both TBA and SK&F 96365 inhibited the intracellular Ca\(^{2+}\) signal, whereas NPA was without any effect up to 100 \(\mu\)mol/L, i.e., at a concentration that clearly inhibited histamine-induced currents. SK&F 96365 at a concentration of 25 \(\mu\)mol/L almost completely abolished the histamine-induced sustained rise in [Ca\(^{2+}\)] while leaving the initial transient response unaffected, indicating that the imidazole derivative has little effect on Ca\(^{2+}\) mobilization from intracellular stores but effectively blocks the histamine-stimulated Ca\(^{2+}\) entry pathway. In contrast, the K\(^+\) channel blocker TBA blunted both the initial transient and the sustained intracellular Ca\(^{2+}\) signal. Inhibition of the sustained histamine response by TBA is consistent with the idea of a contribution of the agonist-induced K\(^+\) current to Ca\(^{2+}\) entry via membrane hyperpolarization, as previously suggested.5-7 The observed inhibition of the initial intracellular Ca\(^{2+}\) transient may be explained by either (1) TBA inhibition of Ca\(^{2+}\) release or (2) the existence of a Ca\(^{2+}\) permeability that is sensitive to TBA but largely insensitive to SK&F 96365. As to the latter alternative, our results do not support the idea of a Ca\(^{2+}\) conductance that is rapidly activated by histamine. However, a possible explanation for TBA inhibition of the initial histamine response might be the existence of a leak Ca\(^{2+}\) conductance. Ca\(^{2+}\) entry via a leak pathway is expected to be considerably enhanced by histamine because of the rapidly induced initial membrane hyperpolarization and the consequently enhanced electrochemical gradient, which, in turn, is known to be inhibited by TBA. Indeed, a passive Ca\(^{2+}\) leak pathway that is insensitive to SK&F 96365 has recently been demonstrated in endothelial cells.48

In an additional set of experiments, we studied the effects of TBA, NPA, and SK&F 96365 on one specific functional response of histamine stimulation, i.e., enhanced NO synthesis. NO formation within the cells was monitored by measuring cellular cGMP levels, which are well known to correspond to agonist-induced NO formation in the endothelium.28 The observed changes in cGMP levels were in perfect agreement with the data obtained in experiments measuring [Ca\(^{2+}\)]. Both TBA and SK&F 96365 blocked the histamine-induced rise in cGMP, whereas NPA was ineffective at least up to 300 \(\mu\)mol/L.

In summary, our results demonstrate that histamine enhances the membrane permeability for K\(^+\), Ca\(^{2+}\), and Cl\(^-\) in human vascular endothelial cells. Evidence is presented for a histamine-induced Ca\(^{2+}\) entry pathway based on a slowly developing cation conductance with moderate selectivity for Ca\(^{2+}\) over K\(^+\). The histamine-induced membrane response comprises a rapidly induced membrane current that is primarily carried by K\(^+\) and a sustained current that is largely due to an enhanced permeability of the membrane for Ca\(^{2+}\) and Cl\(^-\). Our results suggest that the histamine-induced Cl\(^-\) conductance is not involved in regulation of endothelial NO production, whereas histamine-induced K\(^+\) and Ca\(^{2+}\) currents together mediate cell activation in terms of stimulated NO synthesis.

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