Membrane Potential as a Modulator of the Free Intracellular Ca^{2+} Concentration in Agonist-activated Endothelial Cells

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Abstract. We have used combined patch clamp and fura-2 fluorescence to elucidate the role of membrane potential in the regulation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a human umbilical vein derived endothelial cell-line, EA.hy926 (EA cells) stimulated with vasoactive agonists, such as ATP, histamine and bradykinin. This stimulation caused hyperpolarization and sustained Ca^{2+} plateau in non-clamped cells. Clamping agonist-stimulated cells at negative potentials enhanced the amplitude of this plateau, whereas it was smaller at more depolarized potentials, indicating that Ca^{2+} influx follows its driving force. Depolarization of the membrane by increasing extracellular K^+ or by applying charybdotoxin, a blocker of big conductance Ca^{2+}-dependent K^+ channels during agonist stimulation diminished the plateau rise in $[Ca^{2+}]_i$. It is concluded that the membrane potential is an efficient regulator of Ca^{2+} influx during the plateau phase of agonist-mediated Ca^{2+} signals. In addition, the modulating effects on Ca^{2+} signals should be interpreted with caution if the membrane potential of the cells is not controlled.

Key words: Membrane potential — Ca^{2+} influx — Endothelium — Ca^{2+} activated K^+ channels

Introduction

Elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in various cellular functions, including the synthesis and release of vasoactive substances in vascular endothelium (Inagami et al. 1995). Agonists have been shown to induce a rapid increase in $[Ca^{2+}]_i$ in endothelial cells followed by a Ca^{2+} plateau or – in a narrow concentration window – by Ca^{2+} oscillations (for a review see Nilius et al. 1997). The initial

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Ca^{2+} peak is caused by Ca^{2+} released from inositol (1,4,5)-trisphosphate sensitive Ca^{2+} stores; the plateau phase of $[Ca^{2+}]_i$ elevation mainly by a transmembrane influx of extracellular Ca^{2+} (Clapham 1995). This sustained influx of Ca^{2+} in the endothelium is the key event that drives the NO production and enhances the release of prostacyclin (PGI2), Von Willebrand factor (WvF), and platelet activating factor (PAF) (Prescott et al. 1984; Birch et al. 1992; Carter and Ogden 1992; Iouzalen et al. 1996; Nilius et al. 1997; Lantoine et al. 1998). The nature of Ca^{2+} influx pathways in endothelial cells is still a matter of discussion. Likely, it is caused by activation of store-operated Ca^{2+} channels (Fasolato and Nilius 1998) and non-selective cation influx pathways (Nilius et al. 1997).

The membrane potential is supposed to modulate the Ca^{2+} influx by changing the driving force for Ca^{2+} in endothelium. It has been reported that vasoactive substances induce transient hyperpolarization followed by depolarization, sustained hyperpolarization or membrane potential oscillations (Marchenko and Sage 1993,1994; Mehrke and Daut 1990; Mehrke et al. 1991; Usachev et al. 1995; Vaca et al. 1996). A correlation between membrane potential and changes in $[Ca^{2+}]_i$ has already been reported, however, from separate measurements (Laskey et al. 1990; Luckhoff and Busse 1990). A direct correlation from simultaneous measurements between changes in membrane potential and Ca^{2+} influx has to our knowledge never been reported for endothelium. We show here a close correlation between $[Ca^{2+}]_i$ and membrane potential in endothelial cells stimulated with vaso-active agonists. These findings not only demonstrate a key role of the membrane potential for fine-tuning of the Ca^{2+} influx in endothelial cells, but also point to obvious problems in the interpretation of $[Ca^{2+}]_i$ measurements in non-voltage-clamped cells.

Materials and Methods

Cell Culture

A permanent human cell line, Ea.hy926 (EA cells), established by hybridization of human umbilical vein endothelial cells (Edgell et al. 1983) was grown in DMEM containing 20% fetal calf serum plus 10% HAT 50X supplement (Life Technologies). Cell cultures of both types were maintained at 37°C in a fully humidified atmosphere of 10% CO_2 in air. They were then detached by exposure to 0.05% trypsin in a Ca^{2+} and Mg^{2+}-free solution, reseeded on gelatin-coated coverslips, and kept in culture for 2-4 days before use. Only non-confluent cells were used.

Electrophysiology

Electrophysiological methods and $[Ca^{2+}]_i$ measurements have been described in detail elsewhere (Nilius et al. 1994). We used the patch clamp technique in the whole cell configuration using ruptured or nystatin-perforated patches. For perforated

patches, the tip of the pipette was filled with a nystatin-free solution and then filled from the shank with a nystatin-containing solution (100 mg /ml). Currents and voltages were monitored with an EPC-9 (List Electronic, Germany, sampling rate 1 ms, 8-Pole Bessel filter 2.9 kHz).

Solutions

The standard external solution (modified Kreb's solution) contained (mmol/l): 150 NaCl; 6 KCl; 1.5 $CaCl_2$; 1 $MgCl_2$; 10 HEPES; 10 glucose. The osmolality of this solution was 310 ± 5 mosmol/kg as measured with an osmometer (Wescor 5500 osmometer, Schlag Instruments, Gladbach, Germany). The standard internal pipette solution contained (mmol/l): 40 KCl; 100 potassium aspartate; 2 $MgCl_2$; 0.1 EGTA; 10 HEPES and 4 Na_2ATP. In some experiments, internal 100 mmol/l potassium aspartate was replaced by equimolar KCl. In 150 mmol/l external K^+ solution, 150 mmol/l NaCl in Krebs solution was replaced by equimolar KCl. The pH of the Krebs and 150 mmol/l K^+ solution were adjusted to 7.4 with NaOH and KOH, respectively, that of the internal solutions to 7.2 with KOH.

ATP and charybdotoxin (Sigma, St. Louis, USA) were added to the external solution. Nystatin (Sigma, St. Louis, USA) was dissolved in DMSO as a stock and added to the internal solution.

$[Ca^{2+}]_i$ measurement

For $[Ca^{2+}]_i$ measurements, cells were loaded with fura-2/AM (the acetoxymethyl ester form). Fura-2/AM (2 μmol/l) was added to the bath and the cells were incubated for 25 min at 37°C. After loading, the cells were washed with Krebs solution. The loaded cells were illuminated at wavelengths of 360 and 390 nm through a filter wheel rotating with a frequency of 2 cycles/second. The diaphragm allowed to limit the area from which the fluorescent light was collected from a disc of approximately 20 mm excluding the tip of the pipette. In this area the endothelial cell studied was placed. The fluorescence was measured at 510 nm. Fluorescence was also measured beside the cell and is considered as a reliable estimate of cell autofluorescence. This approach was justified in experiments on endothelial cells (thickness between 200 and 800 nm) where the dye was included into the pipette and the cell autofluorescence was measured in the cell-attached configuration before establishing the whole cell configuration. The autofluorescence was abolished by means of two offsets downstream from the sample and hold circuits (for details see Neher 1989). Apparent free Ca^{2+} concentration was calculated from the fluorescence ratio R by $[Ca^{2+}]_i = K_{eff}(R - R_0)/(R_1 - R)$, where K_{eff} is the effective binding constant, R_0 the fluorescence ratio at zero Ca^{2+}, and R_1 that at high Ca^{2+}. These calibration constants were determined experimentally for the given set-up and the actual experimental conditions used. None of the applied vasoactive agonists induced changes in fluorescence at the excitation wavelength used.

All experiments were performed at room temperature (20–22 °C) Pooled data are given as mean ± S E M

Results

Ca^{2+} transients during agonist stimulation

Figure 1 shows a typical example of the Ca^{2+}-transients in EA cells stimulated by three different agonists The response consists of a fast elevation of $[Ca^{2+}]_i$ which is mainly released from intracellular stores followed by a sustained plateau (Fig 1*A*) Under resting conditions, $[Ca^{2+}]_i$ was 64 ± 5 nmol/l in EA cells (n = 12) When 10 μmol/l ATP was applied to EA cells, $[Ca^{2+}]_i$ rapidly increased to a Ca^{2+} peak (586 ± 51 nmol/l, n = 12) and afterwards declined to a sustained plateau of 434

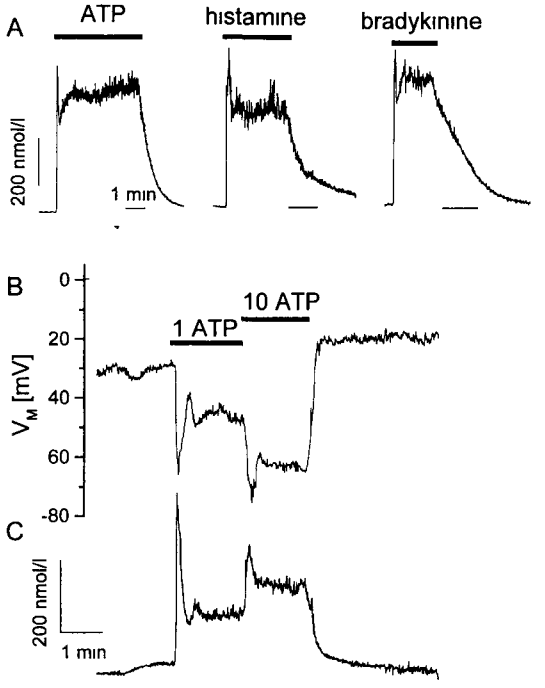

Figure 1. *A* Ca^{2+} responses of non-voltage clamped macrovascular endothelial cells (EA, HUVEC derived cell line Ea926 hy) to stimulation with various vasoactive agonists (ATP 10 μmol/l, histamine 100 μmol/l, bradykinin 100 nmol/l) *B* Change in the membrane potential, V_M, during stimulation of EA cells with different ATP-concentrations *C* Changes in free intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ recorded simultaneously with V_M The dashed lines indicate zero $[Ca^{2+}]_i$ level The signals were sampled at 2 Hz

± 57 nmol/l (n = 12) Similar responses could be observed by stimulation with 100 μmol/l histamine (peak 632 ± 64 nmol/l, plateau 405 ± 46 nmol/l, n=4) and 100 nmol/l bradykinin (peak 605 ± 56 nmol/l, plateau 512 ± 39 nmol/l, n = 3) As shown previously, the Ca^{2+} plateau is caused by Ca^{2+} influx (Kamouchi et al 1997, Viana et al 1998) Here, we show that the Ca^{2+} plateau is closely related to the membrane potential in these cells

Electrical response in EA cells

To study the electrical response in relation to Ca^{2+} signaling, the combined patch clamp and the fura-2 Ca^{2+} fluorescence methods were used Membrane potential was measured using the ruptured-patch, whole cell configuration in current clamp mode EA cells exhibited resting potentials between −38 and −3 mV with an average of −21 4 ± 1 9 mV, n = 25) Under these experimental conditions, the resting Ca^{2+} concentration was 66 ± 5 nmol/l (n = 25)

The effects of ATP stimulation on the membrane potential and the Ca^{2+} response in EA cells are shown in Fig 1*B* and *C* ATP (1 μmol/l) evoked a transient Ca^{2+} peak (503 ± 54 nmol/l, n = 9) and concomitant hyperpolarization (−49 3 ± 4 3 mV, n = 9) due to activation of BK_{Ca} channels (Viana et al 1998) The initial transient Ca^{2+} peak was followed by a decline of $[Ca^{2+}]_i$ to a plateau level (272 ± 30 nmol/l, n = 7) and stabilization of the membrane potential at a less negative value (Fig 1*B*, −37 ± 3 mV, n = 7) Increasing the ATP concentration to 10 mmol/l , enhanced the plateau level of $[Ca^{2+}]_i$ (325 ± 31 μmol/l, n = 7) and hyperpolarized the membrane to more negative potentials (−44 ± 3 mV, n = 7) These differences were statistically significant ($p > 0$ 05)

Effects of membrane potential on plateau Ca^{2+}

Further, the relationship between membrane potential and plateau phase of the agonist induced increase in $[Ca^{2+}]_i$ was analyzed by clamping the membrane potential at various voltages and measuring the amplitudes of the corresponding $[Ca^{2+}]_i$-levels In these experiments nystatin-perforated patches were used to prevent interference with the Ca^{2+}-buffering capacity of the cell This method also prevents Ca^{2+} entry at the rim between the patch pipette and the cell membrane As shown in Fig 2*A* and *B*, it is obvious that the Ca^{2+} plateau was enhanced at more hyperpolarized potentials and reduced at depolarized potentials The correlation between $[Ca^{2+}]_i$ and membrane potential is shown in Fig 2*B* The slope of the regression line amounts to 2 9 nmol l^{-1}/mV, the intercept with the abscissa at +82 mV (Fig 2*C*)

Modulation of plateau Ca^{2+} by membrane potential

Charybdotoxin (CTX), a blocker of BK_{Ca} (50 nmol/l) rapidly and reversibly depolarized the membrane from −36 7 ± 3 0 to −16 4 ± 3 2 mV (n = 5) during

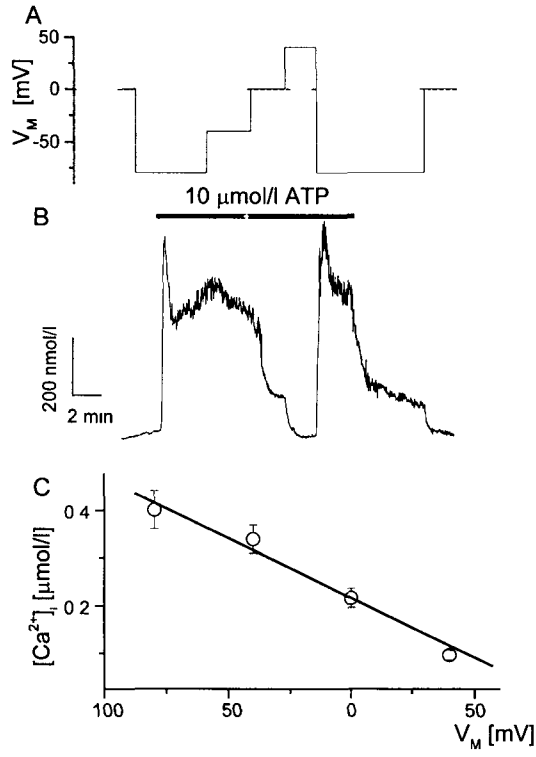

Figure 2. Modulation of $[Ca^{2+}]_i$ by the membrane potential in voltage-clamped cells *A* The holding potential (V_M) was changed as indicated *B* $[Ca^{2+}]_i$ measured at various potentials in voltage-clamped cells as in panel *A* (nystatin perforated patches) ATP was applied as indicated by the horizontal bar *C* Correlation between membrane potential and $[Ca^{2+}]_i$ in voltage clamped cells Open circles indicate the average $[Ca^{2+}]_i$ during the plateau phase at various membrane potentials from 8 11 cells stimulated with 10 μmol/l ATP The solid line represents a linear fit through the data points (slope 2 9 nmol l^{-1}/mV, intercept with the voltage axis +82 mV, $r = -0\ 98$)

the plateau Ca^{2+} induced by 10 μmol/l ATP During the application of CTX, the plateau Ca^{2+} was reduced from 321 ± 32 to 223 ± 11 nmol/l This decrease correlated with the depolarization of the membrane potential (Fig 3*A*, $n = 5$)

When the external K^+ concentration was raised from 6 to 150 mmol/l, the resting membrane potential depolarized to 2 ± 6 mV ($n = 4$) This depolarization was also induced during agonist stimulation In the presence of 10 μmol/l ATP the membrane potential was shifted toward more negative values (−41 ± 11 mV) but

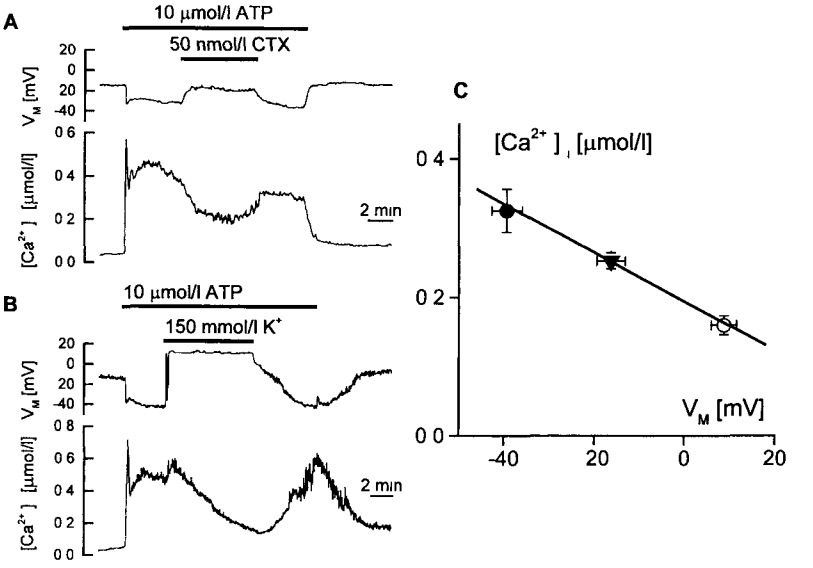

Figure 3. Modulation of $[Ca^{2+}]_i$ by the membrane potential in non-voltage clamped cells *A, B* The dependence of the membrane potential (upper traces) on $[Ca^{2+}]_i$ (lower traces) measured simultaneously in current clamp mode The membrane was depolarized either by application of CTX (*A*) or by increasing external K^+ from 6 to 150 mmol/l (*B*) *C* The relationship between $[Ca^{2+}]_i$ and membrane potential The average value of $[Ca^{2+}]_i$ at the plateau phase in cells stimulated with 10 μmol/l ATP is plotted as a function of the corresponding membrane potential (closed circle) During the plateau induced by 10 μmol/l ATP, $[Ca^{2+}]_i$ was also measured after application of 50 nmol/l charybdotoxin (CTX, closed down triangle) or 150 mmol/l extracellular KCl (open circle) The continuous line is obtained from a linear fit (slope 3 4 nmol l^{-1}/mV, intercept +58 mV, $r = -0\ 99$)

depolarized during application 150 mmol/l KCl to 9 ± 4 mV ($n = 4$) During this depolarization, the plateau Ca^{2+} decreased from 411 ± 28 nmol/l before KCl application to 127 ± 14 nmol/l ($n = 5$, Fig 3*C*) The relationship between membrane potential and $[Ca^{2+}]_i$ during the plateau phase is shown in Fig 3*C* The membrane potential correlated with plateau Ca^{2+} after stimulation with 10 mmol/l ATP under control conditions (solid circle), in the presence of ChTx (solid triangle) and elevated extracellular $[K^+]$ (open circles) The slope of the regression line was 3 4 ± 0 5 nmol l^{-1}/mV, the intercept at +58 mV These values are comparable to those in voltage clamped cells Thus, membrane potential and plateau $[Ca^{2+}]_i$ strongly correlate also under non-voltage clamp conditions

Discussion

Ca^{2+} entry in endothelial cells is one of the most important mechanisms which control the synthesis and release of NO (Lantoine et al 1998) and other vasoactive compounds such as prostacyclin, platelet activating factor (PAF), endothelium-derived hyperpolarizing factor (EDHF), endothelins, natriuretic peptide, small signaling molecules such as substance P, ATP, growth factors, steroids, and even larger receptors and proteins involved in the blood clotting cascade (e g von Willebrand factor, tissue factor inhibitor) (Inagami et al 1995, Nilius et al 1997) In the present study, we focused on the role of the membrane potential for Ca^{2+} influx in the endothelium

The resting membrane potential of most endothelial cells scatters enormously between -10 and -70 mV in the endothelium (Nilius et al 1997) Similar results were obtained in EA cells, although the range was narrower In EA cells used in this study, the resting membrane potential in non-stimulated cells scatters between -38 and -3 mV During stimulation of these cells, BK_{Ca} is the dominant current (Viana et al 1998) which induces hyperpolarization in the presence of the agonist It has been reported in other endothelial cell types that vasoactive agonists induce transient hyperpolarization followed by sustained hyperpolarization or depolariza tion (rabbit aorta (Busse et al 1988), bovine aorta (Vaca et al 1996), rat aorta EC (Marchenko and Sage 1993, 1994, Usachev et al 1995), guinea-pig coronary EC (Mehrke and Daut 1990, Mehrke et al 1991)) In EA cells, ATP induced a peak hyperpolarization followed by a sustained negative potential which mirrored the change in $[Ca^{2+}]_i$ Voltage clamp experiments showed that the hyperpolarization is caused by activation of BK_{Ca} (data not shown) and can be abolished by applica tion of charybdotoxin During the plateau phase, the negative membrane potential is still due to activation of BK_{Ca} by an increased $[Ca^{2+}]_i$

In EA cells, agonist stimulation causes an initial peak in $[Ca^{2+}]_i$ due to release of Ca^{2+} from $Ins(1,4,5)P_3$ sensitive stores followed by a $[Ca^{2+}]_i$ plateau which depends on Ca^{2+} influx (Kamouchi et al 1997, Madge et al 1997, Viana et al 1998) The plateau disappears after withdrawal of extracellular Ca^{2+} Obviously, this plateau is the balance between Ca^{2+} influx via not yet identified Ca^{2+} permeable cation channels and Ca^{2+} extrusion via plasma membrane Ca^{2+} pumps (PMCA) and probably also Na^+/Ca^{2+} exchangers (for a detailed discussion see Nilius et al 1997) The peak depolarization induced by high K^+ external solution or application of charybdotoxin (CTX) significantly reduced the plateau Ca^{2+} In addition, voltage clamp experiments demonstrated that $[Ca^{2+}]_i$ during the plateau was modulated by changes in the membrane potential These results clearly indicate that membrane potential plays a key role in the Ca^{2+} signaling in stimulated endothelium

In a previous report, we demonstrated that *hslo*, a pore forming α-subunit

of BK_{Ca} which was heterologously expressed in CPAE cells modulated the Ca^{2+} signaling in endothelial cells (Kamouchi et al 1997). Interestingly, the relationship between membrane potential and plateau Ca^{2+} in EA cells is similar to that in *hslo* expressing CPAE cells (compare Fig 2 with Fig. 5*C* in Kamouchi et al. (1997)) These results suggest the importance of endogenous BK_{Ca} in determining electrical response and Ca^{2+} signaling in the endothelium.

In conclusion, $[Ca^{2+}]_i$ induces changes in membrane potential which feedback on Ca^{2+} influx and might be important for fine-tuning of $[Ca^{2+}]_i$ during endothelial cell stimulation In addition, these results clearly show that the interpretation of the modulating effects on $[Ca^{2+}]_i$ in non-clamped cells must take into account possible changes in membrane potential

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