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REGULATION OF ION PERMEABILITY IN FROG BRAIN VENULES. SIGNIFICANCE OF CALCIUM, CYCLIC NUCLEOTIDES AND PROTEIN KINASE C

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SUMMARY

1. The effect on ionic permeability of frog brain endothelium of various second messengers was studied by a technique based on continuous recording of the electrical resistance of the venular endothelium *in vivo*.

2. Augmentation of the cytosolic Ca^{2+} concentration in endothelial cells induced with the ionophores ETH 1001 and A23187 increased ion permeability significantly as reflected in the reduced electrical resistance.

3. The electrical resistance fell reversibly within 1–2 s after administration of Ca^{2+}-activating agents. The maximal effect was a reduction to about 0·70 times the pre-experimental resistance value. The resistance decrease was similar to that induced by several inflammatory mediators (Olesen & Crone, 1986).

4. Administration of the following agents did not change the electrical wall resistance: 8-bromo-cyclic AMP, dibutyryl-cyclic AMP, forskolin, 8-bromo-cyclic GMP, dibutyryl-cyclic GMP, sodium nitroprusside, phorbol myristate acetate (a protein kinase C stimulator). Changes in cytosolic Mg^{2+} did not affect permeability.

5. Ca^{2+} may be an important cytosolic signal in the endothelial cell, acting as an intracellular mediator for several permeability-augmenting substances.

INTRODUCTION

Many chemical substances elicit an increase in permeability of microvascular endothelium. The permeability changes induced by many of these substances have similar time courses and about the same maximal effects (Olesen, 1985; Olesen & Crone, 1986), indicating that the effects may be mediated via one or a few shared cellular signal transduction systems, activated by stimulation of cell membrane receptors or by initiation of other membrane reactions. Endothelial cells are endowed with many different receptors that are currently being identified (Grega, Maciejko, Raymond & Sak, 1980; Furchgott, 1983; Vanhoutte, Rubanyi, Miller & Houston, 1986).

Intracellular signal pathways activated by autacoids, hormones, neurotransmitters and other stimuli are well characterized in cell types such as vascular smooth muscle cells, thrombocytes and secretory cells (Berridge & Irvine, 1984; Rink & Hallam, 1984; Rasmussen & Barrett, 1984; Exton, 1985). Important intracellular signals

include Ca^{2+}, cyclic nucleotides and degradation products of phosphatidyl inositol.

Since the existence of these second messengers seems to be ubiquitous, the aim of this study was to determine whether an increase in the intracellular concentration in endothelial cells of any of these substances augments venular permeability. Of the substances investigated, only those increasing the cytosolic Ca^{2+} activity augmented permeability.

METHODS

Theory

The determination of the d.c. resistance of pial venular endothelium is based on classic cable theory (Hodgkin & Rushton, 1946; Crone & Olesen, 1982). Sine-wave current (0·1–1·0 μA, 2 Hz) is injected into the microvessel via a glass micro-electrode, and the ensuing intravascular potential profile is determined by means of two other micro-electrodes positioned in the vessel lumen some distance from the current source. The spatial decline in potential is characterized by the 'length constant', λ. The electrical wall resistance, R_m, or conductance, G_m, of the vessel can be expressed as:

$$R_m = 1/G_m = 2\rho\lambda^2/\alpha, \quad (1)$$

where ρ is the resistivity of blood and α is the vessel radius.

The electrical current is carried through the vascular wall by the small ions in plasma and interstitial fluid, and the electrical resistance may be interpreted in terms of permeability to these ions. The relation between the total conductance, G_m, and the individual ion permeability, P_i, is:

$$G_m = (F^2/RT)\sum_i z_i^2 c_i P_i, \quad (2)$$

where F is the Faraday number, R the gas constant, T the absolute temperature, z_i the valency of the ith ion, and c_i the ion concentration.

Should an agent induce a change in the electrical resistance of the venular endothelium, the change in ion permeability could be calculated according to eqn. (2). The ion concentrations (mequiv/l) used in the calculations were: Na^+, 110; K^+, 2·5; Cl^-, 74; HCO_3^-, 25. The individual permeabilities are proportional to the relative conductances of the ions which are: Na^+, 1·00; K^+, 1·47; Cl^-, 1·52; HCO_3^-, 0·89 (Harned & Owen, 1958; table 6.8.2). If the resistance was reduced from, for example, 2000 to 1500 Ω cm^2 the potassium would increase from $7{\cdot}7 \times 10^{-7}$ to $10{\cdot}3 \times 10^{-7}$ cm/s.

Chemicals and solutions

All vasoactive agents were dissolved in a Ringer solution immediately before use. Initially the substances were dissolved in 96 % (v/v) ethanol (final ethanol concentration < 1 %) except PMA (phorbol-12-myristate-13-acetate), which was first dissolved in 20 mM-Tris–HCl (pH = 7·4) at a concentration of 1×10^{-4} M, and sodium nitroprusside, which was dissolved directly in Ringer solution. The calcium ionophore ETH 1001, which is sensitive to oxygen, was kept under nitrogen.

The substances were obtained from various sources: forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-14-ene-11-one), Calbiochem-Behring, La Jolla, CA, U.S.A.; calcium ionophore ETH 1001 ((−)-(*R*,*R*)-*N*,*N*′-bis-((11-ethoxycarbonyl)-undecyl)-*N*,*N*′,4,5-tetramethyl-3,6-dioxaoctan-diamid), Fluka AG, Buchs, Switzerland; ionophore A23187, Boehringer GmbH, Mannheim, F.R.G.; MS222, Sandoz Ltd, Basle, Switzerland. All other agents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. BW755C (3-amino-1(*m*-(trifluoromethyl)-phenyl)-2-pyrazoline) was a gift from Dr McHale, Wellcome Research Laboratories, Beckenham, U.K.

The preparation was superfused with a frog Ringer solution containing: KCl, 2·5 mM; NaCl, 110 mM; $CaCl_2$, 1 mM; $MgCl_2$, 1 mM; sodium phosphate buffer, 3 mM; glucose, 5 mM; and bovine serum albumin, 0·01 % (w/v). pH was adjusted to 7·4 by titration. In particular experiments the content of Ca^{2+} or Mg^{2+} was changed as described below. The solution used in perfusion experiments contained 0·02 % (w/v) MS222.

Resistance recording

The experiments were carried out on adult frogs (*Rana temporaria*), anaesthetized by a subcutaneous injection of 10 mg MS222/100 g body weight. The plexus of pial venules covering

the telencephalic brain surface was exposed by removing the frontoparietal bones, and the vessels were superfused with frog Ringer solution.

The brain was transilluminated and observed through a Leitz stereo-microscope (magnification: 40–100 ×). In a few experiments, where the aim was to detect whether the vasoactive agents induced a change in venular diameter, the brain surface was viewed in a binocular compound microscope (Laborlux II, Leitz, Wetzlar, F.R.G.) at a magnification of 320 ×.

The head was fastened and the micro-electrodes positioned by means of micromanipulators as described earlier (Olesen, 1985). The intravascular potentials were recorded on-line on a PDP 11/34 computer (Digital Corp., MA, U.S.A.). The signals were sampled fifty times per second, and the resistance, R_m, was calculated every second. After the experiment average values of the vessel wall resistance in 2–10 s periods were calculated, and time–resistance curves were drawn using a Calcomp 81 plotter (California Computer Products Inc., Anaheim, CA, U.S.A.).

The electrical resistance of the venular endothelium was recorded for a control period of 5 min. Then the superfusion was stopped and the test substance was applied to the brain surface at a similar rate (1·0 ml/min) for 3 min. Finally, the test substance was removed by resuming superfusion with the normal Ringer solution. One experiment was performed in each animal.

Four experimental series were conducted.

(1) The effect of increasing the intracellular concentration of cyclic nucleotides in the endothelial cells was investigated by administration of 8-bromo-cyclic AMP (1×10^{-3} M), dibutyryl-cyclic AMP (5×10^{-4} M), forskolin (1×10^{-5} M), 8-bromo-cyclic GMP (1×10^{-3} M), dibutyryl-cyclic GMP (5×10^{-4} M), and sodium nitroprusside (1×10^{-6} M) to the autoperfused frog brain.

(2) The effect of stimulating the intracellular mediator protein kinase C was studied by administration of PMA (1×10^{-6} M) to the autoperfused frog brain.

(3) The level of free cytosolic Ca^{2+} or Mg^{2+} in the endothelial cells of the autoperfused frog brain was augmented by various agents. The intracellular Ca^{2+} concentration was selectively increased by the Ca^{2+} ionophore ETH 1001 (3×10^{-4} M), and the Ca^{2+} and Mg^{2+} concentrations were increased by the ionophore A23187 (10^{-6} M). Thirteen experiments were performed with A23187 on non-pre-treated frogs and six experiments on animals treated with 1·0 mg of the combined cyclo- and lipoxygenase inhibitor BW755C per 100 g body weight I.V. 1 h prior to the experiment and 25 μM-BW755C in the superfusion solution. A23187 is known to degranulate mast cells, and the significance of mast cell degranulation was investigated by superfusing the brain with compound 48/80 (5 μg/ml) for 10 min.

(4) The effect of ionophore A23187 was studied in perfusion experiments. Ionophore A23187 transports extracellular Ca^{2+} as well as Mg^{2+} into the cytosol. The effect of one of the two ions could be studied selectively by reducing the extracellular concentration of the other ion to a level where there is no net transport into the cell. The ionophore A23187 mediates electroneutral exchanges of Ca^{2+} and Mg^{2+} for protons, and accordingly the ionophore-mediated equilibrium distribution of divalent cations across the cell membrane is equal to the proton distribution (r) raised to the second power (Vestergaard-Bogind & Stampe, 1984). At an extracellular pH of 7·4 and an assumed intracellular pH in endothelial cells of 7·0, $r^2 = 6{\cdot}3$. The free intracellular Ca^{2+} concentration in endothelial cells is about 70 nM (Lückhoff & Busse, 1986), and the free Mg^{2+} concentration of most cells is 10000 times higher, about 0·6 mM (Flatman & Lew, 1977; Alvarez-Leefmans, Gamino & Rink, 1984). Under these conditions there will be no net transport of Ca^{2+} and Mg^{2+} into the endothelial cell by the ionophore at or below extracellular concentrations of 10 nM and 100 μM, respectively, but a significant influx of Ca^{2+} and Mg^{2+} at extracellular concentrations of 1 mM. The extracellular concentrations of divalent cations were controlled by simultaneously superfusing and perfusing the vessels with Ringer solution. Artificial perfusion of the intact cardiovascular system was performed via a catheter placed in the aortic trunk (Olesen, 1985).

The Ringer solution used for superfusion and perfusion of the venules employed water rinsed through two ion exchangers and a charcoal filter (Milli-Q water system, Millipore, Bedford, MA, U.S.A.). Three types of solutions were prepared: (a) normal Ringer solution containing 1 mM-Ca^{2+} and 1 mM-Mg^{2+}, (b) a low-Ca^{2+} solution containing 1 mM-Mg^{2+} and < 1 μM-Ca^{2+} plus 50 μM-EGTA (ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetic acid) which reduces the free Ca^{2+} concentration to less than 1 nM, and (c) a low-Mg^{2+} solution containing 1 mM-Ca^{2+} and 20 μM-Mg^{2+}. Ca^{2+} and Mg^{2+} concentrations in effluents from the brain surface were determined using an Atomic Absorption Spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Perfusion experiments were performed with the three different Ringer solutions.

Normal Ringer solution. The brain was both superfused and perfused with the normal Ringer solution. Following a 5 min control period, A23187 was administered to the superfusate for 3 min.

Low-Mg^{2+} solution. The brain was superfused and perfused with the normal Ringer solution for 5 min, then the endothelium was exposed to the low-Mg^{2+} solution at both surfaces from minute 5 to minute 10. Superfusion with ordinary Ringer solution was re-established from minute 10 to minute 15. From minute 15 to minute 17, the venules were exposed to the low-Mg^{2+} solution, and finally the ionophore A23187 was administered in the low-Mg^{2+} solution from minute 17 to minute 20.

Low-Ca^{2+} solution. The experiments were performed as the preceding low-Mg^{2+} experiments using low-Ca^{2+} solutions instead of low-Mg^{2+} solutions.

In a control series, the vehicle, ethanol (1 %) in Ringer solution plus albumin (0·1 %), was administered for 3 min.

The obtained results are given as mean values ±S.E. of mean. n is the number of animals on which one experiment was performed. Statistically significant differences between mean values were assessed using Student's unpaired t test at a 5 % limit.

RESULTS

The vessels investigated were post-capillary venules with an inner diameter ranging from 26 to 74 μm. No constriction or dilation of the venules was observed upon administration of the vasoactive agents. The average electrical wall resistance, corrected for branching vessels (Olesen, de Saint-Aubain & Bundgaard, 1984), was 2240 Ω cm^2 (S.E. of mean = 99 Ω cm^2, n = 121). When an agent exerted an effect, the ratio of the minimal electrical resistance recorded following stimulation, to the average pre-experimental resistance value, was used to express the effect.

Cyclic nucleotides

This series showed that administration of the lipophilic cyclic AMP analogues 8-bromo-cyclic AMP and dibutyryl-cyclic AMP were without effect on the electrical resistance of venular endothelium (Table 1). Forskolin, which is known to activate the adenylate cyclase directly (Seamon, Padgett & Daly, 1981), was also without effect. Increasing the intracellular level of cyclic GMP in the endothelial cells with 8-bromo-cyclic GMP, dibutyryl-cyclic GMP or sodium nitroprusside (Gruetter, Gruetter, Lyon, Kadowitz & Ignarro, 1981) did not change the electrical resistance.

Protein kinase C

Administration of PMA, an activator of the 'second messenger' protein kinase C (Nishizuka, 1984), did not change the electrical resistance in a detectable way.

Transport of divalent cations in autoperfused frog brain

The calcium ionophore ETH 1001 (Hinds & Vincenzi, 1985) induced a significant effect reducing the electrical wall resistance to 0·84 times control value (S.E. of mean = 0·038, n = 20). The resistance decreased within 1–2 s after administration, and maximal effects were seen after 3 min (Fig. 1). The effect was reversible within 20 min after the administration was stopped. The divalent cation ionophore A23187 induced a response with a similar time course (Fig. 2), but it was more potent and more powerful (resistance decrease to 0·69 times control value, S.E. of mean = 0·054, n = 13). The resistance change induced by A23187 in non-pre-treated frogs and in frogs pre-treated with BW755C (maximal resistance decrease to 0·66 times control

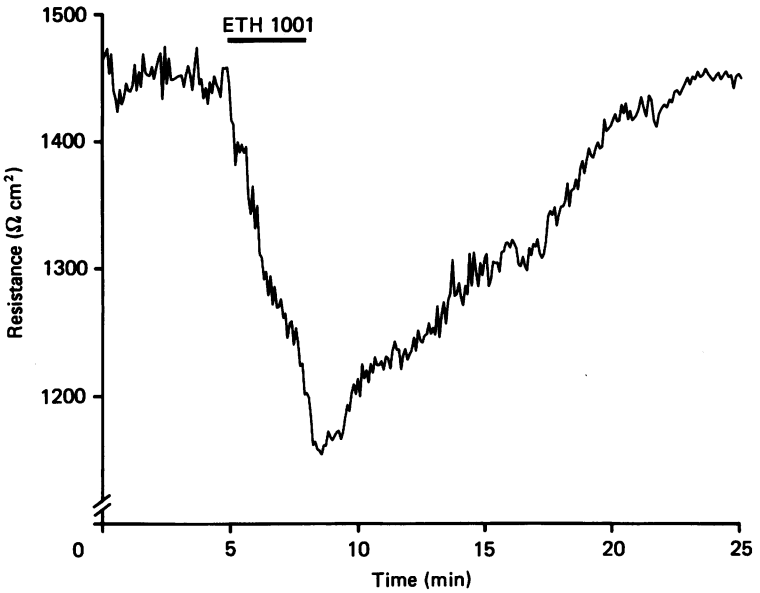

Fig. 1. Decrease in the electrical resistance of the pial venular wall induced by administration of the selective Ca^{2+} ionophore ETH 1001 (3×10^{-4} M) plus Ca^{2+} (1×10^{-3} M) to the exposed brain surface of the frog. Note the rapid initial effect and the slower reversal to pre-experimental resistance after the administration is stopped.

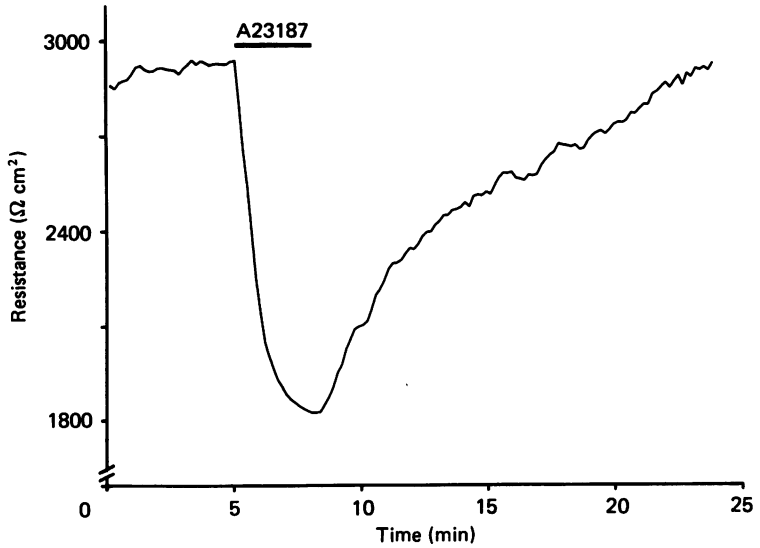

Fig. 2. Change in the electrical resistance of the venular endothelium after superfusion with the ionophore A23187 (1×10^{-6} M) together with Ca^{2+} (1×10^{-3} M) and Mg^{2+} (1×10^{-3} M). The frog brain is autoperfused.

value, S.E. of mean = 0·050, $n = 6$) was not different, indicating that secondary production of cyclo- and lipoxygenase metabolites was of no importance for the ionophore effect. Administration of compound 48/80 to the brain surface did not change the electrical wall resistance ($n = 6$).

Transport of divalent cations in artificially perfused frog brain

The effect of A23187 on vessels perfused with a solution containing 1 mM-Ca^{2+} and 1 mM-Mg^{2+} was a resistance reduction to 0·74 times control value (S.E. of mean = 0·057, $n = 7$). This is not significantly different from the value obtained in the autoperfused frog brain. In perfusion experiments where Ca^{2+} was high and Mg^{2+} low in the extracellular medium (Ca^{2+}, 1 mM; Mg^{2+}, 20 μM), a similar response was recorded (resistance reduction to 0·73 times control value, S.E. of mean = 0·069, $n = 9$). In experiments where extracellular Mg^{2+} was high and Ca^{2+} low, application of the ionophore A23187 did not significantly change the resistance (resistance decrease to 0·95 times control value, S.E. of mean = 0·030, $n = 9$). Reducing either the extracellular Ca^{2+} or Mg^{2+} concentration for a period of 5 min did not in itself change the electrical resistance. In control experiments performed with 1 % ethanol in Ringer solution, the average resistance after 3 min was 1·01 times control value (S.E. of mean = 0·007, $n = 9$).

DISCUSSION

The effect on brain venular permeability of augmenting the intracellular concentration of various second messenger molecules in endothelial cells was studied. It was consistently found that permeability increased following administration of agents elevating the free cytosolic Ca^{2+} concentration. The permeability increase was similar to that induced by serotonin and other inflammatory mediators.

The cytosolic concentration of second messengers was not determined in this study, but the endothelial cells were stimulated with chemical agents known to increase the intracellular level of these molecules in many other cell types, and it was assumed that the substances activate similar messenger systems in endothelial cells. In the case of ionophore A23187 it was directly shown that removal of extracellular Ca^{2+} inhibited the response.

Nicolaysen (1971) demonstrated that the hydraulic conductivity of rabbit pulmonary microvessels was augmented after 40 min of perfusion with a Ca^{2+}-free medium. In frog, exposure of mesenteric capillaries to Ca^{2+}-free conditions for up to 20 min did not affect permeability (Mason, Curry & Michel, 1977). In the present study, permeability did not change within 5 min of perfusion with a low-Ca^{2+} solution.

Stimulation of the intracellular level of cyclic nucleotides and protein kinase C did not change permeability *per se*. However, it cannot be excluded that these mediators play a role in a control system regulating vascular permeability by potentiating or inhibiting the Ca^{2+}-mediated response. Interaction between second messengers has been described in several other cells (Rasmussen & Barrett, 1984; Rink & Hallam, 1984).

Michel (1985) suggested that increasing the cytosolic concentration of ionized

magnesium in endothelial cells with ionophore A23187 induced an increase in permeability of frog mesenteric capillaries. Experiments in the present series on perfused brain vessels showed that mobilization of extracellular Mg^{2+} did not change permeability significantly. A weak effect observed in a few experiments might as well

TABLE 1. Agents decreasing electrical resistance of venular endothelium

Agent	Fractional resistance decrease	Concentration (M)	No.
8-Bromo-cyclic AMP	Nil	1×10^{-3}	8
Dibutyryl-cyclic AMP	Nil	5×10^{-4}	6
Forskolin	Nil	1×10^{-5}	8
8-Bromo-cyclic GMP	Nil	1×10^{-3}	8
Dibutyryl-cyclic GMP	Nil	5×10^{-4}	6
Sodium nitroprusside	Nil	1×10^{-6}	5
Phorbol-12-myristate-13-acetate	Nil	1×10^{-6}	7
ETH 1001	$0{\cdot}84 \pm 0{\cdot}038$*	3×10^{-4}	20
A23187	$0{\cdot}69 \pm 0{\cdot}054$*	1×10^{-6}	13
A23187 + BW755C	$0{\cdot}66 \pm 0{\cdot}050$*	1×10^{-6}	6
A23187 (1 mM-Ca^{2+}, 1 mM-Mg^{2+})	$0{\cdot}74 \pm 0{\cdot}057$*	1×10^{-6}	7
A23187 (1 mM-Ca^{2+}, 20 μM-Mg^{2+})	$0{\cdot}73 \pm 0{\cdot}069$*	1×10^{-6}	9
A23187 (< 1 nM-Ca^{2+}, 1 mM-Mg^{2+})	$0{\cdot}95 \pm 0{\cdot}030$	1×10^{-6}	9

The table shows the decrease in electrical resistance of frog brain venular endothelium induced by various chemicals. All experiments were conducted in autoperfused frogs except the lowermost three series, which were performed in frogs vascularly perfused with Ringer solution containing Ca^{2+} and Mg^{2+} in the concentrations indicated. Fractional resistance decrease indicates minimal resistance value recorded after stimulation relative to the pre-experimental resistance value. The values are given as mean $\pm$S.E. of mean. * Indicates that the effect was significant ($P < 0{\cdot}05$). No. gives number of animals in which one experiment was performed.

be due to release of Ca^{2+} from intracellular stores (Itoh, Kanmura & Kuriyama, 1985). Thus, the present findings do not comply with the view that microvascular permeability is changed by increasing the cytosolic Mg^{2+} concentration in endothelial cells above resting level.

Stimulation of endothelial cells with histamine, bradykinin, ATP, ADP, AMP and melittin causes an increase in the free cytosolic Ca^{2+} concentration from a resting level of about 70 nM to several hundred nM (Lückhoff & Busse, 1986; Rotrosen & Gallin, 1986; Izzo, Loeb, Johns & Peach, 1986; Morgan-Boyd & Hassid, 1986). Inhibition of the mobilization of extracellular Ca^{2+} attenuates the permeability-augmenting effect of histamine, serotonin and free oxygen radicals (Mayhan & Joyner, 1984; Olesen, 1985; Shasby, Lind, Shasby, Goldsmith & Hunninghake, 1985). These findings indicate that a rise in free Ca^{2+} concentration in endothelial cells constitutes an important part of the signal transduction leading to an increase in microvascular permeability.

In the present study, the intracellular Ca^{2+} concentration in endothelial cells was increased directly by use of ionophores. The magnitude and typical asymmetrical time course of the permeability changes induced by the ionophores A23187 and ETH

1001 were similar to those of serotonin, ATP and oxygen radical fluxes (Olesen, 1985; Olesen & Crone, 1986; Olesen, 1987). A23187 was a more potent and powerful ionophore than ETH 1001 (Table 1), but ETH 1001 is more selective for Ca^{2+}. This ionophore has 16000 times higher affinity to Ca^{2+} than to Mg^{2+}, whilst A23187 binds Ca^{2+} only 2·6 times better than Mg^{2+} (Hinds & Vincenzi, 1985).

The experiments indicate that the primary source of activator Ca^{2+} in endothelial cells is the extracellular medium, but liberation from intracellular stores may also be significant. In this study, A23187 induced a weak effect in the absence of extracellular Ca^{2+} in some experiments. ATP, histamine and bradykinin elicit a small increase in the cytosolic Ca^{2+} concentration in endothelial cells in a Ca^{2+}-free medium, but the endothelial responsiveness declines after continuous or repetitive stimulations (Lückhoff & Busse, 1986; Rotrosen & Gallin, 1986; Morgan-Boyd & Hassid, 1986). A cisternal system situated just beneath the plasmalemmal invaginations of endothelial cells has recently been found by Bundgaard (1986) and it may be relevant to view this system as analogous to the Ca^{2+}-containing sarcoplasmic reticulum of smooth muscle cells.

The rise in cytosolic Ca^{2+} concentration may elicit a change in the cell shape by stimulating a 'contraction' (Majno, Shea & Leventhal, 1969) of the actin and myosin filaments in endothelial cells (Drenckhahn, 1983). The increase in intracellular Ca^{2+} concentration could also activate Ca^{2+}-dependent actin-fragmenting proteins (gelsolin, fragmin and villin), which are present in almost every type of vertebrate cell (Weeds, 1982). The permeability-increasing effect of actin-disrupting agents such as cytochalasin B is interesting in this context (Cereijido, Meza & Martinez-Palomo, 1981; Shasby, Shasby, Sullivan & Peach, 1982). Both activities – contraction of actomyosin filaments and activation of actin-severing proteins – may be involved in the changes of endothelial cell shape, as is the case in non-muscle-cell locomotion (Stossel, Hartwig, Yin, Southwick & Zaner, 1984).

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