Influence of vasoactive agents on cytoplasmic free calcium in vascular endothelial cells

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RYAN, UNA S., PAVEL V. AVDONIN, EUGENE YA. POSIN, EUGENE G. POPOV, SERGEY M. DANILOV, AND VSEVOLOD A. TKACHUK. *Influence of vasoactive agents on cytoplasmic free calcium in vascular endothelial cells.* J. Appl. Physiol. 65(5): 2221–2227, 1988.—The regulation of cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in endothelial cells (EC) derived from human umbilical vein, aorta, and pulmonary artery, or from bovine pulmonary artery, was studied by means of the fluorescent Ca^{2+} indicator indo-1. Histamine and thrombin caused a rapid transient elevation in $[Ca^{2+}]_i$ in the EC of all the human blood vessels tested. In aortic EC, $[Ca^{2+}]_i$ also rose in response to ATP and bradykinin. It was shown that in bovine pulmonary artery EC $[Ca^{2+}]_i$ rises in response to platelet-activating factor (PAF) and thrombin. For a more detailed investigation of the receptor-mediated mechanism of $[Ca^{2+}]_i$ increase in EC we used histamine as a stimulating agent. Histamine effects were seen at concentrations ranging from 5×10^{-7} to 10^{-4} M [50% effective dose (ED_{50}) ~2–4 μM)] and were mediated by H_1-receptors. The histamine-induced increase in $[Ca^{2+}]_i$ was not markedly diminished when the extracellular calcium was bound by excess ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). The data obtained indicate that the histamine effect is best explained by Ca^{2+} mobilization from intracellular stores. The histamine-induced increase in $[Ca^{2+}]_i$ was not influenced by elevating the intracellular levels of adenosine 3′,5′-cyclic monophosphate (cAMP) or cyclic guanylic acid (cGMP) by use of isobutylmethylxanthine and forskolin or by nitroprusside preincubation, respectively. However, the protein kinase C stimulator, phorbol myristate acetate (PMA), strongly inhibits $[Ca^{2+}]_i$ elevation. It is assumed that a negative feedback mechanism that blocks receptor-mediated $[Ca^{2+}]_i$ increase is triggered as a result of the activation of protein kinase C.

indo-1; histamine; thrombin; platelet-activating factor; adenosine triphosphate; bradykinin; phorbol ester; cyclic nucleotides

MANY VASOACTIVE SUBSTANCES only cause relaxation of blood vessels when the vascular endothelium is intact (8, 10, 20, 32). Binding of these agents with their specific receptors on the endothelial cell (EC) plasma membrane causes the release of two active substances; prostacyclin (PGI_2) (7, 25, 38) and another factor(s) termed endothelium-derived relaxing factor (EDRF) (11) likely to be nitric oxide (30). These substances cause the relaxation of vascular smooth muscle cells and inhibit platelet aggregation (10, 25, 27). Their release in blood vessels is observed in response to histamine, thrombin, bradykinin, substance P, ATP, platelet-activating factor (PAF), and other compounds. Though the complete sequence of reactions leading to the synthesis and release of PGI_2 and EDRF is not completely known, it has been suggested that these processes are triggered by an increase of cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in EC. The stimulation of production of PGI_2 and EDRF by calcium ionophores supports this concept (12). Furthermore, the agonist-induced release of these compounds in several cases is inhibited by removing the extracellular $[Ca^{2+}]_i$ (9, 20, 37).

To test the hypothesis of a triggering role for calcium ions it is necessary to determine whether vasodilators are responsible for increasing $[Ca^{2+}]_i$ in EC. Progress in this field has been restrained by limitations in the published methods of $[Ca^{2+}]_i$ measurement applicable to cultured EC. With the widely used fluorescent Ca^{2+} indicator quin2, elevation of $[Ca^{2+}]_i$ in these cells has been shown in response to several vasodilators (5, 16, 22, 33). Use of this dye, however, is complicated by the fact that quin2 induces a substantial buffering capacity for Ca^{2+} in cytoplasm. Besides this, quin2 is not convenient for cells cultivated on flat surfaces because its fluorescence is registered at one wavelength and affected not only by $[Ca^{2+}]_i$ but also by the intactness of the cell layer and other factors (13). Recently, newly synthesized fluorescent Ca^{2+} indicators such as fura-2, indo-1, and others (13), have overcome many of the difficulties. The new generation of dyes have 1–2 orders of magnitude higher fluorescence than quin2 and have the additional advantage that the fluorescence of Ca-bound and Ca-free forms are registered simultaneously. $[Ca^{2+}]_i$ is calculated by a function dependent on the ratio of the forms independent of the total fluorescence. In the present study, we determined $[Ca^{2+}]_i$ by means of indo-1 and a multiwavelength method of fluorescence measurement (32) to investigate the regulation of $[Ca^{2+}]_i$ in EC of pulmonary and systemic vessels in response to several vasoactive agents.

MATERIALS AND METHODS

Medium 199 (modified) with Earle's salts and 2.2 g/l sodium bicarbonate without glutamine, and *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) were obtained from Flow Laboratories, UK. Phosphate-

buffered saline without Ca and Mg was obtained from GIBCO, UK. Bovine thrombin, ATP Na_2, PAF, histamine 2HCl, 4-β-phorbol-12 β-myristate-13-α-acetate, isobutilmethylxanthine, pyrilamine, cimetidine, $CaCl_2$, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and carbachol were obtained from Sigma Chemical. 4-α-Phorbol-12,13-didecanoate was from P-L Biochemicals. Forskolin, indo-1/AM, indo-1, angiotensin II, [arg^8]-vasopressin, and ionomycin were from Calbiochem. Nitroprusside Na was from Fluka. U46619 was a generous gift from Upjohn. PGI_2 was from "Reachim" USSR. Disposable polystyrene cuvettes were obtained from LKB, Sweden. Atrial natriuretic factor was from Nova Biochem, Lanfelfingen, Switzerland. Synthetic bradykinin was obtained from Professor M. I. Titov.

Culture of human and bovine endothelial cells. EC from human aorta and human and bovine pulmonary artery were isolated without exposure to proteolytic enzymes by scraping the vessels with a scalpel (35) and were cultured in Ryan Red medium as previously described (35, 36). EC of human umbilical veins were isolated by collagenase treatment (17). Isolated human EC were cultured in medium 199 (M199) supplemented with 20% heat-inactivated pooled human serum and 0.2 μg/ml EC growth supplement from human brain (23), 0.1 mg/ml heparin, and antibiotics (1). Human EC were passaged using 0.05% trypsin and 0.02% EDTA and were seeded in flasks coated with 0.2% gelatin solution in phosphate-buffered saline (PBS). Bovine EC were passaged mechanically and seeded into uncoated flasks. One or two days before the experiments, the cells were harvested from flasks using trysin and EDTA (human EC) or with a rubber policeman (bovine EC) and seeded into cylindrical polystyrene cuvettes (previously coated with 0.2% gelatin solution in PBS) at a density of ~20–50 $\times$ 10^3 cells per tube, stoppered and incubated at a slight angle. Thus the cells formed a confluent monolayer on the lower third of the tube (Fig. 1).

Loading of endothelial cells with indo-1. A 4 mM solution of the acetoxymethyl ester of indo-1 (indo-1/AM) in dimethylsulfoxide was dissolved in M199 to a final concentration of 20 μM. Three-hundred μl of this solution was added to the cuvette with EC growing on the wall (Fig. 1). The cells were then incubated for 45 min at 37°C with gentle shaking. After incubation with indo-1/AM the medium was aspirated and the cells were washed twice with M199. After the first wash, the cells were incubated for 30 min at 37°C to obtain a more complete hydrolysis of indo-1/AM in EC. As shown (21), the fluorescence of nonhydrolyzed indo-1/AM considerably complicates determination of Ca^{2+}-free indo-1 fluorescence. Then 1 ml of M199 (containing 1.8 mM Ca^{2+}) was added to each cuvette. Just before measurement, an additional 2 mM of exogenous Ca^{2+} was added to the cuvettes. In some experiments $[Ca^{2+}]_i$ measurements were performed in PBS containing 1 mM $MgCl_2$ with 2 mM $CaCl_2$ or 1 mM of EGTA. In these cases M199 was aspirated and PBS was added to cuvettes just before $[Ca^{2+}]_i$ measurement. Each experiment was performed at least two times.

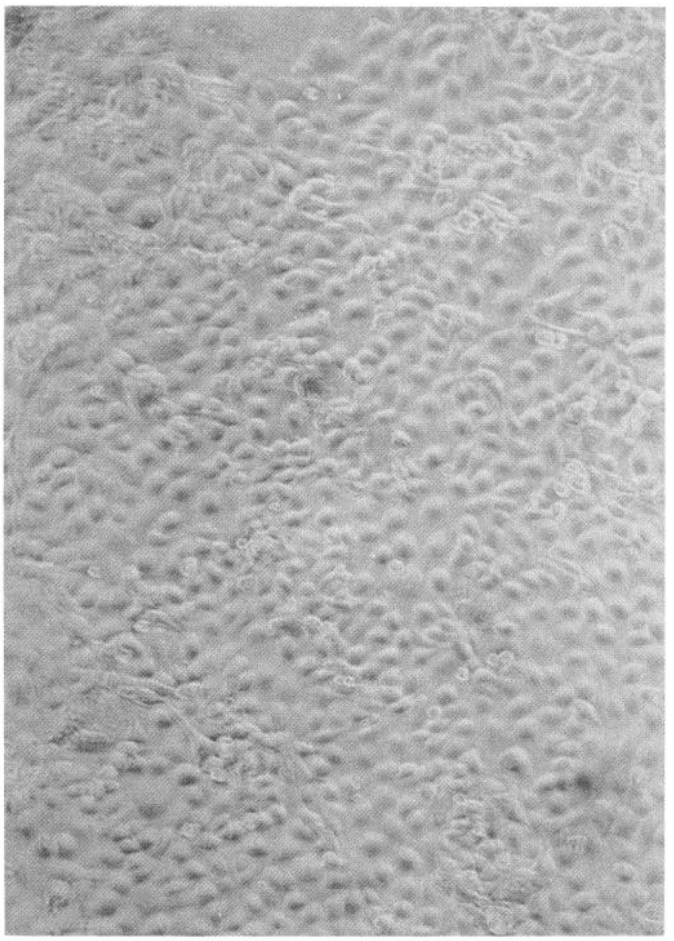

FIG. 1. Phase contrast micrograph of bovine pulmonary artery endothelial cells forming a monolayer on the side of a disposable polystyrene cuvette. Original magnification ×260.

Measurement of $[Ca^{2+}]_i$. The Ca^{2+}-sensitive fluorescent probe indo-1 (13) was used to determine cytosolic free Ca^{2+} concentration. The multiwavelength method of measurement was applied and has been described in more detail elsewhere (32). A M2100 pulsed laser (PARC, wavelength 337 nm, pulse duration 10 ns, pulse energy 550 μJ) was used for fluorescence excitation. The beam was focused on the front side of the tube with attached indo-1 loaded EC, (spot diameter 0.2 mm). The fluorescence spectrum was registered at wavelengths 350–600 nm by means of a 1254EW vidicon detector (PARC). The interval between each determination was 3 s. During $[Ca^{2+}]_i$ measurement the content of the cuvette was stirred using a motor-driven bar. The background fluorescence of the cuvette and autofluorescence of the cells were automatically subtracted. These parameters were determined after quenching of indo-1 fluorescence by 1 mM $MnCl_2$ in the presence of 10 μM ionomycin. $MnCl_2$ and ionomycin were added at the end of each experiment and the background for every individual cell preparation was measured. The resulting fluorescence spectrum of indo-1 was deconvoluted into two spectra corresponding to Ca^{2+} bound and Ca^{2+} free indo-1, this calculation is described in (32). $[Ca^{2+}]_i$ concentration in EC was deter-

mined from the equation

$$[Ca^{2+}]_i = Kd \cdot \frac{C_b}{C_f}$$

where K_d is the apparent dissociation constant, and C_f and C_b are concentrations of free and Ca^{2+}-bound indo-1 forms, respectively. To calculate $[Ca^{2+}]_i$ we used the K_d of 270 μM previously determined (32). This value accords well with previous data (13). All values of $[Ca^{2+}]_i$ given are means ± SD. Statistical significance was calculated according to Student's t test.

RESULTS

Figure 2 shows the influence of vasocative agents on $[Ca^{2+}]_i$ in cultured EC from human umbilical vein (*A*), aorta (*B*), human pulmonary artery (*C*), and bovine pulmonary artery (*D*). The measurement of $[Ca^{2+}]_i$ was carried out in M199 in the presence of 2 mM added Ca^{2+}. Under these conditions the resting level of $[Ca^{2+}]_i$ ranges from 100 to 200 nM. No significant differences in the basal level of $[Ca^{2+}]_i$ were found in EC from different vessels. The indo-1 fluorescence of several dozen cells in the monolayer is registered simultaneously and $[Ca^{2+}]_i$ is the mean level obtained from all these cells. In some cases the initial $[Ca^{2+}]_i$ was higher than 200 nM and occasionally exceeded 500 nM but fell to normal levels after a short period of mixing of the cuvette contents. The initial fall in $[Ca^{2+}]_i$ level was accompanied by a significant decrease in the total fluorescence of the cells. These effects are probably due to a small population of cells with high $[Ca^{2+}]_i$ that detach from the cuvette wall and are washed away during mixing. Furthermore, it cannot be excluded that the rise in initial resting $[Ca^{2+}]_i$ is due to activation of EC by stirring. In our experiments we waited until $[Ca^{2+}]_i$ decreased to the normal level and then added the solution of the test compound into the cuvette. From these experiments (Fig. 2), the following data were obtained. Basal levels of $[Ca^{2+}]_i$ in endothelial cells of human umbilical vein, aorta, pulmonary artery, and bovine pulmonary artery were 112 ± 8 (n = 6), 120 ± 23 (n = 7), 125 ± 17 (n = 6), and 104 ± 13 (SD) nM (n = 4), respectively. The peak levels of $[Ca^{2+}]_i$ in the presence of histamine and thrombin were 431 ± 30 (n = 3) and 452 ± 17 nM (n = 3), respectively, in umbilical vein endothelial cells, 461 ± 3 nM (n = 3) and 575 ± 35 (n = 3) in EC of human aorta, and 697 ± 30 nM (n = 3) and 994 ± 66 nM (n = 3) in EC of human pulmonary artery. We tested the effects of PAF and thrombin in EC of bovine pulmonary artery. The peak levels of $[Ca^{2+}]_i$ in response to PAF and thrombin in bovine EC were 453 ± 62 nM (n = 2) and 667 ± 60 nM (n = 2), respectively. Introduction of an agonist (Fig. 2) caused a rapid elevation of $[Ca^{2+}]_i$ (in most cases within several seconds). Afterwards, $[Ca^{2+}]_i$ decreased to a base-line level within 1–2 min (Fig. 2, *B–D*). However, in the case of EC from umbilical vein the fall of $[Ca^{2+}]_i$ was slower and sometimes did not reach a resting level within 5 min. In all human EC $[Ca^{2+}]_i$ rises in response to thrombin and histamine were recorded. In cultured EC from human aorta $[Ca^{2+}]_i$ also increased in response to ATP and bradykinin. The responses to histamine and thrombin were stable and observed in all preparations of human EC tested, whereas responses to ATP and bradykinin were expressed variably by human aortic cells. The effect of ATP was studied in five preparations of human aortic endothelial cells. Three of them responded to ATP. The level of $[Ca^{2+}]_i$ in response to this agonist was 255 ± 21 (SD) nM. The action of bradykinin was tested in four preparations of cells and was detected in three preparations. The peak level of $[Ca^{2+}]_i$ in the presence of bradykinin in these three preparations was 371 ± 39 (SD) nM. Typical results are shown in Fig. 2*B*. In bovine EC we have observed the effects of PAF and thrombin (Fig. 2*D*). We found no effect of carbachol (10^{-4} M), angiotensin II (40 μM), prostaglandin endoperoxide H_2 analogue U46619 (0.5 μM), vasopressin (1 μM), or atrial natriuretic factor (1 μM) on the $[Ca^{2+}]_i$ of cultured human EC.

As mentioned above, the histamine-induced increase in $[Ca^{2+}]_i$ as well as the thrombin effect were marked and stable. Therefore, we used histamine as a tool for a more detailed study of the mechanism of the receptor-mediated increase in $[Ca^{2+}]_i$ in EC. Figure 3 shows the dose-dependent curve for histamine-induced increase in $[Ca^{2+}]_i$ in EC from human umbilical vein. The effect is expressed in concentrations ranging from 5×10^{-7} to 10^{-4} M. As is seen in Fig. 3, higher doses of histamine resulted in a rise in both the peak level of $[Ca^{2+}]_i$ and an increase in the initial rate of $[Ca^{2+}]_i$ elevation. At 50–100 μM the $[Ca^{2+}]_i$ increase was maximal. The histamine-induced rise in $[Ca^{2+}]_i$ is mediated by H_1-receptors. As is shown in Fig. 4, pyrilamine, a blocker of H_1-receptors, progressively decreased the response to histamine while cimetidine, a specific H_2-receptor antagonist had no effect.

Receptor-mediated increases of $[Ca^{2+}]_i$ can result from Ca^{2+} influx from the extracellular medium and mobilization from intracellular nonmitochondrial stores. To evaluate the contribution of each source to the histamine-induced $[Ca^{2+}]_i$ rise, we measured the effect of histamine in medium without extracellular calcium in the presence of 1 mM EGTA or with 2 mM Ca^{2+} in the extracellular medium (Fig. 5). In Ca^{2+}-free medium the resting level of $[Ca^{2+}]_i$ was 124 ± 6 (SD) nM (n = 3) and the increment of $[Ca^{2+}]_i$ due to histamine was 295 ± 23 nM (n = 3). In the presence of external calcium the basal $[Ca^{2+}]_i$ level was 132 ± 11 nM (n = 3) and the increase after histamine administration was 315 ± 23 nM (n = 3). As is seen from these data, there was no marked decrease in histamine effect when the extracellular calcium was bound by excess EGTA, indicating that histamine-induced $[Ca^{2+}]_i$ increase can be accounted for by Ca^{2+} mobilization from intracellular stores.

In a number of types of cells it has been shown that their sensitivity to agonists may be regulated from the inner side of the plasma membrane. The key role in such regulation apparently is played by second messengers. For instance, in platelets the receptor-mediated increase in $[Ca^{2+}]_i$ is blocked when cAMP or cyclic guanylic acid (cGMP) levels are elevated (28, 39, 40). The Ca^{2+} blocking effect is also observed when endogenous protein kinase C is stimulated by phorbol myristate acetate

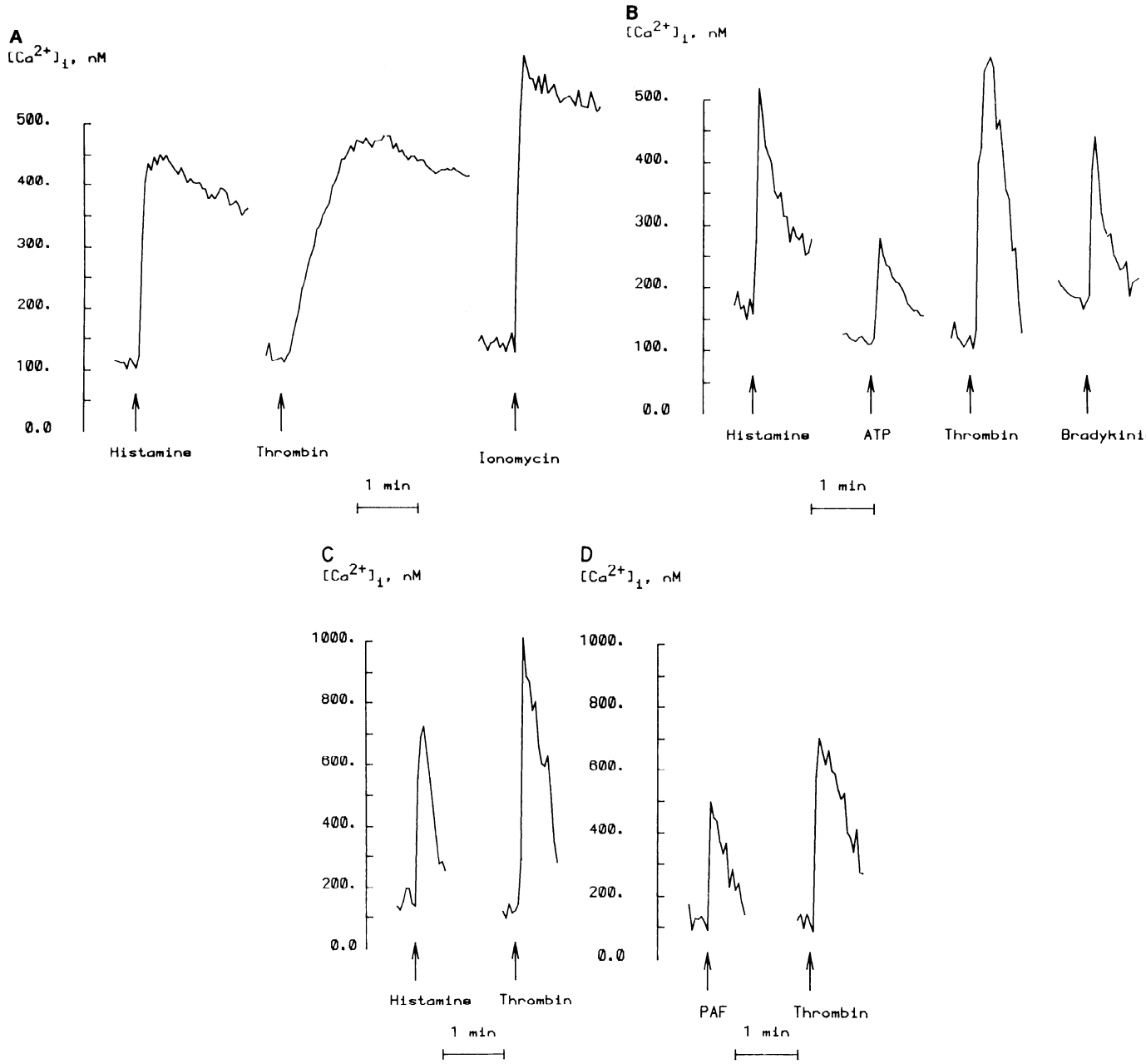

FIG. 2. The influence of histamine (100 μM), thrombin (1 U/ml), ATP (200 μM), bradykinin (10 μg/ml), or platelet-activating factor (PAF, 10^{-7} M) on cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in cultured endothelial cells of human umbilical vein (*A*), aorta (*B*), pulmonary artery (*C*), or bovine pulmonary artery (*D*). Cells were incubated in medium 199 with 2 mM additional exogeneous Ca^{2+} at 37°C. Traces are representative of several measurements.

(PMA) (2, 24). In the case of vascular EC, the question of how the receptor-mediated $[Ca^{2+}]_i$ increase is regulated by second messengers is unresolved. The results of the experiment in which we have studied the role of cyclic nucleotides and protein kinase C in $[Ca^{2+}]_i$ regulation are shown in Table 1. Human aortic EC were preincubated in the cuvette for 5 min at 37°C with PMA, 10^{-7} g/ml, or preincubated in the presence of 10^{-4} M isobuthylmethylxanthine (IBMX) with 5×10^{-5} M forskolin or 5×10^{-5} M nitroprusside to elevate intracellular levels of cAMP and cGMP, respectively, or under control conditions. In experiments with IBMX and forskolin preincubation, IBMX was added 30 s before forskolin. The values of $[Ca^{2+}]_i$ presented in Table 1 were obtained after preincubation just before histamine administration and when the peak response to histamine was reached. As shown from these results, neither IBMX with forskolin nor nitroprusside influenced basal $[Ca^{2+}]_i$. The small increase of $[Ca^{2+}]_i$ in the presence of PMA was not statistically significant. As seen in Table 1, preincubation with PMA considerably inhibits histamine-induced $[Ca^{2+}]_i$ elevation.

These data indicate that activation of endogenous protein kinase C blocks the receptor-mediated elevation of $[Ca^{2+}]_i$. Similar results were obtained previously with other cells (2, 24, 29). In addition to the supression of the histamine effect, a small elevation of basal $[Ca^{2+}]_i$ was sometimes observed. This increment was consider-

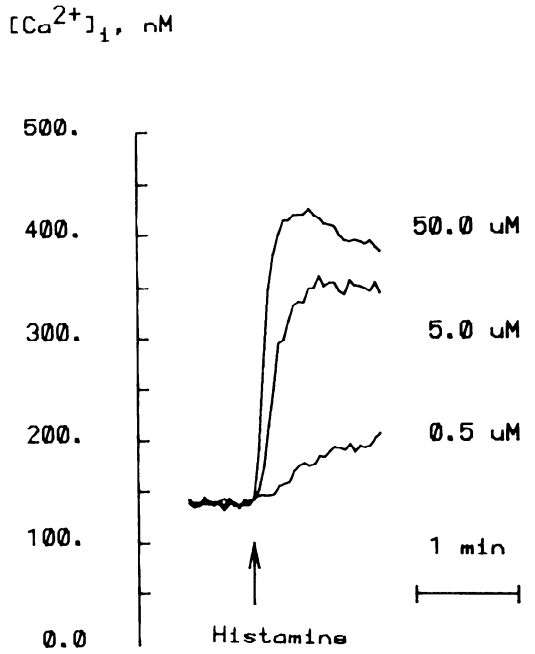

FIG. 3. Influence of different histamine concentrations on cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in endothelial cells from human umbilical vein. Each concentration was tested in 2 separate cuvettes. Cells were incubated in phosphate-buffered saline in presence of 1 mM $MgCl_2$ and 2 mM $CaCl_2$ at 20°C.

ably smaller than agonist-induced $[Ca^{2+}]_i$ increase. In contrast to the PMA effect, elevation of intracellular levels of cAMP and cGMP did not markedly decrease the histamine response (Table 1). Preincubation of EC with the adenylate cyclase stimulator. PGI_2, also had no effect on histamine-induced $[Ca^{2+}]_i$ elevation.

DISCUSSION

A new multiwavelength method described elsewhere (32) for measuring the concentration of $[Ca^{2+}]_i$ using the fluorescent probe indo-1, has been introduced in our study. The method is based on measurement of the indo-1 total fluorescence spectrum in the cell cytoplasm with its subsequent deconvolution into two components, representing the Ca^{2+} bound and free-probe forms. Another advantage of this method is the possibility of subtracting the whole spectrum of the background fluorescence that we measured by adding ionomycin and manganese at the end of each experiment. Using this method, we have studied the influence of different vasoactive substances on $[Ca^{2+}]_i$ in cultured EC from human umbilical vein, aorta, pulmonary artery, and bovine pulmonary artery. Histamine and thrombin caused a rapid elevation of $[Ca^{2+}]_i$ in EC of all human blood vessels tested. We have shown that in aortic EC $[Ca^{2+}]_i$ also rose in response to ATP and bradykinin. The effects of ATP and bradykinin were not so stable as those of histamine and thrombin, perhaps as a result of sensitivity of these receptors to culture conditions such as trypsinization. Earlier, studies using fura-2 and quin2 have shown that ATP increases $[Ca^{2+}]_i$ in piglet and calf aortic EC (14, 22). Regulation of $[Ca^{2+}]_i$ in pulmonary EC has not been previously studied. We have shown that EC of bovine pulmonary artery respond to PAF and thrombin by transient elevation of $[Ca^{2+}]_i$ (Fig. 2*D*). All the agents used in this study are known to induce release of PGI_2 and EDRF (7, 10, 25, 34, 38). Thus the data obtained support the hypothesis that elevation of $[Ca^{2+}]_i$ triggers these processes. Our results on the regulation of $[Ca^{2+}]_i$ in human umbilical vein EC by histamine confirm the recently published data (15, 33) showing that this agonist increases $[Ca^{2+}]_i$ in these cells via H_1-receptors. In our experiments (Fig. 5), as well as in experiments with quin2 loaded EC (33), binding of extracellular Ca^{2+} by EGTA did not influence the maximal $[Ca^{2+}]_i$ increase in response to histamine, whereas in (15), it was shown that binding of extracellular Ca^{2+} by EGTA decreases both

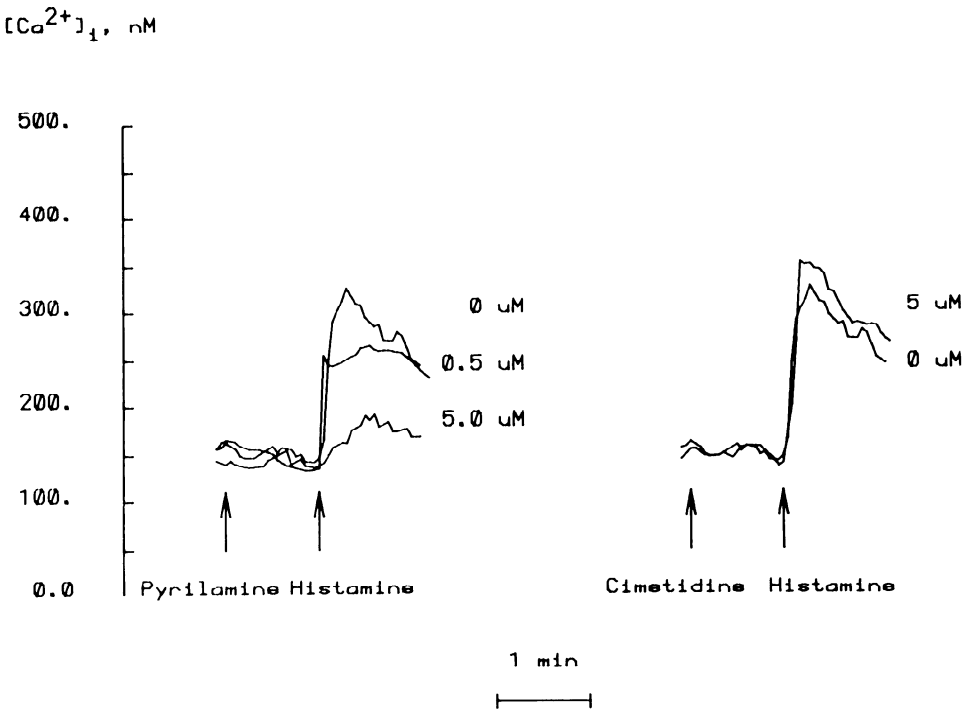

FIG. 4. Influence of pyrilamine and cimetidine on histamine-induced cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) elevation in human umbilical vein endothelial cells. Histamine concentration was 5 μM. Concentration of antagonist is shown at *right* of each curve. Incubations were performed (in replicates of 3) in medium 199 at 20°C in presence of 2 mM additional exogeneous Ca^{2+}.

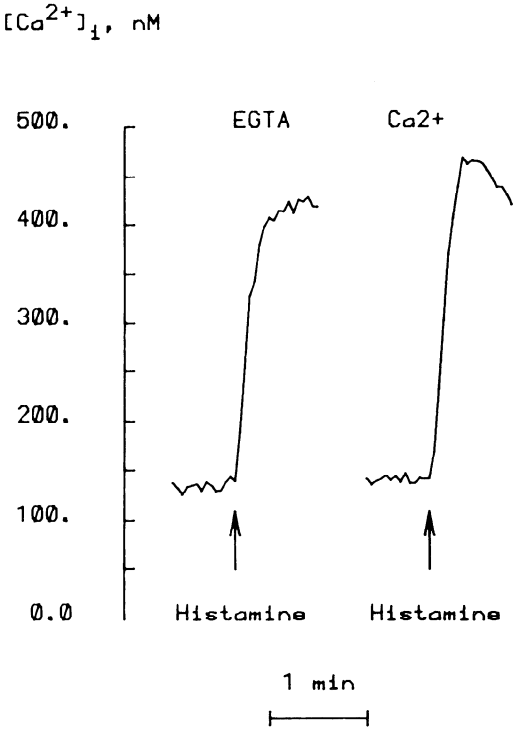

FIG. 5. Influence of histamine (50 μM) on cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in human umbilical vein endothelial cells in presence of 1 mM EGTA or 2 mM Ca^{2+}. $[Ca^{2+}]_i$ measurement was performed in phosphate-buffered saline in presence of 1 mM $MgCl_2$ at 20°C.

resting $[Ca^{2+}]_i$ level and the histamine-induced rise. Influx of extracellular Ca^{2+} is reportedly required for sustained elevation of $[Ca^{2+}]_i$ (33). We did not carry out extensive studies of the kinetics of $[Ca^{2+}]_i$ decrease.

In addition to the agonists mentioned above we also tested other vasoactive agents such as carbachol, atrial natriuretic peptide, thromboxane A_2 mimetic U46619, and angiotensin II on human EC. None of these compounds increased $[Ca^{2+}]_i$i in our experiments. This may be explained either by the absence of receptors for these agents coupled with Ca^{2+} mobilizing systems or by loss of receptors during culture.

It has been shown that stimulation of endogeneous protein kinase C in EC blocks the histamine-induced rise in $[Ca^{2+}]_i$. The inactive phorbol ester 4α-phorbol didecanoate did not influence the response of EC to histamine. Similar effects of PMA were observed on other types of cells (2, 24, 29). At least two hypotheses have been proposed to explain the mechanism of inhibition by protein kinase C on Ca^{2+} mobilizing mechanisms. Connolly et al. (6) suggested that feedback suppression of the rise in $[Ca^{2+}]_i$ can be ascribed to the stimulating effect of protein kinase C on inositol 1,4,5-trisphosphate 5′-phosphomonoesterase. An alternative hypothesis suggests that protein kinase C inhibits signal transmission from agonist receptors via the plasma membrane due to inactivation of GTP binding protein (3, 19). Both these hypotheses are based on data obtained from experiments using human platelets. Further experiments are necessary to establish the mechanism responsible for the Ca^{2+} blocking effect of protein kinase C in EC.

We have shown that cyclic nucleotides do not block the receptor-mediated Ca^{2+} increase in endothelial cells, and in this respect, endothelial cells differ from some other cell types such as smooth muscle cells where cyclic GMP blocks $[Ca^{2+}]_i$ increase (4), platelets where both cAMP and cGMP block $[Ca^{2+}]_i$ increase (28, 39, 40).

All in all, the data reported here are consistent with the hypothesis that signal transduction in endothelial cells involves a receptor mediated rise in $[Ca^{2+}]_i$. In complementary studies using combined techniques for calcium flux measurement and patch clamping, we have suggested that the initial rise is probably a result of mobilization from intracellular stores that are subsequently replenished by inward calcium movement from extracellular sources via receptor operated ion channels (18).

TABLE 1. *Effects of PMA, IBMX and forskolin, and nitroprusside on histamine-induced $[Ca^{2+}]_i$ elevation in human aortic endothelial cells*

	$[Ca^{2+}]_i$, nM	
	Before histamine	After histamine
Preincubation without effectors	113±24	397±25
PMA (10^{-7} g/ml)	147±20†	206±16*
Forskolin (5×10^{-5} M) and IBMX (10^{-4} M)	124±9†	375±18†
Nitroprusside (5×10^{-5} M)	108±14†	339±21†

Values are means ± SD of 3 parallel determinations. $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; PMA, phorbol myristate acetate; IBMX, isobutylmethylxanthine. * $P < 0, 0.01$; † not significant.

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