Nitric oxide production in human endothelial cells stimulated by histamine requires Ca^{2+} influx

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The causal relationships between cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) increases and production of nitric oxide (NO) have been investigated mostly with indirect methods and remain unclear. Here we demonstrate, by direct real-time measurements of [NO] with a porphyrinic microsensor, that Ca^{2+} entry, but not an increase in $[Ca^{2+}]_i$, is required for triggering of NO production in human endothelial cells. Histamine, ranging from 0.1 to 100 μM, increased both NO production and $[Ca^{2+}]_i$ when given in a single dose. However, histamine caused increased NO release but induced progressively smaller $[Ca^{2+}]_i$ changes when cumulatively added. In the absence of a transmembrane Ca^{2+} gradient, no significant NO release was detectable, despite the marked Ca^{2+} peak induced by histamine. Inhibition of Ca^{2+} entry by SK&F 96365 abolished histamine-elicited NO production but only reduced the transient $[Ca^{2+}]_i$ rise. The suppression of the sustained $[Ca^{2+}]$i response under these two conditions suggests that NO release was closely associated with Ca^{2+} entry from the extracellular space. In addition, membrane depolarization, achieved by increasing the extracellular K^+ concentration from 5 to 130 mM, reduced both the amplitude of histamine-induced sustained $[Ca^{2+}]_i$ elevation and NO production. These results lead us to propose that the availability of numerous Ca^{2+} ions around the internal side of the plasma membrane would promote the association between nitric oxide synthase and calmodulin, thereby activating the enzyme.

INTRODUCTION

The vascular endothelium responds to various agonists and shear flow by producing a potent vasodilator, first called endothelium-derived relaxing factor by Furchgott and Zawadzki [1], and then identified as nitric oxide (NO) by Ignarro et al. [2] and Palmer et al. [3]. NO also prevents platelet adhesion to endothelial cells and inhibits their aggregation [4]. Endothelial cells constitutively express NO synthases (NOS), which synthesize NO from L-arginine [5]. It is now well established that the activation of constitutive NOS depends on the presence of various cofactors and on a conformational change induced by the interaction of the Ca–calmodulin complex with the enzyme [6]. Studies *in vitro* on purified enzymes indicate that such a mechanism occurs at free Ca^{2+} concentrations above 100 nM [7], suggesting that NO production is controlled by increases in the cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$). Endothelial cells respond to various agonists by a biphasic $[Ca^{2+}]_i$ variation: a transient peak followed by a sustained elevation, corresponding respectively to Ca^{2+} release from internal stores and Ca^{2+} entry from the extracellular space [8]. These agonist-induced $[Ca^{2+}]_i$ increases have been reported to control NO production. However, due to the difficulties in directly measuring NO, most of the previous studies on the relation between cytosolic Ca^{2+} movements and NO release have investigated the accumulation of nitrites, one of its oxidation metabolites [9], or of cGMP, considered to be its cytosolic effector [10–12]. Only a few studies have investigated this relation by directly measuring NO with electrochemical [13–15] or chemiluminescence methods [16], and the results remain controversial. Some authors observed a parallel between NO concentration and cytosolic Ca^{2+} level [14,15], whereas others demonstrated a major role either for Ca^{2+} release from internal stores [16] or for Ca^{2+} influx [9,11].

The present study was undertaken to investigate, by real-time measurement of [NO] with a porphyrinic microsensor, the role of the cytosolic Ca^{2+} level in the regulation of NO production elicited by histamine in human umbilical vein endothelial cells (HUVEC). The work provides evidence that NO release increases with histamine concentration, independently of the cytosolic Ca^{2+} levels reached, and demonstrates that Ca^{2+} influx from the extracellular space is required to initiate NO synthesis.

MATERIALS AND METHODS

Materials

Histamine dihydrochloride, EGTA, Hepes, thapsigargin, NO gas and Nafion film were from Sigma-Aldrich Chimie (St Quentin Fallavier, France). Collagenase A was obtained from Boehringer (Mannheim, Germany). Medium 199 was obtained from Eurobio (Les Ulis, France). RPMI 1640 medium, Hanks balanced salt solution, fetal calf serum, L-glutamine, penicillin/streptomycin, fungizone and PBS were from Gibco-BRL (Cergy Pontoise, France). Fura2 acetoxymethyl ester (fura2 AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Tetrakis(3-methoxy-4-hydroxyphenyl)nickel(II) porphyrin was obtained from Interchim (Paris, France).

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free-Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; HUVEC, human umbilical vein endothelial cells; NOS, nitric oxide synthase.

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Cell culture

Endothelial cells were isolated from segments of human umbilical cord vein and cultured in medium 199 (40 %) and RPMI 1640 (40 %) containing fetal calf serum (20 %, v/v), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and fungizone (2.5 μg/ml), as previously described [17]. The medium was renewed every 2 days until confluence (4–5 days), cells were then detached by incubation in PBS containing 0.01 % trypsin and 0.004 % EDTA for 1–2 min at room temperature, washed by centrifugation and re-seeded onto 35-mm plastic culture dishes (Corning, New York, NY, U.S.A.) for NO measurements and onto glass coverslips (9 × 35 mm) in Leighton tubes for [Ca^{2+}] determinations. The cells were then grown to confluence (3–5 days) until use.

Measurement of NO

NO release was monitored with an NO-selective microsensor [18]. The working electrode was made of carbon microfibre (8 μm diameter, approximately 1 mm length), coated by tetrakis(3-methoxy-4-hydroxyphenyl)nickel(II) porphyrin and Nafion films. Differential-pulse amperometry was performed with a three-electrode potentiostatic biopulse system (Tacussel, Lyon, France) as described previously [18].

The cells were incubated in PBS medium complemented with 5 mM glucose/0.5 mM $MgCl_2$/1 mM $CaCl_2$. The use of a micromanipulator (Biologic Instruments, Claix, France) attached to the stage of an inverted microscope (Zeiss, Germany) allowed the probe to be positioned 10–15 μm above the cell surface, all the apparatus being enclosed in a Faraday chamber. At the end of each experiment, the electrochemical sensor was calibrated by addition of NO standard solutions *in situ*, as detailed previously [18]. The sensitivity of electrodes varied from 0.5 to 2.6 nM NO/pA (mean 1.6 ± 0.4 nM NO/pA, $n = 9$) with a detection limit varying from 2 to 5 nM NO.

The identification of NO as being responsible for the amperometric signal induced by histamine was assessed through the effects of preincubation with L-arginine and N^G-monomethyl-L-arginine as previously described [19].

To suppress the transmembrane Ca^{2+} gradient, experiments were performed in the presence of 50 nM external Ca^{2+} obtained by adding 0.1 mM EGTA and 42 μM $CaCl_2$ to PBS. The presence of 0.1 mM EGTA did not modify the amperometric signal of 20 nM NO standard solutions (14.8 ± 2.1 versus 14.4 ± 0.8 pA in the presence and absence of EGTA, $n = 8$ for each).

Determination of cytosolic Ca^{2+} concentration

$[Ca^{2+}]_i$ was determined as described previously [17]. Briefly, cells grown on glass coverslips in Leighton tubes were transferred into buffer A (in mM: NaCl 136, KCl 5, NaH_2PO_4 2, $MgSO_4$ 0.4, $NaHCO_3$ 4, $CaCl_2$ 1, glucose 8, Hepes 25, pH 7.4, at 37 °C) containing 20 % (v/v) fetal calf serum, a cocktail of amino acids and 2 mM glutamine. They were first equilibrated in buffer A for 30 min at 37 °C, then loaded with 2 μM fura2 acetomethoxy ester for 25 min. De-esterification was completed in fresh buffer A containing 20 % (v/v) fetal calf serum by a further 25 min incubation at 37 °C. To record fluorescence signals, cells were washed twice with buffer A, and the coverslip was inserted into a special quartz-suprasil cuvette thermostated at 37 °C [20]. Fura2 was excited at 340 and 380 nm and emission was collected at 505 nm on a spectrofluorimeter SPEX CMIII (ISA-Jobin-Yvon, Longjumeau, France). $[Ca^{2+}]_i$ was calculated from the 340/380 ratios of fluorescence intensities using the equation of Grynkiewicz et al. [21] and the K_d and calibration parameters previously determined [17].

Statistical analysis

Results are expressed as means ± S.E.M. Dose–response effects were analysed by one-way analysis of variance, and the variance (F) with degrees of freedom as subscripts were given. Significance of difference between control and treated cells was assessed by using unpaired Student's t-test with $P < 0.05$ considered statistically significant.

RESULTS

Effect of histamine on NO production and $[Ca^{2+}]_i$ in HUVEC

The real-time measurement of NO production with a porphyrinic microsensor positioned close to the cell surface showed that, in HUVEC, histamine (1–10 μM) induced a rapid and transient NO release, reaching a peak within 20 s (Figure 1A). Histamine, at a concentration of 100 μM, increased NO production up to 80 nM, with maintenance of high NO levels for more than 2 min. When cells were repeatedly stimulated with increasing concentrations of histamine, profiles of NO production were similar (Figure 1B). The dose–response curves to single or repeated

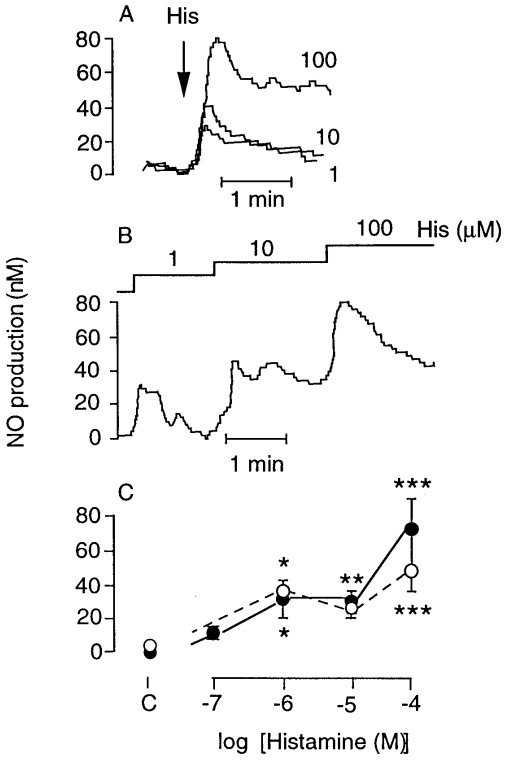

Figure 1 Effect of histamine on NO production in human endothelial cells

Cells were washed then incubated in PBS containing 1 mM external $CaCl_2$. (**A**) Single doses of different histamine concentrations were added as indicated by the arrow. (**B**) Cumulative doses of histamine were successively given. (**C**) Dose-dependent curves of cumulative (○) and independent stimulations by histamine (●) on NO production with respectively, $F_{3,27} = 6.29$, $P = 0.02$ and $F_{4,22} = 9.43$, $P < 0.001$. Data were from 4–15 independent experiments, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with control 'C' values determined in the absence of histamine.

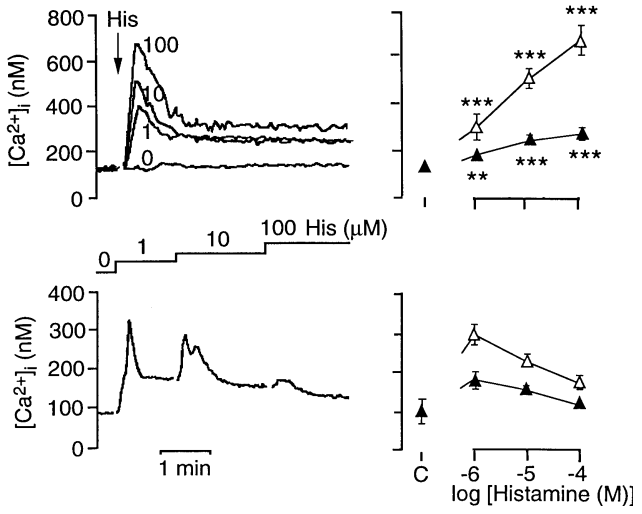

Figure 2 Effect of histamine on $[Ca^{2+}]_i$ in human endothelial cells

$[Ca^{2+}]_i$ was determined in fura2-loaded cells incubated in buffer A containing 1 mM $CaCl_2$. Upper panel: time-courses of $[Ca^{2+}]_i$ increases elicited by single doses of histamine (His) at different concentrations (left) and dose–response curves for Ca^{2+} peak (△) and plateau (▲) ($F_{3,40} = 42.78$ and 33.67, $P < 0.001$ respectively) (right). $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with control (C) values determined in the absence of histamine. Lower panel: time-courses of $[Ca^{2+}]_i$ increases induced by cumulative doses of histamine (left) and dose–response curves for Ca^{2+} peak (△) ($F_{3,6} = 11.54$, $P = 0.009$) and plateau (▲) (right).

stimulations did not significantly differ as assessed by two-way analysis of variance ($P > 0.05$) (Figure 1C).

In contrast to NO production, the histamine-elicited Ca^{2+} movements differed depending on whether single or cumulative doses were added (Figure 2). When cells were stimulated with a single dose, histamine induced a biphasic increase in $[Ca^{2+}]_i$, with a transient peak occurring 15–20 s after stimulation, followed by a long-lasting plateau (Figure 2, upper panel). Under these conditions, the histamine effects were dose-dependent on both peak and plateau. When cells were cumulatively stimulated, both the transient and sustained $[Ca^{2+}]_i$ rises decreased progressively with increasing histamine concentrations (Figure 2, lower panel).

This constitutes the first evidence for a lack of an association between NO production and cytosolic Ca^{2+} levels in stimulated endothelial cells.

Influence of Ca^{2+}-influx on NO production and $[Ca^{2+}]_i$ variation

To investigate in more detail the link between the levels of NO and those of Ca^{2+} in the cytosol, [NO] and $[Ca^{2+}]_i$ were determined in HUVEC treated with single doses of 10 μM histamine in both the presence and absence of a transmembrane Ca^{2+} gradient [1 mM and 50 nM extracellular calcium concentration ($[Ca^{2+}]_o$) respectively]. At 50 nM $[Ca^{2+}]_o$, the sustained $[Ca^{2+}]_i$ increase was suppressed and the marked transient Ca^{2+} rise was not associated with NO production, although its amplitude is similar to that obtained at 1 mM $[Ca^{2+}]_o$ (Table 1). Such results were also observed when HUVEC were pretreated with SK&F 96365, an inhibitor of receptor-activated Ca^{2+} influx, which, at 30 μM, totally inhibited histamine-activated Ca^{2+} entry but elicited Ca^{2+} release from internal stores [22,23]. Both the histamine-induced NO production and sustained $[Ca^{2+}]_i$ responses were suppressed, whereas the amplitude of the Ca^{2+} peak was only reduced (Table 1). These results indicate that, in histamine-stimulated HUVEC,

Table 1 Influence of external Ca^{2+} and SK&F 96365 on histamine-induced NO production and $[Ca^{2+}]_i$ increases

Cells were incubated for 6 min at 37 °C in buffer A containing 1 mM or 50 nM $[Ca^{2+}]_o$ with or without 30 μM SK&F 96365. The effects of 10 μM histamine on [NO] and $[Ca^{2+}]_i$ are expressed as the differences between values of maximum or sustained increases and of basal levels. Results are from 4–12 independent experiments. Statistical analysis: $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with control values determined in 1 mM $[Ca^{2+}]_o$ only.

		$[Ca^{2+}]_i$ increase (nM)	
$[Ca^{2+}]_o$	NO production (nM)	Peak	Plateau
1 mM	24 ± 5	442 ± 41	153 ± 18
50 nM	2 ± 2**	385 ± 40	6 ± 5***
1 mM + SK&F 96365	Below detectable threshold	116 ± 20***	11 ± 7***

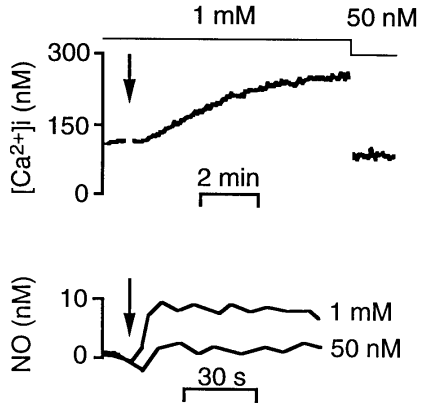

Figure 3 Influence of the extracellular Ca^{2+} concentration on ryanodine-induced $[Ca^{2+}]_i$ increase and NO production

$[Ca^{2+}]_i$ and NO production were determined in cells incubated in the presence of 1 mM or 50 nM $[Ca^{2+}]_o$. Ryanodine (20 μM) was added as indicated by the arrow. Traces are representative of five independent experiments.

NO release is closely associated with the sustained $[Ca^{2+}]_i$ response.

The role of Ca^{2+} influx in NO production was also demonstrated by experiments performed with ryanodine. In endothelial cells of various origins, ryanodine activates Ca^{2+} release from Ins(1,4,5)P_3-sensitive Ca^{2+} pools [24]. As shown in Figure 3, suppression of the transmembrane Ca^{2+} gradient decreased the ryanodine-induced $[Ca^{2+}]_i$ increase, indicating the activation of Ca^{2+} entry from the extracellular space. The ryanodine-activated Ca^{2+} influx was associated with slight but significant NO production, which was abolished in the absence of a transmembrane Ca^{2+} gradient (Figure 3).

The close relation of NO production to Ca^{2+} influx was confirmed by the effect of membrane depolarization in cells stimulated by histamine. Membrane depolarization by incubating HUVEC in high-K^+ buffer, before histamine stimulation, did not significantly affect the amplitude of the Ca^{2+} peak but decreased both the sustained $[Ca^{2+}]_i$ increase and NO production (Table 2).

DISCUSSION

Our results demonstrate that NO production by endothelial cells requires Ca^{2+} entry from the extracellular space and does not

Table 2 Influence of the external K^+ concentration on histamine-induced NO production and $[Ca^{2+}]_i$ increases

Cells were incubated for 5 min at 37 °C in either 5 mM or 135 mM external K^+ concentration, then stimulated with 10 μM histamine. The effects of histamine on [NO] and $[Ca^{2+}]_i$ are expressed as differences between values of peak or plateau values and basal levels. Results are from six independent experiments. Statistical analysis: $^*P < 0.05$ when compared with values determined in 5 mM external K^+ concentration.

External K^+ (mM)	NO production (nM)	$[Ca^{2+}]_i$ increase (nM)	
		Peak	Plateau
5	16.3 ± 2.7	250 ± 36	109 ± 4
135	6.5 ± 3.0*	230 ± 12	88 ± 6*

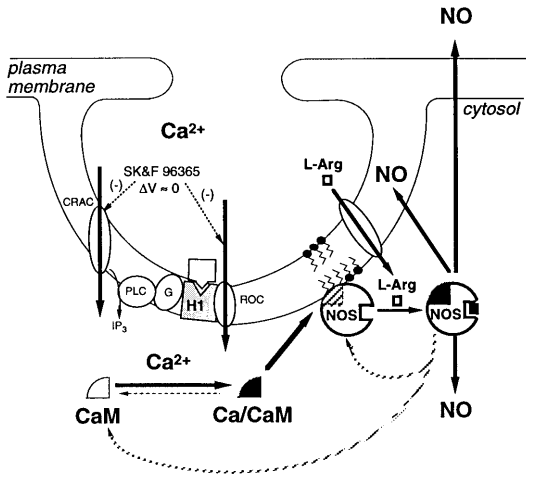

Figure 4 Model for the regulation by Ca^{2+} influx of endothelial NOS in caveolae

CaM, calmodulin; Ca/CaM, Ca–calmodulin complex; Ca^{2+}, free Ca^{2+} concentration; CRAC, Ca^{2+}-release-activated channel; NOS, nitric oxide synthase; G, G-protein; H1, histamine receptor; IP_3, Ins(1,4,5)P_3; L-Arg, L-arginine; PLC, phospholipase C; ROC, receptor-operated channel.

depend on the global increase in cytosolic Ca^{2+} concentration. This proposal is based on the following convergent observations: (1) the histamine-induced NO release was not associated with the cytosolic Ca^{2+} levels reached, since NO production continued increasing while $[Ca^{2+}]_i$ rises decreased during repeated stimulations; (2) independently of the way by which Ca^{2+} influx was activated, NO production was abolished when the transmembrane Ca^{2+} gradient was suppressed; (3) irrespective of the way by which Ca^{2+} influx was reduced, NO release was decreased.

In the present study, single or cumulative doses of histamine had a similar activating effect on NO production. In contrast, the effects of histamine on $[Ca^{2+}]_i$ differed between the two modes of histamine administration. The lack of parallelism between $[Ca^{2+}]_i$ and NO responses under repeated stimulations could be explained by the following hypothesis. At high doses of histamine, minimal Ca^{2+} requirements may be satisfied and other processes may now control NOS activation. The desensitization associated with receptor internalization during repeated stimulation [25] could also explain the trend towards the lowered NO production observed with 100 μM histamine during repeated stimulation. Another possibility is the control of NO synthesis by Ca^{2+} entry itself, or by the resulting submembrane local Ca^{2+} concentration. In HUVEC, it has been demonstrated that the progressively reduced amplitudes of $[Ca^{2+}]_i$ peaks during repeated stimulation by histamine reflected the filling-state of internal Ca^{2+} stores which were not refilled as long as the agonist was bound to its receptor [26]. The decreased $[Ca^{2+}]_i$ responses we observed during repeated stimulation by histamine may also reflect the activation of Ca^{2+} recapture or extrusion mechanisms, thereby masking the increasing activation of Ca^{2+} entry observed in cells stimulated with single doses. In cumulatively stimulated bovine coronary venular endothelial cells, the bradykinin-induced decreases in sustained $[Ca^{2+}]_i$ increases have been demonstrated to be associated with hyperpolarized membrane potentials and to reflect activation of Ca^{2+} extrusion [26]. Bradykinin induces membrane hyperpolarization by opening K^+ channels responsible for Ca^{2+} entry, but also activates cGMP formation [27]. Thus, the similar profiles of NO production observed under the two conditions of histamine stimulation could reflect a comparable activation of Ca^{2+} currents.

The role of ionic currents in stimulation of NO synthesis by endothelial cells has mostly been investigated with indirect methods and remains unclear. Cell membrane depolarization with high-K^+ buffer, or the selective blockage of agonist-activated K^+ channels with tetraethyl- or tetrabutyl-ammonium, suppressed the bradykinin-induced sustained $[Ca^{2+}]_i$ increases, the stimulation of guanylate cyclase activity and the production of cGMP [28–30]. In HUVEC, both tetrabutyl-ammonium and SK&F 96365 suppressed the histamine-induced cation currents, increases in $[Ca^{2+}]_i$ and cGMP formation [29]. This differs from the recent observation by Kishi et al. [15] using electrochemical methods, who showed that pig aortic endothelial cells stimulated by high histamine concentration produced NO in both the presence and absence of Ca^{2+} influx. In bovine aortic endothelial cells, the nitrite production induced by bradykinin, ATP or thapsigargin was reduced to basal levels by the suppression of the transmembrane Ca^{2+} gradient or by the addition of Ni^{2+}, a non-specific divalent cation-channel blocker [9]. The thapsigargin-activated NO formation evaluated by chemiluminescence was also greatly reduced in the absence of the transmembrane Ca^{2+} gradient [15]. Our study demonstrates by direct real-time measurement of [NO] that membrane depolarization reduces both the histamine-elicited NO production and the sustained $[Ca^{2+}]_i$ increases, and that the suppression of the transmembrane Ca^{2+} gradient, as well as SK&F 96365 treatment, abolishes them. We also observed that neither thapsigargin [23] nor ryanodine activated NO production in the absence of a transmembrane Ca^{2+} gradient. This suggests that NO synthesis at the endothelial cell surface could be directly controlled by Ca^{2+} entry from the extracellular space.

NO is synthesized by NOS [5]. This enzyme is translocated from membrane to cytosol during stimulation of endothelial cells by agonist [31] and activated by the Ca–calmodulin complex [6]. The recent demonstrations of involvement of the calmodulin-binding domain of NOS in its association with membrane [32,33], and the strict relation of NO synthesis to Ca^{2+} influx observed here, led us to propose a model for regulation of endothelial NOS (Figure 4). In activated endothelial cells, the local availability of numerous Ca^{2+} ions around the internal side of the plasma membrane increases the occupancy of Ca^{2+}-binding sites in calmodulin. This could induce the formation of the NOS–Ca–calmodulin complex, thereby dissociating the enzyme from the plasma membrane and activating its catalytic activity. Such a mechanism is supported by the previous observation that agonists concomitantly increase both L-arginine uptake and

cGMP formation in endothelial cells [34]. The increase in L-arginine concentration in a submembrane domain close to NOS may provide its calmodulin-activated form with substrate (Figure 4). The recent demonstrations of NOS localization in caveolae [35,36] are consistent with the existence of an NOS regulatory system in such specialized membrane areas, where different proteins involved in signal transduction are also concentrated [37,38].

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