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Identification of Membrane Calcium Channels Essential for Cytoplasmic and Nuclear Calcium Elevations Induced by Vascular Endothelial Growth Factor in Human Endothelial Cells

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Vascular endothelial growth factor (VEGF) is mitogenic for endothelial cells and has been shown to induce angiogenesis and endothelial cell migration through stimulation of endothelial tyrosine-kinase receptors. Here, using confocal microscopy and the patch-clamp technique on endothelial cells, membrane permeability to calcium as well as cytoplasmic and nuclear free calcium levels have been investigated in the first stages of tyrosine-kinase receptor activation by VEGF. VEGF (0.5 nM) as well as inositol trisphosphate (IP_3) induced an activation of membrane calcium-permeable channels exhibiting a similar low conductance in the range of 10 pS. The VEGF-triggered activation of these calcium channels, mediated by IP_3 and involving the intracellular calcium stores, results in an increase in both cytoplasmic and nuclear calcium levels in endothelial cells, potentially modulating gene expression. Finally, the effect of Ni^{2+}, a calcium channel blocker, on endothelial cell proliferation has been studied. The results show that inhibition of extracellular calcium influx significantly inhibits VEGF-induced cell proliferation. In the process of cell stimulation by VEGF, and possibly by other growth factors, activation of calcium channels could then be a key step in calcium-regulated gene expression and cell activation. These results suggest that the use of calcium channel blockers could be a novel way of prevention or reversion of VEGF-induced tumoral angiogenesis.

Keywords: Vascular endothelial growth factor, human umbilical venous endothelial cells, calcium channels, nucleus, proliferation

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INTRODUCTION

Vascular endothelial growth factors (VEGFs), also known as vascular permeability factors, are a family of small dimeric glycoprotein presenting some homology to the platelet-derived growth factor (Ferrara *et al.*, 1992) and secreted by a wide variety of cell types, including keratinocytes and cells from the lung, kidney, heart and other organs (Brown *et al.*, 1992; Berse *et al.*, 1992). VEGFs have been shown to induce blood vessel permeability and strong proliferation of vascular endothelial cells through a specific binding to endothelial membrane receptors. This is different from other growth factors, such as fibroblast growth factors 1 and 2, which exert their mitogenic actions on a wide variety of cell types (Ferrara *et al.*, 1992; Toi, 1995). In particular, VEGFs action is essential in embryonic vascularization and tumoral angiogenesis (Breier and Risau, 1996; Ferrara and Davis-Smith, 1997), as well as in the angiogenic activity occurring during the proliferative phase of wound healing (Nissen *et al.*, 1998).

Physiological or pathological angiogenesis is a complex multistep process resulting from local activation of genes encoding diffusible angiogenic factors or from release of stored presynthesized factors, particularly growth factors (Hanahan, 1997; Klagsburn and D'Amore, 1991; Vernon and Sage, 1995). It has been shown that VEGFs are essential in vascular signalling of these processes (Nicosia *et al.*, 1994). Two high affinity tyrosine-kinase receptors for VEGFs have been characterized (VEGFR1/Flt-1 and VEGFR2/Kdr/Flk-1) (Mustonen and Aliato, 1995), and binding of VEGFs to their receptors initiates tyrosine phosphorylation of cytosolic target proteins and generates activation of several cellular pathways (Soltoff and Cantley, 1988). One of the major events in growth factor-stimulated cells is activation of a cascade of kinases and phosphatases leading to: (i) the translocation into the nucleus of proteins inducing oncogene expression, and (ii) the stimulation of phosphatidylinositol biphosphate degradation and release of diacylglycerol (a protein kinase C stimulator) and inositol trisphosphate (IP_3) (Berridge and Irvine, 1988; Jaye *et al.*, 1992), both being implicated in growth stimulation. IP_3 releases calcium from internal stores and also stimulates extracellular calcium influx through plasma membrane channels, thereby raising the intracellular free calcium concentration ($[Ca^{2+}]i$) (Vaca and Kunze, 1995; Kuno and Gardner, 1987; Merle *et al.*, 1997; Selinfreund and Blair, 1994). In many cell types, elevation of $[Ca^{2+}]i$ has been shown to be one of the early events immediately following the binding of growth factors, including VEGFs (Vaca and Kunze, 1995; Merle *et al.*, 1997; Brock *et al.*, 1991; Merle *et al.*, 1995). Free calcium, playing the role of third messenger controlled by its binding to calmodulin, then initiates and regulates subsequent phosphorylation of calcium-dependent kinases or phosphatases (Delphin *et al.*, 1997), and activates gene transcription via cyclic-AMP-response element stimulation (Hardingham *et al.*, 1997). In addition, it has been reported that induction of replicative competence by growth factors is dependent upon the maintenance of a sustained increase in the $[Ca^{2+}]i$ due to the influx of extracellular calcium (Estacion and Mordan, 1993; Mogami and Kojima, 1993).

In this context, the present study was undertaken to determine the action of recombinant human VEGF (rhVEGF), whose sequence is identical to one of the most active VEGFs ($VEGF_{165}$) (Ferrara *et al.*, 1992), on the permeability of membrane calcium channels and subsequent $[Ca^{2+}]i$ increase in human endothelial cells. We have shown that human umbilical venous endothelial cells (HUVECs) express VEGF-activated calcium permeable channels, whose activation could be, at least in part, triggered by IP_3. In addition, we have observed that VEGF triggers IP_3 production as well as cyclic elevations of the free calcium concentration ($[Ca^{2+}]$) in both the nucleus and the cytosol. Finally, neither depletion of intracellular calcium stores nor blockage of extracellular calcium influx were able to abolish VEGF-induced $[Ca^{2+}]i$ increase, although absence of extracellular

calcium influx was the most efficient inhibitor, suggesting that the contribution of extracellular calcium influx is major. In accordance with previous studies (Vaca and Kunze, 1995; Merle *et al.*, 1997; Selinfreund and Blair, 1994; Merle *et al.*, 1995), these results confirm that one of the first steps of tyrosine-kinase receptor activation in endothelial cells is the increase in $[Ca^{2+}]i$ via mobilization of calcium from both internal stores and, mainly, extracellular origin.

EXPERIMENTAL PROCEDURES

Cell Culture

Human umbilical venous endothelial cells (HUVEC) were isolated using the Jaffe technique, adapted as described (Jaffe *et al.*, 1973; Faury *et al.* 1998). Cells were removed from the umbilical vein by a 10 minute collagenase 1A incubation. The endothelial cells were pelleted an resuspended in medium 199 containing 22% human serum, 0.1 mg ml^{-1} streptomycin, 100 UI ml^{-1} penicillin and 2 mM L-glutamine, then placed in 0.25 mg ml^{-1} fibronectin-pre-coated dishes. Only first to third passage HUVEC were then used in the experiments.

VEGF

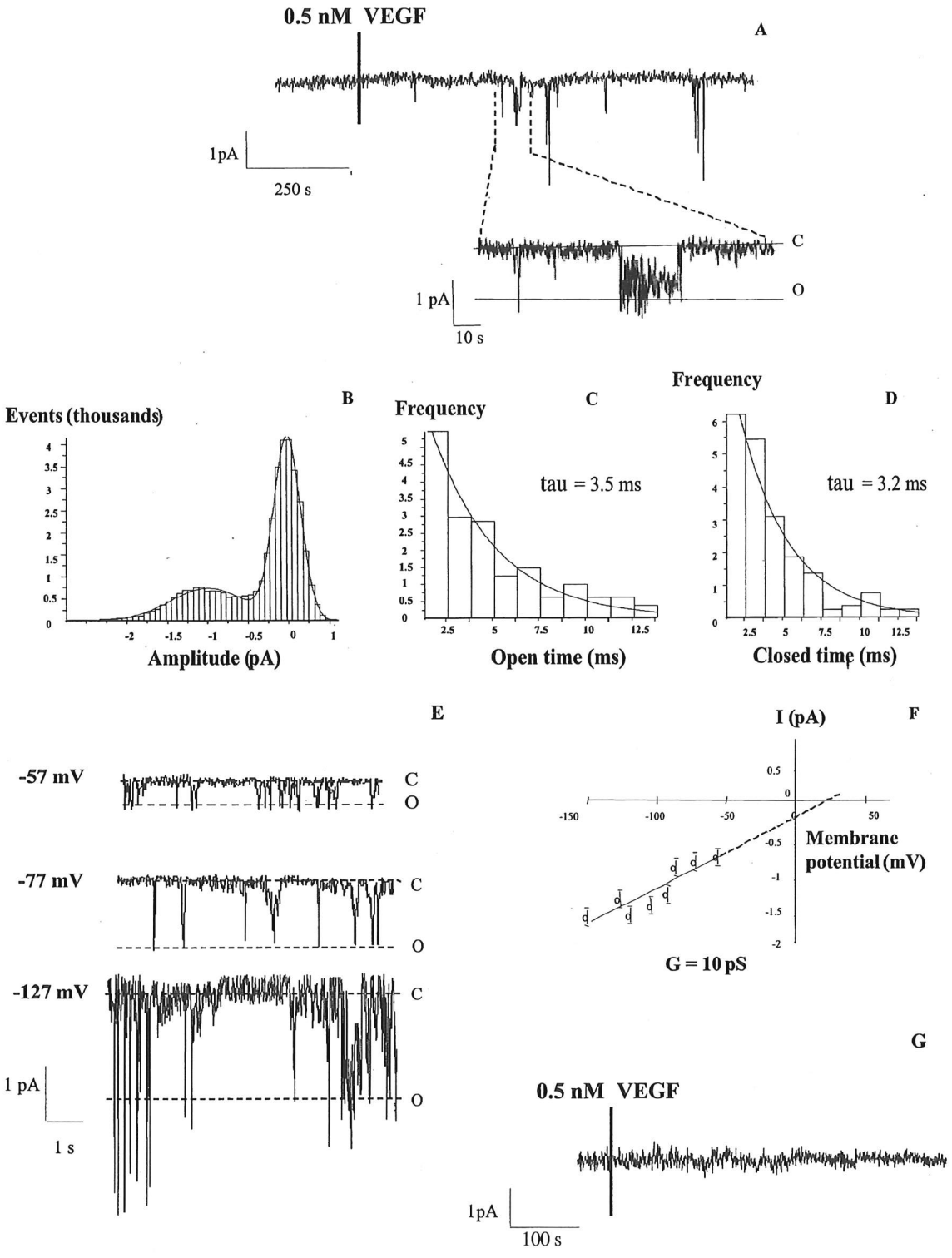
The VEGF type used was recombinant human vascular endothelial growth factor (rhVEGF), in its homodimeric glycosylated 165 amino-acid form, with a given ED_{50} in the range of 0.1 nM. To prepare a VEGF stock solution (200 nM) kept at −20 °C, the VEGF solvant used was composed of: 154 mM NaCl, 0.1% BSA, 10 mM HEPES, pH 7.4.

Electrophysiological Recording

Recordings of calcium currents were performed and analyzed using the cell-attached and inside-out single channel patch-clamp techniques and materials previously described (Merle *et al.*, 1995; Faury *et al.*, 1998). For single channel current recorded from cell-attached patch, cells were immerged in a Tyrode solution containing: 125 mM, NaCl; 5.6 mM KCl; 2.4 mM $CaCl_2$; 1.2 mM $MgCl_2$; 11 mM N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES); 11 mM Glucose, 0.1% bovine serum albumin (BSA), pH 7.4. The Tyrode solution includes BSA to mask the non-specific growth factor binding sites and the potential effect of the BSA present in the solvant of VEGF. When single channel currents were recorded from excised inside-out membrane patches, cells were bathed in an intracellular medium containing: 150 mM KCl; 0.55 mM $CaCl_2$; 2 mM $MgCl_2$; 10 mM HEPES; 1.1 mM EGTA, pH 7.35. In both cases the patch pipette contained: 110 mM $Ba(CH3COO)_2$; 10 mM HEPES, pH 7.4. Barium was used instead of calcium because calcium channels are generally more permeable to this ion, and barium is known to inhibit potassium currents (Tsien *et al.*, 1987).

Adhering HUVEC Cytoplasmic and Nuclear Free Calcium

Measurements and analyses were performed by confocal laser scanning microscopy (Zeiss LSM 410, Carl Zeiss, Iena, Germany) using the calcium-sensitive fluorescent dye FLUO 3 (excitation: 488 nm; emission: 540 nm), according to the procedures previously described (Faury *et al.*, 1998). During the measurements, cells were bathed in a saline physiological solution of the following composition: 118 mM NaCl; 5.6 mM KCl; 2.4 mM $CaCl_2$; 1.2 mM $MgCl_2$; 10 mM HEPES, 0.1% bovine serum albumin (BSA), and 11 mM D-Glucose (pH 7.4). Some experiments were performed in a low-calcium physiological solution (final calcium concentration in the range of 10^{-7}M), composed of: 125 m NaCl; 5.6 mM KCl; 1.2 mM $MgCl_2$; 0.2 mM EGTA; 10 mM HEPES, 0.1% BSA and 11 mM D-Glucose (pH 7.4). Other experiments were performed after preincubation of the cells with 0.2 μM thapsigargin for 45 minutes, or with 1 mM $NiCl_2$ for 6 minutes, these products


0.5 nM VEGF
A
1pA
250 s
C
O
1 pA
10 s
B
Events (thousands)
Amplitude (pA)
C
Frequency
tau = 3.5 ms
Open time (ms)
D
Frequency
tau = 3.2 ms
Closed time (ms)
E
-57 mV
-77 mV
-127 mV
1 pA
1 s
F
I (pA)
Membrane potential (mV)
G = 10 pS
G
0.5 nM VEGF
1pA
100 s

remaining in the medium during the experiment. The solutions include BSA to mask nonspecific growth factor binding sites and the potential effect of the BSA present in the solvant of VEGF. The optical slices passed through the nuclei. *F/Fo* is the ratio of current fluorescence to initial fluorescence and is assumed to represent nuclear and cytoplasmic $[Ca^{2+}]$.

Inosital Phosphates (IPs)

Dosage was adapted from a protocol previously described (Diserbo *et al.*, 1995). HUVEC, grown in Petri dishes (diameter: 60 mm), were pre-labeled with *myo*-[2-^{3}H]- inositol (4 μCi ml^{-1}, 3 ml) for 72 hours in the culture medium described above (see *cell culture* section). The cells were washed 3 times with a Krebs-Ringer Henseleit buffer (KRH) containing (mM): NaCl 118; KCl 4.6; $NaHCO_3$ 24.9; KH_2PO_4 1; $MgSO_4$ 1.1; $CaCl_2$ 1; HEPES 5, Glucose 11.1. KRH was supplemented with 0.1% BSA and adjusted to pH 7.0 with NaOH. The cells were preincubated for 10 minutes at 37 °C in 4 ml culture medium without serum containing 10 mM LiCl and 0.1% BSA. Cells were then stimulated by 2 nM rhVEGF for 30–45 s. The reaction was terminated by addition of 400 μl IGEPAL 10% and 400 μl trichloroacetic acid (TCA) 100%. The samples were maintained on ice for 20 minutes and the cells were then scraped and transferred into a 15 ml polypropylene centrifuge tube. After centrifugation, the supernatant was removed and poured into a glass tube. TCA was extracted from the supernatant by 6 consecutive extractions with 6 ml of water-saturated diethylether. Excess diethylether was evaporated under nitrogen, and the samples were neutralized with 30 μl OF 0.5 M Tris base (final pH: 7). Samples were then stored at −80 °C until the inositol phosphates were separated by ion exchange chromatography. The samples were mixed with 6 ml of 5 mM sodium tetraborate in 0.5 mM EDTA and 5 ml of sample were poured into disposable poly-prep columns containing 0.5 g of Dowex AG1X8 anion exchange resin (Bio-Rad, formate form, 200–400 mesh). *Myo*-[2-^{3}H]- inositol was then eluted with 20 ml of 20 mM ammonium formate. [^{3}H]-InsP1 (IP_1), [^{3}H]-InsP2 (IP_2), [^{3}H]-InsP3 (IP_3) and [^{3}H]-InsP4 (IP_4) were sequentially eluted with 20 ml of 0.2, 0.4, 0.8 and 1.2 M ammonium formate containing 0.1 M formic acid, respectively. A 5 ml aliquot of each fraction was mixed with 10 ml scintillation solution (ACS, Amersham) in a vial, and the radioactivity was determined by liquid scintillation counting using a Tricarb 2000 CA counter (Packard).

Cell Proliferation

HUVEC were trypsinized and resuspended in culture medium 199 containing 5% human serum, 0.1 mg ml^{-1} streptomycin, 100 UI ml^{-1} penicillin and 2 mM L-glutamine, then plated in 0.25 mg ml^{-1} fibronectin-pre-coated dishes (diameter: 35 mm), with an approximate density of 2500 cells/cm^2. The medium contained only 5% human serum to limit spontaneous cell proliferation. 0.5 nM or 2 nM VEGF (3 dishes each), or

FIGURE 1 Effect of VEGF on Ca^{2+} channel currents. (A) Representative tracings of VEGF-induced Ca^{2+} currents recorded from HUVEC, using the cell-attached configuration and barium pipette solution. At basal level, membrane permeability was low and stable. Addition of 0.5 nM VEGF to the bath solution enhanced inward currents 5–7 minutes later. The delay is partly due to the diffusion of VEGF across the medium. Open and closed states of the VEGF induced channels are indicated in the enlargement (membrane potential = − 77 mV, recordings sampled at 1 kHz). (B) Amplitude distribution of the observed openings reveals an inward unitary current of 1.2 pA. (C) and (D) Histogram distributions of the open and closed times. Constants are 3.5 and 3.2 ms, respectively. (E) Representative tracings of 0.5 nM VEGF-induced Ca^{2+} currents recorded from HUVEC at different membrane potentials (cell-attached configuration). (F) The current-voltage relation was plotted, with mean values, after conversion of the holding potentials into membrane potential according to the resting potential of −57 mV measured in 15 independent experiments. The conductance is 10 ± 3 pS. The tracings presented are representative of 21 different experiments. (G) Representative tracing showing that 0.5 nM VEGF is unable to enhance Ca^{2+} currents on HUVEC preincubated for 18 hours with 200 μM suramin (cell-attached configuration, $n = 4$).

the same volume of solvant of VEGF (6 dishes) were then added to the cells for 96 hours. Also, 1 mM $NiCl_2$ was added to 3 other dishes for 10 minutes prior to addition of 0.5 nM VEGF and was removed and washed 1 hour after addition of VEGF, before refilling the dishes with new medium containing 0.5 nM VEGF until 96 hours. The dishes were then washed 4 times with saline physiological solution containing: 140 mM NaCl; 4 mM KCl; 7.6 mM D-glucose; 15 mM HEPES, 0.1 mg ml^{-1} streptomycin, 100 UI ml^{-1} penicillin and 0.1% phenol red, pH 7.4, and refilled with fresh culture medium supplemented with the corresponding concentration of VEGF or solvant of VEGF (same product as initially added in the dish). This time was considered as time 0 hour. Medium, and VEGF or solvant of VEGF were replaced after 48 hours. Adhering cells were counted on video images of 5 randomized fields (3.875 mm^2 per field) per dish (3 dishes per group), at 0 and 96 hours. As a result, 15 randomized cell counts were obtained and compared at each time (0 and 96 hours) for each one of the groups (0.5 nM VEGF, 2 nM VEGF, solvant of VEGF, 0.5 nM VEGF + 1 mM $NiCl_2$).

Statistical Analyses

Analyses of patch-clamp signal were performed using the software Biopatch (Biologic, Claix, France). Comparisons of IP dosages and cell proliferation counts were performed using 2-way ANOVAs followed by Fisher's LSD tests for paired comparisons. $p < .05$ was considered as statistically significant.

Materials and Chemicals

Recombinant human vascular endothelial growth factor (rhVEGF) was from Calbiochem (La Jolla, CA, USA). Collagenase 1A, thapsigargin, INDO 1-AM, FLUO 3-AM, digitonin, EGTA, inositol 1,4,5-trisphosphate, suramin, IGEPAL and all components of saline solutions were from Sigma (St Quentin-Fallavier, France). HEPES was from Merck (Nogent-sur-Marne, France). *Myo*-[2-^{3}H]-inositol was from Amersham (England). Cell culture dishes were Falcon dishes (Becton-Dickinson, Plymouth, England). Bovine serum albumin was fraction V, fatty acid free from Boehringer-Mannheim (Germany).

RESULTS

Effects of VEGF on Membrane Channel Activation

In order to record changes in membrane permeability for calcium after VEGF stimulation, patch-clamp experiment were carried out on human umbilical vein endothelial cells (HUVEC). The resting potential was -57 ± 3 mV, as measured using intracellular microelectrodes filled with 3 M KCl, from 15 independent experiments. As shown in figure 1A, after a few minutes delay probably due to the diffusion of VEGF (0.5 nM) in the bath, a transient inward current was observed from cell-attached patch held at a membrane potential of -77 mV (membrane potential = resting potential – holding potential). The amplitude histogram determines the amplitude of the inward current of 1.2 pA ($n = 18$) (Fig. 1B). The open time constant is 3.5 ms and the closed time constant is 3.2 ms (Figs. 1C and 1D). Figure 1E shows a representative inward current from a cell-attached patch at different membrane potentials. Mean values from 8 independent experiences indicate that VEGF-activated channels have a conductance of 10 ± 3pS (Fig. 1F). Incubation (for 18 hours prior to experiment) of HUVEC with suramin, a conformational inhibitor of the VEGF receptor (Waltenberg *et al.*, 1996; Middaugh *et al.*, 1992), abolished VEGF-induced channels activation ($n = 4$) (Fig. 1G).

Effects of IP_3 on Excised Inside-out Patch

Inositol 1,4,5 trisphosphate (IP_3) has previously been proposed as a messenger in mediating the opening of plasma membrane calcium channels (16, 18,21). Consequently, the inside-out configuration

of the patch-clamp technique was used to determine the effects of IP_3 (5 μM) on the channel activity of membrane excised patches. As shown in Fig. 2A, IP_3-induced transient inward currents exhibited an amplitude of 1.2 pA ($n = 14$) in 70% of the experiments (Fig. 2B). Based on seven independent experiments, the open time constant is 3.7 ms and the close time constant is 8.1 ms (Figs. 2C and 2D), and the conductance is 13 ± 1 pS (Figs. 2E and 2F).

Effect of VEGF on Nuclear and Cytoplasmic Free Calcium Levels

Addition of the solvant of VEGF (which includes BSA) alone induces little effect on HUVEC free calcium concentration ($[Ca^{2+}]$), i.e. only a slight and sustained increase, variable from cell to cell, in the range of 0–20% in the cytoplasm and 0–40% in the nucleus. On the contrary, addition of 2 nM VEGF first induced a fast, large, transient – which was repetitive in some cells – elevation of both cytoplasmic and nuclear $[Ca^{2+}]$, in ≈80% of the cells, in the range of 2–6 fold in the cytoplasm and 2–8 fold in the nucleus. In both the nucleus and the cytoplasm, this initial fast response is followed by a slower $[Ca^{2+}]$ decrease before returning to normal or, for some cells, to a sustained slightly elevated $[Ca^{2+}]$ as compared to initial level (+10–20%) (Figs. 3A–3D). The respective contributions of intracellular calcium stores and extracellular calcium influx in the VEGF-induced $[Ca^{2+}]$ increase were then investigated. First, nuclear and cytoplasmic $[Ca^{2+}]$ were measured in HUVEC bathing in a low calcium physiological solution, i.e. with no possibility of extracellular calcium influx. In this condition, 2 nM VEGF induced a slower very small increase in nuclear and cytoplasmic $[Ca^{2+}]$, as compared to the experiments performed in the presence of extracellular calcium. The maximum $[Ca^{2+}]$ elevation was in the range of 1.1–1.3 fold in the cytoplasm and 1.1–2.3 fold in the nucleus, before returning to normal or, for some cells, to a sustained slightly elevated $[Ca^{2+}]$ level as compared to initial level (+ 10–20% in the cytoplasm and + 10–80% in the nucleus) (Figs. 4A and 4B). Second, to investigate the role of intracellular calcium stores in VEGF-produced signaling, we then performed measurements on HUVEC treated with 0.2 μM thapsigargin, an endoplasmic reticulum calcium ATPase blocker (Thastrup *et al.*, 1989) that induces depletion and blocks restoration of the endoplasmic reticulum calcium, a store potentially mobilizable by IP_3. The 2 nM VEGF-induced calcium elevation was also reduced in this condition, but less than in the absence of extracellular calcium. In the presence of thapsigargin, the maximum increase in $[Ca^{2+}]$ was in the range of 2 fold in both the cytoplasm and nucleus, as opposed to the minimum two fold increase observed in the absence of thapsigargin (Figs. 4C and 4D). Also, experiments performed both in the presence of thapsigargin and no extracellular calcium show that maximum increase in nuclear and cytoplasmic $[Ca^{2+}]$ induced by 2 nM VEGF was lower than two fold, indicating a cumulative effect of the two pathways. Nevertheless, in this last condition, VEGF still produced a slight increase in $[Ca^{2+}]$, suggesting that other intracellular calcium stores, not mobilized by thapsigargin, are also involved in the transduction pathway (Figs. 4E and 4F). Finally, since VEGF-signaling is largely dependent upon extracellular calcium influx, we have used $NiCl_2$ (1 mM), a large spectrum calcium channel blocker (Narahashi and Herman, 1992), to determine whether VEGF-induced calcium signaling could be chemically inhibited. 2 nM VEGF induced, for most cells, an elevation of nuclear and cytoplasmic $[Ca^{2+}]$ globally lower than in the absence of nickel, i.e. 1.1–1.6 fold in the cytoplasm (vs 2–6 fold in the absence of nickel) and 1.2–2 fold in the nucleus (vs 2–8 fold in the absence of nickel). Only one cell cytoplasm and two cell nuclei still presented a virtually normal response under this test condition (Figs. 5A and 5B).

Effect of VEGF on Inositol Phosphate Turnover

Inositol phosphate dosages (IP_1, IP_2, IP_3 and IP_4), performed in HUVEC stimulated with 2 nM VEGF,

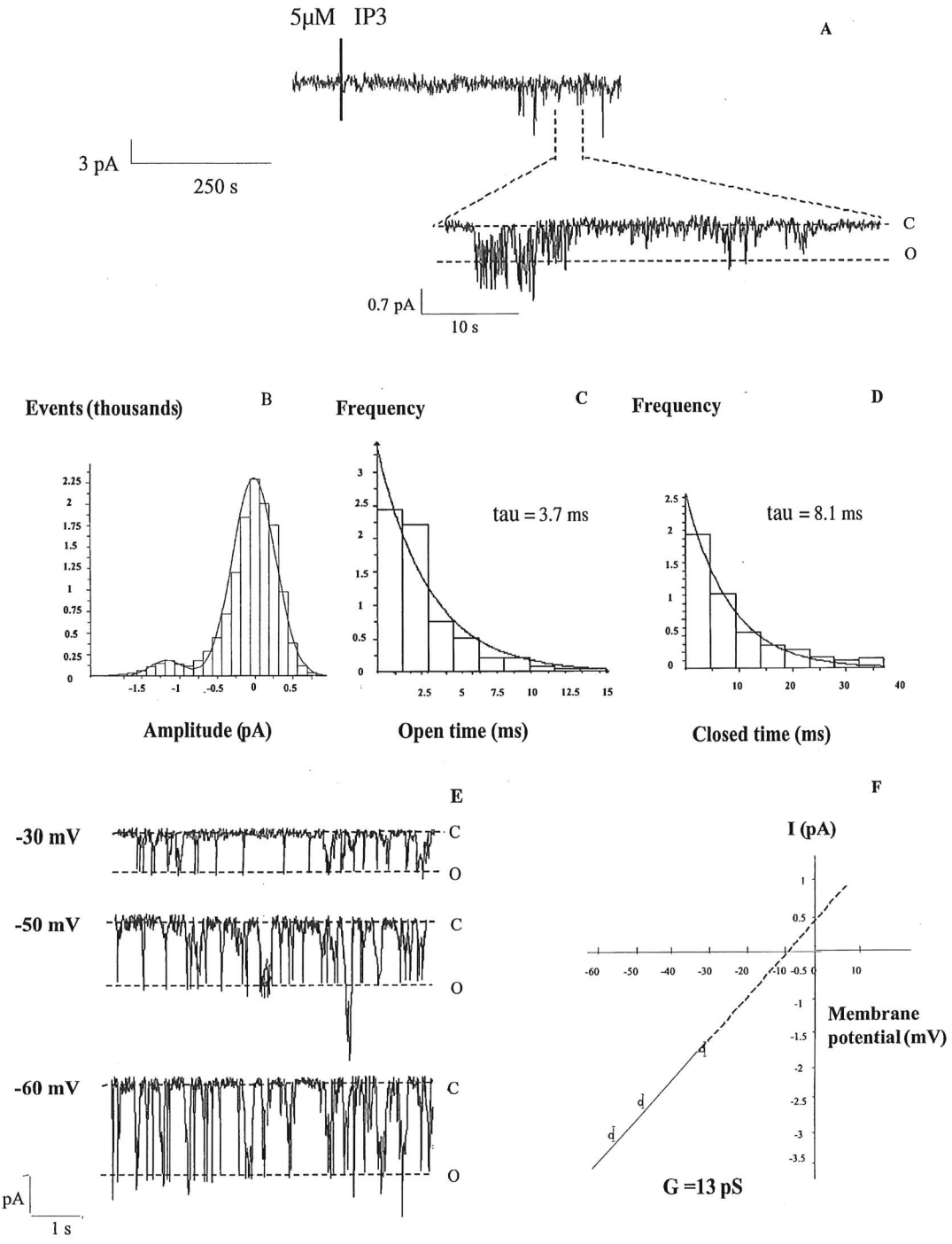


FIGURE 2 Effect of IP_3 on Ca^{2+} channel currents. (A) Representative example of IP_3-induced currents recorded from HUVEC inside-out patch membrane held at −20 mV. From 1–5 minutes after 5 μM IP_3 addition into the bath, downward deflections were observed corresponding to open and closed states as indicated in the enlargement. (B) The resulting density histogram of current amplitudes revealed an inward unitary current of 1.2 pA. (C) and (D) Histogram distributions of the open and closed times. Constants are 3.7 and 8.1 ms, respectively. (E) Representative tracings of 5 μM IP_3-induced Ca^{2+} currents recorded from HUVEC at different membrane potentials (inside-out configuration). (F) The slope of the current-voltage relation reflects a conductance of 13 ± 1 pS. The tracings presented are representative of 12 different experiments.

show that VEGF induces a global significant increase in all four dosed IP levels. In particular, IP_3, which is largely responsible for mobilization of intracellular calcium stores and for activation of membrane calcium channels, is found to be significantly increased by at least 20% very early after stimulation by VEGF, as compared to control experiments (Table I).

Effect of VEGF and Nickel on HUVEC Proliferation

In order to investigate the involvement of the extracellular calcium influx on cell proliferation, we tested the effect of a large spectrum calcium channel blocker (Ni^{2+}) on the proliferation of VEGF-stimulated HUVEC. As compared to HUVEC cultured in the presence of the solvant of VEGF alone, 0.5 nM and 2 nM VEGF induced a significantly increased proliferation of HUVEC bathing in 5% serum culture medium after 96 hours (+53% and +180%, respectively). In addition, an 1 hour application of 1 mM $NiCl_2$, limited in time to minimize the global cellular effects of calcium influx blockage, was performed during the early period of application of VEGF. Under this condition, proliferation of HUVEC stimulated by 0.5 nM VEGF was significantly reduced after 96 hours

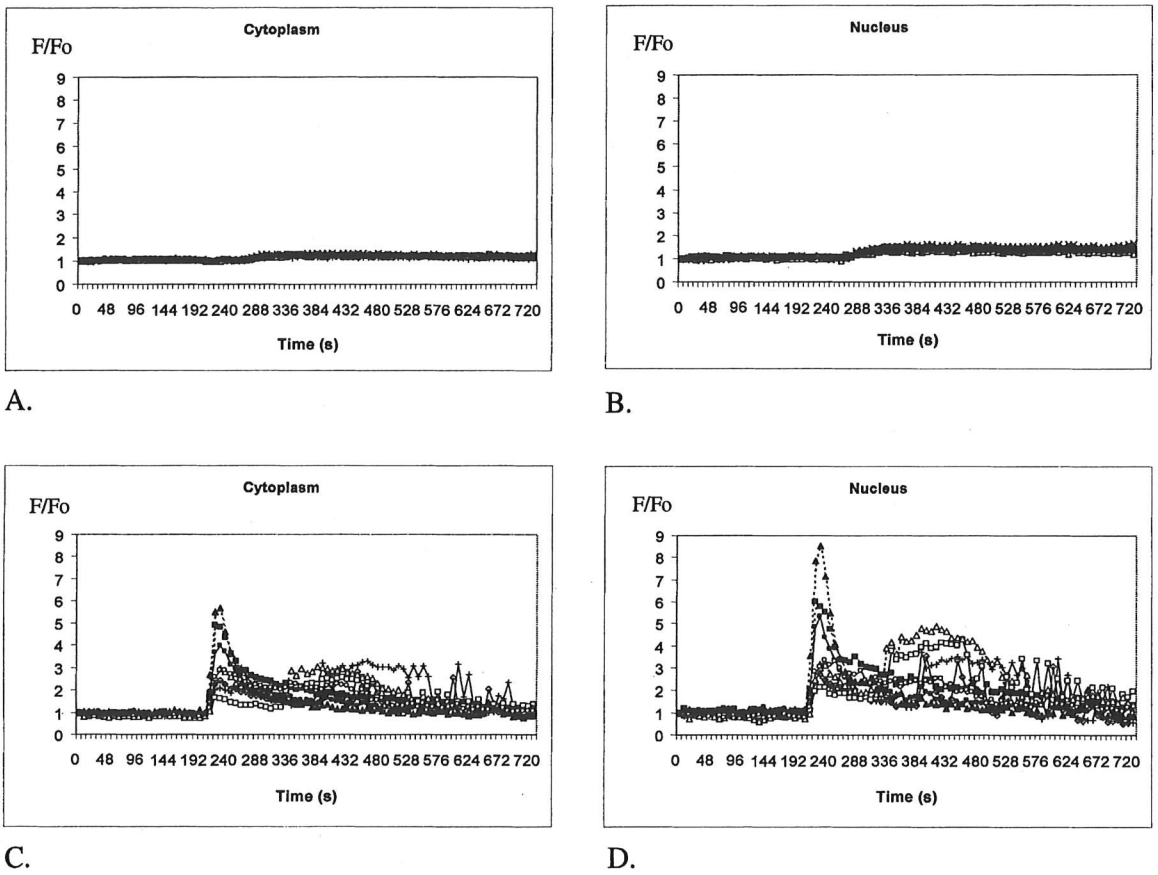


FIGURE 3 Effect of VEGF on the cytoplasmic and nuclear [Ca^{2+}] of HUVECs. Control experiments show that addition of solvant of VEGF (at ≈200 s) alone induces little effect on cytoplasmic (A) and nuclear (B) [Ca^{2+}] ($n=7$ cells). On the contrary, addition of 2 nM VEGF at the same time induces a fast, large and transient elevation of [Ca^{2+}], cyclic in some cells, in both the cytoplasm (C) and the nucleus (D) of most cells ($n=8$ cells). The tracings presented are representative of the results obtained in all the experiments, that is 57 cells from 7 different dishes for the action of VEGF, and 22 cells from 4 different dishes for the action of the solvant of VEGF alone.

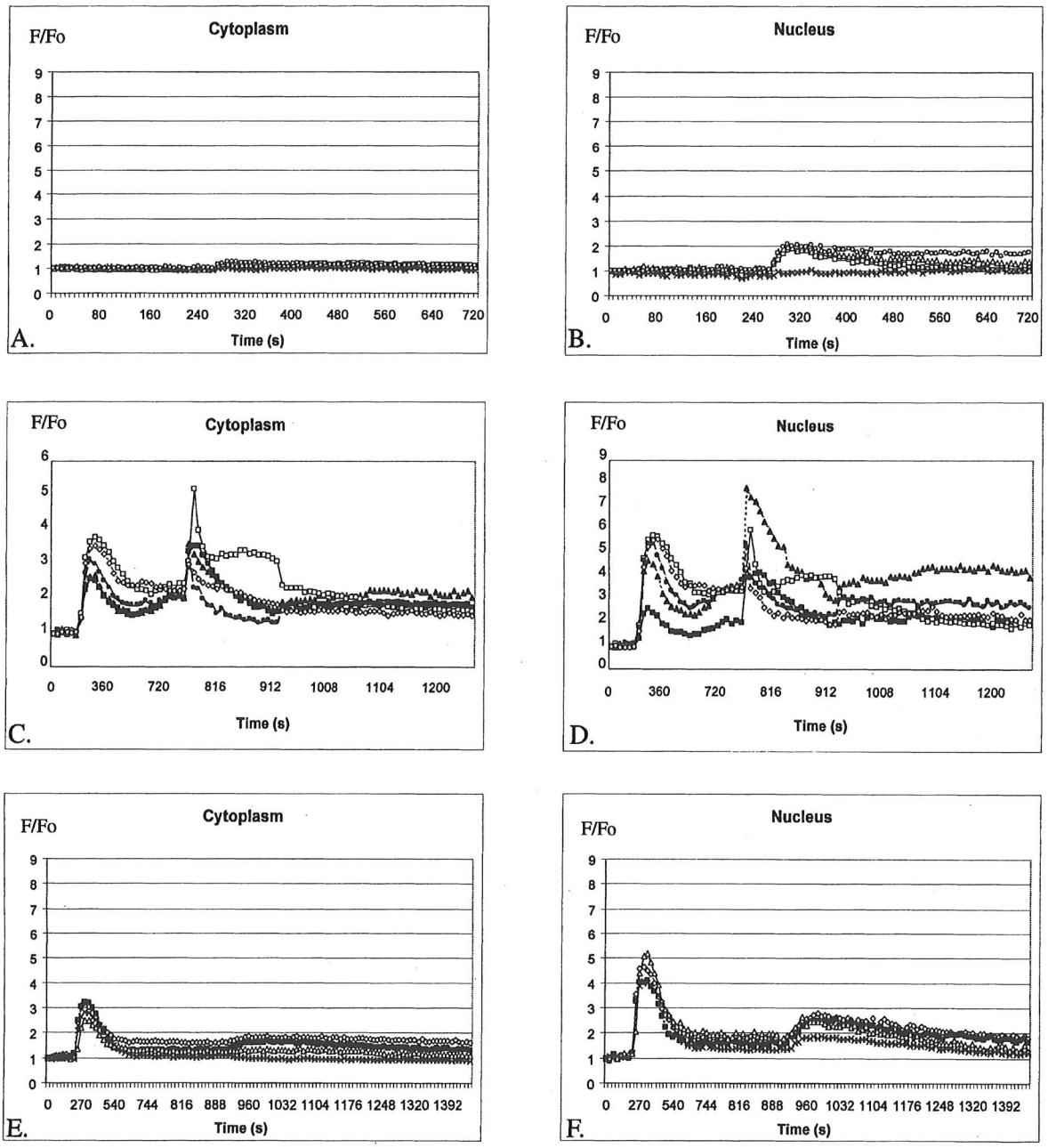
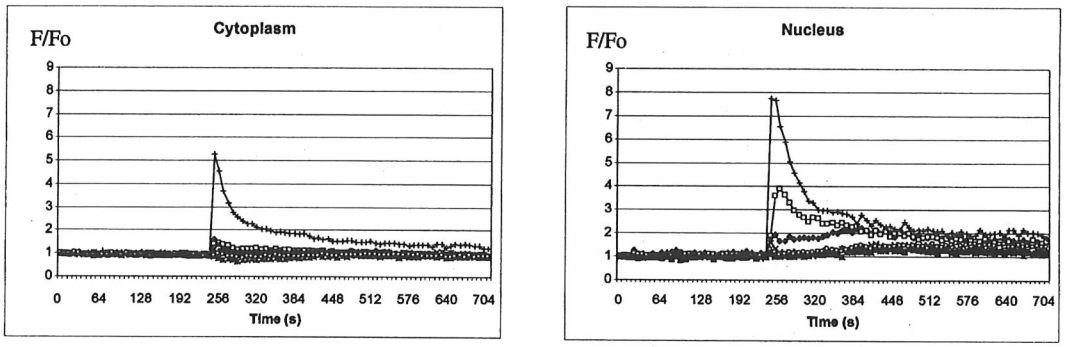


FIGURE 4 Effect of prevention of mobilization of extracellular or intracellular calcium on VEGF-induced $[Ca^{2+}]$ increase in HUVECs. Absence of extracellular calcium dramatically, but not totally, inhibits the cytoplasmic (A) and nuclear (B) increases in $[Ca^{2+}]$ due to the action of 2 nM VEGF applied at 240 s ($n=4$ cells). Depletion of intracellular calcium stores, produced by preincubation of HUVECs with 0.2 μM thapsigargin (from 180 s until 750 s), also inhibits the VEGF-induced elevation of $[Ca^{2+}]$ (VEGF applied at 750 s) in the cytoplasm (C) and the nucleus (D), down to a maximum of two fold ($n=5$ cells). In the absence of extracellular calcium and after preincubation with 0.2 μM thapsigargin (from 180 s until 850 s), 2 nM VEGF (at 850 s) still produces a slight increase in cytoplasmic (E) and nuclear (F) $[Ca^{2+}]$ ($n=4$ cells), suggesting that VEGF also mobilizes non-thapsigargin-sensitive intracellular calcium stores. The tracings presented are representative of the results obtained in all the experiments performed in different dishes, that is 7 cells in the absence of extracellular calcium, 25 cells for preincubation with thapsigargin, and 4 cells for extracellular calcium depletion coupled to preincubation with thapsigargin.



A. B.

FIGURE 5 Effect of preincubation with nickel, a calcium channel blocker, on the elevation of $[Ca^{2+}]$ triggered by VEGF in HUVECs ($n=7$ cells). (A) Presence of 1 mM $NiCl_2$ significantly inhibits the effect of 2 nM VEGF, in particular in the cytoplasm: only 1 cell out of 7 presented a strong increase in $[Ca^{2+}]$ (5 fold), whereas 6 out of 7 presented $[Ca^{2+}]$ increases in the range of only 0–60% maximum. (B) In the nucleus, 4 cells presented $[Ca^{2+}]$ increases of 40% maximum, whereas 3 cells presented increases of higher amplitude: 2, 4 and 8 fold, respectively.

to +12%, as compared to HUVEC cultured in the presence of the solvant of VEGF alone (Fig. 6).

DISCUSSION

The observed VEGF-induced elevation of $[Ca^{2+}]i$ can be explained by at least two different mechanisms. Like most growth factors, VEGF acts through membrane tyrosine-kinase receptors. Activation of these receptors induces multiple intracellular phosphorylation cascades, as well as synthesis of inositol 1,4,5 trisphosphate (IP_3) which lead to an increase in the intracellular free calcium concentration ($[Ca^{2+}]i$) (Heldin and Westermark, 1989). First. IP_3 can trigger the release of IP_3-sensitive intracellular calcium stores, such as in the response to VEGF and platelet-derived growth factor (PDGF) (Berridge and Irvine, 1988; Brock *et al.*, 1991). Second, the extracellular calcium influx induced by growth factors, as demonstrated on cells stimulated with basic fibroblast growth factor (FGF2), epidermal growth factor (EGF), PDGF or VEGF (Merle *et al.*, 1997; Brock *et al.*, 1991; Estacion and Mordan, 1993; Mogami and Kojima, 1993; Bates and Curry, 1997; Moolener *et al.*, 1986), is able to induce elevations of $[Ca^{2+}]i$ (Brock *et al.*, 1991;

TABLE I IP level increase (% ± SEM) 30–45 s after application of 2 nM VEGF, as compared to corresponding controls

IP1	IP2	IP3	IP4
19 ± 16	21 ± 15	20 ± 11	16 ± 12

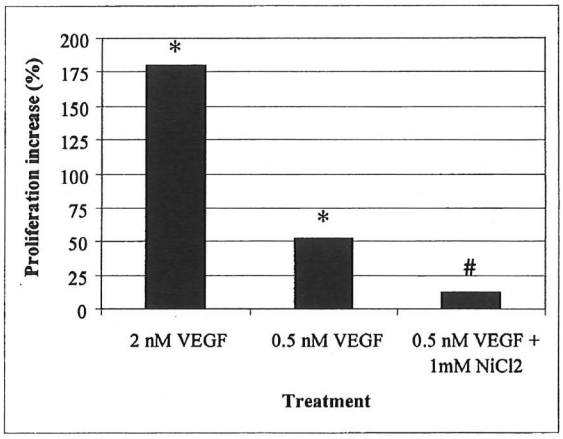


FIGURE 6 Percentage of additional proliferation when HUVEC were cultured for 96 hours in the presence of VEGF, as compared to the proliferation in the presence of solvant of VEGF alone. Inhibition by nickel of additional proliferation due to 0.5 nM VEGF, as compared to the proliferation due to the presence of solvant of VEGF alone. Initial cell density at 0 hour was identical between the test groups and the corresponding control groups (P non significant). * indicates a significant difference with solvant alone ($p<.05$). # indicates a significant difference ($p<.05$) with the corresponding group (0.5 nM VEGF) in the absence of nickel.

Estacion and Mordan, 1993) and could result from the activation of IP_3-sensitive membrane calcium channels (Merle *et al.*, 1995; Merle *et al.*, 1997).

Here, we have identified calcium permeable channels present on the endothelial cell membrane which are activated by VEGF, resulting in calcium influx. Activation of calcium channels is mediated by the tyrosine-kinase VEGF-receptor KDR/VEGFR2 since incubation of cells with suramin, a VEGF-receptor KDR inhibitor acting by conformational changes of the receptor structure (Waltenberg *et al.*, 1996; Middaugh *et al.*, 1992), abolishes this effect of VEGF. The VEGF-activated calcium permeant channels have a conductance of about 10 ± 3 pS. In addition, the reversal potential of the observed current is lower than the theoretical calculated calcium reversal potential by the Nernst-Planck equation, indicating that the selectivity of the channel for calcium is low (Fig. 1). Nevertheless, our results are consistent with the observation of Estacion (1993) who proposes that the rise in intracellular calcium concentration following the stimulation of PDGF-tyrosine-kinase receptors is due, in part, to influx of extracellular calcium and release of calcium from intracellular store (Estacion and Mordan, 1993).

Since, in cell-attached patch-clamp configuration, substances are unable to diffuse from the bath to the inside of the patch pipette, it could be suspected that the VEGF action is mediated by a second messenger rather than direct receptor operated-calcium entry. Several studies (Vaca and Kunze, 1995; Merle *et al.*, 1995; Merle *et al.*, 1997) have shown that IP_3 is able to activate calcium channels located on the surface of plasma membrane. Thus, we have tested the role of IP_3, because it is one of the second messengers produced during the cascade induced by the tyrosine-kinase receptor activation, including the VEGF receptor activation (Brock *et al.*, 1991). We have observed a very early (significant) 20% increase in IP_3 level (Table I), 30 s after cell stimulation by VEGF, and other authors showed that this increase reaches a plateau after 2 minutes in the range of a 100% increase (Brock *et al.*, 1991). Accordingly, in HUVECs, we have demonstrated the presence of IP_3-activated membrane calcium permeable channels (Fig. 2) whose properties were comparable to those of the VEGF-activated channels, in particular conductances (about 10 pS). This leads to think that VEGF-induced IP_3 production could, in part, result in calcium permeant channel activation. However, this direct activation of membrane channels by IP_3 does not exclude that IP_3-induced calcium release from store results in membrane calcium activated current (Icrac) (Li *et al.*, 1997). Nevertheless, using inside-out configuration, we have not observed direct calcium activated permeable channels (results not shown). Furthermore our study is in agreement with previous studies showing that IP_3 produced after tyrosine-kinase receptor activation by other growth factors is responsible for activation of membrane calcium channels and/or elevation of $[Ca^{2+}]i$. Some of these studies using growth factors (FGF-2, EGF, ...) and different cell types (T-lymphocytes, endothelial cells, cardiomyocytes, ...) describe calcium permeable channels with characteristics identical to those observed in our study: low conductance, permeability to calcium, low calcium specificity, activation by both growth factors and IP_3 (Vaca and Kunze, 1995; Merle *et al.*, 1995; Merle *et al.*, 1997; Selinfreund and Blair, 1994; Estacion and Mordan, 1993; Mogami and Kojima, 1993). IP_3 may be able to trigger the release of calcium and activate membrane channels, eventually resulting in endothelial $[Ca^{2+}]i$ increase. This hypothesis is further supported by our results showing that the VEGF-induced $[Ca^{2+}]i$ elevation (Fig. 3) is inhibited by previous depletion of the intracellular calcium stores, and almost abolished by the absence of extracellular calcium or addition of a calcium channel blocker (Figs. 4 and 5). In addition, in HUVECs stimulated with 0.5 nM VEGF, inhibition of the calcium influx using the calcium channel blocker Ni^{2+} limits cell proliferation (Fig. 6), suggesting a potential way for new therapies in which treatments with such calcium channel blockers would prevent or reverse VEGF-related tumoral angiogenesis.

Taken together, these observations suggest that membrane calcium permeable channel activation is one of the first and major steps resulting from tyrosine-kinase receptors stimulation by growth factors. Our results also suggest that activation of calcium channels by growth factors is essential to trigger the increase in intracellular calcium concentration and, thus, the growth factor action. $[Ca^{2+}]i$ plays the role of a major second messenger regulating many intracellular pathways and biological activities, such as the enhancement of the PDGF mitogenic action by the extracellular calcium influx-mediated $[Ca^{2+}]i$ increase (Mogami and Kojima, 1993). It has recently been shown that nuclear and cytoplasmic $[Ca^{2+}]$ have different functions on gene expression control, acting through two different calcium-response elements (Hardingham *et al.*, 1997). Since VEGF induces a rise in both cytoplasmic and nuclear $[Ca^{2+}]$, it is suggested that VEGF acts at different levels to trigger calcium-dependent enzymatic cascades in the cytoplasm, leading to cell activity modulation and modulation of expression of certain genes, as well as direct calcium-dependent nuclear action on gene expression. In particular, the extracellular calcium influx could play a major role in the modulation of VEGF-driven cell regulation, proliferation, and angiogenesis.

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