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Role of Na^+/H^+ Exchange in Thrombin-induced Platelet-activating Factor Production by Human Endothelial Cells*

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Thrombin-stimulated endothelial cells produce platelet-activating factor (PAF) in a dose-dependent manner: the activation of a Ca^{2+}-dependent lyso-PAF acetyltransferase is the rate-limiting step in this process. The present study shows that acetyltransferase activation and consequent PAF production induced by thrombin in human endothelial cells are markedly inhibited in Na^+-free media or after addition of the amiloride analog 5-(*N*-ethyl-*N*-isopropyl)amiloride, suggesting that a Na^+/H^+ antiport system is present in endothelial cells and plays a prominent role in thrombin-induced PAF synthesis. Accordingly, thrombin elicits a sustained alkalinization in 6-carboxyfluorescein-loaded endothelial cells, that is abolished in either Na^+-free or 5-(*N*-ethyl-*N*-isopropyl)amiloride-containing medium. Extracellular Ca^{2+} influx induced by thrombin (as measured by quin2 and ^{45}Ca methods) is completely blocked in the same experimental conditions, and monensin, a Na^+/H^+ ionophore mimicking the effects of the antiporter activation, evokes a dose-dependent PAF synthesis and a marked Ca^{2+} influx, which are abolished in Ca^{2+}-free medium. An amiloride-inhibitable Na^+/H^+ exchanger is present in the membrane of human endothelial cells, its apparent K_m for extracellular Na^+ is 25 mM, and its activity is greatly enhanced when the cytoplasm is acidified. These results suggest that Na^+/H^+ exchange activation by thrombin and the resulting intracellular alkalinization play a direct role in the induction of Ca^{2+} influx and PAF synthesis in human endothelial cells.

Thrombin elicits a variety of responses on human endothelial cells, including platelet-activating factor (PAF)[1] synthesis (1–3). The rise in cytosolic calcium ($[Ca^{2+}]_i$) plays a fundamental role in transduction of the thrombin stimulus in many of these responses, as most effects of thrombin can be mimicked by calcium ionophore(s) (4, 5), and thrombin elicits a dose-dependent increase in $[Ca^{2+}]_i$ (6–8). Both calcium influx across plasma membrane and calcium mobilization from intracellular stores are involved (6–8). Since thrombin triggers phosphoinositide turnover (7, 8) and $[Ca^{2+}]_i$ rise in EGTA-containing medium with superimposable time course and concentration dependence, inositol-1,4,5-trisphosphate is the likely mediator of the discharge from intracellular stores. As far as the Ca^{2+} influx from the extracellular medium is concerned, the mechanisms by which thrombin acts are still unknown.

In platelets and fibroblasts (9) thrombin is known to induce the activation of Na^+/H^+ exchange. This is a transport system in the plasma membrane of most mammalian cells that mediates the electroneutral, amiloride-inhibitable exchange of external Na^+ for internal H^+; it responds to a fall in intracellular pH (pH_i) by rapidly extruding protons (10) and can be modulated by a variety of growth-promoting agents (11). Some recent reports (12) demonstrate that activation of Na^+/H^+ exchange is a prerequisite for thrombin-induced Ca^{2+} mobilization from intracellular stores (12, 13) and for thrombin- and arachidonic acid-mediated Ca^{2+} influx (14) in human platelets.

Thrombin-stimulated human endothelial cells produce platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (1–3), that may mediate some of the responses of the endothelium to inflammatory injuries. The kinetics of thrombin effect are rather fast and PAF synthesis is maximal within a few minutes. It seems that short-term stimulation of PAF synthesis in inflammatory cells involves two sequential enzymatic steps: 1) activation of phospholipase A_2 which acts on pre-existing 1-*O*-alkyl-2-acyl-glycerophosphocholine (-GPC) (for review see Ref. 15) to increase the levels of precursor 1-*O*-alkyl-lyso-GPC (lyso-PAF) and 2) stimulation of an acetyltransferase activity (1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine: acetyl-CoA acetyltransferase; EC 2.3.1.67) (16) which transfers acetate from acetyl-CoA to lyso-PAF to produce PAF. Both enzymes are calcium-dependent (17, 18); moreover, acetyltransferase activation, which appears to be the rate-limiting step, requires Ca^{2+} entry, and Ca^{2+} uptake correlates well with the extent of enzyme activation (18).

With the purpose of understanding the mechanism(s) of thrombin-induced PAF synthesis and Ca^{2+} influx in endothelial cells, in this study we investigated: 1) the thrombin-dependent PAF production in human endothelial cells in culture, in conditions of altered gradients of Na^+, H^+, and K^+ across the plasma membrane, and in the presence of specific inhibitors on Na^+/H^+ exchange and Na^+ channels; 2) the Na^+/H^+ exchange and extracellular Ca^{2+} dependence of the activation of the acetyltransferase; 3) the thrombin-elicited

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[1] The abbreviations used are: PAF, platelet-activating factor; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; $[Ca^{2+}]_i$, cytosolic calcium; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GPC, glycerophosphocholine; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TLC, thin layer chromatography; 6-CFDA, 6-carboxyfluorescein diacetate; HPLC, high performance liquid chromatography; 6-CF, 6-carboxyfluorescein.

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$[Ca^{2+}]_i$ rise and $^{45}Ca^{2+}$ influx in Na^+-free media and in the presence of Na^+/H^+ exchange inhibitors; and 4) the presence of an amiloride-inhibitable Na^+/H^+ exchanger in human endothelial cells and the role of Na^+/H^+ exchange in thrombin-induced Ca^{2+} influx.

EXPERIMENTAL PROCEDURES

Materials

The chemicals used and their sources were as follows: PAF (1-*O*-octadecyl-2-acetyl-GPC) and lyso-PAF (1-*O*-octadecyl-2-lyso-GPC) were from Bachem Feinkemikalien (Bubendorf, Switzerland); Iscove's medium and fetal calf serum from Flow Laboratories, United Kingdom (U. K.); bovine serum albumin fraction V (BSA), L-α-phosphatidylcholine (from bovine brain), L-α-lysophosphatidylcholine (lyso-phosphatidylcholine, from bovine liver), phospholipase A_2 (from pig pancreas), lipase A_1 (from *Rhizophus arrhizus*), phospholipase C (from *Bacillus cereus*), acetyl-CoA, Triton X-100, butylated hydroxytoluene, HEPES, thrombin (Human), nigericin, and monensin from Sigma; collagenase (from *Clostridium histolyticum*), phospholipase D (from cabbage), and tetrodotoxin from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany (F. R. G.)); CV-3988 from Takeda Chemical Ind. (Osaka, Japan); 1-*O*-[^{3}H]alkyl-2-acetyl-GPC ([^{3}H]PAF, 120 mCi/mmol), 1-[^{14}C]palmitoyl-2-lyso-GPC (55 mCi/mmol), [^{3}H]acetyl-CoA (1 mCi/mmol; the specific activity was adjusted by addition of unlabeled acetyl-CoA), quin2 and quin2-tetraacetoxymethyl ester (quin2-AM) from Amersham International (Bucks, U. K.); pyridine, thin layer chromatography (TLC) plates (60F254), acetic anhydride, and dimethyl sulfoxide (Me_2SO) from Merck (Darmstadt, F. R. G.); ionomycin and 6-carboxyfluorescein diacetate (6-CFDA) from Behring Diagnostics, $^{45}CaCl_2$ (2 mCi/ml, 93 μg/ml) from Du Pont-New England Nuclear, EGTA from Fluka, top purity grade. Amiloride, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), and benzamil were prepared specifically for this study by previously described methods (19); BN52021 was a gift of Dr. P. Braquet (Institut Henri Beaufour, Le Plessis Robinson, France); other reagents and solvents were analar grade or of the highest purity available.

All products for the cell culture were from Corning Glass Works (Corning, NY). All solvents used for the extraction and characterization of the lipids contained 50 mg/liter butylated hydroxytoluene. Before use, all lipids were TLC-chromatographed using chloroform/methanol/water (65:36:6, v/v) as solvent system. 1-[^{14}C]palmitoyl-2-acetyl-GPC was prepared by reacting 1-[^{14}C]palmitoyl-2-lyso-GPC with acetic anhydride in the presence of catalytic amount of pyridine as previously described (20). PAF was dissolved in Iscove's medium containing 0.25% BSA. Stock solutions of BN52021, EIPA, and benzamil were prepared in Me_2SO and then solubilized in 150 mM NaCl containing 0.1% BSA. 6-CFDA, quin2-AM and ionomycin were prepared in Me_2SO, and nigericin and monensin were solubilized in ethanol. In each experimental condition, control and samples received the same volume addition of solvent, and the final solvent concentration never exceeded 0.1%. CV-3988 was solubilized immediately before use in 150 mM NaCl by heating at 60 °C and then buffered to pH 7.4.

Na^+-solution contained 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose, 1 mM $CaCl_2$, pH 7.4, at 37 °C. Choline$^+$ and *N*-methyl-D-glucamine$^+$ solutions were prepared by total isoosmotic replacement of NaCl by choline-Cl and the chloride salt of *N*-methyl-D-glucamine$^+$, respectively, but were otherwise identical. K^+ solutions were prepared by replacing NaCl with KCl, partially (56 mM KCl + 94 mM NaCl) or totally (150 mM KCl). Mannitol solution contained 300 mM mannitol, 10 mM HEPES/Tris, 10 mM glucose, pH 7.4, at 37 °C.

Human Endothelial Cell Cultures

Endothelial cells were obtained by treating human umbilical cord veins with collagenase, cultured in Iscove's medium containing 20% fetal calf serum and characterized as previously described (1). Primary cultures were plated in 25-cm^2 plastic flasks and refed with the same medium every 2 days. After 4–7 days, when the cultures were at confluence, 1×10^5 cells and 3×10^5 cells were plated on 35-mm diameter wells for the experiments of PAF production and on 25-cm^2 flasks for the measurement of acetyltransferase activity, respectively. The cultures were grown until the confluence and the cell number was $5.1 \pm 0.24 \times 10^5$ and $9.8 \pm 0.42 \times 10^5$ cells, respectively ($n = 14$). For $[Ca^{2+}]_i$ transients, ^{45}Ca influx and pH_i measurements, coverslip-adherent cells were used. 3.5×10^4 cells were seeded for a single glass coverslip (12-mm diameter) and grown to confluence. At the time of the experiment, the cell number/coverslip was $5 \pm 0.3 \times 10^4$. No significant variations were observed after manipulations for the different measurements.

The viability of endothelial cells was determined by trypan blue exclusion and ranged 95–98% after 10 min of incubation under the different conditions described.

PAF Production and Acetyltransferase Activity: Experimental Protocol

PAF production and acetyltransferase activity were measured after cell stimulation at 37 °C with thrombin (0.2 units/ml) in the following different conditions: 1) in Na^+ solution containing 0.25% BSA; 2) in Na^+-free media (choline$^+$, methylglucamine$^+$, or K^+ solution) containing 0.25% BSA; 3) after 10 min of incubation in Na^+ solution + 0.25% BSA with different concentrations of EIPA, amiloride, benzamil, or tetrodotoxin; 4) in Na^+ solution + 0.25% BSA containing different Ca^{2+} concentrations. To prepare a Na^+ solution nominally without $CaCl_2$ the deionized water used was passed through a Milli-Q system (Millipore Chromatography Division, Waters Associates, Milford, MA) (resistivity < 0.01 μSiemens) and then through a Chelex 100 column (Bio-Rad). Ca^{2+} content was assessed, by using atomic absorption spectrometer Perkin-Elmer 300 (Perkin-Elmer) technique, to be lower than 42 nM. The desired Ca^{2+} concentrations were obtained by adding different amounts of $CaCl_2$ to the Ca^{2+}-free Na^+ solution. PAF production was also measured in endothelial cells stimulated by thrombin in Na^+ solution containing 0.25% BSA at pH ranging from 7.0 to 7.8.

PAF Production and Characterization—Endothelial cells were washed three times with the different solutions described above to remove fetal calf serum, then stimulated with thrombin, according to the experimental protocol. The reaction was blocked by cooling the wells in an ice bath. The medium was removed and centrifuged for 1 min in an Eppendorf 5412 microcentrifuge (Brinkman Instruments Inc., Westbury, NY) at room temperature. The lipids were extracted from the medium and from the cells according to a modified procedure of Bligh and Dyer (21) in which formic acid was added to lower the pH of aqueous phase to 3.0 ± 0.1 (20). The cells were harvested by using a rubber policeman in the presence of 1 ml of acidified methanol. The wells were washed with 2 ml of acidified methanol and the washings were pooled.

In preliminary experiments no PAF release was detected in the medium after thrombin stimulation according to the previous observation reported by Prescott *et al.* (2). For this reason, PAF production was routinely measured only in cell extract after thrombin stimulation. To check overall recovery of PAF 10 nCi of [^{3}H]PAF were added to the supernatants or to the cells. The extracted lipids were submitted to TLC with chloroform/methanol/water (65:35:6, v/v) as solvent system (22). The lipid material having an R_F from 0.15 to 0.22 was extracted (20) and used for biological assay and characterization. The recovery of added labeled PAF from the supernatants and the cells ranged from 93 to 95% after TLC purification.

PAF was detected by aggregation of washed rabbit platelets (2). The amount of PAF was expressed in pmol/3×10^5 cells and calculated over a calibration curve of standard PAF constructed for each series of assays (1). The specificity of platelet aggregation was inferred from the inhibitory effect of 5 μM CV-3988 and 5 μM BN52021 (23), two well-known PAF antagonists. PAF was characterized as previously described (21) according to the behavior under high performance liquid chromatography (HPLC) (24) and chemical (22) and lipase (25) treatments. Briefly, TLC-purified PAF dissolved in 0.2 ml of chloroform/methanol (1:1, v/v) was resolved by HPLC apparatus (Millipore Chromatography Division, Waters Associates, Milford, MA) equipped with a microPorasil column. The mobile phase consisted of chloroform/methanol/water (60:55:5, v/v) at a flow rate of 1.0 ml/min (24). Standard PAF (retention time = 20 min), phosphatidylcholine (retention time = 10 min) and lyso-phosphatidylcholine (retention time = 26 min) were used as references. The recovery of 5 nCi [^{3}H]PAF was 91.3 ± 2.5% (mean ± S.D. from four experiments). By HPLC, PAF from endothelial cells was eluted in a single, distinct peak between phosphatidylcholine and lyso-phosphatidylcholine after 20 min. HPLC- and TLC-purified PAF were characterized by the following criteria: sensitivity to phospholipase A_2, C, and D, and to base-catalyzed methanolysis; insensitivity to lipase A_1 and acidic and weak base treatment (22).

Acetyltransferase Assay—To test acetyltransferase activity, unstimulated and stimulated endothelial cells were processed as previously described (26) according to Wykle *et al.* (16). Proteins from lysed cells were determined by the method of Lowry *et al.* (27). The standard reaction mixture contained 40 μM lyso-PAF, 200 μM [^3H] acetyl-CoA (0.5 μCi), 40 μg of lysate protein, in 0.5 ml of Tris-HCl 0.1 M, pH 6.9. The reaction was allowed to continue for 15 min at 37 °C. The enzymatic activity was linear as a function of lysate protein (up to 60 μg) and the incubation time (up to 20 min). The reaction was stopped by lipid extraction according to Bligh and Dyer (20). The lipids were TLC-chromatographed by using chloroform/methanol/water (65:35:6, v/v) as solvent. The radioactivity corresponding to the R_F of standard PAF (0.21) was used to measure the enzymatic activity. No other areas of the TLC plate contained radioactivity. To correct the radioactivity losses during lipid extraction and TLC purification, 0.05 μCi of 1-[^{14}C]palmitoyl-2-acetyl-GPC was extracted and TLC-chromatographed in separated samples. The absolute recovery of 0.05 μCi 1-[^{14}C]palmitoyl-2-acetyl-GPC after these procedures was 89.2 ± 5.9% (mean ± S.D. from three experiments). Acetyltransferase activity was expressed as nmoles of [^3H]acetate transferred to lyso-PAF/min/mg protein.

Measurement of $[Ca^{2+}]_i$ and ^{45}Ca Influx

3.5×10^4 cells were seeded for a single glass coverslip (12-mm diameter) and grown to confluence. At the time of the experiment, the number of cells on each coverslip was estimated by a fluorimetric determination of the DNA content (28) and found to be $5 \pm 0.3 \times 10^4$ cells/coverslip. No significant variations of these values were observed after manipulations for $[Ca^{2+}]_i$ and ^{45}Ca influx measurements. The integrity of the monolayer was also assessed by light microscopy examination. $[Ca^{2+}]_i$ transients were measured by using the calcium-sensitive fluorescent probe quin2 (29). Coverslips were incubated for 45 min in the presence of 6 μM quin2-AM in culture medium at 37 °C and 5% CO_2 atmosphere. After loading, coverslips were washed with culture medium. Viability of cells after quin2 loading was confirmed by Trypan blue exclusion. Fluorescence of the intracellular quin2 was measured in a Perkin-Elmer LS-5 spectrofluorimeter. The standard monochromator settings were 339 nm excitation (5-nm slit width) and 492 nm emission (10-nm slit width). For the test, the coverslip was firmly positioned in a quartz cuvette (1 cm) containing 1 ml of Na^+ solution so that emission radiation reached the coverslip surface at a 45° angle. Approximately 2×10^4 cells were within the excitation light path. The cuvette holder was thermostatted at 37 °C. Fluorescence spectral analysis of adherent cells after loading revealed a peak at 492 nm, demonstrating intracellular accumulation of quin2. The signal-to-noise ratio was more than satisfactory. For calibration, the fluorescence of Ca^{2+}-saturated dye (F_{max}) was taken as the maximal emission from cells treated with 200 μM ionomycin in Ca^{2+}-containing medium. 2 mM $MnCl_2$, which enters the cells via the ionophore and quenches quin2 fluorescence completely, was then added. The resulting fluorescence (FMn) was essentially equal to the background signal obtained before quin2 loading. Since quin2 fluorescence increases 6-fold upon binding with calcium (29), F_{min} was taken as F_{min} = FMn + 1/6(F_{max} − FMn), as suggested by Rink and Pozzan (30). $[Ca^{2+}]_i$ was calculated from the observed fluorescence F as $[Ca^{2+}]_i = 115\ nM\ (F - F_{min})/(F_{max} - F)$, with 115 nM being the apparent dissociation constant for Ca^{2+}/quin2 at 37 °C. For ^{45}Ca influx studies, coverslips were rinsed three times with Na^+ (or choline$^+$) solution and incubated in the same solution at 37 °C with a tracer quantity (~5 μCi/ml) of $^{45}CaCl_2$ for a 20–30-min period, during which three control samples were removed. When equilibration was reached, thrombin was added and coverslips were removed at intervals over 60 s. Each coverslip was rapidly washed three times with ice-cold saline containing 2 mM $CaCl_2$ (300-ml total rinse), and dropped in 5% Triton X-100 solution for 3 min. Cell-associated $^{45}Ca^{2+}$ was measured by scintillation counting by using Instagel counting solution (Amersham Corp., U. K.).

Cytoplasmic pH Measurements and Manipulation

For fluorimetric determination of cytoplasmic pH (pH_i), coverslips were washed twice with Na^+ solution and then incubated for 8 min in a 37 °C water bath with 30 μM 6-CFDA in Na^+ solution. 6-CFDA readily enters the cell where esterases remove the two acetate groups; the resulting impermeable 6-carboxyfluorescein (6-CF) is trapped in the cytoplasm. When excited at 490 nm, the intensity of its emission at 520 nm is a function of pH. After loading, the coverslips were washed twice with Na^+ solution and used for fluorescence determination in 1 ml of the indicated medium by using a Perkin-Elmer LS-5 spectrofluorimeter equipped with a thermostatically controlled (37 °C) cell holder. The monolayer was positioned so that the incident light reached the coverslip at a 45° angle, and the same cells were studied throughout the experiment. Approximately 2×10^4 cells were within the excitation light path. Excitation band widths (490 and 430 nm) and the emission band (520 nm) width were set at 5 nm. The ratio of the emitted fluorescence signals (520 nm) permits calculations of resting pH_i and rates of change in pH_i which are independent of cell number, dye loading, and dye bleaching. The fluorescence signal was calibrated as a function of pH_i by the H^+ equilibration method of Thomas *et al.* (31) using the H^+/K^+ ionophore nigericin (4 μM; Sigma). The ratio of the signal at 490 nm to that at 430 nm as a function of pH_i followed a standard pH titration curve. Leakage of dye during the time of measurement, estimated from the fluorescence of the solution in which the monolayers had been placed, was negligible. Cytoplasmic acid loading was obtained by treating 6-CF-loaded monolayers placed in choline$^+$ or mannitol solution, pH 7.4, with 4 μM nigericin. The ionophore exchanges inner K^+ for outer H^+, resulting in cytoplasmic acidification. Acid loading could be stopped readily at the desired pH_i by addition of albumin (5 mg/ml final concentration), which presumably binds nigericin and stops its further action (32).

Determination of intracellular buffering power was carried out by NH_4^+ titration, as described (32), in 6-CF-loaded monolayers placed in choline solution. To determine the pH dependence of the intracellular buffering power, the starting pH_i was manipulated by the nigericin-albumin technique in choline$^+$ solution, 2 mM NH_4Cl was added and pH_i was measured. The buffering capacity was calculated as $\Delta[NH_4^+]_i/\Delta pH_i$ (33). The concentrations of intracellular NH_4^+ ($[NH_4^+]_i$) were calculated by using a p*K* value of 9.21 and assuming that the uncharged species NH_3 is in equilibrium across the membrane. The buffering power was found to increase slightly as pH_i decreased in the range 7.3–6.2 and the range of values was 23–47 mmol/liter/pH unit. An average value of 32 mmol/liter/pH unit was used for calculations of H^+ equivalent fluxes throughout the pH_i 7.4–6.2 range. The acid extrusion rates (H^+ efflux: mmol/liter/min) were calculated as the product of the rate of pH_i change (in pH units/min) times the buffering power (in mmol/liter/pH unit). The rates of pH_i change were measured directly from the fluorescence recordings.

RESULTS

Thrombin-induced Activation of Acetyltransferase and PAF Production by Endothelial Cells: Effect of Extracellular Ca^{2+}

In endothelial cells human thrombin (0.2 units/ml) rapidly enhanced acetyltransferase activity about 7-fold and increased the PAF content 8-fold (Fig. 1*A*). The peak level in both events was reached within 5 min and declined to the basal value within 10 min. Acetyltransferase activation and PAF production strongly depended on the extracellular Ca^{2+} concentration, as they were inhibited at 0–10 μM extracellular Ca^{2+} and progressively increased up to 1 mM outer Ca^{2+} (Fig. 1*B*). Accordingly, a complete inhibition was observed in Ca^{2+}-free medium with 2 mM EGTA or EDTA (data not shown).

Thrombin-induced Activation of Acetyltransferase and PAF Production by Endothelial Cells: Effect of Na^+-free Media and Na^+/H^+ Exchange Inhibitors

When thrombin (0.2 units/ml) stimulation was performed in 1 mM Ca^{2+}-containing media where Na^+ was replaced with choline$^+$ or *N*-methyl-D-glucamine$^+$, there were observed a 88–95% decrease of PAF production (Fig. 2, *upper panel*) and a 88% inhibition of acetyltransferase activation (Fig. 2, *lower panel*). The absence of extracellular Na^+ did not modify the basal activity of acetyltransferase (3.41 ± 0.22 nmol/min/mg protein in the presence of extracellular Na^+ *versus* 3.26 ± 0.15 nmol/min/mg protein in Na^+-free medium, *n* = 3) and the content of PAF in resting cells (1.53 ± 0.3 pmol/5 × 10^5 cells in the presence of extracellular Na^+ *versus* 1.61 ± 0.7 pmol/5

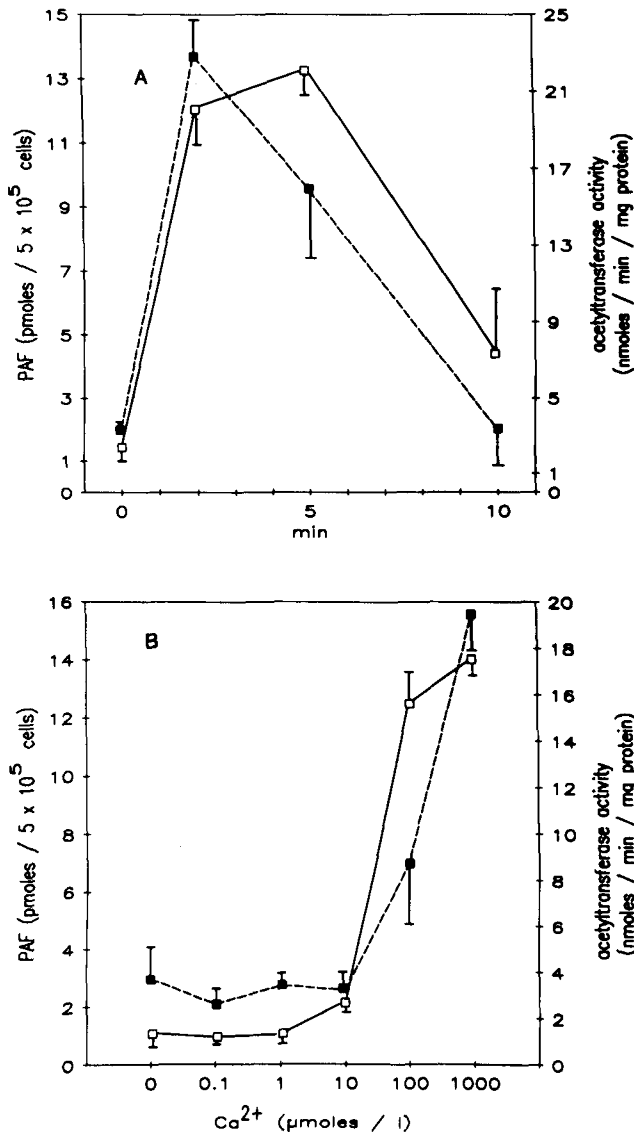

FIG. 1. **Effect of thrombin on PAF synthesis and acetyltransferase activity in human endothelial cells.** *A*, endothelial cell monolayers (cell count = 5.1 ± 0.24 × 10^5 for PAF measurement and 9.8 ± 0.42 × 10^5 for detection of acetyltransferase activity), incubated at 37 °C, were stimulated with 0.2 units/ml thrombin: PAF production (□——□) and acetyltransferase activity (■– – –■) were measured as described under "Experimental Procedures" at various intervals of time. Data are expressed as the mean ± S.D. of duplicate and quadruplicate determinations, respectively. *B*, in the same experimental conditions, PAF production (□——□) and acetyltransferase activation (■– – –■), induced after 5 min of thrombin (0.2 units/ml) stimulation, were determined under various $CaCl_2$ concentrations in the medium. Acetyltransferase activity was expressed as nmoles of [^{3}H]acetate transferred to lyso-PAF/min/mg protein. Results are presented as mean ± S.D. from two and three experiments, respectively.

× 10^5 cells in Na^+-free medium, $n = 4$). EIPA, an inhibitor of Na^+/H^+ exchange caused a dose-dependent inhibition of thrombin-evoked PAF synthesis and acetyltransferase activation (Fig. 2), after 10 min of preincubation of the cells in the Na^+,Ca^{2+}-containing medium. Maximal inhibition was achieved at 100 µM EIPA, that *per se* did not cause any effect on the basal values nor reduced viability of endothelial cells (which was 93–96%, as inferred by trypan blue exclusion). 0.5–1 mM amiloride, a Na^+/H^+ exchange inhibitor less selective than EIPA (34), blocked (in a dose-dependent manner) the effect of thrombin on PAF production (data not shown).

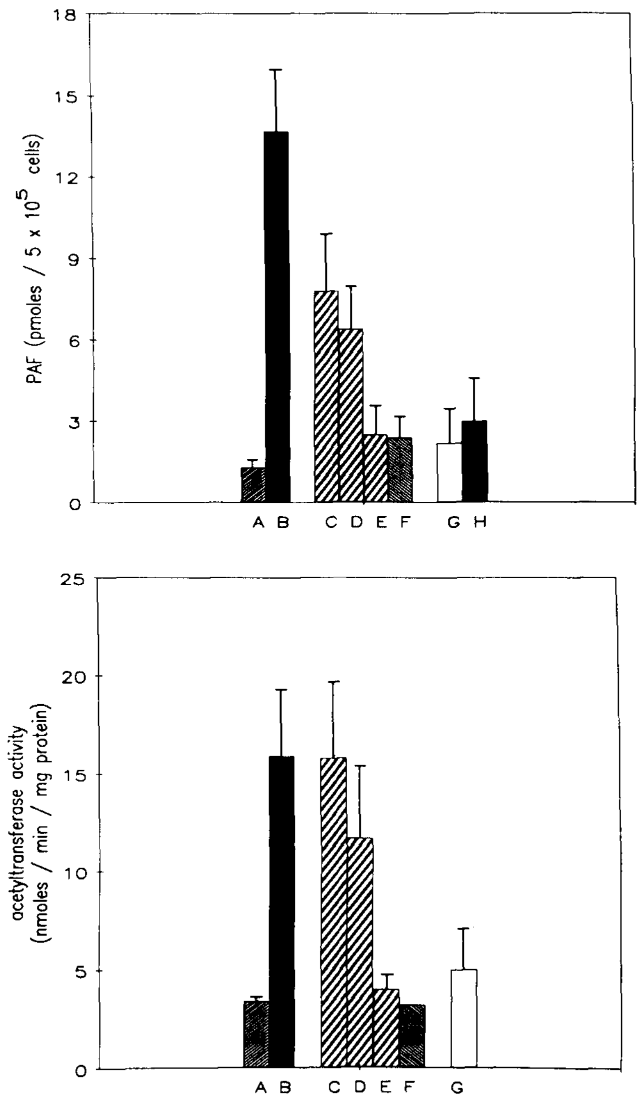

FIG. 2. **Effect of Na^+/H^+ exchange impairment on thrombin-induced PAF synthesis and acetyltransferase activation in human endothelial cells.** *Upper panel*, PAF production in endothelial cell monolayers (5.1 ± 0.24 × 10^5 cells) was measured in the following different experimental conditions. *A*, control; *B*, 5 min of thrombin stimulation (0.2 units/ml); *C*, 5 min EIPA (10 µM) followed by 5 min of thrombin stimulation (0.2 units/ml); *D*, 5 min EIPA (50 µM) followed by 5 min of thrombin stimulation (0.2 units/ml); *E*, 5 min EIPA (100 µM) followed by 5 min of thrombin stimulation (0.2 units/ml); *F*, 5 min of incubation with 100 µM EIPA alone; *G*, 5 min of thrombin stimulation (0.2 units/ml) in a medium where Na^+ was replaced with choline$^+$; *H*, 5 min of thrombin stimulation (0.2 units/ml) in a medium where Na^+ was replaced with *N*-methyl-D-glucamine$^+$. Results are expressed as the mean ± S.D. from four experiments. *Lower panel*, acetyltransferase in endothelial cell monolayers (9.8 ± 0.42 × 10^5 cells) was measured in the following different experimental conditions: *A*, control; *B*, 5 min of thrombin stimulation (0.2 units/ml); *C*, 5 min of EIPA (5 µM) followed by 5 min of thrombin stimulation (0.2 units/ml); *D*, 5 min of EIPA (50 µM) followed by 5 min of thrombin stimulation (0.2 units/ml); *E*, 5 min of EIPA (100 µM) followed by 5 min of thrombin stimulation (0.2 units/ml); *F*, 5 min of incubation with 100 µM EIPA alone; *G*, 5 min of thrombin stimulation (0.2 units/ml) in a medium where Na^+ was replaced with *N*-methyl-D-glucamine$^+$. Results are expressed as the mean ± S.D. from three experiments.

Thrombin-induced PAF Production by Endothelial Cells: Effect of Changing Extracellular pH (pH_o)

The effect of changing pH_o on thrombin-induced PAF production by endothelial cells was measured as follows. PAF

production was determined after 3 min of cell stimulation at 37 °C with thrombin (0.2 units/ml) in Na^+ solution + 0.25% BSA at different pH_o values (7.0, 7.4, and 7.8). No effect of changing pH_o on PAF basal values was observed (0.81 ± 0.27, 0.85 ± 0.37, 0.72 ± 0.39 pmol/5 × 10^5 cells at pH 7.0, 7.4, and 7.8, respectively). The net thrombin-evoked PAF production showed a strong dependence on pH_o (3.91 ± 0.74, 7.26 ± 0.85, and 9.71 ± 0.71 pmol/5 × 10^5 cells at pH 7.0, 7.4, and 7.8, respectively). This observation stresses the involvement of Na^+/H^+ exchange in thrombin-induced PAF production, as an increase of pH_o enhances the activity of the exchanger (10).

Thrombin-induced PAF Production by Endothelial Cells: Effect of Membrane Depolarization

To test if depolarization of endothelial cells may account for thrombin-induced PAF production, in the Na^+ solution Na^+ was partially or totally replaced by K^+. A 1–3-min incubation of endothelial cells with 56 and 150 mM KCl did not significantly modify basal values of PAF production (Table I). When thrombin (0.2 units/ml) stimulation was performed in 56 mM KCl-containing medium, PAF production was 81% of that obtained in Na^+ solution, while a 91% inhibition was observed in 150 mM KCl-containing medium, a condition similar to the total replacement of Na^+ with choline$^+$ or *N*-methyl-D-glucamine$^+$. Depolarization with K^+-rich solution did not affect the subsequent effect of thrombin on PAF production, when measured in Na^+ solution (Table I).

To rule out the involvement of the "fast" Na^+ channels and the epithelial Na^+ channels in the thrombin-induced PAF synthesis in endothelial cells, the cells were preincubated in Na^+ solution + 0.25% BSA for 10 min with tetrodotoxin and benzamil (Table II): neither 0.3–3 μM tetrodotoxin nor 0.01–100 μM benzamil produced any effect on the PAF synthesis elicited by 3 min of stimulation with 0.2 units/ml thrombin.

Thrombin-induced $[Ca^{2+}]_i$ Rise and ^{45}Ca Influx in Endothelial Cells: Effect of Na^+-free Media and Na^+/H^+ Exchange Inhibitors

Endothelial cells grown on coverslips, preloaded with quin2 and placed in Na^+ solution containing 1 mM $CaCl_2$, were exposed to 0.2 units/ml thrombin. The agonist evoked a large increase in $[Ca^{2+}]_i$ within a few seconds. In the presence of 1 mM extracellular Ca^{2+}, $[Ca^{2+}]_i$ rose from the basal level of 0.166 ± 0.051 to 0.864 ± 0.138 μM (n = 12). This increase was transient: it reached its maximum within about 30–45 s and then fell, within a few minutes, to base-line-like values. In Ca^{2+}-free medium with 1 mM EGTA, the peak level was very much smaller: 0.350 ± 0.085 μM (n = 5). EGTA was added to the Ca^{2+}-free medium 30–40 s before the agonist addition, and during this time there was little fall in resting $[Ca^{2+}]_i$, judged from the quin2 signal (<10%). The difference between the two transients is likely due to an influx of Ca^{2+} across the plasma membrane, the rise observed with 1 mM extracellular EGTA being attributable to the release of Ca^{2+} from intracellular stores. When endothelial cells on coverslips were stimulated with 0.2 units/ml thrombin in a medium where Na^+ was completely and isoosmotically substituted by choline$^+$, a complete inhibition of $[Ca^{2+}]_i$ transient was observed. This suggests that thrombin-mediated Ca^{2+} movements in endothelial cells are strongly dependent on the presence of extracellular Na^+. As the fluorescence of the Na^+/H^+ exchange inhibitors (amiloride and EIPA) interfered with measurements of quin2 fluorescence, we were not able to test responses to thrombin in the presence of EIPA or amiloride by using quin2-loaded endothelial cells. In order to overcome this difficulty, the time course of Ca^{2+} influx into endothelial cells was measured by observing $^{45}Ca^{2+}$ uptake after thrombin stimulation (0.5 units/ml) in cells pre-equilibrated with $^{45}Ca^{2+}$ at 37 °C (Fig. 3). Cell-associated $^{45}Ca^{2+}$ peaked at 10 s, and then fell to base-line values within 60 s. The peak was completely abolished when Na^+ was totally replaced by choline$^+$, and when Na^+/H^+ exchange was impaired by addition of 100 μM EIPA (5-min preincubation). As the $^{45}Ca^{2+}$ method measures Ca^{2+} influx from extracellular medium exclusively, these results suggest that thrombin-elicited Ca^{2+} influx depends on Na^+/H^+ exchange activation. Countertransport of external Na^+ for internal H^+ can be artificially obtained by addition of the cation exchanger monensin. When added to 6-CF-loaded endothelial cells placed in Na^+ solution, 10 μM monensin induced a sustained alkalinization (ΔpH_i = 0.16 ± 0.04 units; n = 12), which reached the maximum in 1–2 s. When added to quin2-loaded cells suspended in Na^+ solution, 10 μM monensin was found to elicit a clear-cut increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$ = 1.164 ± 0.422 μmol; n = 7), which reached the maximum in 20–30 s. Interestingly enough, $[Ca^{2+}]_i$ level did not fall to base-line-like values, but remained near to maxi-

TABLE I

Effect of membrane depolarization on PAF synthesis by human endothelial cells

PAF was extracted from human endothelial cells (5.0 ± 0.18 × 10^5) as described under "Experimental Procedures," after 1–3 min of preincubation in Na^+ solution + 0.25% BSA at different concentrations of external K^+ (K^+_o) in the absence (resting cells) and in the presence (stimulated cells) of 0.2 units/ml thrombin (3 min). Data are means ± S.D. of the number of determinations (n) indicated.

K^+_o	PAF		n
	Resting cells	Stimulated cells	
mM	*pmol/5 × 10^5 cells*		
0 (control)	0.85 ± 0.23	8.30 ± 0.90	3
56 (1 min)	1.26 ± 0.28		3
150 (1 min)	0.66 ± 0.31		3
56 (3 min)	1.14 ± 0.55	7.18 ± 0.27	3
150 (3 min)	0.79 ± 0.37	1.56 ± 0.22	3
56 (3 min)[a]		7.94 ± 0.99	3
150 (3 min)[a]		7.1 ± 0.37	3

[a] After 3 min of incubation in K^+ solutions, cells were washed and stimulated for 3 min with 0.2 units/ml thrombin in Na^+ solution.

TABLE II

Effect of Na^+ channel inhibitors on PAF synthesis by human endothelial cells

PAF was extracted from human endothelial cells (5.0 ± 0.18 × 10^5) as described under "Experimental Procedures," after 10 min preincubation in Na^+ solution + 0.25% BSA with 50 μM EIPA or with different concentrations of benzamil and tetrodotoxin in the absence (resting cells) and in the presence (stimulated cells) of 0.2 units/ml thrombin (3 min). Data are means ± S.D. of the number of determinations (n) indicated.

Conditions	PAF		n
	Resting cells	Stimulated cells	
μM	*pmol/5 × 10^5 cells*		
Control	1.50 ± 0.20	5.17 ± 0.98	3
Benzamil 0.01		6.30 ± 0.80	3
Benzamil 0.1		5.70 ± 0.60	3
Benzamil 1		4.30 ± 0.75	3
Benzamil 10		5.65 ± 0.55	3
Benzamil 50		5.00 ± 1.10	3
Benzamil 100	1.17 ± 0.21	5.05 ± 0.25	3
Tetrodotoxin 0.3		5.55 ± 0.65	3
Tetrodotoxin 3	0.87 ± 0.12	5.70 ± 1.40	3
EIPA 50		1.62 ± 0.32	3

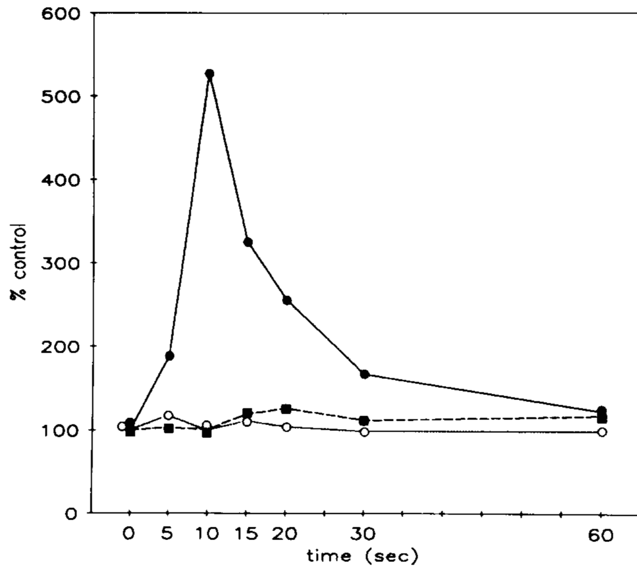

FIG. 3. **Time course of $^{45}Ca^{2+}$ influx into human endothelial cells stimulated with thrombin and effect of Na^+/H^+ exchange inhibition.** Endothelial cells were allowed to grow on sterile glass coverslips until confluence ($5 \pm 0.3 \times 10^4$ cells/coverslip), and were equilibrated with $^{45}Ca^{2+}$ by incubating them with 5 μCi/ml $^{45}CaCl_2$ for a 20–30-min period, then 0.5 units/ml thrombin was added (time 0) and the cell-associated $^{45}Ca^{2+}$ was measured at various intervals of time up to 1 min, as described under "Experimental Procedures." Every experiment was performed in the following conditions: Na^+-containing medium (●——●), medium containing choline$^+$ instead of Na^+ (○——○), and Na^+-containing medium + 100 μM EIPA (5-min preincubation) (■——■). The results, expressed as percentage of control radioactivity, are representative of four experiments with similar results (basal cpm/coverslip = 720 ± 64 in Na^+-containing medium, 890 ± 69 in choline$^+$-containing medium, 490 ± 63 in Na^+/EIPA-containing medium; n = 4).

TABLE III
Effect of thrombin and monensin on PAF synthesis by human endothelial cells

PAF was extracted from human endothelial cells ($5.1 \pm 0.24 \times 10^5$) and their supernatants as described under "Experimental Procedures," after 3 min of preincubation with medium alone, thrombin, or two different concentrations of monensin. Data are means ± S.D. of the number of determinations (n) indicated.

Conditions	PAF		
	Cell extract	Supernatant extract	n
	pmol/5 × 10⁶ cells		
Control	0.85 ± 0.49	0	3
Thrombin (0.2 units/ml)	7.05 ± 0.49	0	3
Monensin (5 μM)	3.30 ± 0.42	0	3
Monensin (10 μM)	8.98 ± 2.90	2.44 ± 1.01	3

mum for a prolonged time. Monensin did not increase $[Ca^{2+}]_i$ if choline$^+$ replaced Na^+. The monensin-induced $[Ca^{2+}]_i$ rise was completely eliminated when external Ca^{2+} was removed (Ca^{2+}-free medium plus 1 mM EGTA), suggesting that the major component of monensin-induced $[Ca^{2+}]_i$ rise is the Ca^{2+} influx from extracellular medium. Monensin-stimulated endothelial cells produced PAF in a concentration-dependent way, when Ca^{2+} (1 mM) was present in the extracellular medium (Table III). The response elicited by 10 μM monensin was superimposable to the response obtained with 0.2 units/ml thrombin, but in addition the ionophore, at this higher concentration, induced also a PAF release in the medium. The monensin-dependent Na^+/H^+ exchange activation results in increased intracellular Na^+ concentration and cytoplasmic alkalinization. In order to understand which of the two events was the likely trigger of extracellular Ca^{2+} influx, 20 mM NH_4Cl, pH 7.4, was used to raise intracellular pH without modifying cellular Na^+ content. NH_4^+, which is in equilibrium with the permeating weak base NH_3 (33), elicited a very rapid alkalinization in 6-CF-loaded endothelial cells placed in Na^+-solution (ΔpH_i = 0.34 ± 0.09 units; n = 3). When added to quin2-loaded cells placed in Na^+ solution, NH_4Cl evoked a $[Ca^{2+}]_i$ transient ($\Delta[Ca^{2+}]_i$ = 1.410 ± 0.220 μmol; n = 6), which was decreased by 80% in Ca^{2+}-free medium containing 1 mM EGTA. These results strongly suggest that cytoplasmic alkalinization is responsible for the Ca^{2+} influx across the plasma membrane that accompanies activation of the Na^+/H^+ exchange. The possible role of depolarization in thrombin-induced Ca^{2+} influx was ruled out, since when quin2-loaded endothelial cells were exposed to 56 and 150 mM KCl-solutions, no rise in $[Ca^{2+}]_i$ was observed (data not shown). Pretreatment of the cells with the same solutions for 3 min did not modify the thrombin-induced $[Ca^{2+}]_i$ rise when measured both in Na^+ solution containing 1 mM $CaCl_2$ and in Ca^{2+}-free medium with 1 mM EGTA (data not shown).

Evidence for a Na^+/H^+ Exchange in Endothelial Cells

In four experiments, the pH_i of 6-CF-loaded endothelial cells on coverslips placed in Na^+ solution at 37 °C averaged 7.37 ± 0.03. The presence of a Na^+/H^+ antiport in endothelium was assayed by acid-loading the cells to pH_i 6.2 with nigericin in choline$^+$ or mannitol solution (Fig. 4). The ionophore had no effect on endothelial cells viability following a 30-min incubation. Nigericin was then scavenged by adding albumin (32), and medium was replaced with medium containing 50 mM NaCl, which brought about a rapid cytoplasmic alkalinization, consistent with Na^+_o/H^+_i countertransport. The Na^+-induced alkalinization followed an exponential course and ceased when pH_i returned to 7.0–7.2. The rate of

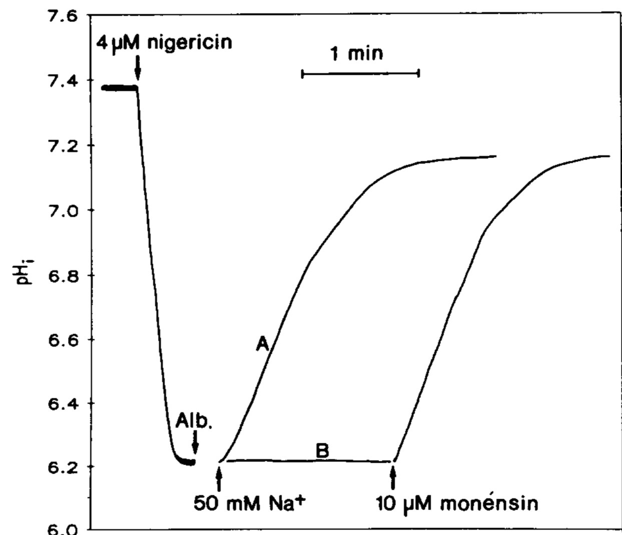

FIG. 4. **Na^+/H^+ exchange activation in acidified human endothelial cells.** Endothelial cells grown on sterile coverslips and loaded with 6-carboxyfluorescein (as described under "Experimental Procedures") were rapidly acidified by incubating them with 4 μM nigericin in a medium containing choline$^+$ or mannitol instead of Na^+. Acidification was stopped at pH ~6.2 by removing nigericin via albumin (*Alb.*, 5 mg/ml) addition. *A*, replacement of medium with a buffer solution containing 50 mM NaCl evoked a rapid alkalinization due to Na^+/H^+ exchange activation. *B*, after 10 min of incubation with 10–100 μM EIPA cells were acid-loaded by nigericin addition, then the medium was replaced by a buffer solution containing 50 mM NaCl + 10–100 μM EIPA; alkalinization occurred only when blockade of Na^+/H^+ exchange was bypassed by 10 μM monensin addition.

alkalinization depended on the starting pH_i and on the external Na^+ concentration ($[Na^+]_o$) (see below). If endothelial cells were treated with 10–100 μM EIPA or 0.5–1 mM amiloride while in choline$^+$ or mannitol solution (Fig. 4), there was complete inhibition of alkalinization upon substituting Na^+-free with Na^+-containing solution. Addition of the inhibitor itself had no significant effect on pH_i, and subsequent addition of monensin bypassed the inhibition, resulting in cytoplasmic alkalinization. The dependence of cytoplasmic alkalinization upon extracellular Na^+ and inhibition by EIPA provide strong evidence for mediation of this alkalinization by Na^+/H^+ exchange.

Properties of Na^+/H^+ Exchanger of Endothelial Cells

$[Na^+]_o$ Dependence—To study the Na^+_o dependence of endothelial cells antiport, the endothelial cells on coverslips were initially acid-loaded with nigericin while pH_i was fluorimetrically monitored. When pH_i reached 6.2–6.3 the ionophore was scavenged, and Na^+/H^+ exchange was initiated by replacing choline$^+$ or mannitol medium with media of varying concentrations of Na^+_o (osmotically balanced with choline$^+$ or mannitol, pH 7.4). The rate of H^+ equivalent efflux was then determined from the rate of Na^+-induced ΔpH_i, by using the value for the buffering capacity determined above (see "Experimental Procedures"). The rate of alkalinization increased with increasing $[Na^+]_o$, as illustrated in Fig. 5. The maximal rate of Na^+/H^+ exchange (measured at $[Na^+]_o = 100$ mM, pH 7.4) was 41.22 ± 5.76 mmol H^+/liter/min and half-maximal stimulation occurred at 25 mM $[Na^+]_o$.

pH_i Dependence—The rate of Na^+-induced alkalinization depended critically on the pH_i (Fig. 6). This was determined by acid loading endothelial cells on coverslips to varying degrees with nigericin/albumin, followed by replacing choline$^+$ or mannitol medium with medium of constant (50 mM) Na^+_o concentration. All the determinations were carried out at a constant pH_o (7.4). As shown in Fig. 6 the rate of Na^+-induced alkalinization increased approximately linearly as pH_i decreased in the 7.2–6.2 range. No net Na^+-induced H^+ efflux was detectable at $pH_i \geq 7.2$.

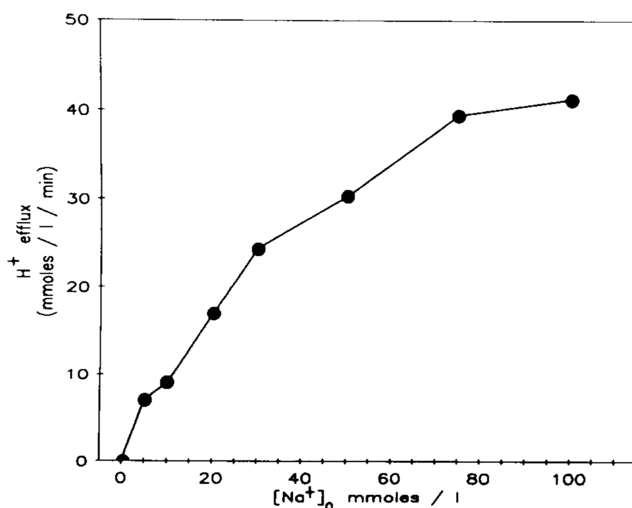

FIG. 5. **Dependence of Na^+/H^+ exchange on extracellular Na^+.** After acid-loading was performed as described in Fig. 4, the medium was replaced by isoosmotic buffer solutions containing progressively increasing concentrations of NaCl. The rate of H^+ equivalent efflux was determined from the rate of Na^+-induced ΔpH_i, by using the value for the buffering capacity (see "Experimental Procedures"). $[Na^+]_o$, extracellular Na^+ concentration. The curve is representative of three experiments with similar results.

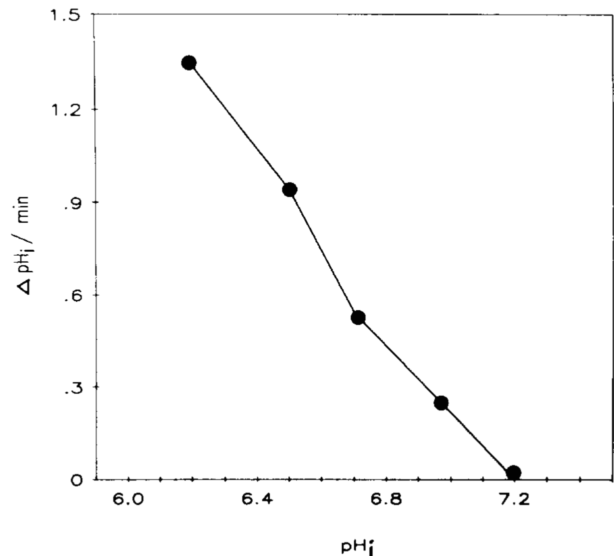

FIG. 6. **Dependence of Na^+/H^+ exchange on intracellular pH.** 6-Carboxyfluorescein-loaded endothelial cell monolayers were placed in media containing 120 mM K^+ at different pH, then 4 μM nigericin was added. Due to the absence of transmembrane K^+ gradient, in these experimental conditions nigericin mediates a downhill passage of H^+ through the membrane until intracellular and extracellular pH are equal; by manipulating pH of K^+ medium it is possible to obtain cells with different intracellular pH (pH_i). When the desired pH_i was achieved, nigericin was scavenged by addition of 5 mg/ml albumin and the medium was replaced by a buffer solution containing 50 mM Na^+: the alkalinization rate was measured as ΔpH_i/min and plotted against pH_i. The curve is representative of two experiments with similar results.

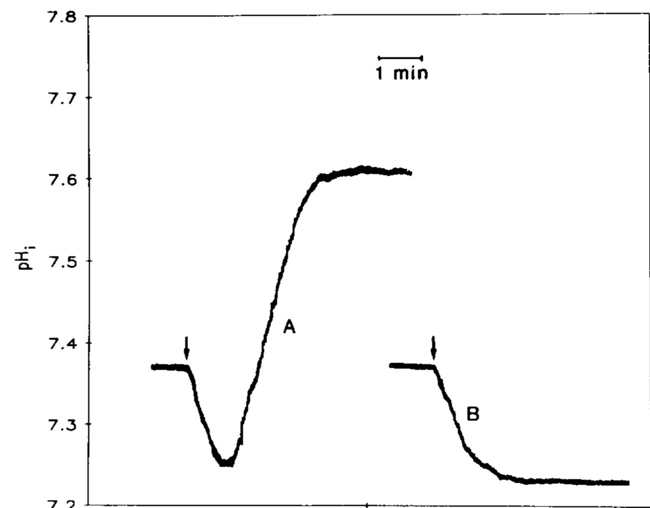

FIG. 7. **Effect of thrombin on intracellular pH of human endothelial cells.** 6-Carboxyfluorescein-loaded endothelial cell monolayers were stimulated with 0.5 units/ml thrombin (*arrow*) in Na^+ solution (*A*) and in Na^+ solution + 100 μM EIPA (10 min preincubation) or in Na^+-free choline$^+$ solution (*B*). Representative tracings of five experiments with similar results.

Effects of Thrombin on Cytoplasmic pH_i of Endothelial Cells

The effects of thrombin (0.5 units/ml) on the pH_i of endothelial cell monolayers are shown in Fig. 7. When thrombin was added to cells placed in Na^+ solution, pH 7.4 (resting pH_i = 7.37), a transient cytoplasmic acidification (−0.1–0.2 pH units) was observed, followed by a sustained alkalinization (total rise = 0.359 ± 0.039 pH units; $n = 5$). The acidification phase became more pronounced and sustained when the Na^+/H^+ exchange was impaired by removal of extracellular Na^+

(stimulation with thrombin in choline$^+$ solution) or when the cells were pretreated with 100 μM EIPA; the alkalinization response was inhibited in both cases.

DISCUSSION

The results of the present work confirm that, as a consequence of thrombin stimulation, human endothelial cells are able to synthesize PAF in a concentration-dependent way and that thrombin-induced PAF remains bound to the cells. The kinetics are rather fast and the effect is maximal within 5 min. These results are in keeping with previous results of Prescott *et al.* (2) and with more recent observations of Hirafuji *et al.* (35). PAF synthesis depends strongly on the concentration of free Ca^{2+} in the medium, as it is inhibited at 0–10 μM extracellular Ca^{2+}, and it is fully expressed at 1 mM outer Ca^{2+}.

Stimulation of human endothelial cells by thrombin elicits a rapid increase of lyso-PAF acetyltransferase activity, which peaks within 2 min, returns to basal values within 10 min, and shows a time course parallel to that of PAF production. Acetyltransferase activation depends on extracellular Ca^{2+}, being maximal at 1 mM Ca^{2+} and half-maximal at about 100 μM Ca^{2+}. The Ca^{2+} requirement of acetyltransferase activation has been described in rat macrophages (18, 36), in human leukocytes (37), in human platelet microsomes (38), and in rat splenic microsomes (39). It has been reported that Ca^{2+} at micromolar concentrations regulates the acetyltransferase activity by decreasing the K_m for acetyl-CoA (39). In addition, the enzyme seems to be modulated by reversible phosphorylation (40, 41) in rat spleen microsomes. Recently, a calcium/calmodulin-dependent protein kinase has been described as the most likely candidate for the short-term regulation of acetyltransferase activity in intact parotid gland acinar cells (42). Whether such a mechanism is operating in thrombin-stimulated endothelial cells remains to be determined. Our results are consistent with those of Hirafuji *et al.* (35), who reported a similar Ca^{2+}-dependent acetyltransferase activation after thrombin challenge; moreover, when using the cell lysates from stimulated and unstimulated endothelial cells, these authors showed that thrombin induces a three to four times increase in the V_{max} of the acetylation reaction with no change in the apparent K_m value of the enzyme for acetyl-CoA, suggesting an increase in the number of the active enzyme molecules.

Our results additionally demonstrate that when thrombin stimulation of human endothelial cells is performed in Ca^{2+}-containing media where Na^+ is isoosmotically replaced with choline$^+$ or *N*-methyl-D-glucamine$^+$, a 90–95% inhibition of PAF production and acetyltransferase activation is observed. Moreover, preincubation of endothelial cells with the amiloride analog EIPA elicits a dose-dependent inhibition of thrombin-mediated PAF production and acetyltransferase activation. Dependence of both processes on extracellular Na^+, their inhibition by EIPA, and the observation that thrombin-induced PAF synthesis is a function of extracellular pH point to an involvement of Na^+/H^+ exchange. This electroneutral transport system that exchanges extracellular Na^+ for intracellular H^+ appears to be a ubiquitous component of plasma membranes of nucleated mammalian cells (for reviews see Refs. 9, 43) and is believed to play a major role in regulating cell volume, intracellular pH (pH_i) and transepithelial ion transport. Since it can be activated by a variety of growth factors (9, 43), it has also been suggested that it may participate in the initiation of cell proliferation. Na^+/H^+ exchange is inhibited by potassium-sparing diuretics such as amiloride and EIPA: at concentrations that inhibit Na^+/H^+ exchange, the selectivity of amiloride is poor, since it also inhibits protein kinase C (Ca^{2+}/phospholipid-dependent enzyme) (44), protein synthesis (45), Na^+/K^+-ATPase (46), and Na^+/Ca^{2+} exchange (47). EIPA, being 140 times more potent than amiloride (34) and having no significant effect on protein kinase C (44), is believed to be a specific inhibitor of Na^+/H^+ exchange system.

Thrombin is known to depolarize target cells, such as platelets (48), and the membrane potential change is inhibited by a fast-acting amiloride analog (48). The possible role for depolarization in thrombin-induced PAF production by endothelial cells was examined by determining the effect of increasing extracellular K^+ concentrations. Treatment of endothelial cells with 56 mM KCl or with 150 mM KCl solutions up to 3 min did not evoke any significant increase of PAF synthesis in comparison with controls. Depolarization of endothelial cells with 56 mM KCl + 94 mM NaCl-containing solution inhibited subsequent thrombin-induced PAF production by 19%; the 91% inhibition observed with 150 mM KCl-containing medium is attributable to the absence of Na^+. No change of PAF production was observed when endothelial cells, soon after depolarization, were put in Na^+ solution and then challenged with thrombin.

Guanidinium-substituted analogs of amiloride, such as benzamil, which are potent inhibitors of the epithelial sodium channel (49, 50) have been shown to inhibit a number of other membrane transporters, which include Na^+/H^+ exchanger, Na^+/Ca^{2+} exchanger, Na^+/K^+-ATPase, Na^+-alanine and Na^+-glucose cotransporters, voltage-gated sodium channel, and nicotinic acetylcholine receptors (51). As far as fast Na^+ channels are concerned, it has been demonstrated that guanidinium ions are not only impermeant through but also block fast Na^+ channels (52). Due to the existence and the role of guanidinium groups in tetrodotoxin and saxitoxin molecules, it has been suggested that guanidinium ions interact with the tetrodotoxin-binding site and block fast Na^+ channels which are more sensitive to tetrodotoxin than slow Na^+ channels (53). We therefore tested the effect of tetrodotoxin and benzamil (10 min of preincubation) on thrombin-induced PAF production in endothelial cells. As shown in Table II, tetrodotoxin was without action up to a concentration of 3 μM, and benzamil had no effect up to a concentration of 100 μM. In the same set of experiments, preincubation with 50 μM EIPA completely blocked the thrombin-induced PAF production. These results show that 1) membrane depolarization does not evoke PAF production in endothelial cells; 2) depolarization is not essential for thrombin-induced PAF production; and 3) inhibitors of fast Na^+ channel and/or epithelial Na^+ channel do not affect thrombin-induced PAF production.

We looked to see which was the link between thrombin-mediated PAF production through acetyltransferase stimulation and Na^+/H^+ exchange activity in endothelial cells, and we focussed our attention on thrombin-elicited Ca^{2+} influx. In human endothelial cells, thrombin produces a rise in $[Ca^{2+}]_i$ in both the presence and the absence of external Ca^{2+} (6–8). The much larger rise in the presence of external Ca^{2+} indicates a substantial Ca^{2+} influx across the plasma membrane, additional to the release from internal stores observed in its absence. The measurement of Ca^{2+} release from internal stores, which necessitates the prevention of influx, was done by chelating external Ca^{2+} with EGTA. Theoretically, this procedure could induce (*a*) depletion of intracellular Ca^{2+} and/or (*b*) disruption of receptor-ligand interaction or subsequent signal transduction. These possible artifacts are ruled out by the following evidences: (*a*) EGTA was added to the Ca^{2+}-free medium 30–40 s before the agonist addition, and

during this time the decrease in resting $[Ca^{2+}]_i$, measured from the quin2 signal, resulted very small; furthermore, in a previous experiment (54) we demonstrated that addition of 200 nM ionomycin to endothelial cells suspended in a Ca^{2+}-free 2 mM EGTA-containing medium was able to elicit a mobilization of Ca^{2+} from internal stores and that this $[Ca^{2+}]_i$ rise was largely superimposable to the values obtained with thrombin stimulation in Ca^{2+}-free medium; in addition, it has been shown in fura2-loaded platelets that the thrombin-evoked $[Ca^{2+}]_i$ rises observed in EGTA-containing medium were superimposable to the responses obtained in the presence of 1 mM external Ni^{2+} (55), which is known to block receptor-mediated Ca^{2+} entry across the platelet plasma membrane (56); (*b*) in our previous work (54) we observed that 2 mM EGTA in Ca^{2+}-free medium does not modify [^{3}H]inositol-phosphates formation in response to 0.05–1 units/ml thrombin.

While it is demonstrated that inositol-1,4,5-trisphosphate generated at the plasma membrane after thrombin stimulation diffuses to intracellular storage pools to trigger Ca^{2+} release (8), so far no evidence exists to link inositol-1,4,5-trisphosphate (and other cyclic or more phosphorylated intermediates) to receptor-mediated Ca^{2+} influx in endothelial cells. The evidence available is against the presence of voltage-gated Ca^{2+} channels in endothelial cells membrane: (*a*) depolarization of the membrane by K^+-rich solutions does not induce a rise in $[Ca^{2+}]_i$; (*b*) treatment with verapamil, an inhibitor of calcium influx via voltage-dependent calcium channels, has no effect on either basal- or thrombin-induced levels of $[Ca^{2+}]_i$ (8). Our experiments show that thrombin-induced Ca^{2+} influx in endothelial cells closely depends on the presence of Na^+ in the extracellular medium, as in quin2-loaded cells the influx is completely inhibited when Na^+ is replaced by choline$^+$. These results are confirmed by $^{45}Ca^{2+}$ experiments, in which only the $^{45}Ca^{2+}$ influx from extracellular medium is measured (Fig. 3); the influx elicited by thrombin in the presence of 145 mM extracellular Na^+ is completely blocked when Na^+ is totally replaced by choline$^+$; moreover EIPA, the specific inhibitor of Na^+/H^+ exchange, abolishes the $^{45}Ca^{2+}$ peak in thrombin-stimulated endothelial cells. This strongly suggests that *Na^+/H^+ exchange activation* by thrombin is a prerequisite for Ca^{2+} influx in human endothelial cells. These data are in agreement with recent reports (12–14) demonstrating that activation of Na^+/H^+ exchange is needed for thrombin-induced Ca^{2+} mobilization and for thrombin- and arachidonic acid-elicited Ca^{2+} influx in human platelets.

Na^+/H^+ exchange activation has been already described for phorbol esters, synthetic diacylglycerols, growth factors, and a variety of extracellular signals on different cell types (for reviews see Refs. 9, 43); since most of these agonists act through the phospholipase C-mediated breakdown of phosphoinositides and the formation of diacylglycerol, a putative second messenger activating protein kinase C, it has been suggested that agonist-stimulated Na^+/H^+ exchange results from activation of protein kinase C (9, 43). Our results show that thrombin, an agonist known to activate phospholipase C in human endothelial cells (8), elicits a sustained alkalinization in cultured human endothelium (Fig. 7): the involvement of Na^+/H^+ exchange in this response is stressed by the acidification observed when exchange is impaired by choline$^+$ substitution for Na^+, or by addition of the specific inhibitor EIPA. Moreover, when control and thrombin-stimulated cells are acid-loaded to the same pH_i (6.2), thrombin elicits a dose-dependent increase in the alkalinization rate when Na^+-free medium is replaced with Na^+-containing medium. These results demonstrate that thrombin induces Na^+/H^+ exchange activation in human endothelial cells.

To acquire further evidence that Na^+/H^+ exchange activation may be the trigger of Ca^{2+} influx, monensin was used to induce countertransport of external Na^+ for internal H^+ and cytoplasmic alkalinization in quin2- and 6-CF-loaded endothelial cells. Monensin is known to induce $[Ca^{2+}]_i$ transient in rat lymphocytes (57), in a transformed murine pre-B lymphocyte cell line (58), in sea urchin sperm cells (59), and in human platelets (14).

Our experiments show that in endothelial cells monensin elicits a sustained alkalinization, which is immediately followed by a clear-cut increase in $[Ca^{2+}]_i$. The major component of monensin-induced $[Ca^{2+}]_i$ rise is a Ca^{2+} influx from extracellular medium. The results obtained with monensin in the absence of extracellular Ca^{2+} rule out the possibility that the increases in $[Ca^{2+}]_i$ observed in the presence of 1 mM Ca^{2+} merely reflect pH-dependent changes in the affinity of the indicator; moreover, the presence of monensin-evoked Ca^{2+} influx is indirectly confirmed by the strong production of PAF which can be observed in monensin-stimulated endothelial cells. Monensin-induced PAF production strongly supports the link between Na^+_o/H^+_i countertransport-dependent cytoplasmic alkalinization and Ca^{2+} influx.

When NH_4Cl is used in quin2-loaded endothelial cells to elevate pH_i independently of Na^+ translocation, a rapid $[Ca^{2+}]_i$ transient is observed, which is 80% decreased in Ca^{2+}-free medium. This result indicates that Ca^{2+} influx is linked to cytoplasmic alkalinization and not to the concomitant Na^+ influx. Evidence that an amiloride-sensitive Na^+/H^+ exchange system exists in the membrane of human endothelial cells comes from the following observations: (*a*) Na^+ induces a rapid cytoplasmic alkalinization in cells that have been acidified with nigericin; (*b*) if cells are treated with EIPA while in Na^+-free medium, there is complete inhibition of alkalinization upon substituting Na^+-free with Na^+-containing solution.

The properties of the endothelial cells' Na^+/H^+ exchange are similar to those described for other cell types (10). At pH 7.4, the antiport in acid-loaded endothelial cells has an apparent K_m for Na^+_o of ~25 mM (Fig. 5). This value is intermediate between those reported for epithelial membranes (*e.g.* 6–7 mM for renal brush border vesicles) (60) and those observed in cultured cells (*e.g.* 42 mM) (61) or thymic lymphocytes (59 mM) (32). The Na^+/H^+ antiport of endothelial cells is nearly quiescent at physiological pH_i, as no Na^+-induced or amiloride-sensitive pH_i changes are observed at $pH_i \geq 7.2$. In contrast, the exchanger, as in other cells (10, 32, 62), is greatly activated when the cytoplasm is acidified (Fig. 4). This peculiar behavior is thought to reflect its role in pH_i homeostasis and appears to be determined by a pH_i-sensitive allosteric activator or "modifier" site (10). The existence of such a modifier would account for the steep dependence of the rate of transport on pH_i (Fig. 6). In summary, the present study demonstrates that 1) thrombin-induced PAF production in human cultured endothelial cells is linked to a Ca^{2+}-dependent activation of a lyso-PAF:acetyl-CoA acetyltransferase; 2) Ca^{2+} influx from extracellular medium and the consequent PAF production can be triggered by a cytoplasmic alkalinization, but not by membrane depolarization; 3) alkalinization of endothelial cells cytoplasm is elicited by a thrombin-dependent activation of a Na^+/H^+ exchange system; 4) such a Na^+/H^+ exchange system exists in the plasma membrane of human endothelial cells and is clearly detectable in unstimulated cells only if the cytoplasmic pH is driven below the physiological level. This further insight into the mechanism of thrombin-

induced PAF production in human endothelial cells (and into its possible regulation) may be important, as PAF generated by endothelial cells is believed to influence locally the function of circulating blood cells such as platelets and leukocytes, and actively participate in pathophysiological conditions (such as thrombosis, atherosclerosis, or inflammation) characterized by the interaction between the endothelium and these cells.

REFERENCES

1. Camussi, G., Aglietta, M., Malavasi, F., Tetta, C., Piacibello, W., Sanavio, F., and Bussolino, F. (1983) *J. Immunol.* **131,** 2397–2403
2. Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3534–3538
3. Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1985) *J. Clin. Invest.* **73,** 2235–2246
4. Brotherton, A. F. A., and Hoak, J. C. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 495–499
5. Singer, H. A., and Pech, M. J. (1982) *Hypertension* **4** (Suppl. II), 495–499
6. Brock, T. A., Capasso, E. L., and Gimbrone, M. A. (1986) *Fed. Proc.* **45,** 1072 (abstr.)
7. Hamilton, H. H., and Sims, P. J. (1987) *J. Clin. Invest.* **79,** 600–608
8. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G., and Watanabe, K. (1987) *J. Biol. Chem.* **262,** 8557–8565
9. Grinstein, S., and Rothstein, A. (1986) *J. Membr. Biol.* **90,** 1–12
10. Aronson, P. S. (1985) *Annu. Rev. Physiol.* **47,** 545–560
11. Moolenar, W. H., Tsein, R. Y., van der Saag, P. T., and de Laat, S. W. (1983) *Nature* **304,** 645–648
12. Siffert, W., and Akkerman, J. W. N. (1987) *Nature* **325,** 456–458
13. Siffert, W., Siffert, G., Scheid, P., Riemens, T., Gorter, G., and Akkerman, J. W. N. (1987) *FEBS Lett.* **212,** 123–126
14. Ghigo, D., Treves, S., Turrini, F., Pannocchia, A., Pescarmona, G. P., and Bosia, A. (1988) *Biochim. Biophys. Acta* **940,** 141–148
15. Snyder, F. (1985) *Med. Res. Rev.* **5,** 107–140
16. Wykle, R. L., Malone, B., and Snyder, F. (1980) *J. Biol. Chem.* **255,** 10256–10260
17. Van den Bosch, H. (1980) *Biochim. Biophys. Acta* **604,** 191–246
18. Gomez-Cambronero, J., Inarrea, P., Alonso, F., and Sanchez-Crespo, M. (1984) *Biochem. J.* **219,** 419–424
19. Cragoe, E. J., Woltersdorf, O. W., Bicking, J. B., Kwong, S. F., and Jones, J. H. (1967) *J. Med. Chem.* **10,** 66–75
20. Bussolino, F., Gremo, F., Tetta, C., Pescarmona, G. P., and Camussi, G. (1986) *J. Biol. Chem.* **261,** 16502–16508
21. Bligh, E. G., and Dyer, W. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
22. Pinckard, R. N., Farr, R. S., and Hanahan, D. J. (1979) *J. Immunol.* **123,** 1847–1857
23. Braquet, P., Shen, T. Y., Touqui, L., and Vargaftig, B. B. (1987) *Pharmacol. Rev.* **39,** 97–145
24. Pirotzky, E., Ninio, E., Bidault, J., Pfister, A., and Benveniste, J. (1984) *Lab. Invest.* **51,** 567–572
25. Benveniste, J., LeCouedic, J. P., Polonsky, J., and Tence, M. (1977) *Nature* **269,** 170–171
26. Bussolino, F., Breviario, F., Tetta, C., Aglietta, M., Mantovani, A., and Dejana, E. (1986) *J. Clin. Invest.* **77,** 2027–2033
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
28. Labarca, C., and Paigen, K. (1980) *Anal. Biochem.* **102,** 344–352
29. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1982) *J. Cell. Biol.* **94,** 325–334
30. Rink, T. J., and Pozzan, T. (1985) *Cell Calcium* **6,** 133–144
31. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) *Biochemistry* **18,** 2210–2218
32. Grinstein, S., Cohen, S., and Rothstein, A. (1984) *J. Gen. Physiol.* **83,** 341–369
33. Roos, A., and Boron, W. F. (1981) *Physiol. Rev.* **61,** 269–434
34. Vigne, P., Frelin, C., Cragoe, E. J., and Lazdunski, M. (1984) *Mol. Pharmacol.* **25,** 131–136
35. Hirafuji, M., Mencia-Huerta, J. M., and Benveniste, J. (1987) *Biochim. Biophys. Acta* **930,** 359–369
36. Ninio, E., Mencia-Huerta, J. M., and Benveniste, J. (1983) *Biochim. Biophys. Acta* **751,** 298–304
37. Jouvin-Marche, E., Ninio, E., Beauzain, G., Tence, M., Niaudet, P., and Benveniste, J. (1984) *J. Immunol.* **133,** 892–898
38. Alam, I., and Silver, M. J. (1986) *Biochim. Biophys. Acta* **884,** 67–72
39. Gomez-Cambronero, M., Nieto, L. M., Mato, J. M., and Sanchez-Crespo, M. (1985) *Biochim. Biophys. Acta* **845,** 511–515
40. Lenihan, D. J., and Lee, T. C. (1984) *Biochem. Biophys. Res. Commun.* **120,** 834–839
41. Gomez-Cambronero, J., Velasco, S., Mato, J. M., and Sanchez-Crespo, M. (1985) *Biochim. Biophys. Acta* **845,** 516–519
42. Domenech, C., Machado-DeDomenech, E., and Söling, H.-D. (1987) *J. Biol. Chem.* **262,** 5671–5676
43. Seiffer, J. L., and Aronson, P. S. (1986) *J. Clin. Invest.* **78,** 859–864
44. Besterman, J. M., May, W. S., Jr., LeVine, H., III, Cragoe, E. J., and Cuatrecasas, P. (1985) *J. Biol. Chem.* **260,** 1155–1159
45. Leffert, H. L., Koch, K. S., Fehlmann, M., Heiser, W., Lad, P. J., and Skelly, H. (1982) *Biochem. Biophys. Res. Commun.* **108,** 738–745
46. Soltoff, S. P., and Mandel, L. J. (1983) *Science* **220,** 957–959
47. Dethmers, J. K., Cragoe, E. J., and Kaczorowsky, G. J. (1983) **42,** 2245 (abstr.)
48. Greenberg-Sepersky, S. M., and Simons, E. R. (1984) *J. Biol. Chem.* **259,** 1502–1508
49. Frelin, C., Vigne, P., Barbry, P., and Lazdunski, M. (1987) *Kidney Int.* **32,** 785–793
50. Li, J. H.-Y., Cragoe, E. J., and Lindemann, B. (1987) *J. Membr. Biol.* **95,** 171–185
51. Kleyman, T. R., Yulo, T., Ashbaugh, C., Landry, D., Cragoe, E., J., Jr., Karlin, A., and Al-Awqati, Q. (1986) *J. Biol. Chem.* **261,** 2839–2843
52. Benoit, E., and Dubois, J. M. (1987) *J. Physiol.* **391,** 85–97
53. Benoit, E., Corbier, A., and Dubois, J. M. (1985) *J. Physiol.* **361,** 339–360
54. Lampugnani, M. G., Pedenovi, M., Dejana, E., Rotilio, D., Donati, M. B., Bussolino, F., Garbarino, G., Ghigo, D., and Bosia, A. (1988) *Thromb. Res.*, in press
55. Sage, S. O., and Rink, T. J. (1987) *J. Biol. Chem.* **262,** 16364–16369
56. Hallam, T. J., and Rink, T. J. (1985) *FEBS Lett.* **186,** 175–179
57. Grinstein, S., and Goetz, J. D. (1985) *Biochim. Biophys. Acta* **819,** 267–270
58. Rosoff, P. M., and Cantley, L. C. (1985) *J. Biol. Chem.* **260,** 9209–9215
59. Schackmann, R. W., and Chock, P. B. (1986) *J. Biol. Chem.* **261,** 8719–8728
60. Kinsella, J. L., and Aronson, P. S. (1980) *Am. J. Physiol.* **238,** F461–F469
61. Rindler, M. J., and Saier, M. H., Jr. (1981) *J. Biol. Chem.* **256,** 10820–10825
62. Grinstein, S., and Furuya, W. (1986) *Am. J. Physiol.* **250,** C283–C291