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Amiloride Analogs Induce the Phosphorylation of Elongation Factor-2 in Vascular Endothelial Cells

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SUMMARY

5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA), a potent inhibitor of Na^+/H^+ antiport, reduced [^{35}S]methionine incorporation in proteins and induced the phosphorylation of a M_r 95,000 protein in bovine aortic endothelial cells. This protein was previously shown to become phosphorylated in response to ATP, bradykinin, and A23187 (1) and was identified as elongation factor-2 (2). The action of EIPA was independent of changes in cytosolic pH, because it was neither mimicked by sodium acetate nor inhibited by ammonium chloride, and it was reproduced by 2′,4′-dimethylbenzamil, an analog of amiloride that is inactive on the Na^+/H^+ antiport. Furthermore, EIPA enhanced the Ca^{2+}-dependent phosphorylation of a similar M_r 95,000 protein in a cell-free system, rabbit reticulocyte lysate, where an inhibitory effect of amiloride on protein synthesis has already been described (3). Because phosphorylation decreases the activity of elongation factor-2, our observation might explain why amiloride analogs inhibit protein synthesis.

Adenine nucleotides, ATP and ADP, released during platelet aggregation are able to modulate the activity of aortic endothelial cells; they induce an immediate stimulation of prostacyclin (prostaglandin I_2) release (4) and a delayed mitogenic effect.[3] The responses of endothelial cells to ATP are mediated by P_{2y} purinergic receptors, which are coupled to the hydrolysis of phosphatidylinositol bisphosphate, generating inositol trisphosphate and a subsequent increase of cytosolic calcium (5). This is accompanied by the phosphorylation of multiple proteins (1), probably via the activation of CaM kinases II and III. A biphasic effect of ATP on pH_i has been observed recently in BAECs (6). ATP induces a transient acidification, followed by a sustained alkalinization resulting from the activation of the Na^+/H^+ antiport (6). An alkalinization, via the activation of the Na^+/H^+ antiport, has been described in many systems in response to various mediators and growth factors (for review see Ref. 7) and might play a permissive role for initiation of DNA synthesis (8–10). One step in the process of cell replication, which is controlled by pH_i, seems to be the phosphorylation of a few key proteins; in particular, the phosphorylation of ribosomal protein S6 in response to mitogens is decreased by inhibitors of the Na^+/H^+ antiport (11–13).

The initial purpose of our study was to analyze the relation between pH_i and protein phosphorylation in BAECs, a system that we have previously characterized in detail (1). The amiloride analog EIPA, a potent inhibitor of the Na^+/H^+ exchanger, was the main tool used in our study (14, 15). Surprisingly, we found that EIPA per se induced the phosphorylation of a M_r 95,000 protein, which was identified as EF-2. This phosphorylation seems to be independent of changes in pH_i, because it was also observed in an acellular system, rabbit reticulocyte lysates.

Materials and Methods

Culture of endothelial cells. BAECs were obtained by collagenase digestion of aorta excised from a freshly slaughtered cow, as previously described (4). The cells were seeded in 100-mm Petri dishes and incubated at 37° under an atmosphere of 5% CO_2/95% air, in the following medium: 80% (v/v) MEM with D-valine, 20% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B. The medium was changed the following day and then every 3 days. After 4 or 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5-min incubation in a Ca^{2+}- and Mg^{2+}-free Hanks' buffer

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ABBREVIATIONS: CaM, calmodulin; pH_i, intracellular pH; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EF-2, elongation factor-2, BAEC, bovine aortic endothelial cell; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MIBA, 5-(*N*-methyl-*N*-isobutyl)amiloride; HMA, 5-(*N*,*N*-hexamethylene)amiloride; DMB, 2′,4′-dimethylbenzamil; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

containing trypsin (100 ng/ml) and EDTA (1 mM). Thereafter, they were seeded in 35-mm Petri dishes and the culture was continued in the following medium: 60% (v/v) DMEM, 20% (v/v) Ham's F12, 20% (v/v) fetal calf serum, with the same concentrations of penicillin, streptomycin, and amphotericin B as mentioned above.

[^{32}P]Phosphate incorporation in endothelial cells. Cultured endothelial cells were washed three times in phosphate-free MEM and incubated for 5 hr, under 5% CO_2/95% air, in the same medium containing [^{32}P]phosphate (10 μM; 50 μCi for samples analyzed by one-dimensional electrophoresis or 0.5 mCi for samples analyzed by two-dimensional electrophoresis). The tested agents were added after this period for specified times (1 to 30 min). Incubations were stopped by removal of the medium and addition of 400 μl of lysis buffer [62 mM Tris·HCl, pH 6.8, 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS], in the case of one-dimensional electrophoresis. For two-dimensional electrophoresis, the cells were lysed in 250 μl of the following lysis buffer: 9.5 M urea, 2% (w/v) CHAPS, 1.6% (v/v) Servalyt, pH 5–7, 0.4% (v/v) Servalyt, pH 2–11, 5% (v/v) β-mercaptoethanol (16). The lysis buffers were supplemented with protease inhibitors, 0.1 mg/ml trypsin inhibitor, 2.5 μg/ml leupeptin, and 50 μg/ml pheylmethylsulfonyl fluoride. The lysates were immediately frozen in liquid nitrogen.

[^{35}S]Methionine incorporation in endothelial cell proteins. Confluent BAECs were washed and incubated in methionine-free DMEM, in the presence of EIPA. [^{35}S]Methionine (25 μCi/dish, 1477 Ci/mmol) was added 10 min after EIPA for specified times. The incubations were stopped by removal of the medium and lysis of the cells in 400 μl of lysis buffer, for one-dimensional electrophoresis (see above). Protein labeling was determined in the trichloroacetic acid pellet, after sequential extraction with ethanol, ethanol/chloroform/ether (2:1:2, v/v/v), and diethyl ether (17).

Incubation of reticulocyte lysate with [^{32}P]ATP. The rabbit reticulocyte lysate was prepared by a modification of the method described by Pelham and Jackson (18). This procedure involves the addition of micrococcal nuclease to remove endogenous mRNA; this enzyme is then inactivated by EGTA (2 mM). The reaction mixture for protein phosphorylation contained 22 μl of reticulocyte lysate, 20 mM HEPES, pH 7.6, 1 mM [^{32}P]ATP (10 μCi, 5000 Ci/mmol), 0.2 mM GTP, 0.5 mM spermidine, 1.0 mM dithiothreitol, and 0 to 1 mM $CaCl_2$, in a final volume of 30 μl. The samples were incubated for 5 to 10 min at 37°. A 5-μl aliquot was resuspended in 40 μl of lysis buffer [62 mM Tris·HCl, pH 6.8, 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS], boiled 3 min at 100°, and subjected to one-dimensional electrophoresis.

One-dimensional gel electrophoresis. Aliquots containing 25–50 μg of protein were analyzed by electrophoresis in polyacrylamide slab gels containing 0.1% SDS, using a linear gradient of 6.5 to 20% acrylamide, with a 4% stacking gel and the discontinuous buffer system of Laemmli (19). Electrophoresis was performed for 9 hr at a voltage starting from 200 V and reaching 360 V after 50 min of migration. The electrode buffer contained 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% (w/v) SDS. After protein fixation in a mixture of acetic acid, methanol, and water (1:3:6, v/v/v), the gels were dried and exposed to β-Max Amersham films, at room temperature, for 3 to 5 days. Densitometric scanning of the films was carried out with a LKB Ultroscan.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to the method of O'Farrel (16), except that Servalyt, pH ranges 5–7 and 2–11, was used instead of ampholines, pH ranges 5–8 and 3–10 (20). Proteins were separated by isoelectric focusing on a cylindrical gel in the first dimension (pH range 5–7), in the presence of CHAPS (instead of Nonidet P-40), and according to molecular weight on linear gradient (6–16%) polyacrylamide slab gels containing 0.1% SDS (total length of separation gel, 20 cm) in the second dimension. After protein fixation, the gels were dried and exposed to β-Max Amersham films at room temperature, for a period calculated to reach a total of 4×10^9 disintegrations. For molecular weight determination, a representative sample was subjected to isoelectric focusing, as described above. Thereafter, the gel was cut into two parts, which were separately placed on the top of the slab gel on each side of a well where ^{14}C-labeled molecular weight markers were placed. Apparent molecular weights were determined by using a spline function to describe the relationship between standard molecular weights and migration distances. The autoradiographs were subjected to a visual examination.

Immunoprecipitation of EF-2. EF-2 was immunoprecipitated as described (2). Endothelial cells were labeled with [^{32}P]phosphate and incubated with or without EIPA (50 μM) for 10 min. The incubations were stopped by removal of the medium and lysis of the cells in 400 μl of 1% SDS. These extracts were diluted 2-fold with a concentrated stock solution to give the following final concentrations: 25 mM Tris (pH 7.5), 200 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 100 mM NaF, 20 mM sodium pyrophosphate, 2.5 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, and 0.1 mg/ml trypsin inhibitor (wash buffer). The diluted samples were incubated with 50 μl of Protein A-Sepharose (2.5 mg) for 15 min and centrifuged at 10,000 × g for 15 min, and the supernatants were incubated with 20 μl of rabbit antiserum to EF-2 for 90 min, with mixing by inversion (turbula; Bachofen AG, Basel). The samples were then incubated with 100 μl of Protein A-Sepharose (5 mg) for 30 min and mixed as above. Before use, Protein A-Sepharose was swollen and washed three times in wash buffer. The immunoprecipitate (Protein A-Sepharose-antibody-antigen complex) was centrifuged at 10,000 × g for 15 min and the pellet was washed twice with wash buffer. The pellets were resuspended in 120 μl of one-dimensional electrophoresis lysis buffer. Samples were centrifuged at 10,000 × g for 15 min to remove Sepharose beads and were loaded (40 μl) onto an SDS-gel. The gel was fixed and dried and autoradiography was performed as described above.

Materials. All the amiloride analogs tested were prepared especially for this study by previously published methods (21, 22). These analogs are EIPA, MIBA, HMA, and DMB. cAMP was purchased from Boehringer Mannheim Diagnostics.

ATP and protease inhibitors were obtained from Sigma Chemical Co. [^{32}P]Phosphate, [^{32}P]ATP, [^{35}S]methionine, ^{14}C-labeled molecular weight markers (myosin, M_r 200,000; phosphorylase b, M_r 92,500; bovine serum albumin, M_r 69,000; ovalbumin, M_r 46,000; carbonic anhydrase, M_r 30,000; and lysosyme, M_r 14,300), ^{14}C-labeled Rainbow markers (as above plus trypsin inhibitor (M_r 21,500), and rabbit reticulocyte lysate were obtained from Amersham. DMEM, Ham's F12, MEM with D-valine, fetal calf serum, penicillin, streptomycin, amphotericin B, and glutamine were from GIBCO. Trypsin and phosphate-free MEM were from Flow Laboratories. Collagenase type I was from Cooper Worthington. Protein A-Sepharose CL-4B was obtained from Pharmacia LKB. Glycine and SDS were obtained from Bio-Rad, whereas all the other electrophoresis reagents were purchased from Serva.

Results

EIPA (50 μM) induced the phosphorylation of a M_r 95,000 protein in BAECs (Fig. 1). This protein was similar to a substrate phosphorylated in response to ATP (10 μM) in these cells (1). In two-dimensional gel electrophoresis, this protein migrated around a pI of ±6.8 and exhibited some streaking (Fig. 2). The M_r 95,000 protein phosphorylated in BAECs exposed to either EIPA (Fig. 3) or ATP (data not shown) was immunoprecipitated by using rabbit antibody against EF-2 (2). The kinetics of phosphorylation were quite different for ATP and EIPA. Phosphorylation of the M_r 95,000 protein was detectable within 30 sec of ATP addition and was maintained for only 2 min (1), whereas the effect of EIPA was slower in onset (Fig. 2) and was sustained for at least 30 min (Fig. 1). Except for the M_r 95,000 protein, EIPA and ATP produced completely different patterns of protein phosphorylation in BAECs (Fig.

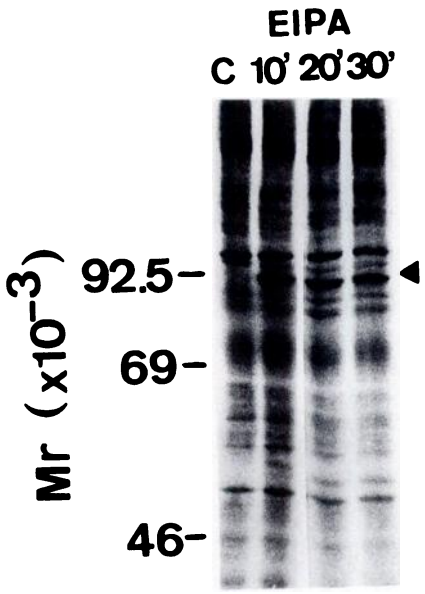

Fig. 1. Kinetics of the M_r 95,000 protein phosphorylation induced by EIPA. Cells were preincubated with [^{32}P]phosphate (50 μCi/ml) for 5 hr and then exposed to EIPA (50 μM) for 10, 20, or 30 min. The incubation was stopped by removing the medium, lysing the cells, and immediately freezing the lysates in liquid nitrogen (see Materials and Methods). The proteins were analyzed by SDS-polyacrylamide gel electrophoresis (on a linear 6.5–20% gradient), followed by autoradiography. Only the M_r 46,000–92,500 region of the gel is shown. *Arrowhead*, position of the M_r 95,000 band. The precise molecular weight of the labeled band depends on the weight of the phosphorylase *b* standard, 92,500 or 97,000 as deduced from the amino acid sequence. This autoradiograph is representative of four separate experiments, where each condition was tested in duplicate. *C*, control.

4). We have previously shown that, in addition to the M_r 95,000 protein, ATP enhances or induces the phosphorylation of at least 10 substrates; these phosphorylations are detectable within 1 min and maintained after 10 min (1). Only three of these substrates were phosphorylated 10 min after EIPA addition, with a lower intensity than with ATP, a M_r 36,000 protein and two proteins of M_r 18,000 (Fig. 4). No change in the phosphorylation of any protein was detectable 1 min after EIPA addition (not shown).

Acidification of the BAECs by exposure to sodium acetate (6) did not lead to the phosphorylation of the M_r 95,000 protein (Fig. 5). Furthermore, the effect of EIPA on this phosphorylation was not prevented by incubation of the cells in the presence of NH_4Cl (20 mM), which is known to increase pH_i independently from the Na^+/H^+ exchanger activity (6) (Fig. 5). In some experiments (four of nine), we were able to detect EIPA stimulation of the phosphorylation of a M_r 16,500 protein. In contrast to that of the M_r 95,000 protein, this phosphorylation was abolished by NH_4Cl (20 mM) (data not shown).

MIBA, HMA, and DMB, at 50 μM, also induced the phosphorylation of the M_r 95,000 substrate in BAECs (Fig. 6).

EIPA (50 μM) reduced the incorporation of [^{35}S]methionine into the proteins of BAECs to 68 ± 5% of the control (mean ± SD of three experiments). This inhibition was sustained with time (Table 1).

Several proteins are phosphorylated in the presence of [^{32}P]ATP in a cell-free reticulocyte lysate; among them, a M_r 95,000 protein has been identified as EF-2 (23). Addition of EIPA to the lysate enhanced the phosphorylation of this M_r 95,000 protein (Fig. 7). The effect of EIPA was inhibited by cyclic AMP, which is known to induce the dephosphorylation of EF-2 (data not shown). Phosphorylation of the M_r 95,000 protein and its enhancement by EIPA were dependent on the concentration of Ca^{2+}; however, the exact requirement for $CaCl_2$ varied from one preparation of lysate to the other (not shown).

Discussion

EIPA induced the phosphorylation of a M_r 95,000 protein in BAECs. We have previously reported that ATP, bradykinin, and A23187 also induce this phosphorylation (1), most probably via an increase of cytosolic Ca^{2+} and activation of CaM kinase III, which is present in BAECs (25). Mackie *et al.* (2) made similar observations in human umbilical vein endothelial cells stimulated by thrombin and histamine; they identified the M_r 95,000 substrate as EF-2. According to the same immunological criterion, the M_r 95,000 protein that is phosphorylated in EIPA-treated BAECs can also be identified as EF-2. Whereas ATP and bradykinin (1) or thrombin and histamine (2) induce a rapid and transient phosphorylation of the M_r 95,000 substrate, the effect of EIPA was slow in onset and sustained. This particular time course might be related to the slow accumulation of EIPA into the cytoplasm, as described in neutrophils (26).

This effect of EIPA on the phosphorylation of EF-2 is unlikely to result from a decrease in pH_i due to blockade of the

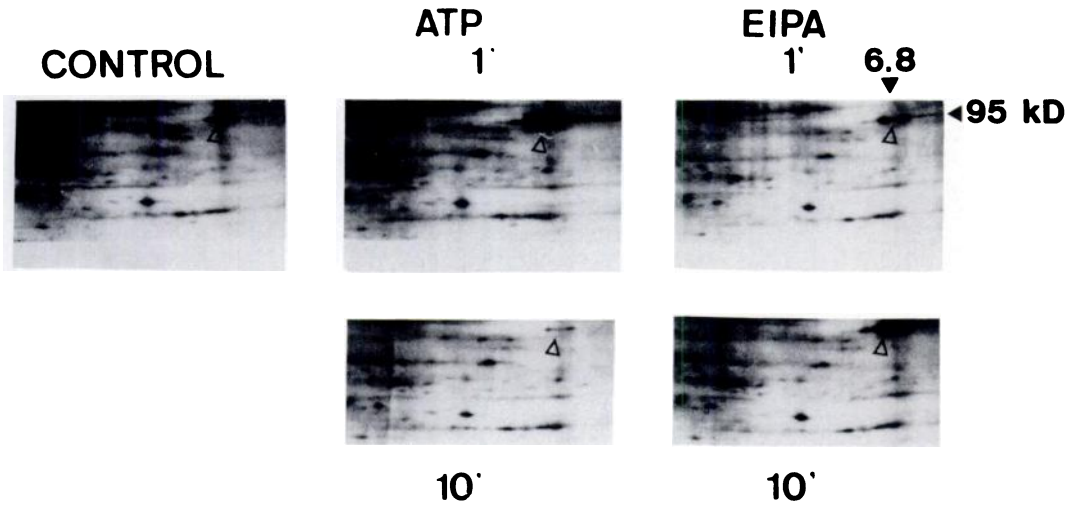

Fig. 2. Analysis of phosphoproteins from BAECs in two-dimensional gel electrophoresis: comparison of the kinetics of ATP and EIPA action on the M_r 95,000 protein. The cells were labeled for 5 hr with [^{32}P]phosphate (500 μCi/ml). ATP (100 μM) and EIPA (50 μM) were added for 1 or 10 min at the end of this period. Samples were subjected to electrophoresis in two dimensions, as described in Materials and Methods. *Arrowhead*, M_r 95,000 band. This autoradiograph is representative of one of two experiments.

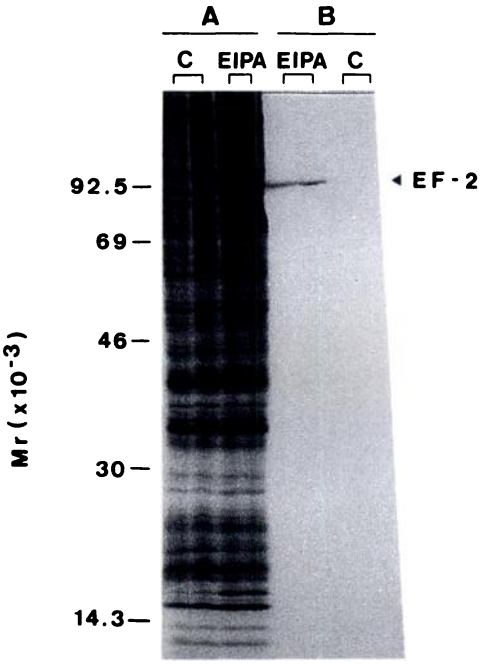

Fig. 3. Immunological identification of the M_r 95,000 phosphoprotein from EIPA-treated BAECs as EF-2. After [^{32}P]phosphate labeling, the cells were incubated for 10 min with or without EIPA (50 μM). Cell lysis, immunoprecipitation of EF-2, SDS-polyacrylamide gel electrophoresis, and autoradiography were performed as described in Materials and Methods. A, SDS-polyacrylamide gel electrophoresis analysis of the whole BAEC lysates. B, SDS-polyacrylamide gel electrophoresis analysis of the immunoprecipitates. *C*, control.

Na^+/H^+ exchanger. Indeed, it was not reproduced by sodium acetate, which is known to transiently lower the pH_i of BAECs (6). Furthermore, it was not inhibited by NH_4Cl, which alkalinizes BAECs independently of the Na^+/H^+ antiport; the validity of this argument is supported by the observation that NH_4Cl inhibited the enhancement of a M_r 16,500 protein phosphorylation that is produced by EIPA in some experiments. Another argument against the involvement of the Na^+/H^+ exchanger in this action of EIPA is that it was reproduced by DMB, an analog of amiloride bearing substituents on the terminal guanidino nitrogen atom. This compound inhibits the Na^+/Ca^{2+} exchanger but is inactive on the Na^+/H^+ antiport (27). Theoretically, the effect of EIPA could be due to an increase of cytosolic Ca^{2+} resulting from an inhibition of the Na^+/Ca^{2+} exchanger (27). This seems unlikely, because EIPA had a rather selective action on the M_r 95,000 protein and did not reproduce the effect of ATP (and A23187) on several other substrates, but it cannot be entirely excluded.

The most compelling argument against a role of pH_i or $[Ca^{2+}]_i$ change in this action of EIPA is that it is reproduced in a cell-free system, rabbit reticulocyte lysate. This last observation suggests that EIPA stimulates directly CaM kinase III, possibly by increasing its sensitivity to the CaM-Ca^{2+} complex. Because EIPA did not prevent the dephosphorylation of EF-2 by cyclic AMP (24), it seems unlikely that it exerts its effect by inhibiting a phosphatase.

This stimulatory effect of EIPA on EF-2 phosphorylation contrasts with the inhibitory effect of amiloride analogs on several kinases, including protein kinase C (28), tyrosine kinase (29), and cAMP-dependent kinase (30).

Phosphorylation of EF-2 decreases the rate of translation and inhibits protein synthesis (31). It is known that amiloride inhibits protein synthesis (3, 32), and we have checked that EIPA exerts such an effect in BAECs. Amiloride inhibits protein synthesis in lysates of rabbit reticulocytes incubated under the same experimental conditions as those used in this study (3). Thus, it is tempting to speculate that inhibition of protein synthesis by amiloride and its analogs results, at least partially, from an increased phosphorylation of EF-2.

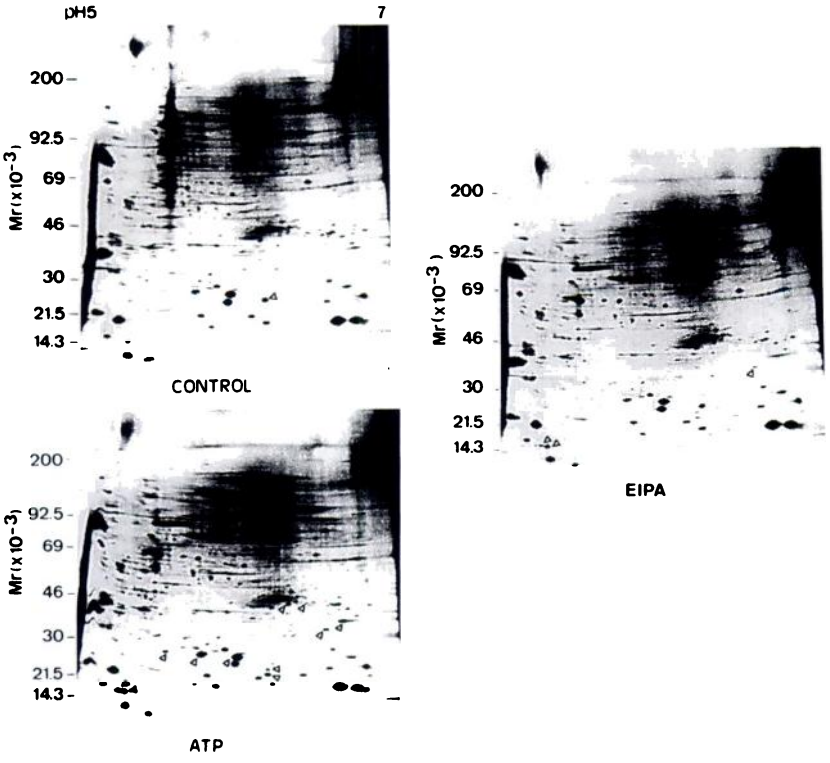

Fig. 4. Analysis of protein phosphorylation in BAECs by two-dimensional gel electrophoresis: patterns of phosphorylation induced by EIPA or ATP after a 10-min incubation. *Arrowheads*, different substrates whose phosphorylation is increased by the agents. This autoradiograph is representative of one experiment of two.

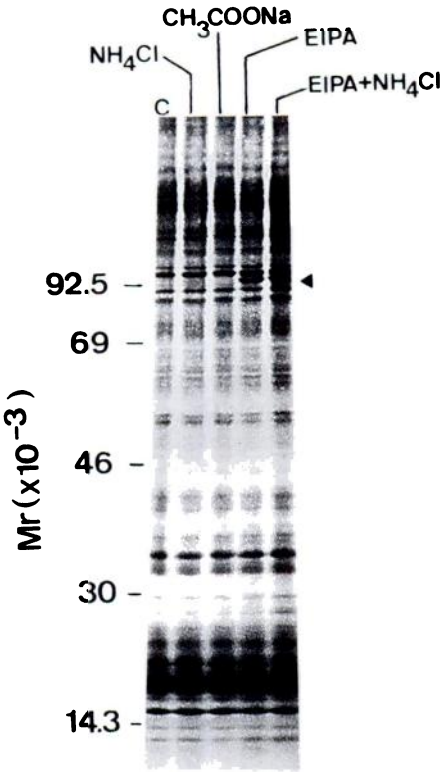

Fig. 5. Phosphorylation of the M_r 95,000 protein: comparison of the effect of EIPA, NH_4Cl, and CH_3COONa. EIPA (50 μM), NH_4Cl (20 mM), and CH_3COONa (20 mM) were added for 10 min; when tested in combination with EIPA, NH_4Cl (20 mM) was added 10 min before EIPA (50 μM). Labeling, lysis, and electrophoresis were performed as described in Materials and Methods. This autoradiograph is representative of three experiments, where each condition was tested in duplicate. *C*, control. *Arrowhead*, M_r 95,000 protein.

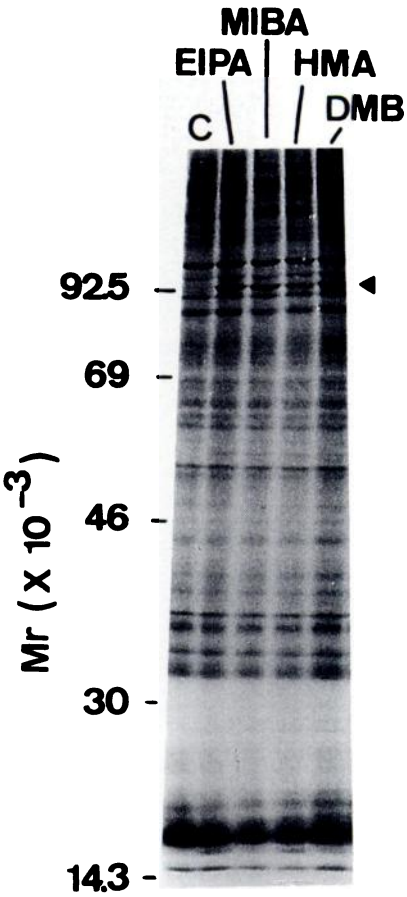

Fig. 6. Phosphorylation of the M_r 95,000 protein in BAECs: comparison of the effects of EIPA, MIBA, HMA, and DMB. The drugs were added at a final concentration of 50 μM for 10 min. Labeling, lysis, and electrophoresis were performed as described in Materials and Methods. This autoradiograph is representative of five experiments, where each condition was tested in duplicate. *Arrowhead*, M_r 95,000 substrate. *C*, control.

Acknowledgments

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TABLE 1

[^{35}S]Methionine incorporation in proteins from BAECs: effect of EIPA

The cells were incubated as described in Materials and Methods. The results are expressed as cpm/dish (× 10^{-3}) (mean ± standard deviation of triplicates in one representative experiment of two).

	Methionine incorporation	
	Control	EIPA (50 μM)
	cpm/dish (×10^{-3})	
20 min	456 ± 120	328 ± 32
40 min	1680 ± 212	1345 ± 59
4 hr	9933 ± 843	7310 ± 432

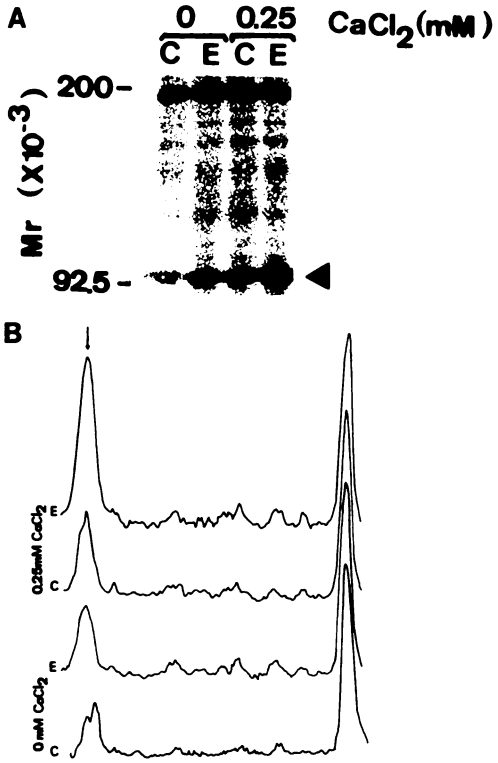

Fig. 7. Phosphorylation of the M_r 95,000 protein in rabbit reticulocyte lysate: stimulation by EIPA. EIPA (*E*) was added at a final concentration of 50 μM for 5 min. Labeling, lysis, and electrophoresis were performed as described in Materials and Methods. A, Autoradiograph; B, densitometric scan. *Arrowhead*, M_r 95,000 protein. This autoradiograph is representative of two experiments. *C*, control.

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