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Effect of the Na^+/H^+ antiport inhibitor Hoe 694 on the angiotensin II-induced vascular smooth muscle cell growth

[1]Agapios Sachinidis, Claudia Seul, Yon Ko, Rainer Düsing & Hans Vetter

Medizinische Universitäts-Poliklinik, Wilhelmstr. 35-37, 53111 Bonn, Germany

1 Hoe 694 (3-methylsulphonyl-4-piperidinobenzoyl)guanidine methanesulphonate) was characterized as a new, potent, non-amiloride inhibitor of the Na^+/H^+ exchanger. In order to elucidate the role of the Na^+/H^+ exchanger isoform 1 (NHE-1) in the regulation of vascular smooth muscle cell growth, we investigated the effects of different amiloride analogues and of Hoe 694 on angiotensin II-induced cell growth. Since intracellular pH, the intracellular free Ca^{2+} concentration and the expression of the transcription factor c-fos seem to be involved in the regulation of cell growth, the effects of the amiloride analogues and Hoe 694 on the angiotensin II-induced changes in these three parameters were examined.

2 Measurement of cytosolic Ca^{2+} and pH in cell monolayers was performed using fura-2/AM and BCECF/AM, respectively. The effect of angiotensin II on cell growth was examined using (1) [^{3}H]-thymidine incorporation, (2) the bromo-2-deoxyuridine (BrdU) immunfluorescence assay, (3) the colorimetric determination of cell mitochondrial dehydrogenase activity and (4) determination of cell number. Total RNA was extracted from cells by the guanidinium isothiocyanate/CsCl procedure. The expression of c-fos was quantitated by Northern blotting.

3 Various amiloride analogues inhibited the angiotensin II-induced stimulation of the Na^+/H^+ exchanger, the increase in cytosolic Ca^{2+} and cell growth but not the induction of c-fos mRNA. Hoe 694 (1-25 μM) dose-dependently inhibited the angiotensin II-induced stimulation of the Na^+/H^+ exchanger but had no significant effects on cytosolic Ca^{2+}, c-fos mRNA levels or cell growth.

4 Our findings support the concept that activation of the Na^+/H^+ exchanger is not essential for angiotensin II-induced vascular smooth muscle cell growth.

Keywords: Amiloride; Hoe 694; cytosolic free Ca^{2+}; cell growth; c-fos; Na^+/H^+ exchanger; angiotensin II

Introduction

The regulation of vascular smooth muscle cell (VSMC) growth is of interest because of the central role of smooth muscle cell proliferation in atherogenic plaque formation (Schwartz & Reidy, 1987) and in the pathogenesis of hypertension (Schwartz *et al.*, 1986). The isoform 1 of the Na^+/H^+ exchanger (NHE-1) is a ubiquitously expressed membrane transport protein which it has been proposed may be involved in the regulation of cell growth following cell stimulation with various growth factors (Sardet *et al.*, 1989; 1990). The NHE-1 is an important regulator of intracellular pH (pH_i) via an electroneutral 1:1 exchange of intracellular H^+ for extracellular Na^+ (Aronson, 1985; Mahnensmith & Aronson, 1985). The pyrazine diuretic, amiloride and several of its analogues have been extensively employed as inhibitors of the Na^+/H^+ exchanger to study the physiological role of this membrane transport system. In the last decade, many studies have shown that stimulation of VSMC with angiotensin II (AII) activates Na^+/H^+ exchange (Smith *et al.*, 1984; Berk *et al.*, 1987; Hatori *et al.*, 1987). It is well established that vasoconstrictors such as AII activate the phosphatidylinositol-specific phospholipase C resulting in an elevation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). It is assumed that DAG stimulates protein kinase C (PKC) resulting in an activation of Na^+/H^+ exchange (Rana & Hokin, 1990). Recently, it has been demonstrated that Ca^{2+} calmodulin also stimulates NHE-1. In this context it has been proposed that direct binding of Ca^{2+} calmodulin to region A (amino acid 636 to 656) of the cytoplasmic domain of the NHE-1 isoform results in its stimulation (Betrand *et al.*, 1994; Wakabayashi *et al.*, 1994; Siffert & Düsing, 1995).

Conflicting reports exist on the effects of amiloride analogues on cell proliferation. Since numerous studies demonstrated that amiloride analogues inhibit the proliferation of several cell types (for review Grinstein *et al.*, 1989), it was attractive to speculate that stimulation of the Na^+/H^+ exchanger may be implicated in the regulation of VSMC growth. However, the importance of the Na^+/H^+ exchanger in the regulation of cell growth is unclear since amiloride analogues, in addition to their ability to inhibit the Na^+/H^+ exchanger, exert other effects on cell function which may be responsible for their antiproliferative effects (Grinstein *et al.*, 1989). Therefore, it is unclear whether the inhibitory effects of the amiloride analogues on cell growth are due to selective inhibition of the Na^+/H^+ exchanger.

More recently, Hoe 694 (3-methylsulphonyl-4-piperidinobenzoyl)guanidine methanesulphonate) was characterized as a new potent non-amiloride inhibitor of the Na^+/H^+ exchanger in erythrocytes, platelets, and endothelial cells (Scholz *et al.*, 1993). In order to elucidate the role of the Na^+/H^+ exchanger in the regulation of VSMC growth, we investigated the effects of different amiloride analogues and Hoe 694 on the AII-induced VSMC growth. Since pH_i (Rozengurt, 1986), intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (Hepler, 1994) and the expression of the proto-oncogene c-fos (Verma & Sassone-Corsi, 1987) might be signals for cell growth, the effects of the amiloride analogues 5-(N,N-dimethyl)amiloride hydrochloride (DMA), N-(ethyl-N-isopropyl)amiloride (EIPA), 5-N(-methyl-N-isobutul) amiloride (MIA) and 5-(N,N-hexamethylene) amiloride (AH) and of the non-amiloride derivative Hoe 694 on the AII-induced changes in the above mentioned parameters were examined.

Methods

Culture of vascular smooth muscle cells

Rat aortic smooth muscle cells were isolated from thoracic aortae from Wistar-Kyoto rats (6-8 weeks old, Charles River

[1] Author for correspondence.

Wiga GmbH, Sulzfeld, Germany) by enzymatic dispersion using a slight modification of the method of Chamley *et al.* (1979) as described previously (Sachinidis *et al.*, 1995). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Eggenstein, Germany) supplemented with 10% foetal calf serum (FCS) (Boehringer Mannheim, Germany), nonessential amino acids, penicillin 100 iu ml^{-1} and streptomycin 100 $\mu g\ ml^{-1}$ (Gibco BRL, Eggenstein, Germany). Cells (3×10^6) in 75 cm^2 flasks (Falcon, Heidelberg, Germany) were grown to confluence over 4–5 days. The purity of VSMC cultures was confirmed by immunocytochemical localization of α-smooth muscle actin using a specific fluoresceinisothiocyanate (FITC)-conjugated monoclonal anti-α-smooth muscle actin (Sigma, Deisenhofen, Germany) plus a second FITC-conjugated F (ab')2 fragment of goat anti-mouse immunoglobulins (Dako GmbH, Hamburg, Germany). Experiments were performed using cells between passages 5 to 20.

Measurement of pH_i

For the measurement of pH_i, confluent cells were detached with 0.04% trypsin/0.02% EDTA in Dulbecco's phosphate-buffered saline (PBS) (w/v) after 5 to 10 min at 37°C. Then cells were cultured on round glass microscope slides (diameter 12 mm) under normal tissue culture conditions. When the cells became confluent they were incubated with the fluorescence pH indicator 2',7'-*bis*(carboxyethyl)-5,6-carboxyfluorescein (BCECF) at a concentration of 4 μM for 20 min at 37°C in HEPES buffer (20 mM HEPES, 16 mM glucose, 130 mM NaCl, 1 mM $MgSO_4$, 7 H_2O, 0.5 mM $CaCl_2$, Tris-base, pH 7.4) supplemented with 1% bovine serum albumin (BSA) (w/v). Just prior to the measurements, the cell monolayer was rinsed with HEPES buffer and the glass slide was positioned diagonally in the cuvette. Measurements were performed in HEPES buffer without BSA in the LS50 luminescence spectrofluorometer equipped with the fast filter application (Perkin Elmer, Überlingen, Germany). For the fluorescence measurements the following wavelengths were set: Excitation wavelengths: 492 and 438 nm; emission wavelength 525 nm. Measurements of pH_i in Na^+-free medium were performed in Na^+-free HEPES buffer in which NaCl was isotonically replaced with choline chloride.

Calibration of BCECF fluorescence was performed in HEPES buffer in which NaCl was replaced by KCl by permeabilizing the cells with the K^+/H^+ ionophore nigericine (1 $\mu g\ ml^{-1}$) in the presence of 2-(N-morpholino)ethane sulphonic acid (MES) as previously described (Berk *et al.*, 1987). The fluorescence of BCECF was approximately linear between pH_i 7.4 and 6.4.

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

VSMC were cultured on round glass microscope slides (diameter 12 mm) under normal tissue culture conditions. Confluent cells were incubated with 2 μM fura-2 pentaacetoxymethyl ester at 37°C for 20 min in HEPES buffer (20 mM HEPES, 16 mM glucose, 130 mM NaCl, 1 mM $MgSO_4$, 7 H_2O, 0.5 mM $CaCl_2$, Tris-base, pH 7.4) supplemented with 1% BSA (w/v). Measurements were performed in 2 ml HEPES buffer, containing 1 mM $CaCl_2$. The Ca^{2+}-fura-2 fluorescence was measured at 37°C in a Hitachi fluorescence spectrofluorometer (Hitachi, Ratingen, Germany) at excitation wave lengths of 340 and 380 nm and at an emission wavelength of 505 nm (Grynkiewicz *et al.*, 1985). Maximum (R_{max}) and minimum (R_{min}) fluorescence was determined by adding digitonin at a final concentration of 3×10^{-5} M followed by the addition of Tris-base/EGTA at a final concentration of 0.1 M Tris-base/25 mM EGTA.

Analysis of c-fos mRNA

The expression of c-fos mRNA was studied in confluent cells in 75 cm^2 flasks starved of serum for 24 h. Then cells were preincubated with either DMA (50 μM) or Hoe 694 (50 μM) for 10 min and then stimulated with AII (100 nM) for 30 min. Total RNA was extracted from VSMC by the guanidinium isothiocyanate/CsCl procedure (Chirgwin *et al.*, 1979): 10 μg of total RNA were separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel, blotted on Hybond N^+ membranes (Amersham, Little Chalfont, England), washed at room temperature in 5 × SSC (1 × = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min, and fixed with u.v. irradiation. After fixing, the blots were washed at 60°C in 0.1 × SSC, 0.1% SDS (sodium dodecylsulphate) for 5 min. Prehybridization and hybridization were performed overnight at 60°C in 5 × SSC, 0.2% SDS, 50 mM sodium phosphate, 10 × Denhardt's solution (Sigma Chemical, Deisenhofen, Germany) 200 $\mu g\ ml^{-1}$ salmon sperm (ss)DNA. The DNA probes were labelled with [α-^{32}P]-deoxycytidine triphosphate ([^{32}P]-dCTP) by random oligonucleotide priming to a specific activity of $2\text{-}4 \times 10^9$ d.p.m. μg^{-1} DNA (Amersham Buchler, Braunschweig, Germany). The stringency of the final wash was 0.2 × SSC containing 0.1% SDS at 65°C for 2 × 45 min. Blots were exposed to Kodak films (Kodak X-OMAT, 8 × 10 inch, Rochester, USA) for 3-7 days at −70°C. Blots were standardized using a 0.77 kb cDNA probe for β-actin (Dianova/Oncor, Hamburg, Germany). The size in kilobases (kb) of the detected mRNA was calculated from the 18S (1.8 kb) and 28S (4.6 kb) ribosomal RNA bands.

Determination of [^{3}H]-thymidine incorporation

The effect of AII on [^{3}H]-thymidine incorporation into DNA was assessed as previously described (Sachinidis *et al.*, 1995). VSMC were seeded in 24-well culture plates and grown to confluence. Then the medium was replaced by serum-free medium consisting of a mixture of DMEM and Ham's medium (1 : 1, v/v). Following another 24 h cultivation in serum-free medium, cells were exposed to AII in the presence and absence of drugs for 20 h prior to the addition of [^{3}H]-thymidine (3 $\mu Ci\ ml^{-1}$) for 4 h. Acid-insoluble [^{3}H]-thymidine was extracted in 0.5 M NaOH and quantified by use of a Packard Instrument liquid scintillation counter model Beckmann, LS3801, (Düsseldorf, Germany). Cell protein was quantified by the Bio-Rad (Bio-Rad, Munich, Germany) protein assay (Bradford, 1976).

5-Bromo-2-deoxyuridine (BrdU) immunofluorescence assay

VSMC (5×10^4 cells/well) were cultured on round glass cover slips in 24-well culture plates (Falcon, Heidelberg, Germany) and cultivated until they reached about 50% confluence. Following 24 h in serum-deprived medium, amiloride analogues or Hoe 694 were added to the cells and 10 min later AII (100 nM) was added. After 24 h detection of BrdU was performed according to the instructions of the '5-Bromo-2'-deoxyuridine Labelling and Detection Kit I' from Boehringer Mannheim (Mannheim, Germany). Briefly, cells were incubated for 40 min in DMEM and Ham's medium (1 : 1, v/v) containing 10 μM BrdU. After fixing with 70% ethanol (in 50 mM glycine buffer, pH 2.0) for 20 min at −20°C cells were incubated with a mouse anti-BrdU antibody for 30 min at 37°C followed by an anti-mouse-Ig-fluorescein antibody for 30 min at 37°C. BrdU was visualised using an immunfluorescence microscope (Leitz DMRB, Leica, Köln, Germany).

Determination of VSMC proliferation using the XTT-colorimetric assay

The determination of VSMC proliferation was performed according to the instructions of the cell proliferation kit (XTT) from Boehringer Mannheim Biochemical (Mannheim, Germany). The colorimetric detection of cell proliferation is based on the conversion of the yellow sodium 3'-[1-(phenyl-aminocarbonyl)3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sul-

phonic acid (XTT) to the orange formazan salt. This reaction is catalysed by the mitochondrial dehydrogenase of living cells. Briefly, approximately 2×10^3 cells in culture medium (0.2 ml/well) were cultured in microtitre plates (96 wells, flat bottom) for 24 h. The medium was then replaced by serum-free medium consisting of DMEM and Ham's F-10 (1 : 1, v/v) and the inhibitors of the Na^+/H^+ exchanger. Following a 10 min incubation period AII (100 nM) was added. After a further 24 h, 50 μl of the XTT labelling mixture consisting of 50 μl N-methyl dibenzopyrazinemethylsulphate (PMS) (1.25 mM) and 10 ml XTT in serum-free medium (1 mg ml^{-1}) were added to the medium. After 2 h measurement of the spectrophotometrical absorbance of the samples was performed using a microtitre plate (ELISA) reader (Flow laboratories) at 450 nm.

Cell number determination

For cell number determination, VSMC were seeded in 24-well culture plates (5×10^4 cells/well, well diameter 12 mm) and cultured in DMEM, supplemented with 10% FCS, non essential amino acids, penicillin 100 iu ml^{-1} and streptomycin 100 μg ml^{-1} at 37°C for 24 h. Under these conditions, once cells attained approximately 70% confluence the medium was replaced by serum-free medium consisting of DMEM and Ham's F-10 (1 : 1, v/v) and containing the Na^+/H^+ exchange inhibitor. Following a 10 min incubation period AII (100 nM) was also added. After a further 24 h, cells were trypsinized, resuspended in DMEM plus Trypan blue and counted with a Neubauer-cell-box by light microscopy.

Materials

Amiloride, DMA, EIPA, MIA and AH were obtained from BIOTREND (Cologne, Germany). DMEM, Ham's F-10 and PBS were obtained from Gibco BRL (Eggenstein, Germany). A0.77 kb fragment cDNA for β-actin was obtained for (Dianova/Oncor Science, Hamburg, Germany). Hybond N^+ membranes, and [^{32}P]-deoxycytidine triphosphate ([^{32}P]-dCTP) were from Amersham Buchler/Braunschweig, Germany). Kodak X-OMAT, 8×10 inch, films were obtained from Kodak (Rochester, U.S.A.). Molecular weight standard RNA was obtained from Boehringer Mannheim (Mannheim, Germany). PVDF membranes were obtained from Millipore (Bedford, U.S.A.). Fura 2/AM and BCECF/AM were obtained from Calbiochem (Bad Soden/TS, Germany). Hoe 694 was kindly provided by Hoechst AG (Frankfurt/M, Germany).

Statistics

Values are expressed as the arithmetic mean $\pm$ s.e.mean. Statistical analysis of the data was performed by the one factor Anova-Scheffe F-test (StatView 512^+, version 1.0, Apple Computer, Inc.). Triplicate wells were analysed for each [^{3}H]-thymidine incorporation experiment and each experiment was performed independently at least three times. $P<0.05$ was considered to be statistically significant.

Results

The resting value of pH_i in VSMC was 7.13 ± 0.005 ($n=18$). AII induced an initial rapid cytosolic acidification which peaked at 30 s and which was followed by a weak alkalinization above baseline within 5 min (Figure 1). Maximal acidification was 0.12 ± 0.015 pH units below the basal pH_i and maximal alkalinization was 0.04 ± 0.005 pH units above baseline ($n=4$, $P<0.05$). Following incubation with Hoe 694 (10 to 25 μM), AII elicited only intracellular acidification of 0.13 ± 0.01 pH units below basal value ($n=4$, $P<0.05$). Secondary alkalinization, attributable to activation of the Na^+/H^+ exchanger, was not observed. Hoe 694 at a concentration of 1 μM had no effect on the AII-induced pH_i changes. Similar

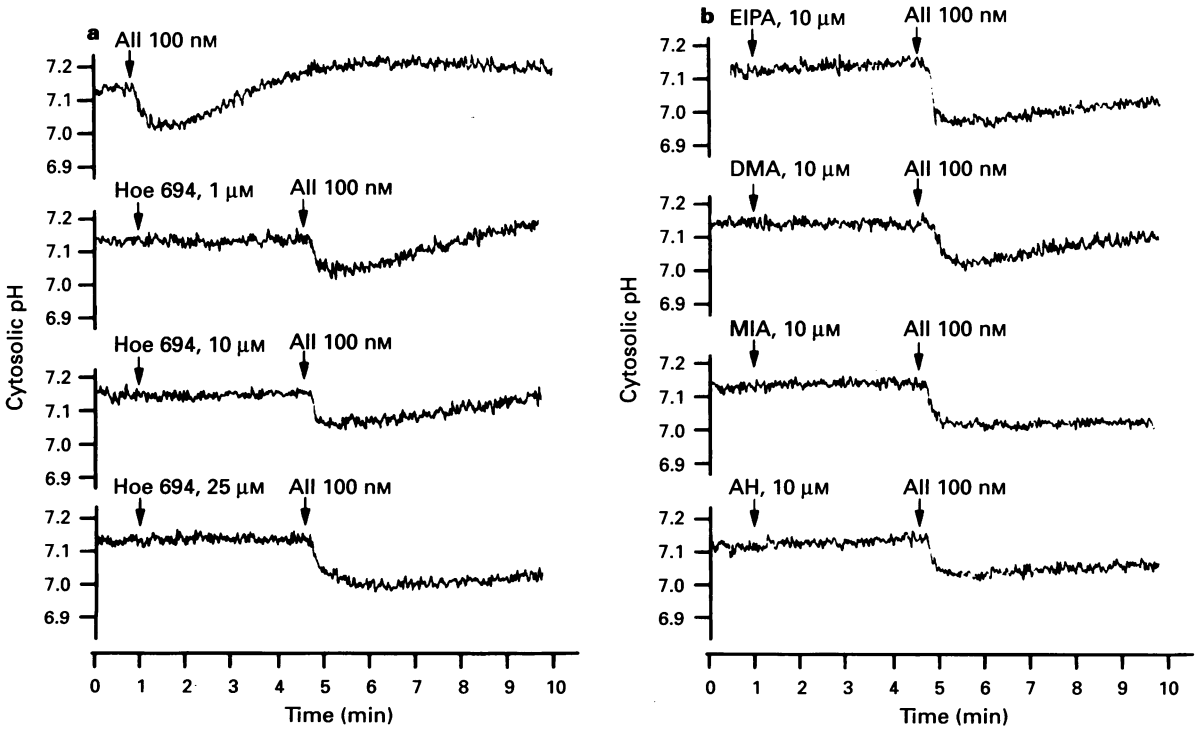

Figure 1 Effect of the amiloride analogues and Hoe 694 on the AII-induced intracellular pH_i changes. Arrows indicate addition of AII (100 nM). Measurements were performed in HEPES buffer. AII was applied to the BCECF-loaded cells in the presence and absence of the Na^+/H^+ exchanger inhibitors. After calibration of the fluorescence signal by permeabilizing the cells with nigericine (1 μg ml^{-1}), changes in 492/438 nm excitation wavelength ratio by the emission wavelength 525 nm were converted into corresponding levels of pH_i.

results were obtained with the amiloride analogues. All of the amiloride analogues tested blocked the Na^+/H^+ exchanger activity at a concentration of 10 μM.

In order to ensure that Hoe 694 specifically blocks the AII-induced stimulation of the Na^+/H^+ exchanger, pH_i measurements were also performed in Na^+-free medium in the presence and absence of Hoe 694 (representative curves from three separate experiments are presented in Figure 2). Figure 2 (top curve) shows the effect of AII on pH_i in the presence of 130 mM NaCl. Again, the AII-induced acidification was followed by the alkalinization towards the basal value. Stimulation of VSMC with AII in the absence of Na^+ resulted in a marked acidification by 0.2 pH_i units (first curve from the top). Addition of NaCl to the medium at a final concentration of 5 mM resulted in an 0.08 pH units increase above basal values within 3 min. Preincubation of the AII-stimulated cells in

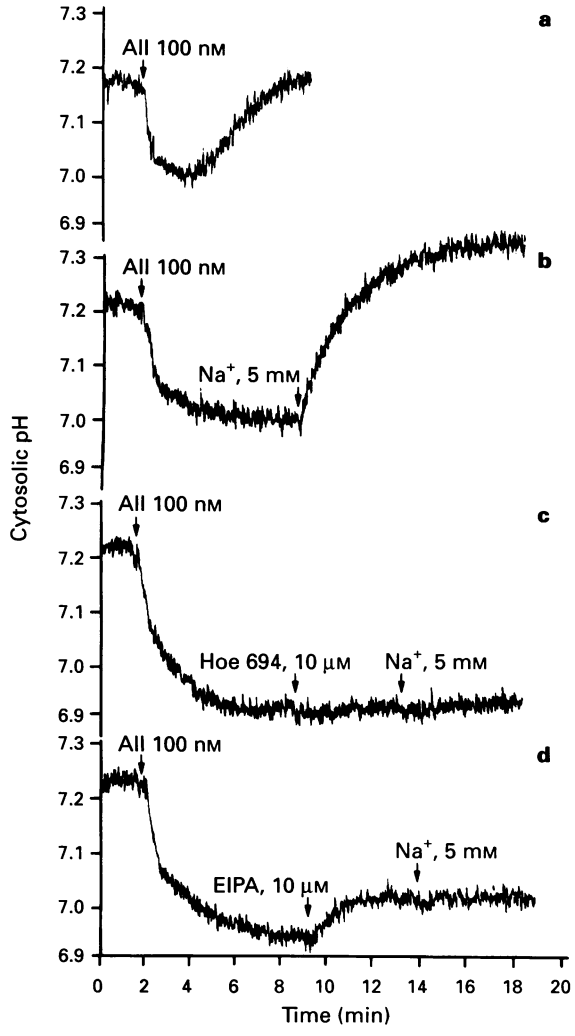

Figure 2 Effect of EIPA and Hoe 694 on the AII-induced intracellular pH_i changes in the presence and absence of extracellular Na^+. Arrows indicate addition of AII (100 nM), Na^+ (5 mM), Hoe 694 (10 μM) and EIPA (10 μM). AII was applied to the BCECF-loaded cells in HEPES buffer (130 mM Na^+) and in Na^+-free HEPES (NaCl was replaced with 130 mM choline chloride). (a) Stimulation of the cells with AII in HEPES buffer; (b) Cells were stimulated with AII in Na^+-free HEPES buffer and after 7 min, NaCl was added at a final concentration of 5 mM; (c) Cells were stimulated with AII in Na^+-free HEPES buffer and after 7 min they were preincubated with Hoe 694 (10 μM) for 5 min before addition of NaCl (5 mM); (d) Cells were stimulated with AII in Na^+-free HEPES buffer and after 7 min cells they were preincubated with EIPA (10 μM) for 5 min before addition of NaCl (5 mM). After calibration of the fluorescence signal by permeabilizing the cells with nigercine (1 μg ml^{-1}), changes in 492/438 nm excitation wavelength ratio by the emission wavelength 525 nm were converted into corresponding levels of pH_i.

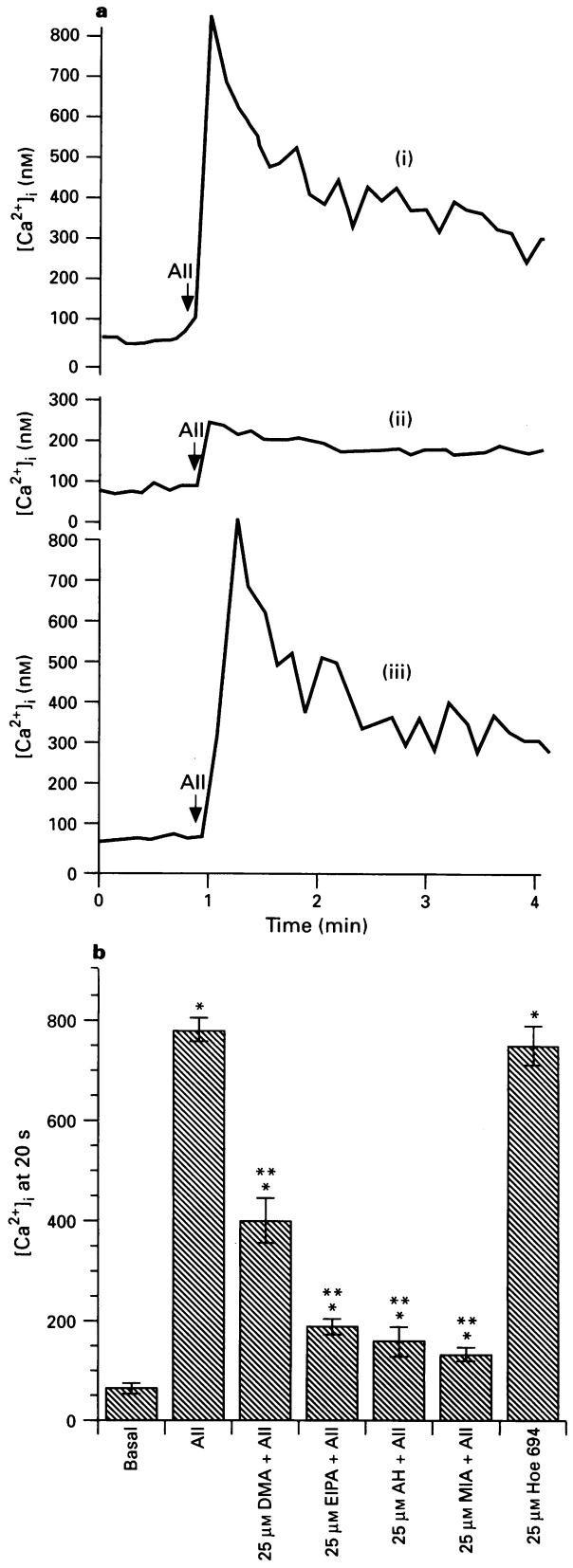

Figure 3 Effect of AII on $[Ca^{2+}]_i$ in VSMC in the presence and absence of the amiloride analogues and Hoe 694. Arrows indicate addition of AII (100 nM). (a) AII was added to fura-2-loaded vascular smooth muscle cells after preincubation of the cells with Na^+/H^+ exchanger blockers for 5 min and changes in fluorescence were monitored. After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio by the emission wavelength of 505 nm were converted into corresponding levels in $[Ca^{2+}]_i$; (b) As $[Ca^{2+}]_i$ was maximal within 20 s, maximal $[Ca^{2+}]_i$ were plotted and given as means ± s.e.mean, $n=3$. $^*P<0.05$ for (amiloride analogues + AII) effect vs. AII effect. $^{**}P<0.05$ for (DMA + AII) effect vs. (EIPA + AII) or (MIA + AII) or (AH + AII) effect.

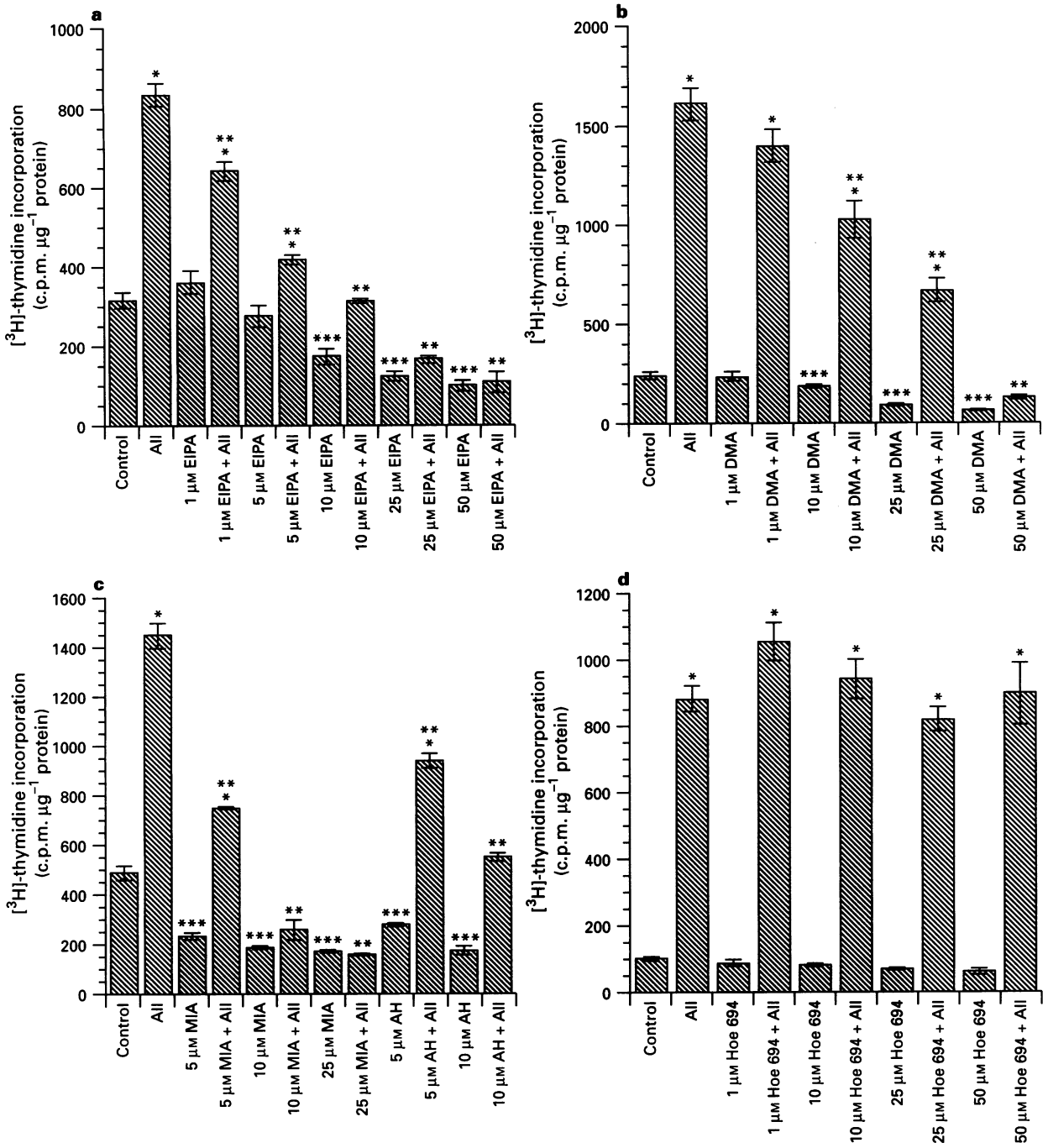

Figure 4 Effect of increasing concentrations of amiloride analogues or Hoe 694 on the AII-induced increase in VSMC DNA synthesis. Confluent cells (24-well plates) were precultured for 24 h in the serum-free medium. Then the Na^+/H^+ exchanger blockers EIPA (a), DMA (b), MIA and AH (c) or Hoe 694 (d) in various concentrations and AII (100 nM) 10 min later were added to the serum-free medium. Following another 20 h incubation, cells were exposed to 3 μCi ml^{-1} [^{3}H]-thymidine. Four hours later, the reaction was terminated and cell protein and [^{3}H]-thymidine incorporation into cell DNA were quantified. Data represent the means ± s.e.mean of a representative experiment in triplicate determination. *$P<0.05$ for AII or for the (1, 10, and 25 μM DMA + AII) effect vs. basal value. *$P<0.05$ for AII or for the (1 and 5 μM EIPA + AII) effect vs. basal value. *$P<0.05$ for AII or (5 μM MIA + AII) effect vs. basal value. *$P<0.05$ for AII or (5 μM AH + AII) effect vs. basal value. **$P<0.05$ for (amiloride analogue + AII) effect vs. AII effect. ***$P<0.05$ for amiloride analogue effect versus basal values. $P>0.05$ for (AII + Hoe 694) effect vs. AII effect).

Na^+-free buffer with Hoe 694 at final concentration of 10 μM for 3 min abolished the Na^+-dependent increase in pH_i (second curve from the top). These findings clearly demonstrate that Hoe 694 inhibits intracellular uptake of extracellular Na^+. Control experiments were performed with 10 μM EIPA (bottom curve). Like Hoe 694, EIPA (10 μM) abolished the Na^+-dependent increase in pH_i. These results demonstrate that amiloride analogues as well as Hoe 694 effectively blocked the activity of the Na^+/H^+ exchanger in VSMC.

As demonstrated in Figure 3a (i), AII (100 nM) induced a maximal elevation in $[Ca^{2+}]_i$ from approximately 60 to 750 nM within 20 s. Thereafter $[Ca^{2+}]_i$ declined toward a stable value of approximately 200 nM within 2 min. Preincubation of cells for 5 min with MIA (25 μM) resulted in a reduction of the maximal effect of AII at 20 s to 200 nM [(Figure 3a(ii))]. In contrast, Hoe 694 (25 μM) had no effect on the AII-induced increase in $[Ca^{2+}]_i$ [(Figure 3a(iii))]. Since the maximal AII-induced increase in $[Ca^{2+}]_i$ occurred within 20 s, the effects of

the different Na^+/H^+ exchange inhibitors on $[Ca^{2+}]_i$ were evaluated by calculating the maximal AII-induced increase in $[Ca^{2+}]_i$ within the first 20 s in the presence and absence of the drugs. As illustrated in Figure 3b, AII induced an increase in $[Ca^{2+}]_i$ at 20 s from 65 ± 6 (basal value, mean $\pm$ s.e.mean, $n=9$) to 779 ± 18 nM (mean $\pm$ s.e.mean, $n=3$). MIA, DMA, EIPA and AH inhibited this AII-induced Ca^{2+} peak by approximately 83%, 50%, 75% and 80%, respectively, whereas Hoe 694 failed to affect the AII-induced increase in $[Ca^{2+}]_i$. None of the Na^+/H^+ exchanger inhibitors tested had any effect on basal $[Ca^{2+}]_i$.

Next we examined the effects of the amiloride analogues and Hoe 694 on the AII-induced [^{3}H]-thymidine incorporation (Figure 4). EIPA (10 to 50 μM) effectively reduced the basal synthesis of DNA from 313 ± 20 to 99 ± 25 c.p.m. μg^{-1} protein (50 μM) (Figure 4a) and concentration-dependently inhibited the AII-induced [^{3}H]-thymidine incorporation. The maximal concentration of EIPA used (50 μM), decreased the AII-induced [^{3}H]-thymidine incorporation from 835 ± 28 to 107 ± 25 c.p.m. μg^{-1} protein. Similar results were obtained after treatment of the VSMC with DMA (1 to 50 μM) (Figure 4b). Again, DMA (10 to 50 μM) concentration-dependently re-

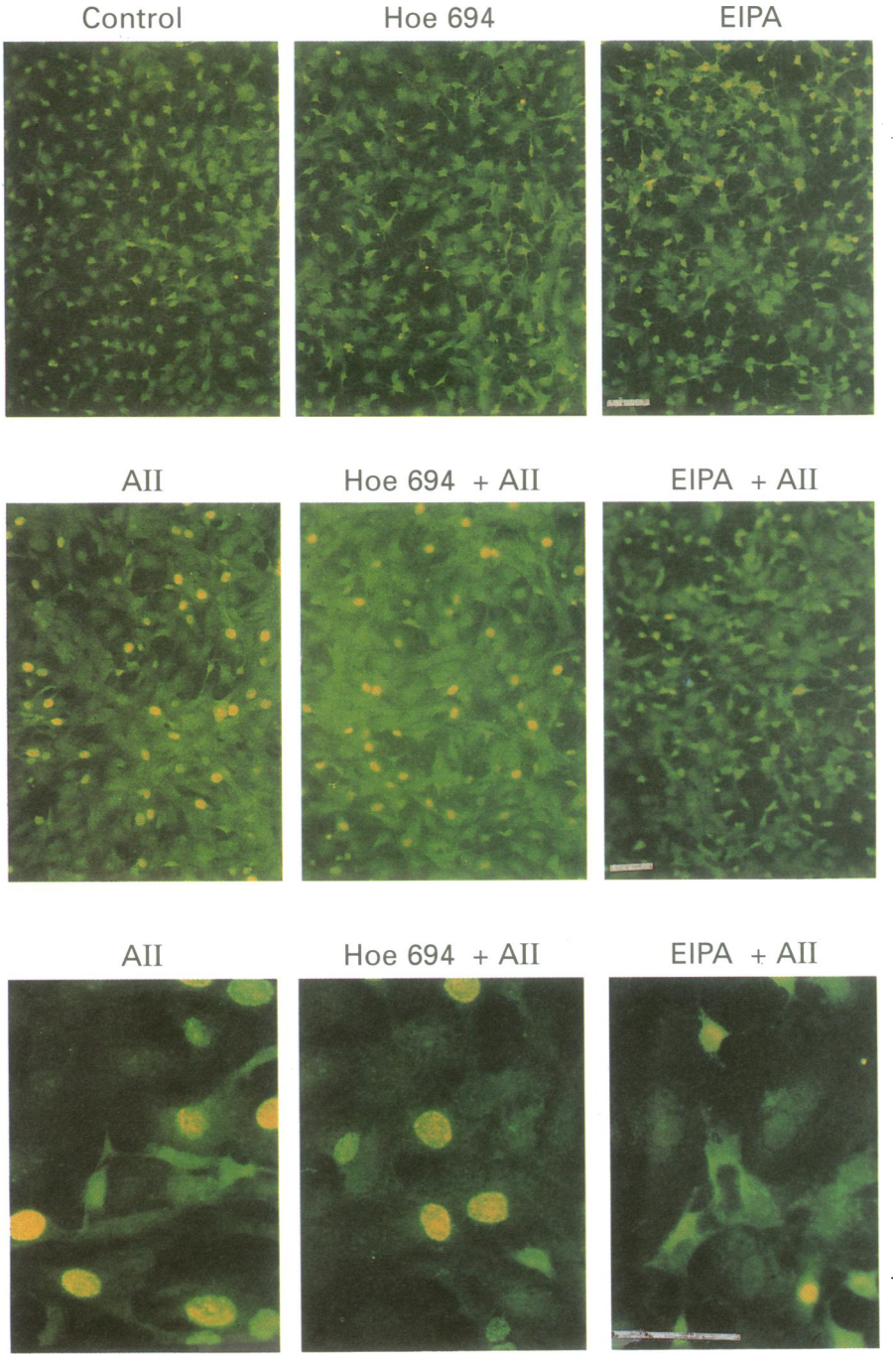

Figure 5 Immunofluorescence micrographs of the nuclei with increased DNA synthesis after stimulation of VSMC with AII in the presence and absence of EIPA and Hoe 694. VSMC (5×10^4 cells/well) were cultured on round glass cover slips (diameter: 12 mm) in 24-well culture plates (Falcon, Heidelberg, Germany) and cultivated until they have reached about 50% confluency. Then the medium was replaced by serum-free medium. Following another 24 h cultivation period in serum-free medium EIPA (25 μM) or Hoe 694 (25 μM) and 10 min later AII (100 nM) was added to the cells for 24 h. Then cells were labelled with 5-bromo-2'-deoxy-uridine for 40 min. Thereafter, detection of BrdU was performed according to the instructions of the '5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I'. (Calibration bar represents 100 µm).

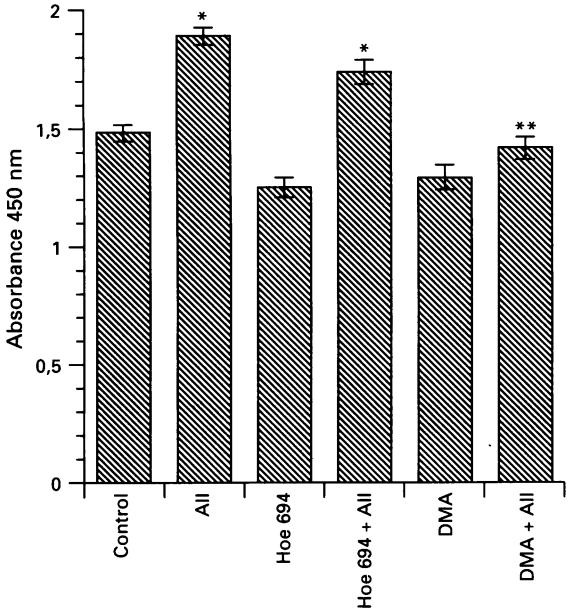

Figure 6 Determination of VSMC proliferation using the XTT-colorimetric assay after cell stimulation with AII in the presence and absence of Hoe 694 or DMA. Approximately 2×10^3 cells in 0.2 ml culture medium per well were cultured in microtitre plates (96 wells) for 24 h. The medium was then replaced by serum-free medium containing Hoe 694 (50 μM) or DMA (50 μM), respectively and 10 min later AII (100 nM) was added to the medium. After a 24 h incubation period, 50 μl of the XTT labelling mixture consisting of 50 μl PMS (1.25 mM) and 10 ml XTT in serum-free medium (1 mg ml^{-1}) was added to the medium. Measurement of the spectrophotometrical absorbance of the samples was performed after 2 h using a microtiter plate at 450 nm (means ± s.e.mean, $n=8$). *$P<0.05$ for AII effect vs. control. *$P<0.05$ for (Hoe 694+AII) effect vs. control. **$P<0.05$ for (DMA+AII) effect vs. AII effect. $P>0.05$ for (Hoe 694+AII) effect vs. AII effect.

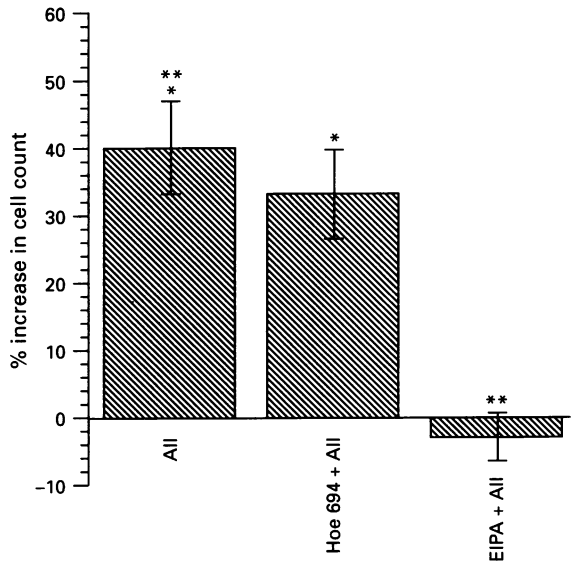

Figure 7 Effects of EIPA and Hoe 694 on the AII-induced increase in cell number. Cells (5×10^4) were seeded per well (24-well plates) in culture medium containing 10% FCS. After 24 h approximately 70% confluence was reached. Then, the medium was replaced with serum-free medium and cells were preincubated with Hoe 694 and EIPA (50 μM) for 10 min and immediately stimulated with AII (100 nM). After 24 h cells were trypsinized and counted as described in Methods. Data represent the means ± s.e.mean of the % increase in cell number from three separate experiments each performed in triplicates. *$P<0.05$ for AII vs. control (=0%). *$P<0.05$ for (Hoe 694+AII) effect vs. control. **$P<0.05$ for (EIPA+AII) effect vs. AII effect.

duced basal [^{3}H]-thymidine incorporation. As demonstrated in Figure 4c, MIA and AH (10 μM) completely abolished the effect of AII on DNA synthesis. In summary, EIPA, MIA, AH (10 μM) and DMA at (50 μM) completely abolished the effect of AII on [^{3}H]-thymidine incorporation. Pretreatment of cells with Hoe 694 (1 to 50 μM) did not influence the AII-induced [^{3}H]-thymidine incorporation (Figure 4d).

To determine whether the AII-induced [^{3}H]-thymidine incorporation truly reflects increases in DNA synthesis we investigated the effects of AII on BrdU incorporation into DNA after pretreatment with EIPA as well as Hoe 694. BrdU can be incorporated instead of thymidine into cell DNA and thus cells in DNA synthesis phase (S-phase) can be visualized by immunofluorescence microscopy using monoclonal antibodies directed against BrdU and an anti-mouse Ig-fluorescein-conjugated second antibody. Figure 5 demonstrates that after stimulation of VSMC with AII the number of cells actively synthezising DNA was increased. The stimulatory effect of AII on DNA synthesis was prevented in the presence of EIPA (25 μM) but was unaffected by Hoe 694 (25 μM).

Next, we investigated the effects of Hoe 694 on the AII-induced VSMC proliferation using the colorimetric XTT cell proliferation-assay. Stimulation of cells with AII resulted in an approximately 30% increase of the mitochondrial dehydrogenase activity. Hoe 694 failed to exert significant effects on the enzyme activity of either control or AII-stimulated cells (Figure 6). DMA abolished the AII-induced increase in dehydrogenase activity.

Next, we investigated the effects of Hoe 694 on the AII-induced increase in cell number. As illustrated in Figure 7, AII induced an approximately 40% increase in cell number. Hoe 694 did not influence the AII-induced increase in cell count whereas EIPA at the same concentration completely inhibited the AII effect on cell number (Figure 7).

Finally, we investigated whether the amiloride analogues and Hoe 694 would inhibit the expression of the immediate-early gene c-fos which is regarded an early marker of cell proliferation. As demonstrated in Figure 8, neither DMA nor Hoe 694 influenced the maximal induction of c-fos mRNA which occurred within 30 min after cell stimulation with AII.

Discussion

It has been proposed that AII may participate in the pathogenesis of cardiovascular diseases such as hypertension and atherosclerosis by promoting VSMC growth (Owens, 1985; Berk *et al.*, 1989; Daemen *et al.*, 1993). Although the predominant opinion of many investigators is that AII induces cellular hypertrophy, defined as increase in total cellular protein of VSMC, but not proliferation, defined as increase in DNA synthesis with cell division, (Berk *et al.*, 1989; Turla *et al.*, 1991) there are several studies demonstrating that AII also induces VSMC proliferation (Daemen *et al.*, 1991; Weissberg *et al.*, 1993; Bagby *et al.*, 1993; Sachinidis *et al.*, 1992; 1996). Using several different methods, we have been able to demonstrate clearly that AII stimulates proliferation of VSMC. Using the BrdU-immunfluorescence assay we have shown that the AII-induced rise in [^{3}H]-thymidine incorporation truly reflects increases in DNA synthesis. Finally, cell number measurements indicate that AII also induced VSMC division.

A number of effects of amiloride and its analogues which are unrelated to inhibition of the Na^+/H^+ exchanger have been described (Presek & Reuter, 1987; Grinstein *et al.*, 1989). In this context, it has been demonstrated that these compounds, at concentrations known to inhibit the Na^+/H^+ antiporter (i.e., 50 μM to 1 mM), inhibit transport systems such as the Na^+/K^+-ATPase and the Na^+/Ca^{2+} exchanger and affect the activity of several protein kinases including tyrosine and

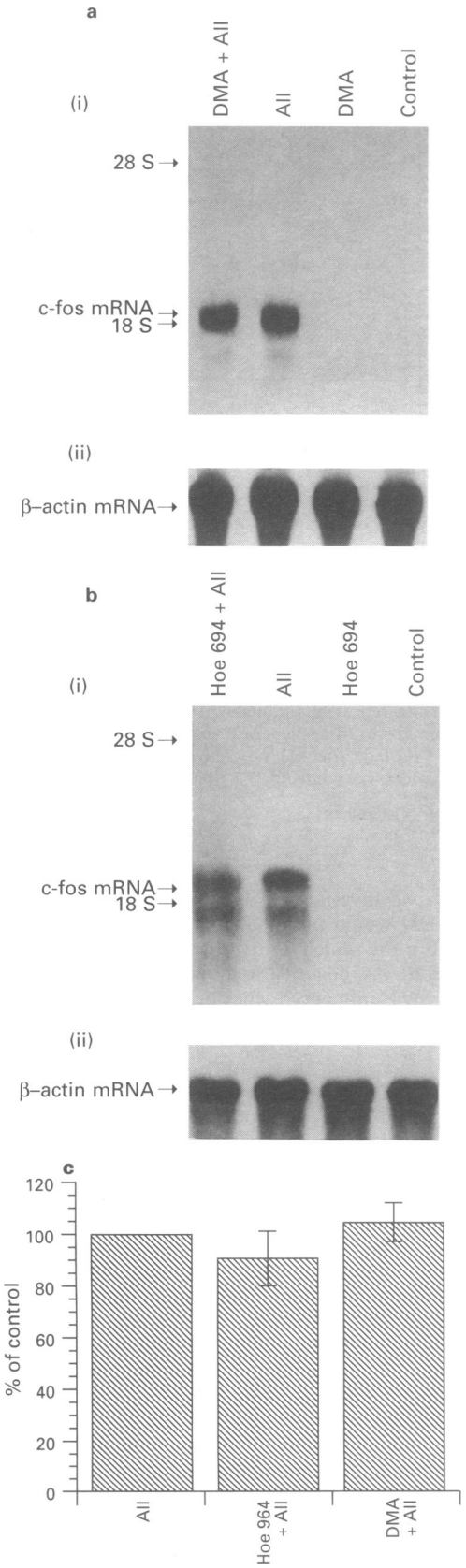

Figure 8 Effect of DMA or Hoe 694 on the AII-induced induction of c-fos mRNA in VSMC. Confluent cells in 75 cm^2 flasks were precultured in serum-free medium for 24 h. Then cells were preincubated with either DMA (50 μM) (a) and Hoe 694 (50 μM) (b) for 10 min and stimulated with AII (100 nM) 30 min; 10 μg of total RNA was electrophoresed on formaldehyde-agarose gels, blotted onto Hybond N^+ membranes and probed with a ^{32}P-labelled 1.0 kb v-fos fragment. The same blot previously hybridized with 1.0 kb v-fos was rehybridized 0.77 kb cDNA probe for β-actin mRNA. Arrows indicate the 28S (4.6 kb), the 18S ribosomal RNA (1.8 kb), the 2.2 kb c-fos mRNA and the 2.0 kb β-actin mRNA. (c): Densitometric analysis from three separate experiments. Standardization of experiments was performed by calculation of the relative changes in the AII-induced increase in c-fos mRNA (100%) in the presence and absence of DMA or Hoe 694. Data are expressed as means ± s.e.mean, $n = 3$.

serine-threonine kinases (Presek & Reuter, 1987; Grinstein *et al.*, 1989). Thus, these substances are unsuitable tools to investigate the role of Na^+/H^+ exchanger in the regulation of cell proliferation. *In vitro* experiments have shown that amiloride analogues also act as Ca^{2+} channel blockers inhibiting L-type Ca^{2+} channels and prevent the binding of diltiazem and nitrendipine to cardiac sarcolemma membrane vesicles with K_i values of 2.0×10^{-5} and 3.0×10^{-5} M, respectively (Garcia *et al.*, 1990). Criscione *et al.* (1989) have shown that the amiloride analogue, dichlorobenzamil, blocks the endothelin-induced contraction of intact aortic rings probably via blockade of Na^+/Ca^{2+} exchange. It has also been reported that EIPA completely inhibits thrombin-induced $[Ca^{2+}]_i$ increase in human platelets (Siffert & Akkermann, 1987) as well as the thrombin-induced Ca^{2+}-influx into human endothelial cells (Ghigo *et al.*, 1988). We have also demonstrated that several amiloride analogues inhibited the AII-induced increase in $[Ca^{2+}]_i$.

Recently, it has been demonstrated that Hoe 694 (10 μM) significantly attenuated the rapid and transient increase of the bradykinin-induced increase in $[Ca^{2+}]_i$ in endothelial cells without affecting the sustained increase in $[Ca^{2+}]_i$ or the basal $[Ca^{2+}]_i$ (Fleming *et al.*, 1994). In the present study however, no attenuation of the rapid and transient increase in $[Ca^{2+}]_i$ could be observed with Hoe 694 in VSMC using AII as the agonist. The inconsistencies between our results and those reported by Fleming *et al.* (1994) most likely reflect differences in the cell type or in the agonist used to stimulate the cells.

Based on such observation it has been proposed that the activation of the Na^+/H^+ exchanger is a prerequisite for the agonist-induced increase $[Ca^{2+}]_i$ in these cells (Siffert & Akkerman, 1987; Ghigo *et al.*, 1988). Since Hoe 694 effectively blocked the activity of the Na^+/H^+ exchanger but did not influence $[Ca^{2+}]_i$ we are able to conclude that the AII-induced activation of the Na^+/H^+ exchanger does not play an essential role in the AII-induced increase in $[Ca^{2+}]_i$. Our findings are in line with those from other groups suggesting that Na^+/H^+ exchanger activation is not essential for agonist-induced Ca^{2+} mobilization or the subsequent Ca^{2+} influx (Sage *et al.*, 1990; Kimura *et al.*, 1994).

It is widely thought that $[Ca^{2+}]_i$ plays a substantial role in the regulation of cell growth. Since all of the amiloride analogues tested reduced the AII-induced increase in $[Ca^{2+}]_i$ it is possible that the inhibitory effects of amiloride and its analogues on VSMC growth may be attributed, at least in part, to their effects on Ca^{2+} signalling and/or to some other non-specific effects. However, future experiments will be required to elucidate exactly the cellular mechanism by which amiloride analogues attenuate the AII-stimulated Ca^{2+} response in VSMC. Mitsuka *et al.* (1993) demonstrated that EIPA inhibits the migration of mutant VSMC that lacking the NHE-1 and reported that the inhibitory effects of EIPA on VSMC growth appear to be mediated by more than one mechanism and must likely comprises inhibition of the Na^+/H^+ exchanger as well as inhibition of protein tyrosine kinases. That the Na^+/H^+ exchanger is not essential for cell proliferation is also supported by the findings of Rosskopf *et al.* (1995) obtained in human lymphoblasts. In the later study EIPA, at a concentration of 1 μM, completely blocked the Na^+/H^+ exchanger but was without effect on cell proliferation. Only at higher concentrations i.e., 10 μM, did EIPA exert an inhibitory effect on cell proliferation.

Although in the present study, the amiloride analogues inhibited AII-induced VSMC proliferation they failed to inhibit the AII-induced expression of c-fos mRNA expression. It is widely accepted that the proto-oncogene c-fos is a marker for cell proliferation. Assuming that the amiloride analogues are able to penetrate into the cells it is conceivable that they may inhibit the replication of cell DNA via intercalation into or binding to DNA without affecting the expression of c-fos. Indeed, intercalation or binding of amiloride to DNA and inhibition of topoisomerase II by amiloride has been reported (Grinstein *et al.*, 1989). It is also possible that the expression of c-fos is not sufficient to elicit the AII-induced growth promoting effect in VSMC thus other factors such, as an elevated $[Ca^{2+}]_i$, may be essential for the cell proliferation induced by AII. Our data suggest that the AII-induced expression of c-fos mRNA in VSMC is not mediated by the stimulation of the Na^+/H^+ exchanger or by an elevated $[Ca^{2+}]_i$. These observations are in concordance with the results of Nishimura *et al.* (1992) who reported that the expression of c-fos and c-myc in VSMC is independent of $[Ca^{2+}]_i$. Data obtained in the present study demonstrate that Hoe 694 inhibits Na^+/H^+ exchange in VSMC but not the AII-induced proliferation, c-fos mRNA induction or Ca^{2+} response. These findings suggest that Hoe 694 is a more specific blocker of the Na^+/H^+ exchanger in VSMC than the amiloride analogues.

In summary, our results suggest that, at least *in vitro*, effective inhibition of the Na^+/H^+ exchanger by Hoe 694 does not attenuate AII-induced cell proliferation. Therefore AII seems to promote VSMC growth mainly by mechanisms other than activation of Na^+/H^+ exchanger. It is therefore likely that nonspecific cellular 'side effects' of amiloride and its analogues including their capacity to attenuate the AII-induced increase in $[Ca^{2+}]_i$ may be responsible for the inhibitory effects of the amiloride on the AII dependent cell proliferation.

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