Na\textsuperscript{+}-H\textsuperscript{+} Exchange Inhibitors Decrease Neointimal Formation After Rat Carotid Injury
Effects on Smooth Muscle Cell Migration and Proliferation

Masayuki Mitsuka, Mieko Nagae, Bradford C. Berk

The presence of multiple growth stimuli at the sites of vascular injury following angioplasty suggests that therapies targeted toward common growth pathways will be more effective than therapies that inhibit only a single growth factor. We tested this hypothesis using amiloride and ethyl isopropyl amiloride (EIPA), which is inhibited by the Na\textsuperscript{+}-H\textsuperscript{+} exchanger, whose activity is believed to be required in many cells for proliferation and migration. In the rat carotid injury model, EIPA (100 \( \mu \)g/h for 15 days) significantly decreased intimal area and the ratio of intimal to medial area, whereas amiloride (25 \( \mu \)g/h) showed an inhibitory trend that was similar to that observed for captoril (80 mg/kg per day) and heparin (25 U/h). EIPA and amiloride inhibited rat vascular smooth muscle cell DNA synthesis, with \( IC_{50} \) values of 8.8 and 82.2 \( \mu \)M, respectively. Using platelet-derived growth factor as a chemoattractant, EIPA caused a concentration-dependent inhibition of migration (\( IC_{50} \approx 60 \) \( \mu \)M). Because amiloride and EIPA have nonspecific effects on cellular function (especially inhibition of tyrosine kinases), we sought to characterize the specific role of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger in vascular smooth muscle cell proliferation and migration. We generated a Na\textsuperscript{+}-H\textsuperscript{+} exchanger-deficient mutant cell line [RNHE(-)]. Studies with these cells suggested that the inhibitory effects of EIPA and amiloride were mediated only in part via Na\textsuperscript{+}-H\textsuperscript{+} exchange because (1) RNHE(-) cells grew well at pH 6.8 to 7.5 in bicarbonate-containing medium, and (2) there was no difference in migration in response to platelet-derived growth factor in the RNHE(-) cells. In summary, these data indicate that amiloride and EIPA inhibit neointimal formation in the rat carotid after injury. However, the mechanism of inhibition is likely to involve cellular events other than Na\textsuperscript{+}-H\textsuperscript{+} exchange, such as an effect on tyrosine kinases. (Circulation Research 1993;73:269-275)

**Key Words** • Na\textsuperscript{+}-H\textsuperscript{+} antiporter • amiloride • tyrosine kinases

vascular smooth muscle cell (VSMC) growth is tightly controlled in adult blood vessels. In response to injury, the cells proliferate, whereas in response to hemodynamic stress, the cells may proliferate and/or undergo hypertrophy.\(^1\) Recent work in several models of vascular injury has shown that the process of vessel wall repair involves both migration and proliferation of VSMCs.\(^2\) This process has a great impact on clinical restenosis following coronary angioplasty.\(^3\) Several inhibitors of cell growth as well as Na\textsuperscript{+}-H\textsuperscript{+} exchange have been found to inhibit neointimal formation in the rat carotid balloon injury model,\(^4\) but none of these approaches has been successful in humans. Possible explanations include species differences, difficulty achieving adequate drug concentrations in humans, changes in the growth characteristics of VSMCs in atherosclerotic vessels, and the likely fact that multiple autocrine and paracrine growth stimuli cause human restenosis.\(^5\)

The failure of therapy targeted to individual growth factors suggested that an approach to limiting VSMC growth and migration based on a mechanism that was common to all growth factors might be successful. The amiloride-sensitive Na\textsuperscript{+}-H\textsuperscript{+} exchanger (specifically the NHE-1 isoform) is a ubiquitous membrane transport protein that exchanges intracellular H\textsuperscript{+} for extracellular Na\textsuperscript{+} in its normal transport mode. The Na\textsuperscript{+}-H\textsuperscript{+} exchanger is activated by all growth factors as one of the earliest events associated with entry of cells into the cell cycle.\(^6\) Amiloride and its derivatives are potent inhibitors of Na\textsuperscript{+}-H\textsuperscript{+} exchange.\(^7\) These compounds inhibit cell growth in a concentration-dependent fashion that correlates with their potency as inhibitors of Na\textsuperscript{+}-H\textsuperscript{+} exchange.\(^8\) Recently, Bobik et al\(^9\) have shown that growth of cultured VSMCs is inhibited by these compounds. Vairo et al\(^10\) have shown that inhibition of Na\textsuperscript{+}-H\textsuperscript{+} exchange prevents the induction of the M1 and M2 subunits of ribonucleotide reductase, an essential enzyme for DNA synthesis. In addition, Na\textsuperscript{+}-H\textsuperscript{+} exchange appears to be important for cell migration. Simchowitz and Cragoe\(^11\) have shown that Na\textsuperscript{+}-H\textsuperscript{+} exchange is activated when polymorphonuclear white blood cells migrate and that Na\textsuperscript{+}-H\textsuperscript{+} inhibitors block chemotaxis. Thus, amiloride and its derivatives, which may block both VSMC migration and proliferation, appear to be logical agents to prevent neointimal formation in the rat carotid injury model.

Amiloride and its derivatives have effects on cell function other than blockade of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger,
such as inhibition of protein synthesis and tyrosine kinases. To study the mechanisms by which amiloride-like compounds may inhibit VSMC growth and migration, we mutagenized VSMCs and prepared a clonal cell line deficient in Na\(^+\)-H\(^+\) exchanger activity [RNHE(−) cells]. This allowed us to investigate specifically the role of the Na\(^+\)-H\(^+\) exchanger in VSMC growth and migration. The results of our studies show that amiloride and its derivatives are potent inhibitors of neointimal formation after balloon injury in the rat carotid. Two mechanisms appear responsible: a Na\(^+\)-H\(^+\) exchange-dependent inhibition of VSMC growth and a Na\(^+\)-H\(^+\) exchange–independent inhibition of VSMC migration.

Materials and Methods

Carotid Injury and Measurements of Neointimal Formation

The left carotid artery of male Sprague-Dawley rats (450 to 600 g) was injured using a 2F Fogarty catheter balloon as described by Clowes et al. 1 The schedules for drug administration, dosages, and routes of administration are detailed in the figures and tables. Before death, each rat was killed by cervical dislocation. The carotid arteries were harvested, fixed in buffered 10% formalin, and imbedded in paraffin. After sectioning, the tissue was stained with elastica–van Gieson, and morphometry was performed with a digitizing pad. To assess toxicity, animals were treated with increasing concentrations of amiloride and ethyl isopropyl amiloride (EIPA) both orally and intravenously. Amiloride was well tolerated (no weight loss or death) at concentrations <300 mg/kg orally or ≤25 mg/kg intraperitoneally, whereas EIPA was tolerated at concentrations <50 mg/kg orally and ≤50 mg/kg intraperitoneally.

Cell Culture and Preparation of RNHE(−) Cells

Rat VSMCs were isolated from rat thoracic aorta by enzymatic digestion as described previously. 14 Bovine thoracic aortic cells were isolated by the explant technique as described by Ross. 17 All cells were harvested twice a week using trypsin/versene and passed at a 1:4 ratio in 80-cm\(^2\) culture flasks. For experiments, cells at passage levels 4 to 20 were replicated-plated into 100- or 35-mm culture dishes (2\(\times\)10\(^4\) cells/cm\(^2\)), refed every other day, and used at confluence. Cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin.

Cells deficient in the Na\(^+\)-H\(^+\) exchanger [RNHE(−) cells] were prepared by the acid-suicide technique described by Pouyssegur et al. 18 Briefly, clonal VSMCs were prepared from rat aorta by serial dilution techniques and characterized as VSMCs by \(\alpha\)-actin expression and growth in hills and valleys. RNHE(−) cells were prepared by mutagenesis of clone 413SC13 with ethyl methanesulfonate (0.25 \(\mu\)M/mL) during logarithmic growth. Mutagenized cells were grown to confluency, trypsinized, and exposed to 130 mM LiCl for 2 hours to cause intracellular loading. The cells were then subjected to acid suicide (in a Na\(^-\)- and Li\(^+\)-free solution, pH 5.5) at 37°C for 30 minutes. Surviving cells were characterized by measurement of amiloride-sensitive 22Na\(^+\) influx and amiloride-sensitive pH recovery as described below. EIPA-sensitive Na\(^+\)-H\(^+\) exchange was decreased =80% after one round of selection, and additional rounds of selection yielded cells completely devoid of Na\(^+\)-H\(^+\) exchange activity.

Cell Migration

Cell migration was performed as previously described. 19 In brief, the lower chamber of a 48-well microchemotaxis chamber was filled with DME supplemented with 0.2% bovine serum albumin (BSA) and 10 ng/mL platelet-derived growth factor (PDGF) B chain plus or minus drug. The chamber was sealed with a collagen-coated filter (pore diameter, 8 \(\mu\)m). The upper chamber containing bovine VSMCs (10\(^5\) cells in 50 \(\mu\)L, passages 5 to 7) in DME supplemented with 0.2% BSA plus or minus drug was then placed on the assembly. Migration was performed at 37°C in an air-CO\(_2\) incubator for 4 hours. Migrated cells were fixed and stained with Diff Quik and then quantitated using a scanning densitometer (CS-9000, Shimadze Co., Japan).

Measurement of Na\(^+\)-H\(^+\) Exchange Activity

The activity of Na\(^+\)-H\(^+\) exchange was measured by 22Na\(^+\) influx and by pH recovery from an acid load as previously described. 16 For both assays, cells were maintained in DME supplemented with 0.4% serum until assayed. For 22Na\(^+\) influx, the dishes were washed with an HCO\(_3\)\(^-\) and Na\(^+\)-free balanced salt solution (Na\(^+\)-free buffer) at 30°C containing (mM) choline chloride, 130; KCl, 5; CaCl\(_2\), 2; MgCl\(_2\), 1; and Tris-HEPES, 20 (pH 6.9). Cells were preincubated in this solution containing 10 mM glucose and 1 mg/mL BSA for 30 minutes with 1 mM ouabain; 0.1 mM bumetanide was added for the final 5 minutes. To initiate influx, the medium was replaced by a HCO\(_3\)-free influx medium of the same composition except for the addition of 1 \(\mu\)Ci/mL 22Na\(^+\), 1 mM ouabain, 0.1 mM bumetanide, and 130 mM NaCl (plus choline chloride), with or without 60 \(\mu\)M EIPA (pH 7.4). The concentration of EIPA chosen was based on previous studies indicating a K\(_i\) in VSMCs of 22 \(\mu\)M for amiloride. 16 The influx was terminated by rapid aspiration followed by six washes of ice-cold 100 mM MgCl\(_2\). Incorporated 22Na\(^+\) was measured in a gamma counter and corrected for cell protein and counter efficiency. The fluorescent pH indicator 2',7'-bis(carboxyethyl-5(6)-fluorescein) (BCECF) was used to measure pH, as previously described. 16 Briefly, cells were loaded for 30 minutes in a 37°C water bath with 3 \(\mu\)M BCECF AM in Hanks' solution. Fluorescent measurements were performed in an SLM DX-1000 spectrofluorometer equipped with a beam splitter, two excitation monochromators, and a dual chopping mechanism to allow rapid alternating excitation of BCECF at 500 and 438 nm. The emission ratio of the fluorescence signals at 530 nm was used to determine pH, after calibration of the pH, relative to fluorescence intensity using 10 \(\mu\)M nigericin in 135 mM KCl, 10 mM HEPES, and morpholinooethanesulfonic acid for coverslips. Buffering capacity was determined by NH\(_4\)Cl alkalization of cells acid loaded to pH 6.6. There was no significant difference in buffering capacity between a clonal VSMC cell line [RNHE(+)] and RNHE(−) cells (42±6 and 46±8 mmol H\(^+\)/L cells per pH unit, respectively).
Effect of Amiloride, Ethyl Isopropyl Amiloride, Captopril, and Heparin on Neointimal Formation in the Rat Carotid Artery: Percent Inhibition

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Intimal area (mm²)</th>
<th>Intima/media</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.142±0.008</td>
<td>1.07±0.08</td>
<td>6</td>
</tr>
<tr>
<td>Amiloride</td>
<td>25 mg/h</td>
<td>0.106±0.014</td>
<td>0.83±0.10</td>
<td>6</td>
</tr>
<tr>
<td>EIPA</td>
<td>100 mg/h</td>
<td>0.085±0.002*</td>
<td>0.68±0.10*</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.154±0.012</td>
<td>1.16±0.09</td>
<td>8</td>
</tr>
<tr>
<td>Captopril</td>
<td>80 mg/kg per day</td>
<td>0.096±0.013†</td>
<td>0.74±0.08†</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.056±0.014</td>
<td>0.40±0.10</td>
<td>6</td>
</tr>
<tr>
<td>Heparin</td>
<td>25 U/h</td>
<td>0.035±0.012</td>
<td>0.30±0.10</td>
<td>5</td>
</tr>
</tbody>
</table>

EIPA, ethyl isopropyl amiloride. Values are mean±SEM. Male Sprague-Dawley rats (500 to 700 g) were injured on day 0 as described in “Materials and Methods.” All animals received drug continuously until death 12 days after injury. Amiloride and EIPA were administered subcutaneously by osmotic pump (Alzet 2ML2, Alza Corp, Palo Alto, Calif) beginning 3 days before injury (day −3). Captopril was administered in the drinking water beginning at day −7. Heparin was administered by osmotic pump (Alzet 2ML2) beginning at day 0.

*P<.05 and †P<.01 vs control value.

DNA and Protein Synthesis

Two protocols were used. RNHE(−) and clonal cells were grown in six-well cluster dishes (Costar Corp, Cambridge, Mass) to 70% confluence as above and growth-arrested by incubation for 48 hours in DME containing 0.4% calf serum. The medium was aspirated and replaced with fresh DME containing 1 μCi/mL [³H]thymidine or [³H]leucine (New England Nuclear, Boston, Mass). After 24 hours, the medium was aspirated; cells were washed, fixed with 10% trichloroacetic acid, and washed with 95% ethanol; and the incorporated radioactivity was measured by liquid scintillation spectrometry. The effects of amiloride and EIPA were studied using bromodeoxyuridine (BrDU) and a proliferation assay kit (RPN.210, Amersham Corp, Arlington Heights, Ill). For these experiments, VSMCs (5×10⁴ cells in 100 μL) were plated in 96-well plates in 10% fetal calf serum/DME. The next day the medium was removed, cells were rinsed with 5% platelet-depleted serum (PDS/DME), and grown in 5% PDS/DME for 24 hours. On day 3, fresh 5% PDS/DME was added containing 10 ng/mL PDGF plus or minus drug. After growth for 16 hours, cells were labeled with BrDU per manufacturer’s instructions for 2 hours. Incorporated BrDU was determined by enzyme-linked immunosorbent assay in a microplate reader (absorption at 405 nm).

Materials

Choline chloride was from Calbiochem Corp, La Jolla, Calif. Versene and DME were from Gibco, Grand Island, NY. EIPA was prepared by Mitsubishi Kasei. BCECF was obtained from Molecular Probes Inc, Eugene, Ore. Heparin was from Novo Co. Radioisotopes were from New England Nuclear.

Statistical Analysis

All Na⁺ influx experiments were performed using four wells for total Na⁺ influx and two wells for EIPA-sensitive influx. Other results are presented as mean±SEM for two or three experiments using the largest SEM of either control or treatment groups. We compared the effect of treatment by a one-factor analysis of variance (ANOVA). When a significant effect was observed (P<.05), we compared group means using the residual variance of the ANOVA by Fisher’s protected least significant difference test (STATVIEW), which is equivalent to a multiple t test between all means.

Results

Recent studies have suggested that inhibition of VSMC proliferation and migration results in decreased neointimal formation following arterial injury.6,7,20 We tested this hypothesis both in vivo and in vitro using amiloride and EIPA, inhibitors of Na⁺-H⁺ exchange, which is required in many cells for proliferation and migration. We also studied the specific role of the Na⁺-H⁺ exchanger in VSMC proliferation and migration by creating mutant cells lacking the exchanger. In vivo, we examined the effect of amiloride and EIPA on neointimal formation in response to balloon injury by administering these compounds at 25 and 100 μg/h, respectively, for 15 days, beginning 3 days before injury. In animals treated with EIPA, there were significant (P<.05) decreases in intimal area (41% inhibition) and the ratio of the intimal to medial area (37% inhibition), whereas amiloride showed an inhibitory trend (26% and 19% inhibition, respectively), which was not significant (Table and Fig 1). We also tested the effectiveness of amiloride and EIPA as compared with well-established inhibitors of neointimal formation; they were as effective as captopril (80 mg/kg per day, 48% inhibition of intimal area, P<.01) and heparin (25 U/h, 38% inhibition of intimal area, P>.05) (Table and Fig 1).

We tested the relative effects of amiloride and EIPA on VSMC proliferation and migration in vitro in a cultured rat aortic VSMC model. Inhibition of rat VSMC DNA synthesis by both EIPA and amiloride was potent, with IC₅₀ values of 8.8 and 82.2 μM, respectively (Fig 2). Using PDGF as a chemoattractant, we found a concentration-dependent increase in VSMC migration (maximum at 10 ng/mL [data not shown]). EIPA showed a concentration-dependent inhibition of migration (IC₅₀, Ê 60 μM) (Fig 3, solid symbols). To verify that this effect of EIPA was not restricted to rat VSMCs, we studied its effects on bovine VSMCs. EIPA caused a concentration-
dependent inhibition of migration, with an IC\textsubscript{50} value of 68 \textmu M (Fig 4). Amiloride was much less potent, as expected, with an IC\textsubscript{50} of \approx 800 \textmu M. These findings indicate that both amiloride and EIPA are potent inhibitors of VSMC migration and DNA synthesis.

Because amiloride and its derivatives have many nonspecific effects on cellular function, we sought to characterize the specific role of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger in VSMC proliferation and migration. We generated a Na\textsuperscript{+}-H\textsuperscript{+} exchange-deficient mutant cell line [RNHE(−)]. Initially, a clonal VSMC cell line [RNHE(+)] was prepared. These cells were then mutagenized and selected by acid suicide to generate the Na\textsuperscript{+}-H\textsuperscript{+} exchange–deficient mutant cell line RNHE(−), as described in “Materials and Methods.” We confirmed the lack of Na\textsuperscript{+}-H\textsuperscript{+} exchange activity in RNHE(−) cells by pH recovery and Na\textsuperscript{+} influx: RNHE(−) cells failed to recover from an acid load in the absence of bicarbonate (Fig 5) and also showed no significant EIPA-sensitive \textsuperscript{22}Na\textsuperscript{+} influx (Fig 6).

To study the role of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger in cell proliferation, we compared the ability of RNHE(+) and RNHE(−) cells to grow in the presence or absence of bicarbonate. Both cell types grew well in DME containing bicarbonate (pH 6.8 to 7.5) (Fig 7). When placed in a medium lacking bicarbonate (L-15), there was no significant change in growth at pH 7.5. In contrast, at pH 6.8 cell growth was inhibited to a much greater extent in RNHE(−) cells than in RNHE(+) cells: 57% versus 25% inhibition, respectively. The growth response of RNHE(−) and RNHE(+) cells was quantified further using [\textsuperscript{3}H]leucine incorporation as a measure of protein synthesis.\textsuperscript{16} The growth responses of RNHE(−) and RNHE(+) cells to 10% serum were similar in DME, pH 7.5: 10% serum stimulated a 119±4% increase in [\textsuperscript{3}H]leucine incorporation compared with 0.4% serum in the RNHE(−) cells and a 92±10% increase in the RNHE(+) cells (P>0.05, n=2) (Fig 8). These findings demonstrate that in a bicarbon-
Note that EIPA- and amiloride-sensitive 22Na+ uptake in RNHE(+) and RNHE(−) cells. Cells were preincubated in HCO3- and Na+-free solution (pH 6.8) as described in "Materials and Methods." Influx medium contained (mM) NaCl, 100; KCl, 5; CaCl2, 2; MgCl2, 1; ouabain, 1.0; bumetanide, 0.1; and HEPES-Tris, 20 (pH 7.4) at 30°C. 22Na+ uptake was measured at 1 minute as described. 22Na+ influx was linear for 2 to 2.5 minutes. Values are mean ± SEM of four determinations (total) or two determinations (+EIPA). EIPA (60 μM) dissolved in ethanol was added concurrently with addition of influx medium. No effect of vehicle (0.5% ethanol) was observed.

50±19% increase over that produced by 0.4% serum in the RNHE(−) cells and a 143±6% increase in the RNHE(+) cells (P<.05, n=2). These findings demonstrate that, in the absence of bicarbonate, the Na+-H+ exchanger is required for growth but only at relatively acidic pH values.

To study the role of the Na+-H+ exchanger in cell migration, we tested the migration of RNHE(+) and RNHE(−) cells in response to platelet-derived growth factor (PDGF). In the absence of PDGF, there was no significant difference in the migration of RNHE(+) and
RNHE(−) cells: $3.6 \pm 1.6$ and $2.9 \pm 1.2$ area units/1000, respectively (Fig 9). Although PDGF stimulated a concentration-dependent increase in cell migration (data not shown), this did not differ significantly between RNHE(+) and RNHE(−) cells (Fig 9 shows results for 10 ng/mL PDGF). The Na⁺-H⁺ exchanger therefore has no apparent role in PDGF-stimulated migration of VSMCs. EIPA inhibited RNHE(−) migration with a $K_i$ of $\approx 60$ µM (Fig 3, open symbols), which was not significantly different from that found in RNHE(+) cells or bovine VSMCs (Fig 3, solid symbols; and Fig 4). These findings indicate that EIPA inhibition of VSMC migration is independent of its effects on Na⁺-H⁺ exchange.

Discussion

Restenosis remains the major complication following coronary angioplasty. Despite almost a decade of investigation into this problem, no completely effective therapy has been found.⁶,⁷ VSMCs, the target of intensive research, respond to a wide range of growth factors. Thus, therapies that inhibit intracellular events common to many growth factors appear promising. For this reason, we chose to study the role of the Na⁺-H⁺ exchanger, specifically investigating two of its inhibitors, EIPA and amiloride. Although EIPA was clearly effective in inhibiting restenosis (and amiloride showed an inhibitory trend), our experiments suggest that amiloride and EIPA exert their effects independent of the Na⁺-H⁺ exchanger.

The present work produced two useful findings: (1) EIPA significantly inhibits neointimal formation in the rat carotid after injury. EIPA and, to a lesser degree, amiloride were as effective as captopril and heparin at nontoxic doses. (2) Knowledge of the fact that certain drugs inhibit neointimal formation is not enough; the precise mechanisms by which this occurs need to be studied and defined. The combined data of our tissue culture and in vivo studies yield an interesting result: the inhibition of neointimal formation by amiloride and EIPA is apparently independent of the effects of these drugs on the Na⁺-H⁺ exchanger.

In seeking to determine the mechanism by which EIPA and amiloride inhibit neointimal formation in vivo, we carried out several in vitro experiments. The results suggest that EIPA and amiloride decrease neointimal formation via inhibition of both VSMC proliferation and migration. Inhibition of VSMC proliferation appears to be largely due to inhibition of the Na⁺-H⁺ exchanger. Data to support a predominant role for the Na⁺-H⁺ exchanger include the following: (1) DNA synthesis was reduced by these agents in a concentration-dependent manner that paralleled their potency as Na⁺-H⁺ exchange inhibitors. (2) RNHE(−) cells failed to grow in the absence of bicarbonate at pH <6.8. (3) VSMC growth was inhibited in a concentration-dependent manner by amiloride-like drugs.¹¹ (4) Inhibition of Na⁺-H⁺ exchange prevents induction of genes required for DNA synthesis and cell cycle progression.¹² However, there is also evidence that EIPA and amiloride inhibit proliferation by mechanisms other than blockade of the Na⁺-H⁺ exchanger. Specifically, the requirement for Na⁺-H⁺ exchange is weakened by the fact that RNHE(−) cells grew as well as the RNHE(+) cells in the presence of bicarbonate and in the absence of bicarbonate at pH 7.5. Although it is possible that pH might be <7.0 in an injured vessel, it seems likely that CO₂/bicarbonate would always be present. The most likely alternative mechanism of action for EIPA and amiloride would be inhibition of cell protein kinases. Recent data¹⁵,²¹ suggest that amiloride and EIPA inhibit tyrosine kinases, which are critical mediators of many growth signals. In summary, VSMC growth inhibition by EIPA and amiloride appears to be mediated by more than one mechanism, including inhibition of Na⁺-H⁺ exchange and possibly protein tyrosine kinases.

Inhibition of VSMC migration by amiloride and EIPA appears to be largely independent of the Na⁺-H⁺ exchanger. We found that RNHE(−) cells exhibited no
increase in migration in response to PDGF as compared with RNHE(+) cells and that the concentrations of amiloride and EIPA required for inhibition of migration were significantly greater than those required for growth inhibition. For example, the IC₅₀ of EIPA for migration was 68 μM but only 8.8 μM for growth. This is in contrast to findings reported for other cell types. Simchowiz and Cragoe found that the Na⁺−H⁺ exchanger is required for migration of white blood cells, and several investigators have suggested that changes in intracellular sodium are important in migration, specifically in the shape changes required for cell motility. Thus, it appears that VSMCs can migrate in response to PDGF in the absence of Na⁺−H⁺ exchanger activity. It should be noted that the RNHE(−) cells are clonal in origin. Because it has been suggested that there is heterogeneity in VSMCs present in normal vessels, it is possible that the results obtained may pertain to only a subset of VSMCs in vessels.

Inhibition of neointimal formation in vivo by EIPA and amiloride may also be due to inhibition of autocrine growth mechanisms. For example, these agents probably decrease VSMC protein synthesis. This effect may be particularly important in vivo, since autocrine growth mechanisms, such as production of angiotensin II, fibroblast growth factor, and PDGF by VSMCs, may contribute importantly to VSMC growth and neointimal formation. EIPA and amiloride, by decreasing protein synthesis, would block these autocrine growth pathways. Further research will be needed to examine these possibilities.

Our hypothesis in doing the present work was based on the idea that inhibiting cell signaling events common to all growth factors would be an effective therapy against restenosis. We targeted the Na⁺−H⁺ exchanger using the specific inhibitors EIPA and amiloride. Although they were effective in inhibiting cell migration and proliferation, the inhibitors appeared to work independent of the Na⁺−H⁺ exchanger. Given these findings, we see two possible directions for future studies: (1) to define the cellular substrates of EIPA and amiloride action, especially the role of kinases, and (2) to determine whether there are VSMC-specific targets for drugs of the amiloride class, especially autocrine growth pathways.

Acknowledgments

This study was supported in part by Grants HL-44721 and P50 DK-45215 from the National Institutes of Health (Dr Berk). The authors thank Lester Gamble for technical assistance.

References


Na(+)–H+ exchange inhibitors decrease neointimal formation after rat carotid injury. Effects on smooth muscle cell migration and proliferation.
M Mitsuka, M Nagae and B C Berk

doi: 10.1161/01.RES.73.2.269

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/73/2/269

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/