Na+/H+ Exchange in Vascular Smooth Muscle Cells Is Controlled by GTP-Binding Proteins

Sergei N. Orlov, Sergei L. Aksentsev, Nickolai I. Pokudin, Johanne Tremblay, Pavel Hamet

Abstract—This study examines the involvement of GTP-binding proteins (Gps) in the regulation of Na+/H+ exchange and Ca2+ influx in SHR VSMCs. Increased Na+/H+ exchange has also been observed in blood cells and immortalized lymphoblasts from patients with essential hypertension, suggesting that this intermediate phenotype is caused by intrinsic factors rather than by a hypertensive milieu that might influence the carrier in vivo. The molecular mechanisms of these ion transport abnormalities remain unclear. Thus, neither Northern blot nor Western blot analysis has revealed overexpression of NHE-1 in SHR or normal VSMCs. Using single-stranded conformational polymorphism analysis of cDNA from SHR and stroke-prone SHR, we also did not find any mutation in the NHE-1 coding sequence (unpublished data). These observations are in agreement with the negative results of linkage analysis in affected sib-pairs, arguing against mutation of the NHE-1 in essential hypertension. The latter hypothesis is supported by numerous data on the regulation of permeability of ion channels by purified Gps, which are involved in Ca2+ transport pathways via the cytosolic signaling cascade and modulation of the activity of protein kinases or directly by the interaction of Gp subunits with ion transporters. Earlier we had shown that NaF drastically increases the rate of Ca influx and EIPA-inhibitable 22Na entry into VSMCs and that this effect is potentiated by the addition of AlCl3. It is well documented that fluorde anions activate Gps via the formation of AlF3 complexes whose conformation resembles that of the terminal phosphate group in GTP. Data obtained in several laboratories indicate that Gp function is altered in SHR VSMCs. These results suggest that Gps could be involved in the abnormal functioning of Na+/H+ exchange and Ca2+ transport pathways via the cytosolic signaling cascade and modulation of the activity of protein kinases or directly by the interaction of Gp subunits with ion transporters.

Key Words: vascular smooth muscle cells; Na+/H+ exchange; Ca2+ uptake; fluoride; antisense oligodeoxynucleotides; GTP-binding proteins

In the last decade, several laboratories have reported heightened Na+/H+ exchange and Ca2+ influx in SHR VSMCs. Increased Na+/H+ exchange has also been observed in blood cells and immortalized lymphoblasts from patients with essential hypertension, suggesting that this intermediate phenotype is caused by intrinsic factors rather than by a hypertensive milieu that might influence the carrier in vivo. The molecular mechanisms of these ion transport abnormalities remain unclear. Thus, neither Northern blot nor Western blot analysis has revealed overexpression of NHE-1 in SHR or normal VSMCs. Using single-stranded conformational polymorphism analysis of cDNA from SHR and stroke-prone SHR, we also did not find any mutation in the NHE-1 coding sequence (unpublished data). These observations are in agreement with the negative results of linkage analysis in affected sib-pairs, arguing against mutation of the NHE-1 in essential hypertension. The latter hypothesis is supported by numerous data on the regulation of permeability of ion channels by purified Gps, which are involved in Ca2+ transport pathways via the cytosolic signaling cascade and modulation of the activity of protein kinases or directly by the interaction of Gp subunits with ion transporters. Earlier we had shown that NaF drastically increases the rate of Ca influx and EIPA-inhibitable 22Na entry into VSMCs and that this effect is potentiated by the addition of AlCl3. It is well documented that fluorde anions activate Gps via the formation of AlF3 complexes whose conformation resembles that of the terminal phosphate group in GTP. Data obtained in several laboratories indicate that Gp function is altered in SHR VSMCs. These results suggest that Gps could be involved in the abnormal functioning of Na+/H+ exchange and Ca2+ transport pathways via the cytosolic signaling cascade and modulation of the activity of protein kinases or directly by the interaction of Gp subunits with ion transporters. The latter hypothesis is supported by numerous data on the regulation of permeability of ion channels by purified Gps subunits. To test the involvement of Gp in the regulation of Na+/H+ exchange and Ca2+ influx in VSMCs, we studied the kinetic characteristics of 22Na and 45Ca uptake modulated by NaF and compared the results with those obtained using nonhydrolyzable analogues of GTP and GDP as well as...
Selected Abbreviations and Acronyms

α-comm, β-comm = antisense oligodeoxynucleotides
complementary to the conserved part of DNA encoding α- and β-subunits of GTP-binding proteins

DMEM = Dulbecco’s modified Eagle’s medium

EIPA = ethylisopropyl amiloride

Gp = GTP-loading protein

LDH = lactate dehydrogenase

NHE-1 = ubiquitous form of Na+/H+ exchanger

ODN = oligodeoxynucleotides

SHR = spontaneously hypertensive rat

VSMC = vascular smooth muscle cell

Antisense ODNs complementary to the conserved regions of α- and β-subunits of Gp. The effects of these substances on the well-characterized signaling cascade of adenylate cyclase activation by β-adrenoceptors served as a control. Our results provide evidence of the nonspecific character of increases in 45Ca uptake under treatment of cells with NaF and suggest Gp participation in the regulation of Na+/H+ exchanger in VSMCs.

Materials and Methods

Cell Culture

VSMCs were obtained by explant methods from the aortas of 10- to 13-week-old male Brown Norway (BN) (Institute of Biology, Faculty of Medicine, Charles University, Czech Republic) and Wistar-Kyoto rats (Cardiology Center of the Ministry of Public Health, Moscow, Russia) as described in detail previously. They were seeded and grown in DMEM with 10% calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. When they reached confluency in 7 to 10 days, they exhibited a hill-and-valley pattern, which is typical of smooth muscle cells in culture and reacted positively to specific smooth muscle myosin antibodies, as verified by fluorescence microscopy. The VSMCs were passaged by treatment with 0.05% trypsin (Gibco) in Ca2+- and Mg2+-free Dulbecco’s phosphate-buffered saline and incubated in 80-cm2 tissue culture flasks at a density of 105 cells/ml. This investigation was performed on VSMCs after 12 to 16 passages. Before the experiments, the cells were plated in 24-well plates (25 K ca influx and cAMP content study) or in 24-well dishes (45Ca and 86Rb influx measurements) and allowed to grow in DMEM containing 10% calf serum for 20 to 24 hours. Then, to establish quiescence, they were incubated for 48 hours in DMEM containing 0.2% fetal calf serum. This medium was aspirated and changed with a physiologically balanced salt solution immediately before the experiments. Cell protein content was determined by a modified Lowry method. LDH activity was measured by spectrophotometric assay (Sigma Chemical Co).

Loading of VSMCs With GTPγS, GDPβS, and ODN

To modulate Gp activity, we loaded VSMCs with nonhydrolyzable GTP and GDP analogues (GTPγS and GDPβS, respectively) and with antisense or nonsense ODNs for α- and β-subunits of Gp under reversible permeabilization with streptolysin O. The sequence of α-comm antisense ODN (5’TCTATGCTTCACAATGGT-3’, where Y is T or C) corresponds to nucleotides 133 to 151 of the identical strand of the α4 gene sequence. The sequence of β-comm ODN (5’TTCGAGTGGATTCTCCTGA-3’, where R is G or A) corresponds to nucleotides 825 to 844 of the identical strand of the β2 gene sequence. α-comm, β-comm antisense and nonsense 20-mer ODN (5’GTTATGGATTCACATATGC-3’) were synthesized using a Genex Assembler (Pharmacia). The cells were washed twice with 2 mL of medium A containing 130 mmol/L NaCl, 8 mmol/L Na2SO4, 5 mmol/L glucose, 0.1 mmol/L EGTA, 1 mmol/L MgCl2, 1 mmol/L ATP, 10 mmol/L HEPES-Tra (pH 7.4), and 0.15% bovine serum albumin and then incubated at room temperature in the same medium (vehicle) or in medium A containing 0.5 U/ml streptolysin O with or without 0.6 mmol/L GDPβS, GTPγS, or 75 μmol/L ODNs. To estimate the efficiency of loading, GTPγS was added to part of the samples containing nonlabeled GTPγS and streptolysin O. In 10 minutes, these media were aspirated, and the VSMCs were washed twice with DMEM containing 10% calf serum and used for the determination of ion fluxes, cAMP production, or GTPγS uptake. To study the effect of ODNs, after the first 10 minutes of permeabilization with ODNs, the VSMCs were washed twice with DMEM and incubated for 24 hours in the presence of 10% calf serum. The permeabilization procedure was repeated, and the cells incubated for 48 hours in DMEM containing 0.2% calf serum.

To estimate the effect of permeabilization on the properties of VSMCs, LDH release and passive permeability of intact and permeabilized cells to potassium were compared. As can be seen from Table 1, permeabilization with streptolysin O led to a 3-fold increase of GTPγS uptake. The intracellular concentration of GTPγS calculated as an increment of GTPγS uptake per VSMC water volume (3.85 μl/mg of protein) was increased up to 0.3 mmol/L in permeabilized cells. Neither passive permeability to 45Ca nor LDH release was affected by the permeabilization procedure (Table 1).

45Ca Influx

VSMCs prepared in 24-well dishes were washed twice with 2-mL aliquots of 150 mmol/L NaCl, 10 mmol/L HEPES-Tris (pH 7.4) and then premixed for 30 minutes in 0.5 mL of medium B containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L CaCl2, 5 mmol/L d-glucose, and 20 mmol/L HEPES-Tris (pH 7.4). In part of the experiments, the precipitation medium was also supplied with compounds mentioned in the figure and table legends. This medium was then aspirated, and 0.25 mL of medium B was

<table>
<thead>
<tr>
<th>Type of Cells</th>
<th>Passive Permeability to Potassium, mmol (mg protein)−1 min−1</th>
<th>GTPγS Uptake, mmol (mg protein)−1</th>
<th>Extracellular LDH Activity, %</th>
<th>Protein Content, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpermeabilized</td>
<td>2.73±0.22</td>
<td>587±66</td>
<td>3±2</td>
<td>100±3</td>
</tr>
<tr>
<td>Streptolysin-treated</td>
<td>2.89±0.30</td>
<td>1626±106*</td>
<td>4±3</td>
<td>95±5</td>
</tr>
</tbody>
</table>

Passive permeability to potassium was measured as a rate of 86Rb influx in the presence of 1 mmol/L ouabain and 10 μmol/L bumetanide. GTPγS was added in streptolysin O-containing medium as indicated in “Methods.” Permeability for K+, lactate dehydrogenase activity, and protein content in permeabilized cells were measured after a 1-hour preincubation in medium B. Protein content in control (untreated) wells and the values of total (extracellular + intracellular) LDH activity were taken as 100%. Total LDH activity in control and streptolysin-treated cells was not significantly different. Intracellular LDH was measured after treatment of VSMCs with 0.5% Triton X-100. The mean±SE of two experiments performed in quadruplicate are given.

P<0.001 compared with the controls.
added with different concentrations of CaCl₂ and the compounds mentioned in the figure and table legends. Ca²⁺ uptake was initiated by adding 0.25 mL of medium B with 2 µCi/ml ⁴⁵CaCl₂. Previously, it was demonstrated that the kinetics of Ca²⁺ uptake by cultured VSMCs are linear up to 7 minutes. In NaF-treated VSMCs, these kinetics were linear up to 15 minutes (data not shown). In this study, ⁴⁵Ca uptake was terminated after 5 minutes by adding 2 mL of ice-cold medium C containing 100 mM MgCl₂ and 10 mM/L HEPES-Tris (pH 7.4). The VSMCs were washed four times with 2 mL of ice-cold medium C and lysed with 1 mL of a 1% sodium dodecyl sulfate/4 mM/L EDTA mixture. ⁴⁵Ca uptake (V, nmol/mg protein) was determined as V= A/am, where A is radioactivity of the cell lysate (cpm) and m is protein content of the cell lysate (mg).

Na⁺ Uptake

VSMCs were washed twice with 2-mL aliquots of 150 mM/L NaCl, 10 mM/L HEPES-Tris (pH 7.4) and preincubated for 30 minutes with 1 mL of medium B with the additions mentioned in the figure and table legends. This medium was then aspirated, and 0.5 mL of medium B containing 1 mM/L ouabain and 20 µM/L bumetanide was added. In part of the samples, this medium also contained 20 µM/L EIPA and other additions listed in the figure and table legends. ²²Na uptake was initiated by adding 0.5 mL of 150 mM/L choline chloride with 4 µCi/ml ²²NaCl. Previously, it was demonstrated that the kinetics of ²²Na uptake by VSMCs are linear up to 5 minutes. In NaF-treated VSMCs, the kinetics were linear up to 15 minutes (data not shown). In the present study, ²²Na uptake was terminated after 5 minutes and calculated, using the same procedures as for ⁴⁵Ca influx. The values of ouabain+bumetanide-resistant, EIPA-inhibited components of ²²Na uptake were used as a measure of Na⁺/H⁺ exchange activity.

TABLE 2. Effect of NaF on ⁴⁵Ca and ²²Na Uptake by VSMCs

<table>
<thead>
<tr>
<th>Additions in Preincubation Medium, mmol/L</th>
<th>Additions in Incubation Medium, mmol/L</th>
<th>Ca²⁺ Uptake</th>
<th>Na⁺ Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>EIPA</td>
<td>EIPA Resistant</td>
<td>EIPA-Sensitive (Na⁺/H⁺ Exchange)</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>0.9 ± 0.08</td>
<td>12 ± 1.6</td>
</tr>
<tr>
<td>20</td>
<td>None</td>
<td>3.6 ± 0.2#</td>
<td>10 ± 1.0</td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>9.6 ± 1.4‡</td>
<td>20 ± 1.8</td>
</tr>
<tr>
<td>20</td>
<td>1.2</td>
<td>1.02 ± 0.14</td>
<td>19 ± 0.3</td>
</tr>
</tbody>
</table>

VSMCs were preincubated in 1 mL of medium B with or without EGTA and NaF. After 30 minutes, this medium was aspirated, and 0.25 ⁴⁵Ca²⁺ uptake or 0.5 mL Na⁺ uptake of medium B containing 1 mM/L ouabain, 20 µM/L bumetanide with or without 20 µM/L EIPA (for the study of Na⁺ uptake only), and with or without EGTA and NaF were added. ⁴⁵Ca uptake was initiated after 10 minutes by the addition of 0.25 mL of medium B with 2 µCi/ml ⁴⁵Ca²⁺ NaCl. ²²NaCl uptake was initiated after 5 min by the addition of 0.5 mL of 150 mM/L choline chloride containing 4 µCi/ml ²²NaCl. The means ± SE of three (Ca²⁺ uptake) or two (Na⁺ uptake) experiments performed in quadruplicate (Ca²⁺ uptake) or triplicate (Na⁺ uptake) are given.

**P < 0.01, **P < 0.001, respectively, compared with the controls.

Effect of NaF

Table 2 shows that preincubation of VSMCs in the presence of 20 mM/L NaF resulted in a 3- to 4-fold increase of ⁴⁵Ca influx. When NaF was added to the incubation medium 10 minutes before ⁴⁵Ca addition, there was a 10-fold elevation of ⁴⁵Ca uptake. A similar increment of ⁴⁵Ca uptake was observed with simultaneous addition of NaF and isotope (data not shown). The decrease of [Ca²⁺]₀, in the preincubation medium from 1 mM/L to 1 µM/L by the addition of 12 mM/L EGTA did not modify ⁴⁵Ca uptake measured at [Ca²⁺]₀ = 1 mM/L (data not shown) but completely abolished the effect of NaF on ⁴⁵Ca (Table 2). These results suggest that potentiation of Ca²⁺ uptake by NaF was caused by accumulation of calcium-fluoride complexes rather than by accumulation of intracellular F⁻ and subsequent activation of the Ca²⁺ transport system. To examine this hypothesis, we studied the dependence of ⁴⁵Ca uptake by VSMCs on NaF and CaCl₂.
concentration and compared these results with the formation of calcium-fluoride complexes measured by light scattering of incubation media.

Fig 1a shows that in the presence of 1 mmol/L CaCl₂, ⁴²Ca uptake by VSMCs was sharply increased at >5 mmol/L NaF and did not reach saturation up to 20 mmol/L. A similar dependence was observed earlier for ⁴²Ca transport in endothelial cells.³⁶,²⁷ The addition of 10 mmol/L NaF to the incubation medium did not significantly affect ⁴²Ca uptake at [Na⁺]₀ < 0.5 mmol/L. However, above this value, NaF-induced ⁴²Ca uptake was increased sharply and reached 25 ± 6 ⁴²Ca mmol (mg protein)⁻¹ 5 minutes⁻¹ at [Na⁺]₀ = 3 mmol/L (Fig 1b). In both cases, dependence of ⁴²Ca uptake was correlated with light scattering of media, suggesting that the NaF-induced increment of ⁴²Ca uptake was caused by the formation of calcium-fluoride complexes.

Because NaF evoked a sharp rise of Ca accumulation at [Ca²⁺]₀ > 0.5 mmol/L (Fig 1b), Na⁺/H⁺ exchange was studied in a Ca²⁺-free medium as seen in Table 2, 12 mmol/L EGTA ([Ca²⁺]₀ < 1 μmol/L) increased EIPA-resistant ²²Na influx by 50% to 60% and had no effect on the EIPA-sensitive component (Na⁺/H⁺ exchange). A 30-minute preincubation of VSMCs with 10 mmol/L NaF did not change EIPA-insensitive ²²Na influx but increased the rate of Na⁺/H⁺ exchange by 5-fold. When NaF was introduced directly into the incubation medium 5 minutes before measurement of ²²Na entry at final concentration of 10 mmol/L, the Na⁺/H⁺ exchange rate was augmented 4-fold. Dependence of the Na⁺/H⁺ exchange rate on NaF concentration at [Na⁺]₀ = 10 mmol/L (b) to measure ⁴⁵Ca uptake, 0.25 mL of medium B with 2 μCi/ml ⁴⁵Ca without (3) or with NaF (1) was added. The values of light scattering in the absence of NaF (a) or CaCl₂ (b) were taken as 1 0.0 The means ± SE of three (a) or 2 (b) experiments performed in quadruplicate are given.

**Effect of GTPγS and GDPβS**

Cell permeabilization in the absence of nonhydrolyzable analogues of guanylnucleotides caused no change in basal or isoproterenol-induced cAMP production by VSMCs (Table 4). The addition of GTPγS to permeabilized cells elevated basal cAMP production by 15-fold. Replacement of GTPγS by GDPβS had no influence on basal levels of cAMP production compared with control values. Unlike basal cAMP, the isoproterenol-induced cAMP response was increased up to 2-fold by GTPγS and decreased by 4-fold in VSMCs loaded with GDPβS (Table 4). The nonhydrolyzable analogues of guanylnucleotides had no effect on the rate of ⁴²Ca uptake by VSMCs (Table 4). Unlike NaF, the rate of Na⁺/H⁺ exchange was increased by 50% and decreased by 2-fold under cell permeabilization in the presence of GTPγS and GDPβS, respectively (Table 4). EIPA-insensitive ²²Na influx was not affected by permeabilization and did not depend on the presence of nonhydrolyzed analogues of guanylnucleotides (data not shown).

**Figure 2.** Dependence of EIPA-inhibited ²²Na uptake (V) by VSMCs on NaF concentration/VSMCs were preincubated for 30 minutes in Ca²⁺-free medium B with NaF at a range of 0 to 10 mmol/L. This medium was then aspirated, and 0.25 mL of medium B with 1 2 mmol/L EGTA, 1 mmol/L ouabain, 20 μmol/L bumetanide with or without 20 μmol/L EIPA was added. After 5 minutes of preincubation, ²²Na uptake was inhibited by the addition of 0.5 mL of 150 mmol/L choline containing 4 μCi/ml ²²NaCl. The means ± SE of three experiments performed in quadruplicate are given. b. Data from Fig 2a are presented for the NaF-dependent component of EIPA-inhibited ²²Na influx in Lineweaver-Burk plots.
The exchange rate on NaF concentration followed a saturation curve with half-maximal activation at 13 mmol/L NaF (Fig 2). In NaF m Ca2+-free medium, whereas its subsequent introduction into the medium during measurement of 22Na influx was inhibited by the addition of 0.5 ml of 150 mmol/L choline chloride containing 4 μCi/ml 22NaCl. The means±SE of three experiments performed in triplicate are given.

TABLE 3. Effect of AlCl₃ and Deferoxamine on Basal and NaF-Induced ²²Na Uptake by VSMCs

<table>
<thead>
<tr>
<th>Additions in Preincubation</th>
<th>²²Na Uptake, nmol (mg protein⁻¹ 5 minutes⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EIPA-Resistant</td>
</tr>
<tr>
<td>None (control)</td>
<td>13.7±2.1</td>
</tr>
<tr>
<td>NaF, 10</td>
<td>15.8±2.2</td>
</tr>
<tr>
<td>NaF+ AlCl₃, 0.1</td>
<td>14.4±3.0</td>
</tr>
<tr>
<td>NaF+ deferoxamine, 0.1</td>
<td>14.7±4.0</td>
</tr>
</tbody>
</table>

VSMCs were preincubated in 1 ml of Ca²⁺-free medium B (medium B+1.2 mmol/L EGTA, [Ca²⁺]<1 μmol/L) with the addition of compounds listed in the left column. After 30 minutes this medium was aspirated, and 0.5 ml of medium B containing 1 mmol/L ouabain, 20 μmol/L bumetanide, and 1.2 mmol/L EGTA±20 μmol/L EIPA was added. After 5 minutes, ²²Na uptake was initiated by the addition of 0.5 ml of 150 mmol/L choline chloride containing 4 μCi/ml ²²NaCl. The means±SE of three experiments performed in triplicate are given.

Discussion

The data obtained in the present study show that Na⁺/H⁺ exchange but not the activity of ion transporters involved in Ca²⁺ uptake by VSMCs is under the control of Gps. Several lines of evidence support this conclusion. Thus, to activate Na⁺/H⁺ exchange, it was enough to preincubate cells with NaF in Ca²⁺-free medium, whereas its subsequent introduction into the medium during measurement of ²²Na influx had no additional influence (Table 2). Dependence of the Na⁺/H⁺ exchange rate on NaF concentration followed a saturation curve with half-maximal activation at 13 mmol/L NaF (Fig 2).

The addition of AlCl₃ to Ca²⁺-free medium potentiated the action of NaF on EIPA-inhibitable ²²Na influx whereas chelation of endogenous Al³⁺ with deferoxamine decreased this component (Table 3). These results are in accordance with the hypothesis of activation of Na⁺/H⁺ exchange by NaF via AlF₃⁻-induced dissociation of Gp heterotrimers.

Unlike Na⁺/H⁺ exchange, Ca influx was activated by preincubation of VSMCs with NaF in Ca²⁺-containing medium only (Table 2). These results indicate that in contrast to Na⁺/H⁺ exchange, for activation of ⁴⁵Ca influx, the presence of F⁻ and Ca²⁺ in the incubation medium is sufficient. The extracellular effect of NaF on ⁴⁵Ca influx seems to be associated with complex formation involving F⁻, Ca²⁺, and other polyvalent cations. The formation of these complexes was demonstrated directly by measuring the free Ca²⁺ concentration, giving Kᵥ=‎[Ca²⁺]×[F⁻]=0.3 mmol/L for monovalent non-depleted media. The involvement of calcium-fluoride complexes in NaF-induced Ca²⁺ uptake follows from a correlation of the dependence of ⁴⁵Ca influx on NaF and CaCl₂ concentration and complex formation measured in the same media by light scattering (Fig 1). This dependence took a hyperbolic shape that is also in accordance with the kinetics of complex formation at a concentration range close to the value of the solubility product. The measurement of light scattering demonstrated that both AlCl₃ and deferoxamine modulated the formation of calcium-fluoride complexes (data not shown) which complicates the use of these compounds in studying the role of Gp in the regulation of Ca²⁺ uptake. To overcome this problem, we loaded VSMCs with nonhydrolyzable analogues of GTP and GDP and antisense ODNs for Gp subunits. Neither approach revealed an involvement of Gps in the regulation of Ca²⁺ uptake by VSMCs (Tables 4 and 5).

Unlike data on the activation of ⁴⁵Ca influx by NaF observed in all types of nucleated cells studied so far, those on activation of amiloride-sensitive ²²Na influx, apart from VSMC, are limited to fibroblasts, enteric endocrine cells, and L-6 myoblasts. In thymocytes and platelets, NaF did not change the Na⁺/H⁺ exchange rate while in renal brush border membrane vesicles an opposite inhibitory effect was noted, indicating a specific regulatory mechanism. To further
evaluate the role of Gps in regulation of the activity of Na+/H+ exchanger in VSMCs, we treated cells with nonhydrolyzable guanine nucleotides VSMC permeabilization in the presence of GTPγS resulted in the activation of basal cAMP production and Na+/H+ exchange, whereas GDPβS decreased both the isoproterenol-induced component of cAMP production and Na+/H+ exchange (Table 4) Data on the modulation of cAMP production by nonhydrolyzable guanine nucleotides suggest that these compounds affected VSMC Na+/H+ exchange via modulation of Gp activity Previously, it was shown that the nonhydrolyzed analogue of GTP caged guanosine-5'-[(3-thio-phosphate)-3-5(4,5-dimethoxy-2-nitrobenzyl)thioester, loaded into enterc endocrine cells by reversible hypotonic shock, activated Na+/H+ exchange light dependently, also in accordance with the hypothesis of regulation of this carrier by Gp subunits We tested this method of VSMC permeabilization Unlike streptolysin 0 (Table 1), this method produced a 2- to 3-fold increase in passive permeability of membranes to potassium and a 30% to 40% decrease in intracellular LDH content (data not shown)

To modulate the content of Gp subunits in VSMCs, we used α-comm and β-comm ODNs As shown by Kleuss et al,43,55,56 α-comm can be hybridized with mRNAs of all known rat variants of α2, α3, and α4 and with the α-subunit of transducin, while β-comm can hybridize with mRNAs of all known β-subunits of Gps Loading of streptolysin-O (Table 1), this method produced a 2- to 3-fold increase in passive permeability of membranes to potassium and a 30% to 40% decrease in intracellular LDH content (data not shown)

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Isoproterenol was used at a final concentration of 10 μmol/L The means±SE of three experiments performed in triplicate (cAMP content) and quadruplicate (ion fluxes) are given

* P< 05 compared with the controls

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Based on these results, we applied ODNs to study the involvement of Gps in the regulation of Na+/H+ exchange by NaF Both α-comm and β-comm decreased basal Na+/H+ exchange by 40% to 50% (Table 5) These results suggest that both α- and β- subunits of Gps are involved in the regulation of the basal activity of VSMC Na+/H+ exchange This conclusion is supported by recent data on this activation of NHE-1 by α13, subunit59 and by transducin βγ-dimer60 in the human embryonic kidney cell line and in Xenopus oocytes, respectively In contrast to basal Na+/H+ exchange, the NaF-induced increment of carrier activity was drastically inhibited by β-comm only (Fig 3b), thus indicating that NaF mainly stimulates this transporter via an increase of the content of activated βγ-dimer

In conclusion, our results show that VSMC Na+/H+ exchange is under the control of Gps and its activation by NaF is mediated via the Gp β-subunit Recently, it was reported that the transient expression of mutationally-activated αγ, α12, and α2γ-subunits of Gps stimulates Na+/H+ exchange in HEK29339 and COS-14 cells Stimulation of Na+/H+ exchange in these cells by αγ and α2γ was completely abolished after downregulation of protein kinase C whereas the effect of α12 was protein kinase C independent It should be also underlined that the relative contribution of Gp subunits in the regulation of Na+/H+ exchanger seems to be dependent on the cellular milieu Thus, in COS-1 cells both α12 and α2γ increased the basal activity of Na+/H+ exchange 44 In contrast, in HEK293 cells and in CCL39 fibroblasts α12 did not modify basal activity of the carrier but abolished its activation by serum and α13

Further studies should clarify the relative contribution of different

**Table 5. Effect of α-comm, β-comm, and Nonsense ODNs on 4Ca Uptake, Na+/H+ Exchange, and Basal and Isoproterenol-Induced cAMP Production in VSMCs**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Nonsense ODN</th>
<th>α-comm ODN</th>
<th>β-comm ODN</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP production, pmol (mg protein)⁻¹ hour⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>3.00±0.29</td>
<td>2.88±0.40</td>
<td>2.77±0.56</td>
<td>3.03±0.33</td>
</tr>
<tr>
<td>Isoproterenol-induced</td>
<td>96.8±5.9</td>
<td>105.7±11.6</td>
<td>47±15.8 *</td>
<td>178.0±17.9 *</td>
</tr>
<tr>
<td>4Ca influx, nmol (mg protein)⁻¹ 5 minutes⁻¹</td>
<td>11.0±0.11</td>
<td>1.00±0.12</td>
<td>1.24±0.14</td>
<td>1.18±0.16</td>
</tr>
<tr>
<td>Na+/H+ exchange, nmol (mg protein)⁻¹ 5 minutes⁻¹</td>
<td>13.4±2.0</td>
<td>14.7±2.0</td>
<td>7.9±1.5 *</td>
<td>8.7±1.7 *</td>
</tr>
</tbody>
</table>

* P< 05 compared with the controls
forms of Gps as well as protein kinase-mediated and direct membrane-delimited signal transduction pathways in the regulation of the activity of Na\(^+\)/H\(^+\) exchanger in VSMCs. Both the ODN approach and the search for quantitative trait loci using F\(_2\) hybrids of normotensive and hypertensive rats can be used to study the role of Gps in the enhanced activity of VSMC Na\(^+\)/H\(^+\) exchanger in primary hypertension.

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