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Proximal Nephron Na^+/H^+ Exchange Is Regulated by α_{1A}- and α_{1B}-Adrenergic Receptor Subtypes

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SUMMARY

Activation of α_1-adrenergic receptors (α_1-AR) increases Na^+/H^+ exchange (NHE) in proximal tubule. NHE mediates the majority of active Na^+ absorption in the proximal tubule. Three α_1-AR subtypes have been detected in kidney by molecular and binding techniques. We detected message for all three α_1-AR subtypes in mouse proximal tubule cells through reverse transcription-polymerase chain reaction and Northern analysis. To determine the α_1-AR subtypes that regulate NHE in mouse proximal tubule cells, two strategies were used: (i) antisense oligodeoxynucleotides (ODNs) to selectively inhibit expression of α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes and (ii) subtype-selective α_1-AR antagonists. Streptolysin-*O* permeabilization was used to introduce antisense and sense ODNs into cells three times over 72 hr. Western blot analysis of membranes prepared from cells treated with α_{1B}-AR antisense ODN demonstrated that α_{1B}-AR protein expression was reduced by 90% at 72 hr compared with control or sense ODN treatments. Functional regulation of NHE by α_1-ARs was determined by α_1-AR agonist changes in intracellular pH (pH_i) in cells grown on coverslips and loaded with 2′,7′-bis(2-carboxyethyl)-5(6)carboxyfluorescein-acetoxymethyl ester. Antisense ODNs for α_{1B}-AR significantly reduced phenylephrine (PHE)-induced maximal changes in pH_i by 49%. The PHE-induced changes in pH_i observed in cells treated with α_{1A}-AR antisense ODNs was reduced by 42%. The selective α_{1A}-AR antagonist WB-4101 and the α_{1B}-AR antagonist spiperone reduce PHE-induced pH_i increases to a comparable extent. No significant changes in pH_i were observed with cells treated with α_{1D}-AR antisense ODNs or the α_{1D}-AR antagonist BMY 7378 compared with untreated cells. Combined treatment with α_{1A}- and α_{1B}-AR antisense ODNs and antagonists additively inhibits PHE-induced ΔpH_i by 90%. We conclude that α_{1A} and α_{1B}-AR but not α_{1D}-ARs regulate NHE in proximal tubule cells.

Luminal NHE accounts for the bulk of active Na^+ reabsorption in the PT (1). The NHE functions in the reabsorption of HCO_3^- and secretion of H^+ (2). Catecholamines, peptide hormones, and a number of pharmacological agents regulate proximal nephron NHE (3, 4).

Numerous studies indicate that antidiuretic and antinatriuretic effects in response to low frequency renal nerve stimulation are mediated by α_1-AR (see Ref. 5 for a review). The α_1-ARs activated during renal nerve stimulation are postulated to be located postsynaptically (6). Stimulation of PT α_1-ARs increase NHE in addition to water, chloride, and bicarbonate reabsorption (7, 8). The tubular effects on transport occur independently of changes in glomerular filtration rate, renal blood flow, or the intrarenal redistribution of blood flow (9).

Three α_1-AR subtypes, designated α_{1A}-, α_{1B}-, and α_{1D}-AR, have been cloned (10, 11). α_{1A}-ARs have a high affinity for 5-methyurapidil, WB-4101, and (+)-niguldipine and are sensitive to the alkylating agent SZL-49 (12). α_{1B}-ARs have a low affinity for these agents but are highly sensitive to CEC and spiperone (11). In comparison, α_{1D}-ARs are selectively antagonized by BMY 7378 and SK&F 105854 (13). In human kidney, expression of α_1-AR protein is quite low relative to that of α_2-ARs. The localization of α_1-ARs in kidney remains somewhat controversial. Studies using *in situ* hybridization or message expression often arrive at different distributions compared with pharmacological binding or autoradiography experiments (14–16). In general, the consensus for α_1-AR localization seems to be PT and thick ascending limb/early distal convoluted tubule, where there is direct innervation by renal nerves (5, 17). Recent studies identify α_{1A}- and α_{1B}-AR subtypes in PTs (18, 19), whereas others have identified all three subtypes in PT cells (15). The purpose of this investigation was to identify the subtypes of α_1-ARs present in PT cells that regulate NHE.

Message for all three α_1-AR subtypes was observed

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ABBREVIATIONS: NHE, Na^+/H^+ exchange; AR, adrenergic receptor; ODN, oligodeoxynucleotide; pH_i, intracellular pH; PHE, phenylephrine; PT, proximal tubule; CEC, chloroethylclonidine; SDS, sodium dodecyl sulfate; 5-HT, 5-hydroxytryptamine; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

through the use of RT-PCR and Northern analysis with RNA obtained from mouse PT cells. A comparison of the nucleotide sequences obtained from the partial clones of the RT-PCR products of mouse PT cells with published rat cDNA sequences indicates >94% identity at the nucleotide level and >98% identity at the amino acid level for the three subtypes of α_1-ARs. Use of antisense ODNs selective for α_{1A} and α_{1B}-AR subtypes inhibit protein expression and α_1-AR agonist-induced increases of pH_i in PT cells by 42% and 49%, respectively. Similar levels of inhibition in agonist-induced pHi were observed with the α_{1A}-AR antagonist WB-4101 and the α_{1B}-AR antagonist spiperone. There was no functional reduction of agonist-induced pH_i in cells treated with antisense ODN or the antagonist BMY 7378 to the α_{1D}-AR subtype. Combined treatment with α_{1A} and α_{1B} antisense ODNs resulted in additive inhibition of α_1-AR-induced increases of intracellular pH_i by ≈90%. In summary, message for three subtypes of α_1-AR is expressed in PT cells, but only α_{1A}- and α_{1B}-AR subtypes regulate NHE.

Experimental Procedures

Preparation of Primary Cell Cultures and Immortalized Proximal Convoluted Tubule Cells

Primary cultures of mouse PT (proximal convoluted and straight tubule) cells were prepared as reported previously (20). An immortalized mouse S1 PT cell line was established as described previously (21). The S1 proximal cells exhibit the phenotype of the proximal convoluted S1 portion of the nephron that includes (i) functional Na/P_i cotransport, (ii) formation of cAMP in response to parathyroid hormone, (iii) alkaline phosphatase activity, and (iv) gluconeogenic activity (21).

Primary cultures of mouse proximal cells and immortalized S1 cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Sigma Chemical, St. Louis, MO) supplemented with 5% heat-inactivated fetal calf serum (Sigma) and PSN antibiotic mixture (50 μg of penicillin, 50 μg of streptomycin, 100 μg of neomycin/100 ml of media; GIBCO BRL, Gaithersburg, MD) in a humidified atmosphere of 95% O_2/5% CO_2 at 37°. Cells were placed in serum-free Dulbecco's modified Eagle's medium/Ham's F-12 medium for 16 hr before use.

RNA Isolation, RT-PCR, and Design and Introduction of Antisense ODNs

RNA isolation. Culture dishes (100 mm) of PT cells were washed twice with 5 ml of Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution. Cells were solubilized and scraped in the presence of 1 ml of 1 M guanidine isothiocyanate layered onto a 1.5-ml CsCl gradient and overlaid with 0.15 ml of 20% sarkosyl. Gradients were centrifuged for 2 hr at room temperature, and pellets were washed with 70% ethanol and resuspended in 100 μl of sterile water. Quantification of yield was determined by absorbance at 260 and 280 nm.

RT-PCR. One microgram of total RNA from PT cells was reverse-transcribed using MuMLV reverse transcriptase and the reverse primers for each subtype (GeneAMP RNA-PCR Kit; Perkin-Elmer Cetus, Norwalk, CT) for 15 min at 42°. The cDNA was then amplified with *Taq* polymerase. Mouse heart and kidney was used as positive controls for appropriately sized PCR products. Primer sequences specific for each α_1-AR subtype are presented in Table 1. PCR was performed at 94° for 1 min, annealed at the temperature indicated for each primer set in Table 1 (22) for 1 min, and extended for 1 min at 72° for 35 cycles, with a final extension of 5 min. The products were electrophoresed on a 4% low-melting agarose gel and stained with ethidium bromide. Products were cut from a low-melt agarose gel, the cDNA was eluted, and 30 ng of each product was directly sequenced with 3.2 pmol of the forward or reverse PCR primers using the PRISM DyeDeoxy Sequencing Kit (Applied Biosystems, Foster City, CA) as described by the manufacturer. To control for nucleotide incorporation errors introduced by *Taq* polymerase, multiple RT-PCR reactions were sequenced. The cDNAs from two to four independent reactions were sequenced in both forward and reverse directions. Comparisons between cDNA products and published α_1-AR subtype sequences were carried out with GCG (Genetics Computer Group, Madison, WI) and GeneWorks (Intelligenetics, Mountain View, CA) software.

Design and introduction of antisense ODNs. To design antisense ODNs that were highly specific for each α_1-AR subtype, sequences were chosen to span the third intracellular loop of the α_1-AR DNA sequence. This region was chosen since it is the most divergent among the three α_1-AR subtypes. Antisense and sense ODNs were designed from the cDNA sequence obtained for each α_1-AR subtype in the mouse PT cells used in this study. Antisense and sense 18-mers that were phosphorothioate-substituted in each position were prepared using a low pressure reverse-phase purification system (Molecular Resources, Fort Collins, CO). The sequence for each of the subtypes was as follows: α_{1A}-AR antisense ODN (positions 96–113), 5′-CTTATTCTTGGCACTGCT-3′; α_{1A}-AR sense ODN, 5′-AGCAGTGCCAAGAATAAG-3′; α_{1B}-AR antisense ODN (positions 36–53), 5′-CTTGGTGGTCCTCTTGGC-3′; α_{1B}-AR sense ODN, 5′-GCCAAGAGGACCACCAAG-3′; α_{1D}-AR antisense ODN (positions 212–229), 5′-GCGGGAAAACTTGAGCAG-3′; and α_{1D}-AR sense ODN, 5′-CTGCTCAAGTTTTCCCGC-3′. Antisense and sense ODNs were reconstituted in PBS and stored at −20°. Final concentrations of 5 μM ODNs were introduced into cells grown onto 30-mm dishes or glass coverslips with transient streptolysin-*O* permeabilization (20 units/ml; GIBCO BRL) for 10 min at 37° as described previously (23). Control cells were permeabilized, but no ODNs were added. Time and concentration dependence were determined with Western blot analysis for the α_{1B}-AR subtype. In preliminary studies, we determined that multiple treatments with antisense ODNs (three antisense ODN treatments over 72 hr) were required for maximal inhibition of protein expression.

Western Blot Analysis

Control and ODN-treated cells were collected in lysate buffer (50 mM Tris·HCl, pH 8.0, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS,

TABLE 1

Primers used for amplification of α_1-AR subtype transcripts by RT-PCR

The sequences of the primers (listed from 5′ to 3′) used for RT-PCR analysis are shown. The optimized annealing temperature for the amplification of mouse transcripts by each primer pair is also shown. The primer sequences were determined from rat in a previous report (22).

Primer	Sequence	Position	Annealing temperature
			°
α_{1A}-AR forward	5′-GTAGCCAAGAGAGAAAGCCG-3′	628–647	50
α_{1A}-AR reverse	5′-CAACCCACCACGATGCCCAG-3′	820–839	50
α_{1B}-AR forward	5′-GCTCCTTCTACATCCCGCTCG-3′	629–649	58
α_{1B}-AR reverse	5′-AGGGGAGCCAACATAAGATGA-3′	908–928	58
α_{1D}-AR forward	5′-CGTGTGCTCCTTCTACCTACC-3′	759–779	58
α_{1D}-AR reverse	5′-GCACAGGACGAAGACACCCAC-3′	1042–1062	58

1 μg/ml aprotinin, 75 μg/ml phenylmethylsulfonyl fluoride) and centrifuged at 15,000 × *g* for 10 sec. The supernatant was stored at −80°. Samples containing ≈100 μg of protein/well were boiled in loading buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, bromphenol blue) for 5 min and separated on 7.5% SDS-polyacrylamide gels. The fractionated proteins were transferred to a nitrocellulose membrane. The blots were blocked with 5% Blotto in TBS (137 mM NaCl, 20 mM Tris, pH 7.4) at 4° overnight. After three washes, the blots were incubated with 0.5 μg/ml of goat anti-α_{1B}-AR polyclonal antibody (Santa Cruz Biochemicals, Santa Cruz, CA) in 1% Blotto in TBS for 90 min at room temperature. The blot was washed and incubated with 1:3000 peroxidase-conjugated rabbit lgG fraction to goat lgG (CAPPEL, Durham, NC) in TBS for 60 min. The antibody binding was detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) and exposed on Kodak X-OMAT film. Mouse anti-β-actin monoclonal antibody (Sigma) was used as a control. Western blots were scanned with a laser densitometer, and the density of each band from antisense-treated cells was compared with that of control and sense ODN treated cells. The final values were normalized with the density units obtained with β-actin in the corresponding sample.

Northern Blot Analysis

Total RNA (20 μg) or mRNA (1 μg) from the PT cells was electrophoresed on a 1% agarose/formaldehyde gel and electrophoretically transferred to GeneScreen Plus membrane (Dupont-NEN, Wimington, DE). The blots were prehybridized in a solution of 1 M NaCl, 1% SDS, and 10% dextran sulfate for 60 min at 60°. The cDNA products for each of the α_1-AR subtypes were randomly primed with 2×10^6 cpm/ml [^{32}P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA) and used to probe RNA from mouse PT cells. The blots were washed at high stringency with 50 ml of 2× sodium chloride/sodium citrate containing 0.1% SDS three times at room temperature followed by three washes with 0.1× sodium chloride/sodium citrate containing 0.1% SDS at 60° and exposure to Kodak X-AR film for 24–48 hr at −70° (1× sodium chloride/sodium citrate = 150 mM NaCl, 15 mM sodium citrate).

Fluorescent BODIPY FL Prazosin Binding

To determine relative amounts of α_1-AR subtypes and examine antisense ODN inhibition of receptor protein expression, the fluorescent ligand BODIPY FL prazosin (Molecular Probes, Eugene, OR) was used for labeling α_1-ARs. Cells were grown on eight-well Falcon culture slides (Becton Dickinson Labware, Franklin Lakes NJ); when the cells were treated with ODNs, they were permeabilized on the slide, with streptolysin-*O* and ODNs introduced as described above. BODIPY FL prazosin (30 nM) was incubated with cells on slides for 4 hr at 4°. For competitive binding studies with pharmacological antagonists, cells were preincubated with antagonists at 4° for 15 min before incubation with antagonists plus BODIPY FL prazosin for 4 hr. After binding of BODIPY FL prazosin, cells were rinsed three times with 4° PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.40, and fixed for 15 min at room temperature with 3.7% methanol-free paraformaldehyde in PBS. Slides were rinsed three times with PBS, the chamber wells were removed, and a glass coverslip was affixed with Fluorostab mounting solution (ICN, Costa Mesa, CA). Cells were visualized with a Nikon FXA microscope equipped with a B2A filter cube.

Determination of pH_i

Cells grown onto 25-mm glass coverslips, were rinsed three times with a assay buffer containing 140 mM Na^+, 148 mM Cl^-, 5 mM K^+, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 28 mM HEPES, 18 mM Tris, and 10 mM glucose, pH 7.4, and adjusted to 295 mOsmol/kg H_2O). Cells were incubated with the pH-sensitive dye BCECF-AM (10 μM; Molecular Probes, Eugene, OR) for 1 hr at 37°. Cells were rinsed twice with buffer and placed in a temperature-controlled chamber of microincubation system at 37° as described previously (24). A Nikon Photoscan-2 (Natick, MA) was used to measure fluorescence intensity. Each experiment was calculated using equilibration with buffers of varying pH values (6.5–7.6) and treatment with the ionophore valinomycin (10 μM; Calbiochem, San Diego, CA).

Materials, Preparation of Drug Solutions, and Statistical Evaluation of Data

The α_1-AR agonist PHE and the antagonists WB 4101, spiperone, and BMY 7378 were prepared so that the molar concentration indicated in text or figures is the final concentration to which the cells were exposed. Solutions containing drugs were prepared fresh daily. All receptor ligands were purchased from Research Biochemicals (Natick, MA).

All results of Western blot analyses and intracellular pH measurements are presented as mean ± standard error. Comparisons between control and drug-treated groups were examined by *post hoc* analysis of multiple comparisons with the Bonferroni or Newman-Keuls multiple-comparisons tests using the statistical software Instat for MacIntosh (GraphPAD Software, San Diego CA). Values of $p \leq 0.05$ were considered significant.

Results

Analysis of α_1-AR transcripts in PT cells. RNA isolated from primary cultures of PT cells and immortalized S1 cells was reverse-transcribed, and the resulting cDNA was amplified by PCR in separate experiments using ODN primers specific for the three α_1-AR subtypes (22). RT-PCR for each primer set was performed on RNA samples obtained with three or four independent isolations. Primers for the α_{1A}-AR subtype resulted in a product of ≈212 bp with RNA from primary cultures and immortalized PT cells (Fig. 1). Primers specific for the α_{1B}-AR subtype produced a band at ≈300 bp, whereas primers for the α_{1D}-AR subtype resulted in a product of 304 bp. Products of similar size were obtained for each α_1-AR subtype using rat kidney as a positive control (data not shown). For all primer sets and RNAs used, samples analyzed in the absence of RT resulted in no product. These findings suggest that mouse PT cells express transcripts encoding all three α_1-AR subtypes.

To confirm the identity of PT α_1-AR subtypes, the PCR products obtained with each set of primers were sequenced. The mouse PT cell partial clones demonstrated high similarity to the published rat sequence (25–27). The mouse PT cell

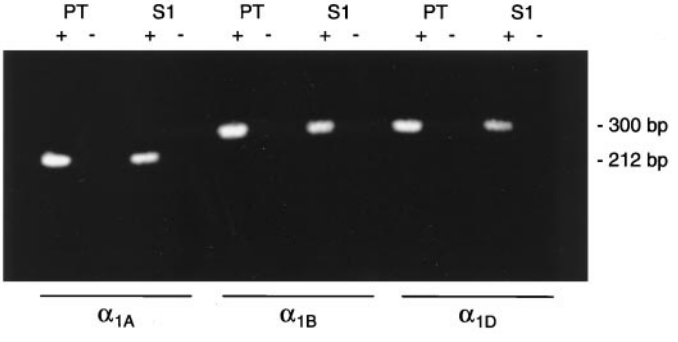

Fig. 1. Analysis of transcripts for α_1-AR subtypes in PT cells. Total RNA obtained from primary cultures (PT) or immortalized (S1) proximal convoluted tubule cells was used to detect message for α_1-AR subtypes with RT-PCR. Samples were determined in the presence (+) or absence (−) of reverse transcriptase. The predicted size for α_{1A}-AR message was 212 bp, and the predicted product was 300 bp for α_{1B}-AR and 304 bp for the α_{1D}-AR.

α_{1A}-AR product was 95% identical to the published rat nucleotide sequence (26). Similarly, high levels of nucleotide identity were observed with sequence comparison of products for α_{1B}-AR (98%) and α_{1D}-AR (94%) subtypes with rat sequence (26, 27). A comparison of amino acid sequences obtained from the partial clones for each α_1-AR subtype indicates >98% identity for the three subtypes. Sequence analysis confirms that the mouse α_1-AR subtypes are highly homologous within the amplified regions to those reported in rat and demonstrates the specificity of the primer sets used for RT-PCR for each specific subtype.

Northern blot analysis was used to determine the sizes of α_1-AR subtypes mRNAs in mouse PT cells. The mouse α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes products were randomly primed, ^{32}P-dCTP labeled, and used as cDNA probes for Northern blot analysis. As shown in Fig. 2, the mouse α_{1A}-AR cDNA probe hybridized with mRNA obtained from PT cells with a transcript size of 2.3 kb. The cDNA probe for α_{1B}-AR hybridized with total RNA for a transcript of 2.7 kb. These transcript sizes are consistent with published observations for kidney and other tissues (14, 25). The cDNA used for labeling the α_{1D}-AR hybridized to two bands of ≈2.6 and ≈2.3 kb (Fig. 2). The major transcript observed at 2.6 kb is similar to that reported in other tissues (25), whereas the 2.3-kb band is consistent with the smaller transcript size observed with hepatocytes (28). The molecular evidence provided by RT-PCR and Northern analysis supports the presence of mRNAs for all three α_1-AR subtypes in mouse PT cells.

Analysis of protein expression for α_1-AR subtypes. To assess protein expression of α_1-AR subtypes, two complementary methods were used: (i) Western analysis was performed on membrane preparations from primary cultures and immortalized S1 PT cells, and (ii) competition with pharmacological antagonists and antisense inhibition of protein expression was determined with the fluorescent α_1-AR ligand BODIPY FL prazosin. Expression of the α_{1B}-AR subtype was examined using a polyclonal antibody corresponding to amino acids 500–517 of the α_{1B}-AR and maps to the carboxyl terminus (Santa Cruz). As depicted in Fig. 3, a band of 60 kDa was observed with membrane from control cells (streptolysin-*O* permeabilized but no ODN treatment). No 60-kDa band was observed when membranes were pretreated with α_{1B}-AR antibody control peptide (Santa Cruz) and confirmed the specificity of this antibody for the α_{1B}-AR (data not shown); similarly, no discernible bands were detected with the rabbit anti-goat secondary antibody alone. Proximal cells treated with 5 μM antisense or sense ODNs to the α_{1B}-AR are also shown. We observed a significant reduction in the intensity of the 60-kDa band with membrane samples obtained from three treatments over 72 hr of antisense- but not sense-treated proximal cells. The level of α_{1B}-AR protein expression in the PT cells treated with sense ODNs was similar to that observed in control cells. Nonspecific bands observed with all three treatment groups did not change relative to the 60-kDa band observed with the α_{1B}-AR antisense ODNs treatment and indicate the specificity of the ODNs. To determine whether the effect was specific for α_{1B}-AR protein expression, the blots were stripped and reprobed with mouse anti-β-actin monoclonal antibody. Similar intensities of the 45-kDa bands

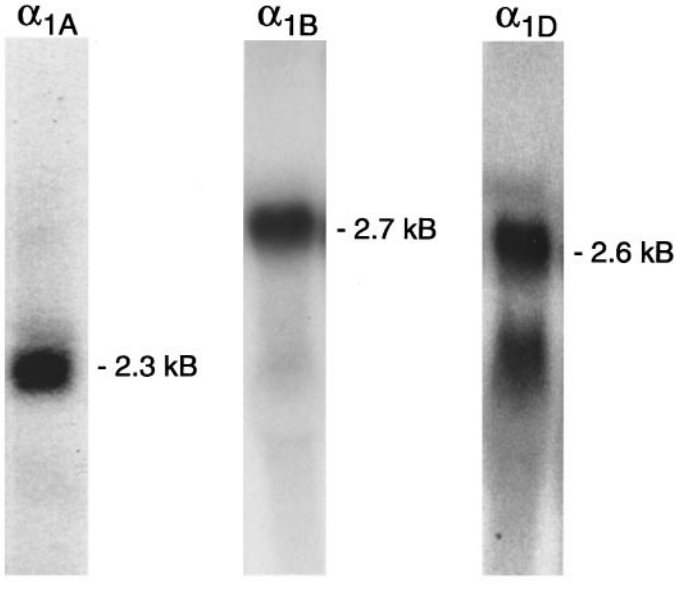

Fig. 2. Northern blot analysis of PT cell RNA for α_1-AR subtypes. Total RNA (20 μg; α_{1B}-AR, α_{1D}-AR) or mRNA (1 μg; α_{1A} AR) obtained from S1 PT cells was probed with randomly primed and ^{32}P-dCTP-labeled α_1-AR subtype PCR products. Transcripts were observed of 2.3 kb for the α_{1A} subtype, 2.7 kb for the α_{1B} subtype, and a major band at 2.6 kb and a minor band at 2.3 kb for the α_{1D} AR subtype. These sizes are consistent with transcripts reported in kidney and other tissues.

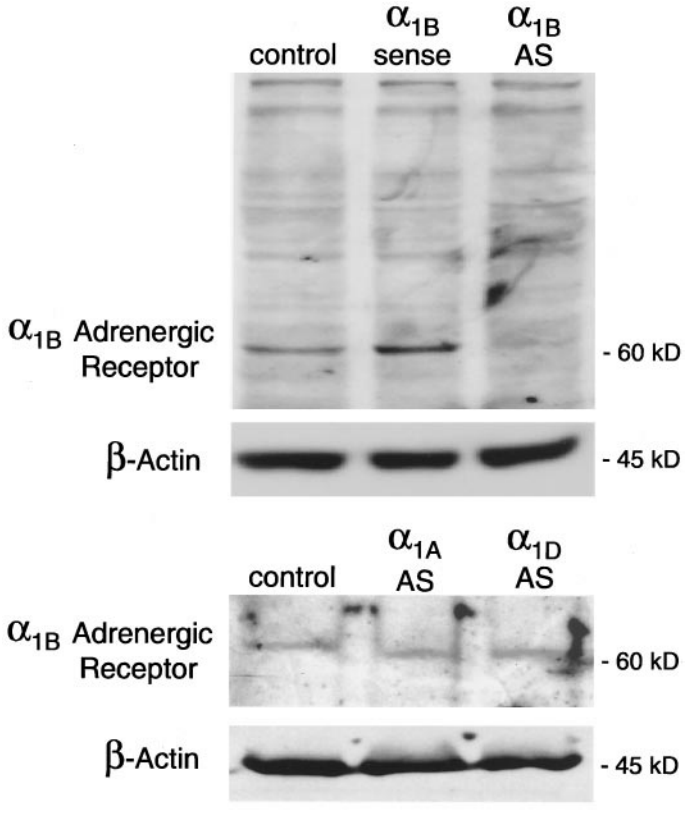

Fig. 3. Analysis of α_{1B}-AR protein expression in PT cells in a Western blot with protein obtained from S1 PT cells. Control cells were permeabilized but received no ODNs, whereas antisense and sense were permeabilized and treated with 5 μM α_{1B}-AR ODNs daily for 3 days (*top*). A band of 60 kDa is observed in control and sense lanes; the blot was stripped and reprobed for β-actin, and a 45-kDa band of similar intensity was observed in each lane. When cells were treated with α_{1A}- and α_{1D}-AR antisense ODNs, there was no change in protein expression of the 60-kDa band of α_{1B}-AR relative to control (*bottom*).

were observed with membranes obtained from control and sense- and antisense-treated cells. Fig. 3 (*bottom*) demonstrates that cells treated with antisense ODNs to α_{1A}- and α_{1D}-AR subtypes had no effect on expression of α_{1B}-AR. The ratio of α_{1B}-AR protein to β-actin was equivalent for all three lanes and supports the specificity of antisense ODN treatment for specific subtypes.

As presented in Fig. 4, the ratio of α_{1B}-AR protein to β-actin for control and sense-and antisense-treated cells for three separate experiments was determined. We observed that α_{1B}-AR protein expression was reduced by ≈64% in proximal cells treated with two antisense ODNs treatments over 48 hr and by ≈90% in cells with three antisense ODN treatments over 72 hr. To examine the relative expression of α_1-AR subtypes, we quantified α_1-AR labeling with the fluorescent ligand BODIPY FL prazosin in cells that were treated with subtype-selective antisense ODNs. As depicted in Fig. 8, α_{1A}-AR antisense treatment significantly reduced fluorescent labeling by 41% and α_{1B}-AR treatment reduced labeling by 34%. Treatment with α_{1D}-AR antisense ODN reduced fluorescent labeling by 18%. Combined treatment with α_{1A}- and α_{1B}-AR antisense ODNs decreased fluorescent labeling by 68%. These findings suggest that the majority of α_1-AR on PT cells are α_{1A}- and α_{1B}-AR subtypes. The finding that combined treatment did not abolish fluorescent labeling may be attributable to incomplete inhibition of expression with antisense ODNs or binding of BODIPY FL prazosin to other surface proteins or receptors (data presented in Fig. 8 were corrected for specific binding by competition with unlabeled prazosin).

Determination of α_1-AR subtypes that regulate NHE. As reported previously, α_1-ARs increase NHE in PT cells (29). Proximal cells treated with selective α_1-AR agonists exhibit a rapid increase in intracellular pH relative to the resting intracellular pH. Although pharmacological receptor antagonists provide some estimate of subtype, they are much less selective than the specific and transient knockout of receptor proteins achieved with antisense oligonucleotides. To assess the α_1-AR subtypes that increase NHE in PT cells, we treated cells with antisense specific for α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes. Cells were treated with 5 μM concentrations of antisense ODNs three times over 72 hr because this treatment produced a maximal reduction in α_{1B}-AR protein expression. As shown in Fig. 5, proximal cells exposed to varying concentrations of the α_1-AR agonist PHE produced concentration-dependent increases in pH_i. Basal pH_i of immortalized proximal cells was 7.08 ± 0.01, and on exposure to 1 μM PHE, a maximal increase of 0.35 ± 0.02 pH units was observed (17 independent experiments). The dose-dependent increase in pH_i was similar for cells treated with α_{1B}-AR sense ODNs compared with control cells but was reduced by ≈46% in cells treated with α_{1B}-AR antisense ODNs.

Representative responses of pH_i to PHE in PT cells treated with antisense to α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes three times over 72 hr are depicted in Fig. 6. Control cells (streptolysin-O permeabilized with no ODNs) and cells that received antisense α_{1D}-AR ODNs responded to PHE with similar increases of pH_i. Proximal tubule cells that received α_{1A}- and α_{1B}-AR ODN treatments displayed approximately half the increase of pH_i in response to PHE compared with control cells. To determine whether regulation of NHE by α_{1A}- and α_{1B}-AR subtypes is independent and additive, we treated cells with both α_{1A}- and α_{1B}-AR antisense ODNs. As presented in Fig. 6, treatment with antisense to both α_{1A}- and α_{1B}-AR subtypes resulted in a significantly reduced response compared with that obtained with antisense treatment with each subtype alone. The PHE-induced increase of pH_i in cells treated with combined α_{1A}- and α_{1B}-AR ODNs was ≈89% of that observed in control cells. The combined use of both α_{1A}- and α_{1B}-AR ODNs was almost 30% greater than that observed with either α_{1A}- or α_{1B}-AR ODN treatment alone. A

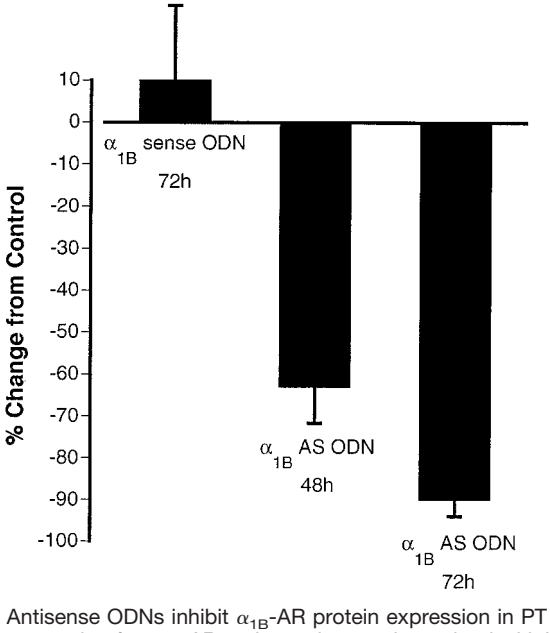

Fig. 4. Antisense ODNs inhibit α_{1B}-AR protein expression in PT cells. Protein expression for α_{1B}-AR and β-actin was determined with Western blot analysis for membrane protein obtained from control and sense- and antisense-treated cells. The intensity of each band was determined densitometrically, and the ratio for α_{1B}-AR to β-actin was calculated for sense ODN-treated cells at 72 hr and antisense to α_{1B}-AR at 48 or 72 hr. *Bars*, percent change from the ratio determined for control cells represents mean ± standard error for three separate determinations.

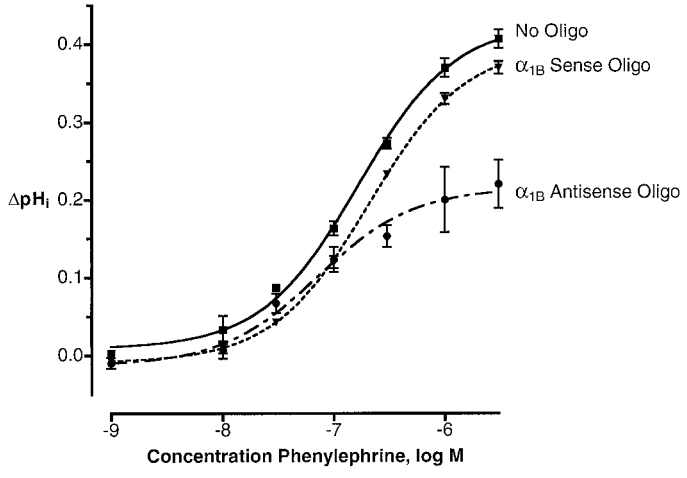

Fig. 5. Dose-response curves for the α_1-AR agonist PHE in control (no ODNs) and α_{1B} antisense and sense ODN-treated cells. Cells were grown onto glass coverslips, permeabilized, and treated with ODNs three times over 3 days. *Points*, ΔpH_i measured with BCECF-AM in response to PHE addition (mean ± standard error for three separate sets of experiments). Maximum increases of pH_i occurred with 1 μM final concentrations of PHE.

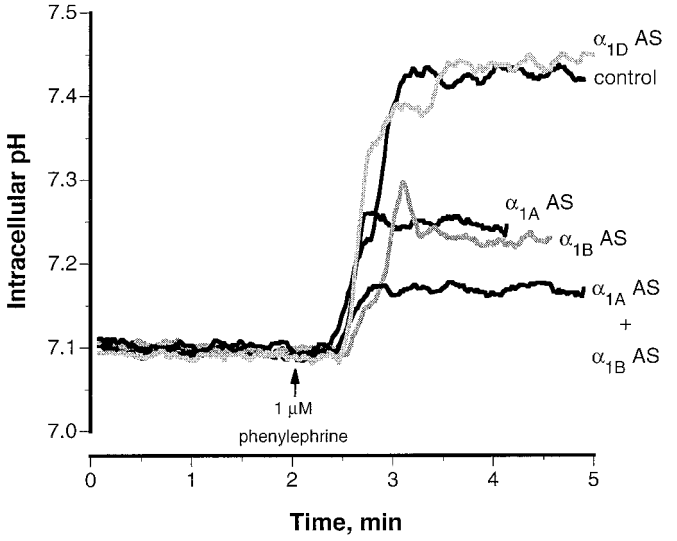

Fig. 6. Tracings of pH_i responses to PHE in control and antisense ODN-treated PT cells. Control cells were permeabilized but received no ODNs. Proximal S1 cells were treated three times over 72 hr with α_{1A}, α_{1B}, α_{1D}, or α_{1A} plus α_{1B} antisense ODNs at final concentrations of 5 μM. A basal pH_i was determined for 2 min before the addition of 1 μM PHE. After exposure to PHE, calibration was performed with 10 μM valinomycin and buffers of pH 6.5–7.6.

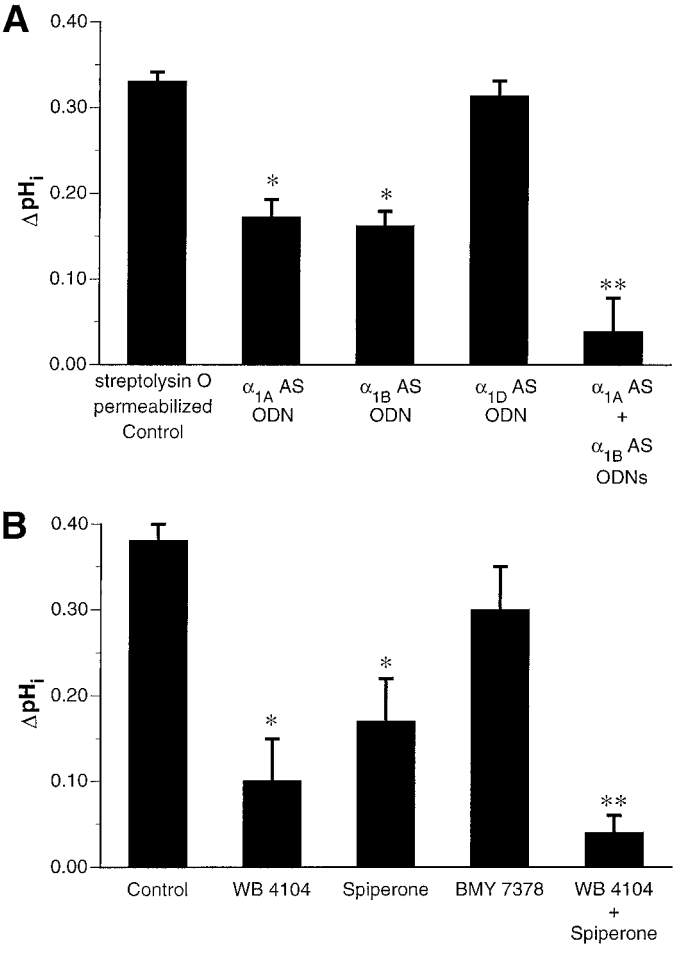

Fig. 7. Summary of basal and PHE-induced pH_i changes in control and antisense (*AS*) ODN-treated cells (A) and treatment with subtype-selective α_1-AR antagonists (B). Cells exposed to antisense ODNs were treated three times over 3 days. *Bars*, mean ± standard error for six to nine separate experiments for basal pH_i and the change of pH_i that occurred with addition of 1 μM PHE. B, Cells were treated with the α_{1A}-antagonist WB-4101 (1 μM), the α_{1B}-antagonist spiperone (1 μM), or the α_{1D}-AR antagonist BMY 7378 (1 μM). *Bars*, mean of four or five independent experiments with each treatment. *, $p < 0.01$ compared with PHE-induced change in control cells. **, $p < 0.05$ compared with PHE-induced change in cells treated with α_{1A} or α_{1B} antisense ODNs or antagonists alone.

summary of these observations in provided in Fig. 7. For each of the α_1-AR subtypes, treatment with sense ODNs was not significantly different from the findings obtained with control cells. The percent changes from control for antisense and sense treatment for the α_1-AR subtypes as well as combined α_{1A}- or α_{1B}-AR ODN treatment are depicted in Fig. 8.

Fig. 7 also provides a summary of the results of experiments performed with subtype-selective α_1-AR antagonists. The α_{1A}-AR antagonist WB 4101 inhibited PHE-induced increases of pH_i by 74%, and the α_{1B}-AR antagonist spiperone inhibited this increase by 55%. The level of inhibition observed with WB-4101 is greater than that determined with α_1-AR antisense ODN treatment; this may in part be due to partial inhibition of α_{1B}-AR as well. The inhibition resulting from spiperone antagonism is comparable to that achieved with α_{1B}-AR antisense ODN treatment. Although spiperone binds to dopamine and 5-HT receptors (30), the use of PHE to selectively activate α_1-AR precludes any confounding influences that may arise from binding to other receptors. The combination of WB-4101 and spiperone to block α_{1A}- and α_{1B}-ARs resulted in an equivalent level of inhibition to that observed with combined α_{1A}- and α_{1B}-AR antisense ODN treatment, ≈90% with antagonists or antisense ODNs. The α_{1D}-AR antagonist BMY 7378 inhibited PHE-induced increases of pH_i by ≈20%; however, this reduction was not significant. The modest inhibition by BMY 7378, although somewhat greater than that observed with α_{1D}-AR antisense ODN treatment, may be due to binding to other α_{1A}-AR subtypes. These data provide additional support for the lack of α_{1D}-AR regulation of NHE in PT.

Discussion

Catecholamines bind and activate adrenergic receptors in the kidney, where they mediate effects on tubular transport, metabolism, blood flow, and release of renin (see Ref. 5 for a review). The localization of α_1-AR expression in the kidney has resulted in conflicting reports concerning the distribution of α_1-AR subtypes. Meister *et al.* (14) report that mRNA for α_{1A}-AR is localized primarily to vessels of the renal parenchyma and α_{1B}-AR mRNA is confined to outer and inner stripe of the medulla in S3 proximal segments and thick ascending limb. Feng *et al.* (15) report that mRNAs for all three α_1-AR subtypes are expressed in PTs. Gopalakrishnan *el al.* (18) identify only α_{1A}- and α_{1B}-AR subtypes with radioligand binding in PTs; subsequently, they reported that α_{1B}-ARs increase Na^+/K^+-ATPase activity in the PT, whereas α_{1A}-ARs are linked to tubular inositol trisphosphate production and protein kinase C activation (19). Earlier studies that demonstrate α_1-ARs increase NHE in PT cells do not identify the particular receptor subtypes that mediate enhanced exchange activity (29). The purpose of the current study was to determine the α_1-AR subtypes that regulate NHE in PT cells.

Three α_1-AR subtypes have been cloned (see Ref. 31 for a

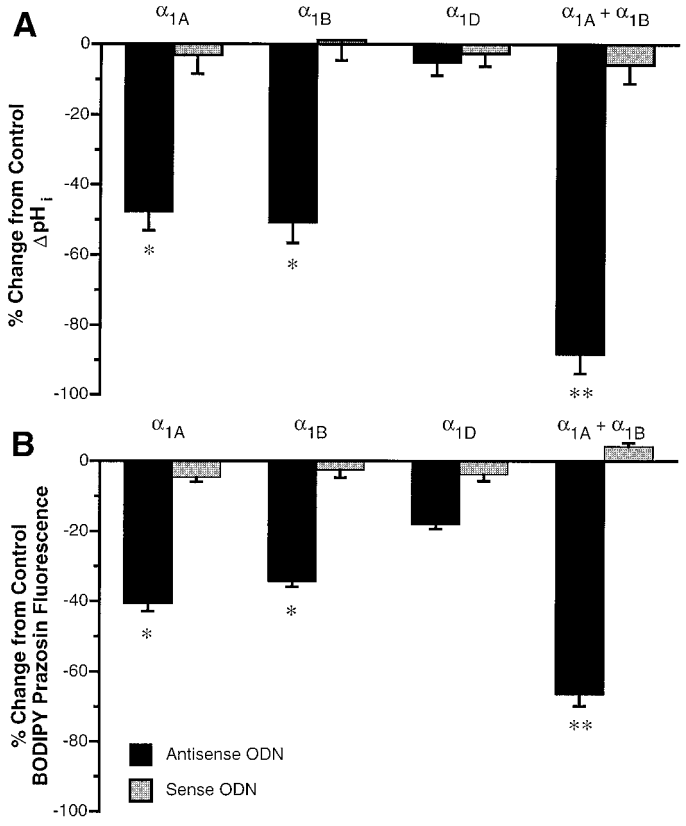

Fig. 8. AR subtypes α_{1A} and α_{1B} but not α_{1D} regulate PHE-induced changes in pH_i (A) and represent the majority of α_1-AR expressed in PT cells (B). *Bars*, mean ± standard error for four to seven separate experiments for inhibition of the PHE-induced increase of pH_i compared with that observed in control cells (no ODN treatment). Sense and antisense ODN-treated cells were permeabilized and incubated with ODNs three times over 3 days. To assess the relative amount of α_1-AR subtypes expressed in PT cells, subtype-selective antisense ODNs were used to inhibit expression of α_1-AR subtypes, and the reduction in labeled binding sites was measured with BODIPY FL prazosin. *Bars*, mean of four to six independent experiments with the fluorescence intensity determined in 10–15 cells on each slide.

review); all three cloned subtypes bind prazosin (11). Drugs with selectivity for α_{1A}-AR over α_{1B}-AR include 5-methylurapidil, (+)-niguldipine, SZL-49, and WB 4101. There is some indication that BMY 7378 may exhibit selectivity for α_{1D}- over α_{1A}- and α_{1B}-AR, whereas only CEC seems to exhibit selectivity for α_{1B} relative to α_{1A} and α_{1D}, with the profile of alkylation and inactivation: $\alpha_{1B} > \alpha_{1D} > \alpha_{1A}$ (11). The pharmacological agents currently available do not sufficiently discriminate α_1-AR subtypes. Two agents that bind with selectivity to α_{1A}-AR (i.e., niguldipine and 5-methylurapadil) also bind L-type Ca^{2+} channels and 5-HT_{1A} receptors, respectively (31). Decreases in urine flow rate and Na^+ excretion induced by PHE in Sprague-Dawley rats are abolished by pretreatment with CEC but not SZL-49 and suggest that these effects are mediated by α_{1B}-AR (32). In comparison, PHE-induced reductions in urine volume and absolute and fractional sodium excretion in Wistar and stroke-prone spontaneously hypertensive rats are blocked by 5-methylurapadil (33). These findings suggest that α_{1A}-AR mediate the increase in Na^+ and water absorption. Other subtype-selective effects of α_1-AR in kidney have also been reported (34).

In human kidney, detection of mRNAs for α_1-AR subtypes is somewhat controversial. Some studies discern α_{1A}-AR message by RNase protection assays but not RT-PCR (35, 36). It is estimated that the α_{1A}-AR subtype may constitute up to 45% of all α_1-AR mRNA in the kidney (see Ref. 37 for a review). In rats, message for α_{1B}-AR is detected in outer and inner stripes and PT (14, 15). In comparison, several binding studies detect α_{1A}- and α_{1B}-AR protein in kidney, with a predominate localization on the PT (15, 18). The α_{1D}-AR subtype is the least abundant form in human kidney (37). In rats, message expression of the α_{1D}-AR subtype is detected only in intrarenal blood vessels (14).

As demonstrated in Figs. 1 and 2, we detected transcripts for all three α_1-AR subtypes in primary cultures of PT cells and in the immortalized proximal S1 cell line. These findings are consistent with those reported by Feng *et al.* (15). Through the use of RT-PCR and CEC-sensitive and -insensitive binding, they concluded all three α_1-AR subtypes are expressed in rat PTs. One must note that the findings of Feng *et al.* (15) do not demonstrate protein for the α_{1D}-AR subtype in PT cells, so it is difficult to determine whether protein for this subtype is expressed in PT cells. In comparison, Gopalakrishnan *et al.* (18) provide binding data that only α_{1A}- and α_{1B}-ARs are present in rat renal PTs. Based on [^{3}H]prazosin binding and competition studies with selective antagonists, they report equal distributions of α_{1A}- and α_{1B}-ARs. Although transcripts for all three α_1-AR subtypes are observed, the results of the functional studies support the presence of only α_{1A}- and α_{1B}-ARs.

To identify the α_1-AR subtypes that regulate NHE in PT cells, two strategies were used and involved (i) antisense ODNs designed to regions of poor conservation among the α_1-AR subtypes to inhibit expression of selected receptor subtypes and (ii) subtype-selective α_1-AR antagonists. Antisense ODNs were used to inhibit gene expression of specific α_1-AR subtypes and circumvent the relative specificity of pharmacological antagonists. This problem is noted as being particularly significant for α_{1A}- and α_{1B}-ARs because few ligands exhibit sufficient selectivity for these receptor subtypes to permit unambiguous detection with radioligand binding techniques (10). In the current study, phosphorothioate ODNs were used because the phosphorothioate linkage affords resistance to intracellular nuclease degradation (38). Antisense and sense ODNs of 18 nucleotides were chosen because reductions in length result in both decreased activity and affinity (38). Oligonucleotides were introduced into PT cells using a transient permeabilization with streptolysin-*O* as reported previously (23). In addition, the use of cell-permeabilization reagents may help to release ODNs from endosomal vesicles and enhance entry to the nucleus (38). Based on preliminary dose and time course studies, we determined that a final concentration of 5 μM for ODNs and multiple treatments was necessary to achieve a maximal inhibition of protein expression (Figs. 3 and 4) with a minimal loss of cell number or viability. In general, minimal toxicity is associated with phosphorothioate-substituted ODNs and only at concentrations well above those needed to produce specific effects (38). Based on the unavailability of antibodies selective for α_{1A}- and α_{1D}-ARs and the degree of inhibition observed on α_{1B}-AR protein expression, equivalent ODN treatments were performed for α_{1A}- and α_{1D}-AR subtypes. Preliminary studies for each subtype indicated that

additional treatments or increased concentrations did not enhance the degree of functional inhibition observed for each subtype.

Antisense ODNs designed to a specific sequence of the α_{1B}-AR selectively inhibit the expression of this receptor subtype protein by ≈90% (Figs. 3 and 4). The fact that antisense but not sense ODNs inhibit protein expression and functional changes of pH_i is consistent with the selectivity of the antisense ODN approach. The observation that a virtually complete inhibition of the α_{1B}-AR subtype reduces NHE by only ≈50% suggests more than one α_1-AR subtype mediates stimulation of NHE. Nonselective antagonists, such as prazosin, that completely inhibit α_1-AR agonist-induced increases of NHE presumably do so through actions on more than one α_1-AR subtype (29). The finding that α_{1A}- and α_{1B}-ARs each regulate ≈50% of α_1-AR stimulated NHE (Fig. 7) is consistent with stimulation of Na^+/K^+-ATPase by these subtypes in the PT (19). The finding of α_{1D}-AR message expression in proximal by Feng *et al.* (15) agrees with our observations that α_{1D}-AR transcripts are present in this segment. The data presented in Fig. 7 indicate that antisense ODNs and pharmacological antagonists for the α_{1D}-AR subtype have minimal effects on α_1-AR-stimulated changes in NHE. To estimate the relative expression of α_1-AR subtypes, we treated cells with antisense ODN for each receptor subtype and measured binding of the fluorescent ligand BODIPY FL prazosin. Changes in fluorescence intensity were estimated with image analysis of 10–15 separate cells on four independent slides. Background fluorescence was determined with 100-fold competition with unlabeled prazosin. As depicted in Fig. 8, antisense ODNs reduced BODIPY FL prazosin fluorescence by 41%, 34%, and 18% of α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes, respectively. When cells were treated with combined α_{1A}/α_{1B} AR antisense ODNs, approximately two thirds of labeled sites were reduced. Although these studies do not demonstrate conclusively the presence or absence of α_{1D}-AR protein expression, we provide compelling evidence (Figs. 7 and 8) with antisense ODN inhibition of expression and pharmacological antagonists that this subtype does not seem to have a significant effect on NHE. Whether protein for this subtype is expressed in the PT remains to be determined. Studies in human kidney indicate this is the least abundant subtype and is detected only in intrarenal blood vessels of rat kidney with *in situ* hybridization (14). Hence, the apparent lack of effect of α_{1D}-ARs in regulation of NHE may relate to the very low or lack of protein expression for this subtype in the PT.

The mechanism through which α_1-ARs activate NHE in PT cells is likely to be increases in intracellular Ca^{2+} and inositol trisphosphate formation that lead to activation of protein kinase C (39). Several studies show that NHE in PT cells is regulated by protein kinase C (40). The increase of α_1-AR agonist-induced intracellular second messengers is abolished with the α_1-AR antagonist prazosin or the phospholipase C inhibitor U-73122 but not pertussis toxin (39).

In summary, several studies demonstrate that α_1-ARs increase NHE in PT cells (29). The particular α_1-AR subtypes that regulate NHE have not been identified. We provide pharmacological and molecular classification of α_1-AR subtypes present in mouse PT cells. Although we identified transcripts for all three subtypes in PT cells, the use of antisense ODNs to inhibit protein expression and subtype-selective pharmacological antagonists indicate only α_{1A}- and α_{1B}-ARs regulate NHE. We conclude that message and protein for α_{1A}- and α_{1B}-ARs are expressed in PT cells and activation of these receptors lead to increased NHE. Furthermore, the two subtypes seem to contribute equally to regulation of NHE. Finally, the observation that α_{1D} -ARs do not regulate NHE in these cells probably relates to the absence of protein expression for this subtype in PT cells.

References

1. Preisig, P. A., and F. C. Rector, Jr. Role of Na^+-H^+ antiport in rat proximal tubule NaCl absorption. *Am. J. Physiol.* **255:**F461–F465 (1988).
2. Mahnensmith, R. L., and P. S. Aronson. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. *Circ. Res.* **56:**773–788 (1985).
3. Gesek, F. A., and A. C. Schoolwerth. Hormonal interactions with the proximal Na^+/H^+ exchanger. *Am. J. Physiol.* **258:**F514–F521 (1990).
4. Wakabayashi, S., M. Shigekawa, and J. Pouyssegur. Molecular physiology of vertebrate Na^+/H^+ exchangers. *Physiol. Rev.* **77:**51–74 (1997).
5. Dibona, G. F., and U. C. Kopp. Neural control of renal function. *Physiol. Rev.* **77:**75–197 (1997).
6. Smyth, D. D., S. Umemura, and W. A. Pettinger. Renal nerve stimulation causes α_1-adrenoceptor-mediated sodium retention but not α_2-adrenoceptor antagonism of vasopressin. *Circ. Res.* **57:**304–311 (1985).
7. Chan, Y. L. Adrenergic control of bicarbonate absorption in the proximal convoluted tubule of the rat kidney. *Pflueg. Arch. Eur. J. Physiol.* **388:** 159–164 (1980).
8. Wong, K. R., C. A. Berry, and M. G. Cogan. α_1-Adrenergic control of chloride transport in the rat S1 proximal tubule. *Am. J. Physiol.* **270:** F1049–F1056 (1996).
9. Osborn, J. L., R. J. Roman, and R. W. Harland. Mechanisms of antinatriuresis during low-frequency renal nerve stimulation in anesthetized dogs. *Am. J. Physiol.* **249:**R360–R367 (1985).
10. Guarino, R. D., D. M. Perez, and M. T. Piascik. Recent advances in the molecular pharmacology of the α_1-adrenergic receptors. *Cell. Sign.* **8:**323–333 (1996).
11. Michel, M. C., B. Kenny, and D. A. Schwinn. Classification of α_1-adrenoceptor subtypes. *Naunyn Schmiedebergs Arch. Pharmacol.* **352:** 1–10 (1995).
12. Hieble, J. P., and R. R. Ruffolo. α_1-Adrenoceptors: pharmacological subclassification and therapeutic applications. *Proc. West. Pharmacol. Soc.* **37:**163–167 (1994).
13. Hieble, J. P., D. B. Bylund, D. E. Clarke, D. C. Eikenburg, S. Z. Langer, R. J. Lefkowitz, K. P. Minneman, and R. R. Ruffolo, Jr. International Union of Pharmacology. X. Recommendation for nomenclature of α_1-adrenoceptors: Consensus update. *Pharmacol. Rev.* **47:**267–270 (1995).
14. Meister, B., A. Dagerlind, A. P. Nicholas, and T. Hökfelt. Patterns of messenger RNA expression for adrenergic receptor subtypes in the rat kidney. *J. Pharmacol. Exp. Ther.* **268:**1605–1611 (1994).
15. Feng, F., P. W. Abel, M. A. Scofield, F. Liu, D. W. Wolff, and W. B. Jeffries. Heterogeneous expression of α_1-adrenoceptor subtypes among rat nephron segments. *Mol. Pharmacol.* **44:**926–933 (1993).
16. Cohen, H. T., F. Takemoto, T. Satoh, and A. I. Katz. Renal adrenergic receptors: localization of [^{125}I]prazosin binding sites along the microdissected rat nephron. *Can. J. Physiol. Pharmacol.* **70:**1016–1020 (1992).
17. Barajas, L., K. Powers, and P. Wang. Innervation of the renal cortical tubules: a quantitative study. *Am. J. Physiol.* **247:**F50–F60 (1984).
18. Gopalakrishnan, S. M., C. Chen, and M. F. Lokhandwala. Identification of α_1-adrenoceptor subtypes in rat renal proximal tubules. *Eur. J. Pharmacol.* **250:**469–472 (1993).
19. Gopalakrishnan, S. M., C. Chen, and M. F. Lokhandwala. α_1-Adrenoceptor subtypes mediating stimulation of Na^+,K^+-ATPase activity in rat renal proximal tubules. *Eur. J. Pharmacol.* **288:**139–147 (1995).
20. Pizzonia, J. H., F. A. Gesek, S. M. Kennedy, B. A. Coutermarsh, B. J. Backsai, and P. A. Friedman. Immunomagnetic separation, primary culture and characterization of cortical thick ascending limb plus distal convoluted tubule cells from mouse kidney. *In. Vitro. Cell Dev. Biol.* **27A:**409–416 (1990).
21. Nesbitt, T., M. J. Econs, J. K. Byun, J. Martel, H. S. Tenenhouse, and M. K. Drezner. Phosphate transport in immortalized cell cultures from the renal proximal tubule of normal and *Hyp* mice: evidence that the *HYP* gene locus product is an extrarenal factor. *J. Bone Miner. Res.* **10:**1327–1333 (1995).
22. Scofield, M. A., F. Liu, P. W. Abel, and W. B. Jeffries. Quantification of steady state expression of mRNA for *alpha*-1 adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. *J. Pharmacol. Exp. Ther.* **275:**1035–1042 (1995).
23. Barry, E. L. R., F. A. Gesek, and P. A. Friedman. Introduction of antisense oligonucleotides into cells by permeabilization with streptolysin O. *Biotech.* **15:**1018–1020 (1994).
24. Gesek, F. A., and P. A. Friedman. Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells. *J. Clin. Invest.* **90:**429–438 (1992).

25. Lomasney, J. W., S. Cotecchia, W. Lorenz, W. Y. Leung, D. A. Schwinn, T. L. Yang-Feng, M. Brownstein, R. J. Lefkowitz, and M. G. Caron. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor, the gene for which is located on human chromosome 5. *J. Biol. Chem.* **266:**6365–6369 (1991).
26. Laz, T. M., C. Forray, K. E. Smith, J. A. Bard, P. J. Vaysse, T. A. Branchek, and R. L. Weinshank. The rat homologue of the bovine α_{1c}-adrenergic receptor shows the pharmacological properties of the classic α_{1A} subtype. *Mol. Pharmacol.* **46:**414–422 (1994).
27. Voigt, M. M., J. Kispert, and H. M. Chin. Sequence of a rat brain cDNA encoding an α_{1B} adrenergic receptor. *Nucleic Acids Res.* **18:**1053 (1990).
28. Garcia-Sainz, J. A., M. T. Romero-Avila, R. Alcantara Hernandez, M. Macias-Silva, A. Olivares-Reyes, and C. Gonzalez-Espinosa. Species heterogeneity of hepatic *alpha* 1-adrenoceptors: *alpha* 1A-, *alpha* 1B- and *alpha* 1C-subtypes. *Biochem. Biophys. Res. Commun.* **186:**760–767 (1992).
29. Gesek, F. A., and A. C. Schoolwerth. Effects of *alpha*-adrenergic agonists on intracellular and intramitochondrial pH in rat proximal nephrons. *Am. J. Physiol.* **257:**F623–F630 (1989).
30. Hancock, A. A. α_1 Adrenoceptor subtypes: a synopsis of their pharmacology and molecular biology. *Drug Dev. Res.* **39:**54–107 (1996).
31. Hieble, J. P., W. E. Bondinell, and R. R. Ruffolo, Jr. α- and β-Adrenoceptors. From the gene to the clinic. 1. Molecular biology and adrenoceptor subclassification. *J. Med. Chem.* **38:**3415–3444 (1995).
32. Elhawary, A. M., and C. C. Y. Pang. α_{1B}-Adrenoceptors mediate renal tubular sodium and water reabsorption in the rat. *Br. J. Pharmacol.* **111:**819–824 (1994).
33. Sattar, M. A., and E. J. Johns. α_1-Adrenoceptor subtypes mediating antinatriuresis in Wistar and stroke-prone spontaneously hypertensive rats. *Eur. J. Pharmacol.* **294:**727–736 (1995).
34. Canessa, L. M., M. M. Piccio, P. Vachvanichsanong, A. Sidhu, C. C. Porter, J. E. Robillard, R. A. Felder, and P. A. Jose. α_{1B}-Adrenergic receptors in rat renal microvessels. *Kidney Int.* **48:**1412–1419 (1995).
35. Weinberg, D. H., P. Trivedi, C. P. Tan, S. Mitra, A. Perkins-Barrow, D. Borkowski, C. D. Strader, and M. Bayne. Cloning, expression and characterization of human α-adrenergic receptors α_{1A}, α_{1B} and α_{1C}. *Biochem. Biophys. Res. Commun.* **201:**1296–1304 (1994).
36. Hirasawa, A., K. Horie, T. Tanaka, K. Takagaki, M. Murai, J. Yano, and G. Tsujimoto. Cloning, functional expression and tissue distribution of human cDNA for the α_{1C}-adrenergic receptor. *Biochem. Biophys. Res. Commun.* **195:**902–909 (1993).
37. Michel, M. C., and L. C. Rump. α-Adrenergic regulation of human renal function. *Fundam. Clin. Pharmacol.* **10:**493–503 (1996).
38. Wagner, R. W., M. D. Matteucci, J. G. Lewis, A. J. Gutierrez, C. Moulds, and B. C. Froehler. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science (Washington D. C.)* **260:**1510–1513 (1993).
39. Gesek, F. A. α_2-Adrenergic receptors activate phospholipase C in renal epithelial cells. *Mol. Pharmacol.* **50:**407–414 (1996).
40. Reboucas, N. A., and G. Malnic. Regulation of rat proximal tubule Na/H exchange by protein kinase C. *Renal Physiol. Biochem.* **19:**87–93 (1996).

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