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Research Article

The role of adrenergic agents in augmenting proximal tubular salt and water flux, was studied in a preparation of freshly isolated rabbit renal proximal tubular cells in suspension. Norepinephrine (NE, 10(-5) M) increased sodium influx (JNa) 60 +/- 5% above control value. The alpha adrenergic antagonist, phentolamine (10(-5) M), inhibited the NE-induced enhanced JNa by 90 +/- 2%, while the beta adrenergic antagonist, propranolol, had a minimal inhibitory effect (10 +/- 2%). The alpha adrenergic subtype was further defined. Yohimbine (10(-5) M), an alpha2 adrenergic antagonist but not prazosin (10(-5) M), an alpha1 adrenergic antagonist completely blocked the NE induced increase in JNa. Clonidine, a partial alpha2 adrenergic agonist, increased JNa by 58 +/- 2% comparable to that observed with NE (10(-5) M). Yohimbine, but not prazosin, inhibited the clonidine-induced increase in JNa, confirming that alpha2 adrenergic receptors were involved. Additional alpha2 adrenergic agents, notably p-amino clonidine and alpha-methyl-norepinephrine, imparted a similar increase in JNa. The clonidine-induced increase in JNa could be completely blocked by the amiloride analogue, ethylisopropyl amiloride (EIPA, 10(-5) M). The transport pathway blocked by EIPA was partially inhibited by Li and cis H+, but stimulated by trans H+, consistent with Na+-H+ antiport. Radioligand binding studies using [3H]prazosin (alpha1 adrenergic antagonist) and [3H]rauwolscine (alpha2 adrenergic antagonist) were performed to complement the flux studies. Binding of [3H]prazosin to […]

Alpha$_2$ Adrenergic Agonists Stimulate Na^+-H^+ Antiport Activity in the Rabbit Renal Proximal Tubule

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Abstract

The role of adrenergic agents in augmenting proximal tubular salt and water flux, was studied in a preparation of freshly isolated rabbit renal proximal tubular cells in suspension. Norepinephrine (NE, 10^{-5} M) increased sodium influx (J_{Na}) 60±5% above control value. The alpha adrenergic antagonist, phentolamine (10^{-5} M), inhibited the NE-induced enhanced J_{Na} by 90±2%, while the beta adrenergic antagonist, propranolol, had a minimal inhibitory effect (10±2%). The alpha adrenergic subtype was further defined. Yohimbine (10^{-5} M), an alpha$_2$ adrenergic antagonist but not prazosin (10^{-5} M), an alpha$_1$ adrenergic antagonist completely blocked the NE induced increase in J_{Na}. Clonidine, a partial alpha$_2$ adrenergic agonist, increased J_{Na} by 58±2% comparable to that observed with NE (10^{-5} M). Yohimbine, but not prazosin, inhibited the clonidine-induced increase in J_{Na}, confirming that alpha$_2$ adrenergic receptors were involved. Additional alpha$_2$ adrenergic agents, notably *p*-amino clonidine and alpha-methyl-norepinephrine, imparted a similar increase in J_{Na}. The clonidine-induced increase in J_{Na} could be completely blocked by the amiloride analogue, ethylisopropyl amiloride (EIPA, 10^{-5} M). The transport pathway blocked by EIPA was partially inhibited by Li and *cis* H^+, but stimulated by *trans* H^+, consistent with Na^+-H^+ antiport. Radioligand binding studies using [^{3}H]prazosin (alpha$_1$ adrenergic antagonist) and [^{3}H]rauwolscine (alpha$_2$ adrenergic antagonist) were performed to complement the flux studies. Binding of [^{3}H]prazosin to the cells was negligible. In contrast, [^{3}H]rauwolscine showed saturable binding to a single class of sites, with B_{max} 1678±143 binding sites/cell and K_D 5.4±1.4 nM. In summary, in the isolated rabbit renal proximal tubular cell preparation, alpha$_2$ adrenergic receptors are the predominant expression of alpha adrenoceptors, and in the absence of organic Na^+-cotransported solutes, alpha$_2$ adrenergic agonists enhance ^{22}Na influx into the cell by stimulating the brush border membrane Na^+-H^+ exchange pathway.

Introduction

There is general agreement that catecholamines are antinatriuretic. Both data obtained in whole animal experiments or performed on the isolated perfused kidney tend to support this view (1–4). The enhanced rate of catecholamine-induced solute flux across the tubular epithelial cell is attributed primarily to alpha adrenergic agonist activity. Both alpha$_1$ and alpha$_2$ adrenergic receptors have been invoked (3, 4). However, these types of experiments cannot discern whether the observed change in sodium excretion is exclusively a direct consequence of catecholamine action on renal tubular transport, or an indirect consequence of some other action of the agent, e.g., hemodynamic alterations.

The bulk of the early experimental data dealing with the specificity of the subtypes of adrenergic receptors present on the renal tubule, have been derived essentially by indirect methods. The renal adrenoceptors have been studied by examination of the end-organ response after administration of selective agonists and antagonists (5, 6). Alternately, renal nerves have been electrically excited and similar pharmacological maneuvers performed (1). While this type of approach ensures that the receptors are pharmacologically relevant, it is difficult to pinpoint with any certainty their anatomical distribution within the organ. The subsequent use of radioligand assays to characterize adrenergic receptor subtypes, has proven to be an extremely powerful tool.

This study describes the effect of adrenergic agents on a membrane transport event, and correlates these findings with radioligand binding studies in the same preparation, the intact rabbit renal proximal tubular cell. The results demonstrate that in this preparation, alpha$_2$ adrenergic receptors are the predominant adrenergic subtype, and that in the absence of Na^+-cotransported solutes, alpha$_2$ adrenergic agonists enhance ^{22}Na influx into the cell by stimulation of the brush border membrane Na^+-H^+ exchange pathway.

Methods

Isolation of proximal tubular cells. A suspension of isolated proximal tubular cells was prepared from rabbit kidney as previously described (7). Briefly, the kidneys were perfused with a modified Hanks' solution comprised of (in mM): NaCl 137, KCl 3, $CaCl_2$ 2, $MgCl_2$ 0.5, $MgSO_4$ 0.4, KH_2PO_4 0.5, Na_2HPO_4 0.3, D-glucose 5, L-lactate 4, L-alanine 1, Tris HCl 5, and Tris base 6 titrated to pH 7.4 to which BSA 0.2% (wt/vol) was added. The blanched kidneys were perfused with 2 ml of the above solution containing 0.5% (wt/vol) iron oxide. The large magnetized iron particles are trapped in the glomerular capillary network. The cortex was dissected from the medulla, homogenized, and passed sequentially through a 250-μm and 80-μm nylon mesh screen. The proximal tubules and glomeruli, trapped on the 80-μm mesh, were suspended in the preparative medium, and the iron-laden glomeruli were extracted with a magnet. The proximal tubule suspension was briefly exposed to a hypotonic medium (75 mM) devoid of divalent cations, and mechanically agitated for 60–90 s. Total exposure time to the hypotonic medium was < 2 min. After resuspension in the preparative medium, the suspension was passed through additional nylon meshes and glass wool to remove contaminating tubule fragments. The final cell pellet was suspended in a medium the composition of which

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was determined by the specific experimental condition. Cell counts were obtained by counting appropriate dilutions of cell suspension in a standard hemocytometer chamber. A typical two kidney preparation yielded 5–6 × 10^8 cells, and was completed in 50–60 min. Characterization of the cell suspension as being predominantly of proximal tubule origin, and the functional integrity of the preparation, have previously been described (7).

Transport experiments. Transport experiments with $^{22}Na^+$ to monitor sodium influx (J_{Na})[1] into cells, were performed at 37°C using a rapid filtration procedure (8). Freshly prepared cells were preequilibrated for 20 min at 37°C in a sodium-free buffer comprised of (in mM): tetramethylammonium chloride (TMA-Cl) 55, KCl 80, $CaCl_2$ 2, $MgCl_2$ 1, titrated to pH 7.4 with Tris-base 7.7 and Hepes 12.3 (final osmolarity 296 mosM); 10^{-4} M ouabain was added to the solution. Previous experiments have established that intracellular pH (pH_i) is 7.30–7.35 following incubation under these conditions (7). The transport buffer (37°C) was identical to the equilibration buffer except that 55 mM NaCl replaced TMA-Cl isoosmotically. This concentration of Na^+ was chosen since the amiloride analogue could inhibit J_{Na^+} by at least 50% vs. 10–20% at 140 mM Na^+. Ouabain (10^{-4} M) and 1.5 μCi tracer-free $^{22}Na^+$ were added to the transport buffer. Where indicated, a 1:1,000 dilution of catecholamine (agonist, antagonist, or both) was added to the transport buffer alone. In experiments where norepinephrine was used, the compound was suspended in a 10^{-3} M ascorbic acid solution immediately before use. Preliminary experiments showed that ascorbic acid had no effect on J_{Na}. In experiments where the amiloride analogue ethylisopropyl amiloride (EIPA) was used, the probe was added to the transport buffer in a 1:1,000 dilution. EIPA was prepared in dimethyl sulfoxide (DMSO). Preliminary experiments established that the vehicle did not influence J_{Na}.

To measure $^{22}Na^+$ uptake, 90 μl of transport buffer were pipetted onto the floor of a 12 × 75-mm polystyrene test tube, and 10 μl of cell suspension (4–6 × 10^6 cells/assay) were positioned adjacent to, but not in contact with the transport buffer. The reaction was initiated by rapid placement of the test tube on a vortex followed by incubation of the test tube in a water bath at 37°C. The reaction was stopped after a timed period with 1 ml of buffered LiCl solution (296 mosM) chilled to 4°C. The mixture was pipetted onto 3.0-μm filters, washed, and the filter with the trapped cells was placed in a scintillation vial and analyzed for radioactivity in a liquid scintillation counter (LS 7500, Beckman Instruments, Inc., Irvine, CA).

Na^+ uptake was expressed in moles per 10^6 cells. A correction for $^{22}Na^+$ binding to the filters was made by subtracting radioactivity measured at zero time, i.e., where the reaction had been stopped before the addition of cells. All experimental points were performed in triplicate or quadruplicate. Data presented are for single representative experiments and all were repeated on two to four preparations of freshly isolated cells. Since there was considerable variation in the control uptake value, the actual rates of uptake are presented for the individual experiments while the mean data is expressed as percentage of control. Therefore the error bars depicted in each illustration pertain to triplicate or quadruplicate determinations in a single experiment.

Determination of intracellular pH. Intracellular pH was determined by the weak acid distribution method using [2-^{14}C]5,5-dimethyloxazolidine-2,4-dione (DMO) as outlined in detail in a previous communication from this laboratory (7). Briefly, proximal tubule cells were preequilibrated for 20 min at 37°C in the transport buffer delineated above, in the presence of 10^{-4} M oubain. Incubation was initiated by addition of 500 μl of cell suspension (2–3 × 10^7 cells/assay) to an equal volume of suspension buffer that contained 0.5–0.6 μCi [^{14}C]DMO, and proceeded at 37°C for 3 min in a metabolic shaking incubator. Previous experiments established that DMO equilibrated with the intracellular fluid within 2 min (7). For determination of intracellular water space 0.5 μCi 3-*O*-methyl-D-[4-^{14}C]glucose (final concentration 5 mM) was used, and the residual extracellular volume was measured using 0.7 μCi [^{3}H]methoxyinulin as an extracellular marker. As previously reported, extracellular water space comprised < 20% of total pellet volume (7).

After incubation with the isotope, the cell suspension was pipetted onto 400 μl of silicon oil mixture consisting of (vol/vol) 80% silicone oil (SG 1.504) and 20% US Pharmacopeia medium mineral oil, and rapidly pelleted in a microfuge. The supernatant was removed, pH determined, and an aliquot taken for determination of radioactivity. The microfuge tube and its remaining contents were immersed in liquid nitrogen (−190°C) and the solidified tip of the tube containing the cell pellet was cut off and placed in a scintillation vial for solubilization and scintillation counting.

Intracellular pH was calculated from the weak acid distribution formula (see Ref. 7). The pK_a of DMO at 37°C was taken to be 6.13.

Radioligand binding studies. [^{3}H]Prazosin and [^{3}H]rauwolscine, selective antagonists of alpha$_1$ and alpha$_2$ adrenergic receptors, respectively (9, 10), were used to characterize binding to receptors on these cells. Nonspecific binding of [^{3}H]prazosin and [^{3}H]rauwolscine was defined in the presence of 10 μM phentolamine, a nonspecific alpha antagonist. In most instances nonspecific binding accounted for up to 30% of total ligand binding, but at higher ligand concentrations approached 50%. Heterogeneity of the cell population (7), and use of an intact cell rather than membrane fragments, increased the nonspecific component of radioligand binding.

Freshly prepared cells were pelleted at 600 *g*, washed twice, and resuspended in binding buffer, comprised of (in mM): NaCl 120, EDTA 0.5 and Tris-HCl 50, titrated to pH 7.5. The radioligand binding studies were performed using this buffer. Assays for estimation of receptor number were conducted in a total volume of 250 μl, and competitive binding studies were conducted in a total volume of 500 μl. Intact cells were incubated with the specific ligands for 1 h at 25°C in a shaking water bath. Preliminary experiments established that equilibrium binding conditions for proximal tubule cells were similar to that described for other tissues (10). Reactions were terminated by dilution with 10 ml of ice-cold buffer containing (in mM): EDTA 0.5, Tris-HCl 50, titrated to pH 7.5. The stopped reaction was immediately passed over glass fiber filters (Whatman, GF/C) that were washed with an additional 10 ml of the chilled buffer. The radioactivity retained on the filters was determined by liquid scintillation spectometry.

Statistical analysis. Differences in J_{Na} were assessed by one or two way analysis of variance, (ANOVA) as appropriate (11).

Materials. $^{22}Na^+$ (carrier free) [^{14}C]DMO and [4-^{14}C]3-*O*-methyl-D-glucose were purchased from Amersham Radionucleotides (Arlington Heights, IL); [^{3}H]rauwolscine and [^{3}H]prazosin from New England Nuclear (Boston, MA); and [^{3}H]methoxyinulin from ICN Radionucleotides (Irvine, CA). Ethylisopropyl amiloride (EIPA) was kindly supplied by Dr. Edward J. Cragoe, Jr., of Merck Sharp Dohme Research Laboratories (West Point, PA). Prazosin was a gift from Eugene M. Weiss of Pfizer Inc. (New York); phentolamine was a gift from Charles A. Brownley, Jr., of Ciba Pharmaceutical Co. (Summit, NJ); alpha-methylnorepinephrine was a gift from Albert E. Soria of Sterling-Winthrop Research Institute (Rensselaer, NY). Silicone oil was purchased from Aldrich Chemical (Milwaukee, WI), and US Pharmacopeia medium mineral oil from J. T. Baker Chemical, Phillipsburg, NJ. All other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest commercial grade available. The 0.3-μm cellulose nitrate filters used in the transport experiments were purchased from Sartorius Filters Inc. (Hayward, CA), and the glass fiber filters used in the radioligand binding studies were purchased from Whatman (Maidstone, England).

Results

Characterization of sodium influx into proximal tubular cells. The time course of uptake of 55 mM $^{22}Na^+$ into sodium-de-

1. *Abbreviations used in this paper:* B_{max}, maximal binding; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione; EIPA, ethyl isopropyl amiloride; G_i, guanine nucleotide inhibitory protein; J_{Na}, sodium influx; J_v, sodium reabsorption; NE, norepinephrine; pH_i, intracellular pH; pH_o, extracellular pH; TMA-Cl, tetramethylammonium chloride.

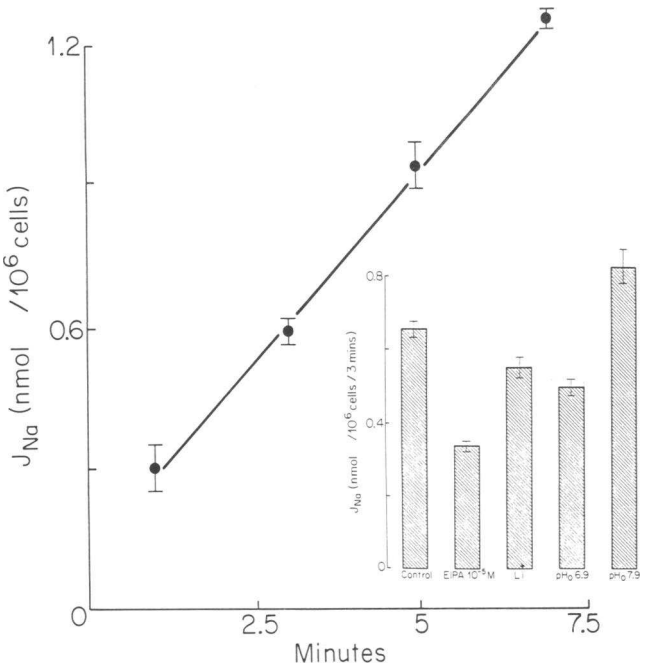

Figure 1. Time course of uptake of sodium by isolated proximal tubular cells. Freshly prepared cells were washed and preequilibrated for 20 min at 37°C in a sodium-free buffer comprised of (in mM): TMA-Cl 55, KCl 80, $CaCl_2$ 2, $MgCl_2$ 1, Tris-base 7.7, and Hepes 12.3 titrated to pH 7.4. Ouabain (10^{-4} M) was added to the buffer. The transport buffer was identical to the equilibration buffer except that 55 mM NaCl replaced TMA-Cl isoosmotically. Ouabain (10^{-4} M) and 1.5 μCi tracer-free $^{22}Na^+$ were added to the transport buffer. Transport proceeded at 37°C, and the reaction was stopped at the indicated time intervals with ice-cold LiCl titrated to pH 7.4 with 1 mM Hepes-Tris-base. The final osmolarity of all solutions was 296 mosM. In this and subsequent figures, where error bars are absent, graphical representation of the mean was larger than the standard error. Unless otherwise stated error bars pertain to triplicate or quadruplicate determinations in a single representative experiment. (*Inset*) EIPA, ethylisopropyl amiloride; Li, (55 mM) lithium chloride; pH_o, external pH.

pleted proximal tubular cells (measured in the absence of both a hydrogen ion gradient and organic solutes) was linear for 1 to 7 min (Fig. 1). All subsequent experiments were performed at 3 min. The inset to Fig. 1 demonstrates that the amiloride analogue, ethylisopropyl amiloride (EIPA), at 10^{-5} M, inhibited the influx of 55 mM sodium (J_{Na}), by 48%: control 0.65±0.02 vs. EIPA 0.34±0.01 nmol · 10^6 cells · 3 min. In seven similar experiments 10^{-5} M EIPA inhibited J_{Na} 47±3% ($P < 0.0001$). Both Li^+ and H^+ (pH 6.9) present in the incubation buffer, blunted J_{Na}, albeit to a lesser extent than EIPA 23±5% (NS) and 28±7% ($P < 0.05$), respectively. Decreasing the extracellular H^+ concentration to pH 7.9 (vs. intracellular pH 7.4) enhanced J_{Na} 30±5% ($P < 0.05$). The inhibition of J_{Na} at the external locus by EIPA, Li^+, and H^+, and enhancement of J_{Na} by an outwardly directed transmembrane H^+ gradient, are documented characteristics of the luminal membrane Na^+-H^+ exchanger (7, 8, 12). Note that at 55 mM Na^+, only ~ 50% of J_{Na} is EIPA inhibitable compared to 90% inhibition of 1 mM Na^+ with 10^{-5} M EIPA in this preparation (7).

Effect of alpha versus beta adrenergic agonist on sodium influx. The effect of norepinephrine (NE) on J_{Na} was tested, and the results of a representative experiment are depicted in Fig. 2. NE (10^{-5} M) enhanced J_{Na} 50% above the control value: NE 0.51±0.02 vs. control 0.34±0.02 nmol · 10^6 cells · 3 min. The mean increment for four experiments was 60±5% ($P < 0.0001$). Phentolamine 10^{-5} M, an alpha adrenergic receptor antagonist, inhibited the NE-induced enhanced J_{Na} by 82%: NE 0.51±0.02 vs. NE + phentolamine 0.37±0.05 nmol · 10^6 cells · min; mean inhibition was 90±2% ($P < 0.005$). Phentolamine alone was without effect on J_{Na}. In contrast to the prominant inhibition of the NE-induced increase in J_{Na} obtained with phentolamine, the beta adrenergic antagonist propranolol had only a marginal inhibitory effect: NE 0.51±0.02 vs. NE + propranolol 0.47±0.03 nmol · 10^6 cells · 3 min; the mean value was 10±2% (NS). Propranolol alone did not influence J_{Na}. Moreover, 10^{-5} M isoproterenol, a beta adrenergic agonist did not stimulate J_{Na}: isoproterenol 0.31±0.04 vs. control 0.34±0.02 nmol · 10^6 cells · 3 min, with a mean value of 8±1% (NS). These data demonstrate that catecholamines enhance the rate of sodium influx into the renal proximal tubule cell, and that their effect is mediated via an alpha adrenergic receptor.

Effect of $alpha_1$ vs. $alpha_2$ adrenergic agonist on sodium influx. To further define the alpha adrenergic receptor subtype that mediates enhanced J_{Na^+} in response to NE, selective $alpha_1$ and $alpha_2$ adrenergic antogonists were employed. Fig. 3 shows that 10^{-5} M yohimbine, an $alpha_2$ antagonist, completely blocked the NE-induced increase in J_{Na^+}. The mean inhibitory effect observed in two experiments was 97±5% ($P < 0.05$). In contrast, the $alpha_1$ antagonist, prazosin (10^{-5} M), was essentially without effect. Mean inhibition was 3±1% (NS). These results indicate that the NE-induced enhanced J_{Na^+} is mediated via $alpha_2$ adrenergic receptors.

$Alpha_2$ adrenergic agonist stimulation of Na^+-H^+ antiport. To further examine the $alpha_2$ adrenergic–mediated effect on J_{Na^+}, the $alpha_2$ agonist clonidine was tested (13). A time

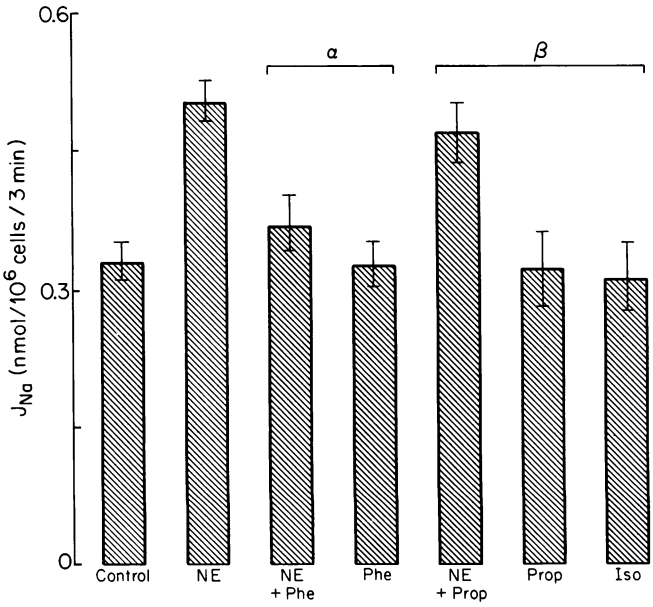

Figure 2. Effect of alpha vs. beta adrenergic agonists on sodium influx. The composition of the buffers used is outlined in the legend to Fig. 1. In all experiments 10^{-5} M adrenergic agonist or antagonist was added to the transport buffer in a 1:1,000 dilution. The three-minute uptake values are depicted. *PHE*, phentolamine; *PROP*, propranolol; *ISO*, isoproterenol.

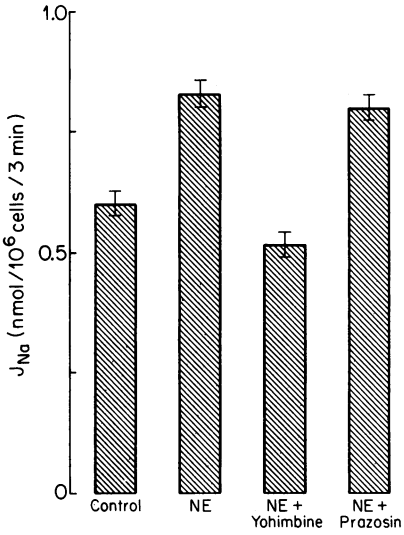

Figure 3. Effect of alpha$_1$ vs. alpha$_2$ adrenergic agonists on sodium influx. Solutions used are described in the legend to Fig. 1. NE, prazosin, and yohimbine were added to the transport buffer in 1:1,000 dilution to yield a final concentration of agonist or antagonist of 10^{-5} M.

course of uptake of Na^+ in the absence and presence of 10^{-5} clonidine is illustrated in Fig. 4. After 1 min, uptake values were identical for control and experimental conditions: control 0.22±0.03 vs. clonidine 0.23±0.01 nmol · 10^6 cells. Beyond this timepoint, clonidine stimulated J_{Na} with the maximal effect observed at 3 min: control 0.50±0.05 vs. clonidine 0.82±0.06 nmol · 10^6 cells. The mean stimulatory effect observed in four experiments was 58±2% ($P < 0.0001$). No attempt was made to follow the time course beyond 7 min. The inset to Fig. 4 provides evidence that clonidine imparted its effect via stimulation of Na^+-H^+ antiport activity since the clonidine-induced enhanced J_{Na} could be completely blocked by the amiloride analogue EIPA (10^{-5} M). In these experiments, EIPA inhibited J_{Na} by 48±2% ($P < 0.0001$) and inhibited the clonidine-induced increase in J_{Na} by 95±4% ($P < 0.0001$). Similar results were obtained for NE (data not shown) and have been previously demonstrated for another alpha$_2$ adrenergic agonist, guanabenz (14).

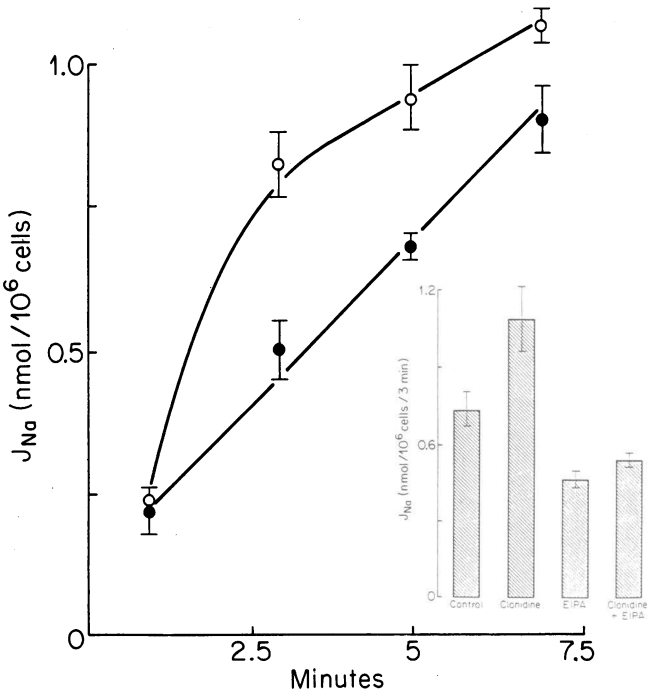

Figure 4. Time course of uptake of sodium in the absence and presence of 10^{-5} M clonidine, an alpha$_2$ adrenergic agonist. See legend to Fig. 1 for composition of buffers. (*Inset*) EIPA, 10^{-5} M.

The effect of different concentrations of clonidine (10^{-10} M–10^{-5} M) on antiport activity was tested and the results are illustrated in Fig. 5. No response was elicited at 10^{-10} M or 10^{-9} M clonidine. At the highest concentration of alpha$_2$ agonist tested (10^{-5} M), J_{Na} reached a maximal value, although a peak value was not attained. This precluded accurate determination of the $K_{0.5}$ for clonidine for the process.

Since high concentrations of a compound-like clonidine may also bind to alpha$_1$ receptors (13), clonidine-stimulated J_{Na} was tested in the presence of prazosin and yohimbine, specific alpha$_1$ and alpha$_2$ adrenergic receptor antagonists, respectively (Fig. 6). In these experiments a single concentration of agonist or antagonist (10^{-5} M) was used throughout. 3-min uptake values are reported. J_{Na} observed in the presence of yohimbine (alpha$_2$ antagonist) or prazosin (alpha$_1$ antagonist) were not different from control values: control 0.49±0.06 vs. yohimbine 0.46±0.01 vs. prazosin, 0.60±0.02 nmol · 10^6 cells · 3 min. In three experiments, J_{Na} was increased 5±2% and 8±3% in the presence of yohimbine and prazosin, respectively (NS). In the experiment depicted in Fig. 6, yohimbine, the alpha$_2$ antagonist, blocked the clonidine-stimulated J_{Na} by 82%: clonidine 0.99±0.02 vs. clonidine + yohimbine 0.58±0.01 nmol · 10^6 cells · 3 min. Prazosin, the alpha$_1$ antagonist, was a poor inhibitor of clonidine-stimulated J_{Na} : clonidine 0.99±0.02 vs. clonidine + prazosin 0.87±0.01 nmol · 10^6 cells · 3 min (24%). The mean inhibitory effect of yohimbine or prazosin on clonidine-stimulated J_{Na} in three experiments was 87±6% ($P < 0.005$) and 20±3% (NS), respectively. The concentration of prazosin used in these experiments has been documented to interact partially with alpha$_2$ adrenergic receptors, but is several orders of magnitude higher than that required to block fully alpha$_1$ adrenergic receptors (14).

Additional alpha$_2$ agonists were tested to demonstrate that alpha$_2$ agonist J_{Na^+} stimulation was not confined to clonidine alone. Fig. 7 shows that in addition to clonidine, *p*-amino-clonidine and alpha-methyl-NE stimulate J_{Na} in a comparable manner. The mean stimulatory effect observed in three experiments was 58±2%, 60±5%, and 70±8% for clonidine, *p*-amino-clonidine, and alpha-methyl-NE, respectively ($P < 0.0001$). Other data indicate that the partial alpha$_2$ adrenergic agonist, guanabenz, shows this effect (15).

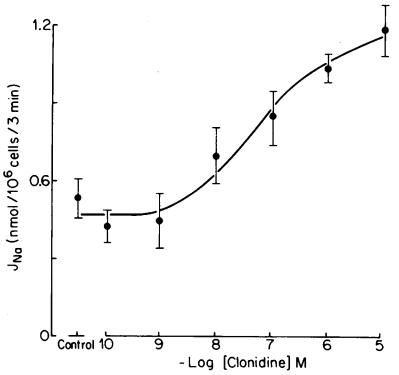

Figure 5. Concentration-dependent stimulation of J_{Na} by clonidine. Assay conditions are detailed in the legend to Fig. 1.

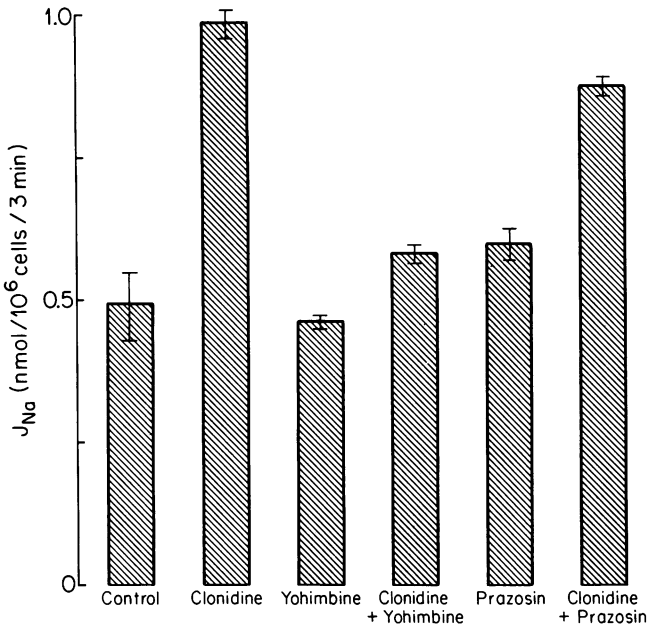

Figure 6. Effect of alpha$_1$ and alpha$_2$ antagonists on alpha$_2$-stimulated J_{Na}. The composition of the buffers is outlined in the legend to Fig. 1. 10^{-5} M agonist or antagonist was used.

Exclusion of alternate processes that might enhance alpha$_2$ adrenergic induced increase in sodium influx. The alpha$_2$ adrenergic enhanced J_{Na} observed in the absence of sodium-cotransported solutes, and inhibition of this process by the amiloride analogue EIPA, is consistent with alpha$_2$ adrenergic stimulation of Na^+-H^+ antiport. It is feasible, however, that under the described experimental conditions, the observed increase in J_{Na} may be a secondary effect consequent to other primary events. In this regard, alpha$_2$ adrenergic agonists might conceivably stimulate metabolic processes that would increase proton production rate, thereby secondarily enhancing antiport activity. Alternately, stimulation of a Na^+-dependent base exit pathway by alpha$_2$ adrenergic agonists could result in a net increase in $^{22}Na^+$ influx into the cell. Stimulation of a Na^+ channel by the catecholamine is a third possibility. Experiments addressing these specific issues are described below.

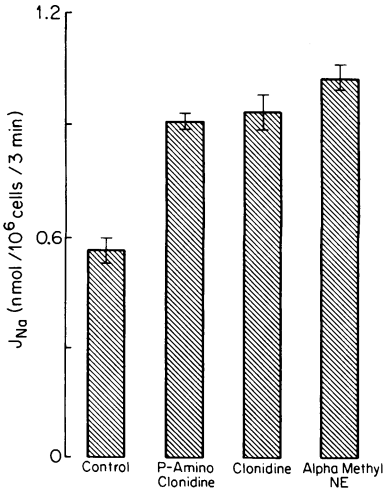

Figure 7. Stimulation of J_{Na} by alpha$_2$ adrenergic agonists (10^{-5} M). See legend to Fig. 1 for buffer composition.

To test whether catecholamines increased proton production rate, cells preequilibrated in the 55 mM NaCl transport buffer in the presence of 10^{-4} M oubain (see Methods) were exposed to 10^{-5} M EIPA, NE or clonidine for 3 min under identical experimental conditions described for the transport experiments, and pH_i derived using the weak acid distribution method (DMO). In additional experiments, NE and clonidine incubation was performed in the presence of EIPA. Note that preequilibration was performed in the 55-mM NaCl buffer in the presence of oubain, so that exposure of cells to the various compounds would differentiate a metabolic from a transport event. The pH_i values derived after 3-min incubations were identical under all conditions. For example when external pH (pH_o) was 7.41, pH_i was 7.41±0.01, 7.40±0.01, and 7.41±0.01 (n = 2) in the presence of EIPA, NE, and clonidine (all 10^{-5} M), respectively. Thus, enhanced antiport activity subsequent to catecholamine-induced increased proton production, cannot explain these data.

A possible secondary effect on EIPA-sensitive $^{22}Na^+$ influx, subsequent to stimulation of a base exit pathway by catecholamines was tested, by monitoring NE and clonidine-enhanced sodium influx in the absence and presence of the stilbene derivative, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS). For this purpose cells were incubated for 30 min in the presence of 50 μM DIDS before monitoring $^{22}Na^+$ influx for 3 min in the presence of 10^{-5} M NE or clonidine. In two independent experiments the catecholamine-induced enhanced J_{Na} was identical in the presence and absence of DIDS. Hence stimulation of a base exit pathway, e.g., Na^+/base symport or Cl^-/base exchange (16) cannot account for the current findings.

Although scant evidence exits for the presence of a sodium channel in the plasma membrane of the proximal tubule cell in situ, it is feasible that the preparative procedure might unmask such a Na^+-entry pathway, and that catecholamines may augment J_{Na} via this mechanism. 3-min $^{22}Na^+$ uptakes in the presence of 10^{-5} M NE or clonidine were monitored with addition of 10^{-5} M or 10^{-7} M EPIA. The catecholamine-induced increase in J_{Na} was completely blocked by 10^{-5} M EIPA (see Fig. 4), however 10^{-7} M EIPA, a concentration that would be expected to block Na^+ channels in the distal tubule (17), was without effect (n = 2). These data render unlikely the possibility that catecholamines impart their effect via stimulation of a Na^+ channel.

Radioligand binding studies. Radioligand binding studies using specific probes for alpha$_1$ and alpha$_2$ adrenergic receptors were performed, to correlate the functional studies described above with a specific adrenergic receptor subtype. Preliminary experiments indicated the absence of [^{3}H]prazosin binding sites, i.e., alpha$_1$ adrenergic receptors on these cells (data not shown). In contrast, [^{3}H]rauwolscine, a selective alpha$_2$ adrenergic antagonist, showed saturable binding to a single class of sites (Fig. 8). The mean (±SEM, n = 3) values for the apparent affinity (K_D, equilibrium dissociation constant) of these sites for [^{3}H]rauwolscine was 5.4±1.4, and the maximal number of binding sites (B_{max}) was 1678±143 binding sites/cell. Moreover, the alpha$_2$ selective adrenergic antagonist, yohimbine, competed for the [^{3}H]rauwolscine binding sites, whereas the alpha$_1$ selective adrenergic antagonist, prazosin (10^{-6}–10^{-8} M), was ineffective in competing for these sites (Fig. 9).

The ability of the amiloride analogue, EIPA, to block Na^+

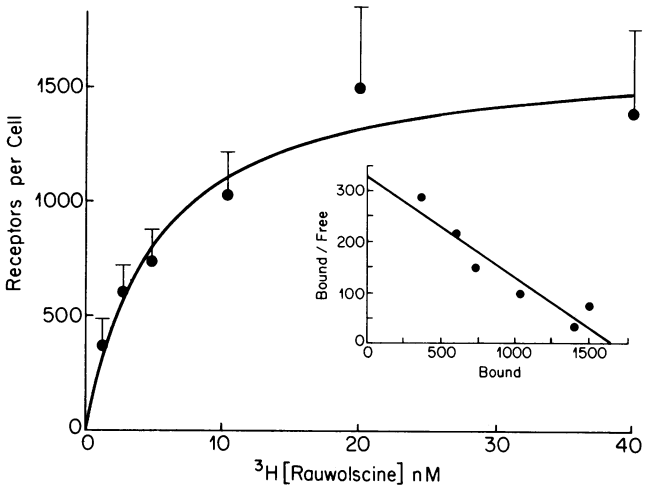

Figure 8. Binding isotherm and Scatchard analysis (*inset*) of [^{3}H]rauwolscine binding to rabbit renal proximal tubule cells. Cells were suspended in a buffer comprised of (in mM): NaCl 120, EDTA 0.5 and Tris-HCl 50 titrated to pH 7.5. The radioligand binding studies were performed in this buffer to which increasing concentrations of radioligand were added in the absence (total binding) or presence (nonspecific binding) of 10 μM phentolamine. Binding proceeded at 25°C for 1 h. The reaction was terminated by dilution with 10 ml of ice cold buffer containing (in mM): EDTA 0.5, Tris-HCl 50, titrated to pH 7.5. The data shown for the isotherm are mean (±SEM) values for receptors/cell obtained in three separate experiments. The inset shows the Scatchard analysis of these mean data.

flux in response to clonidine (Fig. 4, *inset*) might result from an interaction of EIPA at the alpha$_2$ adrenergic receptor. To exclude this possibility [^{3}H]rauwolscine binding was performed in the presence of different concentrations of EIPA (Fig. 10). It is evident that EIPA has only a limited ability to block specific [^{3}H]rauwolscine binding to alpha$_2$ adrenergic receptors in this system, with a $K_{0.5}$ of 20 μM. The $K_{0.5}$ of EIPA for the antiporter under these conditions would be 0.3 μM (7).

Discussion

The present studies demonstrate that in the rabbit renal proximal tubule, alpha$_2$ adrenergic agonists enhance sodium influx

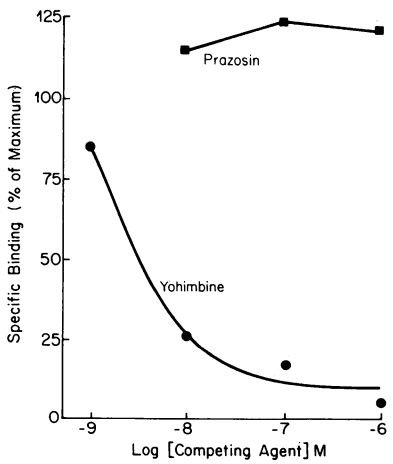

Figure 9. Competition of yohimbine and prazosin for [^{3}H]rauwolscine binding to proximal tubule cells. Assay buffers are detailed in the legend to Fig. 8.

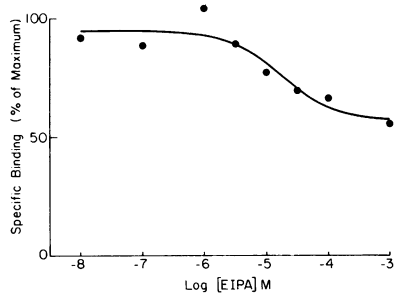

Figure 10. Competition of EIPA for [^{3}H]rauwolscine binding sites in proximal tubular cells. Assay and dilution media are detailed in the legend to Fig. 8. Cells were incubated with 4 μM [^{3}H]rauwolscine alone or in the presence of the indicated concentrations of EIPA.

into the cell, and that an increase in Na^+-H^+ antiport activity can account for the increment in sodium uptake. That the alpha$_2$ adrenergic agonist increase in sodium influx was indeed mediated via Na^+-H^+ antiport activity, is based primarily on the observation that in the absence of Na^+-cotransported substrates, the amiloride analogue EIPA, completely inhibited the alpha$_2$ agonist enhanced rate of sodium influx (Figs. 1 and 4). Since the affinity of EIPA for the alpha$_2$ adrenergic binding site is 100-fold less than its affinity for the antiporter in this preparation, i.e., 20 μM (Fig. 9) vs. 0.3 μM when corrected for pH (7), competition between EIPA and alpha$_2$ adrenergic agonist for the adrenoceptor (18) cannot be invoked as a plausible explanation for these observations.

Two independent lines of evidence confirm that in preference to other catecholamines, alpha$_2$ adrenergic agonists are functionally the predominant adrenergic subtype in the rabbit renal proximal tubule. First, the flux experiments clearly demonstrate that the NE-enhanced increased rate of antiport activity was blocked by yohimbine, a specific alpha$_2$ adrenergic antagonist, but not by prazosin, a specific alpha$_1$ adrenergic antagonist (Fig. 3). Furthermore, clonidine, a partial alpha$_2$ adrenergic agonist, enhanced antiport activity, and this effect could be blocked by yohimbine but not prazosin, thereby rendering unlikely the possibility that clonidine may have imparted its effect via alpha$_1$ adrenergic receptors (Fig. 6). Additional alpha$_2$ adrenergic agonists, *p*-amino clonidine, alpha-methyl-NE (Fig. 7) and guanabenz (14) had similar effects, thereby establishing that the effect of clonidine was shared by several agonists active at the alpha$_2$ adrenergic receptor. Second, radioligand binding studies demonstrated that alpha$_2$ adrenoceptors were the predominant alpha adrenergic receptor in this preparation. In addition, the K_D of [^{3}H]rauwolscine for alpha$_2$ adrenergic binding sites (Fig. 8) was 5.4±1.4 nM, a value similar to that reported in membrane preparations from kidneys of rabbit, rat, dog, and mouse (9). Taken together, the functional studies and radioligand binding data complement one another and provide strong evidence that binding of alpha$_2$ adrenergic agonists to their specific receptors resulted in enhanced Na^+-H^+ antiport activity.

There is general agreement that at the level of the renal proximal tubule, catecholamines enhance salt and water reabsorption (19–21). However, some species difference may exist in the nature of the adrenergic receptor. In vivo micropuncture studies in rat indicate that sodium reabsorption (J_v) is effected by alpha adrenergic agonists (16, 17) although in one study (17) beta adrenergic agonists were also found to stimulate J_V. In rabbit, however, in vitro microperfusion studies suggest that beta adrenergic agonists stimulate J_V, whereas alpha adrener-

gic agonists may in fact slightly inhibit fluid absorption (18). In none of the above studies was the subtype, i.e., $alpha_1$ or $alpha_2$, of the alpha adrenoceptor mediated effect defined. In the current studies in which we used a preparation of isolated rabbit renal proximal tubule cells in suspension, $alpha_2$ adrenergic agonists were shown to stimulate Na^+ influx (Figs. 3 and 6). No evidence was obtained in support of a role for beta adrenergic agonists in enhancing Na^+ influx. The NE-mediated increase in antiport activity was not blocked by the beta antagonist, propranolol, and the beta agonist, isoproterenol, did not stimulate the transport pathway (Fig. 2). The nature of the discrepancy between the current observations and those in the isolated perfused rabbit proximal tubule is not immediately apparent.

Although catecholamines enhance proximal tubule salt and water reabsorption, the stimulated transport pathway whereby sodium crosses the epithelium has hitherto not been described. Since the present studies were performed on a suspension of cells, the paracellular route can obviously be discounted. The observation that the amiloride analogue EIPA (10^{-5} M) completely inhibited the catecholamine-induced increase in sodium influx in the absence of Na^+-cotransported substrate (Fig. 4) indicates that Na^+-H^+ antiport activity was enhanced. The agonists (NE and clonidine) appear to impart their effect directly on antiport activity and not indirectly by primarily lowering pH_i either by stimulation of a base-exit pathway or enhanced proton production accompanying stimulation of intracellular metabolic processes. In support of the observation that catecholamines enhance Na^+-H^+ antiport activity is the report of Chan (16), that alpha adrenergic agonists enhance bicarbonate absorption in rat proximal convoluted tubule. Since bicarbonate reabsorption in this segment is critically dependent upon proton availability in the tubule lumen, and since the major pathway for proton entry into the lumen occurs via exit of H^+ from the cells on the Na^+-H^+ antiporter (22), stimulation of the exchanger by catecholamines would explain the increase in bicarbonate absorption.

The pharmacological characterization of the adrenoceptor involved in the control of renal tubular sodium handling, has been the subject of increasing attention, but has resulted in conflicting results. With regard to rabbit kidney, Neylon and Summers (9) found specific $alpha_2$ adrenergic binding sites in membrane fragments of renal cortex, with little evidence for $alpha_1$ adrenergic sites. Kusano and co-workers (23) using renal tubular fragments from different nephron segments demonstrated that $alpha_1$ adrenergic receptors were present on the proximal convoluted tubule (PCT) but not on the proximal straight tubule (PST). The possibility of the presence of $alpha_2$ adrenergic binding sites could not be excluded since only an $alpha_1$ adrenergic probe was utilized. Preliminary data by Raymond and co-workers (24) indicates the presence of $alpha_2$ adrenergic receptors in basolateral membranes prepared from purified rabbit tubule segments. No data is provided regarding the $alpha_1$ adrenoceptors. The current studies (Fig. 8) indicate the presence of $alpha_2$ adrenergic binding sites with little evidence for $alpha_1$ adrenergic receptors and complement the findings of Neylon and Summers (9). Additional experiments will have to be performed on different populations of the proximal cell preparation, to document whether $alpha_2$ adrenergic receptors are confined to a specific cell type.

The current results provide new insights regarding the anatomical localization of $alpha_2$ adrenergic receptors in the renal cortex. Autoradiographic studies of guinea pig (25) and rat (14, 26) kidney indicate that $alpha_2$ adrenergic receptors confined to the renal cortex are present on proximal tubules, while in dog the receptors appear to be confined to the vasculature (27). Similar studies on rabbit kidney have, to the best of our knowledge, not been performed. However, this methodology fails to distinguish between receptors present on nerve endings versus tubular cells. Our findings that combine functional and radioligand binding techniques provide strong evidence that proximal tubular cells from rabbit (and we would suspect other species as well) possess $alpha_2$ adrenergic receptors. The overall contribution of these receptors to the total population of $alpha_2$ adrenergic receptors in the kidney will require further studies.

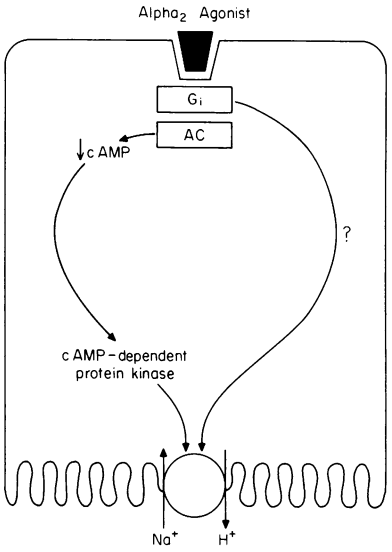

Figure 11. Model for the proposed mechanisms whereby $alpha_2$ adrenergic agonists might stimulate Na^+-H^+ antiport activity. Subsequent to binding of agonist to the receptor, the guanine nucleotide inhibitory component (G_i) of adenylate cyclase might directly activate antiport activity by a yet undescribed mechanism. Alternately G_i depresses the activity of the catalytic subunit of adenylate cyclase (AC) resulting in decreased phosphorylation of cAMP-dependent protein kinase and increased antiport activity.

The molecular mechanisms by which $alpha_2$ adrenergic receptors regulate the Na^+-H^+ antiporter in proximal tubule cells has not been defined. At least two possibilities exist (Fig. 11). Work by Limbird and coworkers has suggested that agonist binding to $alpha_2$ adrenergic receptors, at least in human platelets, may enhance Na^+-H^+ antiport activity either via a direct action of the receptor or guanine nucleotide inhibitory protein (G_i) (28). An alternate explanation would be that $alpha_2$ adrenergic receptor–mediated activation of G_i leads to an inhibition of adenylate cyclase activity, and the resultant decrease in cyclic AMP-dependent protein kinase phosphorylation leads to an enhancement of antiport activity. In this regard, previous evidence indicates that $alpha_2$ adrenergic receptor activation can decrease PTH-stimulated adenylate cyclase activity (29), and that stimulation of cyclic AMP in rabbit proximal tubule can exhibit Na^+-H^+ exchange (30). Since the rabbit proximal tubular cell responds to both parathyroid hormone (7) and $alpha_2$ adrenergic agonists, these cells should provide a useful mode to define further the mechanism whereby several types of hormones modulate Na^+-H^+ antiport activity.

Acknowledgments

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