Evidence for an interaction between adducin and Na\(^+\)-K\(^+\)-ATPase: relation to genetic hypertension

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1Praxis Research Institute Sigma-Tau, 20019 Settimo Milanese; 2Chair of Nephrology, Division of Nephrology and Hypertension, University of Milan and San Raffaele Hospital, 20132 Milan, Italy; and 3Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel 76100

Ferrandi, Mara, Sergio Salardi, Grazia Tripodi, Paolo Barassi, Rodolfo Rivera, Paolo Manunta, Rivka Goldsheger, Patrizia Ferrari, Giuseppe Bianchi, and Steven J. D. Karlish. Evidence for an interaction between adducin and Na\(^+\)-K\(^+\)-ATPase: relation to genetic hypertension. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1338–H1349, 1999.—Adducin point mutations are associated with genetic hypertension in Milan hypertensive strain (MHS) rats and in humans. In transfected cells, adducin affects actin cytoskeleton organization and increases the Na\(^+\)-K\(^+\)-pump rate. The present study has investigated whether rat and human adducin polymorphisms differently modulate rat renal Na\(^+\)-K\(^+\)-ATPase in vitro. We report the following. 1) Both rat and human adducins stimulate Na\(^+\)-K\(^+\)-ATPase activity, with apparent affinity in tens of nanomolar concentrations. 2) MHS and Milan normotensive strain (MNS) adducins raise the apparent ATP affinity for Na\(^+\)-K\(^+\)-ATPase. 3) The mechanism of action of adducin appears to involve a selective acceleration of the rate of the conformational change E\(_2\) (K) \(\rightarrow\) E\(_1\) (Na) or E\(_2\) (K) \(\rightarrow\) E\(_1\) Na·A\(_{\text {TP}}\). 4) Apparent affinities for mutant rat and human adducins are significantly higher than those for wild types. 5) Recombinant human \(\alpha\) and \(\beta\)-adducins stimulate Na\(^+\)-K\(^+\)-ATPase activity, as do the COOH-terminal tails, and the mutant proteins display higher affinities than the wild types. 6) The cytoskeletal protein ankyrin, which is known to bind to Na\(^+\)-K\(^+\)-ATPase, also stimulates enzyme activity, whereas BSA is without effect; the effects of adducin and ankyrin when acting together are not additive. 7) Pig kidney medulla microsomes appear to contain endogenous adducin; in contrast with purified pig kidney Na\(^+\)-K\(^+\)-ATPase, which does not contain adducin, added adducin stimulates the Na\(^+\)-K\(^+\)-ATPase activity of microsomes only about one-half as much as that of purified Na\(^+\)-K\(^+\)-ATPase. Our findings strongly imply the existence of a direct and specific interaction between adducin and Na\(^+\)-K\(^+\)-ATPase in vitro and also suggest the possibility of such an interaction in intact renal membranes.

cytoskeleton; blood pressure; genetics

INTERACTIONS BETWEEN cytoskeletal and integral membrane proteins are fundamental for the maintenance of polarity in epithelial or neuronal cells (25, 39) and the regulation of ion transport (4, 10). The actin-based cytoskeleton has been demonstrated to interact with ion transport proteins such as the band 3-anion exchanger (17), epithelial Na\(^+\) channels (4), the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter (49), and Na\(^+\)-K\(^+\)-ATPase (41, 42). In polarized renal tubular cells, the confinement of Na\(^+\)-K\(^+\)-ATPase to the basolateral surface involves the direct anchorage of the enzyme to ankyrin (28, 43, 50) and, thence, to fodrin (41). The mechanisms of actin polymerization (21) and the formation of the spectrin/fodrin-actin network (2) are regulated by a variety of proteins, including adducin (34). Adducin is a heterodimeric cytoskeletal protein composed of related but nonidentical subunits (\(\alpha\), \(\beta\), or \(\gamma\)). Adducin is involved in signal transduction mechanisms through the modulation of the actin cytoskeleton at cell-cell contact sites (2, 34). Much experimental evidence indicates that adducin may be a candidate protein to explain genetic alterations in ion transport associated with primary or essential hypertension (8). In rats of the Milan hypertensive strain (MHS), a primary increase of renal tubular Na\(^+\) reabsorption (1) is involved in the development of hypertension. In particular, the MHS rat shows an increased activity and expression of Na\(^+\)-K\(^+\)-pump units per cell compared with their Milan normotensive strain (MNS) controls (18). In MHS and MNS rats, two missense point mutations in the \(\alpha\)-subunit (F316Y) and \(\beta\)-subunits (Q529R) of adducin have been demonstrated to be genetically associated with hypertension (8). Moreover, rat renal cells (NRK-52E) transfected with MHS adducin cDNA show a significant increase of Na\(^+\)-K\(^+\)-pump activity and a higher immunohistochemical reactivity for the Na\(^+\)-K\(^+\) pump compared with cells transfected with MNS adducin (47). Finally, adducin polymorphisms in the human \(\alpha\)-subunit (G460W, S586C) have been found to be genetically associated (12, 13, 38) and linked (13) with essential hypertension in humans and to affect the relationship between renal Na\(^+\) excretion and blood pressure (13, 27). Despite the different mutations in hypertensive rat and human adducins, the evidence so far suggests that these mutations produce similar alterations in renal Na\(^+\) handling of hypertensive rats and humans (1, 38). To our knowledge, this is the first demonstration showing that a spontaneous polymorphism in the \(\alpha\)-adducin gene affects blood pressure in two species (rat and human) that diverged ~40 million years ago.

The best characterized functions of adducin are to promote spectrin-actin association and to bind actin and bundle actin filaments (3). The \(\alpha\)-/\(\beta\)-dimer of adducin is arranged so that pairs of head domains form a globular core that caps the end of actin filaments, whereas tails of the \(\alpha\)- and \(\beta\)-subunits participate in the lateral contacts between several actin filaments and...
the β-subunit of spectrin, recruiting additional spectrin units to the actin filaments (26, 35). The increase in Na\(^+-\)K\(^+-\)pump activity at maximal stimulation (V\(_{\text{max}}\)) and the promotion of actin bundling on transfection of cells with adducin may therefore lead to a reasonable assumption that an increase in Na\(^+-\)K\(^+-\)pump density is the result of a change in cellular turnover due to adducin’s property of organizing the cytoskeleton. A direct cytoskeleton-Na\(^+-\)adducin (residues 530–726) were provided by Dr. V. Bennett and Y. Matsuoka (40). The purity of the synthetic peptide was 95% as determined by C18 reversed-phase column HPLC. The concentration of the synthetic peptide was determined by the amino acid composition analysis that confirmed the sequence (40).

Purification and assay of Na\(^+-\)-ATPase. Membranes were prepared from the renal outer medulla of pigs or 3-mo-old MHS rats (29). Na\(^+-\)-ATPase activity was assessed by the release of \(^{32}\)P from \(^{32}\)P-labeled ATP ([\(^{32}\)P]ATP) as described previously (18, 29). Whenever the reaction medium is unspecified, it consisted of 100 mM NaCl, 3 mM MgCl\(_2\), 5 mM KCl, 50 mM HEPES-Tris, 1 mM EGTA, 3 mM Tris-ATP, and 20 mM MgCl\(_2\). The ATPase activities were determined in the absence of added protein. The Na\(^+-\)-ATPase activity was determined by the release of bound phosphate, which was measured by the measurement of the absorbance of the bound phosphate at 412 nm. The Na\(^+-\)-ATPase activity was inhibited 99% by 5 mM ouabain. For the study of effects of adducin, the ionic strength of the medium was maintained constant by the addition of choline chloride. For the study of effects of adducin, the ionic strength of the medium was maintained constant by the addition of choline chloride. For the study of effects of adducin, the ionic strength of the medium was maintained constant by the addition of choline chloride.

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The expressed COOH-terminal domains were obtained after lysis of the bacteria and centrifugation for 20 min at 2,700 g. The supernatant was heated for 20 min at 70°C and centrifuged for 1 h at 30,000 g. The supernatant was diluted with buffer A containing NaCl, 1 mM dithiothreitol, and 100 mg/ml Pefabloc (lysis buffer). The full-length protein was purified from inclusion bodies, which were dissolved in buffer A (10 mM Na-phosphate, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.05% Tween 20, and 40 mg/ml Pefabloc) containing 7 M urea and centrifuged for 1 h at 30,000 g. The supernatant was dialyzed versus buffer A containing 4 M urea and was loaded on a 6-ml Resource-Q column that was eluted with a NaBr gradient (0–300 mM) in buffer A containing 4 M urea.

The expressed COOH-terminal domains were obtained after lysis of the bacteria and centrifugation for 20 min at 2,700 g. The supernatant was heated for 20 min at 70°C and centrifuged for 1 h at 30,000 g. The supernatant was diluted with buffer A and purified on SP-Sepharose and a Resource-Q column as described above. The expressed recombinant protein accounted for roughly 25% of the total protein. The yield was ~15–20 milligrams of protein per liter of culture. Protein contents of the expressed constructs were estimated from SDS-PAGE by comparing the intensities of their Coomassie blue-stained bands with known amounts of BSA. Reflectance densitometry was carried out with a Bio-Rad model 620 densitometer.

A purified 31-aminoc acid synthetic peptide, corresponding to the COOH-terminal residues 696–726 of human β-adducin tail (GSPSPPSKKKFKRTPSFLKKSKKKEvkEs), was a kind gift of Dr. V. Bennett and Y. Matsuoka (40). The purity of the synthetic peptide was ~95% as determined by C18 reversed-phase column HPLC. The concentration of the synthetic peptide was determined by the amino acid composition analysis that confirmed the sequence (40).

Purification and assay of Na\(^+-\)-ATPase. Microsomal membranes and purified Na\(^+-\)-ATPase were prepared from the renal outer medulla of pigs or 3-mo-old MHS rats (29). Na\(^+-\)-ATPase activity was assessed by the release of \(^{32}\)P from \(^{32}\)P-labeled ATP ([\(^{32}\)P]ATP) as described previously (18, 29). Whenever the reaction medium is unspecified, it consisted of 100 mM NaCl, 3 mM MgCl\(_2\), 5 mM KCl, 50 mM HEPES-Tris, 1 mM EGTA, 3 mM Tris-ATP, and 20 mM MgCl\(_2\). The tracer activity was inhibited 99% by 5 mM ouabain. For the study of kinetic effects of adducin, concentrations of Na\(^+-\), K\(^+-\), and ATP were varied at saturating concentrations of the other pump ligands. Where necessary, the ionic strength of the medium was maintained constant by the addition of choline chloride.
10–25 ng of purified Na\(^+-\)K\(^+-\)ATPase in 100 µl of standard assay medium. With the assumption of a relative molecular mass (Mr) for adducin of 160 kDa (α- plus β-subunits), the final concentration was in the range of 3–300 nM.

Gel electrophoresis, blotting to polyvinylidene fluoride, and immunoblotting. Procedures for running 10% tricine (N-tris[hydroxymethyl]methylglycine) SDS-PAGE, electroblotting to polyvinylidene difluoride paper, and immunoblotting have been described in detail previously (11, 24). Immunoblots were developed by enhanced chemiluminescence (ECL; 3–5 µg protein/lane) with the use of anti-rabbit IgG-horseradish peroxidase conjugate and the protocol supplied with ECL reagents from the 1998 Amersham-Pharmacia Life Science Products catalog. For the quantification of bands, the X-ray films were scanned with a Bio-Rad GS-690 imaging densitometer and analyzed with Bio-Rad Multi-Analyst software (version 1.01). For immunoblots, we used 1) an affinity-purified polyclonal antibody referred to as “anti-KETYY,” which recognizes the five COOH-terminal residues of the α-subunit of Na\(^+-\)K\(^+-\)ATPase (1:200 dilution), and 2) a monoclonal antibody raised against the human adducin α-subunit at Prassis laboratories. In brief, mice were immunized with bacterial tail constructs in Freund’s adjuvant (4 boosts every 15 days, 100 µg/boost). The spleen was fused with mice myeloma cells, and hybridomas were grown in a selective medium and screened for a positive reaction with antigen on ELISA plates. Positive clones were subcloned twice.

Kinetic analysis. Kinetic parameters were calculated by nonlinear regression (Enzfitter, Elsevier-Biosoft, Cambridge, UK) and expressed as means ± SE. Statistical comparisons were performed by ANOVA. P < 0.05 was regarded as significant.

RESULTS

Purity of rat and human erythrocyte adducins and Na\(^+-\)K\(^+-\)ATPase. Rat adducin preparations consisted of three bands (Fig. 1, lanes 2–5). The major 105-kDa band contained both α- and β-subunits, which comigrate in this species, and accounted for ~60% of the total. The 70- and 60-kDa bands always copurified with the 105–kDa band and are either proteolytic fragments produced within the erythrocyte or alternatively spliced forms. In preparations of human erythrocyte adducin, α- and β-subunits ran separately, and the 70- and 60-kDa bands were also found, although in much smaller amounts than for rat adducin (Fig. 1, lane 6), as previously shown (22). Several preparations of MHS, MNS, and human adducin were made. Only those batches of adducin showing an electrophoretic pattern like that in Fig. 1 were used for the experiments. The purified MHS rat Na\(^+-\)K\(^+-\)ATPase used for most experiments in this study showed essentially only the two bands of the α- and β-subunits with apparent Mr values of 95 and 55 kDa, respectively, and minor impurities (Fig. 1, lane 1). The specific activity of this preparation was particularly high, 30–35 µmol P\(_i\)·min\(^{-1}\)·mg protein\(^{-1}\), with an estimated sevenfold enrichment compared with the microsomal fraction with a specific

Fig. 1. Coomassie blue-stained SDS-PAGE gel of purified rat Na\(^+-\)K\(^+-\)ATPase and rat and human erythrocyte adducin. Lane 1: Na\(^+-\)K\(^+-\)ATPase purified from Milan hypertensive strain (MHS) renal medulla. Lanes 2 and 3: Milan normal strain (MNS) adducin. Lanes 4 and 5: MHS adducin. Lane 6: human adducin. Values at left are relative molecular mass in kDa.

Materials. All chemicals were reagent grade from Sigma. [\(^{32}\)P]ATP (0.5–3 Ci/mmol) was purchased from Amersham.
activity of 3.5–4.5 µmol P$_i$·min$^{-1}$·mg protein$^{-1}$ (see Ref. 29).

Effects of adducin on Na$^{+}$-K$^{+}$-ATPase activity. Figure 2 shows that both MHS and MNS adducin significantly stimulated MHS rat Na$^{+}$-K$^{+}$-ATPase activity in a medium containing 0.1 mM ATP, 100 mM NaCl, 5 mM KCl, and 3 mM MgCl$_2$. The extent of stimulation was 78.7 ± 3.1% (n = 11) for MHS adducin and 83.4 ± 1.7% (n = 11) for MNS adducin (see also Table 1). Stimulation by adducin was fitted to hyperbolic curves with an apparent affinity (K$_{0.5}$) of 14.2 ± 1.7 mM (n = 11) for MHS and 60.9 ± 11.7 nM (n = 11) for MNS (see Table 1). The stimulation of Na$^{+}$-K$^{+}$-ATPase activity by adducin was 100% inhibited by ouabain, and no hydrolysis of [$^{32}$P]ATP by purified adducin was detected (data not shown). The effect of adducin was lost if it was subjected to extensive proteolysis by proteinase K (56°C for 24 h), followed by heating at 95°C for 1 h. Stimulation of Na$^{+}$-K$^{+}$-ATPase by adducin was largely prevented by preincubation of the adducin (75 nM) with an anti-adducin antibody (1:100 dilution) (Table 2). This confirmed that minor protein contaminants in the adducin preparation were responsible for the stimulation of Na$^{+}$-K$^{+}$-ATPase activity.

The striking difference in K$_{0.5}$ between MHS and MNS adducins for stimulating Na$^{+}$-K$^{+}$-ATPase activity is confirmed by the data in Table 1. Table 1 also shows that the K$_{0.5}$ for both MHS and MNS adducin decreased significantly as ATP concentration was raised from 1 µM through 0.1 mM to 3 mM. Evidently, the percentage of stimulation of Na$^{+}$-K$^{+}$-ATPase activity also increased greatly when the ATP concentration was lowered from 3 mM to 0.1 mM and 1 µM (Table 1). Probably raising the ATP concentration caused adducin to bind more tightly to the enzyme, one could predict that the binding of adducin should also raise the apparent binding affinity of ATP. The data in Fig. 3 are in accord with this prediction. The experiment measured the effect on Na$^{+}$-K$^{+}$-ATPase of either MHS or MNS adducin at a near-saturating concentration of 100 nM over a wide range of ATP concentrations. It is clear that both MHS and MNS adducin induced a substantial increase in the apparent affinity for ATP compared with the control without added adducin [control: 424 ± 27 µM (n = 3); MHS adducin, 209 ± 12 µM (n = 3); MNS adducin, 233 ± 10 µM (n = 3)]. The degree of stimulation at the saturating concentration of 3 mM ATP was ~15–20% in the present series of experiments (Fig. 3). With other preparations of MHS and MNS adducin, a somewhat higher degree of stimulation (30%) was observed (Table 1).

<table>
<thead>
<tr>
<th>ATP Concentration</th>
<th>Na$^{+}$-K$^{+}$-ATPase Stimulation, %</th>
<th>K$_{0.5}$ for Adducin, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mM</td>
<td>30 ± 2.7 (n = 6)</td>
<td>47.9 ± 13.3*</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>78.7 ± 3.1 (n = 11)</td>
<td>83.4 ± 1.7</td>
</tr>
<tr>
<td>1 µM</td>
<td>130 ± 5.2 (n = 4)</td>
<td>137.4 ± 15.9</td>
</tr>
</tbody>
</table>

Increasing concentrations of Milan hypertensive strain (MHS) and Milan normotensive strain (MNS) rat adducin were incubated with 25 ng of rat Na$^{+}$-K$^{+}$-ATPase in presence of 100 mM NaCl, 5 mM KCl, 3 mM MgCl$_2$, and different concentrations of ATP (3 mM, 0.1 mM, and 1 µM). The kinetic parameters of apparent affinity (K$_{0.5}$) for Na$^{+}$-K$^{+}$-ATPase and %Na$^{+}$-K$^{+}$-ATPase stimulation were calculated from nonlinear regression fits to hyperbolic curves. Data represent means ± SE of n different experiments, each run in duplicate. Statistical analysis was performed by ANOVA. *P < 0.05, †P < 0.01, MHS vs. MNS.

Table 2. Anti-adducin antibody counteracts the stimulation of Na$^{+}$-K$^{+}$-ATPase by adducin

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Na$^{+}$-K$^{+}$-ATPase Activity, µmol·min$^{-1}$·mg protein$^{-1}$</th>
<th>+ Adducin/ − Adducin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.9</td>
<td>9.46</td>
</tr>
<tr>
<td>Adducin (75 nM)</td>
<td>4.4</td>
<td>1.25</td>
</tr>
<tr>
<td>Antibody (1:100)</td>
<td>5.5</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Anti-adducin antibody was dialyzed versus 10 mM Tris-HCl, pH 7.4, and dialyzed 1:100 with 0.3% BSA and 10 mM Tris-HCl, pH 7.4. MHS adducin was incubated with anti-adducin antibody for 1 h at 4°C. Stimulation of 10 ng of rat Na$^{+}$-K$^{+}$-ATPase by 75 nM adducin was measured in presence of 100 mM NaCl, 3 mM MgCl$_2$, 5 mM KCl, and 100 µM ATP. The ratio of stimulation in presence of adducin to that in absence of adducin (+ Adducin/− Adducin) was calculated over a respective control sample run in absence or presence of anti-adducin antibody.
We also looked at the effects of MHS and MNS adducin, at a fixed concentration of 100 nM, on the activation of Na\(^+\)-K\(^+\)-ATPase by Na\(^+\) and K\(^+\) (Table 3). The apparent K\(_{0.5}\) value for Na\(^+\) was not changed by MHS or MNS adducin, but a slight increase in the Hill number was observed with either adducin. K\(_{0.5}\) values for K\(^+\) were increased moderately but significantly by both MHS and MNS adducins, with no change in the Hill number.

Tests of the mechanism of stimulation of Na\(^+\)-K\(^+\)-ATPase activity. A large stimulation of Na\(^+\)-K\(^+\)-ATPase activity by adducin at 1 µM ATP implies that the adducin accelerates the rate of the conformational change E\(_2\)(K) \(\rightarrow\) E\(_1\)Na, which is the rate-limiting step of the Na\(^+\)-K\(^+\)-ATPase cycle in this condition (44). This inference can be tested independently by looking at the effect of K\(^+\) on Na\(^+\)-ATPase activity at 1 µM ATP (14, 48). Normally, the addition of K\(^+\) to a reaction medium containing Na\(^+\), Mg\(^2+\), and 1 µM ATP inhibits the rate of ATP hydrolysis because the rate-limiting step E\(_2\) \(\rightarrow\) E\(_1\) for Na\(^+\)-K\(^+\)-ATPase activity is slower than that for Na\(^+\)-ATPase activity in the absence of K\(^+\) (E\(_2\) \(\rightarrow\) E\(_2\)). Thus, in Fig. 4, we compared the effects of K\(^+\) on ATP hydrolysis at 1 µM ATP in the absence or presence of adducin. In the absence of adducin, K\(^+\) inhibited the ATP hydrolysis as expected. In contrast, in the presence of adducin, the addition of K\(^+\) produced a small stimulation first, followed by a reduction of the rate but, overall, no inhibition of the ATPase activity. In the absence of K\(^+\) adducin had little or no effect on the rate of Na\(^+\)-ATPase activity.

Another test of the mechanism of stimulation of adducin utilized prior knowledge that the rate-limiting step of the cycle changes at different pH values. The catalytic cycle is limited partly by E\(_2\)(K) \(\rightarrow\) E\(_1\)Na at pH 6, more so by E\(_1\)-P \(\rightarrow\) E\(_1\)-P at neutral pH, and by the rate of phosphorylation E\(_1\) \(\rightarrow\) E\(_1\)-P at pH > 8 (20). Data in Table 4 show that the degree of stimulation by adducin is greatest at pH 6, somewhat less at pH 7, and altogether nonexistent at pH 9.

The clear-cut conclusion from both of these tests is that adducin accelerated the rate of E\(_2\)(K) \(\rightarrow\) E\(_1\)Na at the low ATP concentration (see Discussion).

Effects of ankyrin and BSA on Na\(^+\)-K\(^+\)-ATPase activity. An indication of the specificity of the adducin-Na\(^+\)-K\(^+\)-ATPase interaction was obtained by comparing the effects with those of another cytoskeletal protein, ankyrin, which is known to bind to the Na\(^+\)-K\(^+\)-ATPase (28, 41–43, 50), and with BSA, which should not bind.

Table 3. Effect of MHS and MNS adducin on the K\(_{0.5}\) and Hill number for Na and K ions of Na\(^+\)-K\(^+\)-ATPase

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>MHS Adducin</th>
<th>MNS Adducin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>K(_{0.5}) mM</td>
<td>11.3 ± 0.38</td>
<td>11.3 ± 0.37</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>1.42 ± 0.06</td>
<td>1.9 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>K(^+)</td>
<td>K(_{0.5}) mM</td>
<td>0.80 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>1.12 ± 0.14</td>
<td>1.23 ± 0.06</td>
</tr>
</tbody>
</table>

Experiments were performed in duplicate in absence (control curves) and presence of 100 nM MHS and MNS adducin with 25 ng of rat Na\(^+\)-K\(^+\)-ATPase. Dose-dependent activation curves as a function of Na\(^+\) and K\(^+\) were measured in presence of increasing concentrations of each ion, while other ligands were maintained at saturating concentrations. The kinetic parameters K\(_{0.5}\) and Hill number (n\(_H\)) were calculated from nonlinear regression fits to Hill equation. Data represent means ± SE of different experiments. Statistical comparisons were performed by ANOVA. *P < 0.05, †P < 0.01, MHS or MNS vs. control.
ADDUCIN MODULATES NA\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was measured at 0.1 mM ATP, with Na\textsuperscript{+}, K\textsuperscript{+} and Mg\textsuperscript{2+} at saturating concentrations and increasing concentrations of adducin, ankyrin, or BSA (Fig. 5). As expected, adducin induced a substantial stimulation of activity ($K_{0.5} = 9.5$ nM, 73% Na\textsuperscript{+}-K\textsuperscript{+}-ATPase stimulation). Purified ankyrin also increased the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, although to a lower degree and with lower affinity than adducin ($K_{0.5} = 110$ nM, 47% Na\textsuperscript{+}-K\textsuperscript{+}-ATPase stimulation). BSA did not affect the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity up to 1 µM. The experiment shown in Fig. 6 looked at the combined effects of adducin and ankyrin on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase using both adducin and ankyrin at saturating or near-saturating concentrations. Evidently the stimulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity by adducin and ankyrin when acting together is much lower than could be expected for the additive effects of the two proteins acting independently (see DISCUSSION).

Effects of human recombinant adducin on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. Further evidence of the specificity of the adducin-Na\textsuperscript{+}-K\textsuperscript{+}-ATPase interaction was obtained by comparing the effects on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase of full-length wild-type or mutant human adducins or truncated forms purified from extracts of E. coli (Fig. 7). Erythrocyte adducin is formed by α- and β-subunits, each composed of three distinct domains (40): a 39-kDa NH\textsubscript{2}-terminal globular protease-resistant head domain connected by a 9-kDa neck domain to a COOH-terminal protease-sensitive tail domain containing the calmodulin binding site and the regions involved in the adducin-spectrin-actin binding (16, 26, 30, 31, 35, 40). The constructs used in this study code for recombinant human full-length α-adducin containing the wild-type G460/S586 and mutant W460/C586 substitutions, wild-type and mutant tail fragments of the α-subunit (residues 430–737 and 530–737), or wild-type full-length β-subunit and tail fragments (residues 530–726 and 696–726). The human α- and β-adducins, added alone or as mixtures, and tail fragments stimulated the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, assayed at 0.1 mM ATP and saturating concentrations of Na\textsuperscript{+}, K\textsuperscript{+} and Mg\textsuperscript{2+} in a dose-dependent fashion (concentration range: 3–1,000 nM) (Fig. 7). The degree of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase stimulation of wild-type and mutant full-length α-subunit or α- plus β-subunit mixtures was ~75%. The $K_{0.5}$ values for the activation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase show that mutant W460/C586 full-length α-subunit or the W460/C586 α- plus β-subunit mixture had a significantly higher affinity than the wild-type full-length G460/S586 α-subunit or wild-type G460/S586 α- plus β-subunit mixture. Both wild-type and mutant tail fragments (residues 430–737) of the α-subunit retained the ability to stimulate the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, although to a lower extent (60%) than the full-length proteins (75%). The apparent affinities of wild-type and mutant α-subunit tail fragments were much lower than those of the full-length α-adducins or α- plus β-subunit mixtures (25 ± 3.9 vs. 408 ± 67 nM and 9.8 ± 1.3 vs. 157 ± 7 nM for full-length α-subunit compared with tail fragments, respectively).

Table 4. Effect of pH on stimulatory effect of adducin

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Activity, µmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.5</td>
<td>1.5</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>3.5</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>2.33</td>
</tr>
</tbody>
</table>

Stimulatory effect of 80 nM MHS adducin on 10 ng of rat Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was measured in presence of 100 mM NaCl, 3 mM MgCl\textsubscript{2}, 5 mM KCl, and 100 µM ATP at pH 6.5, 7.4, and 9.

![Fig. 5. Effects of adducin, ankyrin, and BSA on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase.](image)

![Fig. 6. Combined effects of adducin and ankyrin on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. MHS adducin (30 nM) and ankyrin (400 nM), alone or in combination, were incubated with 7.5 ng of rat Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in presence of 100 mM NaCl, 3 mM MgCl\textsubscript{2}, 5 mM KCl, and 1 µM ATP. Predicted percentage of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activation was calculated over a control sample run in absence of adducin and ankyrin. Predicted percentage of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activation was calculated by assuming an additive effect between the 2 proteins.

![Fig. 7.](image)
It is of interest that the $K_{0.5}$ values of the $\alpha$-subunit tail fragments (residues 430–737) containing both point mutations were significantly reduced compared with those of wild-type $\alpha$-subunit tail fragments (residues 530–726) were obtained as recombinant proteins. The 31-mer peptide corresponding to COOH-terminal residues 696–726 of human $\beta$-adducin tail was obtained as a synthetic peptide. G460W and S586C, point mutations in $\alpha$-subunit of human adducin (G460 and S586, wild types; W460 and C586, mutant substitutions); N, NH$_2$-terminal globular protease-resistant head domain; C, COOH-terminal polylysine basic domain. Apparent affinities ($K_{0.5}$, in nM) for Na$^+$/K$^+$-ATPase stimulation at 0.1 mM ATP in presence of 100 mM NaCl, 5 mM KCl, and 3 mM MgCl$_2$ are reported using 25 ng of rat enzyme. Statistical comparisons were performed using ANOVA. *P < 0.05, mutant vs. wild-type proteins.

For comparison with the recombinant proteins, $\alpha$,$\beta$-adducin purified from human erythrocytes was also tested. Human $\alpha$-adducin genotypes were established (13), and two subjects with the wild-type G460/S586 form and three subjects with the mutant W460/C586 form of adducin were studied. As was found for the recombinant proteins, the human adducins stimulated Na$^+$/K$^+$-ATPase by 75%, and the mutant erythrocyte adducin showed a higher affinity than the wild-type protein (10.2 ± 1.8 vs. 25.7 ± 2.6 nM, n = 3). These findings provide a strong indication that the properties

**Fig. 7.** Domain organization for $\alpha$- and $\beta$-subunit of human adducin, according to Matsuoka et al. (40). Full-length $\alpha$- and $\beta$-adducin and COOH-terminal tails of human $\alpha$- (residues 430–737), $\alpha$- (residues 530–737), and $\beta$-adducin (residues 530–726) were obtained as recombinant proteins. The 31-mer peptide corresponding to COOH-terminal residues 696–726 of human $\beta$-adducin tail was obtained as a synthetic peptide. G460W and S586C, point mutations in $\alpha$-subunit of human adducin (G460 and S586, wild types; W460 and C586, mutant substitutions); N, NH$_2$-terminal globular protease-resistant head domain; C, COOH-terminal polylysine basic domain. Apparent affinities ($K_{0.5}$, in nM) for Na$^+$/K$^+$-ATPase stimulation at 0.1 mM ATP in presence of 100 mM NaCl, 5 mM KCl, and 3 mM MgCl$_2$ are reported using 25 ng of rat enzyme. Statistical comparisons were performed using ANOVA. *P < 0.05, mutant vs. wild-type proteins.
of the recombinant proteins are similar to those of the native proteins and are not artifactual effects of denatured protein.

An adducin-Na\(^+\)-K\(^+\)-ATPase interaction in intact renal membranes? If adducin and Na\(^+\)-K\(^+\)-ATPase interact in kidney in vivo, one could expect that membranes isolated from renal medulla would contain adducin. With the use of the anti-adducin antibody to screen immunoblots, preliminary experiments showed that there is very little intact adducin in rat kidney medulla microsomes, although there appear to be adducin fragments. In contrast, in pig kidney medulla microsomes, we detected a band that runs in the same position as intact human adducin \(\alpha\)-subunit, as well as bands that could be fragments of adducin. The immunoblot in Fig. 8 attempted to quantify the amount of this band in pig kidney medulla microsomes and the pig kidney enzyme with the use of known amounts of human adducin to calibrate the immunoblot. In 30 \(\mu\)g of microsomal protein, the amount of the band corresponding to intact adducin is comparable to \(\sim 1\ \mu\)g of human adducin \(\alpha\)-subunit, and there is also a substantial amount of smaller bands that recognize the antibody. In contrast, in 40 \(\mu\)g of the pig kidney Na\(^+\)-K\(^+\)-ATPase preparation, anti-adducin detected no proteins. For comparison, the specific activity of microsomes (3.5 \(\mu\)mol Pi \(\cdot\) min\(^{-1}\) \(\cdot\) mg protein\(^{-1}\)) of the purified enzyme, consistent with the fivefold lower \(r\) value of 153 kDa (\(\alpha + \beta + \gamma\)-subunits) as 30–45 \(\mu\)g/mg microsomal protein. This value is similar to that of the band tentatively assigned as intact adducin (\(\sim 30\ \mu\)g/mg microsomes). If indeed intact endogenous adducin is present in the microsomes and a significant fraction of the Na\(^+\)-K\(^+\)-ATPase is bound, one could predict that added adducin would stimulate the Na\(^+\)-K\(^+\)-ATPase less than the purified enzyme, which contains no endogenous adducin. This prediction was tested in the experiment shown in Fig. 9. The percentage of control Na\(^+\)-K\(^+\)-ATPase activity in each case was plotted as a function of the added adducin concentration. The curves represent best fits to hyperbolas with the following parameters: enzyme, \(V_{\text{max}} = 387 \pm 32.5\%\) of control and \(K_{0.5} = 30.5 \pm 6.7\) nM adducin; and microsomes, \(V_{\text{max}} = 149.5 \pm 12.8\%\) of control and \(K_{0.5} = 33.5 \pm 7.4\) nM adducin. Thus added human adducin stimulated the Na\(^+\)-K\(^+\)-ATPase activity of the microsomes only onethird as much as purified pig Na\(^+\)-K\(^+\)-ATPase, whereas the apparent affinity for adducin was the same on enzyme and microsomes. These two findings indicate that, in microsomes, part of the Na\(^+\)-K\(^+\)-ATPase is unavailable for interaction with exogenous adducin, whereas the available fraction of Na\(^+\)-K\(^+\)-ATPase interacts with exogenous adducin in the same way as does the purified enzyme preparation.

**DISCUSSION**

In the present study we have provided evidence for a novel interaction between the cytoskeleton and the Na\(^+\)-K\(^+\)-ATPase in vitro. The experiments raise ques-

![Fig. 8. Detection of adducin and \(\alpha\)-subunit of Na\(^+\)-K\(^+\)-ATPase in purified pig kidney Na\(^+\)-K\(^+\)-ATPase and in pig kidney medulla microsomes. Indicated amounts of human adducin, pig kidney medulla microsomes (Micr), and pig kidney Na\(^+\)-K\(^+\)-ATPase (Enz) were applied to lanes of 10% tricine gel. Gels were blotted to polyvinylidene difluoride paper, which was incubated with anti-adducin or anti-KETYY antibody, and immunoblot was developed as described in MATERIALS AND METHODS.](http://ajpheart.physiology.org/)
Mechanism of stimulation of \( \text{Na}^+\text{K}^+\)-ATPase by adducin. The key to an understanding of the mechanism of stimulation of \( \text{Na}^+\text{K}^+\)-ATPase by adducin is the finding that the degree of stimulation increased greatly when ATP concentration was reduced from 3 mM to 0.1 mM and then to 1 \( \mu \text{M} \) (Table 1, Fig. 3). At the lowest ATP concentration (1 \( \mu \text{M} \)), the rate of ATP hydrolysis is limited almost exclusively by a slow rate of the conformational transition \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \), in which occluded \( \text{K}^+ \) is deoccluded and released to the cytoplasmic surface, where \( \text{Na}^+ \) binds. ATP binds with low affinity to \( \text{E}_3\text{(K)} \) and greatly accelerates the rate of the conformational transition from \( \text{E}_3\text{(K)} \cdot \text{ATP} \) to \( \text{E}_1\text{Na} \cdot \text{ATP} \), the form to which ATP is bound with a high affinity and phosphorylates the enzyme. In an ATP hydrolysis experiment, the apparent affinity for ATP reflects its binding affinity to \( \text{E}_3\text{(K)} \) and the relative proportion of the \( \text{E}_2\text{(K)} \) form among the other states of the enzyme (\( \text{E}_1 \), \( \text{E}_1\text{-P} \), \( \text{E}_2\text{-P} \)), which itself is determined by the rate constants of the transitions between the different states. With these kinetic considerations in mind, it is clear that the major effect of both MHS and MNS adducin is to accelerate the conformational transition \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \). The effect on ATP hydrolysis would be to decrease the relative proportion of \( \text{E}_2\text{(K)} \) relative to \( \text{E}_1\text{Na} \) and the other forms (\( \text{E}_1\text{-P} \), \( \text{E}_2\text{-P} \)) and thereby increase the apparent affinity for ATP. At saturating concentrations of ATP, the rate of ATP hydrolysis is only partially limited by the rate of \( \text{E}_2\text{(K)} \cdot \text{ATP} \rightarrow \text{E}_1\text{Na} \cdot \text{ATP} \), thus explaining the much lower degree of stimulation by adducin. A necessary corollary of the proposed action of adducin, to shift the conformational equilibrium between \( \text{E}_3\text{(K)} \cdot \text{ATP} \) and \( \text{E}_1 \) toward the latter form, is that adducin should itself bind more tightly to the \( \text{E}_1\text{-ATP} \) form. This prediction is confirmed by the finding that the apparent affinity for both MHS and MNS adducins is raised when the ATP concentration is raised (Table 1). Independent confirmation that adducin accelerates the rate of the conformational change \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \) at low ATP concentrations (1 \( \mu \text{M} \)) was obtained in the experiment showing no inhibition or even slight stimulation of \( \text{Na}^+\text{K}^+\)-ATPase by \( \text{K}^+ \) in the presence of adducin but showing the expected inhibition by \( \text{K}^+ \) in the absence of adducin. The biphasic effect of \( \text{K}^+ \) observed in Fig. 4 is a reproducible phenomenon. It can be explained by assuming that the binding of only one potassium ion is required to catalyze dephosphorylation of \( \text{E}_2\text{-P} \) and that the rate of the conformational change depends on whether one or two potassium ions are bound in \( \text{E}_2 \). However, this does not affect the conclusion concerning acceleration of the conformational change by adducin. The \( \text{pH} \) dependence of the effect of adducin as shown in Table 4 (strongest at \( \text{pH} 6 \), somewhat less at \( \text{pH} 7 \), and absent at \( \text{pH} 9 \)) is completely in accordance with the stimulation of \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \) or \( \text{E}_2\text{(K)} \cdot \text{ATP} \rightarrow \text{E}_1\text{Na} \cdot \text{ATP} \).

Adducin may stimulate \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \) at low ATP, by either interacting with the ATP binding site and raising the binding affinity of ATP to \( \text{E}_2\text{(K)} \), thus accelerating \( \text{E}_2\text{(K)} \cdot \text{ATP} \rightarrow \text{E}_1\text{Na} \cdot \text{ATP} \), or accelerating the \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \) conformational transition directly, with equivalent results on the kinetics. Stimulation by adducin of \( \text{E}_2\text{(K)} \rightarrow \text{E}_1\text{Na} \) or \( \text{E}_2\text{(K)} \cdot \text{ATP} \rightarrow \text{E}_1\text{Na} \cdot \text{ATP} \) but a lack of effects of adducin on ATP hydrolysis in conditions in which other steps are rate limiting (phosphorylation at \( \text{pH} 9 \) for \( \text{Na}^+\text{K}^+\)-ATPase experiments or \( \text{E}_2\text{P} \rightarrow \text{E}_2 \) for \( \text{Na}^+\text{K}^+\)-ATPase) indicates selectivity of the functional effect. This suggests the presence of a specific structural interaction of adducin with the enzyme.

By assuming that adducin accelerates the rate of \( \text{E}_2\text{(K)} \cdot \text{ATP} \rightarrow \text{E}_1\text{Na} \cdot \text{ATP} \), one could predict secondary effects on the apparent \( \text{K}^+ \) and \( \text{Na}^+ \) affinities for activating ATP hydrolysis due to the changes in the distribution of enzyme forms. The direction of these effects should be to lower apparent \( \text{K}^+ \) affinity and raise apparent \( \text{Na}^+ \) affinity, and the size of such changes should be smaller than the change in apparent ATP affinity. A moderate reduction of apparent \( \text{K}^+ \) affinity was indeed observed (Table 3). No significant effect on apparent \( \text{Na}^+ \) affinity was observed. In view of the lower overall affinity of \( \text{Na}^+ \), the expected change might be too small to be detected.

Specificity of the interaction between adducin and \( \text{Na}^+\text{K}^+\)-ATPase. The results of this study strongly imply that we are dealing with a direct interaction between adducin and \( \text{Na}^+\text{K}^+\)-ATPase. Apart from the inference of a specific structural interaction on the basis of the functional effects, the following features imply that the interaction requires a specific structural domain, or conformation, of adducin. 1) The apparent affinities of MHS and MNS rat and human adducins for stimulating \( \text{Na}^+\text{K}^+\)-ATPase are in the tens of nanomolar concentration range. 2) The apparent affinities are associated with the genetic variants found in both hypertensive (MHS) and normotensive (MNS) rats [\( \alpha \)-F316Y and \( \beta \)-adducin (Q529R)] (8) and in humans (\( \alpha \)-adducins G460W and S586C) (Fig. 7) (12, 13, 38). In particular, the mutant MHS rat adducin stimulates rat renal \( \text{Na}^+\text{K}^+\)-ATPase activity with a higher affinity than does the wild-type MNS rat adducin (Table 1), and a similar phenomenon is observed for wild-type and mutant (G460W, S586C) human adducins (Fig. 7; see also below). 3) The stimulation of \( \text{Na}^+\text{K}^+\)-ATPase is retained by restricted portions of both the \( \alpha \)- and \( \beta \)-adducin COOH-terminal tails, even though it is lower than that of the full-length protein (60 or 30%, according to the length of the fragment, vs. 75%), whereas it is lost when the tail fragment is reduced to 31 amino acids (see Fig. 7 for domain organization). The finding that the affinity of tail fragments is lower than that of full-length adducin (Fig. 7) could indicate that both head/neck and tail regions are required for binding or that removal of the head/neck region affects the structure of the tail region, making it suboptimal for binding. In addition, the mutant tails show a higher
affinity than wild-type tails (Fig. 7). 4) The cytoskeletal protein ankyrin, which is known to bind directly to the Na\(^{+}\)-K\(^{+}\)-ATPase (28, 41, 42, 43, 50), is also able to stimulate the enzyme activity, although to a lower extent (47 vs. 73\%) and with lower affinity than adducin (110 vs. 10 nM), whereas a noncytoskeletal protein (BSA) has no effect (Fig. 5). The finding that the stimulatory effects of adducin and ankyrin are not additive (Fig. 6) implies that these two proteins may interfere with each other on the pump. Ankyrin is known to bind to the \(\alpha\)-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase at two distinct cytoplasmic domains (15). These domains have been identified within residues 140-166 of the minor cytoplasmic loop between transmembrane segments M2 and M3 (50) and also to a motif ALLK in the central cytoplasmic loop between transmembrane segments M4 and M5 (28). Therefore, it is possible that adducin also binds at or near one or both of these cytosplasmic ankyrin binding regions.

Physiological and pathophysiological role of adducin in modulating Na\(^{+}\)-K\(^{+}\)-pump activity. How are the present findings relevant to the functional and pathophysiological roles of adducin polymorphism known to be involved in the mechanisms responsible for genetic hypertension (8, 12, 13, 27, 38, 47)? In genetically hypertensive MHS rats, the development of hypertension is linked to a primary renal defect, “transplantable” with the kidney (6, 7), that consists of an increased tubular Na\(^{+}\) reabsorption (5, 9, 19) associated with higher basolateral Na\(^{+}\)-K\(^{+}\)-pump activity (18). The increase in Na\(^{+}\)-K\(^{+}\)-pump activity in MHS kidney is already present before the development of hypertension, is accounted for by a higher number of functionally active Na\(^{+}\)-K\(^{+}\)-pump sites on the cell membrane surface (18), and is also associated with an increased expression of the \(\alpha\)- and \(\beta\)-subunit mRNA of the Na\(^{+}\)-K\(^{+}\)-pump (18).

In view of the important regulatory role that adducin plays in organizing the actin-spectrin complex and the higher density of Na\(^{+}\)-K\(^{+}\) pumps in MHS compared with MNS kidneys (18) and after transfection of cells with adducin (47), one may assume that, at the cellular level, adducin affects the density of Na\(^{+}\)-K\(^{+}\)-ATPase molecules by altering their retention time on the cell membrane (18). One can envision a mechanism involving indirect modulation of the cytoskeleton, bound to the Na\(^{+}\)-K\(^{+}\) pump via ankyrin (42, 43, 50), or direct binding of adducin to the Na\(^{+}\)-K\(^{+}\) pump. Adducin polymorphisms in rats or humans (8, 12, 13, 47) may differentially affect the rate of membrane cycling.

The findings in Figs. 8 and 9 are compatible with the hypothesis that adducin interacts with the Na\(^{+}\)-K\(^{+}\)-ATPase in intact renal microsomal membranes, although this must be demonstrated independently, for example, in coimmunoprecipitation or cross-linking experiments. Assuming that independent evidence for such an interaction can be demonstrated, stimulation of Na\(^{+}\)-K\(^{+}\)-ATPase activity by adducin in vitro appears to be unnecessary if the primary role of adducin in the physiological context is to anchor the pump to the cytoskeleton. One may note that, at physiological concentrations of ATP (2-3 mM), adducin could have only a minor effect on the Na\(^{+}\)-K\(^{+}\)-ATPase activity. However, it seems paradoxical that a structural interaction should affect ATP binding affinity and stimulate Na\(^{+}\)-K\(^{+}\)-ATPase activity at all. Recent evidence of the structural events accompanying E\(_{1}\)/E\(_{2}\) conformational transitions offers a possible explanation of this paradox. A technique of Fe-catalyzed oxidative cleavage of the Na\(^{+}\)-K\(^{+}\)-ATPase described recently (23) provides information on the spatial organization of the protein. Specifically, we have proposed that the cytoplasmic loops of the Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit, between transmembrane segments M2 and M3 (minor loop) and between M4 and M5 (major loop), interact in the E\(_{2}\)(K) state and move apart in the E\(_{1}\)Na state. A variety of structural modifications, such as proteolytic cleavages or mutations in the interacting sequences, appear to interfere with the loop interactions and thereby stabilize the E\(_{1}\) state. It is easy to imagine that extrinsic proteins that bind to the cytoplasmic loops could also hinder their interactions, thus poising the E\(_{2}\)/E\(_{1}\) equilibrium toward E\(_{1}\) and raising the apparent ATP affinity. This mechanism could apply to both adducin and ankyrin, which is known to recognize both minor and major cytoplasmic loops (Fig. 5) (15, 28, 50). In other words, the in vitro functional effect of adducin or ankyrin on Na\(^{+}\)-K\(^{+}\)-ATPase may be incidental to the physiological role.

The principal significance of the functional effect could be that it indicates the presence of a specific and direct interaction between adducin and the Na\(^{+}\)-K\(^{+}\) pump. In the physiological context this interaction may regulate the cellular cycling of Na\(^{+}\)-K\(^{+}\) pumps and their density. Indeed, the higher affinity for mutant MHS compared with that for wild-type MNS adducin (Table 1) may serve to anchor the cytoskeleton to the Na\(^{+}\)-K\(^{+}\) pump more tightly and reduce the rate of internalization.

The evidence presented here, which suggests the existence of a novel adducin-Na\(^{+}\)-K\(^{+}\)-ATPase interaction, supports previous evidence showing that adducin polymorphisms are involved in genetic alterations of cell Na\(^{+}\) transport and the pathogenesis of primary hypertension in rats and humans. In particular, despite the differences in the mutation positions between rat and human adducins, the “hypertensive” adducin variants of both species affect the Na\(^{+}\)-K\(^{+}\)-ATPase activity similarly. These findings strongly support the notion that rat genetic studies provide relevant information for understanding the genetic and molecular mechanisms of human primary hypertension.

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