Structure/function analysis of Na^+-K^+-ATPase central isoform-specific region: involvement in PKC regulation

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Pierre, Sandrine V., Marie-Josée Duran, Deborah L. Carr, and Thomas A. Pressley. Structure/function analysis of Na^+-K^+-ATPase central isoform-specific region: involvement in PKC regulation. *Am J Physiol Renal Physiol* 283: F1066–F1074, 2002. First published June 4, 2002; 10.1152/ajprenal.00153.2002.—Specific functions served by the various Na^+-K^+-ATPase α-isoforms are likely to originate in regions of structural divergence within their primary structures. The isoforms are nearly identical, with the exception of the NH_2 terminus and a 10-residue region near the center of each molecule (isoform-specific region; ISR). Although the NH_2 terminus has been clearly identified as a source of isoform functional diversity, other regions seem to be involved. We investigated whether the central ISR could also contribute to isoform variability. We constructed chimeric molecules in which the central ISRs of rat α_1- and α_2-isoforms were exchanged. After stable transfection into opossum kidney cells, the chimeras were characterized for two properties known to differ dramatically among the isoforms: their K^+ deocclusion pattern and their response to PKC activation. Comparisons with rat full-length α_1- and α_2-isoforms expressed under the same conditions suggest an involvement of the central ISR in the response to PKC but not in K^+ deocclusion.

α-subunit; rat; α_1- and α_2-isoforms; chimeras; potassium deocclusion

NA+-K+-ATPASE IS CRITICAL for maintaining the ionic gradients across the plasma membranes of animal cells. This enzyme complex extrudes Na^+ from the cell and accumulates K^+, using metabolic energy derived from ATP hydrolysis. The enzyme complex consists of two dissimilar subunits, α and β, that exist in multiple forms. A fundamental question surrounding the pump is the physiological relevance of this subunit diversity. The α-polypeptide is the catalytic subunit and contains the binding sites for ions and substrates. Four distinct isoforms (α_1, α_2, α_3, and α_4) of the Na^+-K^+-ATPase α-subunit have been described so far, with differences in enzyme kinetics and response to second messengers. The primary structures of these isoforms are nearly identical, with the exception of the NH_2 terminus and a 10-residue region near the center of the molecule (19, 24) [isoform-specific region (ISR); K^{489}–L^{499}; Fig. 1]. It

seems likely that the specific functions served by the various Na^+-K^+-ATPase α-isoforms must originate within these regions of structural divergence.

The NH_2-terminal region has been extensively studied. Heterologous expression of NH_2-terminal deletions and chimeric constructs has shown that the mutant α-subunits display changes in kinetics and regulation of ion transport properties. Although the NH_2 terminus of α is not required for Na^+-K^+-ATPase activity (21), a truncated enzyme lacking the first 32 amino acids has distinctive kinetics at micromolar ATP concentrations, conditions in which K^+ deocclusion becomes rate limiting in the overall catalytic cycle. Thus Na^+-ATPase activity of the truncated enzyme is stimulated by low concentrations of K^+, whereas activity of wild-type α_1 is inhibited. Interestingly, the α_2-isoform resembles the truncated enzyme in this respect. However, unexpected characteristics of chimeras resulting from exchanges between α_1 and α_2 NH2-terminal domains suggested that this distinctive kinetic behavior of Na^+-K^+-ATPase α-isoforms is not entirely due to the NH_2-terminal region but rather to its interaction with other ISRs of the α-protein (8).

Another isoform-specific property that clearly involves the NH_2-terminal region is the regulation of Na^+-K^+-ATPase transport activity by PKC. Indeed, we have shown that stimulation of endogenous PKC with phorbol esters increases pump-mediated Rb^+ transport in cultured opossum kidney (OK) cells expressing the exogenous rat Na^+-K^+-ATPase α_1-isoform. This increase was abolished in cells expressing a mutant missing the first 26 amino acids of the rodent α-subunit, consistent with a role for the NH_2-terminal region in PKC regulation. Ser^{16} and Ser^{23}, which are believed to be in vivo targets of PKC phosphorylation, are found within the NH_2-terminal domain of the rat α_1-isoform. We have shown that PKC stimulation of transport is completely abolished in S16A and S23A mutants (9). However, comparisons with other species and experimental systems raise some doubts about the role of these residues. For example, Ser^{16} is well conserved among mammalian α_1-isoforms, but it is only weakly phosphorylated. Ser^{23}, on the other hand, is missing in

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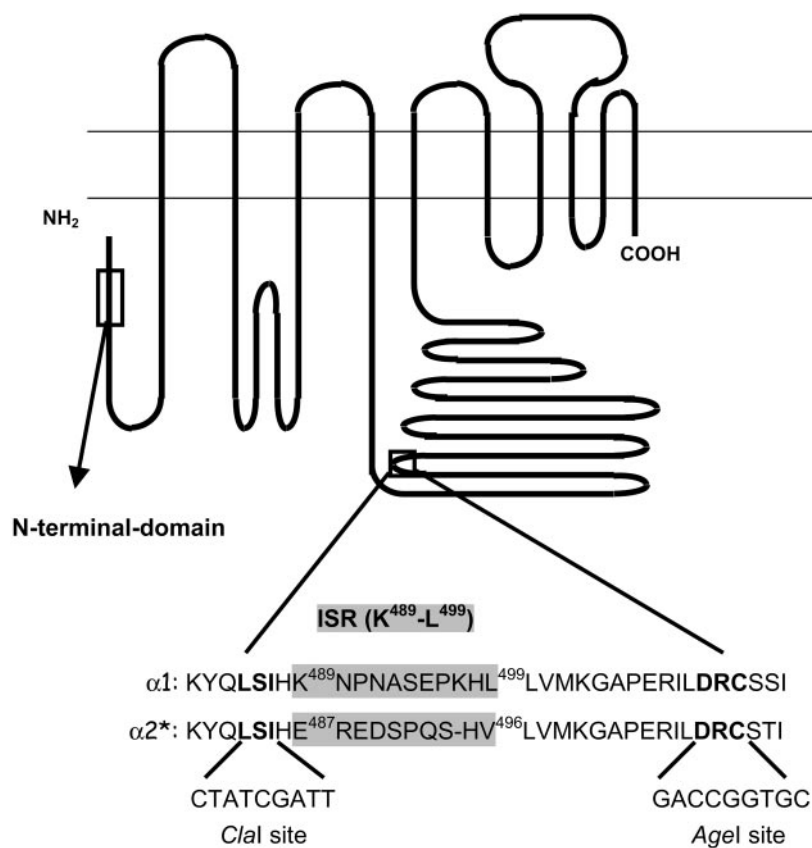
Fig. 1. Structure of the α-subunit. Open rectangle, NH$_2$-terminal domain; small open box, isoform-specific region (ISR) and central ISRs, with aligned amino acid sequences of α_1 and α_2 ISRs appearing as shaded rectangles and the sites of silent mutations in bold.

many species. Neither residue is contained within a PKC consensus sequence, and neither residue is conserved in α_2 or α_3. Nevertheless, there are other serines and threonines in the NH$_2$ termini of these isoforms that may serve as phosphorylation sites. Given the variability of the response and the differences among species and isoforms, it is therefore tempting to speculate that additional regions of the α-subunit are contributing to the effect of PKC.

We hypothesized that the central ISR may be one of these additional regions. To test this hypothesis, chimeric molecules were constructed in which the central ISRs of the rat α_1- and α_2-isoforms were exchanged. After stable transfection into OK cells, the chimeras were characterized for their enzymatic properties. Comparisons of the chimeras with rat full-length α_1- and α_2-isoforms expressed under the same conditions suggest an involvement of the central ISR in the PKC response but not in K$^+$ deocclusion.

METHODS

Preparation of full-length α_1 and ouabain-resistant α_2 sequences. Wild-type α_1 and α_2 cDNAs, a gift from Dr. Jerry B. Lingrel and colleagues (22), were subcloned by our laboratory into pGEM-3Z (Promega, Madison, WI) as described (21). A ouabain-resistant form of the rat α_2-isoform (designated α_2^*) was constructed by site-directed mutagenesis of amino acid residues at the extracellular borders of the first and second transmembrane domains (L111R and N122D) (12). Use of ouabain-resistant α_1 and α_2^* allowed us to employ the ouabain-selection strategy described below.

Preparation of chimeras. The α_1 and α_2^* cDNAs were used to prepare chimeric molecules in which the central isoform-specific domain of α_1 and α_2^* were exchanged. Two silent mutations were introduced in rat α_1 and α_2^* cDNAs by conventional site-directed mutagenesis, creating unique sites for digestion by the restriction enzymes *Cla*I and *Age*I without altering the encoded amino acids. The *Cla*I site was placed within the codons for Leu^{485}, Ser^{486}, and Ile^{487} (LSI), as numbered from the NH$_2$-terminal glycine of the mature polypeptide. The *Age*I site was introduced within the codons for Asp^{511}, Arg^{512}, and Cys^{513} (DRC). This was accomplished with the aid of mutagenic oligonucleotides (Table 1) and their complements, using limited amplification with *Pfu* polymerase, followed by restriction of the original template with *Dpn*I. To perform the exchange of the ISRs, we proceeded in two steps. In the first, the COOH ends of each isoform were swapped, using the introduced *Age*I sites (DRC). The resulting chimeras were then used as starting material for swaps of the NH$_2$ half of the molecule, using the introduced *Cla*I site. The structures of the resulting mutants were then confirmed by restriction analysis and direct sequencing of the altered region.

Expression vectors, gene transfer, and selection. cDNAs encoding α_1, α_2^*, or the chimeras were subcloned into the *Hind*III and *Xba*I sites of the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA), with the aid of a *Sac*I-*Xba*I adaptor. This was prepared by annealing the oligonucleotides CGCGGCCGCT and CTAGAGCGGCCGCGAGCT. The resulting expression vector contained the sequence encoding the α construct downstream of the enhancer-promoter regions of the immediate early gene of human cytomegalovirus, followed by polyadenylation signals from the bovine growth hormone gene.

Table 1. *Characteristics of silent mutation oligonucleotides used for the introduction of ClaI and AgeI sites into rat α₁ and α₂ sequences*

ClaI Target Sequence: AT/CGAT													
α₁	T	N	K	Y	Q	**L485**	**S486**	**I487**	H	K	N	P	N
α₁ WT	5′-ACC	AAC	AAG	TAC	CAG	CTC	TCC	ATT	CAC	AAG	AAC	CCA	AAC-3′
α₁-*ClaI*		3′-G	TTC	ATG	GTC	GA**T**	A**G**C	**TAA**	GTG	TTC	TTG-5′		
α₂	T	N	K	Y	Q	L	S	I	H	E	R	E	D
α₂ WT	5′-ACC	AAC	AAA	TAT	CAG	CTT	TCC	ATC	CAT	GAG	AGG	GAA	GAC-3′
α₂-*ClaI*		3′-G	TTT	ATA	GTC	GA**T**	A**G**C	**TAA**	GTA	CTC	TCC	CT-5′	
AgeI Target Sequence: A/CCGGT													
α₁	P	E	R	I	L	**D511**	**R512**	**C513**	S	S	I	L	L
α₁ WT	5′-CCA	GAA	AGG	ATC	CTG	GAC	CGA	TGC	AGT	TCT	ATC	CTC	CTC-3′
α₁-*AgeI*			5′-C	TAG	GAC	C**T**G	**GCC**	**A**CG	TCA	AGA	TAG-3′		
α₂	P	E	R	I	L	**D**	**R**	**C**	S	T	I	L	V
α₂ WT	5′-CCG	GAG	CGC	ATC	CTG	GAC	CGA	TGC	TCT	ACC	ATC	CTG	GTA-3′
α₂-*AgeI*			3′-CG	TAG	GAC	C**T**G	**GCC**	**A**CG	AGA	TGG	TA-5′		

α₁ WT, wild-type sequence, α₁-*ClaI*, α₁-*ClaI* mutation oligonucleotide sequence, α₂ WT, α₂ wild-type sequence; α₂-*ClaI*, α₂ *ClaI* mutation oligonucleotide sequence. Mutated bases are in bold, and introduced restriction enzyme sites are underlined.

Heterologous expression of the α constructs was achieved by transfection of OK cells (no. CRL-1840, American Type Culture Collection) and subsequent selection of recipients with ouabain. OK cells were routinely maintained at 37°C and 10% CO_2 in DMEM with 10% calf serum. The expression vectors containing rat isoforms or chimeras were introduced into subconfluent cells using the cationic liposome preparation Lipofectin (GIBCO BRL, Grand Island, NY) as described (11). Transfected OK cells expressing the introduced ouabain-resistant Na+-K+-ATPase constructs were selected for their ability to grow in 3 μM ouabain, a concentration sufficient to kill control (i.e., untransfected) OK cells.

Membrane preparations. Crude plasma membranes were isolated from control and transfected OK cells. Confluent monolayers from twelve 100-mm dishes were washed twice with phosphate-buffered saline, and cells were then harvested by scraping with a rubber policeman. Crude membranes were isolated from the resulting cell suspension after hypotonic lysis and differential centrifugation, followed by treatment with sodium iodide as described (21).

Gel electrophoresis and immunoblotting. Expression of introduced α-subunits in transfected OK cells was confirmed by electrophoresis and subsequent immunoblotting of proteins from the isolated membranes. Electrophoresis of samples through SDS-polyacrylamide gels (7.5%), electroblotting, and probing of the blots with appropriate antibodies were performed as described (19). The presence of transferred proteins on the blots and equality of loading among the lanes were confirmed by staining with Ponceau S. For detection and characterization of rat isoform and chimera expression in OK cells, two site-directed rabbit polyclonal antibodies were used. Anti-HERED recognizes the rat α₂ ISR (residues 494–506). Anti-NASE recognizes the rat α₁ ISR (residues 494–505). However, anti-NASE also recognizes the opossum α₁ sequence (19); therefore, it could not be used for confirmation in constructs in which its target sequence was expected.

RNA isolation, reverse transcription, and DNA amplification. In instances where the cross-reactivity of anti-NASE precluded specific detection of the α₁ IRS, we confirmed the structure of the expressed mRNA. Isolation of total RNA from confluent monolayers of cells in 3.5-cm culture dishes was accomplish using a commercial preparation, Ultraspec (Biotecx Laboratories, Houston, TX), a modification of the guanidium thiocyanate-phenol-chloroform protocol of Chomczynski and Sacchi (6). The recovered RNA was dissolved in diethylpyrocarbonate-treated water, and only preparations with an A_{260}/A_{280} amplification ratio >1.5 were analyzed further.

Synthesis of cDNA was achieved by reverse transcription. The RNA mixture was heated for 10 min at 65°C. Reverse transcription was then performed in a final volume of 20 μl containing 50 mM Tris·HCl (pH 8.4), 75 mM KCl, 3 mM MgCl₂, 500 μM of each deoxynucleotides, 10 mM dithiothreitol, 100 pmol of a mixture of random hexamer primers, 60 units of RNAse inhibitor (Promega), and 15 units of Moloney murine leukemia virus RT. (US Biochemical). After 1 h at 37°C, the reaction was terminated by heating for 5 min at 95°C.

Various sets of oligonucleotide primers specific for the α₁ or α₂ sequence were used to confirm the expression of rat isoform and chimera RNAs in OK cells by PCR (Table 2). The design of the primers was based on the location of their target sequence (Fig. 1). Some were derived from regions on the 5′-side of the *ClaI* site (i.e., regions encoding amino acids to the NH₂-terminal side of the ISR), such as "α₁ direct" or "α₂ direct." A second set of primers targeted regions between the *ClaI* and *AgeI* sites (i.e., the ISR itself), such as "α₂ ISR reverse" or "α₂ ISR direct b." Finally, a third set of primers had their target sequences on the 3′-side of the *AgeI* site (i.e., the COOH-terminal side of the ISR), such as "α₁-reverse." For specific amplification, 1 μl of the reverse transcribed sample was added to a PCR incubation mixture containing 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂ (pH 8.3), 0.2 mM of each deoxynucleotide, 0.5 μM of each primer, and 2.5 units of *Taq* polymerase DNA (Roche Diagnostics, Mannheim, Germany) in a final volume of 100 μl. After 2 min at 94°C, samples were submitted to 30 cycles of PCR under the following conditions: 1 min at 94°C, 2 min at an annealing temperature dependent on the characteristics of the primers (Table 2), and 3 min at 72°C. After the final cycle, an additional elongation period of 4 min was performed at 72°C. The amplified products were then analyzed by electrophoresis through agarose gels (1).

Enzymatic activity. The activity of Na+-K+-ATPase in isolated membranes from transfected cells was determined from the hydrolysis of radiolabeled ATP as described (17). Briefly, membranes (1 mg/ml) were treated with 0.4–0.7 mg/ml deoxycholate for 30 min at room temperature to activate latent Na+-K+-ATPase activity. Detergent-treated membranes were then diluted 1:20 and incubated at 37°C for 30 min in a total volume of 0.2 ml containing 20 mM NaCl, 25 mM histidine, 3 mM MgCl₂, 0.2 mM EGTA, and 1 μM ATP (pH

Table 2. *Characteristics of primer pairs used for PCR amplifications*

Panel in Figure 3/Primer Sequence (5′ → 3′)	Position*	GenBank Accession No.	Position (Plasmid)	Fragment Length, bp	Annealing Temperature
A					
α_1 Direct: CCT TGA GTA CAC CTG GCT CG	1196–1215	M28647.1	967–987	747	50°C
α_1 Reverse: GGC AGA AGG AGG TGG CAG	1942–1925	M28647.1	1713–1696		
α_2 Direct: GCT CGA TGA GCT TAA GAA GGA GGT T	185–209	NM 012505.1	98–121	1,425	50°C
ISR Reverse: ATC ACC AGC ACA TGG CTC TG	1609–1590	NM 012505.1	1502–1522		
B					
α_1 Direct: CCT TGA GTA CAC CTG GCT CG	1196–1215	M28647.1	967–987	556	50°C
α_2 ISR Reverse: ATC ACC AGC ACA TGG CTC TG	1609–1590	NM 012505.1	1502–1522		
C					
α_2 IRS Direct b: CAC AAG AAC CCA AAC GCA TC	1737–1756	M28647.1	1481–1500	743	49 °C
α_1 Reverse b: TCA GAG CCA GAG ATG CCC	2308–2291	NM012505.1	2206–2223		

*Sequence given under GenBank Accession No. Annealing temperature was used for PCR.

7.5 at room temperature). To minimize the contribution of endogenous Na$^+$-K$^+$-ATPase, 3 μM ouabain was also included. The Na$^+$-ATPase activity of the introduced enzyme was estimated by the decrement in hydrolysis in the presence of 3 mM ouabain. Various concentrations of KCl, ranging from 0.05 to 5 mM, were also added, and the resulting activity was standardized to the Na$^+$-ATPase estimate measured in the absence of K$^+$.

Active transport. Na$^+$-/K$^+$ pump-mediated transport was assessed by measuring the ouabain-sensitive uptake of the K$^+$ congener ^{86}Rb$^+$ as described (20). Briefly, the difference in accumulation of the radioisotope over 5 min at 37°C was determined in cells exposed to standard culture medium plus 3 μM or 1 mM ouabain. As with the enzymatic analyses, a dose of 3 μM was used to inhibit the ouabain-sensitive endogenous opossum enzyme, whereas the 1 mM ouabain dose was necessary to inhibit the ouabain-resistant enzyme introduced. The effect of PKC activation was determined by treating confluent monolayers for 5 min with the DMSO vehicle or phorbol ester agonist PMA before the addition of radiolabeled Rb$^+$. This was achieved by adding 5 μl of a 10 mM solution of PMA (diluted in DMSO) or 5 μl of DMSO alone (control condition) directly to the 5 ml of culture medium.

RESULTS

Expression of full-length α_1- and α_2^*-isoforms and chimeras. Transfection into OK cells of α_1, α_2^*, $\alpha_1\alpha_2\alpha_1$, and $\alpha_2\alpha_1\alpha_2$ produced ouabain-resistant colonies, indicating that all four exogenous sequences were capable of producing functional Na$^+$-K$^+$-ATPase. To confirm the structure of the introduced subunit, membranes from transfected cells were evaluated by immunoblotting (Fig. 2). As a probe, we used anti-HERED, a polyclonal antibody directed against the α_2 ISR (19). A band corresponding to 116 kDa was detected in $\alpha_1\alpha_2\alpha_1$, as well as rat brain membranes or membranes containing the full-length α_2^* that were included as positive controls (Fig. 2*A*). Anti-HERED did not bind to membranes from rat kidney (known to predominantly express the Na$^+$-K$^+$-ATPase α_1-isoform), α_1-transfected OK membranes, or nontransfected OK membranes that were included as negative controls. No signal was detected in membranes from $\alpha_2\alpha_1\alpha_2$-transfected OK cells (Fig. 2*B*), consistent with an absence of the α_2 ISR in the chimera. Taken together, these data suggest that the structure of the $\alpha_1\alpha_2\alpha_1$ chimera was as intended, with substitution of the α_2 ISR into the α_1-

isoform. Conversely, the α_2 ISR was not detected in membranes expressing $\alpha_2\alpha_1\alpha_2$. The same immunoblots were probed with anti-NASE, a polyclonal antibody directed against the α_1 ISR. Unfortunately, given the cross-reactivity of this antibody with opossum-derived α_1, a band was detected in nontransfected OK cells, precluding any conclusion about exogenous expression in transfected cells (data not shown). Indeed, a previous study has shown this same cross-reactivity with opossum-derived samples (19).

As an alternative to immunological expression, specific oligonucleotide primers were used to confirm ex-
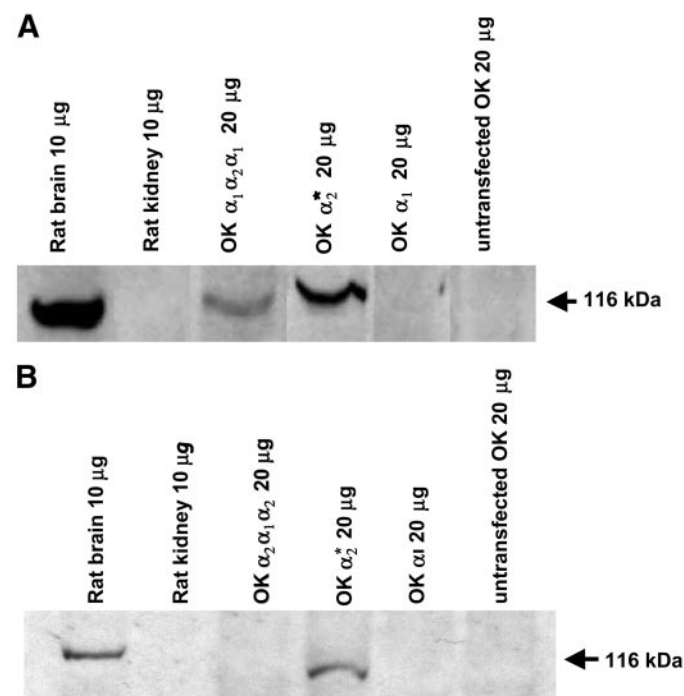

Fig. 2. Immunodetection of HERED epitope. Immunoblot of crude membranes from representative ouabain-resistant colonies was probed with anti-HERED, a polyclonal antibody specific for the ISR of rat α_2-isoform. Membranes from untransfected or α_1-transfected opossum kidney (OK) cells and from rat kidney were included as negative controls. Membranes from ouabain-resistant rat α_2-isoform (α_2^*)-transfected OK cells and from rat brain were included as positive controls. Antibody-epitope interactions were visualized with peroxidase-conjugated goat anti-rabbit IgG and chemiluminescence.

pression of the mRNAs encoding the introduced iso-forms and chimeras. Our strategy was to design primers that recognize α_1 or α_2 nucleic acid sequences encoding regions located before, inside, or after the ISR (Fig. 2). Using reverse-transcribed DNA from OK cells and such primers, we were able to detect the expression of exogenous α_1- and α_2-isoform mRNA after amplification by PCR (Fig. 3). Indeed, bands of 747 and 1,425 bp were readily detected in α_1- and α_2-transfected cells, respectively. No signal was detected in nontransfected OK cells with any primer set. Using a direct primer specific for a region of α_1 before the ISR and a reverse primer specific for the α_2 ISR, we were able to amplify a PCR fragment of the expected mobility (556 bp) using an $\alpha_1\alpha_2\alpha_1$-containing plasmid as a template (positive control) or reverse-transcribed RNA from $\alpha_1\alpha_2\alpha_1$-transfected cells (Fig. 3B). As one would expect, no band of the appropriate mobility was produced when α_1- or α_2-containing plasmids or reverse-

transcribed RNA from α_1- or α_2-transfected cells were used as templates. Finally, Fig. 3C shows the PCR fragment obtained using a direct primer specific for α_2 before the ISR and a reverse primer specific for the α_1 ISR when reverse-transcribed RNA from $\alpha_2\alpha_1\alpha_2$-transfected cells was amplified. As a control, a band of identical mobility (743 bp) was produced from $\alpha_2\alpha_1\alpha_2$-containing plasmid. PCR performed under the same conditions did not reveal any signal from reverse-transcribed RNA of nontransfected cells, α_1- or α_2-containing plasmids, or reverse-transcribed RNA from α_1- or α_2-transfected cells. Taken together, the immunological and PCR analyses strongly suggest that the structures of the various introduced isoforms were correct.

Effect of ISR exchange on enzymatic properties. In micromolar concentrations of ATP sufficient to saturate the high-affinity phosphorylation site, the response of Na$^+$-dependant ATP hydrolysis to varying concentrations of K$^+$ is a convenient and sensitive indication of isoform-specific differences in the E2(K)$\rightarrow$E1 pathway of the Na$^+$-K$^+$-ATPase reaction (8). This part of the reaction becomes rate limiting at low ATP concentration, and K$^+$ inhibits Na$^+$-ATPase activity of the α_1 enzyme. In contrast, α_2 is stimulated. Previous work using chimeric enzymes obtained by exchanges between α_1 and α_2 NH$_2$ termini has suggested that the distinctive kinetic behavior of α_1 and α_2 was not due to the NH$_2$-terminal domain alone but rather to its interaction with other ISRs of the protein (8). Accordingly, a series of experiments was designed to determine whether ISR sequence diversity was involved in the kinetic difference between α_1 and α_2. In Fig. 4, results are presented relative to ouabain-sensitive Na$^+$-ATPase activity, which was 34.8 ± 6.1, 25.2 ± 2.9, 24.0 ± 7.4, and 29.4 ± 1.4 nmol P$_i$·mg protein^{-1}·h^{-1} for α_1, $\alpha_1\alpha_2\alpha_1$, α_2^*, and $\alpha_2\alpha_1\alpha_2$, respectively.

At 1 μM ATP, the K$^+$ activation/inhibition profile of $\alpha_2\alpha_1\alpha_2$ was indistinguishable from that of α_1. Na$^+$-ATPase activity of both α_1 and $\alpha_1\alpha_2\alpha_1$ was inhibited by 0.025–5 mM K$^+$. In experiments with the $\alpha_2\alpha_1\alpha_2$ chimera, low concentrations of K$^+$ stimulated Na-ATPase activity and higher concentrations failed to efficiently inhibit Na$^+$-ATPase activity, resulting in a K$^+$ activation/inhibition profile similar to that of α_2^*. Therefore, switching the ISR between α_1 and α_2 did not alter differences between their K$^+$ activation/inhibition profiles.

Effect of ISR exchange on PKC-dependent activation of cellular Na$^+$-K$^+$-ATPase-mediated Rb$^+$ transport. In basal conditions, Na$^+$-K$^+$-ATPase-mediated Rb$^+$ transport was in the same range for nontransfected and transfected cells, regardless of the construct introduced. Indeed, before PMA-stimulation, Na$^+$-K$^+$-ATPase-mediated Rb$^+$ transport was 8.14 ± 0.6, ($n = 7$) in nontransfected OK cells, 6.44 ± 0.38 ($n = 7$) for α_1, 6.99 ± 0.76 ($n = 7$) for $\alpha_1\alpha_2\alpha_1$, 6.41 ± 1.56 ($n = 11$) for α_2, and 7.99 ± 0.52 nmol·mg protein^{-1}·h^{-1} ($n = 6$) for $\alpha_2\alpha_1\alpha_2$. This strongly suggests that Na$^+$-K$^+$-ATPase was not overexpressed in the transfected cells and that the overall activity was not compromised by the mutations.

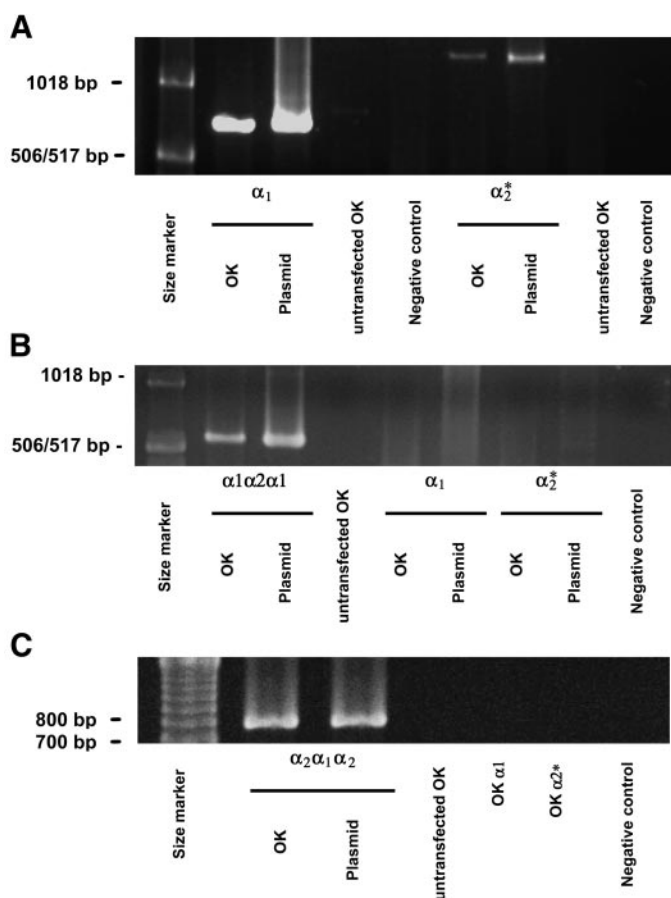Fig. 3. RT-PCR amplification of rat Na$^+$-K$^+$-ATPase $\alpha_1\alpha_2\alpha_1$- and $\alpha_2\alpha_1\alpha_2$-specific sequences. PCR products were obtained with rat Na$^+$-K$^+$-ATPase α_1-, α_2-, $\alpha_1\alpha_2\alpha_1$-, or $\alpha_2\alpha_1\alpha_2$-specific primers (listed in Table 2; see METHODS for details) and plasmid or cellular cDNA as templates. A: α_1- and α_2-specific amplifications. Untransfected OK cell cDNA was used as a negative control. B and C: $\alpha_1\alpha_2\alpha_1$- and $\alpha_2\alpha_1\alpha_2$-specific amplifications, respectively. Untransfected, α_1-transfected, and α_2-transfected OK cell cDNAs were used as negative controls. PCR amplification products were visualized by electrophoresis on ethidium bromide (0.5 μg/ml)-stained 2% agarose gels. Negative control, cDNA was omitted in the PCR mixture.
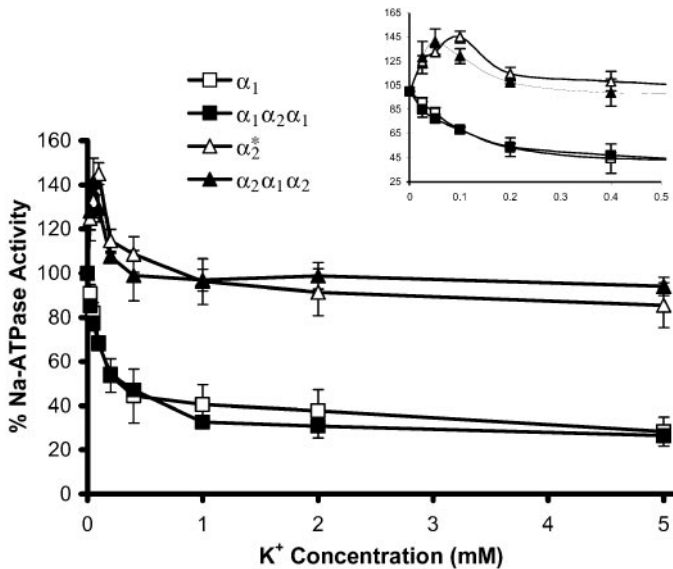
Fig. 4. Effect of α_1/α_2 ISR exchange on K$^+$ sensitivity of Na$^+$-ATPase. ATP hydrolysis was assayed in membranes from transfected OK cells. Hydrolysis of [γ-^{32}P]ATP was measured after 30 min in the presence of 1 μM ATP, 20 mM NaCl, and various concentrations of KCl. Results are presented relative to ouabain-sensitive Na$^+$-ATPase activity for α_1 ($\square$), $\alpha_1\alpha_2\alpha_1$ ($\blacksquare$), α_2^* ($\triangle$), and $\alpha_2\alpha_1\alpha_2$ ($\blacktriangle$), respectively. To inhibit endogenous Na$^+$-K$^+$-ATPase, 3 μM ouabain was present in all reactions. Values are means $\pm$ SE of data obtained from 3 different membrane preparations (assays performed in triplicate for each K$^+$ concentration). *Inset*: shape of the various curves between 0 and 0.4 mM K$^+$ shown in an expanded scale.

We have demonstrated previously that PMA treatment of OK cells expressing the rodent α_1-subunit results in stimulation of Na$^+$-K$^+$-ATPase activity (16). The present study confirms this result for the α_1-isoform and extends it to α_2 (Fig. 5). A significant increase of 15% ($P < 0.01$, paired t-test) in ouabain-sensitive ^{86}Rb$^+$ uptake occurred after 5 min of PMA treatment in both α_1- and α_2-transfected cells. After exchange of the original α_1 ISR for the α_2 sequence (chimera $\alpha_1\alpha_2\alpha_1$), the PMA-induced activation reached 30% ($P < 0.001$, paired t-test). This increase was significantly higher than the increase observed for full-length α_1 ($P < 0.05$, unpaired t-test comparing α_1 and $\alpha_1\alpha_2\alpha_1$ PMA-induced activations). On the other hand, exchange of the original α_2 ISR for the α_1 sequence (chimera $\alpha_2\alpha_1\alpha_2$) completely abolished the PMA-induced activation in the α_2 sequence. These results suggest that the ISR does indeed play a role in PKC-mediated activation of the Na$^+$-K$^+$-ATPase.

DISCUSSION

The primary structures of α-isoforms of the Na$^+$-K$^+$-ATPase are nearly identical, except for the NH$_2$ terminus and an approximately 10-residue region near the center of the molecule, the central ISR (Ref. 19, Fig. 1). We and others have established the influence of the NH$_2$-terminal region on isoform-specific enzyme kinetics and regulation (8, 9, 17, 26). However, this work has also raised suspicions that another site on the catalytic subunit may contribute to these differences. We

thought it likely that this putative site is also a region of structural divergence between the isoforms, and we considered the ISR a prime candidate. To address this question, we produced a pair of chimeric molecules in which the ISRs of α_1 and α_2 were switched. These chimeras were then expressed in opossum renal cells in culture after DNA-mediated gene transfer.

A difficulty with heterologous expression of the Na$^+$-K$^+$-ATPase in mammalian cells is the nearly ubiquitous distribution of this enzyme complex. To distinguish an endogenous from introduced enzyme in the opossum cells, we took advantage of the varied sensitivity of the Na$^+$-K$^+$-ATPase to digitalis glycosides (10, 25). Selection of transfected cells was achieved by growth in a concentration of ouabain sufficient to kill nontransfected cells but not cells that express the more resistant, introduced form. Clearly, this strategy is dependent on the ability of the introduced subunit to sustain active transport despite the presence of digitalis glycoside. Mutations severe enough to inhibit catalytic turnover or to interfere with targeting to the plasmalemma would not produce ouabain-resistant cells and could not be studied with this procedure. For this reason, the successful production of ouabain-resistant colonies after transfection with $\alpha_1\alpha_2\alpha_1$ or $\alpha_2\alpha_1\alpha_2$ suggests that overall enzymatic function was not compromised, despite the switch in ISRs.

Of course, there was always the possibility that the observed ouabain resistance was not a consequence of the transfected α-subunit. Resistance to sublethal concentrations of ouabain is occasionally achieved, for instance, by significant overexpression of pumps rather than the introduction of a resistant enzyme (15).
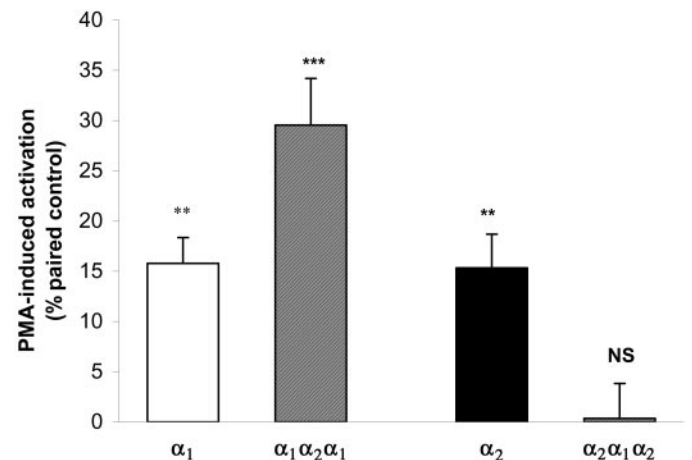

Fig. 5. Effect of α_1/α_2 ISR exchange on PMA-dependent activation of cellular Na$^+$-K$^+$-ATPase-mediated Rb$^+$-transport. Na$^+$-K$^+$-ATPase-mediated transport was assayed in attached cells by measuring the ouabain-sensitive uptake of the K$^+$ congener ^{86}Rb$^+$. PKC activation was induced by a 5-min exposure of the cells to 10 μM PMA before the addition of Rb$^+$ and compared with paired control plates of cells exposed for 5 min to the same amount of vehicle alone (DMSO). Values are means $\pm$ SE ($n = 6$–11) of flux activations induced by PMA exposure, expressed as the percentage of their paired controls (same transfection group, same passage, same day), and were compared using paired 2-tailed Student's t-tests. NS, nonsignificant. **$P < 0.01$; ***$P < 0.001$.

We ensured against this eventuality by selecting cells in ouabain at concentrations well above the $K_{1/2}$ for inhibition of the endogenous enzyme. Moreover, we confirmed the expression and structure of the introduced forms by direct detection of the exogenous polypeptides and mRNAs with specific probes (Figs. 2 and 3).

Having confirmed the successful expression of the introduced isoforms and chimeras, we next evaluated the effect of ISR exchange between α_1 and α_2 on their K^+ activation/inhibition profile, a kinetic parameter known to differ dramatically between these two isoforms. Indeed, the distinctive behavior of the isoforms is apparent when the reaction is carried out under micromolar ATP concentrations because the K^+ deocclusion pathway of the reaction cycle becomes rate limiting (18). Under such conditions, the activity of exogenous α_1 expressed in HeLa and COS-1 cells is inhibited by K^+, whereas α_2 is stimulated (8, 17). The present observations have confirmed these results in OK cells. Daly et al. (8) have argued that it is not solely the difference in amino acid sequences at the NH_2 termini of α_1 and α_2 that is responsible for this kinetic difference. Rather, it is likely to be an interaction of the segment between 24 and 32 of α_1 with some other region of the α_1 protein that determines the K^+-sensitive pattern displayed by this isoform. We hypothesized that the ISR could be this region. However, this seems unlikely because exchange of the α_1 and α_2 sequences did not result in a modification of the K^+ activation/inhibition profile compared with the appropriate unaltered isoform (Fig. 4). Taken together, the results of Daly and co-workers and the present data suggest that it is the interaction of the NH_2-terminal segment with a region of α_1 other than the ISR that is a determinant of the observed K^+ inhibition profile.

Another possible role for the ISR region could be in the isoform-specific response to second messengers. Numerous laboratories have cataloged differences in isoform regulation after stimulation of both protein kinase A and C (reviewed in Refs. 2, 3, and 14). For the present set of experiments, we chose to focus on PKC activation of the Na^+-K^+-ATPase induced by the phobol ester agonist PMA. We observed a 15% stimulation of pump-mediated Rb^+ transport for α_1 and α_2^*. When we replaced the ISR in α_1 with the α_2 sequence, this activation was increased twofold (Fig. 5). Conversely, substituting the α_2 ISR with α_1 in the α_2-isoform resulted in a nonresponding phenotype. These data clearly argue in favor of a role for the ISR in PKC regulation. Moreover, the paradoxical response to the agonist seems to imply an inhibitory effect of the α_1 ISR. When it was removed from α_1, the PMA response was increased ($\alpha_1\alpha_2\alpha_1$), but when it was substituted into α_2 ($\alpha_2\alpha_1\alpha_2$), the previously observed response was blocked.

It is tempting to speculate about what mechanism may underlie this inhibitory effect of the α_1 ISR. Efendiev et al. (9) have shown that PMA-induced activation of Na^+-K^+-ATPase in OK cells is the result of pump translocation from intracellular pools

via clathrin-coated vesicles, resulting in an increased abundance in the plasma membrane (9). This process requires the phosphorylation of Ser^{11} and Ser^{18} (not present in α_2-isoform) of the α_1-isoform by the PKC β-isoform and involves the adaptor activator protein (AP)-1 (9). Adaptors mediate the incorporation of cargo onto transport vesicles by interacting with sorting signals present in the cytosolic domain of transmembrane proteins. Four adaptors (AP-1, AP-2, AP-3, and AP-4) have been described so far. AP-1 and AP-3 mediate sorting events at the level of the *trans*-Golgi network and/or endosomes, whereas AP-2 functions in endocytic clathrin-coated vesicle formation (reviewed in Ref. 4). Recent evidence has shown that AP-4 participates in basolateral sorting in epithelial cells (23). Using the same transfection strategy into OK cells as was used in the present study, investigators have shown that, unlike PMA, dopamine inhibits Na^+-K^+-ATPase activity. Interestingly, this inhibition also involves membrane trafficking, in this case by internalization via clathrin-coated vesicles. This internalization requires activation of the atypical PKCζ and the adaptor AP-2, as well as the binding of phosphoinositide-3 kinase to a proline-rich motif of the α-subunit (5, 26). Little is known about AP-3 and AP-4, but AP-1 and AP-2 are known to recognize their target by consensus signals in the cytoplasmic domain of proteins. These consensus sequences are either di-leucine motifs or tyrosine-based signals, specifically Y-X-X-Ø, where Ø is a bulky hydrophobic amino acid. Recent work by Cotta Doné et al. (7) has identified Tyr^{537} as an essential element for AP-2 binding and the clathrin-dependent endocytosis of Na^+-K^+-ATPase that mediates dopamine-induced inhibition.

In addition to this tyrosine-based signal, the α_1-isoform also displays a di-leucine motif, which appears to be in the ISR but is not present in α_2. The di-leucine motif might represent the molecular basis of the inhibitory effect that we have attributed to the α_1 ISR. It could act as a dynamic retention signal that favors α_1 internalization, even during PMA stimulation. In apparent contradiction, it has been clearly shown that mutating the second leucine of this motif (i.e., Leu^{500}) is not sufficient to alter PMA-induced activation (7). However, this finding may not be inconsistent with an involvement of the first leucine of the motif (i.e., Leu^{499}), which is the last amino acid of the α_1 ISR and is absent from the α_2 sequence. Moreover, dynamic retention signaling can involve an entire region, including several, sometimes redundant, consensus motifs. For instance, insulin-regulated aminopeptidase dynamic retention within the endosomal compartment requires 2 of 3 distinct motifs present in a 30-amino-acid region of its cytoplasmic tail (13). It may be that Leu^{499} is the more important residue of the signaling motif, but other residues might contribute to a more extensive domain that includes the α_1 ISR sequence but is missing in the α_2 sequence.

Along the same lines, it should be kept in mind that although the ISR represents one the most striking sequence variabilities among the isoforms, other regions of the intracellular domain also display various degrees of diversity, especially in the so-called large cytoplasmic loop (between TM4 and TM5; see Fig. 1 in Ref. 2). As mentioned previously, the NH_2-terminal region is also clearly an ISR. By swapping ISRs, we might have disrupted an important interaction with another part of the molecule, thereby interfering with a dynamic retention signal composed of several motifs that may be far apart in the primary structure. In short, it is not clear at this point if ISR swapping is disrupting a signal contained in the sequence itself or rather an important interaction with one or several other motifs within the intracellular domains of the protein.

With previous studies, the present data, and the above-stated hypothesis taken into account, the stimulation of α_1 that we are observing under PMA treatment likely results from the contribution of at least two facilitating components and one inhibitory component. Accordingly, their presence in or absence from the different isoforms would determine the amplitude of the individual response to the phorbol ester. First, an isoform-specific effect is very likely to be involved in the PMA-induced increase observed with α_1 It may involve Ser^{11} and Ser^{18} phosphorylation (which are not present in α_2) and AP-1. The effect is an increase in the number of Na^+-K^+-ATPase complexes expressed at the membrane (9). It is also possible that tyrosine-based motifs such as Tyr^{469}, expressed by α_1 but not α_2, could mediate the interaction. Second, a mechanism of internalization mediated by the di-leucine motif and the surrounding area in the ISR (present only in α_1) may contribute to the response. This effect would be a dynamic retention, taking place even in basal conditions. Third, an activator mechanism seems to be shared by α_1 and α_2. Accordingly, removing the inhibitory ISR of the α_1 sequence and replacing it by the "neutral" α_2 ISR results in an increased response to PMA as shown for $\alpha_1\alpha_2\alpha_1$. On the same basis, the PMA-induced stimulation of α_2 could be compromised by the addition of the inhibitory sequence of the α_1 ISR, as shown by the absence of PMA-induced stimulation in $\alpha_2\alpha_1\alpha_2$.

Clearly, the chimeras used in the present study are just the first step in a systematic evaluation of isoform-specific structure and its influence on function. Future experiments will be needed to determine the individual amino acid residues contributing to the PKC response, as well as the role played by the ISRs of other α-isoforms.

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