Structure/Function Analysis of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Central Isoform-specific Region:

Involvement in Protein Kinase C Regulation.

F-00153-2002, Final Accepted Version

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Running Head: Function of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Isoform-specific Region

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ABSTRACT

Specific functions served by the various Na\(^+\)-K\(^+\)-ATPase \(\alpha\) isoforms are likely to originate within regions of structural divergence within their primary structures. The isoforms are nearly identical, with the exception of the amino-terminus and a ten-residue region near the center of each molecule. Although the amino-terminus has clearly been identified as a source of isoform functional diversity, other regions seem to be involved. We investigated whether the ten-residue region near the center of the molecule (Isoform Specific Region: ISR) could also contribute to isoform variability. We constructed chimeric molecules in which the central ISR of rat \(\alpha_1\) and \(\alpha_2\) isoforms have been exchanged. After stable transfection into opossum kidney (OK) cells, the chimeras were characterized for two properties known to differ dramatically among the isoforms, their K\(^+\) deocclusion pattern and their response to protein kinase C (PKC) activation. Comparisons with rat full-length \(\alpha_1\) and \(\alpha_2\) isoforms expressed under the same conditions suggest an involvement of the central ISR in the response to PKC but not in K\(^+\) deocclusion.

Keywords: \(\alpha\) subunit, rat, \(\alpha_1\) and \(\alpha_2\) isoforms, chimeras, K\(^+\) deocclusion.
INTRODUCTION

The Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is critical for maintaining the ionic gradients across the plasma membranes of animal cells. This enzyme complex extrudes Na\textsuperscript{+} from the cell and accumulates K\textsuperscript{+} using metabolic energy derived from ATP hydrolysis. The enzyme complex consists of two dissimilar subunits, \( \alpha \) and \( \beta \), that exist in multiple forms. A fundamental question surrounding the pump is the physiological relevance of this subunit diversity. The \( \alpha \)-polypeptide is the catalytic subunit and contains the binding sites for ions and substrates. Four distinct isoforms (\( \alpha \textsubscript{1}, \alpha \textsubscript{2}, \alpha \textsubscript{3} \) and \( \alpha \textsubscript{4} \)) of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \( \alpha \) 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 amino-terminus and a ten-residue region near the center of the molecule (24, 20) (Fig. 1, ISR: K\textsuperscript{489}-L\textsuperscript{499}). It seems likely that the specific functions served by the various Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \( \alpha \) isoforms must originate within these regions of structural divergence.

The amino-terminus region has been extensively studied. Heterologous expression of amino-terminal deletions and chimeric constructs has shown that the mutant \( \alpha \) subunits display changes in kinetics and regulation of ion transport properties. Although the amino-terminus of \( \alpha \) is not required for Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (21), a truncated enzyme lacking the first 32 amino acids has distinctive kinetics at \( \mu \)M ATP concentrations, conditions in which K\textsuperscript{+} deocclusion becomes rate-limiting in the overall catalytic cycle. Thus, Na\textsuperscript{+}-ATPase activity of the truncated enzyme is stimulated by low concentrations of K\textsuperscript{+}, whereas activity of wild type \( \alpha \textsubscript{1} \) is inhibited. Interestingly, the \( \alpha \textsubscript{2} \) isoform resembles the truncated enzyme in this respect. However, unexpected
characteristics of chimeras resulting from exchanges between $\alpha_1$ and $\alpha_2$ amino-terminal domains suggested that this distinctive kinetic behavior of Na$^+$-K$^+$-ATPase $\alpha$-isoforms is not entirely due to the amino-terminal region, but rather to its interaction with other, isoform-specific region(s) of the $\alpha$ protein (8).

Another isoform-specific property that clearly involves the amino-terminal region is the regulation of Na$^+$-K$^+$-ATPase transport activity by protein kinase C (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 exogenous rat Na$^+$-K$^+$-ATPase $\alpha_1$ isoform. This increase was abolished in cells expressing a mutant missing the first 26 amino acids of the rodent $\alpha_1$-subunit, consistent with a role for the amino-terminus region in PKC regulation. Ser-16 and Ser-23, which are believed to be in vivo targets of PKC phosphorylation, are found within the amino-terminal domain of $\alpha_1$ rat 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 $\alpha_1$ isoforms, but it is only weakly phosphorylated. Ser-23, on the other hand, is missing in many species. Neither residue is contained within a PKC consensus sequence, and neither residue is conserved in $\alpha_2$ or $\alpha_3$. Nevertheless, there are other serines and threonines in the amino 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 $\alpha$ subunit are contributing to the effect of PKC.
We hypothesized that the central isoform specific region (ISR) may be one of these additional regions. To test this hypothesis, chimeric molecules were constructed in which the central ISRs of the rat α1 and α2 isoforms were exchanged. After stable transfection into opossum kidney (OK) cells, the chimeras were characterized for their enzymatic properties. Comparisons of the chimeras with rat full-length α1 and α2 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 \( \alpha_1 \) and \( \alpha_2^* \) sequences. Wild type \( \alpha_1 \) and \( \alpha_2 \) cDNAs, a gift from Dr. Jerry B. Lingrel and his colleagues (22), were subcloned by our laboratory into pGEM-3Z (Promega, Madison, WI), as described (21). A ouabain-resistant form of the rat \( \alpha_2 \) isoform (designated \( \alpha_2^* \)) 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 \( \alpha_1 \) and \( \alpha_2^* \) allowed us to employ the ouabain-selection strategy described below.

Preparation of chimeras. The \( \alpha_1 \) and \( \alpha_2^* \) cDNAs were used to prepare chimeric molecules in which the central isoform specific domain of \( \alpha_1 \) and \( \alpha_2^* \) were exchanged. Two silent mutations were introduced in rat \( \alpha_1 \) and \( \alpha_2^* \) cDNA by conventional site-directed mutagenesis, creating unique sites for digestion by the restriction enzymes \( ClaI \) and \( AgeI \) without altering the encoded amino acids. The \( ClaI \) site was placed within the codons for Leu-485, Ser-486, and Ile-487 (LSI), as numbered from the amino terminal glycine of the mature polypeptide. The \( AgeI \) 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 \( DpnI \). To perform the exchange of the isoforms specific regions (ISR), we proceeded in two steps. In the first step, the carboxyl ends of each isoform were swapped, using the introduced \( AgeI \) sites (DRC). The resulting chimeras were then used as starting material for swaps of the amino half of the molecule, using the introduced \( ClaI \) 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 α1, α2*, 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 SacI-XbaI adaptor. This was prepared by annealing the oligonucleotides CGCGGCGCGCT and CTAGAGCGGC-GCGAGCT. The resulting expression vector contained the sequence encoding the α construct downstream from the enhancer-promoter regions of the immediate early gene of human cytomegalovirus, followed by polyadenylation signals from the bovine growth hormone gene.

Heterologous expression of the α constructs was achieved by transfection of opossum kidney cells (OK, American Type Culture Collection, ATTC number CRL-1840) and subsequent selection of recipients with ouabain. OK cells were routinely maintained at 37 °C and 10% CO₂ in Dulbecco’s modified Eagle medium 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, N. Y.) 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 (ie., 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 then cells were 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 sodium dodecyl sulfate-polyacrylamide gels (7.5%), electroblotting, and probing of the blots with appropriate antibodies were performed as described (20). 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 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 (20); 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 the 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 diethyl-pyrocarbonate treated water and only preparations with a $A_{260}/A_{280}$ ratio greater than 1.5 were analyzed further.
Synthesis of complementary DNA (cDNA) was achieved by reverse transcription. The RNA mixture was heated for 10 min at 65°C. The reverse-transcription was then performed in a final volume of 20 µl containing 50mM Tris-HCl (pH 8.4), 75 mM KCl, 3 mM MgCl₂, 500 µM each of deoxynucleotides, 10 mM dithiothreitol, 100 pmoles of a mixture of random hexamer primers, 60 units of RNAse inhibitor (Promega) and 15 units of Moloney murine leukaemia virus reverse transcriptase. (United States Biochemical). After one hour at 37°C, the reaction was terminated by heating 5 min at 95°C.

Various set of oligonucleotide primers specific for α1 or α2 sequence were used to confirm the expression of rat isoforms and chimeras RNA in OK cells by polymerase chain reaction (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 Clal site (ie., regions encoding amino acids to the N-terminal side of the ISR), such as “α1 direct” or “α2 direct”. A second set of primers targeted regions between the Clal and AgeI sites (ie., the ISR itself), such as “α2 ISR reverse” or “α2 ISR direct b”. Finally, a third set of primers have their target sequences on the 3’ side of the AgeI site (ie., the C-terminal side of the ISR), such as “α1 reverse”. For specific amplification, 1 µl of 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 each deoxynucleotides, 0.5 µM of each primer, 2.5 units of Taq DNA polymerase (Roche Diagnostics GmbH, 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 to 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 ºC for 30 min in a total volume of 0.2 ml containing 20 mM NaCl, 25 mM histidine, 3 mM MgCl\(_2\), 0.2 mM EGTA, 1 µM ATP (pH 7.5 at room temperature). 3 µM ouabain were also included to minimize the contribution of endogenous Na\(^+\)-K\(^+\)-ATPase. 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 (19). Briefly, the difference in accumulation of radioisotope over 5 min at 37 ºC 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, introduced enzyme. The effect of PKC activation was determined by treating confluent monolayers for 5 min with DMSO vehicle or the phorbol ester agonist, phorbol 12-myristate 13-acetate (PMA), prior to 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 α1 and α2* isoforms and chimeras. Transfection into OK cells of α1, α2*, α1α2α1, and α2α1α2 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 α2 ISR (20). A band corresponding to 116 kD was detected in α1α2α1, as well as rat brain membranes or membranes containing the full length α2* that were included as positive controls (Fig. 2A). Anti-HERED did not bind to membranes from rat kidney (known to express predominantly the α1 Na+-K+-ATPase isoform), α1-transfected or nontransfected OK membranes that were included as negative controls. No signal was detected in membranes from α2α1α2 transfected OK cells (Fig. 2B), consistent with an absence of the α2 ISR in the chimera. Taken together, these data suggest that the structure of the α1α2α1 chimera was as intended, with substitution of the α2 ISR into the α1 isoform. Conversely, the α2 ISR was not detected in membranes expressing α2α1α2. The same immunoblots were probed with anti-NASE, a polyclonal antibody directed against the α1 ISR. Unfortunately, given the cross-reactivity of this antibody with opossum-derived α1, 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 (20).

As an alternative to immunological expression, specific oligonucleotide primers were used to confirm expression of the mRNAs encoding the introduced isoforms and
chimeras. Our strategy was to design primers that recognize $\alpha_1$ or $\alpha_2$ 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 $\alpha_1$ and $\alpha_2$ isoform mRNA after amplification by PCR (Fig. 3). Indeed, bands of 747 bp and 1425 bp were readily detected in $\alpha_1$- and $\alpha_2$-transfected cells, respectively. No signal was detected in nontransfected OK cells with any primer set. Using a direct primer specific for a region of $\alpha_1$ before the ISR and a reverse primer specific for $\alpha_2$ ISR, we were able to amplify a PCR fragment of the expected mobility (556 bp) using $\alpha_1\alpha_2\alpha_1$-containing plasmid as 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 $\alpha_1$- or $\alpha_2$-containing plasmids or reverse-transcribed RNA from $\alpha_1$- or $\alpha_2$-transfected cells were used as templates. Finally, Fig. 3C shows the PCR fragment obtained using a direct primer specific for $\alpha_2$ before the ISR and a reverse primer specific for $\alpha_1$ 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, $\alpha_1$- or $\alpha_2$-containing plasmids, or reverse-transcribed RNA from $\alpha_1$- or $\alpha_2$-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 to Na⁺-dependant ATP hydrolysis to varying concentration of K⁺ is a convenient and sensitive indication of isoform-specific differences in the E2(K)→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 α1 enzyme. In contrast, α2 is stimulated. Previous work using chimeric enzymes obtained by exchanges between α1 and α2 amino-termini has suggested that the distinctive kinetic behavior of α1 and α2 was not due to the N-terminal domain alone, but rather to its interaction with other, isoform-specific region(s) of the protein (8). Accordingly, a series of experiments was designed to determine whether the ISR sequence diversity was involved in the kinetic difference between α1 and α2. In figure 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 Pi/mg protein/h for α1, α1α2α1, α2, and α2α1α2, respectively.

At 1 μM ATP, the K⁺ activation/inhibition profile of α2α1α2 was indistinguishable from that of α1. The Na⁺-ATPase activity of both α1 and α1α2α1 was inhibited by 0.025 to 5 mM K⁺. In experiments with the α2α1α2 chimera, low concentrations of K⁺ stimulated Na-ATPase activity and higher concentrations failed to inhibit efficiently the Na⁺-ATPase activity, resulting in a K⁺ activation/inhibition profile similar to that of α2*. Therefore, switching the ISR between α1 and α2 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 introduced construct. Indeed, prior to PMA-stimulation, Na\(^{+}\)-K\(^{+}\)-ATPase mediated Rb\(^{+}\) transport (nmol/mg protein/h) was 8.14 ± 0.6, (n=7) in nontransfected OK cells, 6.44 ± 0.38 (n=7) for α1, 6.99 ± 0.76 (n=7) for α1α2α1, 6.41 ± 1.56 (n=11) for α2, and 7.99 ± 0.52 (n=6) for α2α1α2. This strongly suggests that Na\(^{+}\)-K\(^{+}\)-ATPase was not over-expressed in the transfected cells and that the overall activity was not compromised by the mutations.

We have demonstrated previously that PMA treatment of OK cells expressing the rodent α1-subunit results in stimulation of Na\(^{+}\)-K\(^{+}\)-ATPase activity (16). The present study confirms this result for the α1 isoform and extends it to α2 (Fig. 5). A significant increase of 15% (p<0.01, paired t-test) after 5 min of PMA treatment occurred in ouabain-sensitive \(^{86}\)Rb\(^{+}\) uptake in both α1- and α2-transfected cells. After exchange of the original α1 ISR for the α2 sequence (chimera α1α2α1), the PMA-induced activation reached 30% (p<0.001, paired t-test). This increase was significantly higher than the increase observed for the full-length α1 (p<0.05, unpaired t-test comparing α1 and α1α2α1 PMA-induced activations). On the other hand, exchange of the original α2 ISR for the α1 sequence (chimera α2α1α2) completely abolished the PMA-induced activation in the α2 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, with the exception of the amino terminus and an approximately ten-residue region near the center of the molecule, the central isoform-specific region (ISR; 20; Fig. 1). We and others have established the influence of the N-terminal region on isoform-specific enzyme kinetics and regulation (8, 17, 9, 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 ISR of α1 and α2 were switched. These chimeras were then expressed in opossum renal cells in culture following DNA-mediated gene transfer.

A difficulty with heterologous expression of the Na⁺-K⁺-ATPase in mammalian cells is the near ubiquitous distribution of this enzyme complex. To distinguish 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 by 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 \( \alpha \) subunit. Resistance to sub-lethal concentrations of ouabain is occasionally achieved, for instance, by significant over-expression of pumps rather than the introduction of resistant enzyme (15). We ensured against this eventuality by selecting 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 \( \alpha_1 \) and \( \alpha_2 \) on their \( K^+ \) activation/inhibition profiles, 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 \( \alpha_1 \) expressed in HeLa and COS-1 cells is inhibited by \( K^+ \), whereas \( \alpha_2 \) is stimulated (8, 17). The current observations have confirmed these results in OK cells. Daly et al. (8) have argued that it is not solely the difference in amino acid sequence at the amino-termini of \( \alpha_1 \) and \( \alpha_2 \) that is responsible for this kinetic difference. Rather, it is likely to be an interaction of the segment between 24-32 of \( \alpha_1 \) with some other region of the \( \alpha_1 \) protein that determines the \( K^+ \) sensitive pattern displayed by this isoform. We hypothesized that the ISR could be this region. However, this seems
unlikely since exchange of the $\alpha_1$ and $\alpha_2$ sequences did not result in a modification of the $K^+$ activation/inhibition profile when compared with the appropriate unaltered isoform (Fig. 4). Taken together, the results of Daly and co-workers and the current data suggest that it is the interaction of the amino terminal segment with a region of $\alpha_1$ 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 ref 2, 3, 14). For the current set of experiments, we chose to focus on PKC activation of the $\text{Na}^+\text{K}^+$-ATPase induced by the phobol ester agonist, PMA. We observed a stimulation of 15% in pump-mediated $\text{Rb}^+$ transport for $\alpha_1$ and $\alpha_2*$. When we replaced the ISR in $\alpha_1$ by the $\alpha_2$ sequence, this activation was increased two fold (Fig. 5). Conversely, substituting the $\alpha_2$ ISR with $\alpha_1$ in the $\alpha_2$ isoform resulted in a non-responding 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 $\alpha_1$ ISR. When it was removed from $\alpha_1$, the PMA response was increased ($\alpha_1\alpha_2\alpha_1$), but when it was substituted into $\alpha_2$ ($\alpha_2\alpha_1\alpha_2$), the previously observed response was blocked.

It is tempting to speculate on what may be the mechanism underlying this inhibitory effect of the $\alpha_1$ ISR. Efendiev et al. have shown that PMA-induced activation of $\text{Na}^+\text{K}^+$-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 $\alpha_2$ isoform) of the $\alpha_1$ isoform by the PKC $\beta$ isoform and involves the adaptor 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 PKC ζ 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 the 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 α1 isoform also displays a di-Leu motif, which appears to be in the ISR but is not present in α2. The di-Leu motif might represent the molecular basis of the inhibitory effect that we have attributed to the α1 ISR. It could act as a dynamic retention signal that favors α1 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 $\alpha_1$ ISR and is absent from the $\alpha_2$ sequence. Moreover, dynamic retention signaling can involve an entire region including several, sometimes redundant, consensus motifs. For instance, insulin-regulated aminopeptidase (IRAP) 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 $\alpha_1$ ISR sequence but is missing in the $\alpha_2$ 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 reference 2). As mentioned previously, the N-terminal region is also clearly an isoform-specific region. By swapping the ISR, 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.

Taking into account the previous studies, present data and the above stated hypothesis, the stimulation of $\alpha_1$ that we are observing under PMA treatment is likely to
result from the contribution of at least 2 facilitating and one inhibitory component. Accordingly, their presence or absence in the different isoforms would determine the amplitude of the individual response to the phorbol ester. 1) An isoform specific effect is very likely to be involved in the PMA-induced increase observed with α1. It may involve Ser 11 and Ser 18 phosphorylation (which are not present in α2), 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 Tyr-based motifs expressed by α1 but not α2 such as Tyr 469 could mediate the interaction. 2) A mechanism of internalization mediated by the di-Leu motif and the surrounding area in the ISR (present only in α1) may contribute to the response. This effect would be a dynamic retention, taking place even in basal conditions. 3) An activator mechanism that seems to be shared by α1 and α2. Accordingly, removal of the inhibitory ISR of the α1 sequence, and replacing it by the “neutral” α2 ISR, results in an increased response to PMA as shown for α1α2α1. On the same basis, the PMA-induced stimulation of α2 could be compromised by the addition of the inhibitory sequence of α1 ISR, as shown by the absence of PMA-induced stimulation in α2α1α2.

Clearly, the chimeras used in the current 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.
Acknowledgments

This work has been supported by grants from the American Heart Association Texas Affiliate, 98G-385, and the National Center for Research Resources, RR-19799.
REFERENCES


FIGURE LEGENDS

Figure 1. Structure of the \( \alpha \) subunit. Dark boxes represent the locations of isoform-specific regions, N-terminal domain and central isoform-specific region (ISR). The aligned amino-acid sequences of \( \alpha_1 \) and \( \alpha_2 \) ISR are \textit{shadowed}, and the sites of silent mutations are in \textit{bold}.

Figure 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 central isoform specific region (ISR) of rat \( \alpha_2 \) isoform. Membranes from untransfected or \( \alpha_1 \)-transfected OK cells and from rat kidney were included as negative controls. Membranes from \( \alpha_2^* \)-transfected OK and from rat brain were included as positive controls. Antibody-epitope interactions were visualized with peroxidase-conjugated goat anti-rabbit immunoglobulin G and chemiluminescence.

Figure 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 \( \alpha_1 \), \( \alpha_2 \), \( \alpha_1\alpha_2\alpha_1 \) or \( \alpha_2\alpha_1\alpha_2 \) specific primers (listed in table 2, see method section for details) and plasmid or cellular cDNA as templates. 3A. \( \alpha_1 \) and \( \alpha_2 \) specific amplifications. Untransfected OK cells cDNA was used as a negative control. 3B. and 3C. \( \alpha_1\alpha_2\alpha_1 \) and \( \alpha_2\alpha_1\alpha_2 \) specific amplifications, respectively. Untransfected-, \( \alpha_1 \)- and \( \alpha_2 \)-transfected OK cells cDNA were used as negative controls. PCR amplification products were visualized by electrophoresis on ethidium bromide (0.5 \( \mu \)g/ml)-stained 2% agarose gel. Negative Control : cDNA omitted in the PCR mixture.
Figure 4. Effect of α1 / α2 ISR exchange on K⁺ Sensitivity of Na⁺-ATPase. ATP hydrolysis was assayed in membranes from transfected OK cells. Hydrolysis of [γ-³²P]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 α1 (□), α1α2α1(■), α2(△), and α2α1α2(▲), respectively. 3 μM ouabain was present in all reactions to inhibit endogenous Na⁺-K⁺-ATPase. Values are means ± SEM of data obtained from 3 different membrane preparations (assays performed in triplicate for each K⁺ concentration). The insert shows, in an expanded scale, the shape of the various curves between 0 and 0.4 mM of K⁺.

Figure 5. Effect of α1 / α2 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, ⁸⁶Rb⁺. PKC activation was induced by a 5 min exposure of the cells to 10 μM PMA prior to the addition of Rb⁺, and compared to paired control plates of cells exposed for 5 min to the same amount of vehicle alone (DMSO). Values are means ± SEM (n = 6 to 11) of flux activations induced by PMA exposure, expressed in percent of their paired controls (same transfection group, same passage, same day). Values were compared using two-tailed Student paired t-test. NS: non significant, ** p<0.01, *** p<0.001.
Table 1. Characteristics of silent mutation oligonucleotides used for the introduction of *ClaI* and *AgeI* sites into rat α1 and α2 sequences.

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<td>TAC</td>
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<tr>
<td>α2 WT</td>
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α1WT: wild type sequence, α1-ClaI: α1-ClaI mutation oligonucleotide sequence, α2WT: α2 wild type sequence, α2-ClaI: α2 Clal mutation oligonucleotide sequence. Mutated bases are in bold. Introduced restriction enzyme sites are shadowed.
Table 2. Characteristics of primer pairs used for PCR amplifications.

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Number in the first column refers to the figure. * The sequence to which the position refers is given under the Genbank “Accession Number” column. Anneal.: Annealing temperature used for the PCR.
FIGURE 1

\[ \text{ISR (K}^{489}\text{-L}^{499}) \]

\[ \alpha_1: \text{KYQLSIHK}^{489}\text{NPNA}^{499}\text{SHKL}^{499}\text{LVMKGAPERILDRCSSI} \]

\[ \alpha_2*: \text{KYQLSIHE}^{487}\text{RDPQS-HV}^{496}\text{LVMKGAPERILDRCSTI} \]

\[ \text{CTATCGATT} \]

\( C_{la}l \text{ site} \)

\[ \text{GACCGGTGC} \]

\( A_{ge}l \text{ site} \)
FIGURE 2

A

<table>
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<td>Rat brain</td>
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<tr>
<td>Rat kidney</td>
<td>10 µg</td>
</tr>
<tr>
<td>OK α1α2α1</td>
<td>20 µg</td>
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<tr>
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<td>OK α1</td>
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116 kDa

B

<table>
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<tr>
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<tr>
<td>Rat brain</td>
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<td>OK α2α2α2</td>
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<tr>
<td>OK α2*</td>
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<tr>
<td>untransfected</td>
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116 kDa
FIGURE 3

A

1018 bp -
506/517 bp -

Size marker  
α1  
untransfected OK  
α2 *  
untransfected OK

B

1018 bp -
506/517 bp -

Size marker  
α1α2α1  
untransfected OK  
α1  
α2*  
untransfected OK  
Negative control

C

800 bp -
700 bp -

Size marker  
α2α1α2  
untransfected OK  
α1  
α2*  
Negative control
FIGURE 4

![Graph showing % Na-ATPase Activity vs. K⁺ Concentration (mM) for different α isoforms. The α1 and α1α2α1 isoforms show higher activity at lower K⁺ concentrations, while the α2* and α2α1α2 isoforms show lower activity.](image-url)
FIGURE 5

PMA-induced activation (% of paired control)

α1  α1α2α1  α2  α2α1α2

***  **  **  NS