Structure/function analysis of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase central isoform-specific region: involvement in PKC regulation

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The NH\textsubscript{2}-terminal region has been extensively studied. Heterologous expression of NH\textsubscript{2}-terminal deletions and chimeric constructs has shown that the mutant \alpha-subunits display changes in kinetics and regulation of ion transport properties. Although the NH\textsubscript{2} 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 micromolar 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 \( \alpha \) is inhibited. Interestingly, the \( \alpha_2 \)-isoform resembles the truncated enzyme in this respect. However, unexpected characteristics of chimeras resulting from exchanges between \( \alpha_1 \) and \( \alpha_2 \) NH\textsubscript{2}-terminal domains suggested that this distinctive kinetic behavior of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \( \alpha \)-isoforms is not entirely due to the NH\textsubscript{2}-terminal region but rather to its interaction with other ISRs of the \( \alpha \)-protein (8).

Another isoform-specific property that clearly involves the NH\textsubscript{2}-terminal region is the regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase transport activity by PKC. Indeed, we have shown that stimulation of endogenous PKC with phorbol esters increases pump-mediated Rb\textsuperscript{+} transport in cultured opossum kidney (OK) cells expressing the exogenous rat Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \( \alpha \)-isoform. This increase was abolished in cells expressing a mutant missing the first 26 amino acids of the rodent \( \alpha \)-subunit, consistent with a role for the NH\textsubscript{2}-terminal region in PKC regulation. Ser\textsuperscript{16} and Ser\textsuperscript{25}, which are believed to be in vivo targets of PKC phosphorylation, are found within the NH\textsubscript{2}-terminal domain of the rat \( \alpha \)-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\textsuperscript{16} is well conserved among mammalian \( \alpha_1 \)-isoforms, but it is only weakly phosphorylated. Ser\textsuperscript{25}, on the other hand, is missing in

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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 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 \( \beta \)-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 isoform-specific domain of \( \alpha_1 \) and \( \alpha_2 \) were exchanged. Two silent mutations were introduced in rat \( \alpha_1 \) and \( \alpha_2 \) cDNAs 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 NH\(_2\)-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 ISRs, we proceeded in two steps. In the first, the COOH ends of each isoform were swapped, using the introduced ClaI sites (DRC). The resulting chimeras were then used as starting material for swaps of the NH\(_2\) half of the molecule, using the introduced AgeI site. The structures of the resulting mutants were then confirmed by restriction analysis and direct sequencing of the altered region.

METHODS

Preparation of full-length \( \alpha_1 \) and ouabain-resistant \( \alpha_2 \) sequences. Wild-type \( \alpha_1 \) and \( \alpha_2 \) 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 \( \alpha_2 \)-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 \( \alpha_2^* \) cDNAs 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 NH\(_2\)-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 ISRs, we proceeded in two steps. In the first, the COOH 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 NH\(_2\) 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 \( \alpha_1 \), \( \alpha_2 \), or the chimeras were subcloned into the HindIII and XbaI 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 CGCGGCGCT and CTAGAGGGCCCGCGA-

GCT. The resulting expression vector contained the sequence encoding the \( \alpha \) 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.
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% CO2 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 transferrred proteins on the blots and equality of loading among the lanes were confirmed by staining with Ponceau S. For detection of proteins on the blots 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 (Biotexc Laboratories, Houston, TX), a modification of the guanidinium thiocyanate-phenol-chloroform protocol of Chomczynski and Sacchi (6). The recovered RNA was dissolved in diethylpyrocarbonate-treated water, and only preparations with an A260/A280 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 isofrom 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 Clal 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 Clal 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 “α2 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
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7.5 at room temperature). To minimize the contribution of endogenous Na\(^+\)-K\(^+\)-ATPase, 3 \(\mu\)M 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 activity in the absence of K\(^+\).

**RESULTS**

Expression of full-length \(\alpha_1\) and \(\alpha_2\)-isoforms and chimeras. Transfection into OK cells of \(\alpha_1\), \(\alpha_2\), \(\alpha_1\alpha_2\), and \(\alpha_2\alpha_1\) 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 \(\alpha_2\) ISR (19). A band corresponding to 116 kDa was detected in \(\alpha_1\alpha_2\), as well as rat brain membranes or membranes containing the full-length \(\alpha_2\) that were included as positive controls (Fig. 2A). Anti-HERED did not bind to membranes from rat kidney (known to predominantly express the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-isoform), \(\alpha_1\)-transfected OK membranes, or nontransfected OK membranes that were included as negative controls. No signal was detected in membranes from \(\alpha_2\alpha_1\)-transfected OK cells (Fig. 2B), consistent with an absence of the \(\alpha_2\) ISR in the chimera. Taken together, these data suggest that the structure of the \(\alpha_1\alpha_2\) chimera was as intended, with substitution of the \(\alpha_2\) ISR into the \(\alpha_1\)-isoform. Conversely, the \(\alpha_2\) ISR was not detected in membranes expressing \(\alpha_2\alpha_1\). The same immunoblots were probed with anti-NASE, a polyclonal antibody directed against the \(\alpha_1\) ISR. Unfortunately, given the cross-reactivity of this antibody with opossum-derived \(\alpha_1\), 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-
pression of the mRNAs encoding the introduced isoforms and chimeras. Our strategy was to design primers that recognize α1 or α2 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 α1- and α2-isofom mRNA after amplification by PCR (Fig. 3). Indeed, bands of 747 and 1,425 bp were readily detected in α1- and α2-transfected cells, respectively. No signal was detected in nontransfected OK cells with any primer set. Using a direct primer specific for a region of exon1 containing plasmid as a template (positive control) or reverse-transcribed RNA from α1,α2α1-transfected cells (Fig. 2B). As one would expect, no band of the appropriate mobility was produced when α1- or α2-containing plasmids or reverse-transcribed RNA from α1- or α2-transfected cells were used as templates. Finally, Fig. 3C shows the PCR fragment obtained using a direct primer specific for α2 before the ISR and a reverse primer specific for the α1 ISR when reverse-transcribed RNA from α1,α2α2-transfected cells was amplified. As a control, a band of identical mobility (743 bp) was produced from α1,α2α2-containing plasmid. PCR performed under the same conditions did not reveal any signal from reverse-transcribed RNA of nontransfected cells, α1- or α2-containing plasmids, or reverse-transcribed RNA from α1- or α2-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+-dependent ATP hydrolysis to varying concentrations 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 NH2 termini has suggested that the distinctive kinetic behavior of α1 and α2 was not due to the NH2-terminal domain alone but rather to its interaction with other IRS of the protein (8). Accordingly, a series of experiments was designed to determine whether ISR sequence diversity was involved in the kinetic difference between α1 and α2. 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 Pi/mg protein/h for α1, α1α2α1, α2, and α2α1α2, respectively.

At 1 μM ATP, the K+ activation/inhibition profile of α1α2α1 was indistinguishable from that of α1. Na+-ATPase activity of both α1 and 1α2α1 was inhibited by 0.025–5 mM K+. In experiments with the α1α2α2 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 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 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 α1, 6.99 ± 0.76 (n = 7) for α1α2α1, 6.41 ± 1.56 (n = 11) for α2, and 7.99 ± 0.52 nmol·mg protein⁻¹·h⁻¹ (n = 6) for α2α1α2. 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 α1,α2α1, and α2α1α2-specific sequences. PCR products were obtained with rat Na+-K+-ATPase α1- and α2-specific primers (listed in Table 2; see METHODS for details) and plasmid or cellular cDNA as templates. A: α1- and α2-specific amplifications. Untransfected OK cell cDNA was used as a negative control. B and C: α1,α2α1- and α2α1α2-specific amplifications, respectively. Untransfected, α1-transfected, and α2-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.
We and others have established the in
center of the molecule, the central ISR (Ref. 19, Fig. 1).

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**DISCUSSION**

The primary structures of \(\alpha\)-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 kinet-
ics 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 \(\alpha_1\) and \(\alpha_2\) 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 ubiqui-
ous distribution of this enzyme complex. To disting-
uish an endogenous from introduced enzyme in the opossum cells, we took advantage of the varied sensi-
tivity 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 digi-
talis glycoside. Mutations severe enough to inhibit catal-
ytic 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-resist-
tant colonies after transfection with \(\alpha_1\alpha_2\alpha_1\) or \(\alpha_2\alpha_1\alpha_2\) suggests that overall enzymatic function was not com-
promised, 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 sublethal concen-
trations of ouabain is occasionally achieved, for instance, by significant overexpression of pumps rather than the introduction of a resistant enzyme (15).

![Fig. 4. Effect of \(\alpha_1/\alpha_2\) ISR exchange on K\(^{+}\) sensitivity of Na\(^{+}\)-ATPase. ATP hydrolysis was assayed in membranes from transfected OK cells. Hydrolysis of [\(\gamma\)\(^{32}\)P]ATP was measured after 30 min in the presence of 1 \(\mu\)M ATP, 20 mM NaCl, and various concentrations of KCl. Results are presented relative to ouabain-sensitive Na\(^{+}\)-ATPase activity for \(\alpha_1\) (○), \(\alpha_1\alpha_2\alpha_1\) (□), \(\alpha_2\) (●), and \(\alpha_2\alpha_1\alpha_2\) (△), respectively. To inhibit endogenous Na\(^{+}\)-K\(^{-}\)-ATPase, 3 \(\mu\)M ouabain was present in all reactions. Values are means ± SE of data obtained from 3 different membrane preparations (assays performed in trip-
licate for each K\(^{+}\) concentration). Inset: shape of the various curves between 0 and 0.4 mM K\(^{+}\) shown in an expanded scale.](http://ajprenal.physiology.org/)

![Fig. 5. Effect of \(\alpha_1/\alpha_2\) 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 86Rb\(^{+}\). PKC activation was induced by a 5-min exposure of the cells to 10 \(\mu\)M PMA before the addition of Rb\(^{+}\) compared with paired control plates of cells exposed for 5 min to the same amount of vehicle alone (DMSO). Values are means ± SE of data obtained from 3 different transfection groups (assays performed in trip-
licate for each K\(^{+}\) concentration). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).](http://ajprenal.physiology.org/)
We ensured against this eventuality by selecting cells in ouabain at concentrations well above the $K_{\text{v}}$ 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 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 $\alpha_1$ expressed in HeLa and COS-1 cells is inhibited by $K^+$, whereas $\alpha_2$ 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 $\alpha_1$ and $\alpha_2$ that is responsible for this kinetic difference. Rather, it is likely to be an interaction of the segment between 24 and 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 because exchange of the $\alpha_1$ and $\alpha_2$ 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 $\alpha_1$ 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 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 $\alpha_1$ and $\alpha_2$. When we replaced the ISR in $\alpha_1$ with the $\alpha_2$ sequence, this activation was increased twofold (Fig. 5). Conversely, substituting the $\alpha_2$ ISR with $\alpha_1$ in the $\alpha_2$-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 $\alpha_1$ 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 about what mechanism may underlie this inhibitory effect of the $\alpha_1$ 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$^{117}$ and Ser$^{18}$ (not present in $\alpha_2$-isoform) of the $\alpha_1$-isoform by the PKC $\beta$-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 PKC$\zeta$ and the adaptor AP-2, as well as the binding of phosphoinositide-3 kinase to a proline-rich motif of the $\alpha$-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-$\Phi$, where $\Phi$ 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 $\alpha_1$-isoform also displays a di-leucine motif, which appears to be in the ISR but is not present in $\alpha_2$. The di-leucine motif might represent the molecular basis of the inhibitory effect that we have attributed to the $\alpha_1$ ISR. It could act as a dynamic retention signal that favors $\alpha_1$ 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 $\alpha_1$ 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 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 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 $\alpha_1$ 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 $\alpha_1$. It may involve Ser$^{11}$ and Ser$^{18}$ phosphorylation (which are not present in $\alpha_2$) 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 $\alpha_1$ but not $\alpha_2$, 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 $\alpha_1$) 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 $\alpha_1$ and $\alpha_2$. Accordingly, removing the inhibitory ISR of the $\alpha_1$ sequence and replacing it by the “neutral” $\alpha_2$ ISR results in an increased response to PMA as shown for $\alpha_2$$\alpha_2$$\alpha_2$. On the same basis, the PMA-induced stimulation of $\alpha_2$ could be compromised by the addition of the inhibitory sequence of the $\alpha_1$ ISR, as shown by the absence of PMA-induced stimulation in $\alpha_2$$\alpha_2$$\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 $\alpha$-isoforms.

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