Sodium pumps (αβ dimers) with the α1 isofrom of the catalytic (α) subunit are expressed in all cells. Additionally, most cells express Na⁺ pumps with a second α isofrom. For example, astrocytes and arterial myocytes also express Na⁺ pumps with the α2 isofrom. The α2 pumps localize to plasma membrane (PM) microdomains overlying “junctional” sacro-/endoplasmic reticulum (S/ER), but the α1 pumps are more uniformly distributed. To study α2 targeting, we expressed α1/α2 and α2/α1 chimeras and 1–90 and 1–120 amino acid N-terminal peptides in primary cultured mouse astrocytes. Immunocytochemistry revealed that α2/α1 (but not α1/α2) chimeras markedly reduced native α2 (i.e. were “dominant negatives”). N-terminal (1–120 and 1–90 amino acids) α2 (and α3), but not α1 peptides also targeted to the PM-S/ER junctions and were dominant negative for native α2 in astrocytes and arterial myocytes. Thus α2 and α3 have the same targeting sequence. Ca²⁺ (fura-2) signals in astrocytes expressing the 1–90 α2 peptide were comparable to signals in cells from α2 null mutants (i.e. functionally dominant negative): 1 mM ATP-evoked Ca²⁺ transients were augmented, and 100 nM ouabain-induced amplification was abolished. Amino acid substitutions in the 1–120 α1 and α2 constructs, and in full-length α1, revealed that Leu-27 and Ala-35 are essential for targeting/tethering the constructs to PM-S/ER junctions.

The sodium pump (Na⁺, K⁺-ATPase) plays a critical role in maintaining a low cytosolic Na⁺ concentration ([Na⁺]ₘ) and a large Na⁺ electrochemical gradient across the plasma membrane (PM) in nearly all animal cells (1, 2). This Na⁺ gradient not only enables the generation of Na⁺−dependent action potentials in excitable cells, but it also drives many solute transport processes, including the Na⁺/Ca²⁺ exchanger that modulates Ca²⁺ signaling.

Functional Na⁺ pumps are αβ dimers (2–4). The catalytic (α) subunits contain the Na⁺, K⁺, ATP, and cardioitonic steroid binding sites (2–4). The α subunits (molecular mass of ~110 kDa) have 10 membrane-spanning helices (M1–M10), cytoplasmic N- and C-terminal segments, and a large cytoplasmic loop between M4 and M5 (Fig. 1A).

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3 The abbreviations used are: PM, plasma membrane; S/ER, sarcoplasmic reticulum; ER, endoplasmic reticulum; SERCA, sarco-/endoplasmic reticulum Ca²⁺ pump; aa, amino acid(s); Adv, adenovirus; GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; WT, wild type; KO, knock-out.

The α subunit tertiary structure has been deduced by homology modeling (5) based on the known structure of the sarco-/endoplasmic reticulum (S/ER) Ca²⁺ pump, SERCA (see Fig. 1B) (6).

There are four isofoms of the α subunit, α1–α4 (2). Most cells express two different α isofoms, α1 and either α2 or α3, but sperm express α1 and α4 (7). For example, astrocytes express α1 and α2, and most neurons express α1 and α3 (8–10). In rodents, α1 has an unusually low affinity for ouabain, whereas the high ouabain affinity binding sites in α2 and α3 have been retained through evolution (2, 11).

Na⁺ pumps with α1 subunits distribute differently from those with α2 subunits in astrocytes or α3 in neurons (9, 13), and they apparently serve different functions (14). In particular, Na⁺ pumps with α2 or α3 subunits cluster, along with the Na⁺/Ca²⁺ exchanger, in PM microdomains at PM-S/ER junctions (9). Moreover, in astrocytes α2 is structurally (13) and functionally (14) linked to the Na⁺/Ca²⁺ exchanger and thereby helps to modulate Ca²⁺ transport and Ca²⁺ signaling. In neurons, α3 likely plays a similar role (13). In contrast, α1 is more uniformly distributed in the PM but may be excluded from PM-S/ER junctional regions (“PlasmERosomes”) (9, 13). Astrocytes (14), like cardiac myocytes (15), express α1 and α2 in a 4:1 ratio, and α1 appears to be the “housekeeper” that maintains the low [Na⁺]ₘ in bulk cytoplasm.

Because α1 and α2 are differently distributed in the PM of astrocytes and other cell types, the two isoforms must be targeted and tethered by different mechanisms. There is no information on the sorting of α2, but immunocytochemical and co-immunoprecipitation data indicate that it is tethered to a complex containing the cytoskeletal protein, ankyrin-B (13, 16). In epithelia, α1 also is tethered to an ankyrin (12, 17), probably ankyrin-G (18).

The sorting of Na⁺ pump α1 and the homologous H⁺, K⁺-ATPase α subunit have been studied extensively in polarized intestinal and renal epithelia. Here, this Na⁺ pump localizes to the basolateral membrane, and the H⁺, K⁺-ATPase sorts to the apical membrane (19). The sorting signal apparently involves a region of transmembrane helix 4 (M4; see Fig. 1A) and the flanking extracellular and cytoplasmic domains (19).

The precise role of the small, highly glycosylated β subunits is uncertain. Experiments on Na⁺ pump α1 indicate, however, that association with the β subunit is required to chaperone α to the PM, as well as for the functional maturation of the pump (20). The β1 subunit (three β isoforms have been identified (21) interacts with a highly conserved amino acid (aa) sequence, SYGQ, in the extracellular loop between M7 and M8 (21) (aa 896–899 in mouse α1; see Fig. 1A). This sequence is present in most α subunit isoforms from chickens (21) to mice and humans.

A sorting mechanism based on interaction with β cannot explain the differences between α1 and α2 localization, because both α1 and α2 can co-assemble with either β1 or β2 (10, 22, 23). This promiscuous co-assemble may be a consequence of the aforementioned conservation of the SYGQ sequence in the α isoforms.

In some epithelia, cycling of α1 subunits into the PM may be governed by protein kinase C-mediated phosphorylation, and several N-terminal poten-
**Na⁺ Pump α2 Subunit Sorting Signal**

Na⁺ pump α2 subunits are sorted to different subcellular compartments, primarily to astrocytes. This sorting is controlled by the Na⁺ pump α2 subunit sorting signal (11) located at the α2 N-terminal cytoplasmic domain (11). Site-directed mutagenesis of the C-terminal Na⁺ pump α2 sorting signal establishes that this domain is involved in the selective sorting and targeting of α2 to astrocytes. The sorted α2 subunit constructs are expressed in astrocytes and not to muscle cells (Fig. 1, A and C). Alternative splicing in astrocytes can also generate constructs with a 120-aa truncated extracellular C-terminal peptide (Fig. 1, A and D). The expression of these constructs is confirmed by immunocytochemistry and Ca²⁺ imaging. In live imaging experiments, these constructs could be visualized in astrocytes.

**EXPERIMENTAL PROCEDURES**

**Primary Cultured Mouse Astrocytes—**Mice with null mutations in both Na⁺ pump α2 alleles (homozygous knockouts, α2−/−; or “KO”) were generated by mating heterozygotes (15). Standard PCR methods were used for genotyping genomic DNA from fetuses. Cortical protoplasmic type-1 astrocytes were prepared from WT and KO mouse fetuses on days 18 or 19 (E18–19) as described (14, 26). The cells were plated onto poly-1-lysine-coated 25-mm glass coverslips (≈50,000 cells/coverslip) for immunocytochemistry and Ca²⁺ imaging, or onto 10-cm Petri dishes for immunoblots. Experiments were performed on subconfluent cultures on days 7–9 in vitro. All animal protocols were approved by our Institutional Animal Care and Use Committee.

**Primary Cultured Rat Arterial Myocytes—**Arterial myocytes were dissociated from the mesenteric arteries of male Sprague-Dawley rats (100–150 g). The cells were plated onto 25-mm coverslips and were grown in primary culture for 4–6 days as described (27).

**Generation of Na⁺ Pump α2 Subunit Chimeras and Related Constructs—**Plasmids containing the DNA for rat Na⁺ pump α1, α2, and α3 subunits (pGEM-1, pBluescript II SK(+); α2 and pGEM-α3 (from Dr. R. Mercer, Washington University, St. Louis)) were used. First, the Na⁺ pump α1 and α2 subunit cDNAs were subcloned into adenosinal (Adv) vectors to generate Adv/α1 and Adv/α2. A “FLAG tag” (f) was added to the C-terminus of each subunit using PCR-based approaches. Second, domain-swaps of α1 and α2 were performed to create the Adv/α1-α2f and Adv/α2-α1f chimeras (e.g. N-terminal α1(1–130) or 1–333 aa) and C-terminal α2 with a FLAG tag; and, conversely, N-terminal α2(1–130) or 1–333 aa and C-terminal α1 with a FLAG tag (Fig. 1, A and C). Chimeras Adv/α1-α2f and Adv/α2-α1f were also subcloned into the pEGFP-C1 vector to generate constructs containing an N-terminal GFP tag (G) as well as the C-terminal FLAG tag (Fig. 1C). All sequences were confirmed by sequencing.

To transfect the α1/α2 or α2/α1 chimera constructs into astrocytes, the cultured cells were treated with purified adenoviral vector solutions. A multiplicity of infection of 10 plaque forming units/cell induced ~80% transfection of cells on coverslips in 48 h. Transfection efficiency was even higher (>80–90%) for cells in 10-cm dishes used for immunoblots. All cells were studied after 48 h.

In a few experiments, we transfected cells with plasmids containing a normal or mutated, full-length α1 with a C-terminal FLAG tag and the Ca²⁺-reporter protein, GCaMP2 (28, 29). A FLAG epitope was also in some cases, inserted at Pro-120 in the first extracellular loop (Fig. 1A). The site-specific mutations (see “Results”) were generated with “QuikChange” (Stratagene, La Jolla, CA) using the manufacturer’s directions, and the constructs were cloned into the pIRE2-DsRed2 vector (Clontech, Mountain View, CA) to facilitate visualization of transfected cells before immunostaining. These constructs were transfected using Lipofectamine 2000 (see next section). The transfected cells expressed the α1f/GCaMP2 construct and DsRed2 independently and simultaneously.

N-terminal Truncations of Na⁺ Pump α2 Subunits—DNA coding for the N-terminal 1–90 and 1–120 aa sequences for α1, α2, and α3 were confirmed by sequencing. These sites are, however, identical in the mouse α1, α2, and α3 isoforms and, thus, cannot explain isoform-specific targeting.

Here we report that α1 and α2 Na⁺ pumps are sorted by different mechanisms in astrocytes. The α2 sorting signal is located in the N-terminal cytoplasmic segment of the molecule. This selective sorting and membrane insertion of α2 apparently does not depend upon phosphorylation or upon association with β.
A. Na⁺ Pump α and β Subunit Topology

B. Mouse Na⁺ Pump α1, α2, α3 and Rabbit SERCA-1a N-terminal Sequences

C. Chimera, Fusion Protein and Truncation Constructs

D. Cross-reactivity of α1, α2 and α1-α2 Chimeras with Two Anti-α2 Antibodies

E. Cross-reactivity of Truncated α1 and α2 Constructs with Anti-Flag Antibody

FIGURE 1. Na⁺ pump transmembrane organization; α subunit N-terminal sequences; fusion, chimera, and truncation constructs; and construct expression. A, diagram of the Na⁺ pump α and β subunits. B, comparison of mouse Na⁺ pump α1, α2, and α3 isoform and rabbit skeletal muscle SERCA1a N-terminal sequences. Numbering is based on the mature α1 protein sequence; the first five residues in α1 and α2, 1–5, are absent in the mature protein. The α3 N terminus is displayed with Pro-7 aligned in all three isoforms. Helices 1 and 2 (aa in green) refer to published data (5, 6); amino acids highlighted in boxes are identical in α2 and α3, but differ in α1. C, diagrams and nomenclature of Na⁺ pump α subunit fusion protein, chimera, and truncation constructs. D, Western blots of α1/α2 and α2/α1 chimeras transfected into WT and α2 (-/-) (KO) astrocytes. Expression of the N- and C-terminal portions were detected with, respectively, two different α2-selective antibodies, Mcb2 and anti-HERED (panel A shows epitope location). All lanes were loaded with 5 µg of protein except KO with α2(1–130)/α1f and α2(1–333)/α1f, which contained 18 µg of protein. E, Western blots of N-terminal α2-truncation constructs identified with anti-FLAG antibody. All lanes were loaded with 10 µg of protein.
RESULTS

The Na\(^+\) pump α2 isoform is localized to PM microdomains that overlie the ER, whereas the α1 isoform is more uniformly distributed in several different cell types (9, 32). This differential distribution is readily apparent when wild-type (WT) astrocytes are double-labeled with α1 and α2 isoform-specific antibodies (Fig. 2A). Based on this observation, our goal was to identify the one or more regions of the Na\(^+\) pump α2 subunit (Fig. 1, A and B) that are involved in targeting and tethering this subunit isoform to its appropriate PM location in astrocytes.

Detection of Native α Subunit Isoforms and Transfected Constructs—To examine the difference between α1 and α2 targeting, we constructed α1/α2 and α2/α1 isoform chimeras and truncated segments (Fig. 1, A and B). To facilitate identification, these proteins were constructed with an N-terminal-fused green fluorescent protein, GFP (G), and/or a C-terminal FLAG tag (F). As we shall see, these tags apparently did not interfere with the targeting, tethering, or function of the constructs. Transfected and native (endogenous) α subunits also could be identified with antibodies raised against isoform-specific epitopes located at the N terminus or in the large cytoplasmic loop between trans-membrane helices 4 and 5 (Figs. 1A, 1D, and 2A) (11, 30, 31). These epitopes were especially useful for studies of transfected astrocytes from KO mice with no native α2, or for detecting repression of endogenous α2 expression.

Western blots probed with anti-α2-selective antibodies (McB2 and HERED; see Fig. 1A) show the expressed proteins in WT and KO cells directly. Thus, following fura-2 loading, Ca\(^{2+}\) signaling in transfected and non-transfected cells could be compared on a single coverslip.

\(\text{Ca}^{2+}\) and GFP Imaging in Living Cells—Cells (on coverslips) were loaded with fura-2 by incubating them (50 min, 20–22 °C) in 1 ml of physiological salt solution (PSS) containing 0.5% bovine serum albumin and 3 μM fura-2/AM (membrane-permeable acetoxymethyl ester). The coverslips were transferred to a tissue chamber mounted on a microscope stage. They were superfused with physiological salt solution (20–30 min, 32–34 °C) to remove extracellular dye and permit intracellular esterases to cleave intracellular ester into active fluorochrome.

Cells were studied for 1–2 h, during continuous superfusion with physiological salt solution (in mM: 140 NaCl, 5 KCl, 5 NaHCO\(_3\), 1.8 CaCl\(_2\), 1.4 MgCl\(_2\), 1.2 Na\(_2\)HPO\(_4\), 11.5 glucose, and 10 HEPES at pH 7.4).

The ratio of fura-2 fluorescent emission (510 nm) at two excitation wavelengths (340 and 380 nm) was used to calculate [Ca\(^{2+}\)] (14). GFP fluorescence was excited at 488 nm and emitted at 525 nm. The imaging system was based on a Nikon Eclipse 2000 inverted microscope equipped with a UV-Fluor 40 X (oil) objective lens and a Hamamatsu ORCA-ER charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ). Illumination was provided by a Sutter DG-4 filter changer (Sutter Instruments, Novato, CA). Images were acquired and analyzed with a Meta Imaging System.

Statistical Analysis—Summarized fura-2 imaging ([Ca\(^{2+}\)]) data are presented as means ± S.E.; n is the number of cells studied. Data comparisons were made with two-way analysis of variance.

FIGURE 2. Expression and distribution of native Na\(^+\) pump α1 and α2 subunits, and of transfected α1/α2 and α2/α1 chimeras in WT and α2\(^{-/-}\) (KO) astrocytes detected by immunocytochemistry. A, different distribution of α1 and α2 anti-α2 (McB2) and (B) anti-α1 (anti-NASE) antibody staining in the same non-transfected WT cell; only a portion of the cell is shown. Boxed area in a and the same region from the equivalent anti-NASE stained image are enlarged in c and b, respectively. Note the reticular distribution of the McB2, but not anti-NASE, stain. B–E, expression of α1 and α2, and transfected α1(1–130)/α2f, in WT (B and C) and KO (D and E) cells. All cells, both transfected and non-transfected, cross-reacted with anti-NASE, indicating the presence of α1. The transfected cells exhibited more intense anti-NASE fluorescence than did the non-transfected cells, however, implying that the NASE epitope is overexpressed in the transfected cells. All WT cells have α2 (detected with McB2), but only the transfected KO cells exhibit the HERED epitope (arrowhead). F and G, expression of α1 and α2, and transfected α2(1–130)/α1f, in WT cells. All cells cross-reacted with anti-NASE. Only the non-transfected WT cells cross-reacted with anti-HERED (G), even though this epitope is normally present in all (non-transfected) WT cells (see panel K). Thus, the α2(1–130)/α1f construct is “dominant negative” for native (full-length) α2 in WT cells (G, arrowheads). Similar results were obtained with α2 (1–333)/α1f transfection (not shown). H and I, expression of transfected Ca2(1–333)/α1f/α1f (H) and α2(1–333)/α1f/α1f (I) in KO cells. The chimeric construct was detected either as GFP fluorescence (H) or by cross-reactivity with McB2 (I). In both cases, the construct distribution pattern is reticular, similar to that of SERCA2b (K). The similarity between the stained structures in the left and right panels in H is readily apparent. K, expression of native α2 detected with anti-HERED in a non-transfected WT cell. The distribution of this epitope, too, is reticular. All scale bars = 10 μm.
(Fig. 1D). Both antibodies detected α2 in non-transfected and full-length α1f-transfected WT cells, but not in KO cells. Both antibodies also detected α2 in KO cells transfected with full-length α2f. When KO cells were transfected with α1(1–130)/α2f or α1(1–333)/α2f, anti-HERED, but not McB2 antibodies, cross-reacted with the membrane proteins (Fig. 1D). Conversely, when KO cells were transfected with α2(1–130)/α1f or α2(1–333)/α1f, McB2, but not anti-HERED, antibodies cross-reacted with the membrane proteins (Fig. 1D). These results are expected because of the different locations of the McB2 and anti-HERED antibody epitopes (Fig. 1A).

Expression and Distribution of α1/α2 and α2/α1 Chimeras in WT and KO Astrocytes—Chimeric constructs (see Fig. 1C for nomenclature) containing the α1 N-terminal segment, α1(1–130)/α2f (Fig. 2, B–E) or α1(1–333)/α2f (not shown) were expressed in WT and KO cells. The FLAG tag in the expressed constructs could be detected with FLAG epitope-specific antibodies in transfected cells (Fig. 2, B–E). The cross-reactivity with anti-NASE antibodies (Fig. 2, B–D) indicates that endogenous α1 is well expressed in all transfected and non-transfected cells even though this epitope is not present in the α1/α2 chimera constructs (Fig. 1, A, C, and D). In KO astrocytes, α2-specific anti-HERED antibodies labeled only the transfected cells (e.g., Fig. 2E, arrowhead in the right-hand panel). The WT cells transfected with these constructs also normally express endogenous α2, which could be labeled specifically with McB2 antibodies (Fig. 2C) that do not cross-react with the α1/α2 constructs (Fig. 1D).

Chimeric constructs with an α2 N-terminal segment, α2(1–130)/α1f, α2(1–333)/α1f, and α2β2(1–333)/α1f also were expressed in WT and KO cells (Fig. 2, F–J). In α2 KO cells, α2/α1 construct expression could be detected with anti-McB2 antibodies (Fig. 2J, see Fig. 1) as well as with FLAG (not shown) or GFP (Fig. 2H).

The HERED epitope (Fig. 1A) detects native α2 in all non-transfected WT astrocytes (Fig. 2K), but is not present in the α2/α1 chimera constructs (Fig. 1, A, C, and D). It was very surprising, however, that staining for this epitope could not be detected in WT cells transfected with either α2(1–130)/α1f (Fig. 2G, arrowheads) or α2(1–333)/α1f (not shown). In other words, either the epitope was “masked,” or these α2/α1 constructs indirectly repressed expression of native, full-length α2 (which we term a “dominant negative” effect). These constructs presumably bind competitively to the targeting complex and thereby lead to degradation of native α2.

The dominant negative effect of the α2/α1 chimeric constructs was confirmed by immunoblot (Fig. 3). When Lipofectamine was used for transfection (~50% transfection efficiency), the expression of native (full-length) α2 was reduced by ~50% (band densities normalized with GFAP, an unrelated glial cell marker, Fig. 3A). In contrast, expression of the McB2 (α2) epitope, which is contained in the α2/α1 construct as well as in native α2 (see Fig. 1, A and C), was comparable in cells transfected with the α2/α1 and the α2/α2 constructs (Fig. 3A). When adenoiral vectors, with much higher transfection efficiency, were used for transfection, native α2 was almost completely suppressed (Fig. 3B).

The α2/α1 chimeras appeared to be expressed primarily in a reticular (honeycomb) pattern (e.g., Fig. 2H), similar to the distribution of native α2 (Fig. 2, A (panels a and c) and K (32)). This distribution resembled the distribution of SERCA2b in the same cells (Fig. 2H), but overlays (not shown) indicated a broader distribution of the α2/α1 chimeras. This suggests that these chimeras are expressed, in part, in PM microdomains that overlie “junctional” (sub-PM) ER (32) or are confined within the ER. In contrast, the α1/α2 chimeras appeared to be more uniformly distributed in the PM (Fig. 2, B and D), reminiscent of the distribution of native α1 (Fig. 2A, panel b (32)).

These results raise the possibility that the α2/α1 chimeras may be sorted and tethered to the normal α2 as well as α1 distribution sites. Accordingly, the α2 sorting sequence may be contained within the N-terminal 1–130 aa segment. In subsequent studies, the dominant negative assay with anti-HERED antibodies (Fig. 2G) was used to identify the α2 sorting sequence.

Expression of α2 N-terminal Segments—The fact that the N-terminal α2 chimeras were dominant negative for native α2 raised the possibility that the N-terminal segment, alone, might have a similar effect. To explore this possibility, the effects of expressing α1 and α2 N-terminal 1–90 and 1–120 aa peptides (Fig. 1, C and E) in WT cells were examined.

The N-terminal 1–120 aa α1 and α2 peptides are both expressed in the WT astrocytes, as indicated by cross-reactivity to anti-FLAG antibodies (Fig. 4A, panels a–e). In both cases, all transfected as well as non-transfected cells cross-reacted with anti-NASE (Fig. 4A, panels a’ and c’), demonstrating the presence of native α1 in all cells. However, only the non-transfected cells and those transfected with α1(1–120)f cross-reacted with anti-HERED antibodies (Fig. 4A, panels b’ and d’). Thus, the α2(1–120)f construct was dominant negative for native α2 (Fig. 4A, panel d’, arrowheads). Neither of these constructs detectably reduced expression of α1 (Fig. 4A, panels a’ and c’).

An even shorter construct, α2(1–90)f, with no transmembrane helices (Fig. 1, A, C, and E) also was dominant negative for native α2 (Fig. 4B, panels a and a’, arrowheads). The implication is that a sequence within the N-terminal 90 amino acids preferentially binds to an appropriate “partner” and thereby displaces native α2, which is then retrieved and degraded.

As a control for the role of the N-terminal sequence in sorting, the entire initial cytosolic segment (i.e., the N terminus, through Gln-90, Fig. 1, A and B) was deleted from α2 (ΔN(1–90)α2f). This peptide, which contains all ten transmembrane helices, also was expressed in WT...
astocytes (detected with anti-FLAG antibodies). ΔN(1–90)α2f, failed to act as a dominant negative for the expression of native α2 (Fig. 4B, panels b and b’, arrowhead). In this case, native α2 was detected with McB2 antibodies, because ΔN(1–90)α2f contains the HERED epitope but lacks the McB2 epitope (Fig. 1A). This is further evidence that only a portion of the N-terminal segment plays an essential role in targeting and tethering of α2; apparently, no other parts of the molecule are required.

The immunocytochemical data in Figs. 2, 4A, and 4B were obtained in permeabilized cells, and expression of the constructs in the surface membrane could not be specifically assessed. To overcome this difficulty, immunocytochemistry on permeabilized and non-permeabilized cells was compared (Fig. 4, A–C). Here, we took advantage of the fact that the FLAG tag at the C termini of the 1–120 aa constructs are expressed in the astrocytes. Anti-NASE antibodies cross-reacted with cells transfected with either α2(1–120)f or α2(1–120)N. Anti-FLAG stain (a–e) indicates that both truncated constructs are expressed in the astrocytes. Anti-NASE antibodies detected α2(1–120)f in cells transfected with α2(1–120)f (d and d’), but not α2(1–120)f (b and b’). Insets in panels c–e (enlargements of the boxed areas) indicate that α2(1–120)f is distributed in a reticular pattern, similar to that of SERCA2a (e’). Panels e* and e** are pseudocolor images (green = anti-FLAG; red = anti-SERCA2b) of enlarged boxes from e and e’, respectively; the yellow, orange and yellow/green areas in the overlay indicate regions of overlap between the two epitopes. α2, anti-FLAG staining in permeabilized cells identifies that both α2(1–90)f (a) and ΔN(1–90)α2f (b) are expressed in WT astrocytes. α2(1–90)f is dominant negative for native α2 (a’, arrowheads), but ΔN(1–90)α2f is not (b and b’, arrowheads). Here, McB2 antibodies were used to detect native α2 (b’) in cells transfected with ΔN(1–90)α2f, because this construct contains the HERED epitope and not the McB2 epitope; the converse is true for α2(1–120)f (Fig. 1, A and C). Inset (a, enlargement of the boxed area) shows that α2(1–90)f distributes in a reticular pattern. C, the α2(1–120)f construct can be detected with anti-FLAG in non-permeabilized cells (d), because the FLAG tag is in the extracellular domain (Fig. 1, A and C). In contrast, the NASE (a’) and SERCA2b (b) epitopes are intracellular and cannot be detected in these cells even though all cells contain these epitopes, α2(1–90)f cannot be detected in non-permeabilized cells with anti-FLAG antibodies, because the FLAG epitope is intracellular in this construct. Nuclei on some coverslips were stained with 4’6-diamidino-2-phenylindole (DAPI) to identify non-permeabilized cells (b’ and c’). All scale bars = 10 μm.

An important question is whether these dominant negative effects are limited to astrocytes. Mouse artery myocytes also express α1 and α2, but not α3 Na⁺ pumps (34, 35); the α2 Na⁺ pumps, but not α1, appear to play a critical role in the long term control of blood pressure and in hypertension (35–37). Therefore, we also expressed the 1–120 aa α1 and α2 peptides in primary cultured mouse mesenteric artery myocytes. Fig. 5 shows that α2(1–120)f (Fig. 5C, panels b and b’, arrowheads), but not α1(1–120)f (Fig. 5B, panels b and b’), was also dominant negative for native α2 expression in arterial myocytes. Neither peptide affected α3 Na⁺ pumps in these cells, which we have called PLasmERosomes (14).

Functional Down-regulation of α2 by Expression of α2 N-terminal Segments—Genetically induced knock-out of α2 (α2−/−) (14) or selective inhibition with 100 nm ouabain (38) sensitizes astrocytes to agonists such as ATP and amplifies the responses to sub-maximal doses of agonists. Accordingly, reduced α2 activity should steepen or left-shift the

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**FIGURE 4.** Expression and distribution of truncated α1 and α2 in permeabilized and non-permeabilized WT astrocytes. A, detection of α1, α2, and SERCA2b in permeabilized cells transfected with α1(1–120)f or α2(1–120)f. Anti-FLAG stain (a–e) indicates that both truncated constructs are expressed in the astrocytes. Anti-NASE antibodies cross-react with cells transfected with either α1(1–120)f or α2(1–120)f as well as with non-transfected cells (a’ and c’). Native α2 is down-regulated (d’, arrowheads) in cells transfected with α2(1–120)f (d and d’), but not α1(1–120)f (b and b’). Insets in panels c–e (enlargements of the boxed areas) indicate that α2(1–120)f is distributed in a reticular pattern, similar to that of SERCA2a (e’). Panels e* and e** are pseudocolor images (green = anti-FLAG; red = anti-SERCA2b) of enlarged boxes from e and e’, respectively; the yellow, orange and yellow/green areas in the overlay indicate regions of overlap between the two epitopes. α2, anti-FLAG staining in permeabilized cells identifies that both α2(1–90)f (a) and ΔN(1–90)α2f (b) are expressed in WT astrocytes. α2(1–90)f is dominant negative for native α2 (a’, arrowheads), but ΔN(1–90)α2f is not (b and b’, arrowheads). Here, McB2 antibodies were used to detect native α2 (b’) in cells transfected with ΔN(1–90)α2f, because this construct contains the HERED epitope and not the McB2 epitope; the converse is true for α2(1–120)f (Fig. 1, A and C). Inset (a, enlargement of the boxed area) shows that α2(1–90)f distributes in a reticular pattern. C, the α2(1–120)f construct can be detected with anti-FLAG in non-permeabilized cells (d), because the FLAG tag is in the extracellular domain (Fig. 1, A and C). In contrast, the NASE (a’) and SERCA2b (b) epitopes are intracellular and cannot be detected in these cells even though all cells contain these epitopes, α2(1–90)f cannot be detected in non-permeabilized cells with anti-FLAG antibodies, because the FLAG epitope is intracellular in this construct. Nuclei on some coverslips were stained with 4’6-diamidino-2-phenylindole (DAPI) to identify non-permeabilized cells (b’ and c’). All scale bars = 10 μm.
agonist dose-response curves. This is, indeed, the case, as illustrated by the comparison of Ca$^{2+}$ transient responses to ATP in WT and α2 KO cells (Fig. 6A).

The dominant negative α2 constructs should have effects similar to α2 KO or low dose ouabain. This is exemplified in WT astrocytes transfected with the Gα2(1–90) construct (Fig. 1C). Fig. 6B (panel a) shows a field with two representative astrocytes: The cell on the right was transfected (indicated by the presence of GFP), whereas the cell on the left was not (Fig. 6B, panel b). ATP (0.1 μM) induced a small Ca$^{2+}$ transient (measured with fura-2) in the non-transfected cell (Fig. 6B, panel c, top

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**FIGURE 5.** Expression of truncated α1 and α2 constructs in permeabilized rat mesenteric artery myocytes. A. non-transfected myocytes: cross-reactivity with anti-NASE (a) and anti-HERED (b) antibodies indicates that these cells express both α1 and α2 Na$^{+}$ pumps. B, myocytes transfected with α1(1–120)f: both transfected cells (detected with anti-FLAG antibodies) and non-transfected cells express native α2 (b+) as well as α1 (a+). C, myocytes transfected with α2(1–120)f; transfected cells (a and b) express α1 (a') but are dominant negative for α2 (b', arrowhead). All scale bars = 10 μm.
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When this cell was treated with 100 nM ouabain, the response to 0.1 μM ATP was greatly augmented. In contrast, the transfected cell exhibited a large Ca²⁺ transient in response to 0.1 μM ATP (as if α2 had already been inhibited), and the response was not augmented by 100 nM ouabain (Fig. 6B, panel c, bottom panel). Summarized data (Fig. 6C) indicate that the response to ATP was significantly augmented in cells transfected with Ga2(1–90), in which native (functional) α2 expression was, presumably, markedly reduced. Also, the usual augmentation of the ATP-evoked response by 100 nM ouabain in WT cells was attenuated in transfected cells (Fig. 6B, panel c, bottom panel versus top panel). This is expected in cells in which expression of the high ouabain affinity α2 receptor is reduced. (Recall that in rodents, α1 has a very low affinity and should not respond to 100 nM ouabain (2, 14, 35).) Thus, the effects observed in Ga2(1–90)-transfected cells were comparable to the results observed in ouabain-treated (i.e., α2-inhibited) cells or in astrocytes from α2−/− mice. Clearly, the reduced α2 function in the cells expressing Ga2(1–90) reflects the dominant negative effect of the non-functional, N-terminal α2 peptide on α2 expression.

It is important to know if the N- and C-terminal fusion peptides, i.e., GFP and FLAG, alter α2 function. This was tested by transfecting Ga2F (with the complete α2 sequence; Fig. 1C) into KO astrocytes. Fig. 6D, panel a, shows two representative KO astrocytes. The one on the left was transfected and expressed Ga2F, as indicated by the GFP fluorescence in panel b. The response of the non-transfected cell to 0.1 μM ATP was not augmented by 100 nM ouabain (Fig. 6D, panel c, bottom panel). This indicates that Ga2F was able to “rescue” the low dose ouabain effect in α2 KO cells. Because ouabain is hydrophilic, the response to ouabain implies that Ga2F was expressed in the PM. Thus, the N-terminal GFP and the C-terminal FLAG tag apparently do not interfere with normal Na⁺ pump sorting and function in the Ga2F construct.

Expression of α3 N-terminal Segments—There is evidence that the expression of Na⁺ pumps with α2 and α3 subunits is mutually exclusive. For example, neonatal rat cardiac muscle expresses α1 and α3, whereas α1 and α2 are present in adult cardiac muscle (39). In neurons, the Na⁺ pump α3 isoform is expressed with a distribution comparable to that of α2 in astrocytes; i.e., α3 is confined to PM microdomains that overlie the sub-PM ER, and it co-localizes with SERCA (9, 32). Neuronal α3 and astrocyte α2 both co-immunoprecipitate comparable cytoskeletal and ER proteins (13). Moreover, the N-terminal sequences of α2 and α3 are very similar (Fig. 1B). For these several reasons, we speculated that the α3 isoform might have the same sorting signal as α2, but a different tissue-specific promoter. Therefore, the effect of transfection with α3(1–120)f was tested on the expression of α2 in astrocytes. As shown in Fig. 7A, panels a–c, α3(1–120)f is expressed in astrocytes, and is distributed in a reticular pattern similar to that of SERCA2b (panels c and c’ insets, and see Fig. 7B, panel a inset). Indeed, the α3 N-terminal segment, like its α2 counterpart, markedly reduced native α2, but not α1 expression (Fig. 7A, panel b’ versus a’). Also, α3(1–120)f experiments on non-permeabilized cells demonstrate that α3(1–120)f is expressed in the PM (Fig. 7B, panel a). The similar distribution patterns of α3(1–120)f and SERCA2b (Fig. 7A, panel c and c’), therefore, indicates that they are expressed in different but adjacent membranes. The implication is that the targeting and tethering mechanisms for α2 and α3 are identical or very similar.

Alteration of Targeting by Site-specific Mutations in the N Termini of α1 and α2 Subunits—This similarity between the effects of the α2 and α3 N-terminal constructs, led us to contrast their amino acid sequences with that of the α1 N terminus. At only three positions are the α2 and α3 N termini identical, and different from α1: residues 27, 35, and 64 (using the numbering for mature α1, Fig. 1B). To test the possibility that these specific amino acid residues (aa) play a critical role in α2 targeting and tethering, each of the three residues in the α2(1–120)f construct was mutated, one at a time, to the corresponding aa in α1, i.e., L27M, A35S, and Q64A. Astrocytes were then transfected with these mutated constructs (e.g., α2(1–120, L27M)). The results are clear: mutations L27M and A35S (Fig. 8A, panels b and c, arrowheads in lower panels), but not Q64A (Fig. 8A, panel d, arrowhead), abolished (“knocked out”) the dominant negative activity of the N-terminal α2 construct. Thus, both Leu-27 and Ala-35 are essential for α2 targeting.

The complementary experiment was then performed: the aforementioned three α2/α3 amino acids were mutated, one at a time and together, into α1(1–120)f. When these constructs were then transfected into astrocytes (Figs. 8B and 9), only the two α1(1–120)f peptides that contained both the M27L and S35A mutations were dominant negative for α2 (Fig. 8B, panels d and f, arrowheads in lower panels); the A64Q mutation was not needed (Fig. 8B, panel e). Furthermore, the two α1(1–120)f constructs with both M27L and S35A were sorted to the PM as indicated by the detection of the FLAG epitope (Fig. 9B, panels a and b), but not the SERCA2b epitope (Fig. 9B, panels a’), in non-permeabilized cells (Fig. 9B shows the three-mutation construct). Also, like native, full-length α2 and α2(1–120)f (Fig. 4A, panels e), α1(1–120, M27L, S35A, and A64Q) were distributed in the PM in a reticular pattern and co-localized with SERCA2b in the underlying ER (detected following permeabilization: Fig. 9A (panel c) and B (panel d)). The introduction of dominant negative activity into α1(1–120)f with the mutations, M27L and S35A, and the sorting to the PM microdomains that overlie adjacent (junctional) ER, confirm the key role of Leu-27 and Ala-35 in the targeting and tethering of α2.

Expression of Wild-type and Mutated Full-length α1 Constructs—We also tested full-length α1 with both the M27L and S35A mutations on α2 expression. This construct should contain the normal α1 targeting
sequence (see "Discussion") as well as the α2 targeting sequence. In this case, we used an α1f construct with a C-terminal Ca^{2+}-sensitive fusion protein (GCaMP2) that is expressed in the PM with the same broad distribution as native α1 (29). Cells transfected with the construct containing the WT α1 sequence (detected with anti-FLAG antibodies), like non-transfected cells, expressed the HERED (α2) epitope (Fig. 10A); i.e. the normal α1 sequence was not dominant negative for α2. In contrast, cells transfected with mutated full-length α1 constructs containing the α2 amino acids, Leu-27 and Ala-35, did not express the HERED epitope (arrowheads in Fig. 10, B and C, panel a). Thus, even the full-length α1 with the M27L and S35A mutations was dominant negative for native α2.

In some experiments, a FLAG epitope was inserted into the mutated, full-length α1 at Pro-120 in the extracellular loop between transmembrane helices M1 and M2. This epitope cross-reacted with anti-FLAG antibodies in non-permeabilized, transfected cells (Fig. 10, C (panel b) and D (panels a and b)). In contrast, the intracellular NASE (α1) pump α2 subunit sorting signal...
epitope, present in all cells (e.g. Figs. 4A (panels a’ and c’), 5A (panel a), and 5C (panel a’)) and included in this mutated α1 construct (Fig. 1A), was not detected in the non-permeabilized cells (Fig. 10C, panel b). Thus, this full-length construct, α1f120(M27L,S35A)f/GCaMP2, was expressed in the PM. The intracellular SERCA2b epitope also did not cross-react with anti-SERCA2b antibodies in non-permeabilized cells (Fig. 10C, panel b). Non-permeabilized cells were cross-reacted with anti-FLAG antibodies (D, panel b) to identify the constructs expressed in the PM. The cells were subsequently permeabilized with Brij 58 (“Experimental Procedures”) and were then cross-reacted with anti-SERCA2b antibodies (D, panel bii) to identify the ER. The insets and color overlay indicate that there is some overlap between the FLAG and SERCA2b labels, but the FLAG tag (green, b*) is more widely distributed than the SERCA2b label (bii*). All scale bars = 10 μm.

**DISCUSSION**

**N-terminal Constructs of α2 Are Dominant Negatives—Na⁺ pumps with α2 subunits in astrocytes are localized to PM microdomains that overlie junctional ER (32). Here, they are part of a complex that includes the Na⁺/Ca²⁺ exchanger and certain cytoskeletal and ER proteins (13). By functionally coupling with the Na⁺/Ca²⁺ exchanger and SERCA, these Na⁺ pumps help regulate Ca²⁺ homeostasis and Ca²⁺ signaling in astrocytes (14). This was confirmed by functional studies (Fig. 6), which show that knockdown of α2 by a dominant negative construct augments Ca²⁺ signaling. To elucidate, further, the central role of the α2 Na⁺ pumps in astrocyte function, we examined the mechanism of targeting and tethering these Na⁺ pumps to their appropriate location in the PM.**

One method that has been employed successfully to study differences in the targeting of membrane proteins with sequence homologies involves the use of chimeras (19). Application of this method to Na⁺ pump α1 and α2 isoforms not only indicated that the α2 N-terminal was important for targeting but, unexpectedly, also revealed that chimeras with an α2 N-terminal were dominant negative for native α2. We then employed this “dominant negative assay” to identify two amino acids, Leu-27 and Ala-35, in the N-terminal segment of the Na⁺ pump α2 subunit that are essential for the targeting and tethering of this subunit to its proper location in the PM. The α1 isoform in these cells is targeted by different but as yet unresolved mechanisms.

The observations, that α1(1–120, M27L, S35A ± A64Q) sorted to the PM microdomains that overlie junctional ER, co-localized with SERCA and was dominant negative for native α2 (Fig. 9), confirm that the α2 N
terminus contains all of the targeting information for this isofrom. These mutated, truncated α2 constructs were apparently excluded from the remainder of the PM (Fig. 9A, panels b and b'). In contrast, the full-length α1f120(M27L, S35A)f/GCaMP2, which also was dominant negative for native α2, should contain the α1 as well as α2 sorting signals, because the α1 sorting signal is not located at the N terminus (19). Thus, this construct apparently sorts to all areas of the PM (Fig. 10D, panel b). A similar explanation may account for the finding that the α2/α1 chimeras did not strictly overlap with SERCA2b even though the distribution of the chimeras appeared to be predominantly reticular (e.g. Fig. 2, H and f).

The fate of the displaced native α2, following transfection with the dominant negative gene constructs, and whether new, full-length α2 is then synthesized, are unknown. The decline of cross-reactivity with anti-HERED antibodies implies that the native α2 is synthesized and either retained within the Golgi-ER network and degraded or is transported to the PM but is rapidly retrieved and degraded. The α2 N-terminal constructs are overexpressed and should be present in relatively high concentration. Thus, it seems reasonable to speculate that, by “mass action,” they displace native α2 from their normal binding (tethering) sites on the cytoskeleton. One possibility is that α2 (or the N-terminal construct) is assembled with its “target protein” within the Golgi-ER network. The target protein (Fig. 11) may then chaperone α2 (or the construct) to its correct location at the PM-S/ER junction. The observations with α2(1–90)f (Fig. 4, B and C) demonstrate that PM insertion is not required for the dominant negative effect. We cannot be certain, however, whether this construct is exported from the ER or resides on the binding site at the PM-ER junction normally occupied by native α2.

Because all of our constructs were tagged with a C-terminal FLAG, and/or an N-terminal GFP fusion protein, it is important to ask whether these tags affected targeting and tethering or function. The evidence indicates that neither tag interfered with the normal distribution of the α2 constructs as illustrated by their dominant negative effects and correct localization (e.g. see Figs. 2H, 6B, 6D, and 10, B–D). Also, the ability of full-length Ga2f to “rescue” normal ouabain-sensitive α2 Na+ pump activity in KO cells (Fig. 6D) indicates that neither tag abolishes α2 Na+ pump function, although we cannot rule out an alteration in kinetics.

Structure and Function of the α2 N-terminal Domain—Critical issues about α2 targeting and tethering concern the precise structure of the N-terminal and the protein(s) to which this terminal is tethered. The crystal structure of the Na+ pump α subunit has not yet been determined, but the structure of the related P-type ATPase, SERCA, is known (6, 40). Moreover, there are extensive sequence, domain, and mechanistic similarities between the Na+ pump α subunit and SERCA. Indeed, homology modeling based on the SERCA structure has been very useful (5).

At the N terminus, the Na+ pump α1 subunit is 34 amino acids longer than SERCA1α (the skeletal muscle isoform). Moreover, sequence homology begins with His-39 (using α1 numbering) and the tetrapeptide, HKLS in α1 and α2, HKMS in α3, and HSKS in SERCA1α (Fig. 1B). The N terminus of SERCA contains two short helical segments that have homologous regions in α1, at amino acids 42–50 (helix 1) and 60–70 (helix 2). Even the homologous 9-aa “break” between these helices has 33–44% sequence identity. These helices and the intervening break form part of the “activator” (A) domain in SERCA and, presumably, in the Na+ pump α subunit. During the transport cycle, the A domain rotates into and out of the pocket between the nucleotide-binding (N) and phosphorylation (P) domains (Fig. 11). This rotation influences the opening and closing of the ion gates, because the A domain moves M1 (aa 91–112 in α1), to which it is attached by a 20-aa stalk (aa 71–90). It is the movement of M1, when the A domain pulls on the stalk, that apparently opens and closes the ion gates. This has been directly demonstrated for the Ca2+ gates in SERCA (40) and likely also occurs in the Na+ pump α subunit.

These homologies and presumed functional similarities between SERCA and the Na+ pump α subunit suggest that N-terminal amino acids 39–90 in the Na+ pump α subunit are not involved in targeting and tethering. Indeed, this helps to explain why the α1–α2 differences at aa 64 (Gln versus Ala within helix 2 in the A domain) does not affect targeting and tethering. Thus, it is not surprising that control of α2 targeting/tethering resides in a portion of the N-terminal first 38 amino acids. The fact that the critical Leu-27 and Ala-35 residues raise the possibility that these two amino acids are two turns apart on another helical segment in the Na+ pump that is lacking in SERCA. Our data demonstrate that these two residues are critically important in targeting/tethering α2 to its appropriate locus on the cytoskeleton. Fig. 11 shows a model that illustrates this relationship.

In epithelia, ankyrin binding to the α1 Na+ pump is required to “assemble and stabilize” this isoform at its location in the basolateral PM (41). This isoform apparently binds to both red cell ankyrin (ankyrin-R, a product of the Ank1 gene) and ankyrin-G (a product of the Ank3 gene (18, 42)). The ankyrin attachment sites are located on both the M2–M3 and M4–M5 cytoplasmic loops (18). Morrow and colleagues (41) suggest that ankyrin binds to a loop formed by α subunit residues 152–158 (MESKFKNM) in the M2–M3 cytoplasmic domain. This sequence is extremely well conserved in vertebrate species (human, rat, mouse, dog, pig, and frog) and isoforms (identical in α1 and α3, and a conservative E–D substitution in α2). However, α2, in astrocytes, and α3, in neurons, complex with ankyrin-B, and not ankyrin-G (13). Moreover, as discussed above, in these cells α2 and α3 are localized to PM microdomains at PM-ER junctions, which is different from the distribution of α1 in these cells. And, as shown here, α2 (and, perhaps α3) is (are) targeted and tethered by an N-terminal sequence located in first 33 amino acids (α2 is 2 amino acids shorter, and α3 is 10 amino acids shorter than α1;
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see Fig. 1B). Whether this N-terminal sequence is an ankryin-B attachment site remains to be determined.

In conclusion, our data and this analysis indicate that the 9-residue sequence, starting at position 27 (α1 numbering), LDELKKEVA, targets and tethers α2 to its appropriate location in the PM. The sequence is identical in mouse, rat, and human α2. The α3 sequence in all three species differs by the conservative substitution of an Asp for the Glu at position 29.

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