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The gamma subunit of the Na,K-ATPase induces cation channel activity

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ABSTRACT **The γ subunit of the Na,K-ATPase is a hydrophobic protein of approximately 10 kDa. The γ subunit was expressed in Sf-9 insect cells and *Xenopus* oocytes to ascertain its role in Na,K-ATPase function. Immunoblotting has shown that the γ subunit is expressed in Sf-9 cells infected with recombinant baculovirus containing the cDNA for the human γ subunit. Confocal microscopy demonstrates that the γ subunit can be delivered to the plasma membrane of Sf-9 cells independently of the other Na,K-ATPase subunits and that γ colocalizes with α1 when these proteins are coexpressed. When Sf-9 cells were coinfected with α1 and γ, antibodies to the γ subunit were able to coimmunoprecipitate the α1 subunit, suggesting that γ is able to associate with α1. The γ subunit is a member of a family of single-pass transmembrane proteins that induces ion fluxes in *Xenopus* oocytes. Evidence that the γ subunit is a functional component was supported by experiments showing γ-induced cation channel activity when expressed in oocytes and increases in Na^+ and K^+ uptake when expressed in Sf-9 cells.**

The Na,K-ATPase (Na pump, or Na,K pump) uses energy provided by the hydrolysis of ATP to establish and maintain the high internal potassium and low internal sodium concentrations characteristic of most animal cells. The Na pump consists of two major noncovalently linked subunits: a 110-kDa multispanning membrane protein termed the α subunit, and a smaller glycosylated polypeptide of 40–60 kDa, the β subunit. Although all catalytic functions of the enzyme have been assigned to the α subunit, both subunits are required for the functional expression of normal Na,K-ATPase activity (1–3). Multiple isoforms of both the α (α1, α2, α3, and α4) and β (β1, β2, and β3) subunits have been identified in mammalian tissues. These isoforms exhibit a tissue-specific and developmentally regulated pattern of expression that may be important in adapting Na pump function to cellular demands (reviewed in ref. 4).

A third protein, termed the γ subunit, also has been identified in purified preparations of the enzyme. The γ subunit is a small, hydrophobic polypeptide of 8–14 kDa and was considered to be a contaminant of purification until it was shown that this protein, along with the α and β subunits, could be covalently labeled by photoaffinity derivatives of the specific inhibitor, ouabain (5, 6). Other evidence that the γ subunit is a component of the Na,K-ATPase is that the subunit colocalizes with the α subunit in nephron segments and coimmunoprecipitates with $\alpha\beta$ complexes (7). The deduced amino acid sequences of γ subunits from rat, mouse, cow, sheep, and *Xenopus laevis* indicate that they are ≈76% homologous (7, 8), whereas the homology among mammalian species is 93%. A search of the DNA database revealed a cDNA representing the human γ subunit (accession no. X86400). This cDNA contains an extended 5′ sequence, leading to an expanded N-terminal domain. The human γ subunit consists of 96 amino acids with a predicted molecular weight of 10690. Computer analysis of the protein sequence predicts a single transmembrane domain of 19 amino acids with an extracellular N terminus and a highly charged intracellular C terminus (9).

The exact role of the γ subunit in Na,K-ATPase function is unknown, although its necessity for both ATPase and transport activity has been questioned experimentally (10–12). For example, detergent extraction of the γ subunit from purified Na,K-ATPase does not affect the ATPase activity of the enzyme (10). Moreover, expression studies have demonstrated that the α and β subunits are sufficient for the functional expression of Na,K-ATPase activity and expression of the γ subunit with the rat α1 and β1 subunits in yeast does not affect the affinities of the enzyme for Na^+ or ouabain (12). Recently, however, it was shown that the γ subunit can modify the voltage dependence of K^+ activation of the $\alpha1\beta1$ isozyme when expressed in *Xenopus* oocytes (8). In addition, it appears that the γ subunit can stabilize the E_1 conformation of the enzyme (13) and may be required for cavitation in mouse embryos (14). The γ subunit belongs to a family of small membrane proteins containing a single transmembrane domain. As shown in Fig. 1, phospholemman (15), channel-inducing factor (CHIF; ref. 16), and Mat-8 (mammary tumor, 8 kDa; ref. 17) share substantial homology with the γ subunit. Phospholemman is a membrane protein that in myocardium is the major plasma membrane substrate for protein kinase A and protein kinase C. CHIF is a corticosteroid-induced protein found in the colon and kidney, whereas Mat-8 is expressed in breast tumors and appears to be a marker of cells transformed by Neu or Ras oncoproteins. When expressed in *Xenopus* oocytes phospholemman and Mat-8 induce hyperpolarization-activated Cl-currents, whereas CHIF evokes slowly activating, depolarization-induced K^+ currents. Early evidence suggested that these proteins may activate endogenous channels, however, it appears that phospholemman forms a taurine-selective ion channel when incorporated into a synthetic lipid bilayer (18). In the absence of taurine, phospholemman may function as either a cation or anion selective channel (19). The channel seems to undergo voltage-dependent transitions among conformations with separate ion selectivity properties. This result may underlie the different apparent ion selectivities of the members of this group of proteins.

To better understand the role of the γ subunit in Na,K-ATPase function we expressed the human γ subunit in *Xenopus* oocytes and Sf-9 insect cells. In this paper we demonstrate

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Abbreviations: HBS, Hepes-buffered saline; IAP, integrin-associated protein.
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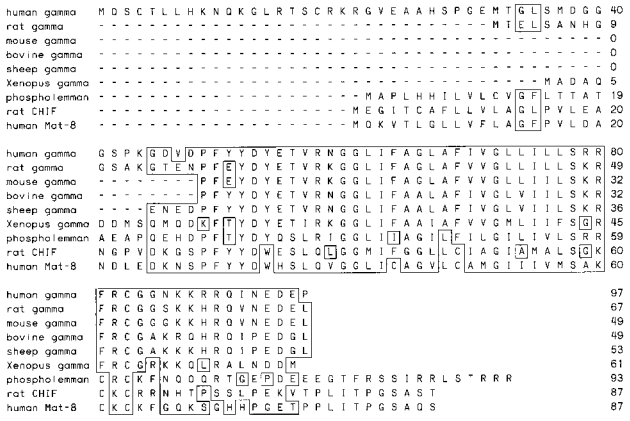

FIG. 1. Alignment of the deduced amino acid sequences of the γ subunit of the Na,K-ATPase from various sources to phospholemman, rat channel-inducing factor, and human Mat-8.

that when expressed in Sf-9 cells the γ subunit is delivered to the plasma membrane independently of the other subunits. Moreover, the γ subunit also can assemble with the α1 subunit in infected Sf-9 cells. When expressed in *Xenopus* oocytes the human γ subunit can induce large inward Na^+ and K^+ currents. Similarly, increases in Na^+ and K^+ fluxes are found when the γ subunit is expressed in Sf-9 cells. These results suggest that the γ subunit is a structural component of the Na,K-ATPase and may participate functionally by inducing ion channel activity.

MATERIALS AND METHODS

DNA and Viral Constructions. The rat α1 and β1 (20, 21) and human γ Na,K-ATPase (Estelle Austruy, Hopital Necker-Enfants Malades, Paris, France; accession no. X86400) cDNAs were subcloned into the baculovirus expression vector p2Bac. Recombinant baculovirus preparation and selection were performed by following standard procedures (22).

Cells and Viral Infections. Uninfected and infected Sf-9 cells were grown in 150-mm Petri dishes in TNM/FH medium (JRH Biosciences, Lenexa, KS), supplemented with 10% (vol/vol) fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone. Viral infections were done at a viral multiplicity of infection ranging from 5 to 10.

PAGE and Immunoblot Analysis. Cells were scraped from the plates in the incubating medium 48–72 hr after infection, centrifuged at 1,500 × *g* for 10 min, and washed three times in 10 mM Tris·HCl, pH 7.4/1 mM EGTA. The final pellet was resuspended in the same solution. Expressed proteins were analyzed by SDS/PAGE and immunoblotting. Proteins were separated by SDS/PAGE (23) and transferred to nitrocellulose (Hybond C^+, Amersham) as described (24). Nitrocellulose blots were blocked in Blotto [5% (wt/vol) nonfat dry milk, 0.1% sodium azide in 150 mM NaCl, 25 mM Hepes, pH 7.4] for 2 hr at room temperature or overnight at 4°C. Primary antibody in 1% Blotto was bound at 37°C for 1–2 hr on a rocking table. After two 10-min washes in 150 mM NaCl, 25 mM Hepes, pH 7.4 (HBS) and one 10-min wash in 0.5% Triton X-100 in HBS, 1–2 μCi of ^{125}I-labeled goat anti-mouse or goat anti-rabbit secondary antibody was added in 1% Blotto. The blots were incubated for 45–60 min at 37°C and washed as above. After washing, the blots were dried and exposed for autoradiography. The α1 subunit was identified with a mAb specific to the α subunit (C464–6B; Michael Caplan, Yale University). The human γ subunit was identified with a γ-specific polyclonal (γ969).

Immunoprecipitations. After 48 hr, α1-, α1γ-, or γ-infected Sf-9 cells growing in 6-well plates were incubated for 1 hr in methionine- and cysteine-free medium. The cells were metabolically labeled for 1 hr with 50 μCi of ^{35}S Translabel in the same medium. The cells then were washed three times with HBS and lysed in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in HBS. After removal of insoluble material in a microcentrifuge (10 min, at 15,000 × *g*), 150–200 μg of total protein was resuspended in 500 μl of 1% CHAPS in HBS. For immunoprecipitation of α1, 25 μl of an anti-α1 monoclonal (C464–6B), and for γ, 30 μl of anti-γ polyclonal (γ 969) were added. One hundred microliters of either goat anti-mouse or goat anti-rabbit coated magnetic beads then were added (BioMag, PerSeptive Diagnostics, Cambridge, MA). After overnight incubation on a rotating stand at 4°C, beads were isolated by holding the tubes to a magnet. The supernatant was aspirated, and the beads were washed three times in 1% CHAPS in HBS. The precipitated proteins were eluted by resuspending the washed beads in sample buffer (100 mM Tris·HCl, pH 6.8/2% SDS/33% glycerol/100 mM DTT) and incubating for 10 min at 65°C. The eluted protein was separated by SDS/PAGE and transferred to nitrocellulose. Nitrocellulose blots were dried and exposed for autoradiography overnight.

γ Antibody Preparation. Polyclonal antibodies to the human γ subunit were prepared by immunizing rabbits with a synthetic peptide of the N terminus of human γ (amino acids 47–60) coupled to hemocyanin or to BSA.

Confocal Microscopy. Cells were seeded onto sterile 12-mm coverslips in 24-well tissue culture plates at a density of 5×10^6 cells/well and allowed to attach for 1 hr. A total of 100 μl of α1β1 and/or γ viral stock was added. After 48 hr, cells were treated for 1 hr with 100 μg/ml cycloheximide. The cells then were fixed for 30 min at room temperature with 220 mM $HgCl_2$/92 mM sodium acetate (B5) and 4% formaldehyde. Cells were permeabilized with rapid washes in 50% and then 100% methanol. The coverslips then were washed four times in HBS and incubated overnight in HBS + 2% goat serum. After three washes with HBS, coverslips were incubated overnight with a polyclonal anti-γ antibody (Ab G17; ref. 7) at a 1:50 dilution in HBS + 0.2% goat serum. For double labeling, coverslips were incubated with a monoclonal ascites specific to the α subunit (5α; Doug Fambrough, Johns Hopkins University) at a 1:10,000 dilution and the G17 polyclonal in HBS + 0.2% goat serum. After three 5-min washes in HBS, fluorescein isothiocyanate-conjugated goat anti-rabbit (Jackson ImmunoResearch) was added at a 1:100 dilution in HBS + 0.2% goat serum and incubated for 1 hr in the dark. For double labeling, Texas red-conjugated anti-mouse secondary (Jackson ImmunoResearch) was included at a 1:100 dilution. After one 15-min wash, coverslips were inverted onto Fluoroguard (Bio-Rad) and sealed with nail polish. Images were analyzed on a Zeiss Axioplan Microscope fitted with a Bio-Rad MRC Confocal Imaging System under a 63× objective. Images are the average of at least five scans.

cRNA Preparation and Oocyte Expression. cDNA for the human γ subunit was subcloned into pXOV-60. This vector contains promoter elements for *Xenopus* globin, which promotes high levels of expression in *Xenopus* oocytes. Capped cRNA was generated by using an SP6 mMessage mMachine *in vitro* transcription kit according to the manufacturer's instructions (Ambion, Austin, TX). Stage V-VI *Xenopus* oocytes were isolated by partial ovariectomy under tricaine anesthesia. Oocytes then were defolliculated by treatment with 1 mg/ml of collagenase (Sigma type 1A) in zero Ca^{2+} ND96 (below) for 1 hr. Between 2 and 24 hr after defolliculation, oocytes were pressure-injected with ≈50 nl of 100 ng/μl of cRNA. Oocytes were maintained in ND96 containing 1.8 mM Ca^{2+}, 100 units/ml of penicillin, and 100 μg/ml of streptomycin for 1–3 days before experimentation.

Electrophysiology. Ionic currents were studied by standard two-microelectrode voltage-clamp technique using an OC-725

voltage-clamp apparatus (Warner Instruments, New Haven, CT). The solutions used in these experiments had the following compositions: ND96 (96 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM Na Hepes, pH 7.5); KD98 (98 mM KCl/1 mM $Mg(OH)_2$/5 mM K Hepes, pH 7.4); Tris98 (98 mM Tris·HCl/1 mM $Mg(OH)_2$/5 mM K Hepes, pH 7.4). Microelectrodes were pulled from thin-walled capillary glass (W-P Instruments, New Haven, CT) on a horizontal puller (Sutter Instruments, Novato, CA), and tips were mechanically broken to bring electrode resistance to 0.5–2 MΩ when filled with 3 M KCl solution. PClamp software and a Digidata 1200 converter were used to generate voltage pulses and collect data. Additionally, data were normally filtered at 5 kHz, digitized at 22 kHz (Neurocorder, Neurodata, NY) and stored on videotape. When necessary, data were redigitized into a microcomputer using Axotape software (Axon Instruments, Foster City, CA).

^{86}Rb and ^{22}Na Uptake. Sf-9 cells, which were infected for 48 or 72 hr and grown in 60-mm dishes, were incubated at room temperature in preincubation medium. For K uptake the preincubation medium contained 150 mM NaCl, 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, and 1% BSA. For the determination of Na uptake, 280 mM sucrose replaced the NaCl. After 1 hr the medium was replaced with identical medium containing 0.1 mM bumetanide. After 10 min the medium was aspirated, and the flux was started by adding 1.5 ml of 100 mM KCl, 50 mM NaCl, 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 1% BSA, 0.1 mM bumetanide, and ^{86}Rb (1 μCi/ml; New England Nuclear). For Na uptake the flux medium contained 100 mM NaCl, 50 mM KCl, 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 1% BSA, 0.1 mM bumetanide, and ^{22}Na (1 μCi/ml). At prescribed intervals, the medium was aspirated, and the cells were washed three times with 3 ml of ice-cold 116 mM $MgCl_2$. The final wash was removed, and the plates were allowed to air dry. The cells were solubilized with 1.5 ml of 0.1 M NaOH, and aliquots were counted by liquid scintillation. Each time point is an average of four replicates.

Biochemical Assays. Protein assays were performed according to the procedure of Smith *et al.* (25), using the bicinchninic acid/copper sulfate solution as described by the supplier (Pierce).

RESULTS

A recombinant baculovirus containing the cDNA for the human γ subunit was used to infect Sf-9 cells, an insect cell line derived from the ovary of the fall armyworm *Spodoptera frugiperda*. To assay the production of γ polypeptides, proteins from infected Sf-9 cells were immunoblotted 72 hr after infection (Fig. 2*A*). In membranes from γ-infected cells, a γ-specific polyclonal antibody (γ 969) recognizes a protein of approximately 12 kDa that is not present in uninfected cells. Preimmune serum does not react to this polypeptide. The high molecular weight band observed in uninfected cells is a nonspecific reaction of the polyclonal antibody. To determine whether the γ subunit can associate with the Na,K-ATPase α subunit, metabolically labeled cells expressing α1, γ, or α1 and γ were lysed with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in HBS for 15 min. After the insoluble material was pelleted, protein from cells infected with the α1 baculovirus were immunoprecipitated with a monoclonal α1 antibody (C464–6B). Protein from cells infected with the α1γ or γ viruses were immunoprecipitated with a polyclonal γ antibody (γ 969). As shown in Fig. 2*B*, cells infected with α1 alone do not contain proteins corresponding to γ, and γ-infected cells do not contain α1 polypeptides. Only in α1γ-infected cells are γ antibodies able to precipitate both α1 and γ, suggesting that these proteins are able to associate in the coinfected cells. Similar results are obtained when the γ subunit is expressed with the α1 and β1 subunits (data not shown).

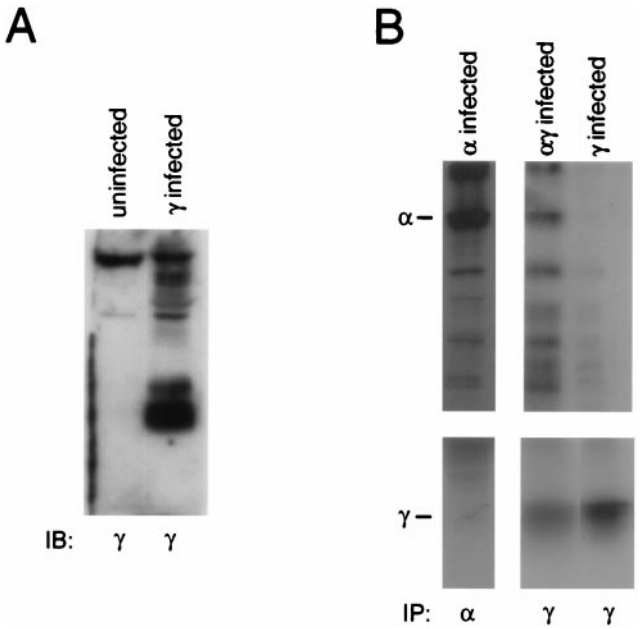

FIG. 2. Expression of the γ subunit in Sf-9 cells and association with the α1 subunit. (*A*) An immunoblot of protein from cells infected with baculoviruses containing the cDNA for the human γ subunit or uninfected cells. The blots were probed with a γ specific polyclonal (γ969). (*B*) An immunoprecipitation of protein from metabolically labeled Sf-9 cells infected with α1, α1γ, or γ baculoviruses. Protein from α1-infected cells was immunoprecipitated with an α1-specific monoclonal (C464–6B). Protein from γ- and α1γ-infected cells was immunoprecipitated with γ969.

The cellular localization of the γ subunit in infected cells was analyzed by using confocal microscopy. To clear the intracellular organelles of proteins in biosynthetic transit, cells were preincubated for 1 hr in cycloheximide, an inhibitor of protein synthesis. Cells were coinfected with α1β1 and γ baculoviruses or individually infected with a γ baculovirus. After 48 hr, cells were fixed, permeabilized, and probed with a γ-specific polyclonal, Ab-G17 (7). For double labeling, cells were probed with the G17 polyclonal and the α-specific (5α) mAbs. Bound antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse secondary antibodies. As shown in Fig. 3*A*, the γ subunit is localized primarily to the plasma membrane in the absence of the other subunits. Moreover, the γ subunit also is located at the plasma membrane in the α1β1γ-coinfected cells (Fig. 3*B*). When the same cells are probed for the α1 subunit, plasma membrane staining is also present (Fig. 3*C*). Fig. 3*D* shows an overlay of *B* and *C*, with yellow indicating the colocalization of the α1 and γ subunits. As shown, the majority of α1 and γ polypeptides colocalize to the plasma membrane. Thus the γ subunit can be delivered to the plasma membrane of Sf-9 cells independently of the other subunits, and the γ subunit colocalizes with the α1 subunit in α1β1γ-coinfected cells.

The γ subunit was expressed in *Xenopus* oocytes to examine the possibility that the subunit mediates ion fluxes (Fig. 4). In two-microelectrode voltage clamp experiments, uninjected oocytes showed only small leakage currents reversing around 0 mV in KD98 solution, with slope conductance (at 0 mV) < 1 μS (n = 25 oocytes). Expression of Na,K-ATPase α subunits alone (n = 8), or together with β subunits (n = 10) had no effect on this leak. However, when γ subunits alone were expressed, steps to voltages positive or negative to the zero current potential caused time-dependent activation of an increasingly larger conductance (Fig. 4, n = 15 oocytes) that did not recover after returning the holding potential to the

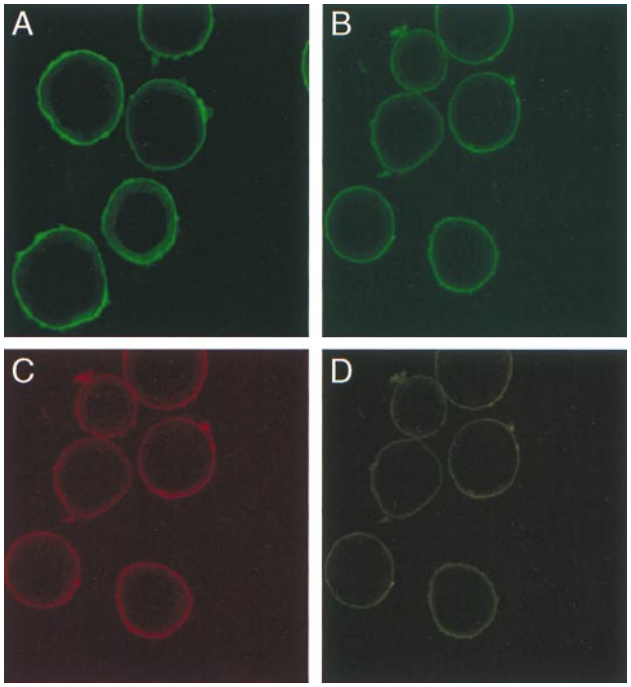

FIG. 3. Localization of the γ subunit in infected Sf-9 cells. Confocal microscopy was used to determine the location of proteins in cells infected with γ or α1β1γ baculoviruses. (*A*) Cells infected with γ baculovirus and probed with G17 polyclonal. (*B–D*) Cells coinfected with α1β1γ baculoviruses and probed with both the G17 polyclonal and 5α monoclonal to detect the γ and α subunits. (*B*) Staining for the γ subunit. (*C*) Staining for the α1 subunit. (*D*) an overlay of *B* and *C*, showing colocalization of γ and α1 in α1β1γ-infected cells.

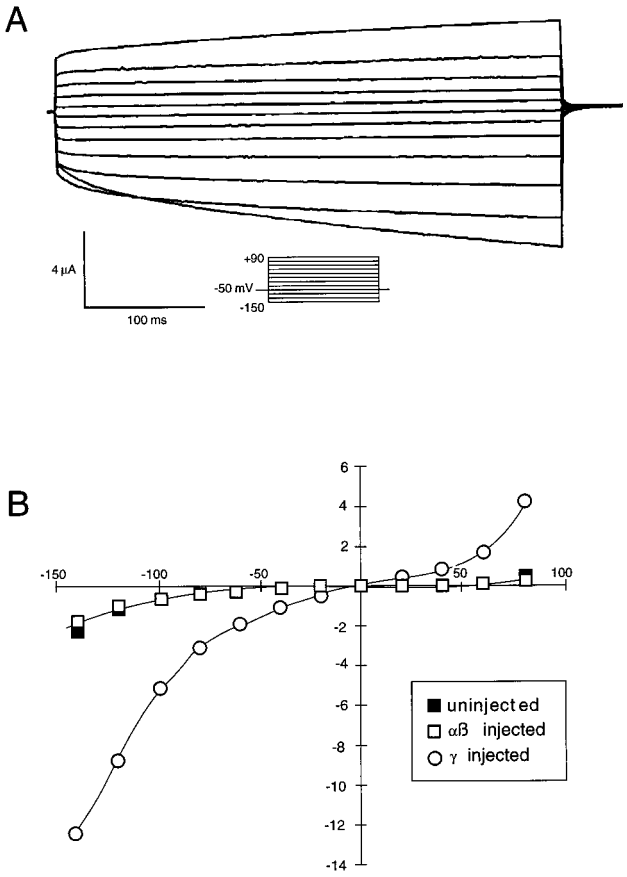

FIG. 4. Ion currents in *Xenopus* oocytes injected with γ cRNA. (*A*) Currents recorded under voltage clamp, from a *Xenopus* oocyte expressing γ subunits. The membrane was stepped from a holding potential of −50 mV to voltages between −150 and +90 mV, for 400 msec, every 5 sec. The record shown is a typical first set of currents after turning on the voltage clamp. (*B*) Typical "steady-state" current-voltage relationships from uninjected oocytes, and oocytes expressing α and β subunits, or γ subunits alone, by using a protocol like that shown in *A*.

zero current potential. Subsequent steps to voltage $> +50$ or < -50 mV caused further increase of conductance. The activated currents showed both positive and negative going rectification (Fig. 4*B*). Typically, the activated conductance after several voltage steps to −150 mV for several seconds was >20 μS (slope conductance at zero mV). This current appeared to be a nonselective cation current carried by K^+ and Na^+ ions, because in ND96 solution ($E_K = -100$ mV), the activated conductance still reversed at 0 mV ($n = 4$), and in Tris98 solution, there was only a slight negative shift in E_{rev} (to ≈ −20 mV, $n = 4$). When γ cRNA was coinjected with either α or β cRNAs alone ($n = 3$ and 5 oocytes, respectively) or together ($n = 4$ oocytes), there was no increase in conductance above background. However, we cannot conclude from these results that α or β subunits are not required or that they suppress the γ-induced conductance. This uncertainty is because *Xenopus* oocytes express endogenous α and β subunits (8) that may associate with the exogenous γ subunit. Moreover, the lack of conductance when cRNAs are coinjected may reflect the reduced expression levels of the γ subunit found when oocytes express multiple polypeptides. Thus, we can only conclude that the γ subunit alone can induce ion currents, and that the induced currents to not require the expression of exogenous α or β subunits.

To further examine the involvement of the γ subunit in the transport of cations, the uptake of Na^+ and K^+ were characterized in Sf-9 cells expressing the γ subunit. Sf-9 cells were infected with integrin-associated protein (IAP, CD47) or γ baculoviruses for 48 and 72 hr. IAP is a 50-kDa multimembrane spanning protein that copurifies with the integrin $\alpha_v\beta_3$ and has been implicated in the regulation of ligand binding by the integrin receptor (26). IAP is a membrane protein unrelated to the Na,K-ATPase and is used as a control for ion flux that may occur as a result of baculovirus infection. Immunocytochemistry using IAP-infected cells indicates that IAP is delivered to the plasma membrane (not shown). Immunoblots indicated that comparable levels of IAP or γ polypeptides were expressed on infection (not shown). As shown in Fig. 5, Na^+ and K^+ uptake is substantially increased in Sf-9 cells expressing the γ subunit but not in cells expressing IAP. When compared

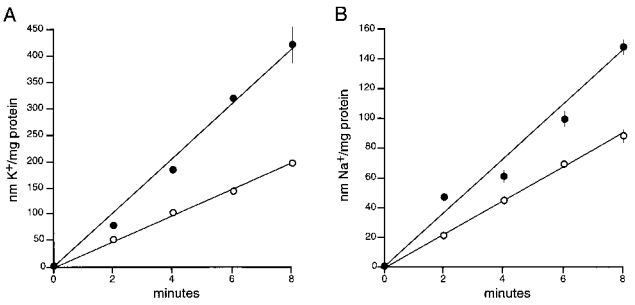

FIG. 5. Na^+ and K^+ uptake into γ-infected Sf-9 cells. Cells infected with γ (●) or IAP (○) baculoviruses were depleted of intracellular K^+ (*A*) or Na^+ (*B*) and uptake was measured by using ^{86}Rb or ^{22}Na as tracer, respectively. Bumetanide (100 μM) was included to inhibit bumetanide-sensitive transport. The rate of Na^+ or K^+ uptake in uninfected cells is approximately 80% of the rate in infected cells (not shown). Each value is the mean with error bars representing the standard error of eight determinations. Similar results were obtained from two different experiments.

with cells expressing IAP, cells expressing the γ subunit exhibit approximately 150% increase in the rate of Na^+ and K^+ uptake. Similar increases in cation uptake were found in cells coexpressing the α and γ subunits. Moreover, 1 mM ouabain did not affect the rate of γ-induced cation uptake (data not shown). These results suggest that when expressed in Sf-9 cells the γ subunit can mediate the transport of Na^+ and K^+.

DISCUSSION

The γ subunit is a small, hydrophobic peptide that copurifies with the α and β subunits of the Na,K-ATPase. Although the γ subunit is not essential for Na,K-ATPase function, the subunit may modify the external cation binding sites of the enzyme when associated with the other subunits (8). In this paper we describe other properties of the γ subunit when expressed in insect cells and *Xenopus* oocytes. For these studies we have used the human γ subunit cDNA. This cDNA extends the N-terminal sequence of the previously described γ subunits. It is not clear whether the human cDNA represents a different gene product or is a result of alternative processing of a single gene. However, it is clear that this cDNA represents the complete coding region of the γ subunit. The predicted size of the subunit is in better agreement with the size of the native protein from the kidney (7). When expressed in Sf-9 insect cells the subunit migrates as a doublet on SDS/PAGE. This observation may be a result of the use of different initiator methionines (8) or alternatively, the larger molecular weight band may represent a dimer of the subunit. Isolation of other rat γ subunit cDNA clones indicates that the cDNA described previously (7) has a divergent 5′ coding region that may be a cloning artifact. Characterization of other cDNAs isolated from libraries and by using reverse transcription–PCR indicate that the deduced amino acid sequence of the rat γ subunit shown in Fig. 1 is correct (unpublished results; Alex Therien and Rhoda Blostein, personal communication).

Coexpression of the human γ subunit with the rat α1 subunit indicates that the subunits can stably associate; however, when expressed alone in Sf-9 cells the γ subunit is delivered to the plasma membrane. Previous results from baculovirus expression studies of the α and β subunits have demonstrated that unassembled Na,K-ATPase polypeptides are delivered to the plasma membrane. These results are in contrast to studies using *Xenopus* oocytes, which have demonstrated that α and β subunits are retained in the endoplasmic reticulum until properly assembled (reviewed in ref. 27). Moreover, when the γ subunit is expressed in *Xenopus* oocytes in the absence of the other subunits, more than 85% of the protein is retained intracellularly (8). Coexpression of the γ subunit with the other subunits leads to stabilization and delivery of the γ polypeptide to the plasma membrane (8). The exact mechanisms that are responsible for the differences in the processing of the Na,K-ATPase in vertebrate and invertebrate cells are unknown.

The γ subunit shares substantial homology with several other small, single transmembrane proteins that are involved in the passage of ions across the plasma membrane. Consistent with findings obtained with other members of this family, the human γ subunit induces cation selective channels when expressed in *Xenopus* oocytes. Similar activity is observed in infected Sf-9 cells. Insect cells expressing the γ subunit exhibit an increase in Na^+ and K^+ influx (Fig. 5). Interestingly, the γ subunit induces channel activity when expressed separately from the other subunits. This result suggests that the γ subunit may have functions independent of those necessary for regulation of Na,K-ATPase activity. On the other hand, we cannot eliminate the possibility that the subunit is associating with endogenous Na,K-ATPase subunits. Thus, the γ subunit may activate cation fluxes only when associated with the other subunits.

Previous studies of the γ subunit failed to uncover a channel-inducing activity in *Xenopus* oocytes (8) or yeast (12). However, this discrepancy may be a result of the differences in the N-terminal regions of the expressed γ subunits; the human γ subunit used in this study has an extended N-terminal sequence. The possibility that this extended sequence is responsible for inducing channel activity is currently under investigation.

Although there is increasing evidence that the γ subunit can modify Na,K-ATPase function, the exact role of the subunit in Na,K-ATPase function awaits further experimentation. In the present study we demonstrate that the γ subunit may have a function separate from normal Na,K-ATPase activity. The physiological significance of this activity is unknown. However, we are now in the position to begin to understand the cellular function of the γ subunit and to determine its role in ion homeostasis.

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