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Agrin Regulation of α3 Sodium-Potassium ATPase Activity Modulates Cardiac Myocyte Contraction*[S]

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Lutz G. W. Hilgenberg[‡], Bryan Pham[‡1], Maria Ortega[‡§1], Saif Walid[‡1], Thomas Kemmerly[‡§1], Diane K. O'Dowd[‡§], and Martin A. Smith[‡2]

From the Departments of [‡]Anatomy and Neurobiology and [§]Developmental and Cell Biology, University of California, Irvine, California 92697

Drugs that inhibit Na,K-ATPases, such as digoxin and ouabain, alter cardiac myocyte contractility. We recently demonstrated that agrin, a protein first identified at the vertebrate neuromuscular junction, binds to and regulates the activity of α3 subunit-containing isoforms of the Na,K-ATPase in the mammalian brain. Both agrin and the α3 Na,K-ATPase are expressed in heart, but their potential for interaction and effect on cardiac myocyte function was unknown. Here we show that agrin binds to the α3 subunit of the Na,K-ATPase in cardiac myocyte membranes, inducing tyrosine phosphorylation and inhibiting activity of the pump. Agrin also triggers a rapid increase in cytoplasmic Na^+ in cardiac myocytes, suggesting a role in cardiac myocyte function. Consistent with this hypothesis, spontaneous contraction frequencies of cultured cardiac myocytes prepared from mice in which agrin expression is blocked by mutation of the *Agrn* gene are significantly higher than in the wild type. The *Agrn* mutant phenotype is rescued by acute treatment with recombinant agrin. Furthermore, exposure of wild type myocytes to an agrin antagonist phenocopies the *Agrn* mutation. These data demonstrate that the basal frequency of myocyte contraction depends on endogenous agrin-α3 Na,K-ATPase interaction and suggest that agrin modulation of the α3 Na,K-ATPase is important in regulating heart function.

Na,K-ATPases, or sodium pumps, are integral membrane enzymes found in all animal cells. Using energy from the hydrolysis of ATP they transport three Na^+ ions out of the cell for every two K^+ ions into the cell, resulting in a transmembrane chemical gradient that is reflected in the resting membrane potential and used to drive a variety of secondary transport processes. Each Na,K-ATPase is a heterodimer consisting of an α- and β-subunit. The α-subunit is the catalytic subunit and contains the binding sites for Na^+ and K^+. The β-subunit is required for pump function and targeting of the α-subunit to the plasma membrane. Four α- and three β-subunit genes have been identified. All combinations of α- and β-subunits form functional pumps, but developmental, cellular, and subcellular differences in expression suggest functional adaptation of the different isoforms (1).

Na,K-ATPases play a central role in regulating the contractile activity of cardiac muscle (2). They are directly responsible for the Na^+ gradient required for propagation of action potentials that initiate myocyte contraction. Moreover, because of the dependence of the Na^+/Ca^{2+} exchanger (NCX)[3] on the Na^+ gradient as the source of counterions for transport of Ca^{2+} out of the cell, they play a critical role in Ca^{2+} homeostasis and excitation-contraction coupling. For example, inhibition of Na,K-ATPases by digoxin, ouabain, or other cardiac glycoside results in a decline of the Na^+ gradient, reducing NCX activity and Ca^{2+} efflux. The inotropic effects of cardiac glycosides result from uptake of this "excess" cytoplasmic Ca^{2+} into the sarcoplasmic reticulum, raising the level of Ca^{2+} in intracellular stores, which, when released during excitation, enhances muscle contraction (3).

In light of the importance of Na,K-ATPases for cardiac muscle function, it is not surprising that mechanisms have evolved to regulate their activity. Na,K-ATPases are susceptible to phosphorylation by either cAMP-dependent protein kinase or protein kinase C, and neurotransmitter- and peptide hormone-dependent activation of these cytoplasmic kinases have been shown to regulate pump activity (4). Other molecules exert their effects through direct interaction with the Na,K-ATPase. For example, phospholemman, a member of the FXYD family of membrane proteins expressed in heart, is tightly associated with the Na,K-ATPase and inhibits its function (5–7). Phosphorylation of phospholemman by either protein kinase C or cAMP-dependent protein kinase, however, relieves inhibition thereby restoring the activity of the pump (8, 9). Endogenous ouabain-like compounds have also been implicated in regulating Na,K-ATPase activity (10). Ouabain, or closely related molecules, is synthesized by the adrenal gland and hypothalamus, and increased circulating levels of these compounds observed in patients with congestive heart failure has been suggested as an adaptive response to improve heart function (11). Recent studies in the central nervous system have identified the protein

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[2] To whom correspondence should be addressed: Dept. of Anatomy and Neurobiology, Joan Irvine-Smith Hall, Rm. 110, University of California, Irvine, CA 92697-1280. Fax: 949-824-0043; E-mail: masmith@uci.edu.

[3] The abbreviations used are: NCX, Na^+/Ca^{2+} exchanger; E18, embryonic day 18; P0, postnatal day 0; ANOVA, analysis of variance; PBS, phosphate-buffered saline; BS^3, bis(sulfosuccinimidyl) suberate; MOPS, 4-morpholinepropanesulfonic acid; DS, dissecting solution; FCS, fetal calf serum; SBFI, sodium-binding benzofuran isophthalate; MuSK, muscle-specific kinase.

agrin as a new endogenous ligand that regulates Na,K-ATPase function through interaction with its extracellular domains (12).

Agrin was first identified as an extracellular matrix protein at the neuromuscular junction where, by signaling through a muscle-specific receptor tyrosine kinase called MuSK, it mediates the motor neuron-induced accumulation of acetylcholine receptors in the postsynaptic muscle fiber membrane (13). Agrin is also expressed in other tissues (14–16), but its function outside of the neuromuscular junction has been less well understood. Recently, however, we showed that agrin plays a role in regulating excitability of central nervous system neurons by binding to and inhibiting the activity of the α3 subunit-containing isoform of the Na,K-ATPase (12). Although both agrin (14, 16) and the α3 Na,K-ATPase (17) are expressed in heart, their potential interaction has not been explored. Here we show that the frequency of cardiac myocyte contraction is modulated by agrin regulation of α3 Na,K-ATPase activity.

EXPERIMENTAL PROCEDURES

Immunohistochemistry—Adult and embryonic day 18 (E18) hearts were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C. Frozen sections (12–15 μm) were mounted on gelatin-coated glass slides and nonspecific binding sites blocked by preincubation for 1 h at room temperature in 4% bovine serum albumin in PBS (PBS/BSA) followed by incubation in the primary antibody in PBS/BSA overnight at 4 °C. Bound antibody was visualized by labeling with an Alexa Fluor-conjugated anti-rabbit or anti-mouse antibody (Molecular Probes). Mouse monoclonal antibodies against the α3 Na,K-ATPase (XVIF9-G10, Research Diagnostics, Inc. (18)) and α1 Na,K-ATPase (clone 9A-5; A275, Sigma-Aldrich (19)) and rabbit serum against the α2 Na,K-ATPase (AB9094, Millipore (20, 21)) were used at a dilution of 1:200. RαAg-1, a pan-specific rabbit anti-agrin serum (22) was used at a dilution of 1:500. Fluorescence images were captured on a Nikon Optiphot 2 using a Spot camera and software.

Biochemistry—Recombinant 15- and 20-kDa COOH-terminal agrin fragments, C-Ag15 and C-Ag20, were expressed and purified as described (22) and stored as a 0.1 μM stock at 4 °C in 20 mM Tris buffer containing 250 mM NaCl and 200 mM imidazole, pH 8.0. Because agrin expressed in cardiac muscle lacks an insert at the alternatively spliced z-site (16), only C-Ag20z_0, hereinafter referred to as C-Ag20, was used. Unless otherwise specified, a saturating (100 pM) concentration of agrin fragments used was used, as determined by bioassay for c-*fos* induction (22).

Chemical cross-linking to either endogenous agrin (*i.e.* saline-treated) or agrin fragments was performed on pieces of ventricular muscle using bis(sulfosuccinimidyl) suberate (BS^3, Pierce). Briefly, ventricles were placed in ice-cold PBS containing 1.8 mM $CaCl_2$ (PBS^{2+}) and cleaned of blood, connective tissue, and major blood vessels. Ventricular tissue was then teased into small pieces (~1 mm^3) and incubated for 5 min in PBS (Ca^{2+}-free) followed by preincubation for 15 min with recombinant agrin or vehicle in 0.9 ml of PBS^{2+} on ice. Cross-linking was started by the addition of 0.1 ml of 1 mM BS^3, and the tissue was incubated for 30 min on ice before the reaction was stopped by washing three times in PBS containing 50 mM ethanolamine. Ten to twenty pieces of tissue were processed per reaction. BS^3 cross-linking of cultured myocytes was performed as described (12).

Following cross-linking, pieces of tissue or cultured cells were collected into ice-cold TI buffer (20 mM Tris, pH 7.4, 10 mM EDTA, protease inhibitors (P8340, Sigma-Aldrich)), and homogenized with a Dounce homogenizer. The homogenate was centrifuged for 5 min at 1000 × *g* to remove debris and the resulting supernatant centrifuged for 1 h at 40,000 × *g* to pellet the membrane fraction. Membrane pellets were resuspended in TI buffer, and protein concentration was determined using the Bio-Rad protein assay. Western blot analysis of α1, α2, and α3 subunits was performed using 9A-5, AB9094, and XVIF9-G10, respectively (18–21). Antibody specificity was confirmed in preliminary experiments in which blots were probed with second antibody alone (data not shown).

Cross-linked agrin-α3 subunit complexes were also analyzed by immunoprecipitation. Following cross-linking, tissue was Dounce-homogenized in immunoprecipitation buffer (20 mM Tris, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) plus protease inhibitors, and the homogenate was cleared by centrifugation for 5 min at 15,000 × *g*. The protein concentration of the resulting supernatant was determined, and primary antibody (10 μl of XVIF9-G10 or 5 μl of RαAg-1) was added to the cleared homogenate (250 μg of protein) in a final volume of 400 μl followed by incubation overnight at 4 °C with gentle agitation. Antigen-antibody complexes were precipitated by incubation for 1 h at room temperature with 20 μl of 1:1 slurry of protein G- or protein A-Sepharose beads (Millipore). The beads were then washed in several changes of immunoprecipitation buffer and eluted in SDS-PAGE sample buffer for Western blot analysis.

Agrin induced phosphorylation of different α-subunits was determined from Western blots of immunoprecipitated phosphoproteins. Briefly, small pieces of tissue were preincubated with recombinant agrin alone or in the presence of 0.1 mM genistein in 250 μl of buffer containing 150 mM NaCl, 5 mM KCl, 3.3 mM Na_2HPO_4, 4.4 mM KH_2PO4, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and then transferred to a water bath for 5 min at 37 °C. Tissue was then cooled on ice and homogenized in ice-cold immunoprecipitation buffer containing 1 mM sodium orthovanadate. Tissue extracts were cleared by centrifugation, and aliquots of the detergent-soluble fraction (250 μg of protein) were incubated with 4 μl of anti-phosphotyrosine antibody (mAb4G10, Millipore) with gentle agitation overnight at 4 °C. Antigen-antibody complexes were precipitated and Western-blotted as described above. Quantitative analysis of Western blots was performed using the gel analyzer routine in the public domain ImageJ software (rsb.info.nih.gov/ij/).

Na,K-ATPase Activity Assay—Cultured cells or pieces of tissue were homogenized in ice-cold SE buffer (318 mM sucrose, 40 mM MOPS, 1 mM EDTA, pH 7.2) and sarcolemmal or other cell membranes recovered by centrifugation as described (23). The resulting membrane pellets were resuspended in SE and stored at −80 °C. Protein concentration was determined using the Bio-Rad protein assay.

To measure Na,K-ATPase activity, aliquots of membrane material (10–25 μg of protein) were preincubated with either 5 mM ouabain (25× stock in H_2O) or recombinant agrin fragment (diluted from a stock solution containing 250 mM NaCl, 200 mM imidazole, 20 mM Tris, pH 8.0) for 1 h in the dark on ice in a buffer containing 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, and 30 mM imidazole, pH 7.4. Samples were then transferred to a 37 °C water bath, and the reaction was started by the addition of ATP to a final concentration of 4 mM (final volume 100 μl). The reaction was stopped after 10 min by the addition of 10 μl of 20% SDS. ATPase activity was determined from the rate of ATP hydrolysis based on release of inorganic phosphate. Briefly, 400 μl of reagent A (1.8% ascorbic acid, 0.3 N HCl, 0.3% ammonium molybdate) and 500 μl of reagent B (2% sodium meta-arsenite, 2% trisodium citrate, 2% acetic acid) was added to each reaction, and the absorbance at 850 nm was measured. The molar concentration of inorganic phosphate was determined by comparison with a NaH_2PO_4 standard. Na,K-ATPase-dependent ATPase activity was defined as the fraction of total ATPase activity inhibited by 5 mM ouabain.

Cell Culture—Primary ventricular myocytes were prepared from E18 mouse hearts using a modification of the protocol as described (24). Hearts were placed into ice-cold dissecting solution (DS; 130 mM NaCl, 23 mM HEPES, 21 mM glucose, 20 mM taurine, 5 mM creatine, 5 mM $MgCl_2$, 5 mM sodium pyruvate, 4.5 mM KCl, 1 mM NaH_2PO_4, 0.75 mM Ca^{2+}, pH 7.3), and the atria, major blood vessels, and connective tissue were removed. Ventricles were washed in fresh DS, minced into small pieces with jewelers forceps, and then washed in Ca^{2+}-free DS containing 3.3 μM EGTA. Tissue pieces were subsequently incubated in DS containing 0.1 mM Ca^{2+}, crude collagenase type 1A (0.5 mg/ml; Sigma-Aldrich), and protease type XIV (0.1 mg/ml; Sigma-Aldrich) for 5 min at 37 °C followed by washing in fresh DS containing 0.1 mM Ca^{2+}. The tissue was gently triturated and the resulting cell suspension transferred to a 15-ml conical centrifuge tube and stored on ice. The cycle of enzyme digestion and trituration was repeated until all of the tissue was dissociated. The pool of dissociated cells was pelleted by centrifugation, resuspended in 199 medium (Sigma-Aldrich) containing 10% fetal calf serum (FCS), 5 mM creatine, 5 mM taurine, 2 mM D-carnitine, 2.5 mM sodium pyruvate, 0.1 μM insulin, 50 IU/ml penicillin, and 50 μg/ml streptomycin, and plated onto laminin-coated culture dishes or glass coverslips. Cultures were maintained in an atmosphere of 5% CO_2, 95% air at 37 °C. BHK cells were grown in minimum Eagle's medium (Sigma-Aldrich) containing 10% FCS and 100 units/ml penicillin/streptomycin. Mouse cerebrocortical glial cells were prepared and maintained as described (12). All handling and treatment of animals was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Na^+ Imaging—Intracellular Na^+ was monitored by ratiometric imaging of the membrane-permeant Na^+ binding dye, SBFI-AM (Invitrogen), essentially as described (12, 25). 4–7-day-old cultured myocytes growing on glass-bottomed culture dishes (MatTek) were loaded with SBFI-AM (5 μg/ml) in HEPES-buffered salt solution (HBSS; 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 15 mM glucose, 20 mM HEPES, pH 7.4) containing 5% fetal calf serum (HBSS-FCS) and 0.1% Pluronic F-127 (Invitrogen) for 2 h at room temperature. Cultures were then washed in fresh HBSS-FCS followed by de-esterification for a further 30 min at room temperature. Recordings were done in HBSS. Excitation wavelengths were selected using band specific filters at 340 and 380 nm, and ratio images at alternating excitation wavelengths were acquired at 510 nm at 1 Hz using an Orca 100 digital CCD camera (Hamamatsu) and analyzed using Metafluor 4.6 software (Molecular Devices). To accommodate for the small spontaneous fluctuations in Na^+, the Na^+ level in normal saline was defined as the average level during the 6-s period prior to treatment with agrin or ouabain. Similarly, the response to each drug was defined as the average response during the 6-s period beginning 1 s prior to the peak response. To control for variation in dye loading, the agrin response of each cell was normalized to the maximal response induced by a mixture 5 mM ouabain and 5 μM gramicidin at the end of each experiment.

Myocyte Contraction Bioassay—4–5-day-old cultured cardiac myocytes were viewed at ×400 magnification, and contractions during 30 s was counted in three to five random fields/culture dish. All counts were performed blind with respect to genotype and treatment.

Data Analysis—Curve fitting was performed using classical nonlinear models provided in Prism 4 (GraphPad Software, Inc.). The concentration dependence of Na,K-ATPase activity on C-Ag20 and C-Ag15 was fit by a variable slope sigmoidal dose-response equation,

$$y = B + \frac{(T - B)}{1 + 10^{(\log EC_{50} - x)\text{HillSlope}}} \quad \text{(Eq. 1)}$$

where B and T are minimal and maximal values for Na,K-ATPase activity, respectively. Data describing the effects of C-Ag20 on ouabain inhibition of Na,K-ATPase activity were fit by a two-site competition model,

$$y = B + (T - B)\left[\frac{F_1}{1 + 10^{x - \log EC_{50_1}}} + \frac{1 - F_1}{1 + 10^{x - \log EC_{50_2}}}\right] \quad \text{(Eq. 2)}$$

where F_1 is the fraction of sites with EC_{50_1} and F_2 is the fraction of sites with EC_{50_2}. All statistical analyses (t test, paired t test, ANOVA) were performed on raw data using DataDesk 6.2 (Data Description, Inc.).

RESULTS

Agrin Binds Specifically to the α3 Na,K-ATPase in Heart—We recently demonstrated that the α3 Na,K-ATPase is a receptor for agrin in central nervous system neurons. Whereas previous studies had shown that agrin and the α3 Na,K-ATPase are expressed in heart, little was known about the cellular distribution of these two proteins and their potential for interaction in cardiac tissue. Using double-label immunohistochemistry we examined the pattern of agrin and α3 Na,K-ATPase expression in sections of embryonic and adult mouse heart. At E18, both agrin and the α3 Na,K-ATPase were readily detectable and relatively evenly dispersed throughout the ventricular and atrial myocardium (Fig. 1, *A* and *A′*). Previous studies in rat have

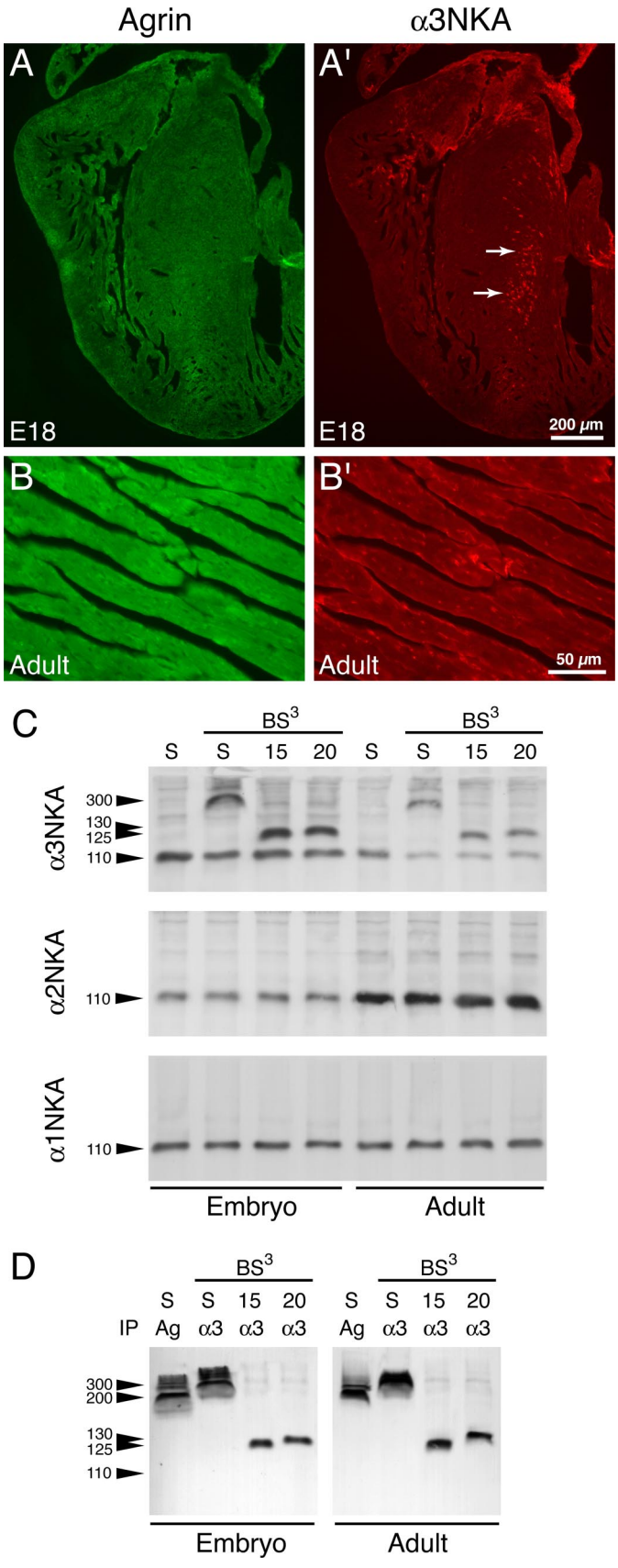

FIGURE 1. **Agrin binds specifically to the α3 Na,K-ATPase in heart.** *A* and *A′*, low power photomicrographs of a frozen section through an E18 mouse heart ventricle double labeled for agrin (*A*) and α3 Na,K-ATPase (*A′*). Both agrin and the α3 Na,K-ATPase are expressed throughout the ventricular myocardium. Higher levels of α3 Na,K-ATPase can be seen in a subpopulation of cells, possibly developing Purkinje fibers, concentrated in the ventricular septum (*arrows*) and scattered throughout the myocardium. *B* and *B′*, agrin (*B*) and α3 Na,K-ATPase (*B′*) are also expressed in adult cardiac muscle fibers. Viewed at higher magnification, agrin appears relatively evenly distributed, whereas α3 Na,K-ATPase is characterized by the presence of more intensely labeled puncta set against a low background. *C*, typical Western blots (50 μg/protein/lane) of embryo and adult ventricular muscle probed with antibodies against the α3, α2, or α1 subunit of the Na,K-ATPase. The α3 subunit migrates as a single band of a 110 kDa in saline (*S*)-treated control tissue and is more abundant in embryos than in adults. Cross-linking saline-treated tissue with BS^3 generates a ≥300-kDa agrin-α3 subunit complex in which formation is blocked by the presence of either C-Ag15 or C-Ag20, resulting instead in 125- and 130-kDa bands, respectively. In contrast, Western blots of aliquots of the BS^3 cross-linked tissue probed with antibodies to α2 or α1 contain only a single band of 110 kDa, evidence that agrin binds specifically to the α3 Na,K-ATPase. Whereas the level of α2 subunit expression increases during development, the α1 subunit is unchanged, confirming similar loading across lanes. *D*, tissue samples, treated as in *C*, were solubilized in detergent-containing buffer and immunoprecipitated (*IP*) with either the anti-agrin serum (*Ag*) or anti-α3 subunit monoclonal antibody (*α3*), and the immunoprecipitates were analyzed by Western blotting with the anti-agrin serum. Native agrin glycoprotein immunoprecipitated by the anti-agrin serum from control tissue treated with saline alone is shown for comparison and appears as a broad band of ≥200 kDa. Consistent with interaction between endogenous agrin and the α3 subunit, cross-linking results in the appearance of a high molecular mass species of ≥300 kDa recognized by both the anti-α3 and anti-agrin antibodies. Formation of the agrin-α3 complex is blocked by cross-linking in the presence of a saturating concentration of either C-Ag15 or C-Ag20.

shown that α3 Na,K-ATPase is expressed in Purkinje and other fibers of the heart conduction system (26). A population of high α3 Na,K-ATPase-expressing fibers, most notable in the ventricular septum, likely represents elements of the developing conducting fiber system. Staining with the anti-agrin serum was specific, based on the low background staining in sections from agrin knock-out ($Agrn^{-/-}$) mice (supplemental Fig. 1). Similar low levels of nonspecific staining were observed in the absence of the α3 Na,K-ATPase monoclonal antibody (not shown). Expression of agrin and α3 Na,K-ATPase was also detected in sections of adult heart (Fig. 1, *B* and *B′*). Whereas staining for agrin was relatively uniform and similar to that seen in embryonic myocytes, α3 Na,K-ATPase appeared as intensely labeled foci distributed within a lower, evenly labeled background. Previous studies have also reported low levels of α3 subunit expression in adult rodent heart (23, 27, 28).

The low resolution of the immunohistochemical studies made it difficult to determine whether agrin and the α3 Na,K-ATPase were present at the cell surface. Therefore, to learn more about their subcellular distribution and potential interaction, we used chemical cross-linking of live cells with the membrane-impermeant bifunctional reagent BS^3 followed by Western blot analysis of partially purified membranes to look for evidence of native agrin-α3 Na,K-ATPase complexes in cardiac tissue (Fig. 1*C*). Western blots of teased pieces of embryo (E18) or adult ventricular muscle probed with the α3 Na,K-ATPase subunit monoclonal antibody revealed a single band with an apparent molecular mass of 110 kDa which, as expected for the α3 subunit, was more abundant in embryo *versus* adult. Cross-linking with BS^3, however, resulted in the appearance of a new α3 antibody cross-reacting band with a molecular mass of ≥300 kDa. Agrin has a predicted molecular mass of 198 kDa, but because of variable glycosylation the native protein runs as a broad band of ≥200 kDa, raising the possibility that the ≥300-

kDa band represents a complex of endogenous agrin and the α3 subunit. Supporting this hypothesis, cross-linking in the presence of a saturating concentration (100 pM) of either of two short COOH-terminal fragments of agrin, C-Ag15 or C-Ag20 (apparent molecular mass of 15 and 20 kDa, respectively), which bind to the α3 Na,K-ATPase on neuron cell membranes (12, 22), blocked the formation of the putative agrin-α3 subunit complex resulting instead in the appearance of 125- and 130-kDa bands predicted for each agrin fragment-α3 subunit adduct.

Because neither the agrin fragments nor BS^3 is hydrophobic, the ability of the agrin fragments to block formation of the high molecular weight complex suggests that agrin secreted by cardiac myocytes binds to one or more extracellular domains of the α3 Na,K-ATPase. Interestingly, not all α3 subunits could be cross-linked even in the presence of a concentration of the agrin fragments sufficient to antagonize cross-linking to endogenous agrin. A portion of the cross-link-resistant pool of α3 subunits may be due to poor penetration of reagents; however, similar results in parallel studies on live cultured cardiac myocytes (supplemental Fig. 2), where access should be greatly improved, suggest that distinct populations of α3 Na,K-ATPases may exist.

Apparent competition between endogenous agrin and the agrin fragments argues for the presence of a limited number of specific agrin binding sites on cardiac myocyte membranes. But cardiac myocytes also express α1 and α2 Na,K-ATPases that are structurally similar to α3, raising the possibility that agrin may also interact with these other pump isoforms. Cross-linking, however, did not affect the electrophoretic mobility of either α1 or α2 (Fig. 1*C*), evidence that agrin binds specifically to the α3 subunit in heart as it does in brain.

Finally, to test directly for interaction between agrin and the α3 subunit, detergent extracts of embryo and adult ventricular myocyte membranes were immunoprecipitated with the anti-α3 monoclonal antibody and the immunoprecipitates analyzed by Western blotting with the anti-agrin serum. Consistent with the results of the Western blots probed with the anti-α3 monoclonal antibody (Fig. 1*C*), cross-linking in saline alone resulted in the appearance of a broad band of ≥300 kDa that contained epitopes for both the α3 subunit and agrin (Fig. 1*D*). Formation of this high molecular mass band was blocked by cross-linking in the presence of either C-Ag15 or C-Ag20, which instead produced the characteristic bands at 125 and 130 kDa expected for the C-Ag15-α3 subunit and C-Ag20-α3 subunit adducts. Taken together, these results provide strong evidence that native agrin and the α3 Na,K-ATPase are expressed and interact in both embryonic and adult heart muscle fibers.

Agrin Induces Tyrosine Phosphorylation of the α3 Subunit—Agrin induces tyrosine phosphorylation of the α3 subunit of the Na,K-ATPase, and inhibition of tyrosine kinase activity blocks agrin signaling in neurons (12, 25). To learn whether agrin might play a similar role regulating Na,K-ATPase activity in cardiac myocytes, we tested the ability of different agrin fragments to modulate tyrosine phosphorylation of each α-subunit in adult heart tissue.

Western blots of phosphotyrosine-containing proteins immunoprecipitated from detergent extracts of small pieces of adult ventricular muscle indicate that some α1, α2, and α3 subunits are phosphorylated even in control, saline-treated tissue (Fig. 2*A*). Treatment with C-Ag15, an agrin fragment that acts as an agrin antagonist (12, 22), reduced phosphorylation of the α3 subunit to barely detectable levels ($p < 0.01$, paired *t* test) but had no effect on α1 or α2 (Fig. 2), evidence that basal levels of cardiac myocyte α3 subunit phosphorylation are due to endogenous agrin-α3 Na,K-ATPase interaction. Conversely, treatment with C-Ag20, an agrin fragment with the same activity as full-length agrin, significantly increased phosphorylation of the α3 subunit ($p < 0.01$, paired *t* test) without affecting α1 or α2. Treatment with 100 μM genistein, a broad-spectrum tyrosine kinase inhibitor, blocked phosphorylation of all three α-subunits, demonstrating the specificity of the anti-phosphotyrosine antibody (Fig. 2). Similar results were obtained in parallel studies in which Western blots of proteins precipitated using antibodies against each α-subunit were probed with the anti-phosphotyrosine antibody (data not shown). Thus, phosphorylation of the α3 Na,K-ATPase in cardiac myocytes appears to be agrin-dependent.

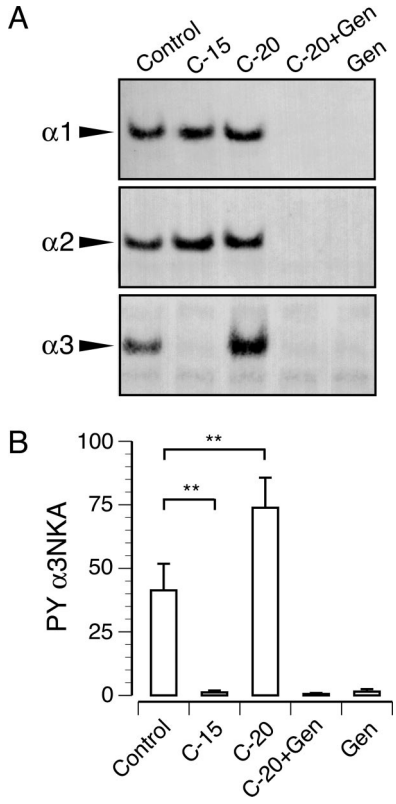

FIGURE 2. **Agrin-dependent tyrosine phosphorylation of the α3 subunit of the Na,K-ATPase.** *A*, typical Western blots show α1, α2, and α3 Na,K-ATPase subunits immunoprecipitated from adult ventricular muscle by an anti-phosphotyrosine antibody. C-Ag15 reduced basal levels of phosphorylation only in the α3 subunit. Treatment with C-Ag20 increased the level of α3 subunit phosphorylation but had no effect on either α1 or α2. Endogenous phosphorylation of all three subunits and C-Ag20-induced phosphorylation of the α3 subunit is blocked by genistein (*Gen*). *B*, densitometric analysis of five independent experiments similar to that shown in *A*. Tyrosine phosphorylation (*PY*) of the α3 subunit is decreased following treatment with C-Ag15 but increased in the presence of C-Ag20. C-Ag20-dependent phosphorylation of the α3 subunit was blocked by genistein, suggesting that agrin interaction with the α3 Na,K-ATPase activates a tyrosine kinase. **, $p < 0.01$; paired *t* test.

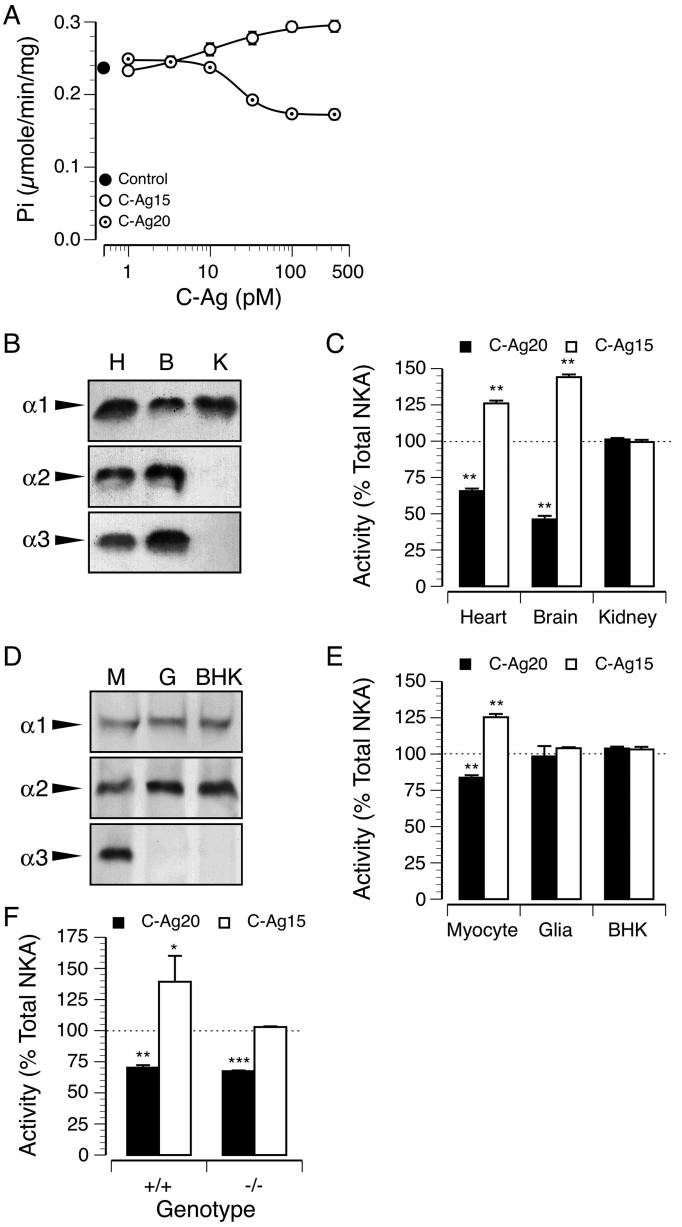

FIGURE 3. **Agrin regulates α3 Na,K-ATPase activity.** *A*, the production of inorganic phosphate from ATP by purified P0 ventricular myocyte sarcolemmal membranes was measured, and background ATPase activity, defined as the ouabain-insensitive component, was subtracted. Na,K-ATPase activity in control, untreated myocyte membranes (*filled circle*) is shown for reference. Measurement of ATP hydrolysis in the presence of different concentrations of the agrin fragments shows that C-Ag20 significantly inhibits ($p < 0.001$; ANOVA), whereas C-Ag15 potentiates ($p < 0.001$; ANOVA) Na,K-ATPase activity in a concentration-dependent manner. Data for both fragments are well fit by a variable slope sigmoidal dose-response curve ($R^2 = 0.98$). Each data point represents the mean ± S.E. of 3 independent membrane preparations. *B*, Western blots of plasma membranes prepared from P0 heart ventricle (*H*), brain (*B*), and kidney (*K*) probed with anti-α1, α2, and α3 subunit antibodies show that expression of the α3 subunit is restricted to heart and brain. Each lane was loaded with 60 μg of total protein. *C*, Na,K-ATPase (*NKA*) activity in sarcolemmal membranes prepared from P0 heart and brain was inhibited by C-Ag20 and potentiated by C-Ag15 but was unchanged by either agrin fragment in kidney. *Bars* show mean ± S.E. for five independent membrane preparations for each tissue. **, $p < 0.01$, paired *t* test. *D*, Western blots of membranes prepared from cultured cardiac myocyte (*M*), glial (*G*), and BHK cells showing pattern of α1, α2, and α3 subunit expression. *E*, effects of C-Ag20 and C-Ag15 on Na,K-ATPase activity in cultured cardiac myocyte, glial, and BHK cell membranes. *Bars* show mean ± S.E. for a minimum of three independent membrane preparations for each cell type. **, $p < 0.01$; paired *t* test. *F*, effects of a saturating concentration of C-Ag20 and C-Ag15 on Na,K-ATPase activity in ventricular myocyte membranes prepared from *Agrn*$^{+/+}$ and *Agrn*$^{-/-}$ E18 mouse hearts. C-Ag20 inhibits Na,K-ATPase activity in both wild type and mutant tissue. In contrast, the increase in Na,K-ATPase activity normally produced by C-Ag15 in wild type tissue is absent in the mutant. Na,K-ATPase activity is expressed as a percent of the ouabain-sensitive fraction in saline-treated control membranes. *Bars* show mean ± S.E. for 3–4 determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *t* test.

Agrin Regulates α3 Na,K-ATPase Activity—The ability of agrin to bind to the α3 subunit of the Na,K-ATPase raises the question of what effect agrin might have on Na,K-ATPase function. Accordingly, we tested the capacity of different agrin fragments to modulate Na,K-ATPase-dependent ATP hydrolysis in mouse heart muscle membranes. Previous studies have shown that α3 Na,K-ATPase expression in rodent heart is highest during early postnatal development; therefore sarcolemmal membranes were prepared from P0 mouse ventricles as described (23). Levels of inorganic phosphate were measured using a colorimetric assay, and Na,K-ATPase activity was defined as the fraction (~60%) of total ATPase activity that could be inhibited by 5 mM ouabain. Estimates of Na,K-ATPase-specific ATPase hydrolysis based on ouabain sensitivity were indistinguishable from those obtained by comparing ATP hydrolysis in the presence and absence of Na^+ and K^+ (data not shown), confirming the efficacy of the ouabain treatment. Moreover, the specific activity of the Na,K-ATPase in control untreated membranes (Fig. 3*A*) was similar to that reported previously for mouse heart (29).

The addition of C-Ag20 resulted in a significant ($p < 0.001$, ANOVA) concentration-dependent inhibition of Na,K-ATPase activity in P0 cardiac sarcolemmal membranes (EC_{50} 21.7 ± 0.4 pM). Maximal inhibition by C-Ag20 was about 30% of total Na,K-ATPase activity (Fig. 3*A*), in accord with estimates of the contribution of α3 Na,K-ATPase to total Na,K-ATPase activity in neonatal rat cardiac sarcolemmal membranes (23). In contrast to the effects of C-Ag20, Na,K-ATPase activity was potentiated (EC_{50} 12.2 ± 2.7 pM; $p < 0.001$, ANOVA) by treatment with the agrin antagonist C-Ag15 (Fig. 3*A*). This observation rules out an effect of the vehicle, as C-Ag20 and C-Ag15 are stored in the same buffer. A similar impact on Na,K-ATPase activity was also observed in membranes prepared from adult mouse brain (EC_{50} C-Ag20 21.5 ± 1.4 pM; EC_{50} C-Ag15 15.3 ± 2.0 pM), evidence that agrin interacts with a common target in the two tissues (supplemental Fig. 3).

The α3 Na,K-ATPase is expressed in heart and brain but not kidney (30). Therefore, to test whether the α3 subunit is required for sensitivity to agrin, we measured the effect of a saturating concentration of each agrin fragment on the rate of Na,K-ATPase-dependent ATP hydrolysis in membrane preparations from P0 heart, brain, and kidney. Western blot analysis showed that the α1 subunit of the Na,K-ATPase was present in membranes of all three tissues, but expression of the α3 subunit was confined to heart and brain (Fig. 3*B*). Consistent with a requirement for α3 subunit, agrin-dependent (C-Ag20 and C-Ag15) modulation of Na,K-ATPase-dependent ATP hydrolysis was observed in membranes prepared from heart and brain but not kidney (Fig. 3*C*). Because the α2 and α3 subunits are co-expressed in heart and brain, we could not rule out the possibility that agrin modulation of ATPase activity might require

the presence of the α2 subunit-containing pump. Therefore, a parallel study was performed on cultured cells in which the pattern of α-subunit expression would allow us to address this question directly. In contrast to cultured cardiac myocytes, which express all three α-subunits, cerebrocortical glial cells and BHK cells express α1 and α2 but not α3 (Fig. 3*D*). Whereas the agrin response of membranes from cultured cardiac myocytes was comparable to P0 myocyte membranes, neither glial nor BHK cell membrane Na,K-ATPase activity was affected by treatment with C-Ag20 or C-Ag15 (Fig. 3*E*). Taken together, the results provide strong support for the conclusion that agrin is a specific inhibitor of the α3 subunit-containing Na,K-ATPase in cardiac myocytes.

How does C-Ag15 increase the activity of the α3 Na,K-ATPase? One possibility is that C-Ag15 directly increases pump rate. A second possibility, suggested by the observation that some α3 Na,K-ATPases are bound by endogenous agrin (Fig. 1, *C* and *D*), is that potentiation of Na,K-ATPase activity reflects disinhibition of existing pumps through competitive displacement of endogenous agrin by C-Ag15. Consistent with this hypothesis, agrin was present in Western blots of membranes prepared from heart tissue or cultured ventricular myocytes (data not shown), and C-Ag15 blocked formation of the endogenous agrin-α3 Na,K-ATPase complex in the cross-linking experiments (Fig. 1, *C* and *D*). To test the hypothesis that basal levels of Na,K-ATPase activity depend on endogenous agrin, we compared the effects of C-Ag20 and C-Ag15 on the Na,K-ATPase activity of sarcolemmal membranes isolated from ventricular myocytes of either wild type ($Agrn^{+/+}$) or agrin mutant ($Agrn^{-/-}$) mouse hearts. Because mutation of both agrin alleles is perinatal lethal, these experiments were performed on tissue isolated from E18 mouse embryos. As expected, a saturating concentration of C-Ag20 inhibited Na,K-ATPase activity in both $Agrn^{+/+}$ and $Agrn^{-/-}$ sarcolemmal membranes (Fig. 3*F*). However, Na,K-ATPase activity was enhanced by C-Ag15 only in membranes from $Agrn^{+/+}$ hearts, whereas $Agrn^{-/-}$ membranes were C-Ag15 -insensitive, ruling out a direct effect of C-Ag15 on Na,K-ATPase activity and supporting the agrin displacement hypothesis.

Agrin Increases Affinity of α3 Na,K-ATPase for Ouabain—The ouabain-like ability of agrin to inhibit α3 Na,K-ATPase activity suggested that agrin and ouabain might interact with a shared site on the α3 pump. To test this hypothesis, we examined the effect of different concentrations of C-Ag20 on ouabain inhibition of Na,K-ATPase activity. To control for the effects of endogenous agrin-α3 Na,K-ATPase interactions, measurements were made on sarcolemma membranes prepared from E18 $Agrn^{-/-}$ hearts. Nonspecific ATPase activity was measured in parallel reactions performed in Na^+/K^+ free solutions.

As expected, ouabain inhibition of Na,K-ATPase activity was well fit by a model assuming two populations of isoenzymes (Fig. 4): a low affinity component (K_i 50.5 ± 4.6 μM) that accounts for about 70% of the activity and corresponds to the α1 Na,K-ATPase; a high affinity component (K_i 59.5 ± 6.5 nM) that accounts for about 30% of the activity and is due primarily to the α3 Na,K-ATPase (23, 31). Consistent with the results of the cross-linking studies and tissue dependence of agrin sensitivity, treatment with C-Ag20 reduced the activity of the high ouabain affinity α3 Na,K-ATPase but had no effect on low ouabain affinity α1 Na,K-ATPase. Interestingly, the effect of C-Ag20 was not simply additive, as would be expected if agrin and ouabain competed for a common binding site, but was due instead to a significant increase ($p < 0.001$, ANOVA) in the apparent affinity of ouabain for the pump. Because ouabain binds to the pump in its E_2P conformation (1), these results suggest that agrin binding to the α3 Na,K-ATPase stabilizes the E_2P state.

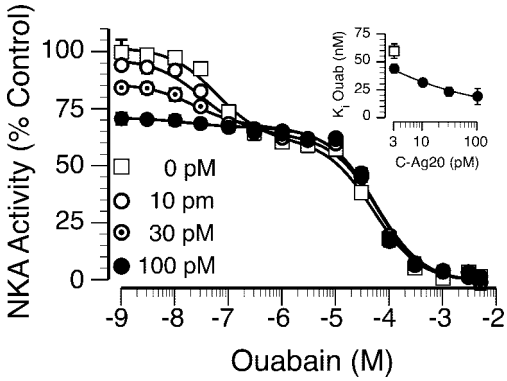

FIGURE 4. **Agrin increases affinity of the α3 Na,K-ATPase for ouabain.** Ouabain inhibition of Na,K-ATPase activity of $Agrn^{-/-}$ sarcolemmal membranes in the presence of different concentrations (0, 10, 30, 100 pM) of C-Ag20. Nonspecific ATPase activity observed in Na^+/K^+-free reaction buffer has been subtracted. Data points (mean ± S.E. of triplicate determinations) have been fit with a two-site competition model ($R^2 > 0.95$). *Inset* shows predicted K_i for the high affinity ouabain binding site plotted as a function of C-Ag20 concentration and includes a data point at 3 pM C-Ag20 that was omitted from the main figure for clarity. C-Ag20 has no effect on ouabain inhibition of the low affinity α1 Na,K-ATPase but significantly ($p < 0.001$, ANOVA) increases the affinity of ouabain for the high affinity Na,K-ATPase, which at this stage in development is predominantly the α3 isoform.

Agrin Modulates Cytoplasmic Na^+ Levels in Cultured Myocytes—The ability of agrin to regulate the activity of the α3 Na,K-ATPase suggests a role in Na^+ homeostasis. To test this hypothesis, ratiometric imaging with the Na^+-sensitive dye SBFI was used to examine the effect of different agrin fragments on cytoplasmic Na^+ levels in cultured cardiac myocytes. Even in normal saline, cardiac myocytes exhibited spontaneous, small amplitude, short duration increases in intracellular Na^+, with similar synchronized activity in other cells within a single field of view, suggestive of extensive electrical coupling between cells. Although cultured cardiac myocytes also exhibit synchronized spontaneous contractions, no simple correlation between the Na^+ spikes and myocyte contraction was observed.

The addition of C-Ag20 (100 pM) sufficient to maximally inhibit α3 Na,K-ATPase-dependent ATP hydrolysis triggered a rapid rise in cytoplasmic Na^+ concentration that was distinct from the spontaneous Na^+ spikes (Fig. 5*A*). The response to agrin was sustained during the period of agrin exposure but returned toward base-line levels upon washing in normal saline. Visual inspection revealed that treatment with agrin also triggered a mild, transient, tetanic contraction, although we did not quantitate this further. Quantitative comparison of the effects of different agrin fragments showed that cytoplasmic Na^+ levels increased rapidly ($t_{1/2}$ 4.2 ± 1.8 s) in the presence of C-Ag20 to a peak level equivalent to about 70% of that seen in the presence of 5 mM ouabain (Fig. 5*B*). Given the relatively small contribution of the α3 pump to total Na,K-ATPase activity of cultured myocyte membranes, the large increase in intra-

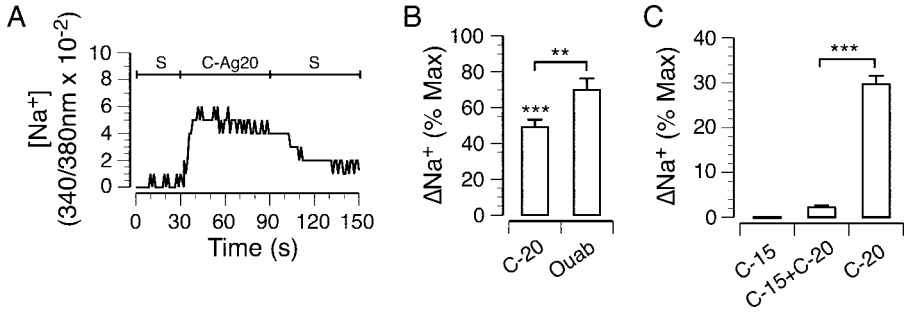

FIGURE 5. **Agrin modulates cytoplasmic Na^+ in cultured cardiac myocytes.** *A*, record from a single cultured cardiac myocyte showing small, spontaneous fluctuations in cytoplasmic Na^+ observed in normal saline (*S*) and larger, sustained increase in Na^+ following treatment with agrin (C-Ag20). *B*, *bars* show mean change in Na^+ concentration in response to saturating concentration of C-Ag20 or ouabain (*Oaub*). To control for differences in SBFI loading between cells and experiments, data for each cell was base line-subtracted and normalized to the maximal response to a mixture of ouabain and gramicidin. Treatment with agrin results in a significant increase in Na^+ levels, albeit lower than that observed in the presence of ouabain, a pan-specific Na,K-ATPase inhibitor. *C*, whereas treatment with C-Ag15 alone had no effect on Na^+, C-Ag15 clearly antagonized the response to C-Ag20. Note that the concentration of C-Ag20 used for the competition assays was 10-fold lower than in *B*, resulting in a smaller maximal response to C-Ag20. *Bars* show data from 14–27 cells from a minimum of two experiments. **, $p < 0.01$; ***, $p < 0.001$, paired *t* test.

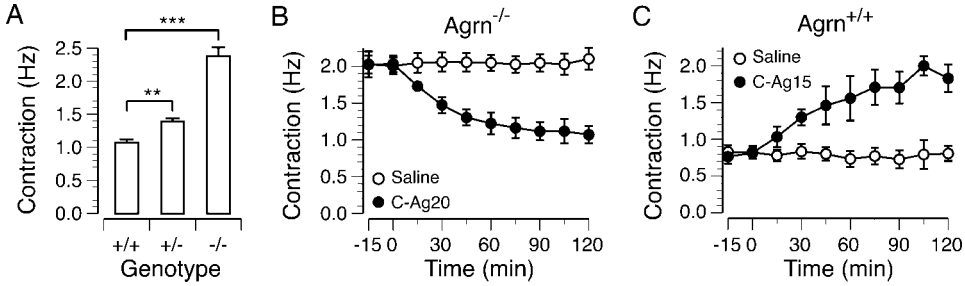

FIGURE 6. **Cardiac myocyte contraction frequency is agrin-dependent.** Cardiac myocytes were prepared from hearts of individual E18 embryos from heterozygous matings. *A*, at 5 days in culture, the frequency of spontaneous contractions was determined by counting five random fields for 30 s at room temperature. The contraction frequency of myocytes that are mutant for either one or both of the *Agrn* alleles is significantly higher (**, $p < 0.01$; ***, $p < 0.0001$; two tailed *t* test) than for wild type cells. *B*, addition of C-Ag20 to $Agrn^{-/-}$ myocytes rescues the mutant phenotype ($p < 0.001$, two-way ANOVA). *C*, treatment of wild type cultures with the agrin antagonist C-Ag15 phenocopies mutation of *Agrn* ($p < 0.001$; two-way ANOVA). Each chart summarizes data from a minimum of three independent experiments. All data were collected blind with respect to genotype and treatment.

cellular Na^+ suggested the presence of a cellular mechanism, such as the activation of voltage-gated sodium channels, capable of amplifying the agrin signal, but this was not pursued further. A similar concentration of C-Ag15 had no detectable effect on either spontaneous Na^+ spikes or mean resting Na^+ levels but blocked the increase normally induced by subsaturating concentration of C-Ag20 (Fig. 5*C*), confirming the specificity of the response to C-Ag20 and not to the vehicle. These data extend our initial observation using biochemical assays of Na,K-ATPase activity by providing evidence that agrin also inhibits α3 Na,K-ATPase activity in intact myocytes.

Cardiac Myocyte Contraction Is Agrin-dependent—The inotropic effects of cardiac glycosides such as ouabain and digoxin derive from their ability to inhibit the Na,K-ATPase. Cardiac myocytes exhibit rhythmic spontaneous contractions when grown in cell culture. Therefore, as a first step toward exploring the role of agrin-α3 Na,K-ATPase interactions in regulating cardiac myocyte function, we examined the effect of mutation of *Agrn* on cardiac myocyte contraction frequency. Analysis of cultured myocytes prepared from E18 $Agrn^{+/+}$, $Agrn^{+/-}$, and $Agrn^{-/-}$ embryos (Fig. 6*A*) showed that the spontaneous contraction frequency is dependent on *Agrn* dosage, with the frequency of $Agrn^{-/-}$ myocytes being approximately twice that of $Agrn^{+/+}$ ($Agrn^{-/-}$ (2.4 ± 0.13 Hz) *versus* $Agrn^{+/+}$ (1.1 ± 0.05 Hz); $p <$ 0.001, *t* test).

To learn whether the mutant phenotype was directly due to the absence of agrin protein in the cultures, we next examined the effects of C-Ag20 and C-Ag15 on myocyte contraction. The contraction frequency of $Agrn^{-/-}$ myocytes declined in the presence of a saturating concentration of C-Ag20 (Fig. 6*B*). The C-Ag20-induced change in contraction frequency was detectable within 15 min of the onset of treatment with C-Ag20. By 45 min the contraction frequency of the C-Ag20-treated $Agrn^{-/-}$ cultures was reduced to 1.3 ± 0.11 Hz, a rate indistinguishable from that of vehicle-treated wild type myocytes. Interestingly, treatment of wild type cultures with C-Ag15 caused an increase in contraction frequency of wild type myocytes to a level similar to that seen in $Agrn^{-/-}$ myocytes (Fig. 6*C*), evidence that the basal contraction frequency of wild type myocytes is regulated by endogenous agrin. A similar increase in contraction frequency (from 1.1 ± 0.02 Hz to 1.9 ± 0.01 Hz; $p < 0.001$, *t* test) was also observed in cardiac myocytes prepared from 129/Swiss mice treated with C-Ag15, ruling out the possibility that agrin regulation of cardiac myocyte rhythmogenicity is strain-specific. Given the relatively short latency of the response to the agrin fragments, the altered contraction frequency of $Agrn^{-/-}$ myocytes is unlikely to be due to the loss of agrin function during development but instead reflects a direct response to a change in α3 Na,K-ATPase function, normally regulated by agrin. Based on these results, we concluded that agrin modulates the contraction frequency of cultured cardiac myocytes.

DISCUSSION

The major finding of this study is that suppression of agrin signaling, either through genetic knock-out or treatment with an agrin antagonist, increases the frequency of spontaneous contraction of cultured cardiac myocytes. Although agrin expression in heart was first documented almost 20 years ago (14), this is the first evidence that cardiac muscle function is agrin-dependent. Cardiac myocyte contractility is critically dependent on Na,K-ATPase function, but the contribution of different Na,K-ATPase isoforms to cardiac muscle function is poorly understood. Our data, showing that agrin exerts its effects through specific interaction with the α3 subunit-con-

taining Na,K-ATPase, suggest that the α3 Na,K-ATPase may a play special role in regulating myocyte contraction. Future studies of conditional *Agrn* mutant mice will be required to determine the role of agrin in regulating heart function *in vivo*. However, our results, particularly in the context of the relatively high levels of α3 Na,K-ATPase expression in human heart (32), suggest that the agrin-α3 Na,K-ATPase signal pathway may be an important therapeutic target for the treatment of congestive heart failure, arrhythmia, and other forms of heart disease.

Agrin binds a number of cell surface proteins including laminin, neural cell adhesion molecule (NCAM), α-dystroglycan, integrins, and MuSK (33). Although, in principle, any one of these proteins could mediate the increased beat frequency associated with mutation of *Agrn*, several lines of evidence suggest that it is the loss of signaling through the α3 Na,K-ATPase that is responsible. With the exception of MuSK, binding sites for the other candidate receptors are absent from the C-Ag20 fragment that effects a complete rescue of the *Agrn* mutant phenotype, ruling out a contribution to the results presented here. A role for MuSK seems unlikely, as a recent study failed to detect MuSK mRNA in mouse heart by PCR (34). Moreover, the C-Ag20 fragment used here lacks an insert at the alternatively spliced z-site that has been shown to be required for MuSK activation (35). Co-localization, cross-linking, and the ability of the C-Ag20 and C-Ag15 to modulate α3 Na,K-ATPase activity and cardiac myocyte contraction frequency all argue that the α3 Na,K-ATPase is an agrin receptor in cardiac muscle. Additional studies in ATP1A3 mutant mice, which lack the α3 subunit, will be important in determining whether this is the primary receptor responsible for the effects of agrin on the heart.

The increased contraction frequency of $Agrn^{-/-}$ myocytes was somewhat unexpected given that the contraction frequency of wild type myocytes increases when Na,K-ATPase activity is inhibited with ouabain (36, 37). We suspect, however, that this is a manifestation of the specificity of agrin for the α3 Na,K-ATPase in contrast to ouabain and other cardiac glycosides, which bind and inhibit α1 and α2 Na,K-ATPases as well. As the second messenger responsible for excitation-contraction coupling, changes in cardiac myocyte function are tightly linked to changes in Ca^{2+} handling. In particular, myocyte contraction frequency is an integral of the velocity of contraction and relaxation, and just as contraction is triggered by a rise in cytoplasmic Ca^{2+}, relaxation depends on its removal. Removal of cytoplasmic Ca^{2+} in cardiac myocytes occurs by two main pathways: re-uptake by the sarcoplasmic reticulum and extrusion by the NCX. Clearly, any increase in the efficiency of either pathway would be expected to increase the rate of relaxation, thereby shortening the refractory period between contractions. One explanation for the chronotropic effects of agrin, therefore, may derive from the functional coupling of the Na,K-ATPase and NCX whereby increased α3 Na,K-ATPase activity, induced by either the agrin antagonist C-Ag15 or mutation of *Agrn*, augments the activity of the NCX, decreasing relaxation time and increasing the overall contraction frequency. This would be consistent with the model proposed by Blaustein and colleagues (38, 39) who showed that α2/α3 subunit-containing Na,K-ATPases and NCX are concentrated in regions of the plasma membrane that overlie the sarcoplasmic/endoplasmic reticulum. Whether agrin affects Ca^{2+} levels in the sarcoplasmic reticulum is not known, but increased NCX activity associated with loss of agrin function or treatment with the agrin antagonist would be expected to reduce the releasable pool of Ca^{2+} in the sarcoplasmic reticulum, resulting in a decrease in the rate and duration of contraction and an increase in contraction frequency.

Numerous studies have implicated nonreceptor tyrosine kinases in regulating Na,K-ATPase function. It is noteworthy, therefore, that endogenous levels of tyrosine phosphorylation of the α3 subunit were increased by C-Ag20 and decreased by C-Ag15, suggesting that tyrosine phosphorylation may inhibit activity of the pump. Curiously, tyrosine phosphorylation has the opposite effect in cortical neurons, where Lyn, a member of the Src family, activates the α3 Na,K-ATPase (40). However, at least for the α1 subunit, *in vitro* studies have shown that the effect of tyrosine phosphorylation is kinase-dependent; Src increases, whereas Fyn and Lyn decrease, pump activity (41). Thus, tissue-specific patterns of kinase expression and/or activation of a specific kinase in response to agrin binding the α3 Na,K-ATPase may play an important role in regulating pump activity. It should be noted, however, that others have suggested that Na,K-ATPase-mediated activation of Src family kinases is evidence of a signaling role that is independent of ion transport (42). Consistent with this possibility, agrin binding to the α3 Na,K-ATPase activates both mitogen-activated protein kinase (MAPK) and calmodulin (CaM) kinase II and induces expression of c-*fos* in cortical neurons (25, 43). Additional studies will be needed to explore the link between the effects of agrin on cardiac myocyte contraction and its ability to modulate α3 Na,K-ATPase ion transport and tyrosine phosphorylation.

Cardiac myocyte contraction is coordinated by extensive electrical coupling through gap junctions located within the intercalated discs connecting one muscle fiber to another. Interestingly, agrin has been shown to trigger a rapid decrease in gap junction-mediated electrical communication between adrenal chromaffin cells (44), raising the possibility that a similar mechanism might be important for the chronotropism of agrin. Two lines of evidence, however, make this unlikely. First, in contrast to the ability of z- agrin to rescue the *Agrn* mutation, regulation of gap junction function in chromaffin cells is specific for the z+ agrin isoform. Second, a decline in the strength of electrical coupling between myocytes would be expected to increase the degree of asynchronous contractile activity within the culture when in fact mutation of the agrin gene was often associated with the appearance of multiple contractile foci within a group of myocytes (supplemental Fig. 4, *A* and *B*).

The use of an alternate start site gives rise to two forms of agrin: a long NH_2-terminal form (LN-agrin), which is secreted and becomes anchored to basal lamina via its laminin binding domain, and a short NH_2-terminal form (SN-agrin), which lacks the laminin binding domain and is expressed as a type II membrane protein. SN-agrin is restricted to the central nervous system neurons, whereas LN-agrin is expressed by motor neurons, skeletal muscle, and other non-neuronal tissues including heart (45). In the present study, changes in spontaneous contraction frequency of cultured myocytes were evident within minutes of treatment with the short, soluble forms of

agrin. Whether native, basal, lamina-associated agrin plays a role in the short latency or in more long-lasting changes in myocyte rhythm remains to be determined. It is conceivable that changes in the rate of agrin synthesis and/or secretion could provide a homeostatic mechanism for control of α3 Na,K-ATPase activity. Why such a mechanism would be selected rather than regulating synthesis and insertion of the α3 Na,K-ATPase itself is unclear, but the presence of a population of α3 subunits that could not be cross-linked to agrin may be evidence that such a pool of α3 Na,K-ATPases exists. An alternate mechanism is suggested by the recent demonstration that agrin is a specific substrate for the serine protease neurotrypsin (46). Interestingly, cleavage of agrin by neurotrypsin results in a 22-kDa COOH-terminal fragment that includes the C-Ag20 fragment used here. Although it remains to be determined whether neurotrypsin is expressed in heart, the possibility that a proteolytic fragment of agrin diffusing within the myocardium, rather than full-length agrin anchored to the basal lamina, is important for regulation of α3 Na,K-ATPase activity warrants consideration.

The inotropic effects of ouabain and other cardiac glycosides stem from their ability to inhibit Na,K-ATPase function. Cardiac myocytes express multiple Na,K-ATPase isoforms, and studies in mouse indicate that ouabain-induced strengthening of muscle contraction depends on inhibition of the α2 Na,K-ATPase isoform (47, 48), evidence that different Na,K-ATPase isoforms serve distinct functions. Further studies will be required to determine whether agrin affects the strength of cardiac myocyte contraction, but the demonstration here that contraction frequency is agrin-dependent raises the possibility that the α3 Na,K-ATPase plays a special role in cardiac rhythmogenicity.

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