Subunit Composition and Role of Na\textsuperscript{+},K\textsuperscript{+}-ATPases in Ventricular Myocytes

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Abstract: Na\textsuperscript{+},K\textsuperscript{+}-ATPases are composed of one \(\alpha\) and one \(\beta\) subunit; four \(\alpha\) and three \(\beta\) isoforms have been found to date. We elucidated which \(\alpha\) and \(\beta\) subunits were present in the ventricular myocytes of rat and guinea-pig and what roles the Na\textsuperscript{+},K\textsuperscript{+}-ATPase isozymes play in cardiac contraction. The presence of the \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) subunits and the \(\beta_1\) and \(\beta_2\) subunits in rat and guinea-pig hearts were confirmed at the protein or mRNA level. Immunocytochemistry showed a patchy presence of \(\alpha_1\) in the transverse tubules and surface sarcolemma, whereas \(\alpha_2\) was distributed continuously in the transverse tubules alone. The \(\alpha_3\) isoform was expressed prominently in the guinea-pig intercalated disc and slightly in the rat. On the other hand, the \(\beta_1\) isoform was located in the transverse tubules and surface sarcolemma, whereas the \(\beta_2\) was mainly located in the intercalated disc. The immunocytochemistry and immunoprecipitation findings indicated that the \(\alpha_1\) and \(\alpha_2\) form heterodimers with \(\beta_1\) and the \(\alpha_3\) with \(\beta_2\) in ventricular myocytes. The application of low concentrations of ouabain enhanced the amplitudes of twitch without a change in resting tension in rat and guinea-pig ventricular strips, whereas that of high concentrations resulted in a decrease in twitch with an increase in the resting tension. We thus conclude that the \(\alpha_2\beta_1\) and \(\alpha_3\beta_2\) isozymes are selectively located in the transverse tubules and intercalated disc of the ventricular myocytes, respectively, and the \(\alpha_2\beta_1\) is involved in the regulation of the Ca\textsuperscript{2+} contents in the SR.

Key words: transverse tubules, intercalated disc, localization, Na\textsuperscript{+} pump, subunits.

\(N\)a\textsuperscript{+}, K\textsuperscript{+}-ATPases are heterodimers comprising \(\alpha\) and \(\beta\) subunits [1]. There are four \(\alpha\) and three \(\beta\) isoforms, and the expression of the \(\alpha\) and \(\beta\) subunits has been reported to be developmentally regulated in a cell-specific manner [2]. Although the expression of \(\alpha\) and \(\beta\) subunits in oocytes [3] or Sf-9 cells [1], an insect cell line, revealed every combination of \(\alpha\) and \(\beta\) subunits was functional, Na\textsuperscript{+},K\textsuperscript{+}-ATPases in cells in situ are thought to exist as a specific combination of \(\alpha\) and \(\beta\) subunits [4, 5]. Furthermore, single cells have shown to have multiple \(\alpha\) subunits, which are distributed differently in the cell membrane [6]. We elucidated that the \(\alpha_1\) subunit, which formed a heterodimer with the \(\beta_3\) subunit, was ubiquitously present in the cell membrane of adrenal chromaffin cells, whereas the \(\alpha_2\) subunit, forming a heterodimer with the \(\beta_2\), was located in the cell membrane in the vicinity of Ca\textsuperscript{2+} store sites [7]. Our functional and morphological analyses revealed that the \(\alpha_2\) subunit was involved in the regulation of the Ca\textsuperscript{2+} contents in intracellular Ca\textsuperscript{2+} store sites and the \(\alpha_3\) subunit was not expressed in chromaffin cells [7]. In astrocytes, the \(\alpha_2\) subunit has also been shown to take part in regulating the Ca\textsuperscript{2+} contents in Ca\textsuperscript{2+} store sites, whereas the \(\alpha_1\) subunit has only been assumed to play a house-keeping role [8]. On the other hand, a recent knock-in experiment revealed that the \(\alpha_1\) subunit could regulate Ca\textsuperscript{2+} contents in the sarcoplasmic reticulum (SR) of mouse cardiac myocytes [9]. This result, however, does not negate the notion that the \(\alpha_2\) subunit specially evolves to regulate Ca\textsuperscript{2+} contents in intracellular Ca\textsuperscript{2+} store sites. The present experiment is aimed first to elucidate whether the \(\alpha_2\) subunit is involved in the regulation of Ca\textsuperscript{2+} contents in SR cardiac myocytes. For this purpose, we investigated the effects of high and low concentrations of ouabain on twitch amplitude and resting tension in guinea-pig and rat cardiac muscle preparations, which were known to differ markedly in affinity of high ouabain affinity sites. The second aim is to elucidate whether the \(\alpha_3\) subunit makes a heterodimer. This information, if the \(\alpha_2\) subunit is involved in the regulation of Ca\textsuperscript{2+} contents in Ca\textsuperscript{2+} store sites, would be helpful to elucidate whether the signal targeting the \(\alpha_2\) subunit to the cell membrane closely associated with Ca\textsuperscript{2+} store sites resides in \(\alpha_2\) or a \(\beta\) subunit coupled. The third aim is to clarify whether the \(\alpha_3\) subunit has a specific function in cells with multiple \(\alpha\) subunits. Our preliminary experiments showed the guinea-pig cardiac myocytes to have \(\alpha_1\), \(\alpha_2\) and \(\alpha_3\) subunits.
Therefore, we examined the distribution of α and β subunits in the cardiac cell membrane and the combination of α and β subunits.

METHODS

Guinea-pigs and rats weighing 250–400 g were used. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of University of Occupational and Environmental Health.

Immunoblot. Guinea-pigs and rats were killed by cervical dislocation. The brains and hearts were homogenized in a Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.4) containing a protease inhibitor cocktail (Calbiochem). Homogenates were spun down at 2,000 × g for 10 min at 4°C, then the post-nucleus supernatants were mixed with twofold concentrated Laemmli sample buffer. Protein concentrations in samples were determined using a BCA protein assay kit (Pierce). Just before electrophoresis, 5% (v/v) 2-mercaptoethanol and 1% (w/v) bromophenol blue were added to the samples, and proteins were separated by SDS-PAGE, followed by a Western blot analysis.

Fractionation study. A crude fraction of intercalated disc was obtained with the method described previously [11]. Each 1 g of guinea-pig hearts was homogenized in 30 ml of 10 mM Tris solution (pH, 7.0), and then was centrifuged at 10,000 × g for 20 min. Each pellet was resuspended in 30 ml of KCl extraction buffer (600 mM KCl, 8% sucrose, and 10 mM Tris, pH 7.0). The resuspended pellets in the high K+ buffer (HK) were pooled and stirred overnight at 4°C, and then were centrifuged at 10,000 × g for 20 min. The pellets were pooled and resuspended in 20 ml of 10 mM Tris solution containing 25% sucrose. This resuspended pellet in 25% sucrose (S) was loaded over a sucrose gradient composed of four steps (37, 45, 50, and 54% sucrose in Tris buffer), and then it was centrifuged at 98,000 × g for 2 h. Materials between 45% and 50% and between 50% and 54% sucrose were collected and designated as F2 and F3, respectively. Proteins in HK, S, F2, and F3 fractions were separated on SDS-PAGE and immunoblotted for N-cadherin, Na+,K+-ATPase α subunits were dissociated from the protein A and Ab mixture by incubation in 45 µl of the Laemmli sample buffer for 30 min at 37°C. The samples were then subjected to SDS-PAGE, followed by a Western blot analysis.

Immunocytchemistry. Guinea-pig and rat hearts, mounted in a Langendorff apparatus, were retrogradely perfused with collagenase-containing Ca2+-free solution, and then with the storage solution containing 50 mM glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH2PO4, 3 mM MgCl2, 10 mM glucose, 10 mM HEPES, and 0.5 mM EGTA (pH, adjusted to 7.4 with KOH), as described previously [10]. The hearts in the storage solution were cut into small pieces with scissors, and ventricular myocytes were dissociated by pipetting. Dissociated myocytes were placed in a dish and fixed in 2% parafomaldehyde in phosphate buffer saline (PBS) for 2 h at 4°C or in methanol at −18°C for 10 min. For indirect immunofluorescence studies, the cells were treated with a primary Ab. After the incubation, the cells were washed three times in PBS and then treated with a respective secondary Ab conjugated with Alexa 488 or 546. The fluorescence was observed using laser confocal scanning microscopy (Zeiss LSM 410). For Alexa 488, a 488 nm laser was used and 510–525 nm emission was observed, whereas for Alexa 546, a 543 nm laser was used and emission above 570 nm was observed.

Immunoprecipitation. Heart post-nuclear supernatants were solubilized in Tris buffer containing 10 µM deoxycholate, 1 mM PMSF, 10 µg ml−1 leupeptin, 10 µg ml−1 antipain, and 10 µg ml−1 pepstatin A to bring the final protein concentrations to 1 µg µl−1. The sample was vortex mixed at 4°C for 15 min before sonication. The insoluble material was then pelleted by centrifugation at 12,000 × g for 3 min at 4°C. The supernatant was removed, and then pre-cleared with 50 µl of immobilized protein A with 15 min vortex mixing at 4°C. The sample mixture was then centrifuged at 12,000 × g for 3 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube, and 5 µg of rabbit anti-β1 Ab, anti-β2 Ab, or anti-β3 Ab was added. The mixture was incubated on a rotating wheel for 3 h at 4°C. After this, 50 µl of immobilized protein A was added and mixed for an additional 4 h on the rotating wheel at 4°C. After the mixture was washed, immunoprecipitated Na+,K+-ATPase α subunits were dissociated from the protein A and Ab mixture by incubation in 45 µl of the Laemmli sample buffer for 30 min at 37°C. The samples were then subjected to SDS-PAGE, followed by a Western blot analysis.

RT-PCR of mRNAs for Na+,K+-ATPase α-subunit isoforms. Brain and heart poly (A)−RNAs were prepared from rat...
and guinea pig using the Micro-fast tract kit (Invitrogen). Oligo dT primer was utilized for reverse transcriptase (RT) reaction to obtain cDNAs. RT reaction was carried out according to the manufacturer's instructions (Super-Script First-Strand Synthesis System for RT-PCR, Invitrogen). Nucleotide sequences of the gene specific primers for α (ATP1A1), α2 (ATP1A2), and α3 (ATP1A3) isoforms were following: 5’-TGACCCAGATGCCAAG-3’ (for ATP1A1 forward); 5’-GAGATGGCGCGAACCATGTC-3’ (for ATP1A1 reverse); 5’-CTGGTCTACACGACAGACGAC-3’ (for ATP1A2 forward); 5’-CATGATGACCTTGATGCCTG-3’ (for ATP1A2 reverse); 5’-CAATATACCAGCTATCCATCC-3’ (for ATP1A3 forward); 5’-CATGATGACCTTGATGCCTG-3’ (for ATP1A3 reverse). The primers for the β-actin gene transcripts (ACTB) were 5’-AGGCACCAGGGTGTT-3’ (forward) and 5’-CTCAAACATGATCTGGGTCATC-3’ (reverse). The calculated sizes of PCR products were 498 bp (ATP1A1), 553 bp (ATP1A2), and 261 bp (ACTB). Each preparation had a primer set for one of α isoforms and a primer set for β-actin as an internal reference. In order to avoid a saturation of the PCR product for β-actin prior to that of the target genes, a primer set for β-actin was added after the first five PCR cycles were complete in which the target gene primers were already contained as described elsewhere [7]. It was confirmed that both the target and the reference gene products were amplified in the exponential range; thus, the relative amount of α-subunit mRNA was measured as a ratio of the α-subunit to the β-actin RT-PCR products. The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide. Each band was scanned with CAMEDIA C-5060 (OLYMPUS), then quantified using Scion Image. RT-PCR reactions without RT were also carried out as negative controls. All experiments were performed in triplicate.

**Antibodies.** The antibodies used were a monoclonal anti-α, β1, and α3 subunit antibody (mAb) (05-369; Upstate Biotechnology) [12], rabbit anti-α, β1, and α3 mAb (MA3-915; Affinity BioReagents) [14], rabbit anti-α, β1, and α3 mAb (06-172; Upstate) [13], anti-α, β1, and α3 mAb (05-382; Upstate) [15], rabbit anti-α, β1, and α3 mAb (06-171; Upstate) [17], rabbit anti-β3 mAb (06-817; Upstate) [7], rabbit anti-SERCA pump mAb (MA3-910; Affinity BioReagents) [18], and anti-N-cadherin mAb (610920; BD) [19].

**RESULTS**

**Immunoblot**

Which α and β subunits were present in guinea-pig and rat hearts was investigated using immunoblotting. An anti-α1 mAb detected 105 kDa proteins in brain and heart homogenates of guinea-pig and rat. The amounts of α1 in the guinea-pig and rat hearts were 219 ± 24% (n = 6) and 50 ± 3% (n = 3) of those in each brain sample. An anti-α2 Ab recognized 105 kDa proteins in brain and heart homogenates of guinea-pig and rat, and the amounts of the α2 subunits in the hearts were 21 ± 8% (n = 3) and 14 ± 7% (n = 3) of those in guinea-pig and rat brains, respectively. An anti-α3 mAb detected about 105 kDa proteins in homogenates of guinea-pig brain and heart and in homogenates of the rat brain, but not the heart. The amount of α3 in the heart was 30 ± 19% that of guinea-pig brain. In contrast to the α subunits, β subunits were present as several forms due to N-glycosylation [17]. An anti-β1 mAb detected three bands with molecular weights of 40 to 50 kDa in the brain and heart homogenates. The major β1 protein in the guinea-pig brain and heart and rat heart was 40 kDa,

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**Fig. 1.** Immunoblots for Na⁺,K⁺-ATPase α and β subunits of the brain and heart homogenates of guinea-pig and rat. Brain (B) and heart (H) homogenates of guinea-pig (g) and rat (r) were subjected to SDS-PAGE and proteins were transferred to the PVDF membranes. Equal amounts of proteins (10 to 25 μg) were loaded. The membrane was treated for 1 h or overnight with an anti-α1 mAb (at a dilution of 1:1,000), rabbit anti-α2 Ab (1:1,000), anti-α3 mAb (1:4,000), anti-β1 mAb (1:500), anti-β2 mAb (1:500), or rabbit anti-β3 Ab (1:500). The immunoreaction was visualized with an anti-mouse IgG Ab or anti-rabbit IgG Ab conjugated with peroxidase and ECL-Plus.
whereas in the rat brains 40 and 48 kDa proteins were equally expressed. The total amounts of β1 proteins detected in the guinea-pig and rat hearts were 121 ± 11% (n = 7) and 50 ± 8% (n = 7) in the corresponding brain. An anti-β2 mAb also detected three bands between 40 and 50 kDa in guinea-pig and rat, and the major band in guinea-pig brain and heart and rat brain was 48 kDa. The total amounts of β2 proteins in guinea-pig and rat hearts were 73 ± 6% (n = 6) and 5 ± 2% (n = 5) in the corresponding brain. In contrast to the β1 and β2 subunits, an apparently single band whose molecular weight extended from 40 to 50 kDa was recognized by an anti-β3 Ab in guinea-pig brain homogenates, whereas three bands were detected between 40 and 50 kDa in rat brain. This Ab did not specifically recognize any band in the heart homogenates of either animal. These immunoblots indicate that the guinea-pig hearts contained α1, α2, and α3 subunits and β1 and β2 subunits, whereas the rat hearts contained at least α1 and α2 subunits and β1 and β2 subunits.

**RT-PCR**

The apparent absence of the α3 subunit in rat hearts led us to study the Na⁺,K⁺-ATPase α subunits at the mRNA level. Figure 2 shows the amounts of α subunit mRNAs expressed as a fraction of the signal level for β-actin in brains and hearts of rat and guinea-pig. In contrast to immunoblotting, rat hearts had mRNAs for the α1, α2, and α3 subunits. The relative values for the α1, α2, and α3 subunits in the rat heart were 163%, 29%, and 32% of those in the brain. On the other hand, the guinea-pig heart also had mRNAs for the three α subunits. The relative value for the α3 subunit was 31% of that in the brain, whereas the relative values for the α1 and α2 subunits in the heart were comparable to those in the brain. The signal level of the α3 subunit in the guinea-pig brain, however, was much smaller than that of the α1 and α3 subunits in the same preparation, thus suggesting that the set of PCR probes for the α2 did not fit with the guinea-pig α2 subunit. We tried several combinations of probes, based on the rat genome database, to amplify the mRNA signal for the guinea-pig α2 subunit, since the guinea-pig Na⁺,K⁺-ATPase α2 subunit has not been cloned. The set of probes used in this figure was best for the amplification among 7

**Fig. 2.** Relative expression levels of Na⁺,K⁺-ATPase subunit mRNAs in rat and guinea-pig hearts. A and B, gel images of RT-PCR products of the α subunit (α1: ATP1A1, α2: ATP1A2, α3: ATP1A3) and β-actin (ACTB) mRNAs of rat and guinea-pig, respectively. Brain and heart mRNAs for α1, α2, α3 and β-actin were amplified with RT-PCR and separated on agarose gels (see METHODS). Upper and lower bands in each gel represent RT-PCR products for α subunits and β-actin, respectively. C, relative signal levels of bands of RT-PCR products for α subunits in rat brain (closed bar) and heart (opened bar) expressed as fractions of those for β-actin in the same preparations. D, relative signal levels of bands of RT-PCR products for subunits in guinea-pig brain (closed bar) and heart (opened bar) expressed as fractions of those for β-actin in the same preparations. Std stands for standard base pairs (100 to 700 or 1000 bp with a step of 100 bp). The data represent means ± SD of three measurements. **P < 0.01; ***P < 0.001 (Student’s unpaired t-test).

**Fig. 3.** Immunocytochemistry for Na⁺,K⁺-ATPase α and β subunits of guinea-pig ventricular myocytes. Images are confocal ones of dissociated ventricular myocytes treated for one or two days with an anti-α1 mAb (at a dilution of 1:100) (A), rabbit anti-α2 Ab (1:50) (B), anti-α3 Ab (1:100) (C), anti-β1 Ab (1:50) (D), anti-β1 Ab (1:50) (E), anti-β2 Ab (1:50) (F), or anti-β3 Ab (1:50) (G). The immunoreactions were visualized with either anti-mouse or rabbit IgG Abs conjugated with Alexa 488. The guinea-pig ventricular myocytes were isolated with collagenase treatment (see METHODS).
sets tested. This result indicates that the guinea-pig heart had mRNAs for the α2 subunit, but the precise amount of the mRNA remains to be determined.

**Immunocytochemistry**

Since α1, α2, and α3 subunits were found to be present at the protein and/or mRNA levels, their distributions were investigated in the isolated ventricular myocytes of guinea-pig and rat using laser scanning microscopy. Immunoreactions to each Ab were visualized as FITC- or rhodamine-like fluorescence. Immunoproducts to the anti-α1 mAb were patchily distributed in the surface sarcolemma and in a striate pattern in guinea-pig ventricular myocytes (Fig. 3A), whereas those to the anti-α2 Ab were distributed only in a striate pattern, and not present in the surface sarcolemma (Fig. 3B). The materials immunoreactive to an anti-α3 Ab were mainly distributed in the intercalated disc (Fig. 3C). An anti-β1Ab produced immunoreactive materials that were distributed in a striate pattern (Fig. 3D) and a little found in the surface sarcolemma (Fig. 3E). On the other hand, immunoproducts to an anti-β2 Ab were mainly distributed in the intercalated disc (Fig. 3F). The immunoreactivity (IR) to the anti-β3 Ab was not consistently observed (Fig. 3G). Figure 4 shows immunocytochemical stainings of rat ventricular myocytes, which were essentially the same as those of guinea-pig myocytes. Although the α3 subunit was not detected in the immunoblots of rat heart homogenates, the ends of ventricular myocytes were slightly stained (Fig. 4C). The anti-β3 Ab did not consistently produce immunoreactive materials in rat myocytes, either (Fig. 4F).

We previously reported that the α2 subunit was preferentially located in the membrane domain closely associated with peripheral Ca2+ store sites in chromaffin cells. Accordingly, the striate distribution of α2-like IR is likely attributed to the α2 subunit in transverse tubules. This notion was supported by double staining experiments. Figure 5 demonstrates that the α2-like IR visible as FITC-like fluorescence alternated with the sarcoplasmic and/or endoplasmic reticulum Ca (SERCA) pump-like IR, which was visualized as rhodamine-like fluorescence.

**Immunoprecipitation**

The immunocytochemical findings suggests that in rat and guinea-pig ventricular myocytes the α1 and α2 subunits and the α3 formed heterodimers with β1 and β2, respectively. These possibilities were examined using an immunoprecipitation technique. The postnuclear homogenates of guinea-pig or rat hearts were mixed with anti-β Abs, and α subunits precipitated with anti-β Abs were explored with anti-α Abs. The treatment of heart preparations with an anti-β1 Ab resulted in the immunoprecipitation of α1 and α2 subunits, whereas that with an anti-β2 Ab led to the immunoprecipitation of the α2 and α3 subunits. Since a heavy chain dimer of IgG has a similar molecular weight to α subunits, immunoblotting was performed without treatment with anti-α Abs. This omission resulted in no band, indicating that bands with about 100 kDa represent α subunits. An anti-β3 Ab did not induce an immunoprecipitation of any α subunit from rat postnuclear heart homogenates, but it did induce the immunoprecipitation of the α3 subunit from rat post-nuclear brain homogenates (not shown). These results suggest the presence...
was preferentially localized in the intercalated disc. These results suggest that the probably due to the degradation during the procedure. subunits were not consistently detected in the fractions, the F3 fraction. A similar enrichment occurred for the which is a marker of the intercalated disc, was enriched in ranging from 1 to 200 µM for rats or from 0.1 to 3 µM for guinea-pigs was field stimulated and contraction was isometrically recorded using a strange gauge. Ouabain ranging from 1 to 200 µM for rats or from 0.1 to 3 µM for guinea-pigs was added cumulatively to a bath solution. In Fig. 8, changes in the twitch tension and resting tension between electrical stimulations 2, 5, and 10 min after the addition of ouabain were expressed as fractions of the control twitch tension before the addition. The twitch amplitude of a rat ventricular strip increased in a concentration dependent manner after the addition of 1, 10, and 100 µM ouabain, whereas the resting tension level between electrical stimulations was not altered significantly. The resting tension, however, markedly increased 5 min after the application of 200 µM ouabain, and the extent of increase in twitch amplitude at 5 min diminished, compared with that at 2 min. This reciprocal change in the resting tension and twitch amplitude also occurred in a guinea-pig ventricular strip. As the concentration of ouabain added increased from 0.1 to 0.5 µM, twitch tension increased in a time-dependent manner with no change in the resting tension. At 10 min after the addition of 1 µM ouabain, however, the resting tension markedly increased and the extent of increase in the twitch tension diminished, compared with that at 5 min. The addition of 3 µM ouabain resulted in a further increase and a further decrease in the resting and twitch tension, respectively.

**DISCUSSION**

**Distribution and combination of a and b subunits**

The anti-α1 mAb used selectively recognized about 105 kDa proteins in brains and hearts of rat and guinea-pig...
reported previously [20]. These results indicate that the
and patchily stained the surface sarcolemma and trans-
verse tubules and a little in the surface sarcolemma, the
ma. Since the
be precipitated only with an anti-
tricular myocytes. This notion was confirmed by immuno-
or anti-
ventricular myocytes differs from the
sarcolemma of rat and guinea-pig ventricular myocytes,
confined to transverse tubules and not found in the surface
sarcolemma of rat and guinea-pig ventricular myocytes.
This notion was further support-
ed by immunoprecipitation experiments which showed the α1 subunit to be
precipitated only with an anti-β1 Ab, but not an anti-β2 or anti-β3 Ab. The combination of α1β1 heterodimer in
ventricular myocytes differs from the α1β3 heterodimer found in adrenal chromaffin cells [7]. In contrast to the
nonspecific distribution of the α1 subunit, α2- and α3-
like IRs were confined to transverse tubules and the intercalated disc in the guinea-pig and rat ventricular myo-
cytes, respectively. The α2 subunit was detected in immu-
oblotts of rat and guinea-pig hearts, whereas the α3 sub-
unit was detected in that of heart of guinea-pig, but not rat. These results might raise the possibility that the α3-like
IR in the rat intercalated disc is not ascribed to the α3 sub-
unit, but to other unknown proteins. This possibility, how-
ever, may not be feasible. First, the α3-like IR was shown
to be present in the junctional site of the rat cardiac con-
duction system [21]. Secondly, RT-PCR revealed the rela-
tive level of PCR products for the α3 in the rat heart to be
about 30% of that in the rat brain, a value which was in
agreement with that for the α3 signal in the guinea-pig
heart. Finally, the α3 subunit was immunoprecipitated
from a rat heart homogenate with the anti-β2 Ab, as was the
case with guinea-pig hearts, and β2-like IRs were con-
fined to the intercalated disc in both the guinea-pig and rat
cardiac myocytes. From these results, we conclude that
the α3 subunit forms a heterodimer with the β2 in hearts
and the α3β2 isozyme is localized in the intercalated disc
of both rat and guinea-pig ventricular myocytes. This no-
tion was consistent with the result of fractionation which
revealed that the distribution of N-cadherin among the
fractions of guinea-pig heart homogenates was similar to
that of the β2 subunit rather than that of the β1. Although
the α3 subunit has been reported to be present in the hearts
of humans [22] and dogs [23], its distribution in the ven-
tricular myocytes and corresponding β subunits for het-
erodimer formation have not yet been elucidated. As a re-
sult, the present experiment extended Zahler et al.’s find-
ings [21] and also demonstrated for the first time, the pres-
ence of the α3β2 isozyme in the intercalated disc of ventricular myocytes.

In contrast to previous reports [20, 24], α2-like IR was
confined to transverse tubules and not found in the surface
sarcolemma of rat and guinea-pig ventricular myocytes,
whereas β1-like IR was mainly distributed in the trans-
verse tubules. These immunocytochemical findings sug-
gest that the α2 subunit formed a heterodimer with the β1
in ventricular myocytes. This notion was further support-
ed by immunoprecipitation experiments which showed
that the α2 subunit was immunoprecipitated from rat and
guinea-pig hearts with an anti-β1 Ab. However, the α2
subunit was also immunoprecipitated from the rat and
guinea-pig heart homogenates with an anti-β2 Ab. This
unexpected findings could not be ascribed to the nonspec-
ificity of the Abs used, because immunoblotting and im-
munocytochemistry indicate that the Abs used were selec-
tive for a respective isofrom. Therefore, part of the Na+,K+-ATPases containing the α2 subunit are likely
present as an α2β2 isozyme as well. We recently elucidat-
ed that the α2 subunit exclusively forms a heterodimer
with the β2 in rat and guinea-pig chromafin cells [7].
Based on the previous findings, it would be natural to as-
sume that the α2β2 combination also occurred in hearts.
One possible explanation for this apparent discrepancy
may be that the α2β2 isozyme is present in cells except
Functions of Na+,K+-ATPase isozymes

Since the \(\alpha_1\beta_1\) isozyme is thought to be distributed in a patchy manner in the sarcolemma, it may play a housekeeping role for the control of intracellular Na\(^+\) ions. The \(\alpha_1\beta_3\) isozyme in adrenal chromaffin cells was also distributed nonspecifically in the plasma membrane [7]. Although the \(\beta_2\) subunit was expressed in both cardiac myocytes and chromaffin cells, the \(\alpha_1\) subunit was not immunoprecipitated with the \(\beta_2\) subunit. In kidney epithelia of autosomal dominant polycystic kidney disease [25], the \(\alpha_1\) subunit formed a heterodimer with \(\beta_2\) and mislocalized in the apical plasma membrane, whereas in normal renal tubules the \(\alpha_1\beta_1\) isozyme was localized in the basolateral plasma membrane and the \(\beta_2\) subunit was not detected on either a protein or mRNA level. It would thus be interesting to explore why the \(\alpha_1\) subunit does not form a heterodimer with the \(\beta_2\) in cardiac myocytes or chromaffin cells, even though the \(\beta_2\) is expressed. One possible explanation would be that the \(\beta_2\) subunit has a lower affinity for \(\alpha_1\) than for \(\alpha_2\); thus when the \(\alpha_2\) is expressed, the majority of \(\alpha_2\) isoforms form a dimer with \(\beta_2\).

The \(\alpha_2\) subunit was consistently found in the plasma membrane closely associated with the Ca\(^{2+}\) store sites in astrocytes [8], chromaffin cells [7] and cardiac myocytes (present experiment). The \(\alpha_3\) subunit was originally indicated to be present in the membrane in the vicinity of Ca\(^{2+}\) store sites in blood smooth muscle cells [6, 26] but a recent knockout experiment suggests the presence of the \(\alpha_2\) in such a membrane domain of smooth muscle cells [27]. As a result, the \(\alpha_2\) subunit localized in the plasma membrane closely associated with Ca\(^{2+}\) store sites may be \(\alpha_2\) irrespective of the cell type. Our present and previous experiments clearly indicated that the \(\alpha_2\beta_1\) and \(\alpha_2\beta_2\) isozymes were localized in the plasma membrane closely associated with the Ca\(^{2+}\) store sites. Therefore, it is likely that the \(\alpha_2\) subunit itself has a targeting signal for the localization in such a membrane domain. Further study is called for to elucidate the molecular mechanism for targeting the \(\alpha_2\) subunit to the membrane domain associated with Ca\(^{2+}\) store sites.

The \(\alpha_2\) isozymes in such a membrane domain may take part in the regulation of Ca\(^{2+}\) contents in Ca\(^{2+}\) store sites, as shown in astrocytes [8] and chromaffin cells [7]. The heterozygous knockout of the \(\alpha_2\) subunit in mice resulted in an increase in the twitch tension of cardiac muscles, which has been ascribed to an increase in Ca\(^{2+}\) contents in the SR [28]. However, the \(\alpha_1\) subunit has also been implicated to be involved in the regulation of Ca\(^{2+}\) contents in the SR in knock-in mice, where the \(\alpha_1\) and \(\alpha_2\) subunits were genetically modified to be ouabain-sensitive and ouabain-resistant, respectively [9]. This study suggests that the \(\alpha_1\) and \(\alpha_2\) subunits are not functionally different at least in the mouse cardiac myocytes. The present immunocytochemical findings of guinea-pig and rat ventricular myocytes show the \(\alpha_1\) subunit to be distributed in a patchy manner in the surface sarcolemma and the transverse tubules, whereas the \(\alpha_2\) subunit was present throughout the whole area of the transverse tubules. It therefore would be rational to assume that the \(\alpha_2\) isoform differentiates to selectively regulate Ca\(^{2+}\) contents in the SR or ER. This notion was supported by the two different effects of ouabain on the twitch and resting tension of cardiac muscles. Application of low concentrations of ouabain resulted in an enhancement of the twitch amplitude with no change in the resting tension in both rat and guinea-pig ventricular strips, whereas that of relatively high concentrations of ouabain led to an increase in resting tension with the concomitant decrease in twitch tension. The order of concentration differences between these two effects in the rat and guinea-pig heart preparations was about two and one, values which roughly agreed with those of the differences between dissociation constants of Na\(^+\),K\(^+\)-ATPases isozymes with high (\(\alpha_2\) and /or \(\alpha_3\) isozymes) and low (\(\alpha_1\) isozyme) affinities for ouabain in rat and guinea-pig hearts [29]. Therefore, the enhancement of the twitch amplitude without any change in the resting tension produced by low concentrations of ouabain may be due to the inhibition of \(\alpha_2\) isozymes, but not to that of a small amount of \(\alpha_1\) isozymes, and an increase in resting tension and the concomitant decrease in twitch amplitude in the presence of higher concentrations of ouabain may be ascribed to the inhibition of \(\alpha_1\) isozymes. From both anatomical and functional studies, we conclude that \(\alpha_2\) isozymes are selectively involved in the regulation of the Ca\(^{2+}\) contents in Ca\(^{2+}\) store sites.

The \(\alpha_3\beta_2\) isozyme was localized in the intercalated disc. The intercalated disc consists of adherens junction, desmosome, and gap junction, and the gap junction is the place where an excitation propagates between cardiac myocytes. As a result, the \(\alpha_3\beta_2\) isozyme may possibly be involved in the regulation of propagation of excitation. The Na\(^+,\)H\(^+\)-exchanger 1 (NHE1) was found to be localized in the intercalated disc [30]. It is known that the conductance of the gap junction decreases with a decrease in pH [31, 32]. Therefore, the putative close association of the \(\alpha_3\beta_2\) isozyme with NHE1 would be expected to efficiently prevent a decrease in pH in the intercalated disc. As a result, the action potentials propagate smoothly across the gap junction. Indeed, exposure to 2 \(\mu\)M ouabain of calf and cow ventricular muscles resulted in a decrease in the conduction velocity, presumably due to a reduced electrical coupling [33]. The cardiac hypertrophy induced by a pressure overload in rats did not result in an alteration of \(\alpha_1\) expression, but led to a decrease in \(\alpha_2\) and an increase in \(\alpha_3\) expression [34]. These changes in hypertrophied hearts may be rational. The decrease in the \(\alpha_2\) subunit expression is expected to result in an increase in Ca\(^{2+}\) con-
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tents of the SR with the consequent increase in contraction strength, thus counteracting the pressure overload. On the other hand, an increase in α3 expression may result in facilitation of excitation spread across the gap junction in hypertrophied cardiac myocytes. The expression of α and β subunits in oocytes revealed that the α3β2 isoform had a higher affinity for Na+ ion than did the α3β1 and α3β3 isoforms [3]. This property of the α3β2 isozone may help in removing Na+ ions efficiently from the cytoplasm in the intercalated disc, and as a result H+ ions may turn out to be efficiently effluxed. The α3 isoform of the Na+,K+-ATPase was reported to diminish in left ventricle of failing human myocardium [35]. Therefore, the diminished expression of the α3 isoform in the intercalated disc might account for the decline in the conduction velocity and the consequent arrhythmia in heart failure [36].

REFERENCES