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# Rat cardiac ventricle has two $Na^+,K^+$-ATPases with different affinities for ouabain: Developmental changes in immunologically different catalytic subunits

(cardiac glycosides/inotropy/sarcolemma/myocardium/ion transport)

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**ABSTRACT** **The mechanism of the inotropic effect of cardiac glycosides on the heart has long been controversial. Inotropic effects at low concentrations of cardiac glycosides indicate more than one class of receptor or more than one cellular mechanism. In the brain of the rat, high- and low-affinity cardiac glycoside receptors have been shown to be associated with two structurally different isoforms of the catalytic subunit of the $Na^+,K^+$-ATPase, termed $\alpha$ and $\alpha(+)$. Evidence is presented here that the high- and low-affinity sites in rat cardiac ventricle are associated with $Na^+,K^+$-ATPase catalytic subunit forms similar to the $\alpha(+)$ and $\alpha$ forms in the brain. Membranes from the rat ventricle contained polypeptides with the electrophoretic mobilities of $\alpha$ and $\alpha(+)$, which could be stained by isoform-specific anti-$Na^+,K^+$-ATPase antibodies on electrophoretic blots. Both polypeptides also displayed $Na^+$-stimulated phosphorylation with [$\gamma$-$^{32}$P]ATP. Inhibition of $Na^+,K^+$-ATPase activity by ouabain demonstrated the presence of both high- and low-affinity ATPases proportional to the presence of the $\alpha(+)$ and $\alpha$ polypeptides. The ratios of the two isoforms changed with postnatal maturation, paralleling known changes in cardiac physiology and cardiac glycoside sensitivity. Cardiac glycoside sensitivity can evidently be regulated at the level of gene expression by developmental signals.**

The $Na^+$- and $K^+$-stimulated ATPase ($Na^+,K^+$-ATPase) is the only known receptor for the cardiac glycosides (reviewed in ref. 1). The mechanism of the inotropic action of these drugs on the heart is still debated because of discrepancies between physiologically effective drug concentrations and the concentrations that inhibit the $Na^+,K^+$-ATPase in membrane preparations (2–4). Adult rat heart has been shown to have more than one class of ouabain binding sites with different affinities, but the majority of the $Na^+,K^+$-ATPase is of low affinity (2–7). Newborn rat heart, in contrast, is more sensitive to ouabain (7–12). Work by Charlemagne *et al.* (7) indicates that a high-affinity form of cardiac $Na^+,K^+$-ATPase like that in newborns is expressed in hypertrophied adult rat heart. Thus, changes in the $Na^+,K^+$-ATPase may reflect and contribute to important alterations in tissue function.

In the brain, ouabain receptors with different affinities can be ascribed to at least two different isozymes of the $Na^+,K^+$-ATPase catalytic subunit, $\alpha$ and $\alpha(+)$ (13). The two isozymes have different mobilities in $NaDodSO_4$/polyacrylamide gel electrophoresis: $\alpha(+)$ has a higher apparent molecular weight than $\alpha$. The two forms have been purified independently from kidney and axolemma and have been found to differ in their ouabain affinities in the rat (13, 14). The kidney isozyme, $\alpha$, is inhibited half-maximally at 30 $\mu$M ouabain, and the axolemma isozyme, $\alpha(+)$, at 0.1 $\mu$M. The two forms also differ in antigenic determinants (15), in N-terminal sequences (16), in gene sequences (17, 18), and in the chromosomal location of their genes (19). In the present work, $\alpha(+)$ is defined as a protein by its mobility and immunoreactivity. It may in reality comprise either or both of the gene products termed $\alpha(+)$ and $\alpha$III by Shull *et al.* (17), or $\alpha$3 and $\alpha$2 by Herrera *et al.* (18).

The presence of a $Na^+,K^+$-ATPase form similar to $\alpha(+)$ in cardiac tissue has been controversial. A polypeptide with the apparent molecular weight of $\alpha(+)$ has been detected in canine (20, 21) and ferret (22) cardiac preparations, but three laboratories have failed to detect $\alpha(+)$ by immunoblot techniques in preparations from rat and guinea pig heart (7, 22, 23). The present communication reports the detection of both $\alpha$ and $\alpha(+)$ forms in rat heart.

## MATERIALS AND METHODS

Ventricles from 150- to 185-g female Wistar rats or 1-day-old rat pups were minced in 0.315 M sucrose/20 mM Tris/1 mM EDTA (pH 7.5 with HCl), homogenized with 15 strokes of a motor-driven Teflon/glass homogenizer (6 adult or 100 newborn ventricles per 120 ml), and centrifuged at 40,000 rpm for 30 min in a Ti 70.1 Beckman rotor. The centrifugation was repeated and the washed pellets (the crude particulate preparation, shown in lane 1 of Fig. 2) were resuspended, layered over 1.4 M sucrose/10 mM Tris/1 mM EDTA (pH 7.5), and centrifuged at 27,000 rpm for 1 hr in an SW27 Beckman rotor. The interface between 0.315 and 1.4 M sucrose solutions (the crude membrane fraction, shown in lane 3 of Fig. 2) was collected. In some experiments, sarcolemma and sarcoplasmic reticulum fractions were separated by centrifuging instead on a step gradient of 1.4 M, 1.2 M, 0.9 M, and 0.75 M sucrose for 6–18 hr at 27,000 rpm. Partial purification of the $Na^+,K^+$-ATPase by extraction with $NaDodSO_4$ was performed as described (15), except that 120 mM NaCl was included during the incubation with $NaDodSO_4$. The final specific activities were 26–90 $\mu$mol of ATP hydrolyzed per hr per mg of protein. The methods for $NaDodSO_4$/polyacrylamide gel electrophoresis and electroblotting (13, 15), for active-site phosphorylation of the $Na^+,K^+$-ATPase (13), and for the assay of $Na^+,K^+$-ATPase activity (13, 14) have been described.

## RESULTS

Evidence for two forms of the $Na^+,K^+$-ATPase in the rat heart was sought by using anti-$Na^+,K^+$-ATPase antisera to stain electrophoretic blots. To avoid the possible pitfall of unequal recovery of $Na^+,K^+$-ATPase isozymes during membrane preparation, initial experiments were performed with the most inclusive possible particulate fractions from adult and newborn ventricles. The preparations were electrophoresed in polyacrylamide gels and transferred to nitrocellulose, along with samples of kidney and axolemma membranes known to contain the $\alpha$ and $\alpha(+)$ isozymes, respectively, of the $Na^+,K^+$-ATPase catalytic subunit. Blots were stained with various anti-$Na^+,K^+$-ATPase antisera (15): Ax2 {against whole purified rat axolemma

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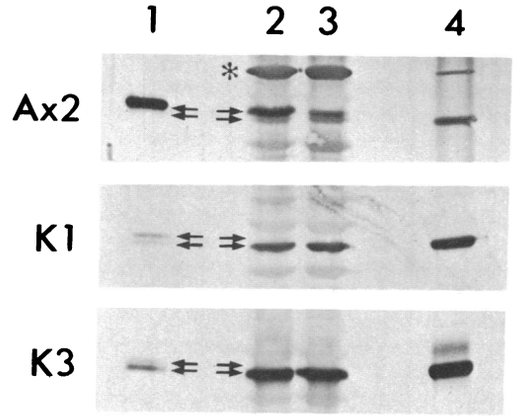


FIG. 1. Antibody stain of blots of axolemma, ventricle, and kidney preparations. Samples were electrophoresed in gels of 5% polyacrylamide and transferred to nitrocellulose. Each blot is labeled with the antiserum used to stain it. Lanes: 1, axolemma; 2, newborn ventricle; 3, adult ventricle; 4, kidney. Arrows mark positions of $\alpha(+)$ (upper arrow) and $\alpha$ (lower arrow). Another anti-$\alpha$ antiserum, K2, gave results essentially identical to those obtained with K3. The Ax2 antiserum contains antibodies that crossreact with the $Ca^{2+}$-ATPase of sarcoplasmic reticulum, which migrates at a somewhat higher molecular weight (asterisk). This protein is known to contain regions of homology with the $Na^+,K^+$-ATPase (24).

$Na^+,K^+$-ATPase [containing $\alpha(+)$]; this antiserum was previously called A2 (15, 16), but we have changed its name to avoid confusion with the nomenclature of the $Na^+,K^+$-ATPase isozymes}; K1 (against the $\alpha$ subunit from rat kidney $Na^+,K^+$-ATPase cut from a gel); and K3 [against whole purified rat kidney $Na^+,K^+$-ATPase (containing $\alpha$)].

The antibodies differed in their relative specificity for the two isozymes (Fig. 1). The K1 and K3 antisera (as well as K2, data not shown) crossreacted only slightly with $\alpha(+)$ and stained a contaminating trace of $\alpha$ in the axolemma preparation, as previously reported (15). No staining of an $\alpha(+)$-like band in heart preparations was detectable above background. The results obtained with K1, K2, and K3 closely resembled the results of Charlemagne *et al.* (7), Ng and Akera (22), and McDonough and Schmitt (23). The Ax2 antiserum, which reacts much more strongly with $\alpha(+)$ than with $\alpha$, detected a band of the same mobility as $\alpha(+)$ in preparations from rat ventricle. Interestingly, the amount of the $\alpha(+)$-like band was apparently greater in newborn than in adult rat heart.

The Ax2 antiserum stained many unrelated proteins in blots of crude cardiac preparations, which makes identification of the $\alpha(+)$-like immunoreactivity only tentative until supported by other evidence. A crucial criterion is that the immunoreactive bands should copurify with the $Na^+,K^+$-ATPase. The crude particulate preparation contained a large amount of myosin, actin, and many other proteins, as seen on polyacrylamide gels stained with Coomassie blue (Fig. 2, lane 1). Nonetheless, bands corresponding in apparent molecular weight to the $Na^+,K^+$-ATPase $\alpha$ and $\alpha(+)$ subunits were resolved (arrows). Crude membrane preparations containing only 15% of the particulate protein were separated from myosin and other sedimentable proteins by a single sucrose density centrifugation step and were greatly enriched in the marked bands (Fig. 2, lane 3). Such membrane-enriched preparations from adult and newborn rat ventricle were compared (Fig. 2, lanes 4 and 5). The marked bands comigrated with authentic $\alpha$ and $\alpha(+)$ purified from rat kidney and axolemma (lanes 6 and 7). That the marked bands included the $Na^+,K^+$-ATPases was verified by antibody staining of replicate blots (data not shown).

A sarcolemma subfraction of cardiac membrane containing only 1% of the particulate protein was examined next (Fig. 2, lane 9). The immunologically crossreactive $Ca^{2+}$-ATPase sedimented, as expected, in the denser sarcoplasmic reticulum fraction in sucrose gradients (data not shown), while both of the putative $Na^+,K^+$-ATPase bands were enriched in the sarcolemma. The proportion of Coomassie blue stain in the $\alpha$- and $\alpha(+)$-like bands changed during purification, however, suggesting that an unrelated protein comigrated with the $\alpha(+)$-

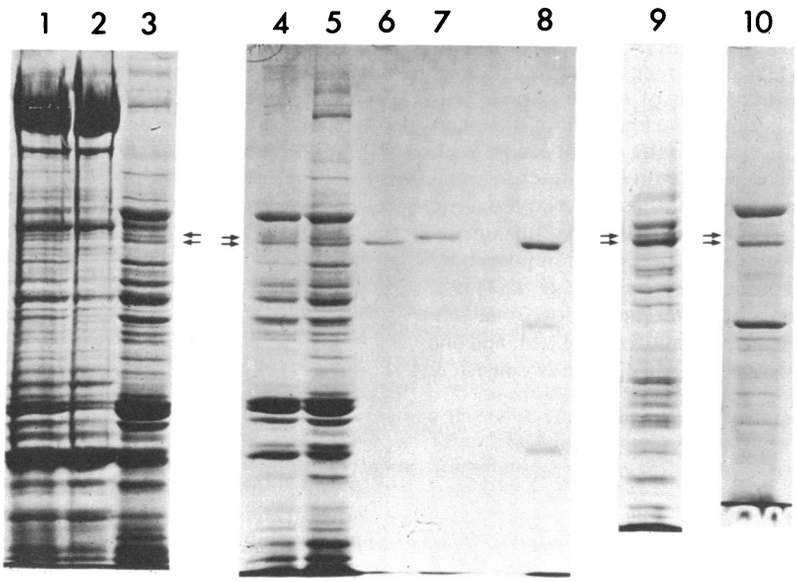


FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of preparations from cardiac ventricle. Samples were electrophoresed in gels of 7.5% polyacrylamide and stained with Coomassie blue. Lanes 1–3 show a preparation from newborn ventricle: lane 1, the crude particulate fraction containing abundant myosin (heavy chain $M_r$ 200,000); lane 2, material that sedimented through 1.4 M sucrose; lane 3, membrane-enriched material collected from the interface between 0.315 M and 1.4 M sucrose. Lane 4: a crude membrane fraction like that in lane 3 but obtained from adult ventricle instead of newborn. Lane 5: for comparison to lane 4, crude membrane fraction from newborn ventricle. Lanes 6 and 7, respectively: purified $\alpha$ and $\alpha(+)$ subunits from kidney and axolemma $Na^+,K^+$-ATPases. Lane 8: molecular weight markers ($M_r$ 97,400, 66,000, and 43,000). Lane 9: sarcolemma-enriched fraction from newborn ventricle. Lane 10: crude membrane fraction from newborn ventricle after partial purification of the $Na^+,K^+$-ATPase by extraction with $NaDodSO_4$. Arrows mark positions of bands that comigrate with $\alpha(+)$ (upper arrow) and $\alpha$ (lower arrow).

like band in the cruder preparations. Much of the $\alpha(+)$-like Coomassie-staining band sedimented to fractions of intermediate density, whereas the $\alpha(+)$-like immunoreactivity cosedimented with the $\alpha$ immunoreactivity in the fractions of lowest density (data not shown). A lower proportion of the $\alpha(+)$-like band was also seen in Coomassie-stained gels of $NaDodSO_4$-extracted crude membrane fractions (lane 10), while again the Ax2 and K3 antibody staining was indistinguishable from that seen in unpurified preparations (data not shown).

As judged by Coomassie blue staining, there appeared to be roughly 20% $\alpha(+)$-like band in the purest newborn ventricle preparations, while in the adult ventricle preparations there was considerably less. More rigorous criteria are needed to measure the true relative amounts of the two bands, however. Coomassie blue stain does not discriminate against contaminants, and the variation in intensity of the relative staining of $\alpha$ and $\alpha(+)$ by the different antibodies makes it difficult to quantitate the two polypeptides with immunostaining.

$Na^+$-stimulated, $K^+$-discharged covalent phosphorylation by ATP is a unique characteristic of the $Na^+,K^+$-ATPase, which permits the catalytic subunits to be identified on electrophoretic blots by autoradiography (13). Partial purification of the $Na^+,K^+$-ATPase by treatment with $NaDodSO_4$ eliminated kinase-mediated background phosphorylation and permitted the detection of the phosphorylated intermediates of the $Na^+,K^+$-ATPase (Fig. 3). Lanes 1–3 show the $Na^+$-stimulated, $K^+$-inhibited phosphorylation of newborn rat ventricle $Na^+,K^+$-ATPase, and lanes 4–6 show the similar phosphorylation of rat brain $\alpha(+)$ and $\alpha$. The proportion of $\alpha(+)$ was lower than that of $\alpha$ in the newborn rat ventricle preparation, whereas the proportion of $\alpha(+)$ was higher than that of $\alpha$ in the brain preparation, as previously described (13). In sarcolemma or detergent-extracted preparations from adult rat heart, $\alpha(+)$ was below the limit of reliable detection either by Coomassie blue stain or by phosphorylation (data not shown), although it could be detected with the Ax2 antiserum (Figs. 1 and 5).

Inhibition of $Na^+,K^+$-ATPase activity by ouabain was used to see whether the $\alpha$ and $\alpha(+)$ forms in newborn rat ventricle have the low and high affinities, respectively, that would be predicted from the characteristics of $\alpha$ and $\alpha(+)$ from kidney and axolemma from the rat. When crude membrane preparations were employed, the rate of hydrolysis of ATP was not linear with time and there was a large background of ouabain-insensitive activity. Extraction of the membranes with $NaDodSO_4$ resulted in linear ATP-hydrolysis rates and removed virtually all of the ouabain-insensitive activity. The $Na^+,K^+$-ATPase of adult rat ventricle was half-maximally inhibited by ouabain at $\approx 10$ $\mu$M (Fig. 4), as reported by Erdmann *et al.* (6). For newborn rat ventricle $Na^+,K^+$-ATPase, the inhibition curve indicates the presence of two ouabain-sensitive components, one with an affinity of $\approx 0.1$ $\mu$M, and one with an affinity of $\approx 10$ $\mu$M. The biphasic curve is consistent

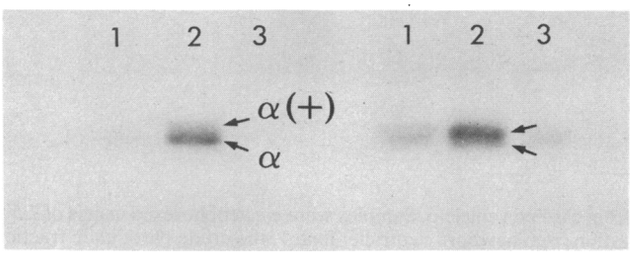


FIG. 3. Active-site phosphorylation of $\alpha(+)$ and $\alpha$. Autoradiogram of samples phosphorylated by [$\gamma$-$^{32}$P]ATP and electrophoresed in a gel of 5% polyacrylamide. Lanes 1–3: newborn rat ventricle $Na^+,K^+$-ATPase. Lanes 4–6: adult rat brain $Na^+,K^+$-ATPase. Lanes 1 and 4: no added $Na^+$ or $K^+$. Lanes 2 and 5: 120 mM $Na^+$. Lanes 3 and 6: 120 mM $Na^+$ plus 5 mM $K^+$.

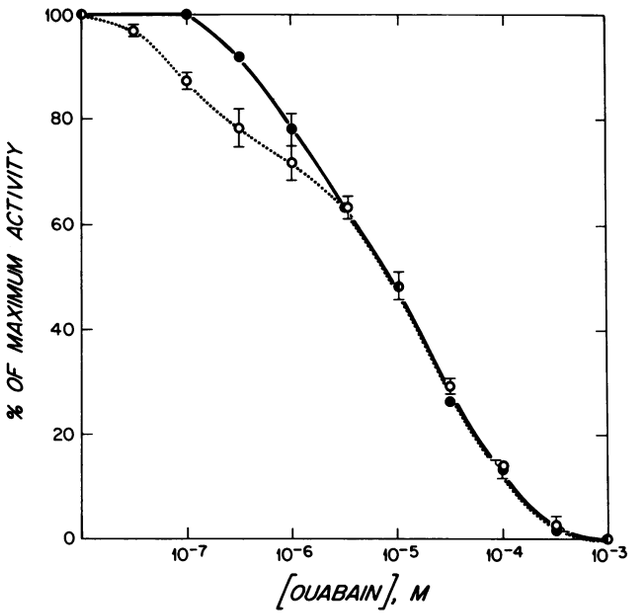


FIG. 4. Inhibition of $Na^+,K^+$-ATPase activity by ouabain. Inhibition was assessed during ATP hydrolysis, in the presence of 120 mM NaCl, 3.6 mM KCl, 3.0 mM $MgCl_2$, 3.0 mM ATP, and the other constituents required to couple the hydrolysis of ATP to the oxidation of NADH (14). After each addition of ouabain, the rate of hydrolysis of ATP approached a new, linear steady state within 10–15 min. Each point is the average of three determinations, and those standard errors not shown by error bars were smaller than the size of the symbols.

with the presence of roughly 20% high-affinity and 80% low-affinity $Na^+,K^+$-ATPase. The data agree qualitatively with the apparent ratios of the $\alpha(+)$ and $\alpha$ polypeptides on Coomassie blue-stained gels. The curve closely resembles the results of Noel and Godfraind (5), who used older rats, however.

To investigate the change in the proportion of the two $Na^+,K^+$-ATPase isozymes during postnatal maturation of the rat heart, ventricles were removed from rats at ages ranging from 0 days to 38 days after birth and from adults. Crude particulate fractions were prepared, samples were electrophoresed and electroblotted, and blots were stained with the Ax2 antiserum or with monoclonal antibody McK1 [which is specific for the $\alpha$ isoform (D. P. Felsenfeld and K.J.S., unpublished data)]. The higher apparent quantity of $\alpha(+)$ was maintained during the first 7 days of life and then declined between days 10 and 17 (Fig. 5 *Upper*). The expression of $\alpha$, on the other hand, did not change obviously (Fig. 5 *Lower*). In a single experiment, the proportion of $\alpha(+)$ and $\alpha$ in hearts from 18-day embryonic rats was indistinguishable from that in newborns (data not shown).

## DISCUSSION

The identification of an $\alpha(+)$ form of the catalytic subunit of the $Na^+,K^+$-ATPase in rat cardiac ventricle is supported by several lines of evidence: there is a polypeptide with the same electrophoretic mobility as $\alpha(+)$ of brain; it is recognized by $\alpha(+)$-specific antibodies; it is enriched in membrane and sarcolemma fractions; it copurifies with $Na^+,K^+$-ATPase during extraction with $NaDodSO_4$; and it exhibits $Na^+$-stimulated phosphorylation with [$\gamma$-$^{32}$P]ATP. The expression of the $\alpha(+)$ catalytic subunit correlates with the expression of a $Na^+,K^+$-ATPase activity with a higher affinity for the cardiac glycoside ouabain. The ratio of $\alpha(+)$ to $\alpha$ is higher in newborns than in adults, as would be predicted from previous studies of ouabain binding sites (7, 11, 12). The results thus demonstrate that there are two $Na^+,K^+$-ATPase catalytic

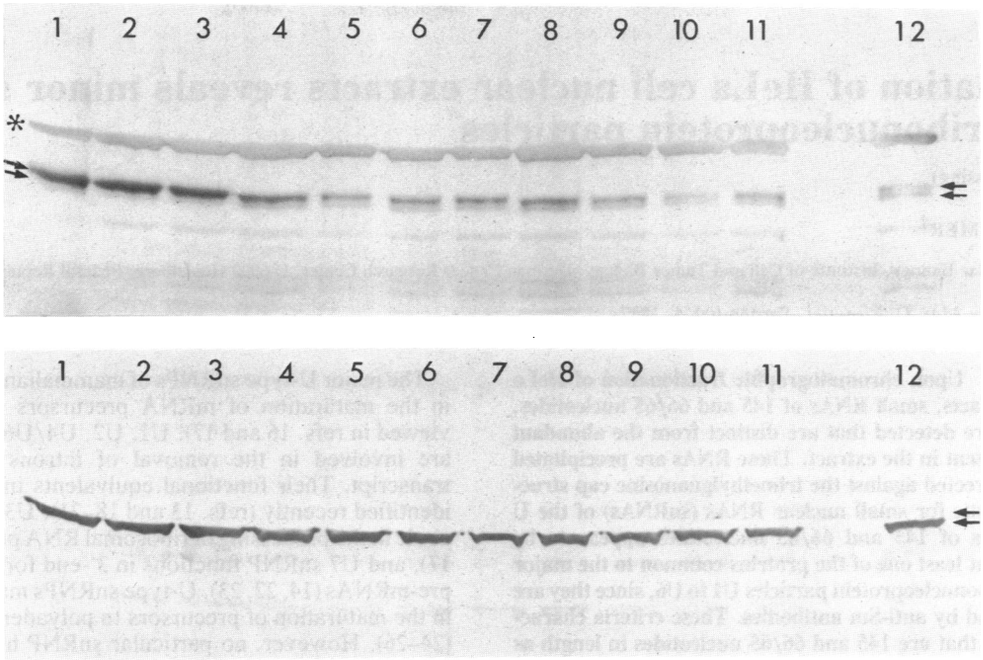


FIG. 5. Alteration of cardiac $Na^+,K^+$-ATPase isozyme expression with postnatal maturation. Crude particulate fractions from three to five ventricles were electrophoresed in a gel of 5% polyacrylamide and transferred to nitrocellulose. The blots were stained with antiserum Ax2 (*Upper*) or monoclonal antibody McK1 (*Lower*). The age (and average weights) of the animals were as follows: lanes 1, 0 days (6.0 g); lanes 2, 7 days (17.7 g); lanes 3, 10 days (26.2 g); lanes 4, 14 days (37.2 g); lanes 5, 17 days (47.5 g); lanes 6, 21 days (38.5 g); lanes 7, 24 days (54.2 g); lanes 8, 28 days (63.5 g); lanes 9, 31 days (86.5 g); lanes 10, 35 days (87.5 g); lanes 11, 38 days (108.5 g); lanes 12, adult (227.5 g). Animals younger than 28 days were of mixed sexes; animals 28 days and older were females. The $Ca^{2+}$-ATPase is marked with an asterisk, while the $Na^+,K^+$-ATPase isozymes are marked with arrows.

subunits that account for the presence of two receptors for cardiac glycosides in rat heart. While this manuscript was under review, evidence was published that the same is true in ferret hearts (22) and canine cardiac myocytes (21) and that mRNA for both $\alpha$ and $\alpha(+)$ forms can be detected in rat heart by blot hybridization (25). To our knowledge, other forms of $Na^+,K^+$-ATPase identified by molecular genetics have not yet been identified as proteins, in the heart or anywhere else.

The change in $Na^+,K^+$-ATPase isozyme expression in the rat heart occurs during the same time period as the shortening of action-potential duration and the alteration of "staircase" contractile properties that occur during maturation between 7 and 21 days after birth (8, 9). The change in $Na^+,K^+$-ATPase isozyme expression also parallels a decrease in sensitivity of the rat heart to the inotropic effect of cardiac glycosides (8, 9), a decrease in sensitivity of its isolated $Na^+,K^+$-ATPase (10), and a decrease in the number of high-affinity ouabain binding sites (11, 12). Although we do not know how the isozymes of the $Na^+,K^+$-ATPase differ functionally and how they contribute to alterations in cardiac physiology, the biochemical demonstration of their existence and variable expression is likely to lead to an improved understanding of many subtle characteristics of cardiac glycoside action.

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