AGE AND MYOCARDIAL Na⁺-K⁺-ATPase/Marsh et al.

SUMMARY Infants and young animals tolerate higher doses of digitalis glycosides, relative to body weight, than adults. One possible explanation for this could be an age-dependent difference in the myocardial digitalis receptor, the Na⁺-K⁺-ATPase. Two functions of this enzyme were studied in adult, 1- and 6-week-old dogs and guinea pigs: in vitro myocardial uptake of rubidium (⁸⁶Rb) and binding of ouabain. In guinea pigs, rubidium uptake (pmol Rb/mg LV per 15 min) was: 1 week old: 100.9 ± 7.1 (mean ± SE); 6 week: 79.8 ± 6.7; adult: 55.2 ± 7.9; (1 week: 6 week: P < 0.025; 1 week: adult, P < 0.001; 6 week: adult, P < 0.025). Similarly in dogs, rubidium uptake was significantly greater at 1 week than at 6 weeks (208 ± 13 vs. 144 ± 9; P < 0.001) and the latter greater than in adults (111 ± 4) (P < 0.005).

Other groups of anesthetized adult and 6-week-old dogs were given digoxin, 0.3 mg/kg, iv. The young dogs took significantly longer to become cardiotoxic (17.3 ± 3.4 min vs. 9.3 ± 1.4 min; P < 0.025), while their myocardial digoxin uptake was at least as great. Rubidium uptake showed an average decrease of 56% after digoxin but residual uptakes were not different in the two groups. Data for ouabain binding showed similar differences between the various groups of dogs studied. Increased myocardial Na⁺-K⁺-ATPase activity, reflected in greater active cation transport and specific enzyme binding, has been demonstrated in young animals and may be partly responsible for their greater tolerance to digitalis glycosides. Circ Res 48: 329-333, 1981

INFANTS tolerate higher doses of digitalis relative to body weight or surface area than adults (Sapin et al., 1956; Neill, 1965; Wettrell and Andersson, 1977), and higher serum levels of digoxin can be maintained in infants and children without the development of cardiac arrhythmias (Rogers et al., 1972; Hayes et al., 1973). Young animals also are relatively tolerant of digitalis, and there is some evidence that the myocardial effects of the cardiac glycosides are age dependent (Wollenberger et al., 1953; Boerth, 1975; Rosen et al., 1975; Berman et al., 1977). The explanation for this remains uncertain, but one possibility would be an age-dependent difference in membrane Na⁺-K⁺-ATPase, which is considered to be the digitalis receptor (Matsui and Schwartz, 1968; Schwartz et al., 1971; Dahl and Hoken, 1974; Wallick et al., 1979).

In this study we examined myocardial Na⁺-K⁺-ATPase activity in dogs and guinea pigs of various ages, measuring the function of the enzyme in vitro in terms of active rubidium uptake (Hougen and Smith, 1978; Hougen et al., 1979) and specific ouabain binding (Schwartz et al., 1971). In other dogs, myocardial enzyme activity and digoxin content were measured after administration of a toxic dose of digoxin.

**Methods**

To determine the influence of age on myocardial Na⁺-K⁺-ATPase activity (protocol A) and susceptibility to digitalis cardiotoxicity (protocol B), myocardial-specific ouabain binding and active rubidium uptake were measured in the following groups of animals.
Protocol A

1. Guinea pigs: 1-2 weeks old (n = 7), 6-8 weeks old (n = 9), and adult guinea pigs (n = 7) were studied. After intraperitoneal administration of pentobarbitone sodium (15-25 mg/kg) and thiopentone sodium (5-10 mg/kg), samples were taken from the free wall of the left ventricle for rubidium uptake estimation.

2. Mongrel dogs: 1-2 weeks old (n = 8), 6-8 weeks old (n = 13), and adult dogs (n = 14) were killed immediately after intravenous administration of the above agents. Samples were taken from the right and left ventricles for estimation of rubidium uptake and from the left ventricle for estimation of specific ouabain binding.

Protocol B

Other groups of 6- to 8-week-old (n = 8) and adult dogs (n = 8) were studied after administration of intravenous anesthesia similar to the above. A toxic dose of digoxin, 0.3 mg/kg, was administered intravenously over 2 minutes. Immediately after the onset of sustained ventricular tachycardia, the heart was excised for measurements of rubidium uptake, specific ouabain binding, and digoxin content.

Rubidium Uptake Measurements

A 3 mm i.d. cork borer was used to obtain 2-5 transmural biopsies of the ventricular free walls. The muscle was rinsed briefly in oxygenated buffer at 30°C and sliced into several strips weighing 2-20 mg each.

The techniques used to measure myocardial uptake of rubidium using 86Rb⁺ as a tracer have been described (Hougen and Smith, 1978; Hougen et al., 1979). Briefly, the samples of myocardium were incubated for 5 minutes at 30°C in medium of the following composition (mM concentrations): KCl, 4.0; NaCl, 120; Na HCO₃, 24; MgCl₂, 2.0; CaCl₂, 2.5; glucose, 5.6; Na H₂PO₄, 1.1; adjusted to pH 7.4 and equilibrated with 95% O₂, 5% CO₂. After this incubation, samples were transferred to flasks containing the same medium except that the KCl concentration was 2.0 mM and 86RbCl (Amersham) was added (5 × 10⁵ dpm/ml), together with unlabeled RbCl to a final concentration of 0.1 mM. Samples were incubated for 15 minutes at 30°C in this medium in the presence or absence of 0.1 mM ouabain. Samples were then rinsed, counted, and weighed as previously described (Hougen and Smith, 1978). Active transport of rubidium, expressed in units of pmol/mg wet weight per 15 min, was calculated as the difference between the average uptake of rubidium in six muscle samples in the presence of 0.1 mM ouabain and the average uptake of six samples in the absence of ouabain.

Ouabain-Binding Studies

Transmural biopsies of the free wall of the left ventricle, weighing approximately 2 g, were taken and homogenized in 15 ml of 0.25 M sucrose, 5 mM Tris buffer, and 1 mM MgCl₂, pH 7.4, using a Polytron PTA 10-35 homogenizer. The homogenate was diluted to a final protein concentration of 2 mg/ml, using the same buffer, and samples were stored at −20°C prior to assay. Protein concentrations were determined by the method of Lowry et al. (1951).

Myocardial ouabain binding was assayed after the method of Schwartz et al. (1971). Triplicate assays were performed on left ventricular homogenates, using 2.0 mg homogenate protein per assay. Homogenate protein in a buffer containing 120 mM NaCl, 50 mM Tris-HCl, 4 mM MgCl₂, 1 mM EDTA, and 4 mM ATP-Tris, pH 7.4, was reacted with ³H ouabain (0.2 μM, 0.5 mCi/μmol) either in the presence or absence of a further excess of unlabeled ouabain (1.8 mM). Reactions proceeded for 10 minutes at 37°C and were terminated by the addition of 9.0 ml of ice cold buffer containing 120 mM NaCl, 50 mM Tris, 5 mM MgCl₂, pH 7.4. The tubes were immediately centrifuged at 105,000 g for 20 minutes at 0°C. The supernatant was discarded and the pellet resuspended in ice cold buffer and again centrifuged as described above. The supernatant was removed, pellet and tube rinsed in buffer, the pellet dissolved in 1 ml of 2% sodium dodecyl sulfate (SDS) with NaOH at pH 11.0 and its tritium activity measured in a final solution of Instagel (Packard). Specific ouabain binding was calculated as the difference between total binding in the absence of excess ouabain and non-specific binding in its presence, and is expressed in fmoles ouabain/mg protein per 10 min.

Digoxin Assay

Left ventricular samples (0.1-0.4 g) were prepared for digoxin assay by the method of Coltart et al. (1972). The tissue was homogenized in chloroform, centrifuged, and the supernatant containing digoxin evaporated to dryness. Chloroform extraction was repeated on the precipitate. The evaporated digoxin was suspended in 70% ethyl alcohol and normal dog serum for assay using radioimmunoassay (Digoxin I 125 Imusay, Abbott Laboratories).

Statistics

Group means were compared using Student’s t-test. In dogs given a toxic dose of digoxin, the relationships between the time to reach toxicity, left ventricular digoxin content, and left ventricular rubidium uptake were analyzed using a multivariate analogue of the t-test (Hotelling’s T²-test), after logarithmic transformation of the data to homogenize variances (Kshirsagar, 1972).

Results

In both guinea pigs and dogs, active rubidium uptake by left and right ventricular myocardium was greater in younger animals. The same was true of ouabain binding in the left ventricle of dogs in which these measurements were made.
The rubidium uptake of guinea pig left ventricle is illustrated in Figure 1. Uptake at 1 week of age is significantly greater than at 6 weeks, which is again greater than in adults (each $P < 0.025$), the adult value being approximately half that of 1-week-old animals.

The findings were directionally similar in dogs, although the absolute values for rubidium uptake were about twice those found in guinea pigs. The rubidium uptake of dog left and right ventricle and ouabain binding of left ventricle are presented in Table 1. The table shows that the values of these three parameters are significantly greater at 1 week than at 6 weeks and these, in turn, are significantly greater than in adults.

**Digoxin Toxicity**

Table 2 shows that 6-week-old dogs took significantly longer to become cardiotoxic than did adult dogs after the administration of digoxin, the dose of which was adjusted on a body weight basis. At the onset of cardiotoxicity, rubidium uptake of right and left ventricular myocardium and ouabain binding of left ventricle (Table 2) were all depressed below the values in dogs not receiving digoxin (Table 1), and the proportionate depression was similar in adults and 6-week-old animals. The left ventricular myocardial rubidium uptake was approximately 40–45% and ouabain binding 37% of that in dogs not receiving digoxin. The residual right ventricular myocardial rubidium uptake was 60% of that in dogs not given digoxin. Although the absolute value of each of these three parameters remained, on the average, higher in the younger dogs than in adults, only right ventricular myocardial rubidium uptake was significantly higher (Table 2).

Multivariate analysis of the left ventricular digoxin level, left ventricular (residual) rubidium uptake, and time to reach cardiotoxicity showed that the two groups of dogs had significantly different vectors for these variables ($P < 0.025$). Further appraisal of this multivariate analysis showed that the differences were due almost entirely to a combination of time to toxicity and digoxin level, with rubidium uptake adding little to the discrimination. A similar conclusion was reached by separate regression analysis of time to toxicity and rubidium uptake on the digoxin level, namely most relevantly, that the relationship between time to toxicity and digoxin level was different in the two groups. Simply stated, although the left ventricular digoxin levels per se were not significantly different between the two groups (Table 2), the mean level was greater in the younger dogs, and yet it was this group that had the greater time to reach cardiotoxicity. That the two age groups constituted two different populations on the basis of time to toxicity and left ventricular digoxin level can be seen by inspection of Figure 2 in which these variables are plotted against each other for individual dogs.

**Discussion**

We found young dogs and guinea pigs to have higher myocardial Na"-K"-ATPase activity, measured in terms of active rubidium uptake and specific ouabain binding, than did their adult counterparts. This agrees with a preliminary report by Miller and Gilliland (1972) who found that newborn dogs had significantly higher myocardial Na"-K"-ATPase activity, measured by phosphate release from ATP, than adult dogs. At the same time Atwood and Dunkley (1972) reported newborn and adult sheep to have the same myocardial enzyme activity, estimated by phosphate release and ouabain binding. The explanation for these diverse findings is not apparent, a species difference probably being unlikely since the sheep fetus, like other young animals, is less sensitive than the adult to digoxin administration (Berman et al., 1977).

It seems clear that the infant and several other species of young animals are less sensitive to a given

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**Table 1. Ouabain Binding and Rubidium Uptake in Dogs of Various Ages**

<table>
<thead>
<tr>
<th>Age</th>
<th>Rubidium uptake (pmol/mg wet wt per 15 min)</th>
<th>Ouabain binding (fmol/mg protein per 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LV</td>
<td>RV</td>
</tr>
<tr>
<td>1-2 weeks</td>
<td>208 ± 13(8)</td>
<td>960 ± 78(8)</td>
</tr>
<tr>
<td>6-8 weeks</td>
<td>144 ± 9(13)</td>
<td>667 ± 63(8)</td>
</tr>
<tr>
<td>Adult</td>
<td>111 ± 4(14)</td>
<td>512 ± 56(5)</td>
</tr>
<tr>
<td>$P^*$</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$P^+$</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>$P^+$</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of dogs is given in parentheses.

$^*P$ value for comparison of 1-2 weeks old with 6-8 weeks old.

$^+P$ value for comparison of 1-2 weeks old with adult.

$^+P$ value for comparison of adult and 6-8 weeks old.

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**Figure 1. Rubidium uptake by guinea pig left ventricle at 1-2 weeks and 6-8 weeks of age and in adults (see text).**
TABLE 2 Cardiotoxic Dogs Given Digoxin (0.3 mg/kg)

<table>
<thead>
<tr>
<th>Age</th>
<th>Rubidium uptake (pmol/mg wet wt per 15 min)</th>
<th>Ouabain binding (fmol/mg protein per 10 min)</th>
<th>Digoxin content (ng/g wet wt)</th>
<th>Toxicity time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LV</td>
<td>RV</td>
<td>LV</td>
<td>LV</td>
</tr>
<tr>
<td>6-8 weeks</td>
<td>65 ± 10(8)</td>
<td>87 ± 13(8)</td>
<td>249 ± 63(7)</td>
<td>1332 ± 248(8)</td>
</tr>
<tr>
<td>Adult</td>
<td>47 ± 6(8)</td>
<td>55 ± 4(8)</td>
<td>190 ± 35(6)</td>
<td>843 ± 96(8)</td>
</tr>
<tr>
<td>P*</td>
<td>NS</td>
<td>&lt;0.025</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of dogs is given in parentheses. NS = not significant.

* P value for comparison of 6-8 weeks old with adult.

dose of digitalis glycoside than adults, although some early evidence in cats was to the contrary (Haag and Corbell, 1940). Young guinea pigs (Wollenberger et al., 1953) and rabbits (Kelliher and Roberts, 1976) require the administration of more ouabain, and young dogs more acetyl strophanthidin (Halloran et al., 1970), digoxin (Vargo et al., 1974), or ouabain (Glantz et al., 1976) before cardiotoxicity is produced. The last of these authors found a lower plasma concentration and larger volume of distribution in young dogs following an intravenous dose of ouabain proportionate to body weight, and they attributed this to the larger vascular and extravascular fluid compartments of the young animals. They found ventricular myocardial ouabain concentrations to parallel the plasma concentrations and considered that a larger volume of distribution substantially contributed to the lesser toxicity to ouabain exhibited by young dogs. Two other aspects of the pharmacokinetics of digitalis, namely, absorption and excretion, do not seem to be greatly different in young and old animals (Wetttrell and Andersson, 1977) or infants and adults (Hernandez et al., 1969; Dungan et al., 1972).

Whereas an altered volume of distribution of the glycosides due to difference in size of fluid compartments may play a role in the age dependence of glycoside action, it cannot be solely responsible as indicated by several lines of evidence. Infants can tolerate higher digoxin plasma levels with a lesser incidence of cardiotoxicity than adults (Rogers et al., 1972; Hayes et al., 1973), despite myocardial: plasma ratios which are as great, and some have thought greater (Hernandez et al., 1969; Gorodischer et al., 1976; Wettrell and Andersson, 1977). Finally, a number of studies have suggested a lesser myocardial effect of glycosides at a cellular level in young animals. Wollenberger et al. (1953) found that the lethal dose of ouabain for the isolated perfused guinea pig heart, per unit of heart weight, was about twice as great in hearts from 3-week-old animals as from adults. They also found the concentration of ouabain necessary to inhibit the oxygen uptake of cardiac muscle slices to be twice as great in week-old guinea pigs as adults. Boerth (1975) found the newborn rabbit myocardium to be less sensitive than adult to the positive inotropic effect of ouabain, and Berman et al. (1977) found the sheep fetus to be less sensitive to the inotropic and arrhythmogenic actions of a given plasma level of digoxin. Rosen et al. (1975) studied the action potential of Purkinje fibers from dogs of various ages and found that the effect of ouabain increased with age although the ouabain content of the perfused fiber bundles was actually greater in the younger animals.

We found that pups took significantly longer to become cardiotoxic after a large dose of digoxin than did adult dogs, despite the pups’ myocardium containing, on the average, more digoxin. The latter, itself, was not significantly different between the two groups, but multivariate analysis indicated a clear difference between the groups in the relation between their toxicity time and myocardial digoxin content. The exact interrelationship between the various observations is, no doubt, open to different interpretations. We suggest that the appearance of toxicity necessitated inhibition of a larger amount of the enzyme Na⁺-K⁺-ATPase in the younger dogs. The data are consistent with the later appearance of cardiotoxicity being associated with the same relative inhibition of an initially greater enzyme activity. However, the data do not convincingly exclude the possibility that suppression of initially
greater enzyme activity to a particular residual level is responsible for toxicity.

Of course, our experiments did not attempt to characterize the membrane distribution or conformation of the enzyme system. It could be that the greater concentration of enzyme per unit mass of myocardium in the young animal is only proportional to the greater surface area of myocardial cells relative to their mass expected in the young, small animal. Also, it is probable that differences in myocardial Na\(^+\)-K\(^+\)-ATPase do not solely explain the relative resistance of young animals to digitalis since there do seem to be differences in digitalis distribution (Glantz et al., 1976), and other physical or biochemical factors, such as myocardial catecholamine concentration (Kelliher and Roberts, 1976), may be relevant.

It does, however, seem reasonably established that the myocardial cellular effects of cardiac glycosides are age dependent and that the age dependency we found in myocardial Na\(^+\)-K\(^+\)-ATPase is at least partly responsible.

Acknowledgments

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