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AGE-DEPENDENT CHANGES IN THE NUMBER OF [^{3}H]OUABAIN-BINDING SITES IN RAT SOLEUS MUSCLE

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The influence of age on the number of (Na^+K^+)-ATPase units in skeletal muscle has been assessed by measurements of [^{3}H]ouabain binding in vitro and in vivo to rat soleus muscle. In vitro measurements showed that from the 2nd to the 28th day of life, the number of [^{3}H]ouabain-binding sites increases from 120 to 580 pmol/g wet wt. This is followed by a decrease, until a plateau between 150 and 200 pmol/g is reached around 150 days after birth. 60 min after intraperitoneal injection of [^{3}H]ouabain (12.5 μmol/kg body weight), the soleus muscles of 28-day-old rats had accumulated 2.4-times more ^{3}H-activity per g wet wt. than muscles of 85-day-old rats and the ^{3}H-activity in plasma was 54% lower. The results may explain the low sensitivity to digitalis glycosides found in infants as compared to premature or adult individuals.

It is well known that 1–2-year-old infants require 4–6-times larger doses of digitalis glycosides per kg body weight than adults to obtain adequate digitalisation [1,2]. Furthermore, infants and young animals can be maintained on a higher plasma digoxin level than adults without developing cardiac arrhythmias [2–5]. This has been understood as the result of a higher myocardial ($Na^+ + K^+$)-ATPase activity, and in keeping with this idea, the myocardium of young guinea pigs and puppies was found to have a larger capacity for [^{3}H]ouabain binding and ^{86}Rb uptake [6]. On the other hand, the decreased sensitivity to digitalis glycosides may as well be explained by a greater volume of distribution [7–9]. In embryonic chicken muscle, ($Na^+ + K^+$)-ATPase activity was found to increase with age [10], and since skeletal muscle constitutes the largest single pool for the peripheral binding of digitalis glycosides, it is of interest to determine whether the number of digitalis receptors in this tissue changes with age. The present study explores this possibility with measurements of [^{3}H]ouabain binding in isolated soleus muscles from rats of various ages. The binding of [^{3}H]ouabain was also assessed in vivo.

All experiments were performed using fed female Wistar rats in the age range 2–600 days corresponding to body weights from 10 to 375 g. Intact soleus muscles were dissected out and equilibrated in Krebs-Ringer bicarbonate buffer as described previously [11]. In the experiments with 2–28-day-old rats (up to 70 g body weight), the entire muscle was incubated. In order to ensure adequate oxygenation in the experiments with larger muscles, the lateral segments (20 to 30 mg wet wt.) of the soleus were carefully dissected out and incubated as described elsewhere [12,13]. It has been shown that such muscle segments maintain almost normal ATP contents for 2 h in vitro [12]. Control experiments with rats weighing 65 and 100 g showed that in muscle segments, the number of [^{3}H]ouabain-binding sites per g wet wt. was the same as in the intact contralateral muscles from the same animals (difference 2.4%, 8 vs. 8 observations).

The total number of [^{3}H]ouabain-binding sites

was determined by measuring the specific (displaceable) binding of [^{3}H]ouabain following 2 h of incubation in a K^+-free Krebs-Ringer bicarbonate buffer containing 5 mM D-glucose [13–16].

The in vivo experiments were carried out by injecting intraperitoneally a 154 mM NaCl solution containing 1.25 mM [^{3}H]ouabain (spec. act. 12 μCi/μmol). 60 min later, the animals were killed by decapitation and blood samples collected from the neck vessels for the measurement of ^{3}H-activity in plasma. Soleus muscles were quickly dissected out and washed with ice-cold saline. In case of the body weight exceeding 70 g, the muscles were divided into segments weighing 20 to 30 mg. All muscles were washed for 120 min in ice-cold buffer so as to remove ^{3}H-activity from the extracellular space. The ^{3}H-activity retained in the tissues following the wash was counted and converted to amount of ouabain using measured values for the specific activity of [^{3}H]ouabain in the injected solution. This procedure is based upon the experience that at 0°C, even cut soleus retain [^{3}H]ouabain that has already been bound to the plasma membrane [14].

Fig. 1 shows the number of [^{3}H]ouabain-binding sites in isolated rat soleus muscles as a function of age. During the first 35 days of life, the total number of [^{3}H]ouabain-binding sites in the entire muscle increases steeply, whereafter a plateau is reached with no significant further rise up to the age of 600 days. In contrast, when expressed as pmol/g wet wt., the early steep rise reaches a maximum around 580 pmol/g at the 28th day of life, followed by a comparable decrease to between 150 and 200 pmol/g, which appears to be the level in rats from 150 to 600 days of age.

In order to determine whether the marked decrease in [^{3}H]ouabain binding measured after the 28th day of life were the result of decreasing affinity for ouabain, the binding was measured in the concentration range $2.5 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ M. As can be seen from a Scatchard plot of the observations (Fig. 2), the affinity constant (K_d) in soleus muscle segments obtained from 65-day-old rats is not different from that measured in intact muscles from 28-day-old rats ($2.2 \cdot 10^{-7}$ M vs. $2.1 \cdot 10^{-7}$ M). However, the total number of binding sites is decreased from 721 to 322 pmol/g wet wt.

In order to examine whether the age-dependent decrease in the total number of [^{3}H]ouabain-binding sites was an artefact resulting from the measurements being performed with segments of larger muscles, the tissue distribution of [^{3}H]ouabain was determined in vivo, where it could be assumed that the intact circulation would allow

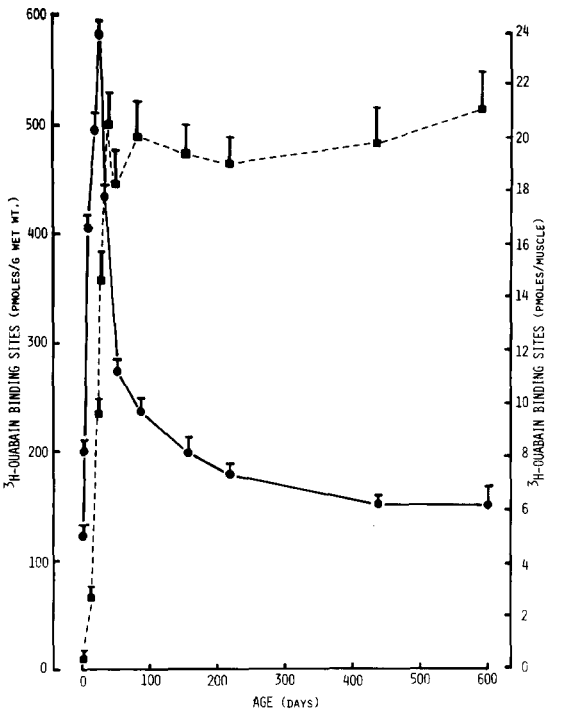

Fig. 1. Effect of age on the total number of [^{3}H]ouabain-binding sites expressed as pmol/g wet wt. (●———●) or pmol/muscle (■------■) in rat soleus muscle. After decapitation, the soleus muscles were dissected out and in case of body weight >70 g, divided into segments. For the determination of the number of [^{3}H]ouabain-binding sites the muscles were incubated for 120 min in K^+-free Krebs-Ringer bicarbonate buffer containing 1.27 mM Ca, 5 mM D-glucose and $2 \cdot 10^{-6}$ M [^{3}H]ouabain (0.6 μCi/ml) at 30°C under continuous gassing with a mixture of 95% O_2 and 5% CO_2. Hereafter, the muscles were washed four times for 30 min at 0°C so as to remove ^{3}H-activity from the extracellular space, blotted, weighed and homogenized in 2 ml of 5% trichloroacetic acid. After centrifugation, 1 ml of the clear supernatant was taken for liquid scintillation counting of the ^{3}H-activity. On the basis of the specific activity of the incubation medium, the amount of ^{3}H-activity retained following the cold wash was expressed as pmol/g wet wt. A minor correction for non-specific retention of ^{3}H-activity was based on measurements performed by incubating the contralateral muscle segments at 10^{-3} M [^{3}H]ouabain (for details see Refs. 14–16).

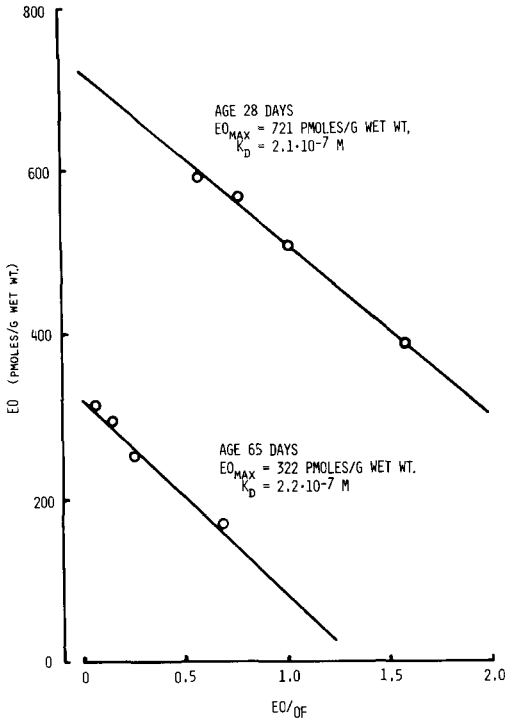

Fig. 2. Plot of 'bound' (EO) versus 'bound/free' (EO/OF) [^{3}H]ouabain in soleus muscles from rats 28 or 65 days old corresponding to 65 or 170 g body weight. Experimental conditions as in Fig. 1, except for the [^{3}H]ouabain concentration range (from $2.5 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ M). The regression lines of the Scatchard-type plot have been constructed using the method of least squares, and the calculated values for the intercepts with the ordinate (EO_{max}) as well as the dissociation constants (K_d) are indicated.

the isotope gaining access to all muscle cells. Following intraperitoneal injection of [^{3}H]ouabain, the ^{3}H-activity in plasma and muscles showed a rapid rise. When the soleus muscles were excised and washed four times for 30 min in ice-cold buffer so as to remove ^{3}H-activity from the extracellular space, the amount of [^{3}H]ouabain activity retained in the tissues corresponds to a value closely similar to the total number of [^{3}H]ouabain-binding sites measured in vitro (see Fig. 1, Table I and Ref. 14). Since the total [^{3}H]ouabain binding estimated in vivo showed clear evidence of satura-

TABLE I

EFFECT OF AGE, TIME AND DOSE ON ^{3}H-ACTIVITY IN RAT SOLEUS MUSCLE AND PLASMA FOLLOWING INTRAPERITONEAL INJECTION OF [^{3}H]OUABAIN

A 154 mM NaCl solution containing 1.25 mM [^{3}H]ouabain (12 μCi/μmol) was injected intraperitoneally in the indicated doses. 30 or 60 min later, the animals were killed by decapitation, the blood from the neck vessels collected into heparinized glass tubes, and the soleus muscles quickly dissected out under wash with ice-cold saline. The entire soleus (formuscles obtained from 28-day-old rats) or the lateral segments of soleus were washed four times for 30 min in ice-cold K^+-free Krebs-Ringer bicarbonate buffer so as to remove ^{3}H-activity from the extracellular phase. Then the muscles were processed for counting of ^{3}H-activity as described for the in vitro experiments. On the basis of the specific activity of the [^{3}H]ouabain injected, the ^{3}H-activity retained in the muscles after the cold wash was converted into pmol ouabain per g wet weight. The results are given as mean values ± S.E. with the number of observations in parentheses.

Ouabain dose (nmol per g body wt.)	Time after injection (min)	^{3}H-activity		P
		28-day-old rats	85-day-old rats	
		Soleus (pmol/g wet wt.)		
0.725	60	148 ± 13 (8)		
1.8	60	318 ± 12 (8)		
5.0	60	477 ± 15 (4)		
12.5	60	583 ± 19 (8)	248 ± 12 (12)	<0.001
20	60	583 ± 23 (4)		
		Plasma (pmol/ml)		
12.5	30	1486 ± 185 (3)	3320 ± 199 (3)	<0.005
12.5	60	874 ± 79 (3)	1914 ± 111 (3)	<0.005

tion (Table I), the ^{3}H-activity retained following the cold wash may for its major part reflect the total number of ouabain-binding sites.

It was found that in the time interval from 30 to 120 min, by far the major part of the ouabain-binding sites in the soleus muscles was occupied by [^{3}H]ouabain, and therefore 60 min was chosen as an appropriate equilibration period for the in vivo experiments.

In good agreement with the in vitro data, the amount of ^{3}H-activity retained in segments of soleus muscles obtained from 85-day-old rats was less than half that measured in muscles from 28-day-old rats (compare Fig. 1 and Table I).

It should be noted that both 30 and 60 min after the injection of [^{3}H]ouabain, the plasma values for ^{3}H-activity were more than 2-fold higher in the 85-day-old rats. Since all animals were given the same dose per kg body weight, this is probably due to a redistribution of ^{3}H-activity and reflects the reduced binding capacity for [^{3}H]ouabain in skeletal muscle.

Measurements of $(Na^+ + K^+)$-ATPase activity in homogenates of chick embryonic skeletal muscle have demonstrated a progressive increase in specific activity up to a few days after hatching. Thereafter, the activity decreased again by about 50% [10]. This pattern corresponds to the results obtained in the present study, even though the relative changes appear to be more pronounced in the rat.

The results emphasize the necessity of strict definition of the age of experimental animals in any quantitative analysis of [^{3}H]ouabain binding or active $(Na^+ + K^+)$-transport in skeletal muscle. Apart from its importance in the design of laboratory experiments, this observation indicates that $(Na^+ + K^+)$-homeostasis and the functions dependent hereon are to a considerable degree influenced by age. As a more specific practical implication, the results provide a likely explanation for the common clinical experience that infants are less sensitive to the inotropic and toxic actions of digitalis glycosides than premature and adult individuals.

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