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A SIMPLE AND RAPID METHOD FOR THE DETERMINATION OF THE NUMBER OF ^{3}H-OUABAIN BINDING SITES IN BIOPSIES OF SKELETAL MUSCLE

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SUMMARY: Based on vanadate facilitation of ^{3}H-ouabain binding to Na-K-ATPase, a method has been developed allowing the measurement of the number of ^{3}H-ouabain binding sites in small rat muscle biopsies (2-14 mg). Comparison with results obtained using intact fibres showed close agreement over the wide range of values for ^{3}H-ouabain binding sites (100-900 pmol per g wet weight) associated with variations in age, thyroid status and K-deficiency.

Measurements of specific displaceable ^{3}H-ouabain binding to isolated muscle preparations indicate that the skeletal muscles contain a large number of ^{3}H-ouabain binding sites, which represent an equivalent number of Na-K-pumps (1-3). This pool of digitalis receptors is around 100 times larger than that of the heart (4-5) and may therefore be decisive for the distribution of digitalis glycosides in plasma and their binding to the myocardium. Furthermore, it has recently been demonstrated that the number of ^{3}H-ouabain binding sites in skeletal muscle shows pronounced variation under a wide range of physiological and pathophysiological conditions such as age (6,7), hypo- and hyperthyreoidism (8), K-depletion (9), denervation (10) and muscular dystrophy (11). This emphasizes the need for a simple assay allowing the quantitative determination of ^{3}H-ouabain binding sites in biopsies obtained from larger muscles. Such samples, however, mostly consist of disrupted fibers, and we have earlier observed that integrity (i.e., undisturbed metabolism and normal intracellular ATP-level) is required for the complete saturation of all ouabain binding sites (1). Likewise, although homogenates of muscle tissue may show specific displaceable binding of ^{3}H-ouabain, a complete quantification has not been ascertained and variable or contradictory results may be obtained with whole and in particular with fractionated homogenates (for a discussion, see 12). Based on the observation that vanadate in the absence of ATP facilitates the binding of ^{3}H-ouabain to the Na-K-ATPase (13), we have

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therefore developed a simple method allowing the determination of the total number of ^{3}H-ouabain binding sites in biopsies of muscles.

METHODS

All experiments were performed using fed female Wistar rats in the age range 28-85 days corresponding to 65-200 g body weight. K-deficiency was induced by maintaining the animals on Altromin pellets containing 0.75 mmol K per kg and distilled water (9). Hypothyreoidism was induced by adding $KClO_4$ (2%) to the drinking water, and hyperthyreoidism by daily intraperitoneal injection of 500 §g triiodothyronine per kg body weight. K-depletion was ascertained by determination of the K-content in the gastrocnemius muscle and hypothyroidism by measurement of T_3 and T_4. The K-content and T_4 were reduced by 45 and 84%, respectively.

In order to allow direct comparison between results obtained using intact muscle fibres and biopsies, the soleus muscle was chosen for most of the measurements, but in a few instances the extensor digitorum longus (e.d.l.) muscle was used. The rats were killed by decapitation, and the soleus muscle exposed. The entire muscle or its lateral segments were prepared from one side and used for the determination of the number of ^{3}H-ouabain binding sites as earlier described (1,9). From the contralateral soleus, biopsies weighing 2-14 mg were taken using a biopsy needle designed for small human muscles (14). These samples were immediately transferred to a buffer containing tris chloride (10 mM), H_3PO_4 (3 mM), $MgSO_4$ (3 mM), tris vanadate (1 mM) and sucrose (250 mM). pH was adjusted to 7.3 with tris. All biopsy samples were contained in a polyethylene cylinder closed with a nylon net at the bottom and attached to a gas inlet allowing continuous gassing with 100% O_2 to ensure agitation. In order to maintain the concentrations of Na and K in the incubation media at a level which would not interfere with the vanadate facilitated binding of ^{3}H-ouabain, the samples were prewashed 2x10 min at 0°C, and shifted into a new incubation medium whenever the incubation with ^{3}H-ouabain lasted more than 60 min. It should be noted that the ^{3}H-ouabain bound to the biopsies only constitute a minute fraction of the ^{3}H-ouabain present in the incubation medium. Incubation with ^{3}H-ouabain (1.8 §Ci/ml) took place at 37°C in centrifuge tubes and lasted 30-120 min. Unlabelled ouabain was added so as to give final concentrations from $2.5x10^{-7}$ to $5x10^{-6}$M, and in each experiment a set of samples was incubated with the addition of 10^{-3}M ouabain in order to allow correction for uptake of ^{3}H-activity not related to binding. Following the incubation with ^{3}H-ouabain, all samples were washed repeatedly for a total of 120 min at 0°C in unlabelled buffer, blotted on dry filter paper and weighed. Each biopsy was allowed to soak overnight in 1 ml 5% trichloroacetic acid containing 0.1 mM unlabelled ouabain as carrier. After shaking, 0.5 ml was taken for liquid scintillation counting of the ^{3}H-activity. On the basis of the specific activity of the incubation media, the amount of ^{3}H-activity retained in the biopsies following the cold wash was calculated and after correction for non-specific retention (measured following incubation with an excess (10^{-3} M) of unlabelled ouabain) expressed as pmol per g wet weight. ^{3}H-ouabain was from New England Nuclear Corporation (Boston, U.S.A.) and its purity was checked by the Na-K-ATPase extraction method (15). The radiochemical purity was found to be 93%, and this was corrected for in the calculation of specific displaceable ^{3}H-ouabain binding. ^{14}C-sucrose was from Amersham, U.K.

RESULTS

In order to define the conditions under which the distribution of ^{3}H-ouabain in the biopsies had reached a steady state, the time-course of uptake was determined. At the concentration $2.5x10^{-7}$M, the amount of ^{3}H-ouabain taken up by the biopsies and retained following wash in the cold reached a plateau corresponding to 0.9 ml of the incubation medium per g tissue wet weight after 60 min (Fig. 1). At 10^{-6}M, the relative uptake reached equilibrium at a somewhat lower level. These time-courses are closely similar to those

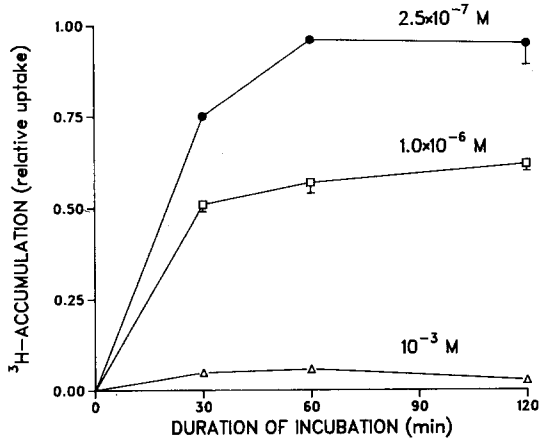

Fig. 1: Time-course of ^{3}H-ouabain accumulation in biopsies from the soleus muscle of 28 day old normal rats. The biopsies were washed 2x10 min at 0°C under continuous gassing with 100% O_2 in a buffer containing tris chloride (10 mM), H_3PO_4 (3 mM), $MgSO_4$ (3 mM), tris vanadate (1 mM) and sucrose (250 mM). This was followed by incubation for 30-120 min at 37°C in the same buffer containing 1.8 µCi/ml ^{3}H-ouabain and unlabelled ouabain to a final concentration as indicated. Hereafter, the biopsies were washed 120 min at 0°C, blotted, weighed and soaked for 24 hrs in 1 ml 5% trichloroacetic acid. 0.5 ml of the TCA extract was taken for counting of the ^{3}H-activity. On the basis of the specific activity of the incubation media, the amount of ^{3}H-activity in the biopsies was expressed as relative uptake (ml/g wet wt.). This value was corrected for the loss of specifically bound ^{3}H-ouabain during the washout by multiplication with 1.15. Each point represents the mean value of 5 observations with bars denoting S.E. when this exceeds the size of the symbols.

obtained using intact soleus muscles and the same concentrations of ouabain (1). When the biopsies were incubated with an excess of unlabelled ouabain (10^{-3}M) the relative uptake corresponded to between 0.025 and 0.050 ml of incubation medium per g wet weight. Parallel experiments carried out with ^{14}C-sucrose (2 §Ci/ml) in the incubation medium showed that using the same cold-wash procedure, the biopsies took up and retained closely similar amounts of this extracellular marker. Thus, after 30, 60 and 120 min, the relative uptake of ^{14}C-activity corresponded to 0.044 ± 0.006, 0.056 ± 0.008 and 0.026 ± 0.001 ml/g wet wt., respectively (n=5 in each instance). These results indicate that the major part of the ^{3}H-activity taken up and retained following incubation with an excess of unlabelled ouabain (10^{-3}M) and washout represents unbound ^{3}H-ouabain. This is in keeping with the earlier observation (1) that in intact soleus muscles, the tissue space available to ^{3}H-ouabain at a concentration of 10^{-3}M was not significantly different from the sucrose space.

It has been shown that during washout at 0°C in Krebs-Ringer bicarbonate buffer, the release of the ^{3}H-ouabain specifically bound to muscle fibres takes place with a half-life of 11 hours (16). Washout experiments performed with biopsies at 0°C in the tris-vanadate

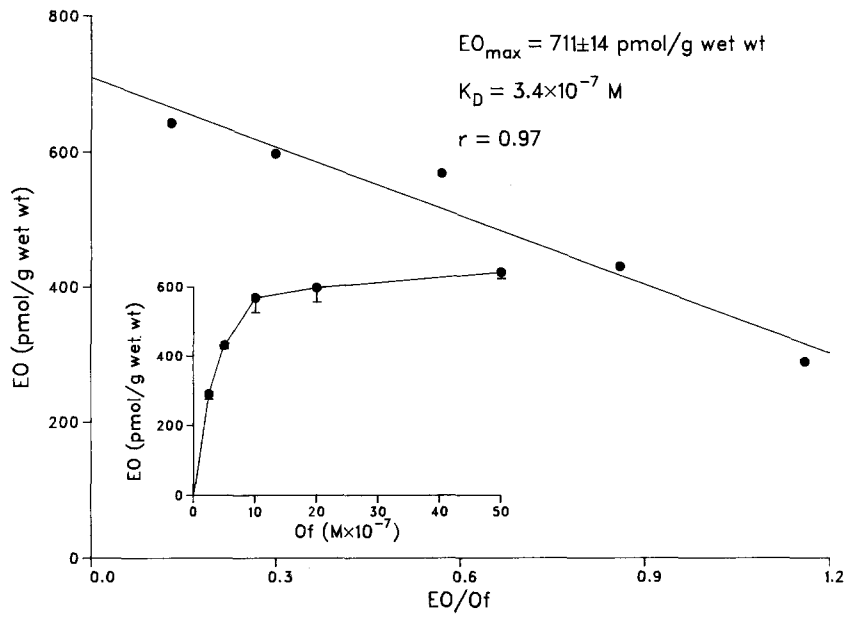

Fig. 2: Plot of "bound" (EO) versus "bound/free" (EO/Of) ^{3}H-ouabain in biopsies from the soleus muscle of 28 day old normal rats. Experimental conditions as in Fig. 1, except that the incubation in all instances lasted for 120 min. The inset shows the amount of ^{3}H-ouabain bound (EO) as a function of the concentration of free ouabain (Of) in the incubation media. A correction for non-specific retention of ^{3}H-ouabain was based on measurements performed by incubation with an excess of unlabelled ouabain (10^{-3}M). Each observation was corrected for the loss of specifically bound ^{3}H-ouabain during the washout. Each point represents the mean of 11 observations with bars denoting S.E. where this exceeds the size of the symbols. The regression line of the Scatchard-type plot was constructed using the method of least squares. The calculated values for the intercept with the ordinate (EO_{max}) ± S.E. as well as for the apparent dissociation constant (K_D) and the regression coefficient (r) are indicated.

buffer described gave a half-life of 10 hours. From these washout curves it could be calculated that using a 120 min washout period, the loss of specifically bound ^{3}H-ouabain could be corrected for by multiplication with 1.15.

Since it could be assumed that an equilibrium level was attained at 120 min this incubation period and the abovementioned corrections for non-specific retention and loss during the cold wash were used as a standard procedure in the following analysis of binding constants, precision and accuracy.

As shown in Fig. 2, the specific binding of ^{3}H-ouabain in biopsies of 28 day old rats was saturable with an apparent dissociation constant of 3.4x10-^{7}M and a maximum number of binding sites corresponding to 711 ± 14 pmol/g wet wt. These values are in good agreement with those obtained using intact soleus muscles obtained from rats of the same age (K_D = 2.1x10-^{7}M and 721 pmol/g wet weight (1)). For homogenates of rat and human

Table 1: The number of ^{3}H-ouabain binding sites in biopsies from soleus and e.d.l. of normal 85 day old rats. Experimental conditions as described in legend to Fig. 1, except that the concentration of ^{3}H-ouabain was 10^{-6} M and that the incubation in all instances lasted for 120 min. Corrections were made as described in legend to Fig. 2. The results are given as mean values ± S.D. and S.E. with the number of observations in parentheses.

PREPARATION	^{3}H-OUABAIN BINDING SITES (PMOL/G WET WT.)	S.D.	S.E.	N
SOLEUS:				
BIOPSIES FROM THE SAME MUSCLE	271	30	12	(5)
BIOPSIES FROM 5 DIFFERENT ANIMALS	296	40	18	(5)
BIOPSIES FROM DIFFERENT ANIMALS (2-8 MG)	274	44	14	(10)
BIOPSIES FROM DIFFERENT ANIMALS (8-14 MG)	258	18	6	(10)
E.D.L.:				
BIOPSIES FROM DIFFERENT ANIMALS	352	41	14	(10)

skeletal muscle, others have reported K_D-values of 1.8×10^{-7}M and 5×10^{-7}M, respectively (17,11). From the Scatchard plot it could be calculated that using a ^{3}H-ouabain concentration of 10^{-6}M, 75 % of the maximum number of ^{3}H-ouabain binding sites would be occupied within 120 min.

The precision of the method was assessed in a series of measurements performed with biopsies obtained from the same rat or from different rats of the same age (85 days). The interindividual scatter was only slightly larger than the intraindividual scatter (Table 1). The wet weight of the biopsies ranged from 2 to 14 mg, but there was no difference between the values obtained with small (2-8 mg) or large (8-14 mg) samples. Control experiments showed that the incubation with ouabain and the subsequent cold wash did not induce any significant change in the wet weight of the samples. For sake of convenience, the wet weight was determined after the incubations. Biopsies from the e.d.l. muscle, which contains predominantly white fibres gave 30 % higher values than the samples of soleus (Table 1). This is in agreement with earlier measurements showing that intact e.d.l. muscles contain 23% more ^{3}H-ouabain binding sites than intact soleus muscles (16).

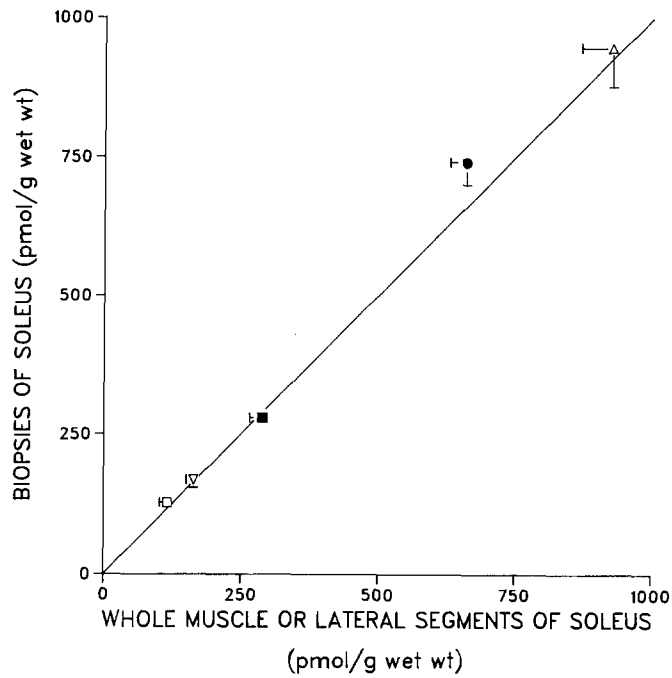

Fig. 3: The correlation between the number of ^{3}H-ouabain binding sites measured in intact soleus muscle fibres and biopsies from the contralateral soleus muscle. (●) normal 28 day old rat, (■) normal 85 day old rat, (□) K-depleted 85 day old rat (Altromin K-deficient fodder for 5 weeks), (▽) hypothyroid 85 day old rat (2% $KClO_4$ in drinking water for 3 weeks) and (▲) hyperthyroid 85 day old rat (500 µg triiodothyronine per kg body weight per day intraperitoneally for 2 weeks). The number of ^{3}H-ouabain binding sites in the biopsies was measured as described in the legend to Fig. 1, except that the concentration of ^{3}H-ouabain was 10^{-6} M and that the incubation in all instances lasted for 120 min. Corrections were made as described in the legend to Fig. 2. The number of ^{3}H-ouabain binding sites in the intact muscle fibres was measured as earlier described (1,8). Each point represents the mean of 6 versus 6 observations with bars denoting S.E. The full drawn line has a slope of 1.

In order to test the accuracy of the method, the number of ^{3}H-ouabain binding sites was measured in biopsies of soleus and compared to the site number measured in the contralateral intact or split preparation of soleus (1,9). It should be noted that using the conventional method with K-free Krebs-Ringer bicarbonate buffer (1), the longitudinal splitting of soleus gave no significant change in the number of ^{3}H-ouabain binding sites (9). From Fig. 3, it can be seen that biopsies of the soleus muscle of young rats (28 days) gave the same result as the intact contralateral muscles. In agreement with earlier observations (6), the lateral segments of soleus muscles from 85 day rats gave considerably lower values, and again the biopsy data were in good agreement. The induction of hypothyreoidism, hyperthyreoidism or K-deficiency gave virtually the same changes in site number in the biopsies and the segments of soleus muscles obtained from 85 day rats. Thus, over the range 100-900 pmol/g wet wt., the two methods gave the same results.

DISCUSSION

Measurements performed in this laboratory over a 10 year period have shown that in the intact soleus muscle obtained from young rats (28 days), the number of ^{3}H-ouabain binding sites can be determined with satisfactory precision and reproducibility when K-free Krebs-Ringer bicarbonate buffer is used for the equilibration with ^{3}H-ouabain (1,2,6,12). The present method offers a simpler procedure allowing the quantification of ^{3}H-ouabain binding sites even in biopsy material, where the cellular integrity is lost. The results obtained with the new biopsy method show the same statistical variation, reproducibility and binding constants as the conventional assay. Comparative measurements performed at a concentration of ^{3}H-ouabain (10^{-6}M) allowing saturation of the major part of the total number of ouabain binding sites show close agreement under a variety of conditions associated with wide variations in the number of ^{3}H-ouabain binding sites. We conclude that the biopsy method offer adequate recovery for the quantitative analysis of changes in the number of ^{3}H-ouabain binding sites in various types of skeletal muscle. Studies in progress have shown that the method allows the determination of ^{3}H-ouabain binding sites in biopsies of human skeletal muscle.

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