Homogeneity of [³H]ouabain-binding sites in rat soleus muscle

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Homogeneity or heterogeneity of rat soleus-muscle Na,K-ATPase (Na⁺+K⁺-dependent ATPase) with respect to affinity for [³H]ouabain was evaluated. Since the standard method for measuring specific [³H]ouabain binding to rat skeletal-muscle samples includes subtraction of a value for non-specific [³H]ouabain uptake and retention, and a wash-out in the cold to remove [³H]ouabain from the extracellular phase, it was possible that these procedures could hide a class of [³H]ouabain-binding sites either with low affinity or with a rapid dissociation of [³H]ouabain. However, measurements of [³H]ouabain uptake and retention over the range 0.1–5 mM, as well as the omission of wash-out, gave no evidence for heterogeneity of [³H]ouabain-binding sites in rat soleus muscle. Furthermore, the observation of agreement between the uptake and retention of non-specific [³H]ouabain and of [¹⁴C]sucrose gave no evidence for the existence of a major pool of [³H]ouabain-binding sites with low affinity for [³H]ouabain. Assuming homogeneity, the total concentration of [³H]ouabain binding sites in soleus-muscle samples from 12-week-old rats is 278–359 pmol/g wet wt.

INTRODUCTION

Heterogeneity of Na,K-ATPase, defined as differences in the affinity of the Na,K-ATPase for [³H]ouabain, has been observed in various tissues such as brain [1–4], heart [5–8] and fat [9–12]. Recent studies have demonstrated two classes of Na,K-pumps also in skeletal muscles: in plasma membranes isolated from human serratus magnus muscle a non-linear Scatchard-type plot with high- and low-affinity sites for [³H]ouabain was obtained [12], and in plasma membranes isolated from rat hindlimbs immunoblotting revealed two classes of Na,K-ATPase [10]. The studies on skeletal muscles have been carried out with purified membrane fractions. However, this procedure gives a recovery of Na,K-ATPase of only 2–8% [13]. Thus it can be argued that a selective enrichment of a minor class of Na,K-ATPase may have taken place.

Measurements of [³H]ouabain binding to non-purified muscle preparations (i.e. to intact rat muscles, as well as to rat muscle samples) have, however, given linear Scatchard-type plots, indicating homogeneity of [³H]ouabain-binding sites [14,15]. Furthermore, homogeneity of [³H]ouabain-binding sites was found in guinea-pig [16] and in human [17,18] skeletal-muscle samples. Also in guinea-pig [16] and human [18,19] heart ventricles [³H]ouabain binding to muscle samples showed homogeneity of [³H]ouabain-binding sites.

The present study was carried out to evaluate the possibility that measurements of [³H]ouabain binding to non-purified muscle samples might overlook a class of binding sites. This was possible for two reasons. (1) Measurements of [³H]ouabain binding include a correction for non-specific uptake and retention of [³H]ouabain, determined by incubation of muscle samples in 1 mM-[³H]ouabain. This could hide a class of [³H]ouabain-binding sites with low affinity for [³H]ouabain [20]. (2) To increase the precision of the [³H]ouabain-binding assay, incubations with [³H]ouabain were followed by wash-out at 0 °C to remove [³H]-

ouabain from the extracellular space. Therefore a class of [³H]ouabain-binding sites with a rapid dissociation of [³H]ouabain may not be detected. In the present study these potential sources of error in the [³H]ouabain-binding assay were evaluated by modifications of the assay.

METHODS

Animals

All experiments were carried out with 12-week-old fed female Wistar rats.

Muscle samples

After the animals had been killed by decapitation, soleus muscles were exposed and samples with a wet weight in the range 4–8 mg were cut out. Muscle samples were either immediately taken for measurements or kept frozen until use, as this does not interfere with the vanadate-facilitated [³H]ouabain binding [17].

[³H]Ouabain binding

These measurements were carried out by the vanadate-facilitated [21] technique [15]. All muscle preparations were incubated in a vanadate/Tris buffer, containing 10 mM-Tris/HCl, 3 mM-MgSO₄, 1 mM-Tris vanadate and 250 mM sucrose; pH was adjusted to 7.3 with Tris/HCl. During incubations, samples were kept agitated by continuous gassing with air. Samples were first prewashed for 2 × 10 min (each in fresh unlabelled buffer) at 0 °C.

In the standard experiments, muscle specimens were equilibrated for 2 × 60 min (each in fresh labelled buffer) at 37 °C in buffer containing 2.08 μCi of [³H]ouabain/ml and unlabelled ouabain added to a final concentration of 1 μM. This was followed by a wash-out in unlabelled buffer for 4 × 30 min (each in fresh unlabelled buffer) at 0 °C. Values for [³H]ouabain binding were generally given as relative uptake expressed as percentage filling, i.e. [³H]ouabain radioactivity taken up per g wet wt. of

tissue expressed as a percentage of the [^3H]ouabain radioactivity per ml of incubation medium. To obtain accurate determinations, a value for the non-specific uptake and retention of [^3H]ouabain was subtracted from the total [^3H]ouabain uptake and retention determined by this standard assay. Furthermore, loss of specifically bound [^3H]ouabain during wash-out, evaporation from muscle samples during weighing, impurity of [^3H]ouabain and incomplete saturation of [^3H]ouabain-binding sites was corrected for by subsequent multiplication by a total correction factor of 1.30 (for details on correction factors see [22]).

The non-specific uptake and retention of [^3H]ouabain was usually evaluated by incubating muscles for 2×60 min at 37°C in buffer containing $2.08 \mu\text{Ci}$ of [^3H]ouabain/ml and ouabain added (final concn. 1 mM), followed by wash-out for 4×30 min at 0°C .

To evaluate the possibility of a population of low-affinity [^3H]ouabain-binding sites being hidden by the correction for non-specific uptake and retention, the [^3H]ouabain uptake and retention were determined by incubating muscle samples for 2×60 min at 37°C in buffer containing [^3H]ouabain ($2.08 \mu\text{Ci}/\text{ml}$) and ouabain added to final concentrations of 0.1–5 mM, followed by wash-out for 4×30 min at 0°C . The non-specific uptake and retention of [^3H]ouabain were compared with the uptake and retention of [^{14}C]sucrose by incubation in 1 mM-[^{14}C]sucrose and 1 mM-ouabain [15]. This is of interest, since sucrose is an extracellular marker of almost the same size as ouabain.

The possibility that a class of [^3H]ouabain-binding sites was undetected, owing to a rapid loss of [^3H]ouabain during the wash-out in the cold, was evaluated by comparing experiments with and without wash-out. Samples were incubated in buffer containing [^3H]ouabain (0.20 – $2.08 \mu\text{Ci}/\text{ml}$) and ouabain added to final concentrations of 10 nM–5 μM for 4×60 min at 37°C with and without a subsequent wash-out for 4×30 min at 0°C . It was previously shown that [^3H]ouabain binding in rat soleus-muscle samples reaches equilibrium after incubation for 2×60 min and 4×60 min at ouabain concentrations of 1 μM and 10 nM respectively [23]. For the Scatchard-type analysis, non-specific uptake of [^3H]ouabain was determined by incubating muscles for 4×60 min at 37°C in buffer containing $2.08 \mu\text{Ci}$ of [^3H]ouabain/ml and ouabain added to a final concentration of 1 mM, with and without a subsequent wash-out for 4×30 min at 0°C . Doubling the incubation time did not increase the non-specific uptake and retention of [^3H]ouabain.

Statistics

Results are given as mean values $\pm$ S.E.M., with the numbers of observations in parentheses. Statistical significance of differences was ascertained by using the two-tailed t test for non-paired observations.

RESULTS

The [^3H]ouabain uptake and retention determined at [^3H]ouabain concentrations in the range 0.1–5 mM constitute 10–14% of the total [^3H]ouabain uptake and retention at 1 μM -[^3H]ouabain. The values for [^3H]ouabain uptake and retention at the high [^3H]ouabain concentrations were in the range 2.5–3.4% filling, and showed no systematic variation with concentration

Table 1. Measurements of the [^3H]ouabain uptake and retention in soleus-muscle samples from 12-week-old female Wistar rats

The standard [^3H]ouabain-binding assay was used unmodified, or with the modification that samples were incubated with [^3H]ouabain and ouabain concentrations of a total of 0.1–5 mM, or with [^{14}C]sucrose and sucrose concentration of a total of 1 mM together with 1 mM-ouabain. In all experiments wash-out was performed for 4×30 min at 0°C . The amount of [^3H]ouabain or [^{14}C]sucrose taken up during incubations and retained after the cold wash was calculated and expressed as relative uptake (% filling), i.e. [^3H]ouabain or [^{14}C]sucrose radioactivity taken up per g wet wt. of tissue, expressed as a percentage of the [^3H]ouabain or [^{14}C]sucrose radioactivity per ml of incubation medium. Values are given as means $\pm$ S.E.M. for five animals. Statistical significance in relation to the values for [^3H]ouabain uptake determined at a concentration of 1 mM was calculated by using the t test for unpaired observations.

Experimental condition	Relative uptake (% filling)	P
[^3H]Ouabain (5 mM)	3.4 ± 0.3	> 0.40
[^3H]Ouabain (2.5 mM)	2.5 ± 0.2	> 0.10
[^3H]Ouabain (1 mM)	2.7 ± 0.3	
[^3H]Ouabain (0.5 mM)	3.0 ± 0.3	> 0.30
[^3H]Ouabain (0.1 mM)	3.3 ± 0.3	> 0.30
[^3H]Ouabain (1 μM)	24.1 ± 1.5	< 0.001
[^{14}C]Sucrose (1 mM)	3.1 ± 0.3	> 0.10

(Table 1). If a population of [^3H]ouabain-binding sites with low affinity for [^3H]ouabain exists, an increase in the relative uptake and retention of [^3H]ouabain should have been seen when the ouabain concentration in the incubation medium was decreased from 5 to 0.1 mM. However, no increase was seen. It should be noted, however, that if the relative uptake and retention at a [^3H]ouabain and ouabain concentration of 0.1 mM had been only 1 %-filling larger than the non-specific uptake and retention at a [^3H]ouabain and ouabain concentration of 1 mM, this would have indicated the possible existence of a specific-binding-site population of 1000 pmol/g wet wt. If a specific pool of [^3H]ouabain-binding sites was hidden in the non-specific uptake and retention of [^3H]ouabain, these values would be larger than that for the uptake and retention of [^{14}C]sucrose. However, agreement between these two sets of values was seen (Table 1). Thus, although the possible existence of a pool of low-affinity [^3H]ouabain-binding sites cannot be entirely excluded by the present measurements, the evaluations provide no support for such a pool being hidden in the correction for non-specific uptake and retention of [^3H]ouabain used in the standard [^3H]ouabain-binding assay.

Fig. 1(a) shows the uptake of [^3H]ouabain as determined over the concentration range 10 nM–5.0 μM with wash-out excluded. In the range 0.1–5 μM , [^3H]ouabain uptake increases linearly with the ouabain concentration. Assuming that the non-specific uptake is directly proportional to the ouabain concentration, the specific uptake of [^3H]ouabain was calculated as the difference between the values measured for the total [^3H]ouabain uptake and those calculated from a line parallel to that measured but with intercept with the ordinate at 0.0.

Fig. 1. Effect of the concentration of ouabain on the binding of [^3H]ouabain in soleus-muscle samples obtained from 12-week-old female Wistar rats

The [^3H]ouabain binding was measured by the standard vanadate-facilitated method [15], except that incubation took place at total concentrations of 10 nM – $5\text{ }\mu\text{M}$ [^3H]ouabain and ouabain for $4 \times 60\text{ min}$ and that wash-out of ouabain from the extracellular space was omitted in some of the experiments. Bound [^3H]ouabain (EO) was plotted as a function of free [^3H]ouabain (O_i) or bound/free (EO/O_i) [^3H]ouabain in the incubation medium. The isotherms of the Scatchard-type plot were constructed by using linear regression analysis, based on unweighted values. Each point represents the mean of observations on five animals, with bars denoting S.E.M. (a, c and e) or $2 \times$ S.E.M. (b, d and f). Panels (a) and (b) show total values for [^3H]ouabain uptake measured without wash-out ($\bullet$). The specific uptake ($\circ$) was then calculated as the difference between values measured for the total [^3H]ouabain uptake and those calculated from a line with intercept with the ordinate at 0.0 and parallel to the straight portion of the uptake curve. The line through 0.0 was constructed by linear regression analysis on the last four values (0.5 – $5\text{ }\mu\text{M}$) for total [^3H]ouabain uptake. Panels (c) and (d) show values determined for the specific [^3H]ouabain binding, calculated as the difference between the uptake measured at 10 nM – $5\text{ }\mu\text{M}$ and that obtained at 1 mM -ouabain, both measured without washout. Panels (e) and (f) show values for specific [^3H]ouabain uptake determined by the standard procedure, i.e. after washout at 0°C for $4 \times 30\text{ min}$. These values were corrected for loss of specifically bound radioisotope during the wash-out in the cold, evaporation from muscle samples during weighing and impurity of [^3H]ouabain by multiplication by a total correction factor of 1.22. Values determined without wash-out were corrected by 1.01 (for details of correction factors, see [22]).

When these values were presented in a Scatchard-type plot (Fig. 1*b*), the maximum [^3H]ouabain-binding-site concentration (EO_{max}) was 289 pmol/g wet wt. and the apparent dissociation constant (K_{D}) 0.04 μM ($r = 0.94$). It should be noted that, because the measured uptake is associated with large absolute variations, especially at high ouabain concentrations, the intercept of the isotherm with the ordinate in the Scatchard-type plot is associated with a larger variation than in the standard experiments with wash-out. Owing to this large variation, the possible error induced by the washout was also assessed in another way. In Figs. 1(*c*) and 1(*d*), values were calculated as the difference between measured total [^3H]ouabain uptake and the uptake measured at 1 mM- ^3H ouabain, both values being measured without wash-out of ^3H radioactivity from the extracellular phase. This gave EO_{max} and K_{D} values of 359 pmol/g wet wt. and 0.06 μM ($r = 0.93$). As the difference was calculated on the basis of two values with large variations, the variation of the intercept in the Scatchard-type plot is large also in this evaluation. In Figs. 1(*e*) and 1(*f*), values were determined by using the normal wash-out for 4×30 min at 0 °C. This gave EO_{max} and K_{D} values of 346 pmol/g wet wt. and 0.07 μM respectively ($r = 0.97$). It should be noted that, since practical considerations limit the above-mentioned evaluations to a maximum ouabain concentration of 5 μM , a receptor with a K_{D} value of, for instance, 50 μM would only be around 10% occupied by ouabain at the highest concentration used. Thus, although the existence of a receptor with low affinity cannot entirely be excluded, the fact that the values for EO_{max} and K_{D} obtained by using the standard wash-out are in agreement with both sets of values obtained without wash-out gives no support for the existence of a large pool of [^3H]ouabain-binding sites which lose a major part of their bound [^3H]ouabain during the washout used in the standard [^3H]ouabain-binding assay.

Accordingly, the standard method for measuring [^3H]ouabain to rat soleus muscle (i.e. incubating samples at 1 μM - ^3H ouabain, followed by a wash-out in the cold), with due corrections (i.e. for non-specific uptake and retention of [^3H]ouabain, loss of specifically bound [^3H]ouabain during wash-out at 0 °C, evaporation from muscle samples during weighing, impurity of [^3H]ouabain and incomplete saturation; for details of correction factors see [22]), can be expected to quantify the total specific [^3H]ouabain-binding capacity. In the present study this value is $(24.1 \pm 1.5 - 2.7 \pm 0.3) \times 1.30\%$ filling, corresponding to 278 ± 20 pmol/g wet wt. (Table 1). Thus the simple standard assay, based on one [^3H]ouabain concentration (1 μM) gives a value for [^3H]ouabain-binding-site concentration which is in the same range as the values based on seven concentrations in the Scatchard-type plots (289–350 pmol/g wet wt., Figs. 1*b*, 1*d* and 1*f*).

DISCUSSION

This occurrence of one or more forms of Na,K-ATPase in skeletal muscle is often debated [10,13,24]. Mammalian brain tissue is well known to contain two isoenzymes of the Na,K-ATPase showing different sensitivity to digitalis glycosides [1–4], whereas other tissues may contain only one population of Na,K-ATPase molecules. Because skeletal muscles contain

nervous tissue, they could be expected to contain different populations of binding sites for [^3H]ouabain. This expectation is in accordance with studies on purified membrane fractions showing that rat skeletal muscle contains two classes of Na,K-ATPase [10,12]. Furthermore, chemical sympathectomy in cats caused a marked (up to 95%) decrease in the [^3H]ouabain-binding capacity of membrane fractions purified from skeletal muscle [25]. However, chemical sympathectomy in rats and mice caused only an insignificant tendency to a decrease of 8% in the [^3H]ouabain-binding-site concentration in intact muscles [26]. That the [^3H]ouabain-binding-site concentration in intact muscles is almost unaffected by sympathectomy indicates that the sympathetic nerve fibres in muscle tissue do not contain a large pool of Na^+/K^+ pumps as compared with the muscle fibres. However, the possible existence in the sympathetic nerve fibres of a small pool of size a few per cent of that in muscle fibres was not excluded.

In the present study no evidence was found for more than one major class of [^3H]ouabain-binding sites in rat skeletal muscles. This is evident from the observation of no systematic variation in the range 0.1–5 mM- ^3H ouabain and the agreement between these values and that determined by incubation with an extracellular-space marker. Furthermore, the linear isotherms in the Scatchard-type plots of [^3H]ouabain binding in soleus muscles, and the agreement in the maximum [^3H]ouabain-binding-site capacity (EO_{max}) determined with and without wash-out, provide no evidence for the existence of more than one major class of [^3H]ouabain-binding sites.

Another argument for homogeneity of Na^+/K^+ pumps in skeletal muscle is the close correlation between the [^3H]ouabain-binding-site occupancy by ouabain and ouabain-suppressible K^+ uptake [27]. This indicates that the present methods for quantification of [^3H]ouabain-binding sites measure the concentration of functional Na^+/K^+ pumps. The observation of a linear relationship between [^3H]ouabain binding and active K^+ uptake is in agreement with observations on human erythrocytes [28] and cardiac fibroblasts [29].

Moreover, the good agreement between measurements of the [^3H]ouabain-binding-site concentration and calculation of the Na,K-ATPase concentration on the basis of 3-*O*-methylfluorescein phosphatase activity measurements in crude rat muscle homogenates [30] indicates that both methods determine the same Na,K-ATPase. However, the latter method for quantifying Na,K-ATPase is not entirely ouabain-independent, because ouabain is used to determine the molecular activity of the 3-*O*-methylfluorescein phosphatase. Thus the agreement does not entirely rule out heterogeneity of Na^+/K^+ pumps.

Finally, recent studies on sheep cardiac Purkinje strands [31] and rat soleus muscles [32] show that the ouabain-suppressible K^+ uptake reaches values corresponding to what can be calculated on the basis of the [^3H]ouabain-binding-site concentration and the molecular activity of the Na,K-ATPase. This indicates that the Na^+/K^+ pumps determined by the [^3H]ouabain binding assay are those that perform the active K^+ uptake. This strongly argues against the possible existence of a major population of low-affinity [^3H]ouabain-binding sites of any physiological importance.

Taken together, there are four major sets of observations that provide no support for the existence of more than one major class of [³H]ouabain-binding sites in mammalian skeletal muscles: (1) the present evaluation of the [³H]ouabain-binding kinetics, (2) the agreement between the occupancy of Na⁺/K⁺ pumps by ouabain and the change in active K⁺ uptake, (3) the agreement between the Na,K-ATPase concentration determined by measuring 3-*O*-methylfluorescein phosphatase in crude muscle homogenates and by measuring [³H]-ouabain binding to intact muscle samples, and (4) that the Na⁺/K⁺-pump concentration determined by the [³H]-ouabain-binding assay agrees with the maximal capacity for active K⁺ uptake. In favour of the opinion that skeletal muscles contain more than one class of Na,K-ATPase are studies on purified membrane fractions. Thus it can be argued that a selective enrichment of a minor class of Na,K-ATPase can have taken place during the purification procedure. This could be from nervous tissue present in the muscles. It is unlikely that it comes from peripheral somatic nerves, since a recent study on rat sciatic nerves has given K_D and EO_{max} values in the same range as found in samples of skeletal muscles [33]. Studies using sympathectomy, however, point to the possibility that the observation of two classes of Na⁺/K⁺ pumps in rat skeletal muscles as found in partially purified membrane fractions can be due to the preferential concentration of a minor class of Na,K-ATPase from sympathetic nerve endings.

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