The Na,K-ATPase system of transport enzymes directly mediates active transport of sodium and potassium and indirectly mediates active calcium transport, thereby playing a key role in cation homeostasis in neural tissue (Albers et al., 1989; Stahl, 1986). The molecular organization of Na,K-ATPase isozymes is necessary to relate biochemical and transport properties to the function of specific CNS cells. Little is known about the precise cellular localization of specific Na,K-ATPase isoforms at the regional or cellular level in CNS. A recent development in methodology permits measurement of Na,K-ATPase molecules in well-defined anatomic regions of the nervous system. (J Histochem Cytochem 40:771-779, 1992)

KEY WORDS: Anatomic localization; Autoradiography; Binding sites; Brain; Cardiac glycosides; Na,K-ATPase; Ouabain; Rat.

Introduction

The Na,K-ATPase system of transport enzymes directly mediates active transport of sodium and potassium and indirectly mediates active calcium transport, thereby playing a key role in cation homeostasis in neural tissue (Albers et al., 1989; Stahl, 1986). The molecular organization of Na,K-ATPase isozymes is complex, in that different isoforms of the catalytic (α1, α2, and α3) and glycoprotein (B1 and B2) subunits (Shyjan et al., 1990; Arystarkhova et al., 1989; Hsu and Guidotti, 1989; Shyjan and Levenson, 1989) have been recently found in CNS. The Na,K-ATPase containing the α2 isoform is insulin sensitive (Lyttón, 1985) raises the possibility that the isoforms may confer unique functional properties to the enzyme. The Na,K-ATPase isoforms have strikingly different sensitivities to cardiac glycosides such as ouabain, which inhibits enzyme and transport activities by interaction with the catalytic subunit (Ruoho and Kyte, 1974). The α2 and α3 isoforms confer high sensitivity to ouabain inhibition, whereas α1 is generally less sensitive to ouabain, especially in rat tissues (Hara et al., 1988; Urayama and Sweadner, 1988; Sweadner, 1979,1985,1989).

Information about the anatomic localization and expression of Na,K-ATPase isoforms is necessary to relate biochemical and transport properties to the function of specific CNS cells. Little is known about the precise cellular localization of specific Na,K-ATPase isoforms at the regional or cellular level in CNS. A complex mosaic distribution of mRNAs (Herrera et al., 1987; Shull et al., 1986) coding for the three different α isoforms has been described in CNS (Stahl and Baskin, 1990; Filuk et al., 1989; Hieber et al., 1989; Urayama and Sweadner, 1988; Sweadner, 1979,1985,1989). Localization of these isoforms in retina has been determined with isoform-specific antibodies (Sweadner, 1989).

Na,K-ATPase molecules can also be detected in tissues using [3H]-ouabain binding in conjunction with autoradiographic methods. An excellent but complex method, introduced by Stirling (1972), has been used in a number of studies and has been reviewed (Stahl and Baskin, 1990; Ernst and Hootman, 1981; Ernst and Mills, 1980). A recent development in methodology permits measurement of Na,K-ATPase molecules in relatively small, anatomically defined regions of the nervous system by use of [3H]-ouabain bind-
ing and quantitative contact film autoradiography (Stahl and Baskin, 1990; Antonelli et al., 1988; Caspers et al., 1987; Benfenati et al., 1985; Spyropoulos and Rainbow, 1984). This approach has relied in part on recent advances in the field of receptor autoradiography (Baskin and Dorsa, 1986; Kuhar et al., 1986) and also on detailed biochemical studies which have established the presence of sites with high and low affinities for ouabain in membrane preparations (Swann, 1987; Hansen, 1984; Sweadner, 1979; Marks and Seeds, 1978a,b).

Recent studies using quantitative autoradiography (QAR) with [3H]-ouabain have shown that high-affinity ouabain binding sites are widely distributed in brain tissue (Antonelli et al., 1989; Caspers et al., 1987; Spyropoulos and Rainbow, 1984). Nevertheless, questions have remained about the specificity of the method for localizing cardiac glycoside binding sites (Stahl and Baskin, 1990). In the present study we demonstrate the feasibility of distinguishing high- and low-affinity binding sites for ouabain in selected regions of neural tissue by QAR. Preliminary reports of this work have appeared (Maki and Stahl, 1990; Maki et al., 1989, 1990).

Materials and Methods
Erythrosin B was purchased from Sigma (St Louis, MO). [3H]-Ouabain (22.1-26.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Ultrafilm was obtained from LKB Instruments (Gaithersburg, MD).

Preparation of Brain Sections. Sprague-Dawley rats (approximately 200 g) were anesthetized with 1 ml pentobarbital (50 mg/ml) and perfused via the left cardiac ventricle with ice-cold heparinized (1 IU/ml) saline. Brains were removed, chilled in 0.9% saline solution at 4°C for 5 min, immersed in Freon (-40°C) for 15-20 sec, and stored at -90°C. Kidneys from Sprague-Dawley rats and New Zealand White rabbits were removed at sacrifice, chilled in saline, and treated as described for brain tissue. Cryostat sections (12 μm) of somatosensory cortex and other regions of brain were cut using the coordinates described by Palkovits and Brownstein (1988). In the case of somatosensory cortex, tissue sections were cut approximately at levels shown in Plates 26 to 39 (Palkovits and Brownstein, 1988). Frozen tissue sections were thaw-mounted onto gelatin-coated slides and dried at 37°C for 30 sec. The slides were stored with desiccant at -20°C for 2-3 weeks.

[3H]-Ouabain Binding and Quantitative Autoradiography. [3H]-Ouabain binding in specific areas of brain was determined following the method of Antonelli et al. (1989). Briefly, sections were pre-incubated in 250 nM sucrose, 0.25 mM EDTA, 30 mM imidazole buffer, 2 mM H3PO4, 3 mM MgCl2, pH 7.4, at 26°C for 10 min with and without 100 μM erythrosin B, an inhibitor of high-affinity [3H]-ouabain binding (Silbergeld et al., 1982; Swann, 1982; Silbergeld, 1981). Sections were transferred to nitric acid-washed plastic incubation chambers (inside dimensions 15 × 25 × 78 mm; four or five slides per chamber) containing 3 ml fresh medium with [3H]-ouabain at various concentrations. Unless indicated otherwise, incubation periods were 3 hr at 26°C. Studies to establish saturation were carried out with 4-3000 nM [3H]-ouabain with and without 100 μM erythrosin B. Specific binding in all experiments is defined as the binding in the presence of Mg2+ and F- (Antonelli et al., 1989).

After incubation the sections were rinsed at 4°C for 10 min in ice-cold fresh medium in the absence of ouabain and were briefly rinsed in cold distilled water. The sections were dried using a stream of air at room temperature and then were placed on a warm plate (60°C) for 30 sec. Radioactivity standards consisting of 14 sections of methacrylate пластик impregnated with tritium (0.02-3.79 mCi/mm2; American Radiolabeled Chemicals, St Louis, MO) were exposed to LKB Ultrafilm at room temperature, together with the tissue sections, for 4 hr to 2 weeks (Antonelli et al., 1989; Baskin et al., 1989). Film was processed in Kodak D19 for 4 min at 21°C.

A microcomputer image analysis system (MCID, Imaging Research, St Catherine’s, Ontario, Canada) was used for quantitative densitometric analysis of autoradiographs by measuring the diffuse optical density (OD) of contact film images from selected regions of the tissue slices (Antonelli et al., 1989; Baskin et al., 1989). Results for each experiment were obtained from brain sections of three or four rats. A minimum of three or four slides, each containing two brain or kidney tissue sections, were incubated under each experimental condition. Ten optical density readings for each tissue section were made in the regions of interest. A mean OD value was obtained for each relevant anatomic area of a tissue section. Results averaged for each group are generally presented as fmol [3H]-ouabain bound per mm2 (Antonelli et al., 1989; Baskin et al., 1989), since the amounts of protein in specific regions of the nervous system were unknown. Data obtained from the quantitative analysis of the autoradiographs of tissue sections were analyzed by double reciprocal analysis as described by Akera and Cheng (1977) to obtain Kd and Bmax values, and are reported as mean ± SEM. Data were analyzed using ANOVA and Fisher’s least significant differences to compare binding parameters in different regions of brain, and the null hypotheses rejected at p>0.05. Hill coefficients were obtained using the EBDA program (Eleveir Biosoft; Cambridge, UK).

Determination of Tritium Quenching Factors for Quantitative Autoradiography. To control for quenching of radioactivity in specific regions in rat brain and rabbit kidney, correction factors were obtained as described by Herkenham and Sokoloff (1984), except that 250 nM [3H]-N-succinimidyl propionate was used so that autoradiographic images could be analyzed within 4 days. Levels of radioactivity were determined from autoradiographs obtained from tissue sections before and after removal of lipid with ethanol. Correction factors for tritium were as follows: somatosensory cortex, Laminae 1-6 = 1.77 ± 0.03; somatosensory cortex, Laminae 1-2 = 1.59 ± 0.11; somatosensory cortex, Laminae 3-4 = 1.49 ± 0.06; somatosensory cortex, Laminae 5-6 = 1.67 ± 0.08; hypothalamic = 1.09 ± 0.04; caudate-putamen = 1.31 ± 0.04; medial forebrain bundle = 1.56 ± 0.06; amygdaloid area = 1.14 ± 0.04; rabbit and rat kidney cortex or outer medulla = 1.06 ± 0.02. Radioactivity levels were corrected by multiplying radioactivity measured by QAR by the relevant quench factor.

Protein and Area Determination in Tissue Sections. To assess [3H]-ouabain binding per mg protein, 30 cortical sections of brain somatosensory cortex and kidney, each 30 μm thick, were collected in a microfuge tube and homogenized in 0.1% sodium dodeyl sulfate. Protein was determined using bovine serum albumin as standard (Bradford, 1976). Every third section was cut at 12 μm from the same block of tissue. The latter tissue sections were used for [3H]-ouabain binding and to determine the area from which protein was obtained in the 30-μm sections.

Results
An autoradiograph of a rat brain tissue section showing [3H]-ouabain binding sites is presented in Figure 1. Equilibrium binding was carried out in 1500 nM [3H]-ouabain and this autoradiograph demonstrates binding to both high- and low-affinity binding sites in rat brain. Very low binding was observed in the absence of the ligands Mg2+ and F- (data not shown). Relatively high levels of specific binding occurred in gray matter and no detectable binding was found in white matter at the exposure period used. In hippocampus, lower levels of binding occurred in pyramidal and granule cells but adjacent regions with high content of synapses exhibited higher levels of [3H]-ouabain binding. [3H]-Ouabain binding was analyzed in more detail in selected regions of brain, especially middle laminae of somatosensory cortex, which exhibit high levels of Na,K-ATPase activity (Stahl, 1986; Stahl and Broderson, 1976; Le- win and Hess, 1964).
Figure 1. Autoradiograph of [3H]-ouabain binding in rat brain. Coronal sections (12 μm) were incubated in the presence of 1500 nM [3H]-ouabain under standard conditions in the presence of 3 mM Mg2+ and 2 mM Pi (see Materials and Methods). The slides were exposed to film for 4 hr and developed. Data reported in this communication were routinely collected in selected regions of brain using an area at least as small as that outlined by the smaller window shown in Laminae 3-4 of cerebral cortex. The larger window indicates the approximate area used for collection of data for Laminae 1-6 of cerebral cortex. The locations of CA3 pyramidal cells of hippocampus (arrowhead) and white matter (asterisk) are indicated. Bar = 3 mm.

Time Course and Specificity of [3H]-Ouabain Binding

Binding of [3H]-ouabain to Laminae 3-4 of somatosensory cortex at 5, 50, and 1500 nM was determined by QAR in tissue sections of rat brain incubated for 10-210 min (Figure 2). Maximum binding was reached within 30-50 min of incubation in 1500 nM [3H]-ouabain and after incubation for 2-2.5 hr at concentrations of 5 or 50 nM.

Specificity of binding was established as shown in Table 1 for low, intermediate, and high concentrations of [3H]-ouabain. Binding at all concentrations of [3H]-ouabain was highest in the presence of Mg2+ and Pi, which promote maximum specific binding of ouabain to the Na,K-ATPase (Siegel and Josephson, 1972; Albers et al., 1968; Schwartz et al., 1968). Deletion of Mg2+ and Pi reduced binding by 98-100%. Lower specific binding of [3H]-ouabain was observed in the presence of ATP, Na+, and Mg2+. In the present QAR study, specific [3H]-ouabain binding was defined as binding that occurs in the presence of Mg2+ and Pi. In addition, excess Na+, which is well known to reduce phosphorylation of the Na,K-ATPase by Pi in the presence of Mg2+, eliminated [3H]-ouabain binding. At 5 and 50 nM [3H]-ouabain the addition of 150-fold excess unlabeled ouabain reduced binding of [3H]-ouabain by 84-95%.

High-affinity [3H]-ouabain binding

In this study we define relative [3H]-ouabain affinities in the following approximate concentration ranges: high, <100 nM; low,
Table 1. Characteristics of [3H]-ouabain binding in cerebral cortex

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[3H]-ouabain concentration</th>
<th>5 nM (%) binding</th>
<th>50 nM (%) binding</th>
<th>1500 nM (%) binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Delete Mg2+, P1</td>
<td></td>
<td>1.9 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>30.5</td>
</tr>
<tr>
<td>Add 150-fold excess unlabeled ouabain</td>
<td>1.6 ± 0.1</td>
<td>5.3 ± 0.4</td>
<td>5.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Add 200 mM Na+</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Add 10 mM K+</td>
<td></td>
<td>18.7 ± 1.8</td>
<td>87.1 ± 1.5</td>
<td>87.1</td>
</tr>
<tr>
<td>Add 100 µM erythrosin B</td>
<td>1.9 ± 0.2</td>
<td>52.9 ± 2.5</td>
<td>52.9 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Add 200 mM Na+ + 100 µM erythrosin B</td>
<td>0</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Delete Pi, add 10 mM ATP</td>
<td>72.5 ± 3.5</td>
<td>89.7 ± 2.0</td>
<td>89.7 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

* Rat brain tissue sections were incubated with [3H]-ouabain with buffer and ligands (3 mM Mg2+, 2 mM Pi) that support specific binding (control). During the pre-incubation period the medium did not contain Mg2+ or Pi. Additions and deletions to the control incubation medium are indicated. [3H]-ouabain binding was determined in three independent experiments in Laminae 3–4 (± SEM) from somatosensory cortex of three to six animals, each assayed on quadruplicate slides. A value of zero indicates background reading for the autoradiograph.

To obtain binding data for cerebral cortex as a whole, Laminae 1–6 of somatosensory cortex were analyzed in one scan and an average [3H]-ouabain binding value of 131 fmol [3H]-ouabain bound/mm² (Table 2) was obtained. We determined the protein content of somatosensory cortical sections and calculated a Bmax for [3H]-ouabain of 110.2 pmol/mg protein. The Kd for somatosensory cortex was 58 nM (Table 2), in good agreement with the value found for Laminae 3–4.

Low- and Very Low-affinity [3H]-ouabain Binding

Low-affinity [3H]-ouabain binding sites were examined in rat brain tissue sections using 1500 nM [3H]-ouabain (Table 1). As found with high-affinity [3H]-ouabain binding, deletion of Mg2+ and Pi decreased low-affinity binding of [3H]-ouabain by 98%. Binding at 1500 nM [3H]-ouabain presumably represented both high- and low-affinity sites. Therefore, total nonspecific binding was approximately 2% of total [3H]-ouabain binding at this concentration. Erythrosin B (100 µM) inhibited [3H]-ouabain binding by 47% (Table 1). These blocked sites presumably were mainly the high-affinity forms of the Na,K-ATPase detected above. Addition of erythrosin B in the presence of excess Na+ blocked total [3H]-ouabain binding (Table 2) by 99%. Therefore, both high- and low-affinity [3H]-ouabain binding sites were blocked by excess Na+.

Dissociation of [3H]-ouabain from low-affinity binding sites was determined at 0°C and 26°C (Figure 4) after labeling of brain tissue sections with 1500 nM [3H]-ouabain in the presence of erythrosin B to block high-affinity sites. After initial loss of radioactivity from slices, probably due to diffusion of unbound ligand trapped in the tissue, dissociation of [3H]-ouabain was relatively slow at 0°C (Figure 4). At 26°C more rapid dissociation occurred, confirming the reversibility of [3H]-ouabain binding and permitting reliable calculation of Kd and Bmax values. An important point is that [3H]-ouabain remained tightly bound during the 0°C washout to the low-affinity binding sites before QAR analysis.

Table 2. Regional localization of high- and low-affinity [3H]-ouabain binding sites in rat brain

<table>
<thead>
<tr>
<th>Region</th>
<th>High-affinity sites</th>
<th>Low-affinity sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd (nM)</td>
<td>Bmax (fmol/mm²)</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lam. 1–6</td>
<td>18.3 ± 2.9</td>
<td>131.1 ± 6.1</td>
</tr>
<tr>
<td>Lam. 1–2</td>
<td>69.3 ± 14.1</td>
<td>107.5 ± 16.0</td>
</tr>
<tr>
<td>Lam. 3–4</td>
<td>68.0 ± 10.2</td>
<td>137.6 ± 14.6</td>
</tr>
<tr>
<td>Lam. 5–6</td>
<td>44.3 ± 7.2</td>
<td>104.1 ± 8.3</td>
</tr>
<tr>
<td>Thalamic nuclei</td>
<td>21.0 ± 2.4</td>
<td>105.0 ± 28.9</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>21.5 ± 4.9</td>
<td>86.5 ± 19.1</td>
</tr>
<tr>
<td>Caudate-puamen</td>
<td>27.8 ± 6.6</td>
<td>84.3 ± 2.5</td>
</tr>
<tr>
<td>Medial forebrain bundle</td>
<td>34.0 ± 11.3</td>
<td>100.1 ± 36.4</td>
</tr>
<tr>
<td>Amygdaloid nuclei</td>
<td>23.3 ± 1.9</td>
<td>76.3 ± 5.3</td>
</tr>
</tbody>
</table>

* Rat brain tissue sections from five or six animals (three or four slides each) were incubated in the presence of control medium containing buffer and ligands to support specific [3H]-ouabain binding. Concentrations of [3H]-ouabain were 4–150 nM for high-affinity binding experiments. For low-affinity binding the [3H]-ouabain concentrations were 200–3000 nM in the presence of 100 µM erythrosin B.

* Ligand binding in somatosensory cortex Laminae 1–6: a larger area than usual was scanned (Figure 1). A protein value of 0.0012 mg protein/mm² (9.9% protein per unit volume) of cerebral cortex was found from analysis of tissue sections. We obtained a Bmax of 266.6 pmol [3H]-ouabain/mg protein for somatosensory cortex for high-affinity binding. A Bmax of 250.6 pmol [3H]-ouabain/mg protein was observed for low-affinity binding for somatosensory cortex.

* p<0.05. Stored (± SEM) for Laminae 3–4 differed from Laminae 1–6. The Kd values were significantly greater (p<0.05) in laminae of cerebral cortex than in other areas studied. For low-affinity sites, Kd values (± SEM) were significantly lower in cerebral cortex and in thalamic nuclei than in the other areas studied (p<0.05).
OUABAIN BINDING SITES IN RAT BRAIN

Figure 4. [3H]-ouabain dissociation from Laminae 3-4 of rat somatosensory cortex and rat kidney medulla. Incubations were in 1500 nM [3H]-ouabain with Mg2+ and Pi, and 100 μM erythrosin B. After binding of [3H]-ouabain at 26°C, tissue sections were washed at 0°C for 10 min before drying for QAR. No binding was observed in the absence of Mg2+ and Pi. To permit calculation of [3H]-ouabain binding on a protein basis, a parallel study was carried out using adjacent 30-μm and 12-μm slices of cortex and outer medulla. Slices were removed, pooled, and analyzed for protein.

The presence in brain slices of very low-affinity [3H]-ouabain binding sites similar to those found in rat kidney (Figure 4) was not evident. The dissociation of [3H]-ouabain from rat kidney was rapid even at 0°C (Figure 4), and 60% of the label was lost within 10 min. All label was lost after an additional incubation for 5 min at 26°C, making it difficult to obtain reliable Kd and Bmax values in rat kidney.

We compared the characteristics of [3H]-ouabain binding at equilibrium in rat brain slices with binding found in both rabbit and rat kidney slices, since kidney from these species is known to contain sites with low and very low affinities for [3H]-ouabain, respectively. These studies were of further help in validating the QAR method for study of low-affinity [3H]-ouabain binding sites. The concentration of [3H]-ouabain binding sites in rabbit kidney was twofold higher in outer medulla than in cortex (Table 3), and Kd values of 308.3 and 690.0 nM (by equilibrium binding) were found for rabbit outer medulla and cortex, respectively, in good agreement with values obtained using other methods (Ducquet and Barlet, 1986; Shaver and Stirling, 1978). Our best estimate (Table 3) indicated that the Kd values in rat kidney were approximately one order of magnitude higher than those observed in rabbit kidney. Bmax values were similar in both tissues.

Characteristics of [3H]-ouabain binding to low-affinity sites were determined in several regions of rat brain (Table 2) and in Laminae 3-4 of somatosensory cortex (Table 1; Figures 4 and 5) were studied in most detail.

Figure 5. Lower-affinity [3H]-ouabain binding in Laminae 3-4 of rat somatosensory cortex. Incubations were carried out in the presence of Mg2+ and Pi with 100 μM erythrosin B for 3 hr. Data were obtained from six or seven animals as in Figure 3 and results were analyzed (inset) as in Figure 5; Kd = 727.8 ± 99.0 nM and Bmax = 304.4 ± 21.4 fmol/mm². Hill coefficient = 1.15.

Table 3. [3H]-ouabain binding sites in kidneya

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mm²)</th>
<th>Bmax (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit cortex</td>
<td>690.0 ± 8.7</td>
<td>116.6 ± 0.6</td>
<td>157.9 ± 2.2</td>
</tr>
<tr>
<td>Rabbit outer medulla</td>
<td>308.3 ± 5.5</td>
<td>236.4 ± 0.4</td>
<td>312.3 ± 0.6</td>
</tr>
<tr>
<td>Rat cortex</td>
<td>10,9006</td>
<td>776</td>
<td>nd^c</td>
</tr>
<tr>
<td>Rat outer medulla</td>
<td>5,0006</td>
<td>1806</td>
<td>nd^c</td>
</tr>
</tbody>
</table>

Note: a Binding was carried out on 12-μm sections for three rabbits (three or four sections per data point) in three independent experiments in the presence of 200-2500 nM [3H]-ouabain, Mg2+, Pi, and 100 μM erythrosin B. After binding of [3H]-ouabain at 26°C, tissue sections were washed at 0°C for 10 min before drying for QAR. No binding was observed in the absence of Mg2+ and Pi. To permit calculation of [3H]-ouabain binding on a protein basis, a parallel study was carried out using adjacent 30-μm and 12-μm slices of cortex and outer medulla. Slices were removed, pooled, and analyzed for protein.

b In this case, rat kidney sections from one animal were assayed in a single experiment over the same [3H]-ouabain concentration range as used with rabbit kidney tissue sections. After [3H]-ouabain binding, tissue sections were washed at 0°C for 1 min before drying and autoradiography. Kd and Bmax values are approximate, since an insufficiently high concentration of [3H]-ouabain was used to saturate very low-affinity binding sites and dissociation from the site is very rapid.

c nd, not determined.

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Low-affinity \( ^{3}H \)-ouabain binding sites were studied using 200–3000 nM \( ^{3}H \)-ouabain in the presence of erythrosin B to block high-affinity binding sites. Low-affinity binding sites in Laminae 3–4 of somatosensory cortex were saturated between 1500–2000 nM \( ^{3}H \)-ouabain (Figure 5). These sites had a \( K_d \) of 727.8 nM and a \( B_{max} \) of 304.4 fmol/mm\(^2\) in Laminae 3–4 of somatosensory cortex (Table 2). A Hill coefficient of 1.15 seemed consistent with a single population of binding sites with low affinity for \( ^{3}H \)-ouabain.

**Distribution of High- and Low-affinity \( ^{3}H \)-ouabain Binding Sites**

Both high- and low-affinity ouabain binding sites were observed in the representative regions studied (Table 2; Figure 1), and a two-fold range of \( K_d \) values was found. Results from several of the nuclei within these broad regions were combined, since no specific differences were observed. In the case of high-affinity \( ^{3}H \)-ouabain binding sites, \( K_d \) values of 44–69 nM were found in somatosensory cortex (Table 2), with the greatest concentration of high-affinity sites in Laminae 3–4. The \( B_{max} \) value of the high-affinity site in Laminae 1–6 of somatosensory cortex was only slightly higher (1.3–1.7-fold) than that measured in other areas of brain (Table 2). \( K_d \) values for the low-affinity \( ^{3}H \)-ouabain binding sites in somatosensory cortex and thalamic nuclei ranged from 728–972 nM and in other regions from 1212–1482 nM (Table 2). These sites were more uniformly distributed in laminae of somatosensory cortex than the high-affinity binding sites. In these representative rat brain regions, the concentration of low-affinity binding sites was about two or three times greater than that of high-affinity \( ^{3}H \)-ouabain binding sites.

**Effects of Potassium on \( ^{3}H \)-ouabain Binding**

The presence of \( K^+ \) in the incubation medium significantly decreased specific \( ^{3}H \)-ouabain binding (Figure 6). The higher-affinity binding site was more sensitive to \( K^+ \), with an IC\(_{50} \) = 1.4 ± 0.2 mM. This site was blocked >90% by 15 mM \( K^+ \). At 1500 nM \( ^{3}H \)-ouabain, and in the presence of erythrosin B to block high-affinity binding, \( K^+ \) inhibited a component with an IC\(_{50} \) of 6.4 ± 2.8 mM, yet about 40% of the low-affinity \( ^{3}H \)-ouabain binding sites were insensitive to 15–90 mM \( K^+ \).

**Discussion**

The main findings from the present study were that (a) two populations of \( ^{3}H \)-ouabain binding sites with \( K_d \) values of 22–69 (high-affinity sites) and 727–1482 nM (low-affinity sites) were distinguished in brain by QAR, and that (b) the distribution of these sites was relatively non-uniform, varying by a factor of two among different regions. Low-affinity \( ^{3}H \)-ouabain binding sites were approximately twofold greater in number than high-affinity binding sites.

**Validity of the Quantitative Autoradiographic Method**

There are two major considerations in establishing the validity of the QAR method as applied to \( ^{3}H \)-ouabain binding: (a) previous biochemical results obtained using filtration assays with membranes; and (b) results from previous studies with tissues utilizing QAR.

Previous biochemical studies identified more than one population of ouabain-sensitive sites in neural tissue (Atterwill and Collins, 1987; Sweadner, 1979,1985; Marks and Seeds, 1978a,b; Taniyaguchi and Iida, 1972). Values of 10–52 nM have been reported for a high-affinity ouabain binding site in rat brain using filtration assays on membranes (Berreb–Bertrand et al., 1990; Hauger et al., 1985; Silbergeld, 1981; Lin and Akera, 1978; Akera and Cheng, 1977; Choi and Akera, 1977). Our observed \( K_d \) values of 22–69 nM for the high-affinity \( ^{3}H \)-ouabain binding site, as measured by QAR (Table 2), are therefore in good agreement with values obtained from the biochemical literature. The study of Akera and Cheng (1977) is most relevant to the present QAR study, since these investigators used a rat brain homogenate rather than a more purified membrane preparation in their studies. Using their method of data analysis in the present QAR study, we found \( K_d \) and \( B_{max} \) values of 58 nM and 110 pmol \( ^{3}H \)-ouabain/mg protein, respectively, in somatosensory cortex (Table 2), which is in reasonable agreement with the values of 25 nM and 64 pmol \( ^{3}H \)-ouabain/mg protein (Akera and Cheng, 1977) for homogenates of whole rat brain. The values obtained by QAR represent specific binding of \( ^{3}H \)-ouabain, since the ligand (Mg\(^2+\), Pi) conditions that we used favor stoichiometric binding of cardiac glycosides to the Na,K-ATPase (Albers et al., 1968; Schwartz et al., 1968). Removal of these ligands or addition of excess Na\(^+\), which blocks phosphorylation of the Na,K-ATPase by Pi, eliminated binding of \( ^{3}H \)-ouabain in tissue sections (Table 1). These results and those of a previous study (Antonelli et al., 1989) support the validity of the QAR method for study of high-affinity \( ^{3}H \)-ouabain binding sites in tissues.

Our results using the QAR method contrast with previous QAR studies on rat brain, especially with regard to dissociation constants. Others have reported only a single population of \( ^{3}H \)-ouabain binding sites in somatosensory cortex and thalamic nuclei ranged from 1212–1482 nM (Table 2).
OUABAIN BINDING SITES IN RAT BRAIN

binding sites in rat brain with a $K_d$ value of 339 nM in one case (Caspers et al., 1987) and 2100 nM in another (Spyropoulos and Rainbow, 1984). The difference between our results and the relatively higher values reported by others may be due to the thicker tissue sections used by other authors. Dohanich et al. (1986) found increased $K_d$ values by QAR as compared with biochemical filtration binding when tissue sections greater than 20 μm thick were used. Our results would not have been influenced by this effect, since we used 12-μm thick sections. In addition, it is often difficult to distinguish multiple binding sites over a wide range of ligand concentrations. A key difference between our results and previous QAR studies lies in our use of erythrosin B, which blocks binding of ouabain to the high-affinity binding site. This permitted analysis of low-affinity ouabain binding without interference from a higher-affinity component.

**Low-affinity $[3H]$-ouabain Binding Sites**

We initially considered the feasibility of characterizing low-affinity cardiac glycoside binding sites by QAR through study of rabbit and rat kidney, since these tissues contain low-affinity binding sites for cardiac glycosides (Sweadner, 1989; Erdmann, 1981; Tobin and Brody, 1972). In accord with results obtained using conventional methods, we found $K_d$ values of 690 and 308 nM in rabbit kidney cortex and outer medulla, respectively, and $K_d$ values of approximately 10.9 μM and 5 μM in rat kidney cortex and outer medulla, respectively (Table 2). These are in the range of values previously reported by Shaver and Stirling (1974) and Doucet and Barlet (1986) for rabbit and for rat kidney. The greater $B_{max}$ for specific $[3H]$-ouabain binding in outer kidney medulla agrees with higher levels of Na,K-ATPase as established by histochemical and immunocytochemical methods (Stahl and Baskin, 1990; Baskin and Stahl, 1982). The QAR data confirm that in kidney more Na,K-ATPase molecules are present in outer medulla than in cortex, and support the validity of the film QAR method for measuring low-affinity $[3H]$-ouabain binding sites.

**Low-affinity $[3H]$-ouabain Binding Sites of Rat Brain**

Low-affinity cardiac glycoside binding sites have not been extensively studied in neural tissue. Berrebi-Bertrand et al. (1990) recently reported that interaction of ouabain with rat brain membranes best fit a three-site model with $K_{150}$ values of 23, 460, and 3200 nM for inhibition of Na,K-ATPase activity. Our present QAR results also identified a low-affinity binding site with $K_d$ values in the range of 727-1482 nM. This site may correspond to the intermediate site found by Berrebi-Bertrand et al. (1990). Our results were achieved after blocking higher-affinity $[3H]$-ouabain binding with erythrosin B. Results from the use of this inhibitor showed that $[3H]$-ouabain binding decreased by 98-100% at low ouabain concentrations and by about 48% at higher levels of ouabain (Table 1), supporting the earlier filtration assay results which showed that this inhibitor blocks high-affinity $[3H]$-ouabain binding (Antonelli et al., 1989; Silbergeld et al., 1982, 1983; Swann, 1982, 1987; Swann et al., 1982; Silbergeld, 1981). As with high-affinity binding sites, specific $[3H]$-ouabain binding to low-affinity sites was found only under conditions favoring phosphorylation of the Na,K-ATPase.

We were unable to resolve further either high- or low-affinity binding sites into subpopulations, and Hill coefficients were consistent with single populations of high- and low-affinity binding sites. However, low-affinity $[3H]$-ouabain binding was incompletely inhibited by $K'$, possibly indicating that a third subgroup of sites insensitive to $K'$ is present. Interaction of $K'$ with the low-affinity sites is complex, however, and antagonism of cardiac glycoside binding by $K'$ is well established (for references see Hansle, 1984). $K'$ increases the $K_d$ of the ouabain-receptor complex but does not alter binding levels at equilibrium (Erdmann and Schoner, 1973). Therefore, as confirmed here, $K'$ has a more profound inhibitory effect on high-affinity ouabain binding sites, since the approach to equilibrium is slower (Figure 2) and ouabain dissociation is slower. In rabbit kidney, which contains low-affinity ouabain binding sites, $K'$ is also relatively ineffective in blocking binding at high concentrations of $[3H]$-ouabain.

In the present QAR study using equilibrium binding of $[3H]$-ouabain, we were unable to identify very low-affinity binding sites in brain. It might be assumed that at the range of concentrations of $[3H]$-ouabain used only a small portion of these sites were probably labeled and the dissociation would be very rapid, as shown for rat kidney (Figure 4). However, even at 0°C at a concentration of 1500 nM $[3H]$-ouabain, at least 40% of the label remained associated in rat kidney tissue sections. Therefore, it remains likely that the low-affinity binding sites found here contain a very low-affinity component. All methodologies, whether involving biochemical filtration assays with membranes or QAR for study of binding in tissue sections, are similarly compromised in defining binding to these very low-affinity sites. A potential strategy for future study of sites with very low affinities for cardiac glycosides might involve use of the radioligand displacement method, short post-incubation washes, and extended autoradiographic exposures. This contrasts with the radioligand saturation method used in the present study.

**Isoforms Involved in $[3H]$-ouabain Binding**

In the present study there is no basis at present for associating high- and low-affinity binding sites with either the α2 or the α3 isoforms. The identity of specific catalytic subunit isoforms of the Na,K-ATPase in tissues that are responsible for high- and low-affinity $[3H]$-ouabain binding sites has received considerable attention in the literature (Sweadner, 1979, 1989). There is little doubt that Na,K-ATPase of rat brain containing the α1 isofrom has very low affinity for binding ouabain compared with the enzyme containing α2 or α3 (Swadevns, 1989), but the affinities of α2 and α3 for cardiac glycosides in tissues remain to be determined. The low-affinity cardiac glycoside binding sites found in rat brain membrane preparations using filtration assays and by QAR in tissue sections have $K_d$ values that are significantly higher than those found in rat kidney. Nevertheless, in the present study the low-affinity site(s) found in rat brain may still receive a contribution from α1, since this isoform in rat brain resembles rat kidney α1 (Swadevns, 1979, 1989). Resolution of very low-affinity ouabain binding sites due to the presence of α1 may not be feasible using the QAR approach.

**Distribution of Ouabain Binding Sites**

Until recently, little was known about the distribution of ouabain binding sites within specific cell groups in the nervous system, al-
though the application of immunocytochemical and histochemical methods has been used to determine relative (non-quantitative) localizations of Na,K-ATPase immunoreactivity and enzymatic activity (for review see Stahl and Baskin, 1990). Results using QAR (Antonelli et al., 1989; Caspers et al., 1987; Spyropoulos and Rainbow, 1984) suggest that a degree of non-uniformity of [3H]-ouabain binding sites exists in rat and rabbit brain, but results on levels of ouabain binding reported by different investigators have differed. In rat brain, Spyropoulos and Rainbow (1984) reported a six-fold difference in ouabain binding among regions, whereas in the present study and that of Caspers et al. (1987), only two- to threefold differences in [3H]-ouabain binding were found between regions. These differences in relative distribution of binding sites reported by different laboratories may be due in part to lack of correction for quenching of [3H]-ouabain radioactivity in specific regions (Baskin et al., 1989) or to differences in sampling. Since QAR data are collected from a series of adjacent tissue sections, anatomic variations between sections could result in significant statistical differences. Apparent regional differences in concentrations of [3H]-ouabain binding sites may also be influenced by the way the results are expressed. We have reported binding in fmol [3H]-ouabain bound per mm² here and elsewhere (Stahl and Baskin, 1990; Antonelli et al., 1989), whereas others have reported binding per mg protein. In those cases an average protein value for brain is used to calculate [3H]-ouabain binding per mg protein in a specific anatomic region. However, protein content of specific nuclei is often unknown and QAR results are often based on brain paste radioactivity standards.

Conclusion

The present work provides evidence for the presence and differential distribution of both high- and low-affinity ouabain binding sites in specific regions of the brain, and validates the use of QAR for measuring these sites. Very low-affinity [3H]-ouabain binding sites were not distinguished in brain using this method, in part due to rapid dissociation of radioligand from these sites. [3H]-Ouabain autoradiography, along with in situ hybridization histochemistry (Stahl and Baskin, 1990), is a powerful quantitative approach for analyzing changes in catalytic subunit molecules of the Na,K-ATPase in small, defined anatomic regions of the brain that are otherwise unsuitable for study using biochemical filtration assays. QAR should be useful in analyzing the response of the Na,K-ATPase system to modulation of function (Antonelli et al., 1991; Swann and Steketee, 1989; Swann, 1987; Swann, 1984a,b; Swann et al., 1982) or to disease states such as epilepsy (Maki et al., 1990).

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OUABAIN BINDING SITES IN RAT BRAIN

779
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