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Distribution of α1 and α2 (Na^+,K^+)-ATPase isoforms between the junctional (t-tubular) and non-junctional sarcolemmal domains of rat ventricle

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In rat ventricle, cardiac glycosides such as ouabain interact with two classes of binding sites (high-affinity, low-capacity and low-affinity, high-capacity, respectively), which have been ascribed tentatively to two isoforms of (Na^+,K^+)-ATPase and related to two saturable components in the inotropic action of ouabain [1–7]. The inotropic efficacy associated with the high-affinity receptor appears greater than that of the low-affinity receptor [1]. More recently, two immunologically distinct isoforms of the α subunit of (Na^+,K^+)-ATPase have been demonstrated in rat heart (reviewed in Ref. 8). Analysis of the α isoform mRNA of adult rat ventricle has confirmed the existence of a major (α1) and a minor (α2) component [9]. In contrast to rat ventricle, rat atrium lacks a significant high-potency component in the inotropic action of ouabain, and high-affinity binding sites are four times less numerous in atrium than in ventricle [1, 2]. Since the t-tubular network is well-developed in adult ventricle but less important in atrium, Finet *et al.* [1] hypothesized that the high-affinity receptor for ouabain could be situated in t-tubules and that this particular localization could be related to its high inotropic efficacy. We were able to test this hypothesis by subfractionating microsomal fractions from rat ventricle in sucrose density gradient. As first described by Brandt [10], this method allows a partial resolution of t-tubular fragments that remain attached by junctional feet to terminal cisternae of the sarcoplasmic reticulum from free, non-junctional sarcolemmal vesicles. Our results suggest that high-affinity ouabain binding sites are mostly associated with the external sarcolemma, in contrast to 1,4-dihydropyridine binding sites, which appear to be highly concentrated in t-tubules.

Materials and Methods

Subcellular fractionation. Ventricles from 10–20 male Wistar rats (300–350 g) were homogenized in 3–5 volumes of ice-cold solution (mM: sucrose 250, histidine 2, dithioerythritol 2, phenylmethylsulfonyl fluoride 0.2 and EDTA 0.2, pH 7.3) by means of an Ultra-Turrax (Janke and Kunkel KG., Staufen i. Br., F.R.G.; three 5-sec bursts at 9000 rpm followed by a 1-sec burst at 20,000 rpm). After centrifugation at 1000 g_{av} for 10 min, the supernatant was collected, and the pellet was resuspended with a Dounce homogenizer. This suspension was recentrifuged at 1000 g_{av}, and the pellet was submitted to two additional cycles of resuspension–centrifugation under the same conditions. The combined supernatants were centrifuged at 10,000 g_{av} for 20 min in a TFT 50.38 rotor (Kontron AG, Zurich, Switzerland), and the pellet was washed once by resuspension and recentrifugation. The combined supernatants were centrifuged at 110,000 g_{av} for 35 min, and the microsomal pellet was resuspended in 0.25 M sucrose (buffered at pH 7.4 with 5 mM Tris–HCl). The microsomal fraction contained 4.4 ± 0.4 mg protein/g tissue wet wt (mean ± SE, N = 4). This microsomal fraction was subfractionated on a linear sucrose gradient as described by Wibo *et al.* [11]. After overnight centrifugation at 100,000 g_{av}, 10 fractions were collected from the gradient and their density (at 0°) was determined by refractometry.

Binding assays. [^{3}H]Ouabain binding was measured as previously described [2]. Tissue samples (0.5–1 mg protein/mL) were incubated with [^{3}H]ouabain (15 Ci/mmol, NEN Research Products, Boston, MA, U.S.A.) at 37° for 20 min in a medium (225 μL) containing 3 mM $MgCl_2$, 3 mM inorganic phosphate, 1 mM EGTA* and 20 mM maleate–Tris, pH 7.4. In some experiments, tissue samples had been preincubated for 20 min at room temperature with saponin (0.5 mg/mL; 0.33 mg/mg protein). Bound radioactivity was determined after filtration on Whatman GF/F filters. Nonspecific binding was estimated from samples incubated in the absence of $MgCl_2$ and inorganic phosphate and in the presence of 0.7 mM ouabain. The specific binding of [^{3}H](+)PN200-110 (isopropyl-4-[2,1,3-benzoxadiazol-4-yl]-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate; 80 Ci/mmol, The Radiochemical Centre, Amersham, U.K.) was measured as described previously [12]. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, 30 Ci/mmol, NEN Research Products) binding was measured at 37° in 10 mM Tris–HCl, pH 7.4. Nonspecific binding was determined in the presence of 1 μM atropine sulfate. After incubation, membranes were collected on GF/F filters and washed twice with 5 mL of ice-cold 10 mM Tris–HCl (pH 7.4). For all binding assays, filters were immersed in Aqualuma (Lumac)/toluene (1/4, v/v) and radioactivity was counted with an efficiency of 40%.

Biochemical determinations. (Na^+,K^+)-ATPase was measured by a coupled optical assay [2]. Samples (0.9 mg protein/mL) were first preincubated for 2 min at room temperature with sodium dodecyl sulfate (SDS) at a concentration of 0.7 mg/mL. Thereafter, 25 μL of this suspension was added to 2.5 mL of assay medium (37°). This SDS treatment provided optimal activation of (Na^+,K^+)-ATPase, while markedly inhibiting the very high basal Mg^{2+}-ATPase activity of rat heart microsomes. Under these assay conditions, the ouabain-inhibitable activity amounted to about 50% of the total ATPase activity. Cytochrome *c* oxidase, 5′-nucleotidase and protein were assayed as reported previously [11].

Results and Discussion

Under our assay conditions, only one class of specific binding sites was detected in microsomal fractions from rat ventricle when [^{3}H](+)PN200-110 and [^{3}H]QNB were used as ligands for, respectively, 1,4-dihydropyridine and muscarinic receptors. Binding parameters estimated from Scatchard plots (not shown) were (mean ± SE, N = 3–4): [^{3}H](+)PN200-110: K_d, 82 ± 8 pM; B_{max}, 663 ± 25 fmol/mg protein; [^{3}H]QNB: K_d, 25 ± 3 pM; B_{max}, 1107 ± 49 fmol/mg protein. Microsomal fractions contained about 20% of the total number of these binding sites in rat ventricle, as compared with 3% of the total sedimentable protein.

A illustrated in Fig. 1, [^{3}H]ouabain binding to microsomal fractions from rat ventricle as a function of ligand concentration was compatible with the existence of two classes of specific binding sites. The K_d values [13] of the high- and low-affinity sites were 0.3 and 12.8 μM, respectively, and the low-affinity sites were seven times

* Abbreviations: EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate; SDS, sodium dodecyl sulfate.

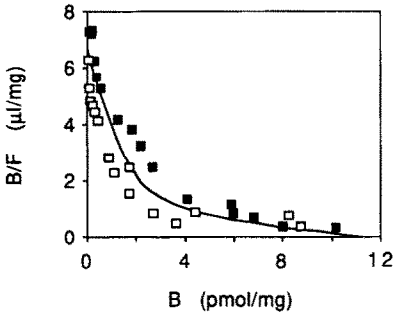

Fig. 1. Scatchard plot of [^{3}H]ouabain binding to microsomal fractions from rat ventricle. Open and filled symbols refer to two different preparations. Binding data from the two experiments were analysed simultaneously by a non-linear least squares curve fitting technique (LIGAND program [13]). The program provides estimates of the number of sites B_{max} and association constant K_a, which is the reciprocal of the K_d value. Final parameter estimates are based on a two-site model and they are given with approximate SE. High-affinity: K_a 3.38 ± 0.86 μM^{-1}, B_{max} 1.42 ± 0.37 pmol/mg protein; low-affinity: K_a 0.078 ± 0.037 μM^{-1}, B_{max} 9.89 ± 2.15 pmol/mg protein.

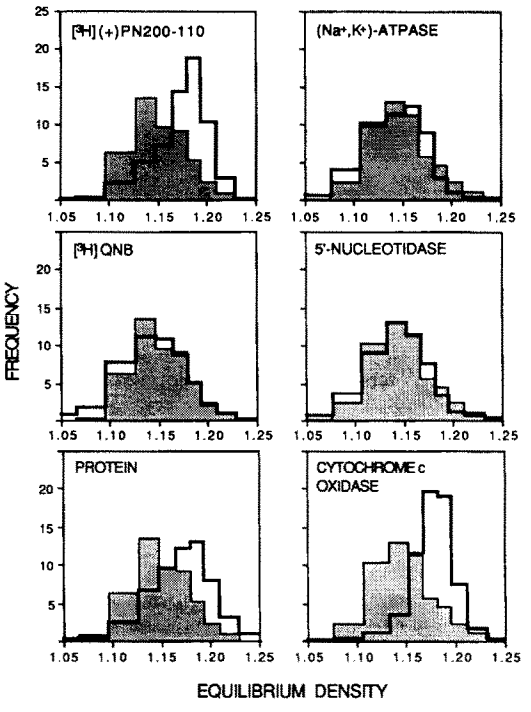

Fig. 2. Density distribution patterns of various binding sites and marker enzymes of the microsomal fraction from rat ventricle. Two 1-mL (10 mg protein) portions of the same microsomal fraction were layered on sucrose gradients (4 mL) prepared in different tubes. Subfractions collected from distinct tubes were used to construct the density frequency histograms shown in the left and right panels. Each subfraction is represented on the abscissa by its density boundaries, which were estimated from the measured average density and volume of adjacent subfractions. The frequency is the fractional amount of specific binding, enzyme activity or protein recovered in a given subfraction, divided by the density increment across that subfraction. The shaded histogram reproduced on each panel is the density distribution of [^{3}H]ouabain binding, which was measured at a free concentration of 21 nM. Total recoveries in gradient subfractions ranged from 92 to 118% of the amounts layered on the gradient. In the SDS-treated microsomal fraction (see Materials and Methods), the activity of (Na^+,K^+)-ATPase was 21.5 μmol/hr/mg protein. Similar density distributions were obtained with other microsomal preparations from rat ventricle.

more numerous than the high-affinity sites. These results are in good agreement with those obtained previously on partially purified (Na^+,K^+)-ATPase preparations from the same tissue [2] and are consistent with the existence in adult rat ventricle of a major and a minor isoform of (Na^+,K^+)-ATPase, designated $\alpha 1$ and $\alpha 2$, respectively [9].

Accurate estimation of the B_{max} values of the high- and low-affinity [^{3}H]ouabain binding sites in the 10 microsomal subfractions separated by density gradient centrifugation was not feasible in view of the limited amounts of tissue material available. Therefore, [^{3}H]ouabain binding at a free concentration of 20 nM was used as an index of the high-affinity ($\alpha 2$) isoform and its distribution in gradient experiments was compared to that of the total (Na^+,K^+)-ATPase activity, which reflected mostly the low-affinity ($\alpha 1$) isoform. As shown in Fig. 2, the distribution of [^{3}H]ouabain binding ($\alpha 2$) was almost superimposable to that of (Na^+,K^+)-ATPase ($\alpha 1$). Their distribution pattern peaked at a density of 1.14–1.15 and was similar to those of 5′-nucleotidase (plasma membrane enzyme) and of [^{3}H]QNB binding (muscarinic receptors). The distribution pattern of [^{3}H]ouabain binding was not appreciably modified when binding was measured on subfractions that had been preincubated with saponin to suppress the membrane permeability barrier (data not shown). This treatment increased the apparent number of [^{3}H]ouabain binding sites by a factor of 2–3.

1,4-Dihydropyridine receptors labeled by [^{3}H](+)PN200-110 were mainly recovered in high-density subfractions (peak at 1.18), but a significant proportion of these receptors was found in the low-density subfractions. Cytochrome *c* oxidase (mitochondrial enzyme) gave a sharp and symmetrical peak around a density of 1.18, which corresponded to the modal density of [^{3}H](+)PN200-110 binding. However, it has been shown conclusively that mitochondria are devoid of high-affinity binding sites for 1,4-dihydropyridines [14]. In close analogy with what has been found in skeletal muscle, the 1,4-dihydropyridine receptors of high-density microsomal subfractions are associated with diadic structures, where they are clearly located in the junctional domain of the sarcolemma and not in the terminal cisternae of sarcoplasmic reticulum [10, 15, 16].

In summary, the $\alpha 1$ and $\alpha 2$ (Na^+,K^+)-ATPase isoforms in microsomal fractions from adult rat ventricle could not be separated by density gradient centrifugation. Both isoforms were mainly recovered in low-density subfractions and their distribution pattern was superimposable to those of other typical plasma membrane constituents (5′-nucleotidase, muscarinic receptors) but differed from that of 1,4-dihydropyridine receptors, which were mainly associated with high-density subfractions. Thus, both (Na^+,K^+)-ATPase isoforms were present essentially in the non-junctional sarcolemmal domain, i.e. at the cell surface, while 1,4-dihydropyridine receptors (voltage-dependent calcium channels) seemed much more concentrated in the junctional domain, which is predominantly of t-tubular origin [17]. Therefore, the high inotropic efficacy of low ouabain concentrations in rat ventricle cannot be explained on the basis of a preferential localization of the high-affinity receptors ($\alpha 2$ isoform) in the vicinity of junctional structures. The difference in inotropic efficacy between high and low ouabain concentrations might be related to

differences in stimulus response coupling associated with $\alpha 1$ and $\alpha 2$ isoforms, as suggested by the greater sensitivity of the effect of low concentrations to ethylisopropylamiloride, an inhibitor of Na^+-H^+ exchange [5].

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Interference of xanthate compounds with phorbol ester TPA-induced changes of phospholipid metabolism: inhibition of prostaglandin production

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Certain xanthate compounds have been shown to act antivirally by inhibition of the transcription and replication of various DNA and RNA viruses without becoming incorporated into viral macromolecules and in the absence of interferon induction [1]. In addition, xanthate compounds have been shown to reverse growth kinetics and cell morphology of transformed cells to that of non-transformed phenotypes [2]. Moreover, these compounds very effectively