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Kinetics of binding of the semi-synthetic α-methyl-digitoxigenin-glucoside to cardiac (Na^+–K^+)ATPase

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3-α-Methyl-digitoxigenin-3-β-glucoside (methyl-dtg-gluc) is a semi-synthetic cardiac glycoside distinguished by two peculiar properties when compared to classic cardiac glycosides: firstly, a fast onset of the positive inotropic effect and a rapid reversibility even of toxic effects upon removal [1–3]; secondly, dose–response curves covering a wider concn range and reaching higher inotropic maxima [1, 2, 4]. In order to characterize the binding properties of methyl-dtg-gluc at the receptor level, equilibrium binding studies were reported with [^{3}H]methyl-dtg-gluc and [^{3}H]ouabain, which revealed that methyl-dtg-gluc binds to ouabain binding sites in cardiac membranes with an affinity more than 10-fold lower than ouabain [5]. Complementary to that, in the present study the kinetic features of the interaction between methyl-dtg-gluc and its binding site were examined, measuring the association and dissociation of [^{3}H]methyl-dtg-gluc with the (Na^+–K^+)ATPase (EC 3.6.1.3) present in a crude membrane suspension of guinea pig ventricular muscle. The results were compared with the kinetic data of [^{3}H]ouabain binding. Methyl-dtg-gluc associated more than 2 times faster, while its dissociation proceeded at least 30 times faster, its affinity thus being more than 10-fold lower. This extraordinarily high dissociation rate is in accordance with the rapid reversibility of inotropic and toxic effects induced by methyl-dtg-gluc.

Materials and methods

[^{3}H]Ouabain (14 Ci/mmole) was obtained from NEN (Dreieich, F.R.G.), [^{3}H]methyl-dtg-gluc (11.1 Ci/mmole) was a gift from Knoll AG (Ludwigshafen, F.R.G.) as well as unlabelled methyl-dtg-gluc. All other chemicals were purchased from E. Merck (Darmstadt, F.R.G.).

Preparation of cardiac membranes. Cardiac ventricles of guinea pigs (either sex, 300–500 g) were frozen and stored at −20°. The duration of storage at −20° up to several weeks was without influence on the experimental results. Compared with hearts not subjected to the freezing procedure the yield of binding sites was a little increased when frozen ventricles were used for the preparation. At a temperature of 4° the ventricles were thawed, minced and homogenized (Waring blender, low speed, 30 sec) in a 0.32 M sucrose solution (20 ml/g wet wt). After treatment with six strokes in a Potter–Elvehjem glass homogenizer with a motor-driven Teflon pestle, the homogenate was centrifuged (10 min at 2000 *g*); the supernatant was recentrifuged (18 min at 30,000 *g*). The pellets were resuspended in 50 mM Tris–HCl adjusted to pH 7.4 (4 ml/g wet wt), frozen in liquid nitrogen and stored at −20° until the day of experiment.

Binding experiments. The equilibrium binding experiments have been described in detail [5]. Similarly, the kinetic experiments were performed at 37° in a medium (total vol. 45 ml) consisting of the membrane suspension (final concn of binding sites about 5 pmoles/ml), of NaCl (80 mM), $MgCl_2$ (16 mM), Tris–HCl (50 mM, final pH 7.3) and of the tritium-labelled cardiac glycoside (about 10 nM).

After a preincubation period of 30 min, ATP was added (final concn 2.5 mM) to initiate specific binding. After 45 min, when binding had attained an equilibrium level, unlabelled ouabain was added in excess (6.3×10^{-5} M) and the dissociation of the radioactive ligand was observed ("chase experiment"). The samples taken from the magnetically stirred medium had a vol. of 1 ml and were filtered under suction through Whatman GF/C filters and rinsed with 2 × 5 ml of ice-cold distilled water within 15 sec. After addition of Soluene 350® and Dimilume 30® (Packard, Frankfurt, F.R.G.) the filter-bound radioactivity was counted.

The unspecific binding was identical within the limits of experimental scattering, whether determined as the ATP-independent binding before addition of ATP or as the "non-displaceable" binding in the presence of the excess of ouabain at the end of the dissociation reaction (Fig. 1); specific binding was the difference between the total and the unspecific binding.

The association rate constant (k_{+1}) was calculated using the equation $d[RL]/dt = k_{+1} \times [L] \times [R]$, which describes the initial association velocity; [L] was considered equal to

the total concn of the radioactive cardiac glycoside and [R] equal to the total concn of binding sites (B_{max}) (B_{max} was smaller than $K_d/10$). d[RL]/dt was determined from a plot of specific binding vs time as the slope of the line connecting the origin and the first value for specific binding. The dissociation rate constant corresponds to $k_{-1} = (\ln 2)/t_{1/2}$ [6, 7].

Results and discussion

Using [^{3}H]ouabain and [^{3}H]methyl-dtg-gluc and the respective unlabelled ligand at increasing concns and applying the filtration technique, equilibrium binding experiments revealed one type of independent binding sites with a maximum number of about 5 pmoles/ml membrane suspension for both ligands; the K_d for ouabain binding amounted to 1.6×10^{-7} M, while the binding of methyl-dtg-gluc was characterized by a K_d of 3.3×10^{-6} M. These data agree quite well with previously published results obtained by centrifugation [5].

The time-course of [^{3}H]ouabain binding is illustrated in Fig. 1B which shows a representative experiment. The association reaction was started by addition of ATP and took about 15 min until the equilibrium was attained. In control experiments, membranes and ATP were preincubated for 75 min before the association was started by addition of [^{3}H]ouabain; the rate of association was found to be identical. As measured by a P_i determination, ATP was almost entirely hydrolyzed after 75 min by an Mg^{2+}-ATPase present in the membrane suspension.

Addition of ADP instead of ATP yielded a decreased association rate; the equilibrium binding, however, attained the same level. Considering these results and taking the absence of potassium into account, it is reasonable to assume that the ouabain binding conformation of the enzyme is induced by a phosphorylation step which is not rate-limiting for binding when ATP is added. Once this confirmation has been formed, it seems to remain stable, since the equilibrium binding stayed unchanged for at least 75 min.

The dissociation of [^{3}H]ouabain followed first-order kinetics as revealed by a plot of log ouabain bound vs time; the half-time of dissociation amounted to more than 3 min. The kinetic data of [^{3}H]ouabain binding are summarized in Table 1; the mean value for K_d of 1.8×10^{-7} M agrees with the K_d of 1.6×10^{-7} M obtained by equilibrium binding experiments.

In the case of [^{3}H]methyl-dtg-gluc (Fig. 1A) equilibrium binding was reached almost immediately after the addition of ATP; in two experiments also the dissociation was complete when the first sample was taken after only 10 sec. The data presented in Table 1 are therefore calculated from 10-sec values, thus providing minimum estimates of the kinetic characteristics of [^{3}H]methyl-dtg-gluc; however, the K_d of 2.3×10^{-6} M calculated from k_{+1} and k_{-1} is close to the K_d obtained in equilibrium binding experiments (see earlier and Ref. 5). Compared with ouabain the association rate constant of methyl-dtg-gluc is more than 2 times and its dissociation rate constant is about 30 times higher. The extraordinarily fast dissociation is in accordance with the rapid reversibility of the inotropic effect of methyl-dtg-gluc;

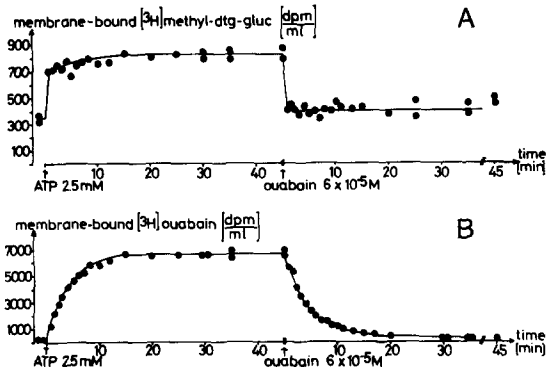

Fig. 1. Time-course of the binding of [^{3}H]methyl-dtg-gluc (10.8 nM, 11.1 Ci/mmole) (A) and [^{3}H]ouabain (10.4 nM, 14 Ci/mmole) (B) to (Na^+–K^+)ATPase-containing membranes of guinea pig ventricular tissue (incubation medium: 80 mM NaCl, 16 mM $MgCl_2$, 50 mM Tris–HCl, final pH 7.3, 37°). Ordinate: total membrane-bound activity (dpm/ml). Abscissa: time (min). Indicated are the start of the association reaction by addition of ATP (2.5 mM) and the initiation of the dissociation process by an excess of unlabelled ouabain (63 μM). The relative unspecific binding of [^{3}H]methyl-dtg-gluc amounted to about 50% of the total binding due to its lower affinity and higher hydrophobicity compared with [^{3}H]ouabain being unspecifically bound to about 5%. The absolute amount of unspecifically bound [^{3}H]methyl-dtg-gluc was about twice as high as that of [^{3}H]ouabain.

also the onset of positive inotropism after administration of methyl-dtg-gluc is faster than in the case of classical cardiac glycosides [1, 2]. The *in vivo* binding conditions with (Na^+–K^+)ATPase facing different environments on the inside and outside of the cell and being active as a pump are quite different from the *in vitro* binding conditions. Nevertheless, the kinetics of the *in vitro* binding of methyl-dtg-gluc match the kinetics of its pharmacological action. As previously reported [5], the binding sites of methyl-dtg-gluc are identical with the binding sites of ouabain, which are probably the receptor sites of "classical" cardiac glycosides. This observation supports the assumption that the kinetics of methyl-dtg-gluc at the binding site described here reflect the kinetics at its receptor site.

Strikingly, this semisynthetic cardiac glycoside distinguished by such a fast turnover at its receptor site has been observed to exert a higher inotropic maximum than usual glycosides before signs of intoxication occur [1, 2, 4]. In view of this remarkable correlation, Lüllmann *et al.* [1] proposed a concept which explains the inotropic effect of cardiac glycosides resulting from the rate of formation of the glycoside–receptor complex and the inhibition of (Na^+–K^+)ATPase depending on the occupation of receptors. Accordingly, a glycoside dissociating rapidly after attachment to the receptor would cause a given increase

Table 1. Binding of [^{3}H]methyl-dtg-gluc and [^{3}H]ouabain

	k_{+1} ($\times 10^{-4}$) ($M^{-1} \times sec^{-1}$)	k_{-1} ($\times 10^3$) (sec^{-1})	K_d ($\times 10^7$) (M)
[^{3}H]Methyl-dtg-gluc	4.6 (3.8, 4.7, 5.2)	100 (130, 50, 130)	23 (33, 11, 24)
[^{3}H]Ouabain	1.9 (1.7, 1.8, 2.1)	3.4 (3.6, 3.3, 3.2)	1.8 (2.1, 1.8, 1.5)

k_{+1}, association rate constant; k_{-1}, dissociation rate constant.
Presented are mean values and the results of three experiments in parentheses.

in contractility with a minimum of (Na^+-K^+)ATPase inhibition.

In conclusion, the unusual properties of methyl-dtg-gluc with respect to the rapid onset and offset of the inotropic response is reflected by unusual kinetic features at the receptor level. The increased turnover rate of the complex between methyl-dtg-gluc and its receptor at a given level of equilibrium binding might have significance for the development of cardiac glycosides with improved therapeutic properties.

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On the mechanism of inhibition of dopamine receptors by fluphenazine*

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It is generally agreed that antipsychotic drugs exert their therapeutic and extrapyramidal effects by blocking dopamine receptors in the brain [1]. It is also often assumed that blockade is due to competitive inhibition of the association of dopamine to its receptors. Although antipsychotic drugs inhibit the specific binding of [^{3}H]dopamine ([^{3}H]DA) to membranes of rat [2–4] or calf [5] brain, the mechanism of this inhibition has not been determined. The finding that antipsychotic drugs inhibit the binding of [^{3}H]spiroperidol by a competitive mechanism [6, 7] cannot be used as evidence that the binding of dopamine to its receptors is also inhibited competitively by these drugs. For example, agonists and antagonists may bind to different sites [8] or to different subunits of a receptor complex [9]. Thus, although interactions within each class of compound may be competitive, interactions between agonists and antagonists may be more complex. Studies showing the multiphasic displacement of ^{3}H-agonists by antipsychotic drugs [2, 10] still do not address the mechanism whereby this displacement takes place.

In this study, we have investigated the inhibition of [^{3}H]DA binding by a potent phenothiazine neuroleptic, fluphenazine. The stereospecific and saturable binding of [^{3}H]dopamine was measured as we have previously described [11]. Briefly, caudate nuclei from adult male Sprague–Dawley rats were homogenized in 100 vol. of 50 mM Tris–HCl buffer, pH 7.0, containing 3.0 mM $CaCl_2$ and preincubated at 37° for 30 min. The homogenate was centrifuged at 20,000 *g* for 15 min at 4°, and the pellet was resuspended in 100 vol. of fresh buffer and recentrifuged. The pellet was washed in the same manner, and the final pellet was resuspended in 20 vol. of 50 mM Tris–HCl, pH 7.1, containing 5.0 mM tetrasodium EDTA, 10 mM $MgSO_4$ and 15 μM pargyline. Samples containing 0.25 mg tissue protein and 0.5 to 10.0 nM [^{3}H]DA (sp. act. 29 Ci/mmole, New England Nuclear Corp., Boston, MA), in a final volume of 0.1 ml buffer, were incubated for 30 min at 23° and rapidly filtered through GF/B filters under suction. The filters were washed with three portions of 5 ml buffer, and bound radioactivity was counted as previously described [11]. Total and nonspecific binding (in the presence of 10 μM *d*-butaclamol) was measured in triplicate. Typical values (± S.E.M.) of total binding were 2450 ± 34 cpm and of nonspecific binding 550 ± 20 cpm using 1 nM [^{3}H]DA or 7100 ± 125 and 2940 ± 60 cpm using 6 nM [^{3}H]DA.

The specific binding of [^{3}H]DA was saturable with a density (B_{max}) for 730 ± 30 fmoles/mg protein and a dissociation constant (K_d) of 1.41 ± 0.17 nM (means ± S.D. of six experiments). The B_{max} was higher that the value we reported previously [4, 11] because the present assay mix contained $MgSO_4$ which increases stereospecific [^{3}H]DA binding [2].

The addition of 5, 20 or 100 nM fluphenazine–HCl to the binding assay lowered the B_{max} progressively without changing the K_d (Fig. 1A). Higher concentrations of fluphenazine (500 or 1000 nM) induced significant increases in the K_d. The results of these and other experiments with additional fluphenazine concentrations are summarized in Fig. 1B. It is clear that concentrations of fluphenazine between 5 and 100 nM inhibited [^{3}H]DA binding by lowering the B_{max}, whereas higher drug concentrations decreased receptor affinity as well.

These results suggested that fluphenazine inhibited [^{3}H]DA binding either noncompetitively or irreversibly. The latter can be evaluated by measuring binding to several receptor concentrations in the presence of a constant concentration of inhibitor. An irreversible inhibitor removes a constant amount of receptors from the binding reaction, shifting the intercept rather than the slope of the protein-binding function (Ref. 12, p. 128). In contrast,

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