Biochemical Pharmacology, Vol. 31, No. 15, pp. 2489–2494, 1982.
Printed in Great Britain.

0006-2952/82/152489-06 $03.00/0

ON THE BINDING OF A 3-α-METHYLATED DIGITOXIGENIN-GLUCOSIDE TO OUABAIN RECEPTORS IN HEART MUSCLE HOMOGENATE

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(*Received* 5 *October* 1981; *accepted* 17 *February* 1982)

Abstract—3-α-Methyl substituted digitoxigenin-3-β-glucoside (methyl-dtg-gluc) displays unusual features, e.g. a high dissociation rate constant from its binding site leading to rapid reversibility of the inotropic and toxic effects, and a flat dose–response curve attaining higher inotropic maxima thus indicating an increased therapeutic index in animal experiments. In order to check whether or not methyl-dtg-gluc is specifically bound to the same receptors as classic cardiac glycosides we compared binding of ouabain and of methyl-dtg-gluc to guinea-pig heart muscle homogenate. For both compounds specific binding required the addition of ATP (2.5 mM). The binding curve for ouabain yielded half maximum binding at 1.3×10^{-7} M and maximum number of binding sites of 6 pmole/mg protein; the corresponding values for methyl-dtg-gluc amounted to 1.4×10^{-6} M and 6 pmole/mg protein, respectively. A mutual competition could be demonstrated between the two compounds. Since the provided data are equilibrium values they do not exclude higher turnover rates of methyl-dtg-gluc in comparison with ouabain at a given glycoside–ATPase complex concentration which can be expected from the fast dissociation rate constant of the methyl-dtg-gluc–ATPase complex. The results are briefly discussed with respect to the molecular mode of action of cardiac glycosides.

Therapy with cardiac glycosides is rendered difficult due to their small therapeutic range. Recently, however, reports have been published about a novel experimental glycoside, 3-α-methyl-digitoxigenin-3-β-glucoside (methyl-dtg-gluc) indicating a wider therapeutic range of this drug compared with 'classical' glycosides in animal experiments [1–5].

The sarcolemmal Na^+,K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is considered to be the receptor for cardiac glycosides [6–8]. Two effects are mediated by the ligand–receptor interaction: the inhibition of the Na^+,K^+-ATPase, and the positive inotropic action. It is still a matter of debate whether these effects are independent from each other or whether the inhibition of the ion pump is causal for the positive inotropism. According to the latter concept, therapeutic and toxic effects are only dependent on the degree of Na^+,K^+-ATPase inhibition. Since the positive inotropism is thought to be inseparably connected to the enzyme inhibition this concept would not allow a cardiac glycoside to have a wider therapeutic range than the classic glycosides as long as it acts at the same receptor site.

We considered it necessary to investigate whether or not methyl-dtg-gluc possesses the same binding site as a classical cardiac glycoside. We, therefore, compared the binding characteristics of ouabain and methyl-dtg-gluc under equilibrium conditions, applying a method similar to those already used for measuring ouabain binding characteristics [e.g. 9–12]. A crude membrane preparation of guinea-pig ventricular muscle was used to determine the binding of the tritium-labelled compounds.

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MATERIALS AND METHODS

The procedure consists of tissue preparation, binding assay and calculation of results, thus following the principle for receptor-binding studies as described, for instance, by Bennett [13].

Chemicals. [^{3}H]Ouabain with a specific activity of 19.5 Ci/mmole (lot-No. 1162-075) and 11.6 Ci/mmole (lot-No. 1191-064) was obtained from N.E.N. (Dreieichenhain, F.R.G.). [^{3}H]Methyl-dtg-gluc had a specific activity of 11.1 Ci/mmole and 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.) unless otherwise indicated.

Preparation of cardiac tissue. The preparation was carried out at 4°. Guinea-pigs (weight range 300–500 g) of either sex were killed by a blow on the head, the hearts were quickly excised, freed from atria and connecting tissue, minced with scissors, and weighed. The tissue was homogenized in a 0.32 M sucrose solution at a volume of 20 ml/g wet wt for 30 sec in a Waring blender at low speed, thereafter with 6 strokes in a Potter–Elvehjem glass homogenizer with a Teflon pestle. The homogenate was centrifuged (Beckman spinco L2 50 B) for 10 min at 2000 *g* (4500 rpm in a Beckman rotor 21); the supernatant was again centrifuged for 18 min at 30,000 *g* (18,500 rpm in a Beckman rotor 30). The pellet was resuspended in 50 mM Tris–HCl pH 7.4 at a volume of 4 ml/g wet wt, distributed into portions of 1 ml, quickly frozen in liquid nitrogen, and stored at −20°. Protein concentrations were measured according to Lowry *et al.* [14], using human serum albumin (Behringwerke AG, Marburg-Lahn, F.R.G.) as the standard.

[^{3}H]*Ouabain and* [^{3}H]*methyl-dtg-gluc binding assay.* The radiochemical purity of the labelled ligands was checked by radiochromatogram scanning of thin-layer chromatograms (Packard model 7201). Incubation was performed in triplicate in thick-wall polyallomer centrifugation tubes (Beckman). The incubation medium consisted of 100 μl of labelled glycoside dissolved in buffer (final concentrations: 5 nM [^{3}H]ouabain and 12 nM [^{3}H]methyl-dtg-gluc), 50 μl of unlabelled cardiac glycoside in various concentrations (all ouabain concentrations dissolved in buffer; the highest concentration of methyl-dtg-gluc dissolved in a mixture of dimethylsulfoxide/propandiole (1 + 9), the lower concentrations diluted in buffer; addition of 50 μl DMSO/PG to a [^{3}H]ouabain binding assay did not influence the results), 250 μl of tissue suspension (final concentration 0.5–0.7 mg protein/ml), 100 μl of ATP–Na_2 (final concentration 2.5 mM) and 1000 μl buffer (final concentrations: 80 mM NaCl, 16 mM $MgCl_2$, 50 mM Tris–HCl; final pH 7.3). Incubation was started by addition of ATP and carried out at 37° for 75 min, this time being sufficient for equilibrium binding as shown by control studies. To terminate the incubation the tubes were cooled on ice. The membrane bound ligand was separated by centrifugation (Kontron ultracentrifuge TGA-65) at 1° for 18 min at 52,000 *g* (28,000 rpm in a Kontron rotor TFT 65.13). After rinsing the pellet three times quickly and carefully with 1 ml of ice-cold buffer, the pellet was resuspended in 1.5 ml buffer by sonication (Braun Labsonic 1510). Ten millilitres of Dimilume® (Packard) was added to a 1 ml aliquot and the radioactivity measured in a Packard Tricarb 460 CD with a counting efficiency of about 34%.

Calculation of results. Unspecific binding of [^{3}H]ouabain and [^{3}H]methyl-dtg-gluc was determined in the presence of 10^{-4} M ouabain and 5×10^{-4} M methyl-dtg-gluc, respectively. Specifically bound radioactivity was considered to be the difference between totally and unspecifically bound activity. If labelled and unlabelled ligand were of the same species the absolute amount of bound glycoside could be calculated taking into account the dilution of the specific activity. The free ligand concentration was assumed to be equal to the initial concentration in the incubation medium since the concentration of receptor was smaller than 10% of the dissociation constant K_d [15] and no binding to the tube wall occurred. Inhibition of the binding of labelled ligand by unlabelled drug was expressed using the IC_{50} value, i.e. the drug concentration which reduced the specifically bound activity by 50%. K_i values with $K_i = IC_{50}/(1 + c/K_d)$ [16] were not calculated since the concentration c of labelled ligand was smaller than its K_d at least by a factor of 20, so that there was hardly any difference between IC_{50} and K_i. For estimation of the dissociation constant K_d and the maximal number of binding sites B_{max} the data were analysed using Scatchard plots.

RESULTS

[^{3}H]*Ouabain as the radioactive ligand*

In order to study whether there was a saturable ouabain binding site present in the heart muscle homogenate, binding of 5 nM [^{3}H]ouabain was measured in the absence and in the presence of 10^{-4} M unlabelled ouabain. Addition of 10^{-4} M ouabain reduced the pellet-bound radioactivity by about 85%. When ATP was omitted, binding of [^{3}H]ouabain was drastically reduced and amounted to the same value as obtained after addition of ATP and 10^{-4} M ouabain. Hence, the saturable, specific binding site was only present, when ATP had been added. However, after the 75 min incubation, ATP was almost entirely hydrolysed as obtained from a P_i determination due to the high Mg^{2+}-ATPase activity. P_i could not replace ATP, but addition of ADP yielded an identical equilibrium binding of [^{3}H]ouabain. The initial rate of association of [^{3}H]ouabain was reduced, when binding was initiated by ADP instead of ATP (determined with a rapid filtration technique). This probably indicates that the rate of formation of the phosphorylated, glycoside binding enzyme conformation was retarded. Since potassium ions were absent, it is likely, that this conformation was preserved under this condition [7, 17].

When ouabain was added in increasing concentrations from 5×10^{-9} M to 10^{-4} M, the specific [^{3}H]ouabain binding attained half maximum values at a concentration of 1.3×10^{-7} M (Fig. 1A). The ouabain binding calculated from these data and analysed by means of a Scatchard plot was characterized by a single binding site with a K_d of 1.3×10^{-7} M and a maximum number of binding sites of about 6 pmole/mg protein (Fig. 2A). The Hill coefficient was 0.93 indicating non-cooperative binding.

To study the affinity of methyl-dtg-gluc to the [^{3}H]ouabain binding site, methyl-dtg-gluc was added in concentrations from 3.3×10^{-8} M to 3.3×10^{-4} M. Methyl-dtg-gluc inhibited the [^{3}H]ouabain binding to half its maximum at a concentration of 1.5×10^{-6} M = IC_{50} (Fig. 1A). 3.3×10^{-4} M methyl-dtg-gluc reduced the [^{3}H]ouabain binding to the same degree as did 10^{-4} M ouabain.

Comparison of the competition of ouabain for the [^{3}H]ouabain binding sites in the absence and in the presence of methyl-dtg-gluc (6.6×10^{-7} M, a concentration reducing [^{3}H]ouabain binding significantly, but leaving it sufficiently high to perform a ouabain binding analysis) displayed a decrease of the ouabain IC_{50} from a control value of 1.3×10^{-7} to 2.2×10^{-7} M (Fig. 3). The Scatchard analysis of ouabain binding revealed a reduced affinity of ouabain to its binding sites while the maximum number of binding sites was not affected. This suggests a competitive type of antagonism between methyl-dtg-gluc and ouabain at the[^{3}H]ouabain binding site.

[^{3}H]*Methyl-dtg-gluc as the radioactive ligand*

In Fig. 4 the binding of 12 nM [^{3}H]methyl-dtg-gluc to the homogenate is presented. The omission of ATP reduced the binding to the same degree as did addition of 5×10^{-4} M methyl-dtg-gluc or 10^{-4} M ouabain. The unspecific binding amounted to 80% of the total binding. When methyl-dtg-gluc was added in increasing concentrations specific binding of [^{3}H]methyl-dtg-gluc was inhibited to half its maximum at 1.2×10^{-6} M = IC_{50}. The competition by

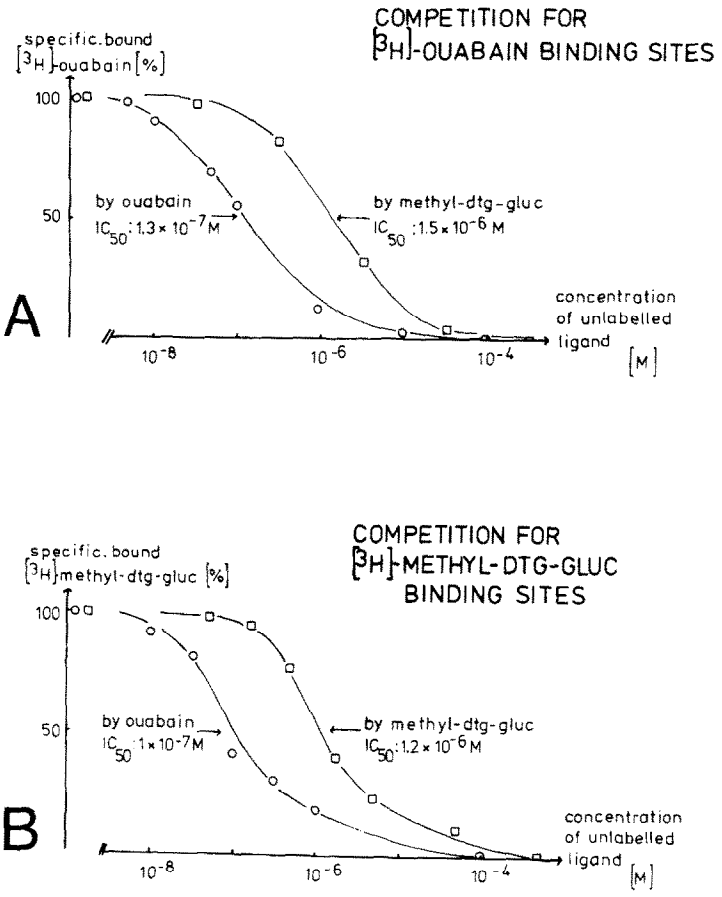

Fig. 1. (A) Competition between the unlabelled ligands ouabain (○—○) and methyl-dtg-gluc (□—□) and the labelled ligand [^{3}H]ouabain (5 nM). Ordinate: amount of [^{3}H]ouabain bound to the specific binding site expressed as per cent of the [^{3}H]ouabain binding in the absence of a competitor. Abscissa: concentration of the competing ligand. (B) Competition between the unlabelled ligands ouabain (○—○) and methyl-dtg-gluc (□—□) and the labelled ligand [^{3}H]methyl-dtg-gluc (12 nM). Ordinate: amount of [^{3}H]methyl-dtg-gluc bound to the specific binding site expressed as per cent of the [^{3}H]methyl-dtg-gluc binding in the absence of a competitor. Abscissa: concentration of the competing ligand.

ouabain revealed an IC_{50} of 1×10^{-7} M (Fig. 1B).

Due to the high unspecific binding and the small portion of specifically bound [^{3}H]methyl-dtg-gluc the calculation of binding data was only possible to a limited extent. In Fig. 2B, however, a Scatchard analysis of methyl-dtg-gluc binding data calculated from the results shown in Fig. 1B is given. The dissociation constant was found to be $K_d = 1.4 \times 10^{-6}$ M, B_{max} amounted to 6 pmole/mg protein. The Hill coefficient of 1.03 indicated non-cooperative binding. When [^{3}H]ouabain was used in this particular homogenate, it displayed a $K_d \approx 1 \times 10^{-7}$M and a $B_{max} \approx 6$ pmole/mg protein.

In the meantime we applied a rapid filtration technique to separate bound from free ligand. Compared to the centrifugation method, the absolute amount of specific binding of [^{3}H]methyl-dtg-gluc was identical but unspecific binding was reduced to 40% of total binding. The binding characteristics of methyl-dtg-gluc were similar to those described above.

DISCUSSION

The purpose of the present paper was to show whether or not the new semisynthetic cardiac glycoside (3-α-methyl-digitoxigenin-3-β-glucoside, methyl-dtg-gluc) specifically binds to the sarcolemmal Na^+,K^+-ATPase which is considered to be the receptor for classical glycosides. The composition of the binding assay (addition of ATP, Na^+ and Mg^{2+}; omission of K^+) has been described to provide conditions for a high affinity binding of cardiac glycosides [18]. It is conceivable to assume that Na^+,K^+-ATPase becomes phosphorylated to the $E_2 \cdot P_i - Na^+$ conformation by addition of ATP in the presence of Na^+ and Mg^{2+}. This enzyme conformation allows binding of cardiac glycosides; since K^+, known to induce a further conformational change, is absent, it seems likely that the enzyme molecules, once phosphorylated, remain in this ouabain binding form [7, 17].

The K_d of the ouabain–receptor interaction which

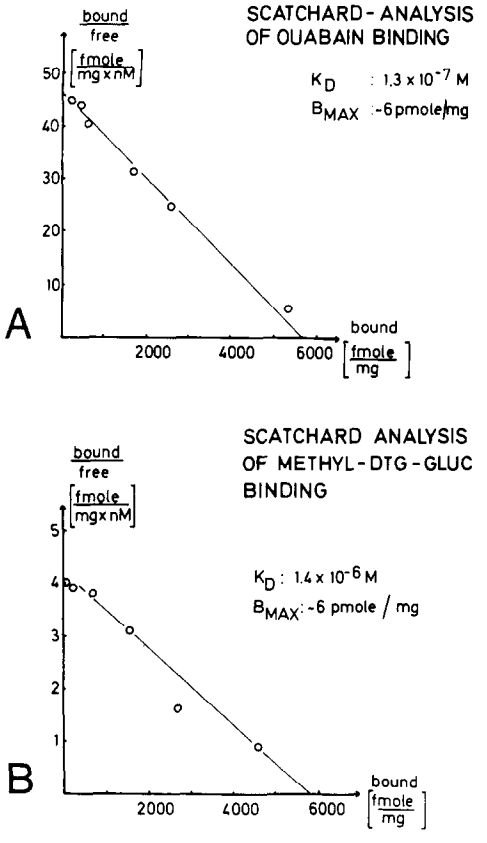

Fig. 2. (A) Scatchard plot of the ouabain binding data from Fig. 1A yielding a single binding site with $K_d = 1.3 \times 10^{-7}$ M and a maximum number of binding sites $B_{max} \simeq 6$ pmole/mg protein. (B) Scatchard plot of the methyl-dtg-gluc binding data from Fig. 1B yielding a single binding site with $K_d = 1.4 \times 10^{-6}$ M and a maximum number of binding sites $B_{max} \simeq 6$ pmole/mg protein. Note the different scales of the ordinates.

was found to be in the range of $1.0–1.3 \times 10^{-7}$ M is in accordance with the K_d of 1.3×10^{-7} M described by Erdmann [19] for ouabain receptors in guinea-pig heart. Yamamoto *et al.* [10] measuring [^{3}H]ouabain binding to a homogenate of guinea-pig ventricular muscle found a K_d of 1.07×10^{-7} M and a B_{max} of 6.75 pmole/mg protein. Gerthoffer and Allen [11] determined $K_d = 1.4 \times 10^{-7}$ M and $B_{max} =$ 3.9 pmole/mg and Yamamoto *et al.* [12] reported $K_d = 1.06 \times 10^{-7}$ M and $B_{max} = 5.66$ pmole/mg in guinea-pig right ventricular muscle. In electrically stimulated guinea-pig atria Busse *et al.* [20] demonstrated a saturable [^{3}H]ouabain binding site with a K_d of about 1.8×10^{-7} M. From these data it is concluded, that the saturable binding site described in the present paper represents the pharmacological receptor for cardiac glycosides.

When the receptor was labelled with [^{3}H]ouabain the affinity of methyl-dtg-gluc was about 10 times lower than that of ouabain, but still sufficiently high to 'displace' the same maximum amount of specifically bound [^{3}H]ouabain as ouabain did itself. Methyl-dtg-gluc inhibited binding of ouabain to its receptor in a competitive manner according to the Scatchard analysis. This result *per se* cannot prove a competition of the two compounds for the same binding site, since, for example, also the local anaesthetic dibucaine decreased the ouabain binding. It reduced the affinity and left the maximum number of binding sites unchanged (unpublished results). Since dibucaine has been demonstrated to interact with phospholipids [e.g. 21, 22] it is unlikely that the inhibition of ouabain binding is the result of a direct competition between ouabain and dibucaine for the receptor.

Accordingly, the competitive inhibition by methyl-dtg-gluc does not necessarily imply an interaction at the same site. It was, therefore, necessary to determine the binding characteristics of methyl-dtg-gluc by using the tritium labelled compound.

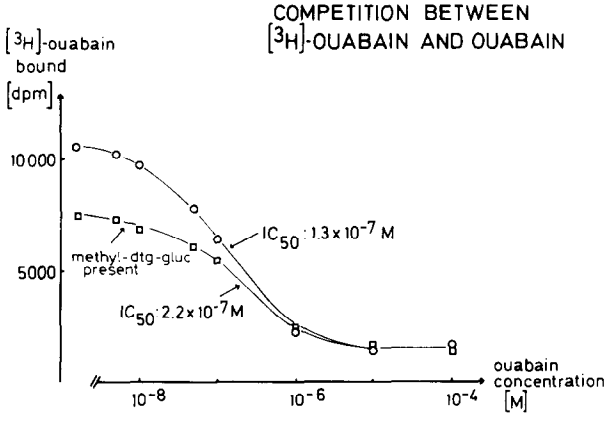

Fig. 3. Competition between [^{3}H]ouabain and ouabain in the presence of methyl-dtg-gluc (6.6×10^{-7} M). Ordinate: total amount of [^{3}H]ouabain bound to the pellet formed after incubation by centrifugation of the tissue homogenate. Abscissa: concentration of unlabelled ouabain present in the incubation medium. Compared to the control value $IC_{50} = 1.3 \times 10^{-7}$ M the presence of methyl-dtg-gluc increased the ouabain concentration necessary for half maximum binding to $IC_{50} = 2.2 \times 10^{-7}$ M.

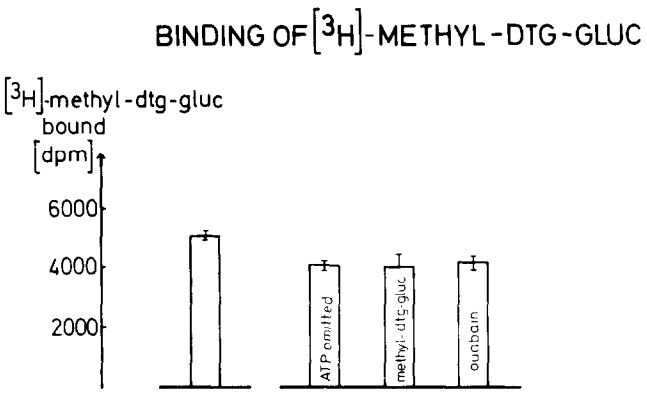

Fig. 4. Binding of [^{3}H]methyl-dtg-gluc (12 nM) measured as the amount of radioactivity bound to the pellet (left, first column). Presented are $\bar{x} \pm$ S.D. of a triplicate determination. Addition of a high concentration of methyl-dtg-gluc (5×10^{-4} M, third column) or of ouabain (10^{-4} M, fourth column) reduced the binding to the same extent as did the omission of ATP (second column).

Also in the case of methyl-dtg-gluc, the addition of ATP was a prerequisite for specific binding. After omission of ATP as well as in the presence of high concentrations of ouabain or methyl-dtg-gluc, the binding was reduced to the same level of unspecific binding (Fig. 4), which was, however, comparably high. At least in part the unspecific binding of the hydrophilic ouabain may be due to supernatant fluid trapped in the pellet. Although the less hydrophilic methyl-dtg-gluc will be trapped in the pellet to the same extent, its unspecific binding to membranes attains higher values than that of ouabain. It is easily understandable that at a given concentration the ratio specific to unspecific binding of methyl-dtg-gluc is much more unfavourable than in the case of ouabain. Because of these difficulties the maximal number of binding sites could not be determined as exactly as for ouabain; however, the B_{max} was also found to be close to 6 pmole/mg protein.

Independent of whether [^{3}H]ouabain or [^{3}H]methyl-dtg-gluc were used to label a binding site, under either condition ouabain had an affinity about ten times higher than methyl-dtg-gluc. Ouabain and methyl-dtg-gluc 'displaced' identical amounts of either [^{3}H]ligand and almost identical B_{max} values were determined. These results indicate, that methyl-dtg-gluc binds to the same site as ouabain, i.e. to the receptor of the classic cardiac glycosides.

As mentioned in the Introduction the positive inotropic effect of cardiac glycosides has been proposed to result from a partial inhibition of the Na^+,K^+-ATPase and a consecutive increase of the intracellular sodium concentration, occurring at least transiently. As long as cardiac glycosides are bound to the same receptor site, a wider therapeutic range as observed with methyl-dtg-gluc cannot be understood on the basis of this hypothesis since therapeutic and toxic effects are thought to be inseparably connected and only dependent upon the degree of Na^+-pump inhibition.

However, experiments on the action of methyl-dtg-gluc in anaesthetized cats [4], in cat heart–lung preparations [5] or in isolated guinea-pig left atria [1–3] yielded dose–response curves with flatter slopes and usually a higher inotropic maximum than obtained with classical glycosides, thus revealing an improved therapeutic index. Stimulated by this unusual behaviour of methyl-dtg-gluc we investigated its binding characteristics. As has been demonstrated in the present paper methyl-dtg-gluc reacts with identical receptors as classic glycosides do. If the increased therapeutic range of methyl-dtg-gluc is taken for granted, the Na^+-pump inhibition hypothesis [6, 17] cannot explain the mode of action of all cardiac glycosides. Lüllmann and Peters postulated, that the positive inotropic action and the inhibition of the Na^+,K^+-ATPase by cardiac glycosides were independent events [8]. With regard to the high dissociation constant of methyl-dtg-gluc [1, 3] the former effect is thought to depend upon the rate of complex formation, the latter upon the occupation of the Na^+-pump, thus being responsible for intoxication [3]. In contrast to the Na^+-pump inhibition hypothesis which necessitates a fixed relationship between therapeutic and toxic concentrations, the concept of Lüllmann and Peters permits the possibility of a large variability in the therapeutic ranges of different cardiac glycosides.

Acknowledgement—We are grateful to Dr. H. P. Albrecht (Knoll AG, Ludwigshafen, F.R.G.), who synthesized 3-α-methyl-digitoxigenin-3-β-glucoside, for providing the compounds and the ^{3}H-labelled samples.

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