Biochemical Pharmacology, Vol. 33, No. 14, pp. 2337–2340, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

PRELIMINARY COMMUNICATIONS

CHRONIC EXPOSURE TO TOXIC BUT NOT TO "THERAPEUTIC" CONCENTRATIONS OF OUABAIN INCREASES CARDIAC GLYCOSIDE RECEPTORS IN CARDIAC MUSCLE CELLS FROM CHICKEN EMBRYOS

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(received: 9 April 1984; accepted: 30 April 1984)

Both an increased number of binding sites for cardiac glycosides (3) and an increased $(Na^+ + K^+)$-ATPase activity (1) have been reported to exist in erythrocytes of patients under chronic treatment with cardiac glycosides. If these findings are also applicable to the heart, then tachyphylaxis of treatment should result (4). In heart tissue, however, experimental findings are controversial (for discussion see 2). Recently, we have characterized the properties of cardiac glycoside receptors in cultured cardiac muscle cells from chicken embryos (5,6). These cells provide a useful model to study possible tachyphylaxis during chronic treatment, by measuring quantitatively the effect of chronic exposure to "therapeutic" as well as to toxic levels of ouabain on the number of cardiac glycoside receptors and sodium pump molecules in the heart.

Methods: Materials and methods used have been described in detail previously (5,6): preparation and cultivation of cardiac muscle cells from 12 - 13 day-old chicken embryos (disaggregation of heart tissue at 37° with trypsin (0.12 %)-collagenase (0.03 %)-salt solution (Ca^{2+},Mg^{2+}free); seeding of the cells ((1,5 - 2,5)x10^5 cells/cm^2 in Nunclon plastic flasks in CMRL medium, supplemented with 0.02 mg/ml gentamycin, 5 % fetal calf serum and 5 % horse serum); determination of cell protein according to the method of Lowry. After the cells have been cultured for 2 days, unlabelled ouabain at the desired concentration has been added to the medium, and cells have been cultured for 3 or 4 days with daily medium change; $[K^+]$ = 3.5 mM. Thereafter, cell-bound ouabain has been replaced by thorough washing of the cells in ouabain-free medium (washing period 2 hours, 37°). 85 - 95 % of unlabelled ouabain could be replaced by this washing procedure, as has been tested by using (^3H)-ouabain

at appropriate concentrations. Subsequently, specific (^{3}H)-ouabain binding to the cells under equilibrium conditions has been determined by adding (^{3}H)-ouabain, as previously described (6): 2.0×10^6 cpm (^{3}H)-ouabain/flask, specific activity 14 - 20 Ci/mmol (NEN Chemicals, D-6072 Dreieich, F.R.G.), 2.0 - 3.0 mg cell protein/flask, $[K^+]$ = 0.75 mM; temp. 37°; incubation period 2 hours; incubation volume 4.1 ml; unspecific (^{3}H)-ouabain binding at 10^{-4}M ouabain: about 8 % of maximal counts bound. Determination of protein/cell ratio (5) after cultivation of the cells for 3 days at 3×10^{-6}M ouabain, and without ouabain, yielded the following results: $(5.7 \pm 1.3) \times 10^6$ and $(5.5 \pm 1.2) \times 10^6$ cells/mg protein (mean+/- SD, n = 4). The data given in this report are mean values from closely correlating triplicates. All experiments have been carried out at least three times.

Results: In the experiment of fig. 1a, specific (^{3}H)-ouabain binding to cardiac muscle cells from chicken embryos has been determined, after acute (2 hours) and after chronic (3 days) exposure of the cells to ouabain (3×10^{-6}M). In comparison with control and acutely ouabain-treated cells, chronically ouabain-treated cells bind about 90 % more (^{3}H)-ouabain at every ouabain concentration chosen. Analysis of these binding data according to Scatchard is consistent with an increase in the number of ouabain binding sites after chronic ouabain exposure without any significant alteration of receptor affinity (fig. 1b).

The slightly higher dissociation constant of ouabain binding to acutely and chronically ouabain-

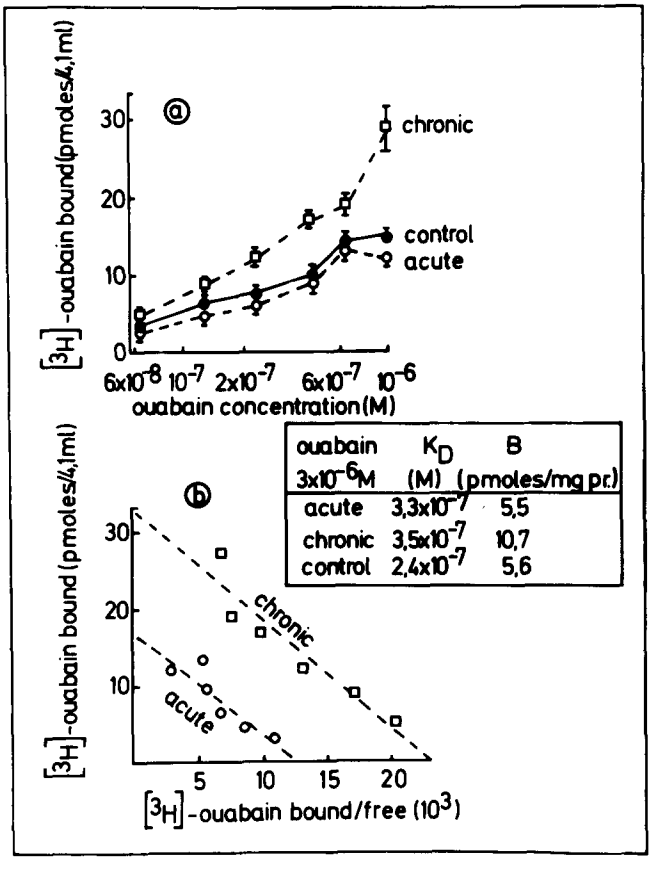

Fig.1: Concentration-dependent, specific (^{3}H)-ouabain binding to cardiac muscle cells from chicken embryos, after acute (2 hours) and chronic (3 days) exposure to 3×10^{-6}M ouabain; 3.1 mg cell protein/flask. For further details see "methods" and "results"; (mean ± SD; n = 3).

treated cells in comparison to control cells result from the small amount of unlabelled ouabain, remaining bound to the cells despite the washing procedure (see methods). Despite this disadvantage, this method is superior to incubation of chronically ouabain-treated cells with (^{3}H)-ouabain at the beginning of the incubation period, as interference with ouabain receptor binding from ouabain internalization would result by application of the latter method (for discussion see (6)). As the protein/cell ratio is not altered by ouabain ($3x10^{-6}$M, see "methods"), the increase in ouabain binding/ mg cell protein reflects an increase of ouabain binding sites per cell. No further increase in the number of sites was observed during a further incubation period of 24 hours. Arrythmias - present within the first hours of exposure- were absent at day 3 of ouabain treat-

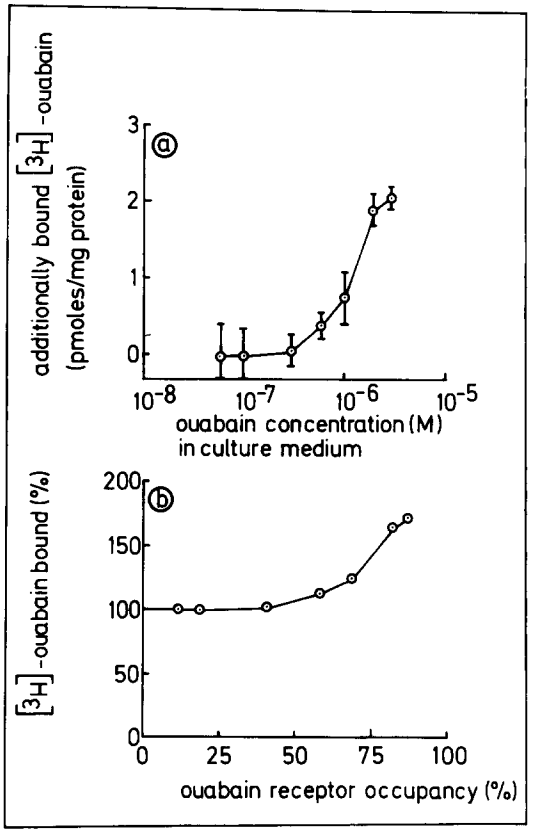

Fig. 2: Influence of chronic ouabain exposure on specific (^{3}H)-ouabain binding ($3x10^{-7}$M) of cardiac muscle cells from chicken embryos. Fig. a: incubation period 3 days; $[K^+]$= 3.5 mM. Values are given as additionally bound (^{3}H)-ouabain (mean ± SD; n = 3) of chronically ouabain-treated cells in comparison with acutely treated cells (for definition see text to fig. 1) at the very same ouabain concentration. (^{3}H)-ouabain binding of acutely treated cells: 3.0 ± 0.2 pmoles/mg protein (n = 7). Fig. b: results of fig. a are given as precentage in increase in specific (^{3}H)-ouabain binding depending on receptor occupancy during ouabain exposure. Acutely ouabain-treated cells at the very same ouabain concentration served as control. Receptor occupancy has been calculated from the dissociation constant (K_D) of ouabain binding at $[K^+]$=3.5 mM, determined as (4.3 +/- 0.2)$x10^{-7}$M (mean +/- SD; n = 5), according to the control experiments of fig. 1 (see also (6)).

ment ($3x10^{-6}$M);chronically ouabain-treated cells had higher ($^{86}Rb^{+}+K^{+}$)-influx rates (for method see (6)) and a higher cell-K^{+} (determined by flame photometry) than acutely treated cells (experiments not shown).

The extent of the rise in receptor density depends on the ouabain concentration chosen during chronic exposure of the cells (fig. 2a). When calculating the receptor occupancy for every ouabain concentration chosen (see legend to fig. 2), the increase in ouabain binding can be plotted as function of receptor occupancy during chronic ouabain exporure (fig. 2b): an increase in ouabain binding only occurs at receptor occupancies $\geq$ 60 %.

Discussion: Chronic exposure of cardiac muscle cells from chicken embryos increases the number of cardiac glycoside receptors on the cell (fig. 1,2; (4)). As this receptor is part of the sodium pump molecule, this finding also demonstrates a higher number of sodium pump molecules per cell. The increased ($^{86}Rb^{+}+K^{+}$)-influx and the higher cell-K^{+} in chronically ouabain-treated - in comparison with acutely ouabain-treated-cells support this suggestion (see text; (4)). However, an induction of additional cardiac glycoside receptors only occurs during chronic exposure to toxic (receptor occupancy $\geq$ 60 %), but not to "therapeutic" ouabain concentrations. This may be due to the capability of these cells to compensate inhibition of about 40 % of sodium pump molecules, as shown during acute ouabain treatment (5). Though an increased receptor density reduces the inotropic action of cardiac glycosides (4), no tachyphylaxis should occur -under our experimental conditions- during chronic exposure to "therapeutic" levels of cardiac glycosides.

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The expert technical assistance of Miss Angelika Thomschke is gratefully acknowledged. This work was supported by Wilhelm-Sander-Stiftung (78.014.3) and by DFG (Er 65/4-3).