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PLASMA MEMBRANE STUDIES ON DRUG SENSITIVE AND RESISTANT CELL LINES—III.

BIPHASIC KINETICS OF OUABAIN BINDING

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Abstract—Ouabain binding to wild type and ouabain resistant cell lines derived from the murine plasmocytoma MOPC 173 was studied on cells grown as monolayers. Biphasic kinetics of ouabain binding was observed; the early phase took place within 30 sec, followed by a spontaneous release of the bound ouabain. Then, the late phase was complete after 1–2 hr. This phenomenon was much more pronounced in fibroblastic than in epithelioid cell lines, regardless of their respective drug sensitivity. Ouabain resistant cells when compared to their wild type counterparts did not exhibit drastic changes either in the number or in the affinity of their respective ouabain binding sites. In contrast, the number and affinity of ouabain binding sites were quite different in fibroblastic and in epithelioid cell lines.

The $(Na^+ + K^+)$ stimulated-Mg^{2+}-ATPase (EC 3.6.1.3) [1–3] could represent one of the major enzymes involved in the regulatory mechanisms of cell division [4]. Ouabain is a specific inhibitor of this enzyme [5]. Ouabain resistance of cells depends upon the species of the cell donors as well as the cell morphology, e.g. human cell growth is inhibited by 10^{-8} or 10^{-7} M ouabain [6, 7] whereas rodent cell growth is inhibited by 10^{-4} to 10^{-3} M ouabain [8, 9]. Using electrophysiological methods, it was shown that the sensitivity of the $Na^+ + K^+$ pump to ouabain was three orders of magnitude higher in the ionically non-coupled epithelioid cells than in the ionically coupled fibroblastoid cells [10]. Since none of these cells have been selected for ouabain resistance, it can be assumed that these differences in ouabain susceptibility may be due either to a different structure of the $(Na^+ + K^+)$-ATPase and/or to a different component of the membrane.

Ouabain resistant mutants have been obtained by a one step selection procedure and shown to have, in most of the cell lines, fewer ouabain binding sites and/or a lower affinity than their wild type counterparts [11–13].

We have obtained from the murine plasmocytoma MOPC 173 two clones in culture: ME_2 which lost its oncogenic properties and exhibited sensitivity to contact inhibition and MF_2 with reverse properties. ME_2 was found to have an $Na^+ + K^+$ ATPase much more sensitive to ouabain inhibition than MF_2 as measured *in vivo* [14] and *in vitro* [15]. From these two clones ouabain resistant cell lines were obtained with the aim of elucidating if two different mechanisms were involved in ouabain resistance when a comparison was made either between normal and transformed cells or between wild type and ouabain resistant cell lines.

During these studies it appeared, in contrast with previous reports [11–12], that ouabain binding kinetics were a two step phenomenon. This paper deals with the two phases of ouabain binding and its expression by wild types as well as their ouabain resistant counterparts.

MATERIALS AND METHODS

Cells

From the murine plasmocytoma MOPC 173 we have isolated two cell lines: MF_2, fibroblastic, not contact-inhibited and able to grow in mice and ME_2, epithelioid, contact-inhibited and unable to grow in mice. Tissue culture conditions were as previously described [16]. From these two cell lines we selected clones resistant to ouabain: MF_2OR_2, fibroblastic and not contact-inhibited and ME_2OR_1 epithelioid and contact-inhibited. The general properties of these cell lines are summarized in Table 1. In order to compare the ouabain binding in the different cell lines, we normalized the cell surface to 2×10^9 μm^2 which was represented by 3×10^6 MF_2 cells, 3×10^6 MF_2OR_2 cells, 2×10^6 ME_2 cells and 2.4×10^6 ME_2OR_1 cells. For each experiment three separate flasks were submitted to the same treatment (incubation in ouabain and washings) and then trypsinized in order to determine the cell number. We determined the number of cells after incubation periods and washings, which was found to be the same as at the beginning of the experiment with the control flasks. Cell viability was checked by trypan blue

Table 1. General properties of the different cell lines

Cell line	Morphology	G 1/2 ouabain (M)	Cell number per plastic flask (25 cm²) at saturation	Cell number per plastic flask (25 cm²) in experimental conditions	Surface per cell (μm^2)
MF_2	Fibroblastic	2×10^{-4}	8×10^6	3×10^6	690
MF_2OR_2	Fibroblastic	2×10^{-4}	8×10^6	3×10^6	690
ME_2	Epithelioid	2×10^{-5}	2×10^6	2×10^6	1020
ME_2OR_1	Epithelioid	2×10^{-4}	2.5×10^6	2.4×10^6	840

G 1/2 = Molarity of ouabain inducing 50% cell growth inhibition.

exclusion. Cell size measurement was performed in a Coultronics Coulter counter.

Ouabain binding studies

Tritiated ouabain (12 Ci/mmole, New England Nuclear Corp.) was dissolved in an Earle's modified medium (5.10^{-6} M KCl and 0.15 M NaCl).

Cells were washed twice with 4 ml of K^+ free Earle's medium and preincubated for 20 min at 37°.

The medium was then removed and replaced by 1 ml of prewarmed Earle's solution containing different ouabain concentrations (10^{-8} to 10^{-5} M) and the flasks were gently rocked. After various periods of time, the medium was aspirated and the cells were rinsed three times by addition of 4 ml of a K^+ free Earle's medium at 4° in less than 40 sec. In the early phase, the first experimental points represented an incubation period of 1–2 sec followed by three washings. The cells were then added to 1.5 ml of a 2% SDS solution and 8.5 ml of Instagel (Packard Co) solution. The radioactivity was measured in an Intertechnique Scintillation Counter. Controls with different amounts of ^3H ouabain showed a 40 per cent quenching. Corrections were made for non-specific binding of ^3H ouabain by assaying parallel incubation in the presence of 1 mM unlabeled ouabain.

Number and affinity of ouabain binding sites

For each cell line, these studies were undertaken after saturation. We used the Scatchard plot method [17] in order to determine the number of binding sites and their affinity.

Specific inhibition of ouabain binding

We used as specific inhibitors of ^3H ouabain binding, 50 mM K^+ [5] or 10^{-4} M unlabeled ouabain, which could be left for more than 3 hr without cell detachment. Higher amounts of K^+ (150 mM) freed the cells in the medium.

Exchangeable ouabain measurements

After incubation with 5.7×10^{-7} M labeled ouabain for various time intervals, at 37° or 4°, cells were washed three times in K^+ ion-free medium. One milliliter of 1 mM unlabeled ouabain was added and the flasks were put under slow rocking conditions for 15–120 min (at the appropriate temperature). The cells were then treated as previously described.

In all experiments, each experimental point was made in duplicate or triplicate and the curves presented below are the mean of 215 experiments.

RESULTS

Ouabain binding

In preliminary experiments, we observed, for all cell lines, two steps or phases of ouabain binding, as shown in Fig. 1 for MF_2 cells.

Early phase

During the early phase the exposure of MF_2 cells

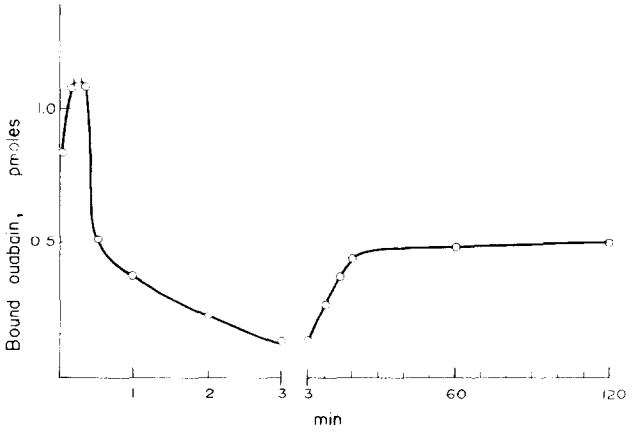

Fig. 1. Ouabain binding on MF_2 cells between 0 and 120 min at 37° (5 μM K^+ ions; 5.7×10^{-7} M ouabain; 2×10^9 μm^2 total cell surface).

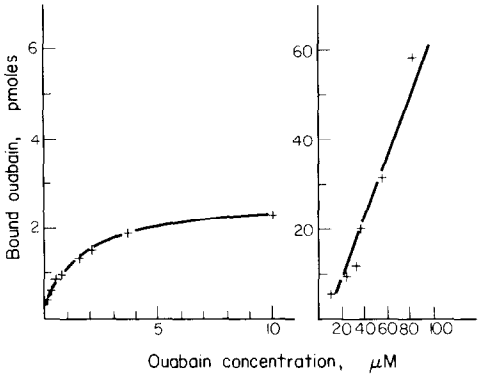

Fig. 2. Binding of ^{3}H-ouabain on MF_2 cells as a function of the concentration of glycoside in the medium (logarithmic scale). (Binding was measured after 15 sec exposure to ouabain, at 37°, in 5 μM K^+ ions, 2×10^9 μm^2 total cell surface.)

to different concentrations of glycoside for 15 sec revealed two different forms of binding: (i) a non-linear component which saturated at 10^{-5} M ouabain, (ii) a linear component which predominated at higher glycoside concentration up to 10^{-4} M (Fig. 2). Therefore, we measured the amount of bound ouabain, during the early phase, with 5.7×10^{-7} M ouabain from 0 to 180 sec.

For all cell lines (Fig. 3), maximum ouabain binding was obtained within 10–50 sec (washes not included). The maximum number of bound ouabain molecules per cell was lower for MF_2 (370,000) than for ME_2 (480,000) and higher for the ouabain resistant cell lines (470,000 for MF_2OR_2 and 670,000 for ME_2OR_1) (Table 2). As expressed in surface unit, it could be seen that the difference found above was still more evident for variants; MF_2 and ME_2 cells (Fig. 3A and 3C) bound about the same amount of ouabain, and resistant cell lines (Fig. 3B and 3D) bound higher amounts. A spontaneous release of

Table 2. Maximum amount of bound ouabain molecules per cell, at 5.7×10^{-7} M ouabain during the early phase (A) and late phase (B)

	A	B
MF_2	370,000	100,000
MF_2OR_2	470,000	110,000
ME_2	480,000	1,000,000
ME_2OR_1	670,000	1,200,000

ouabain was found in all four cell lines with a higher rate in MF_2 (Fig. 3A), for which it was over after 60 sec, than for ME_2 (Fig. 3C); the ouabain resistant cell lines did not exhibit significant difference when compared to their wild type counterparts. After drug release, 12 per cent (MF_2) and 25 per cent (MF_2OR_2, ME_2, ME_2OR_1) of the maximum amount of ouabain bound during the early phase still remained bound to the cells.

At 4°, compared to 37°, there was a 50 per cent decrease in ouabain binding (Fig. 4Ad) and also a drop in velocity of the spontaneous release (120 sec for MF_2 and 300 sec for ME_2) for both wild type and resistant cell lines.

Late phase

The second period of binding took place from 3 min to 2 hr, but 50 per cent of the binding was already reached after 10 min for the four cell lines (Fig. 5). The ouabain binding sites reached equilibrium with the drug between one and two hours. The Scatchard plot method showed (Table 3) that MF_2 had a smaller number of binding sites ($160/\mu m^2$) than ME_2 ($2000/\mu m^2$). Resistant cell lines had a similar number of binding sites as that of their wild type counterparts.

At 4°, equilibrium was not reached after 2 hr. The velocity of ouabain binding was slowed: only 60 per cent (Fig. 4Bd) of the maximum bound ouabain was

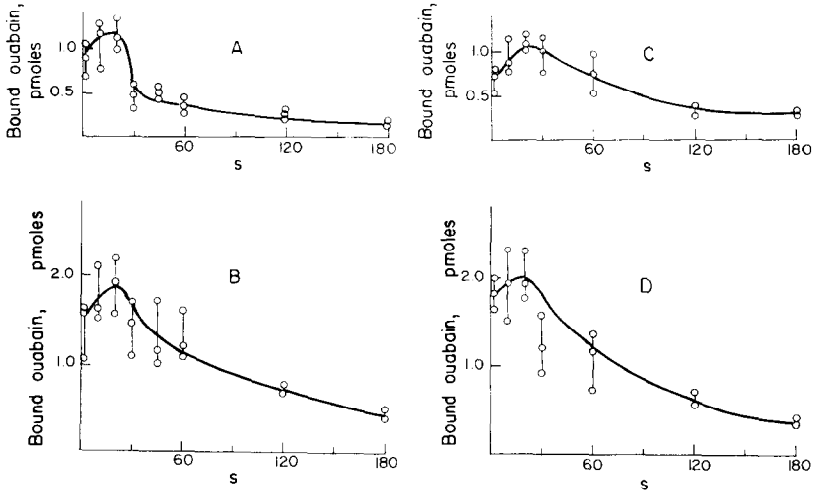

Fig. 3. The early phase of ouabain binding at 37°. Ouabain binding between 0 and 180 sec (5 μM K^+ ions; 5.7×10^{-7} M ouabain; 2×10^9 μm^2 total cell surface).
A = MF_2 cells; B = MF_2OR_2 cells; C = ME_2 cells; D = ME_2OR_1 cells.

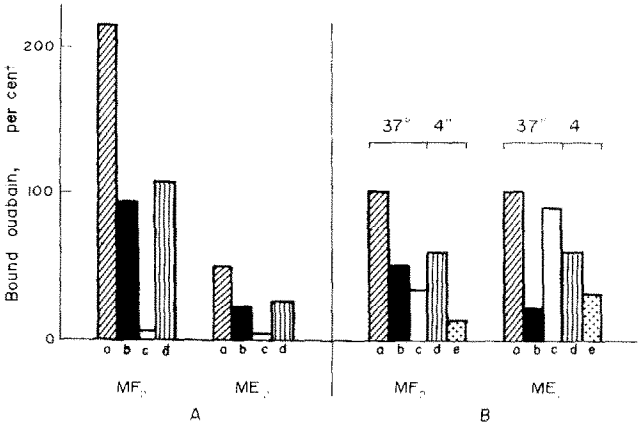

Fig. 4. Influence of time, medium and temperature on ouabain binding. 100 per cent expressed the maximum ouabain molecules bound after one hour in standard conditions (5.7×10^{-7} M ouabain, 5 μM K^+ medium, 37°).
A = Early phase, B = Late phase.
a: standard conditions; b: standard conditions except 50 mM K^+ ions; c: non exchangeable ouabain after incubation in standard conditions and one hour incubation with 1 mM unlabeled ouabain; d–e: standard conditions except temperature at 4°; (d: ouabain binding; e: non exchangeable ouabain).

reached in 60 min, as compared to 100 per cent at 37°C (Fig. 4Ba). The results were similar for wild type and resistant cell lines (data not shown).

Specific inhibition of ouabain binding

During the early phase, substitution of 5 μM K^+ by 50 mM K^+ in the incubation medium led to 50 per cent ouabain binding inhibition for MF_2 and ME_2 (Fig. 4Ab) and for resistant cell lines (data not shown).

During the late phase, a stronger inhibition was observed for ME_2 (Fig. 4Bd) and ME_2OR_1 (80 per cent and 65 per cent respectively) than for ME_2 (Fig. 4Bb) and ME_2OR_1 (50 per cent and 30 per cent respectively).

If tritiated ouabain were trapped in extracellular spaces, addition with the same volume of 10^{-3} M cold ouabain should not modify the amount of labeled drug. Since it was found that in such experiment the amount of labeled ouabain dropped by 90

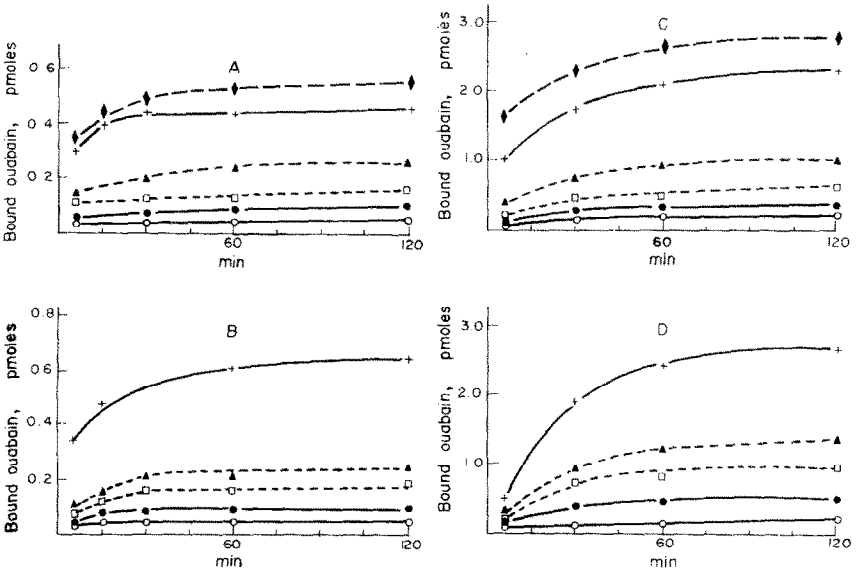

Fig. 5. The late phase of ouabain binding at 37°.
Ouabain binding between 5 and 120 min (5 μM K^+ ions; 2×10^9 μm^2 total cell surface).
Ouabain molarity: ○—○ 1.7×10^{-8} M; ●—● 5.7×10^{-8} M; □- - -□ 1.14×10^{-7} M; ▲- - -▲ 1.7×10^{-7} M; +——+ 5.7×10^{-7} M; ♦- - -♦ 1.14×10^{-6} M.
A = MF_2 cells; B = MF_2OR_2 cells; C = ME_2 cells; D = ME_2OR_1 cells.

Table 3. Ouabain binding sites: number and affinity constant during the late phase

Cell line	Sites/cells	Sites/μm^2	Affinity constant (10^6 M^{-1})
MF_2	110,000	160	~10
MF_2OR_2	120,000	170	~50
ME_2	2,000,000	2000	~1
ME_2OR_1	1,400,000	1600	~2

per cent in all cell lines during the early and late phase, it can be concluded that no labeled ouabain was trapped in extracellular spaces.

Exchangeable bound ouabain

During the early phase, the bound ouabain was completely exchangeable (Fig. 4Ac). During the late phase, only 66 per cent for MF_2 and 10 per cent for ME_2 of the bound ouabain were exchangeable (Fig. 4Bc). Similar results were obtained respectively with MF_2OR_2 and ME_2OR_1 (data not shown).

At 4°, smaller amounts of ouabain bound to the cells and also exchangeable ouabain was higher than at 37° for all cell lines (Fig. 4Bc).

DISCUSSION

We have observed in all four cell lines under study, a biphasic kinetic pattern of ouabain binding. The "early phase" is characterized by an immediate binding (within 1 min) followed by a spontaneous release; the "late phase" starting between the 2nd and the 5th min reached a plateau after 1 hr.

During the early phase the exposure of MF_2 cells to different concentrations of glycoside for 15 sec revealed a saturable binding between 10^{-7} and 10^{-5} M and a non saturable component between 10^{-5} and 10^{-4} M (Fig. 2). The first component of the curve was shown to be inhibited by K^+ ions and by lowering the temperature from 37° to 4° (Fig. 3).

As suggested by Baker and Willis [18] the first component of the curve which is saturable at low glycoside concentration and inhibited by K^+ is specific, while the second non saturable component represents a non specific process. Thus the dose of 5.7×10^{-7} M ouabain was chosen as being in the specific binding dose range and 20 fold lower than the dose required to saturate the membrane receptors.

During the late phase, ^3H ouabain binding was also inhibited by K^+, 10^{-3} M unlabeled ouabain and cold temperature. K^+ has been found to decrease the rate of ouabain binding but does not appear to alter the maximum capacity of the drug bound [7, 19]. This inhibition turned out to be partial as previously shown on HeLa cells, brain microsomes [20] and squid axons [21].

In most of the published data only late binding has been described [9, 12, 18, 22, 23]. However Lindenmayer and Schwartz [24] found ouabain binding in less than 2 min without release of ouabain, on a semi-purified ATPase from beef brain. Recently, we have shown [25] that as in red blood cells [26] incubation of MF_2 cells in a K^+ depleted medium led in 20 min to a 5 fold increase of the $Na^+ + K^+$ pump which in turn would increase the ouabain binding velocity. We would suggest that the binding of a few drug molecules induces a change in the ATPase structure, modifying the interaction between the ATPase itself and inner face proteins [27], which in turn, induces a second change in the ATPase structure leading to a modification of the ouabain binding site. We have already shown, for a different variant cell line MF_2S, that ATPase was 300 fold more susceptible to ouabain, in EDTA treated inside-out vesicles, than in the original inside-out vesicles [27]. Proteins removed by EDTA treatment were found able to restore the original resistance of the enzyme to ouabain in presence of Ca^{2+} [28]. Such inner face proteins might play a major role in the biphasic kinetic of ouabain binding, leading to the release of ouabain during the early phase. Such a desensitization phenomenon would be similar to that described after acetylcholine binding [29].

Comparison among the four cell lines led us to conclude that: a drastic difference was evidenced when contact inhibited cells (ME_2 and ME_2OR_1) and not contact inhibited cells (MF_2 and MF_2OR_2) were compared: more binding sites were found in the former than in the latter as calculated by the Schatchard's plot but higher affinity binding sites were exhibited by the latter than by the former.

This would support in part Hülser's observations [10] on epithelioid and fibroblastic mammalian cell lines that the more the cell growth is susceptible to ouabain at 10^{-7} M ouabain, the higher are the number (2×10^6 molecules on epithelioid and 4×10^4 on fibroblastic cells) and/or affinity of the binding sites.

Previous studies on ouabain resistant cells revealed in most instances a decrease in the number and/or affinity of the ouabain binding sites [12, 13, 23]. In contrast, when the resistant cell lines in the present study were compared to their wild type counterpart, no drastic difference was found either in the number or in the affinity of their ouabain binding sites. In fact, a higher affinity was found in resistant cell lines. Our results suggest that other modifications of the $Na^+ + K^+$ ATPase might be involved in the mechanisms of the ouabain resistance. It has been shown, for instance, that red blood cell ghost exhibited differences in ouabain binding properties depending upon the $[Na]_i$ and the $[K]_i$ [30, 31] and similarly $[K]_o$ is known to compete with ouabain. Changes in the apparent affinity for K^+ and/or Na^+ at the inner and outer faces of the plasma membranes in variant cell compared to their wild type counterpart might explain ouabain resistance. Work is now in progress to shed some light on this problem.

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