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INHIBITION OF LYMPHOCYTE ACTIVATION BY OUABAIN

INTERFERENCE WITH THE EARLY ACTIVATION OF MEMBRANE PHOSPHOLIPID METABOLISM

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Activation of lymphocytes by antigens and mitogens can effectively be prevented by ouabain, a known inhibitor of ($Na^+ + K^+$)-ATPase. Recently it was shown that lowering of intracellular levels of monovalent cations is not involved in the inhibitory effect of ouabain. ($Na^+ + K^+$)-ATPase was found to be closely associated with acyl-CoA : lysophosphatidylcholine acyltransferase in the plasma membrane of lymphocytes. Both enzymes are activated as an immediate consequence of mitogen binding. Human peripheral lymphocytes were stimulated with concanavalin A. Ouabain suppressed the induction of RNA and DNA synthesis in a concentration-dependent way. Increase of RNA synthesis was suppressed only if the glycoside were added within the first hours of activation. If ouabain was added later, incorporation of uridine remained at the rate that was reached at the time of glycoside administration, pointing to an early event where ouabain may be operative. Ouabain, in a dose-dependent manner similar to that affecting RNA and DNA synthesis, inhibited the increase in the incorporation of oleate into phospholipids in stimulated lymphocytes, whereas the turnover of phospholipid fatty acids in resting lymphocytes was unaffected. Increasing extracellular K^+ concentrations reversed the binding of ouabain to lymphocytes. Simultaneously, the inhibition of stimulated RNA synthesis was decreased and the inhibition of oleate incorporation was reversed. These results suggest that the suppression of lymphocyte activation by ouabain is due to the inhibition of membrane phospholipid metabolism mediated by the ($Na^+ + K^+$)-ATPase.

When lymphocytes are activated by antigens or mitogens, a sequence of metabolic events is initiated, leading to growth and finally proliferation. Activation is initiated by binding of mitogens as well as antigens to receptors of the plasma membrane [1]. Since the work of Quastel and Kaplan it is known that activation of lymphocytes can be prevented by the cardiac glycoside ouabain (=g-strophanthin). Ouabain is a known and specific inhibitor of plasma membrane ($K^+ + Na^+$)-ATPase; hence, it appeared to be a straightforward interpretation, to associate the inhibitory action of the glycoside with the function of this enzyme, i.e., the active monovalent cation transport [2]. However, more recent and careful analysis has revealed that shifts of monovalent cation concentrations (predominantly K^+) may not be involved in the initiation of lymphocyte activation [3]. This was corroborated by recent findings that lectin-stimulated mitogenesis of human lymphocytes was inhibited by

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one order of magnitude less ouabain than was K^+ transport [4,5]. These data suggested that ouabain may block lymphocyte activation by a mechanism different from interference with the ion pump activity of membrane $(K^+ + Na^+)$-ATPase. Recently, in the course of experiments analyzing the functional substructure of the lymphocyte plasma membrane, we found that $(K^+ + Na^+)$-ATPase segregated in subfractions of the plasma membrane which also contained the enzyme acyl-CoA: lysophosphatidylcholine acyltransferase [6]. The latter enzyme appears to be a key enzyme in regulating the early changes of the fatty acid moieties of membrane phospholipids in activated lymphocytes [7]. The close spatial association in the plasma membrane between these two enzymes prompted the idea that there may also exist a functional interrelationship between $(K^+ + Na^+)$-ATPase and the membrane phospholipid metabolism which could provide the molecular basis for the inhibition of lymphocyte activation by ouabain.

Human peripheral lymphocytes were isolated by use of a Ficoll/isopaque gradient centrifugation (Lymphoprep, Molter). The mononuclear cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% human pooled AB serum or 2.5 mg/ml defatted albumin (Sigma), as indicated in the experiments, and stimulated with 10 μg/ml (AB serum) or 2 μg/ml (albumin) concanavalin A (Pharmacia, Freiburg, F.R.G.) which resulted in optimal proliferation.

As has been reported earlier, ouabain dose-dependently inhibited induction of DNA synthesis by con-

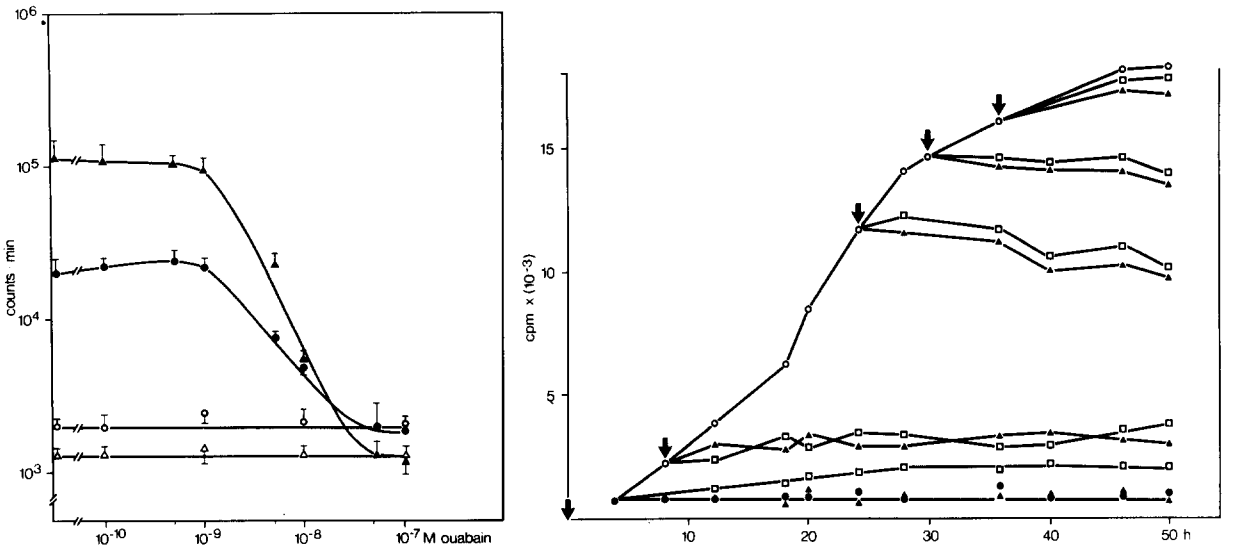

Fig. 1. Effect of ouabain on the incorporation of uridine and thymidine in human lymphocytes. $4 \cdot 10^5$ lymphocytes were cultured in 0.2 ml Dulbecco's modified Eagle's medium, supplemented with 10% human AB serum in microtest II microplates (Falcon, Becton and Dickinson). Concanavalin A and ouabain at final concentrations as indicated on the abscissa were added at the start of the cultures. After 44 h of culture [^{3}H]uridine (specific activity 250 mCi/mmol, made by mixing [^{3}H]uridine (Amersham Buchler) and uridine (Boehringer)) was added and cultures were further incubated for 4 h. After 70 h of culture [^{3}H]thymidine (spec. act. 24 Ci/mmol) was added and cells were incubated for further 2 h. The cells were harvested with an automated cell harvester (Skatron, Flow) on glass fibre filters and the radioactivity measured in a toluene-based cocktail by liquid scintillation counting. All results are the means ± S.D. of triplicates. o———o, uridine incorporation in resting lymphocytes; •———•, uridine incorporation in concanavalin A-stimulated lymphocytes; Δ———Δ, thymidine incorporation in resting lymphocytes; ▲———▲, thymidine incorporation in concanavalin A-stimulated lymphocytes.

Fig. 2. Time kinetics of the effect of ouabain on the incorporation of uridine in human lymphocytes. $4 \cdot 10^5$ lymphocytes were cultured for different lengths of time as in Fig. 1. At the times marked by arrows, α-methyl mannoside (0.1 M) or ouabain ($2 \cdot 10^{-8}$ M) were added. Cultures were pulse-labeled with [^{3}H]uridine during the last 4 h of each culture. Incorporation was measured as in Fig. 1. Results are means of triplicates. •———•, resting lymphocytes; o———o, concanavalin A-stimulated lymphocytes: ▲———▲, concanavalin A-stimulated lymphocytes + ouabain; □———□, concanavalin A-stimulated lymphocytes + α-methyl mannoside.

canavalin A. Complete suppression was observed at concentrations of lower than $5 \cdot 10^{-8}$ M ouabain (Fig. 1), being optimal at $2 \cdot 10^{-8}$ M (see Fig. 2). Over the same dose range, ouabain also suppressed concanavalin A-induced increase in incorporation of [^{3}H]uridine into RNA, measured after 48 h stimulation (Fig. 1).

To determine whether there are defined ouabain-sensitive stages of the cell cycle, lymphocytes were activated with concanavalin A, and then ouabain was added after increasing time intervals. As can be seen in Fig. 2, when ouabain was added together with or shortly after concanavalin A, it completely prevented subsequent increase of RNA synthesis. When, however, ouabain was added later to concanavalin A-activated lymphocytes, lymphocytes continued to incorporate [^{3}H]uridine into RNA at the rate which had been reached at the time of glycoside administration. After 36–40 h stimulation when lymphocytes exhibited maximal RNA synthesis, ouabain was without any further detectable effect. The time-dependent effect of ouabain inhibition was identical to the kinetics of RNA synthesis after removal of concanavalin A by α-methyl mannoside. This shows that activated lymphocytes can be 'frozen' at any stage of activation either by removing the stimulating mitogen (with α-methyl mannoside) or by adding the glycoside ouabain. This, on the other hand, indicates that ouabain interferes with lymphocyte activation at a step which is immediately dependent on the binding of a mitogen, pointing to an early membrane event as the site of action.

Among the earliest changes occurring in activated lymphocytes there is an increase in the turnover of membrane phospholipids [8]. In intact cells, increased oleate uptake is detectable after 1 h of stimulation. In isolated plasma membranes, however, stimulation of [^{14}C]oleate uptake is detected within 10 min after mitogen administration. As can be seen in Fig. 3, ouabain was able to prevent the increase of incorporation of [^{14}C]oleate into phosphatidylcholine of concanavalin A-stimulated lymphocytes. The dose response for inhibition by ouabain was identical to that in the case of macromolecular synthesis, a concentration of about $2 \cdot 10^{-8}$ M being sufficient for complete inhibition. Notably, ouabain had no effect on the incorporation of [^{14}C]oleate into resting lymphocytes, even at high concentrations (Fig. 3).

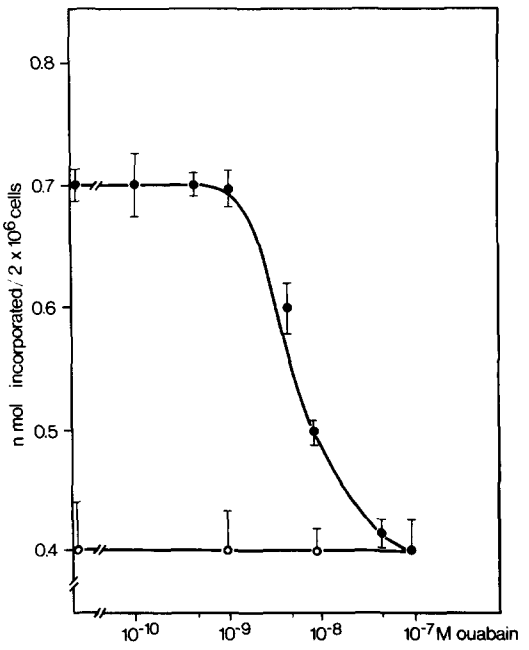

Fig. 3. Effect of ouabain on the incorporation of oleate in human lymphocytes. $2 \cdot 10^6$ lymphocytes were incubated in polypropylene tubes (Falcon) in 1 ml Dulbecco's modified Eagle's medium supplemented with 2.5 mg defatted bovine serum albumin (Sigma) containing a total amount of 10 nmol [^{14}C]oleate (spec. act. 2 mCi/mmol), made by mixing [^{14}C]oleate (Amersham Buchler) and oleate (Sigma)). Concanavalin A and ouabain were added at the beginning of the culture. After culturing for 4 h the lipids were extracted as described earlier [7] and separated by thin-layer chromatography on TLC plastic sheets, silica-gel 60, layer thickness 0.2 mm (Merck) with a mixture of chloroform/methanol/acetic acid/0.9% sodium chloride (50 : 25 : 8 : 4, v/v). After chromatography the individual lipid fractions were visualized by exposure to iodine vapour. Areas containing a lipid fraction were cut out with scissors and counted in a liquid scintillation counter. Incorporation was measured in a fraction containing phosphatidylcholine. Results are mean ± S.D., $n = 6$. o——o, resting lymphocytes; •——•, concanavalin A-stimulated lymphocytes.

To prove that ouabain inhibited activation of membrane phospholipid metabolism by binding to (K^+ + Na^+)-ATPase – and not by an unknown unspecific side effect – we made use of the effect of K^+ on the binding of the glycoside to this enzyme [9]. As can be seen in Fig. 4, increasing concentrations of K^+ prevented dose-dependent binding of labelled ouabain to lymphocytes. Simultaneously inhibition of concanavalin A-stimulated RNA synthesis by ouabain

was decreased, and this was reversed almost completely at 50 mM K^+ (Fig. 4). Increasing K^+ concentrations somewhat decreased incorporation of [^{14}C]-oleate, in resting as well as in concanavalin A-stimulated lymphocytes, the mechanism of this decrease being unknown. However, the degree of concanavalin A-induced stimulation of [^{14}C]oleate incorporation was nearly identical, even at a concentration of 50 mM K^+. On the other hand, whereas $2 \cdot 10^{-8}$ M ouabain completely inhibited the activation of [^{14}C]-oleate incorporation into phosphatidylcholine at 5 mM K^+, increase of K^+ diminuted the effect of ouabain, and at 50 mM K^+ only a marginal effect was apparent (Fig. 4). This suggests that ouabain inhibits early activation of the phospholipid metabolism in mitogen-activated lymphocytes by its action on membrane ($K^+ + Na^+$)-ATPase.

Effects caused by interference with membrane-bound ($K^+ + Na^+$)-ATPase have generally been implicated as involving alterations of the cytoplasmic cation milieu, elaborated by changed ion pump activities [11]. Our results show that the binding of ouabain to membrane-bound ($K^+ + Na^+$)-ATPase also prevents the activation of an early membrane event of lymphocyte activation, the increase in the turnover of phospholipid fatty acids. The inhibitory action of the glycoside on the membrane phospholipid metabolism could be due to cation shifts, or, alternatively, to a linkage of ($K^+ + Na^+$)-ATPase to enzymes involved in membrane phospholipid metabolism. A number of findings argues against the first possibility: (i) at the concentration of ouabain used no change in cellular K^+ concent could be demonstrated [5]; (ii) the activation of the phospholipid metabolism was unaffected

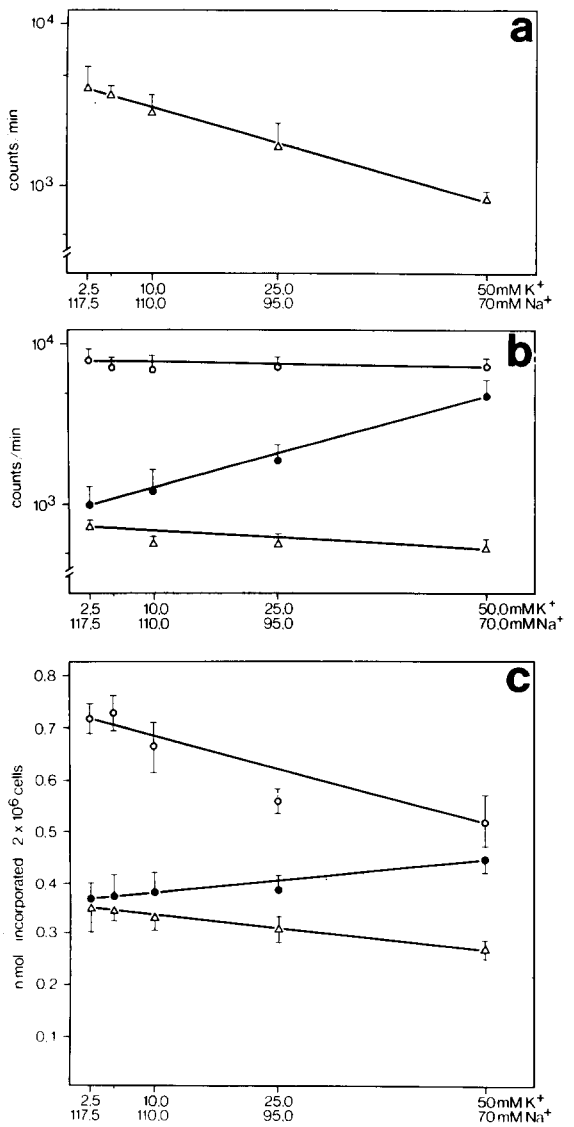

Fig. 4. (a) Effect of various extracellular K^+ and Na^+ concentrations on the binding of ouabain. $2 \cdot 10^7$ cells were incubated in 1 ml Dulbecco's modified Eagle's medium, containing K^+ and Na^+ at concentrations indicated on the abscissa, in the presence of 1 μCi [^{3}H]ouabain (New England Nuclear) and $1 \cdot 10^{-7}$ M ouabain. After incubation for 4 h the cells were spun down and washed twice with physiological saline. The cell pellet was dissolved in 0.1 M NaOH and transferred quantitatively to 9 ml scintillation liquid (Unisolve, Zinsser). Results are means ± S.D. of triplicates. (b) Effect of various extracellular K^+ and Na^+ concentrations on the inhibition of RNA synthesis by ouabain. $5 \cdot 10^4$ lymphocytes were cultured in 0.2 ml Dulbecco's modified Eagle's medium, containing K^+ and Na^+ at concentrations indicated on the abscissa. Ouabain at a final concentration of $2 \cdot 10^{-8}$ M was added at the start of the culture. For more details see Fig. 1. Results are mean ± S.D. of triplicates. △———△, resting lymphocytes; ○———○, concanavalin A-stimulated lymphocytes; ●———●, ouabain-treated concanavalin A-stimulated lymphocytes. (c) Effect of various extracellular K^+ and Na^+ concentrations on the incorporation of oleate. $2 \cdot 10^6$ lymphocytes were incubated in 1 ml Dulbecco's modified Eagle's medium containing K^+ and Na^+ at concentrations indicated on the abscissa. Concentration of ouabain was $2 \cdot 10^{-8}$ M. For more details see Fig. 3. Results are means ± S.D., $n = 6$. △———△, resting lymphocytes; ○———○, concanavalin A-stimulated lymphocytes; ●———●, ouabain-treated concanavalin A-stimulated lymphocytes.

by changing extracellular K^+ concentrations (see Fig. 4c); (iii) ouabain prevented mitogen-induced activation of the key in phospholipid metabolism, lysophosphatidylcholine acyltransferase, measured in isolated plasma membranes at a constant cation concentrations; (iv) the activity of lysophosphatidylcholine acyltransferase proved to be unaffected by varying monovalent cation concentrations [15].

On the other hand, the functional coupling of $(K^+ + Na^+)$-ATPase within the plasma membrane to the membrane phospholipid turnover is corroborated by our finding of a close spatial relationship between $(K^+ + Na^+)$-ATPase and lysophosphatidylcholine acyltransferase, an enzyme involved in the phospholipid fatty acid metabolism [12]. These two enzymes were found to be associated with high-affinity receptors for the mitogen concanavalin A [6]. Binding sites with high affinity for mitogens have been suggested to be responsible for lymphocyte activation, thus representing the biologically active receptors [13]. The selective activation of $(K^+ + Na^+)$-ATPase and lysophosphatidylcholine acyltransferases in the high affinity receptor membrane domains supported the idea that these enzymes are involved in the initiation of lymphocyte activation [14]. The present finding that ouabain suppressed completely the early activation of the phospholipid turnover, but was without any effect on the turnover in resting cells, strongly suggests that activation of $(K^+ + Na^+)$-ATPase and lysophosphatidylcholine acyltransferase constitute essential steps of the triggering process itself. Generally, our results (for the first time to our knowledge) show that ATPases are specifically implicated in the activation of cells by an interrelation with other enzymes of the plasma membrane. $(K^+ + Na^+)$-ATPases may be coupled to other enzymes of the plasma membrane and thus constitute part of functional domains involved in the plasma membrane-dependent control of cell activity, cell growth and division.

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References

1 Resch, K. (1976) in Receptors and Recognition (Cuatrecasas, P. and Greaves, M.F., eds.), pp. 59–119, Chapman and Hall, London
2 Quastel, M.R. and Kaplan, J.G. (1970) Exp. Cell Res. 63, 230–233
3 Segel, G.B. and Lichtman, M.A. (1978) Exp. Cell Res. 112, 95–102
4 Segel, B.G. and Lichtman, M.A. (1980) J. Cell Physiol. 104, 21–26
5 Szamel, M., Somogyi, J., Csukás, I. and Solymosy, F. (1980) Biochim. Biophys. Acta 633, 347–360
6 Resch, K., Loracher, A., Mähler, B., Stoeck, M. and Rode, H.N. (1978) Biochim. Biophys. Acta 511, 176–193
7 Ferber, E. and Resch, K. (1978) in The Lymphocyte: Structure and Function (Marchalonis, J.J., ed.), pp. 593–620, Marcel Dekker, New York
8 Resch, K. and Ferber, K. (1972) Eur. J. Biochem. 27, 153–161
9 Dahl, J.L. and Hokin, L.E. (1974) Annu. Rev. Biochem. 327–356
10 Ferber, E., Reilly, C.E. and Resch, K. (1976) Biochim. Biophys. Acta 448, 143–154
11 Kaplan, J.G. (1978) Annu. Rev. Physiol. 40, 19–41
12 Ferber, E. and Resch, K. (1973) Biochim. Biophys. Acta 296, 335–349
13 Allan, D. and Crumpton, M.J. (1974) Exp. Cell Res. 78, 271–278
14 Rode, H.N., Mähler, B., Loracher, A. and Resch, K. (1979) Eur. J. Immunol. 9, 402–408
15 Szamel, M., Schneider, S. and Resch, K. (1981) J. Biol. Chem., in the press