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# INVOLVEMENT OF TROPOMYOSIN IN THE SENSITIVITY OF $Na^+$+$K^+$ ATPase TO OUABAIN

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**Abstract**— The $Na^+/K^+$ ATPase sensitivity to ouabain was shown to be increased by 300 to 1000-fold after treatment of the plasma membrane by EDTA. Addition of proteins detached from the plasma membrane with $Ca^{2+}$ ions to EDTA treated membranes reconstituted the original $Na^+/K^+$ ATPase resistance to ouabain inhibition. Tropomyosin with $Ca^{2+}$ ions (not with $Mg^{2+}$ ions) induced the same effect. When suboptimal doses of tropomyosin were used for such a reconstitution, the dose–response curve indicated a full reconstitution of a given percentage of enzyme molecules. This observation led us to assume a direct or indirect effect of tropomyosin on $Na^+/K^+$ ATPase functions.

Cardiotonic steroids react with and inhibit the ($Na^+$ + $K^+$) stimulated $Mg^{2+}$ ATPase ($Na^+$ +$K^+$ ATPase E.C.3.6.1.3.) [1] in membranes. A remarkable feature of the inhibitory effect of ouabain is the difference in sensitivity to this inhibitor between species: as a general rule, rodent cells [2–4] are much more resistant to the drug than human [5] and pig [3] cells are. In some experiments on selected ouabain resistant cell lines, obtained by mutagenic agents, the difference in ouabain sensitivity of the enzyme has been related to mutation at the enzyme level [2, 5, 6].

However, from murine plasmocytoma MOPC 173, ouabain resistant variant cell lines are characterized by an increased resistance to *c*AMP, Concanavalin A and theophylline [7], but with almost the same number of ouabain binding sites as in the wild type cells [8, 9].

Furthermore, the sensitivity to ouabain of a plasmocytoma wild type $MF_2S$ was shown to be modified by proteins removed by EDTA treatments from the plasma membrane [10]. Indeed, we have shown these peripheral proteins to be located at the inner face of the plasma membrane and removed from inside-out vesicles leading to a 300 fold increase of the $Na^+$ + $K^+$ ATPase sensitivity to ouabain [11]; moreover, such proteins, after being extensively dialysed against EDTA free medium, led to a complete recovery of the original resistance of the enzyme to ouabain, when added back to EDTA treated inside-out vesicles plus $Ca^{2+}$ ions [11].

Cell movements and other functions involved in the transfer of information through the plasma membrane were shown to be linked in some way to the cytoskeleton [12, 13]. Thus we put forward the suggestion that proteins belonging to the microfilaments and/or microtubules could be present in the EDTA treated membrane supernatant and involved in the shift of the ouabain sensitivity as described above [10, 11]. Proteins belonging to the microfilaments such as actin, myosin, tropomysin and $\alpha$ actinin have been shown to retain similar structure and functions through the different species and organs [14–16].

These observations prompted us to check the possible effect of four rabbit muscle proteins on the sensitivity to ouabain of EDTA treated plasma-membrane bound $Na^+$ + $K^+$ ATPase. Actin (mol. wt 43,000 daltons), troponin C (mol. wt 18,000 daltons), troponin T (mol. wt 37,000 daltons) and tropomyosin (mol. wt 34,000 and 36,000 daltons) were used.

These proteins were chosen either because they bind $Ca^{2+}$ or exhibit molecular weights close to those of the active proteins in the EDTA supernatant (mol. wt 30,000 – 40,000 daltons).

In the following presentation, we demonstrate that only tropomyosin is able to modify the sensitivity of the $Na^+$ + $K^+$ ATPase to ouabain in the EDTA treated plasma membranes.

## MATERIALS AND METHODS

*Cells.* $MF_2S$ cells derived from the MOPC 173 murine plasmocytoma grown as ascites in mice [17] were used.

*Plasma membrane purification.* Cells were lysed in a hypotonic medium and plasma membranes were purified on a discontinuous sucrose gradient and tested for purity as previously described [18]. In fact, these plasma membranes are mixtures of right side-out (RSO) and inside-out (IO) vesicles. RSO were shown to be permeable to ATP and IO to ouabain but both were impermeable to proteins [11]. Characterization of these vesicles have been previously published [19]. Protein composition, determined by

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SDS polyacrylamide slab gel electrophoresis, has been shown to be the same for IO and RSO vesicles.

*EDTA treatment of purified plasma membranes.* Plasma membranes (0.5 mg/ml proteins) were incubated for 15 min with magnetic stirring at 4° in a pH 6.8 buffer containing 250 mM sucrose, 30 mM Imidazole chloride (IS buffer) and centrifuged for 30 min at 31,000 *g*. The pellet was suspended (0.5 mg/ml proteins) in ISE buffer (1 mM EDTA containing IS buffer) and incubated for 60 min at 4° with magnetic stirring. After centrifugation as above, the pellet was again suspended in ISE buffer and submitted to the same treatment. After centrifugation, the pellet suspended in 60 per cent glycerol was stored at −20°.

In all cases, centrifugation of EDTA treated membranes was necessary to affect the ouabain sensitivity [10].

*Assay of* $Na^+ + K^+$ *ATPase.* The activity was assayed according to Ottolenghi [20]. Kinetic studies (0 – 10 min) were performed by transfering aliquots at given times to test-tubes where reaction was stopped by an acidic molybdate solution and inorganic phosphate was determined according to Anner and Moosmayer [21].

The ATPase activity inhibited by 1 mM ouabain was similar to ($Na^+ + K^+$) stimulation of ATP hydrolytic activity of the membranes. Thus we can assume that it represents the $Na^+ + K^+$ ATPase activity.

The ouabain sensitivity of $Na^+ + K^+$ ATPase activity was tested with $10^{-8}$–$10^{-3}$ M ouabain (Calbiochem) prepared daily. The mixture (membrane + ouabain) was pre-incubated for 10 min at 37° in the assay medium. The reaction was initiated with the addition of prewarmed ATP and carried out over 6 points to assess linearity (aliquots taken every 2 min during a period of 10 min). We excluded the possibility that inhibitory activity of ouabain is greatly underestimated at low concentration due to a lag time in the binding of the drug since either a 10 min or a 70 min assay in the presence of varying doses of the drug led to the same inhibition of the enzymatic activity. Furthermore, the amount of Pi liberated over these assays was linear with respect to time, suggesting that enzyme–ouabain equilibrium was reached in less than 2 min (the shortest time used) and that ouabain formed a reversible complex with $Na^+ + K^+$ ATPase as shown by Allen and Schwartz [22] with preparations from rat heart (insensitive species).

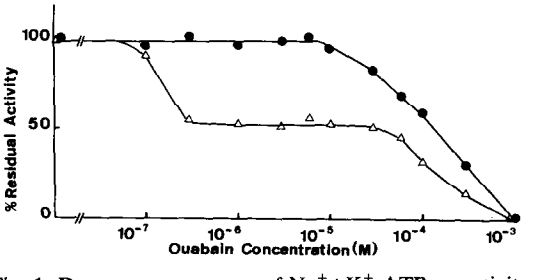


Fig. 1. Dose–response curve of $Na^+ + K^+$ ATPase activity vs ouabain concentration (logarithmic scale) in native membranes (●——●) and EDTA treated membranes (△——△).

The 50 per cent inhibition by ouabain of $Na^+ + K^+$ ATPase activity was symbolized as *E* 1/2 [23]. Figure 1 represents the ouabain sensitivity of $Na^+ + K^+$ ATPase activity in original membranes and in the ISE pellet (membranes treated successively 15 min by IS and twice 60 min by ISE).

*Reconstitution experiments.* EDTA treated membranes (150 μg) were incubated for 30–90 min, at 4°, in 1 ml IS buffer plus $CaCl_2$(0.1 mM) either with 15 μg of dialysed EDTA supernatant proteins or with 10–20 μg of purified proteins, as stated below.

It was shown that reconstitution was only possible with calcium (0.01–0.1 mM) and not magnesium ions and that $Ca^{2+}$, in our experimental conditions, did not inhibit the enzyme nor modify its sensitivity to ouabain [24].

Each experiment was performed several times on 3–5 membrane preparations and 3 different protein preparations.

*Purified proteins.* All proteins were obtained from rabbit white muscle and checked for purity in polyacrylamide slab gel electrophoresis according to Laemmli [25]. Actin was prepared according to Spudich and Watt [26], tropomyosin, troponin C and troponin T according to Harstshorne and Mueller [27]. Protein concentration was determined by the method of Lowry *et al.* [28].

## RESULTS

(1) *Modification of* $Na^+ + K^+$ *ATPase sensitivity to ouabain by EDTA treatment.* In native plasma membranes, the $Na^+ + K^+$ ATPase activity was found to be 50 per cent inhibited by 120 ± 20 μM ouabain (symbolized as *E* 1/2). After EDTA treatment, these plasma membranes, which are mixtures of right-side-out (RSO) and inside-out (IO) vesicles, exhibited a two step dose–response curve to ouabain; the first step is correlated to inhibition by ouabain of $Na^+ + K^+$ ATPase activity of IO vesicles : the *E* 1/2 (0.1–0.3 μM) of this $Na^+ + K^+$ ATPase population was 300 to 1000-fold lower than that of the native membranes. The second step reflects the inhibition of RSO vesicles (*E* 1/2 = 120 ± 20 μM) (Fig. 1) [11]. Furthermore, EDTA treatment removes a considerable fraction of the membraneous proteins. In case of RSO-vesicles, the protective proteins remain caught intravesicularly, thus keeping the ATPase insensitive against ouabain. In contrast the IO-vesicles will be depleted of the protective proteins by dilution, thus becoming more sensitive to ouabain.

(2) *Recovery of the original sensitivity of* $Na^+ + K^+$ *ATPase to ouabain.* It was shown previously that addition of 15 μg of proteins from the dialysed supernatants of EDTA-treated membranes and of 0.1 mM $Ca^{2+}$ to 150 μg of EDTA treated membranes led to the reconstitution of the original $Na^+ + K^+$ ATPase sensitivity to ouabain [10]. Thus, in a first set of experiments, 150 μg of EDTA treated membranes were incubated in the presence of 0.1 mM $Ca^{2+}$ with 10–20 μg of one of the following purified proteins: actin, troponin C, troponin T and tropomyosin. It can be seen in Fig. 2 that none of them was active except tropomyosin. While the *E* 1/2 for the first step was found to be 0.1–0.3 μM for the EDTA treated membranes either alone or incubated

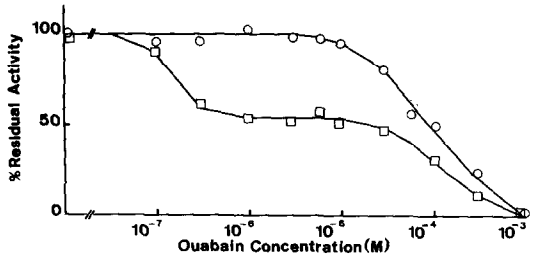


Fig. 2. Dose–response curve of $Na^+ + K^+$ ATPase activity vs ouabain concentration (logarithmic scale) in EDTA-treated membranes (150 $\mu$g) + 0.1 mM $CaCl_2$ + tropomyosin (10 or 20 $\mu$g (○——○); and EDTA-treated membranes (150 $\mu$g) + 0.1 mM CaCl + either actin or troponin C or troponin T (10 or 20 $\mu$g) (□——□).

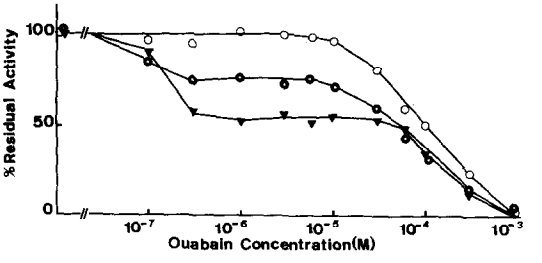


Fig. 4. Dose–response curve of $Na^+/K^+$ ATPase activity vs ouabain concentration (logarithmic scale) in EDTA-treated membranes (150 $\mu$g) + 0.1 mM $CaCl_2$ + tropomyosin (1–10 $\mu$g) (○——○); EDTA-treated membranes (150 $\mu$g) + 0.1 mM $CaCl_2$ + tropomyosin (0.3 0.6 $\mu$g) (●——●); and EDTA-treated membranes (150 $\mu$g) + 0.1 mM $CaCl_2$ + tropomyosin (0.075 $\mu$g) (▼——▼).

with actin, troponin C, troponin T or $Ca^{2+}$ ions alone, it increased to 120 ± 20 $\mu$M with tropomyosin.

In a second set of experiments, 150 $\mu$g of EDTA treated membranes were incubated with 10 $\mu$g of tropomyosin and either no ions or 0.1 mM $Ca^{2+}$ ions or 1 mM $Mg^{2+}$ ions. It can be seen in Fig. 3 that only tropomyosin with $Ca^{2+}$ ions was able to restore the original sensitivity of the enzyme to ouabain, while $Mg^{2+}$ ions are inactive in this phenomenon. In few cases, tropomyosin alone modified the *E* 1/2 from 0.3 $\mu$M up to 2 $\mu$M, probably due to the presence of $Ca^{2+}$ ions in the tropomyosin solution, since replacement of IS buffer by ISE buffer in the incubation suppressed this partial modification.

(3) *Stepwise reconstitution of the ouabain binding site of the* $Na^+ + K^+$ *ATPase.* In order to determine the reconstitution mechanisms induced by tropomyosin, we looked for the restoration capacities of increasing amounts of tropomyosin. It can be seen on Fig. 4 that

(i) amount of tropomyosin up to 0.075 $\mu$g are unable to induce any shift of the sensitivity of the enzyme to ouabain;

(ii) doses of tropomyosin from 0.3 to 0.6 $\mu$g can modify the shape of the dose–response curve of $Na^+ + K^+$ ATPase to ouabain with a stepwise increasing height of the plateau;

(iii) doses of tropomyosin from 1 to 20 $\mu$g lead to a complete restoration of the sensitivity of the enzyme to ouabain identical to original plasma membranes or to EDTA treated membranes reconstituted with $Ca^{2+}$ ions by proteins from ISE supernatant.

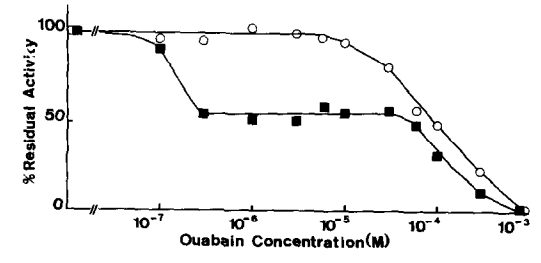


Fig. 3. Dose–response curve of $Na^+ + K^+$ ATPase activity vs ouabain concentration (logarithmic scale) in EDTA-treated membranes (150 $\mu$g) + 0.1 mM $CaCl_2$ + tropomyosin (10 or 20 $\mu$g) (○——○); and EDTA-treated membranes (150 $\mu$g) + tropomyosin (10 or 20 $\mu$g) either with 1mM $MgCl_2$ or without $CaCl_2$ in ISE buffer (■——■).

During stepwise reconstitution, note that the *E* 1/2 values of both steps are never modified (0.3 $\mu$M and 120 $\mu$M).

## DISCUSSION

The susceptibility of the $Na^+ + K^+$ ATPase to ouabain can be drastically increased by an EDTA treatment of purified plasma membranes. The total activity of the enzyme was unaffected by this treatment, in spite of the significant loss (30–40 per cent) of membrane associated proteins [10]. We have shown that the original resistance of the enzyme was recovered by addition of proteins from ISE supernatant together with $Ca^{2+}$. This was also obtained by addition with $Ca^{2+}$ of rabbit muscle tropomyosin, while actin, troponin C and troponin T were revealed to be inactive even at higher doses.

The stepwise reconstitution suggests that there are two populations of ATPase exhibiting a different sensitivity to ouabain (*E* 1/2 = 0.3 $\mu$M and 120 $\mu$M respectively), the sensitive one being shifted by tropomyosin to a resistant one (without any intermediate step).

As proteins from ISE supernatant act only on IO vesicles [11], tropomyosin might be assumed to act at the inner face of the plasma membrane to induce the recovery of the original resistance of the enzyme to ouabain. Thus, the ouabain binding sites expressed at the external face would be modified by events occuring at the inner face. As yet we know nothing about the structure of the reconstituted ouabain binding sites, whether it is similar or mimics the original structure. Until now, tropomyosin was known to bind actin in the microfilaments, but has never been described as a component of the plasma membrane. It is also important to stress the point that tropomyosins are not recognized to be $Ca^{2+}$ binding proteins while here, $Ca^{2+}$ ions are required for the shift experiments and $Mg^{2+}$ cannot substitute $Ca^{2+}$: thus we might hypothetise that there is no direct interaction between $Ca^{2+}$ and tropomyosin and that the way $Ca^{2+}$ is active on the reconstitution between tropomyosin and plasma membranes is indirect.

Several membrane bound enzymes have already been shown to be modulated by proteins which can be removed by EDTA and/or are assumed to belong

to the cytoskeleton: $Ca^{2+}+Mg^{2+}$ ATPase activator protein can be released by EDTA treatment of human erythrocyte plasma membranes and reconstitute the EDTA treated plasma membranes [29]. The ornithine decarboxylase activity is inhibited by colchicine and vinblastine [30], suggesting a regulatory mechanism of the enzyme linked to the integrity of the cytoplasmic microtubules. Taken together with our data reported here and elsewhere [11], these results led us to conclude that proteins located at the inner face of the plasma membrane or belonging to the cytoskeleton may be modulators of membrane bound enzymes and modifiers of specific binding sites located at the external face of the plasma membrane.

The apparent discrepancy between the two steps of ouabain binding on intact cells and the homogeneity of the dose–response curve of the $Na^{+}+K^{+}$ ATPase vs increasing amounts of ouabain in purified plasma membranes is due to the different materials used: in cells the cytoskeleton is intact while in plasma membranes microfilaments disappeared. Thus if the two step phenomenon is due to an interaction between the enzyme and a specific contractile protein of the intact cytoskeleton as suggested by our experiments, it is unlikely to be observed with plasma membranes. In intact cells, ouabain binding will at first stimulate a transmembrane signal leading to a change in the cytoskeleton structure, which in turn could modify the ouabain binding site structure through a change in the relationship between the cytoskeleton and the inner face plasma membrane: thus, we observed a two step binding of ouabain.

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## REFERENCES

1. J. C. Skou, *Biochim. biophys. Acta* **42**, 6 (1960)
2. R. M. Baker, D. M. Brunette, R. Mankovitz, L. H. Thompson, G. F. Whitmore, L. Siminovitch and J. E. Till, *Cell* **1**, 9 (1974).
3. M.R. Quastel and J. J. Vogelfanger, *Cell. Immun.* **2**, 504 (1971).
4. D. F. Hulser, H. J. Ristow, D. J. Webb, H. Pachowsky and W. Franck, *Biochim. biophys. Acta* **372**, 85 (1974).
5. H. M. Rosenberg, *J. Cell. Physiol.* **85**, 135 (1975).
6. R. M. Baker, in *Biogenesis and Turnover of Membrane Macromolecules*, p. 93. Raven Press, Hewlett (1976).
7. A. Paraf, E. Faivre, V. Zilberfarb, A. Zachowski and L. Lelievre, *C. r Acad. Sci. Paris* **281**, 567 (1975).
8. B. Geny, L. Lelievre, D. Charlemagne and A. Paraf, *Expl. Cell Res.* **120**, 383 (1979).
9. D. Charlemagne, L. Lelievre, P. Jolles and A. Paraf, *Biochem. Pharmac.* **28**, 3513 (1979).
10. L. Lelievre, D. Charlemagne and A. Paraf, *Biochem. biophys. Res. Commun.* **72**, 1526 (1976).
11. A. Zachowski, L. Lelievre, J. Aubry, D. Charlemagne and A. Paraf, *Proc. natn. Acad. Sci. U.S.A.* **74**, 633 (1977).
12. T. D. Pollard and R. R. Weighing, *CRC Critical Reviews in Biochemistry* Vol. 2, p.1. Chemical Rubber Company, Cleveland, Ohio (1974).
13. J. H. Ash, D. Louvard and S. J. Singer, *Proc. natn. Acad. Sci. U.S.A.* **74**, 5584 (1977).
14. A. Lazarides, *J. Cell. Biol.* **68**, 202 (1976).
15. E. D. Korn, *Proc. natn. Acad. Sci. U.S.A.* **75**, 588 (1978).
16. R. E. Fine and A. L. Blitz, *J. molec. Biol.* **95**, 447 (1975).
17. A. Zachowski and A. Paraf, *Biochem. biophys. Res. Commun.* **57**, 787 (1974).
18. J. Aubry, A. Zachowski, A. Paraf and J. Colombani, *Anns. Immun. Inst. Pasteur.* **130**, C, 17 (1979).
19. L. Lelievre, *Biochim. biophys. Acta.* **291**, 662 (1973).
20. P. Ottolenghi, *Biochem. J.* **151**, 61 (1975).
21. B. Anner and M. Moosmayer, *Analyt. Biochem.* **65**, 305 (1975).
22. J. C. Allen and A. Schwartz, *J. Pharmac. exp. Ther.* **168**, 42 (1969).
23. L. Lelievre, D. Charlemagne and A. Paraf, *Biochim. biophys. Acta.* **455**, 277 (1976).
24. L. Lelievre, A. Zachowski, D. Charlemagne, R. Laget and A. Paraf, *Biochim. biophys. Acta.* **557**, 399 (1979).
25. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
26. J. A. Spudich and S. Watt, *J. biol. Chem.* **246**, 4866 (1971).
27. D. J. Hartshorne and H. Mueller, *Biochim. biophys. Acta* **175**, 310 (1969).
28. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
29. D. T. Hanakan, R. D. Taverna, D. D. Flynn and J. E. Ekholm, *Biochem. biophys. Res. Commun.* **84**, 1009 (1978).
30. K. Chen, J. Heller, and E. S. Canellakis, *Biochem. biophys. Res. Commun.* **68**, 401 (1976).