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THE FRACTION OF PHOSPHATIDYLINOSITOL THAT ACTIVATES THE ($Na^+ + K^+$)-ATPase IN RABBIT KIDNEY MICROSOMES IS CLOSELY ASSOCIATED WITH THE ENZYME PROTEIN

B. ROELOFSEN and M. VAN LINDE-SIBENIUS TRIP

Laboratory of Biochemistry, State University of Utrecht, Transitorium III, Padualaan 8, De Uithof, 3584 CH Utrecht (The Netherlands)

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1. Extensive treatment of rabbit kidney microsomes with phosphatidylinositol-specific phospholipase C under various conditions never resulted in more than 75% hydrolysis of the substrate. 2. The non-degraded fraction of the phosphatidylinositol (10–12 nmol per mg microsomal protein) could be recovered only by an acidic extraction procedure. 3. The ($Na^+ + K^+$)-ATPase activity found in those membranes was not affected by this treatment. 4. Complete degradation of phosphatidylinositol could be easily achieved when the phospholipase was applied to rat liver microsomes which do not contain any detectable ($Na^+ + K^+$)-ATPase activity. 5. It is concluded that in rabbit kidney microsomes a close association exists between the ($Na^+ + K^+$)-ATPase and that fraction of the phosphatidylinositol that is directly involved in the maintenance of its activity.

In a previous communication from this laboratory [1] it has been shown that phosphatidylinositol serves as the lipidic activator of the ($Na^+ + K^+$)-ATPase present in microsomal membranes prepared from rabbit kidneys. This conclusion has been mainly derived from the following two observations: (i) the enzyme was fully inhibited when those membranes were treated with porcine pancreatic phospholipase A_2 under well-controlled conditions causing a complete degradation of the phosphatidylinositol, whereas (ii) the ($Na^+ + K^+$)-ATPase activity was not appreciably affected by an extensive treatment of the microsomes with a combination of nonspecific phospholipase C (*Bacillus cereus*) and sphingomyelinase C (*Staphylococcus aureus*) which caused a complete degradation of all phospholipid classes with the exception of phosphatidylinositol.

In view of those results, it was thought that phosphatidylinositol-specific phospholipase C would provide a unique tool to obtain further support for the above thesis. This enzyme was isolated from a *B. cereus* culture and purified essentially following the procedure of Sundler et al. [2]. The final product had a specific activity of 35 units per mg protein, assayed at 25°C according to Sundler et al. [2], using unlabeled phosphatidylinositol of which the degradation was determined by the phosphorus assay according to Chen [3]. Microsomes were prepared from fresh rabbit kidneys according to Tashima and Hasegawa [4] and stored in aqueous suspension at −20°C. These preparations exhibited Mg^{2+}-dependent and ($Na^+ + K^+$)-stimulated ATPase activities of about 20 and 42 μmol inorganic phosphate released per mg protein in 1 h, respectively (compare also Table I). All ATPase assays were carried out in triplicate as described by Palatini et al. [5], using the following incubation medium: 100 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 3 mM Tris · ATP, 21 mM histidine, 50 mM sucrose, 0.2 mM EDTA, pH 7.4, and with or without 1 mM ouabain.

Treatment of the microsomes with 1.1 units of phosphatidylinositol-specific phospholipase C per 1 mg of microsomal protein for 60 min did not appreciably affect the level of ($Na^+ + K^+$)-ATPase activity

(Table I). However, about one-fourth of the phosphatidylinositol originally present could still be recovered from the treated microsomes. In a first attempt to degrade this remaining fraction, the phospholipase was added in three successive shots of 1.1 units per mg microsomal protein at time intervals of 30 min (total incubation time 90 min). The final result was essentially the same when compared to that of the former experiment, both with respect to residual $(Na^+ + K^+)$-ATPase activity as well as the amount of undigested phosphatidylinositol (Table I). Although the microsomal suspension had been frozen and thawed once before starting the treatment with the phospholipase, the failure of the enzyme to degrade the last 25% of its substrate could have been due to the presence of considerable quantities of sealed vesicles. Treatment of the microsomes with various amounts of phosphatidylinositol-specific phospholipase C in the presence of 0.03% (w/v) Triton X-100, however, again resulted in exactly the same degree of phosphatidylinositol hydrolysis, leaving the $(Na^+ + K^+)$-ATPase activity unaffected (Table I). A concentration of 0.03% (w/v) Triton X-100 appeared to be the highest one that could be used in those experiments

TABLE I

PHOSPHATIDYLINOSITOL CONTENT AND $(Na^+ + K^+)$-ATPase ACTIVITY OF RABBIT KIDNEY MICROSOMES AFTER TREATMENT WITH PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C UNDER VARIOUS CONDITIONS

Incubations with purified phosphatidylinositol-specific phospholipase C were carried out at 37°C in a medium containing 1 mM Tris · EDTA and 12 mM histidine · HCl, pH 7.4. Further details are as indicated. Incubations were terminated by the addition of a 4-fold volume of ice-cold water and subsequent centrifugation (Beckman Spinco rotor 30, 27 000 rev./min) for 1 h at 4°C. Pellets were resuspended in water (about 2.5 mg protein/ml) and appropriate samples were taken for: (i) protein assay according to Lowry et al. [6]; (ii) assay of ATPase activity in triplicate (see text); and (iii) lipid extraction. Lipids were extracted by vigorous mixing of the aqueous microsomal suspension with three volumes of chloroform/methanol/12 mM HCl (2 : 1 : 0.02, v/v). After phase separation, lipids recovered from the chloroform layer were separated by two-dimensional thin-layer chromatography according to Broekhuyse [7], using silicagel HR 60 (Merck, Darmstadt) containing 6% (w/w) magnesium-silicate. Residual phosphatidylinositol (PI) content was calculated from the releative amount of this compound (determined as described in Ref. 8), using 750 nmol/mg protein as the total phospholipid content of the microsomal preparations [1]. Residual $(Na^+ + K^+)$-ATPase activity in the treated microsomes is expressed as percentage of the specific activity determined in microsomes (control) subjected to the same treatments with the exception of the presence of the phospholipase C. FT, incubation mixture frozen (solid CO_2/acetone) and thawed once after 45 min incubation and prior to the addition of the second shot of the phospholipase; AEE, microsomes previously sujbected to anhydrous diethyl ether extraction [9], thereby removing 20% of the total phospholipid complement, including 19% of the phosphatidylinositol.

Treatment with phosphatidylinositol-specific phospholipase C					Residual PI content (nmol/mg microsomal protein)	Residual $(Na^+ + K^+)$-ATPase activity (% of control)
Enzyme added		Total incubation time (min)	Triton X-100 present (0.03%, w/v)	Other variations		
Amount (units/10 mg microsomal protein)	at times (min)					
none	–	–	–	–	42.2 [a]	100
1 X 1.1	0	60	–	–	10	86
3 X 1.1	0; 30; 60	90	–	–	11	92
2 X 0.4	0; 30	60	+	–	11	88
2 X 0.4	0; 30	60	+	–	10	96
1 X 0.9	0	30	+	–	12	97
2 X 0.9	0; 30	60	+	–	12	89
2 X 0.9	0; 45	105	–	FT	10	87
2 X 1.6	0; 30	60	–	AEE	12	107

[a] S.D. 3.1; $n = 9$.

as a further increase caused an irreversible inhibition of the $(Na^+ + K^+)$-ATPase in the control incubations. Even interruption of the incubation after 45 min by an additional freezing and thawing step, followed by the addition of a fresh shot of the phospholipase and continuation of the incubation for another 60 min, did not lead to results different from the former ones (Table I). Comparable problems have been met before. Extensive treatments of non-sealed human erythrocyte ghosts with phosphatidylserine decarboxylase failed to convert the last 15% of the phosphatidylserine, the endogenous activator of the $(Na^+ + K^+)$-ATPase in that particular membrane (see Ref. 9). Complete conversion of phosphatidylserine into phosphatidylethanolamine, concomitant with a complete loss of $(Na^+ + K^+)$-ATPase activity, could be achieved only after removal of the so-called 'loosely'-bound lipid fraction by extracting the ghosts with ether under anhydrous conditions [9]. However, such a treatment of the microsomal preparation, causing the removal of the cholesterol and 20% of the total phospholipid complement, including 19% of the phosphatidylinositol, still could not create the conditions allowing a complete hydrolysis of the phosphatidylinositol (Table I). It is of interest to note that even after this series of treatments the $(Na^+ + K^+)$-ATPase activity still did not deviate from that of the corresponding control samples. For the sake of completeness, it should be mentioned that in none of the above cases did the phosphatidylinositol-specific phospholipase C treatment have any appreciable effect on the specific activity of the basal Mg^{2+}-ATPase in those microsomes.

From the results presented in Table I it is rather striking that, in terms of absolute amounts, the fraction of phosphatidylinositol that cannot be degraded by the phosphatidylinositol-specific phospholipase C appears to be the same (10–12 nmol/mg protein) in all cases. Regarding our earlier hypothesis of phosphatidylinositol being the endogenous activator of the $(Na^+ + K^+)$-ATPase in this particular membrane [1], it is tempting to suggest that (part of) this fraction of the phosphatidylinositol is so closely associated with the ATPase that it is impossible for the phospholipase to constitute the appropriate interaction with its substrate. The following observation may add an interesting dimension to this suggestion. The same phosphatidylinositol-specific phospholipase C preparation used in the above experiments easily degraded its substrate to completion in rat liver microsomes (prepared as described in Ref. 10), although their phosphatidylinositol content is even higher (approx. 60 nmol/mg protein) when compared to that in kidney microsomes. In this context it is worth noting that even that fraction of the phosphatidylinositol which has been shown to be not transferable by the phosphatidylinositol exchange protein and consequently has been suggested to be bound to membrane proteins [11], could be degraded. The intriguing fact is, however, that those liver microsomes do not contain any detectable $(Na^+ + K^+)$-ATPase activity. Moreover, phosphatidylinositol-specific phospholipase C also caused a virtually complete degradation of its substrate in non-sealed human erythrocyte ghosts in which the $(Na^+ + K^+)$-ATPase activity is believed to be maintained by phosphatidylserine rather than by phosphatidylinositol [9].

For all of the studies mentioned above, we have intentionally made use of an acidic system to extract the lipids from treated as well as non-treated membranes. As is shown in Table I, this system recovered approx. 42 nmol phosphatidylinositol per 1 mg microsomal protein. However, when identical suspensions of native rabbit kidney microsomes were extracted according to Folch et al. [12], the recovery of phosphatidylinositol was only 26.3 (S.D. 1.5, $n = 3$) nmol per mg protein. Hence, it appears that per mg microsomal protein approx. 16 nmol of phosphatidylinositol are not available for the neutral extraction system, this despite the fact that the latter involved much higher quantities of organic solvents than the acidic system used. This difference in ability of extracting the negatively charged phosphatidylinositol becomes of even greater importance when these two extraction methods are applied to the phosphatidylinositol-specific phospholipase C-treated microsomes. It has been shown (Table I) that in all of those cases the acidic extraction system was able to recover 10–12 nmol of undegraded phosphatidylinositol per mg protein. Contrastingly, not a trace of this phospholipid could be detected to be present in the Folch-extract derived from those treated microsomes. It seems likely that the fraction of phosphatidylinositol in rabbit kidney microsomes that is not accessible for the phospholipase forms part of that fraction of this phospholipid which cannot be extracted by a neutral

solvent system. The fact that this fraction can be only extracted under acidic conditions, thus neutralizing the negative charge, indicates that electrostatic interactions do play a role in the anchorage of these molecules in the microsomal membrane. Indeed, Brotherus et al. [13] recently concluded from their ESR experiments that a direct binding between acidic phospholipids and the $(Na^+ + K^+)$-ATPase protein does occur and they suggested such direct binding playing an essential role in activation of the ATPase. Such a specific binding of the polar head group to the protein, which apparently protects the phosphate-glycerol ester linkage from attack by the phosphatidylinositol-specific phospholipase C, may also provide an explanation for the seeming contradiction with the earlier observation [1] that porcine pancreatic phospholipase A_2 is able to degrade the phosphatidylinositol in those microsomes to completion. It should be realized that the latter phospholipase cleaves the ester bond at the 2-position of the glycerol backbone, located at a region of the phospholipid molecule not involved in binding to the protein and apparently therefore still within reach of the phospholipase.

If the lipids in the above experiments had been extracted by using exclusively a neutral system, as for instance that according to Folch et al. [12], one could have erroneously come to the conclusion that the phospholipase had degraded the phosphatidylinositol to completion. Actually, this might have been the reason why de Pont et al. [14] arrived at the conclusion that phosphatidylinositol does not play a role in the $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla. In agreement with our results presented above they observed no inhibition of activity when the purified ATPase was treated with phosphatidylinositol-specific phospholipase C from *S. aureus*. Equally, they could not recover any residual undigested phosphatidylinositol in lipid extracts derived from the preparations thus treated using the method of Folch et al. Alternatively, those conflicting results may be due to the fact that de Pont et al. used negatively charged detergents to purify the ATPase. Earlier reactivation experiments [1] have shown that the lipid requirement of the $(Na^+ + K^+)$-ATPase can be effectively fulfilled by a combination of a negatively charged detergent like cholate and a neutral phospholipid like phosphatidylcholine. It does not seem unlikely that during the isolation and purification of the ATPase from the outer medulla, the negatively charged detergents involved in this process may have replaced those phosphatidylinositol molecules which activated the system in the native membrane. The phosphatidylinositol molecules thus replaced may now be freely available to interact with the phospholipase, ultimately leading to their hydrolysis.

In conclusion, the present experiments indicate that the fraction of phosphatidylinositol which forms the lipidic activator of the $(Na^+ + K^+)$-ATPase in rabbit kidney microsomes occurs in a physical state different from that of the remainder. A close association of this particular fraction with the ATPase protein seems the most plausible explanation. This does not necessarily imply that a fraction of the phosphatidylinositol molecules is permanently bound to the protein. The observations discussed here are not in conflict with the existence of an equilibrium between the molecules bound to the protein and those taking part in the fluid bilayer (compare Ref. 13). However, as the enzyme indeed requires a specific interaction with the phosphatidylinositol, it will keep the corresponding interaction sites saturated with this lipid as long as possible. The polar head groups which are split off by the action of the phospholipase will readily dissolve in the aqueous medium, leaving the diacylglycerols behind in the membrane. Since the latter molecules do not have any charge left, they can no longer compete with the phosphatidylinositol molecules which are linked to the protein. Therefore, such an exchange-equilibrium as mentioned above will drastically shift in favour of the ATPase-bound ones as more of the phosphatidylinositol has been degraded by the phospholipase C.

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