Differential Effects of Lipid Depletion on Membrane Sodium-plus-Potassium Ion-Dependent Adenosine Triphosphatase and Potassium Ion-Dependent Phosphatase

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The phospholipid-dependence of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase (adenosine triphosphatase) (EC 3.6.1.3) and associated K^+ -dependent phosphatase activity (EC 3.6.1.7) have been compared. Unlike the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities, the K^+ -dependent phosphatase activities of a number of different preparations were not closely correlated with their total phospholipid contents. After partial lipid depletion with a single extraction in Lubrol W the residual ATPase and phosphatase activities were correlated, but their magnitudes were quite different: on average only about 5% of the former remained compared with 50% of the latter. A similar differential effect on these activities was found after extraction with deoxycholate. In contrast with the ATPase, consistent restoration of the phosphatase activity of Lubrol-extracted enzymes by added exogenous phospholipids was not observed. We conclude that, although the K^+ -dependent phosphatase may be lipid-dependent, the lipid requirement must be different from that of the complete ATPase system, and this difference should help investigations of their relationship.

A characteristic property of the membrane ($\text{Na}^+ + \text{K}^+$)-dependent ATPase* system (EC 3.6.1.3) is a K^+ -dependent and ouabain-inhibited phosphatase activity (EC 3.6.1.7) which may represent one step of the complete ATPase reaction (see Whittam & Wheeler, 1970). Since the latter has an absolute requirement for phospholipid [see Wheeler *et al.* (1975) for references] the question arises whether the phosphatase is similarly lipid-dependent. An identical requirement for phospholipid by the two enzymic activities would provide strong evidence for the concept that the phosphatase is part of the complete reaction sequence, whereas any difference in their dependence on phospholipid would provide another way of studying their relationship and, possibly, the reaction sequence. Although this question has been considered previously, the phosphatase has not been studied as extensively as the ATPase and the reported results have been even more conflicting than those about the ATPase. For example added exogenous phospholipids activated the K^+ -dependent phosphatase of brain ATPase that had been extracted with deoxycholate to remove lipid (Tanaka & Mitsumata, 1969; Tanaka, 1969; Tanaka & Sakamoto, 1969; Wheeler & Whittam, 1970) but those results could not be repeated with a similar enzyme prepared from kidney tissue (Towle &

Copenhaver, 1970). Neither Kimelberg & Papahadjopoulos (1972), who used the same kind of enzyme preparation as Towle & Copenhaver (1970), nor Palatini *et al.* (1972), who used Lubrol-extracted ATPase from heart, examined the phosphatase activities of their preparations. Rather more thorough studies have been made with the use of enzymes depleted of lipid by phospholipase digestion, but even those gave conflicting results. Thus Taniguchi & Tonomura (1971) found that phospholipase action tended to remove ATPase activity more than phosphatase activity, whereas Stahl (1973) and Goldman & Albers (1973) reported proportional losses of each activity in most experiments. In addition, the ability of different kinds of phospholipid to activate the phosphatase has varied widely among those reports.

A review of the experiments in which lipid was extracted from the enzymes with deoxycholate revealed several factors that might account for the variable and contradictory results recorded in the literature. First, no-one compared the K^+ -dependent phosphatase activities of the various preparations with their lipid content. Secondly, the investigators frequently neglected to compare the phosphatase activities of their lipid-depleted enzymes with those of the original unextracted preparations, so that only activation by added exogenous lipids was examined, not inactivation after removal of lipid (Tanaka & Mitsumata, 1969; Towle & Copenhaver, 1970; Wheeler & Whittam, 1970). Thirdly, only Towle &

* Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethylamine)tetra-acetic acid.

Copenhaver (1970) stored their enzymes as a routine in solutions containing a chelator (EDTA), and their preparations were the ones that were unaffected by added phospholipid. Since those studies were made the possibility of confusing a chelating effect of acidic phospholipids, such as phosphatidylserine, with a specific lipid effect on the ATPase system has been amply demonstrated and emphasized (Ting-Beall & Wells, 1971; Wheeler, 1971; Specht & Robinson, 1973). The development of the Lubrol extraction process for removal of lipid (Palatini *et al.*, 1972; Wheeler *et al.*, 1975) has provided a much more reliable means of examining the lipid requirement of the ATPase system, at least in terms of reproducibility as far as reactivation is concerned, so that we have used this procedure in an attempt to cover some of these deficiencies in earlier work on the phosphatase.

Experimental

Tissue preparations

Microsomal fractions were isolated from rabbit kidney homogenates, treated with 2M-NaI and then partly depleted of lipids by extraction with Lubrol W, by the methods described previously (Wheeler *et al.*, 1975).

Fig. 1. Partial correlation between phosphatase activity and phospholipid content

Microsomal fractions (▲) were isolated from homogenates of rabbit kidney, treated with NaI (■) and then partly depleted of lipid by extraction with Lubrol, either once (●) or twice (□). Both ouabain-sensitive phosphatase activities and total phospholipid contents of all preparations were measured as described in the Experimental section. Mean values $\pm$ S.E.M. are given for the numbers of preparations shown in parentheses.

Phosphatase activity

This was assayed by measuring the initial rate of release of *p*-nitrophenol from *p*-nitrophenyl phosphate, as described by Wheeler & Whittam (1970), except that the incubation media always contained bovine serum albumin (0.5mg/ml) and 0.5mM-EGTA, in addition to the constituents listed before. Ouabain-sensitive or 'K⁺-dependent' phosphatase activity was determined as the difference in activity in the presence and absence of 0.2mM-ouabain. (About 85–95% of the total activity of the NaI-treated preparations was sensitive to ouabain.)

All other materials and methods were exactly as described by Wheeler *et al.* (1975).

Results

Partial correlation between phosphatase activity and phospholipid content

The total phospholipid contents of a number of different ATPase preparations were measured and compared with their K⁺-dependent phosphatase activities. The preparations were microsomal fractions from kidney homogenates before and after the treatments with NaI and Lubrol, as indicated in Fig. 1. In contrast with the findings for the ATPase activity of those preparations (Wheeler *et al.*, 1975) there was only a partial correlation between the phosphatase activity and the total phospholipid content. Thus activation of the phosphatase by exposure to NaI was associated with an increase in phospholipid content, but a single extraction with Lubrol did not then produce proportionate losses of activity and lipid. Instead the activity remained relatively high whereas the total phospholipid content was substantially decreased. As with the ATPase activity, however, a double extraction with Lubrol usually removed all activity but not all of the phospholipids.

Differential effect of lipid depletion on ATPase and phosphatase activities

Extraction with Lubrol. The comparative resistance of the phosphatase to the treatment with Lubrol is shown in Fig. 2. Here the residual phosphatase and ATPase activities, expressed as percentages of the activities of the original unextracted enzyme, are compared for a number of Lubrol-extracted preparations. Clearly, the ATPase activity was much more susceptible than the phosphatase activity to the detergent treatment, the average residual values ($\pm$ S.E.M.) for 16 enzymes being $5 \pm 1\%$ and $49 \pm 6\%$ respectively, after a single extraction with Lubrol. Note also that the phosphatase activity decreased to zero only in the four preparations that had been subjected to multiple extraction with Lubrol; after a single extraction and in two instances after double

extraction, phosphatase activity was present even when no ATPase activity was detectable.

In spite of the clear differential effect shown in Fig. 2, however, it is important to observe the marked correlation between the two residual activities: for all preparations $r = 0.76$ ($n = 23$), whereas for just the singly extracted ones $r = 0.82$ ($n = 16$); both are significant at the 0.001 level. Hence it appears that lipid depletion does decrease both the ATPase and the phosphatase activities in parallel, but that the effect is quantitatively different until both activities are eventually abolished.

Extraction with deoxycholate. A comparison of the effects of lipid depletion by the deoxycholate method on the ATPase and phosphatase activities of a few preparations from kidney is shown in Table 1. Again

Fig. 2. Differential losses of ATPase and phosphatase activities after extraction with Lubrol

Residual ouabain-sensitive ATPase and phosphatase activities of Lubrol-extracted ATPase preparations were measured as described in Fig. 1 and expressed as percentages of the corresponding values for the original unextracted enzymes. Symbols have the same meaning as in Fig. 1.

the differential effect of extraction with detergent is clear: the ouabain-sensitive ATPase activity was decreased to about 11%, but the corresponding phosphatase activity only to 60%. These results thus suggest that the differential effect is not peculiar to the Lubrol method.

Reactivation of lipid-depleted enzymes

After extraction with Lubrol. Consistent restoration of the phosphatase activity of Lubrol-extracted enzymes by added exogenous phospholipids was not observed, in marked contrast with our findings for the ATPase activity (Wheeler *et al.*, 1975). Fig. 3 illustrates the pattern of activity obtained when several preparations were incubated with phospholipid dispersions: most of the phospholipids tested actually inhibited the phosphatase, only phosphatidylserine and phosphatidylcholine showing any ability to reactivate. Also, when phosphatidylserine did reactivate, that effect was obtained only with a limited range of phospholipid concentration, as shown in Fig. 3. Altogether we have tried to reactivate seven singly extracted and six doubly extracted enzymes, using either phosphatidylserine or equimolar mixtures of phosphatidylserine and phosphatidylcholine, with the results shown in Fig. 4. Here the 'restored' activities are either the maxima from curves like that shown in Fig. 3, or the values measured in the presence of 0.1 mM-phospholipid. The broken line in Fig. 4 indicates the values for no reactivation ('restored' activity the same as residual activity). Complete restoration of activity was achieved only with those enzymes in which the residual activity after extraction was high (60–90%) anyway. The other preparations showed either no reactivation at all or, at best, partial reactivation.

There was no correlation between restoration of ATPase and phosphatase activities. In fact, comparison of the ATPase and phosphatase activities of the seven preparations that were extracted once with Lubrol emphasizes the difference in response both to the extraction procedure and to added

Table 1. Differential effects of deoxycholate extraction on phosphatase and ATPase activities

Microsomal fractions were isolated from rabbit kidney homogenates, partly depleted of lipid by extraction with deoxycholate and both phosphatase and ATPase activities of all preparations were measured as described in the Experimental section. Mean values are given for the numbers of preparations indicated in parentheses.

Condition	ATPase activity ($\mu\text{mol of P}_i/\text{min per mg of protein}$)		Phosphatase activity ($\mu\text{mol of } p\text{-nitrophenol/min per mg of protein}$)	
	Initial (3)	Depleted (6)	Initial (3)	Depleted (6)
A Control	0.61	0.12	0.08	0.07
B +Ouabain	0.34	0.09	0.03	0.04
A – B	0.27	0.03	0.05	0.03

Fig. 3. Effects of exogenous phospholipids on the phosphatase activity of lipid-depleted enzymes

ATPase preparations were made from rabbit kidney and partly depleted of lipid by a single extraction with Lubrol, as described in the Experimental section. Pure phospholipids were dispersed by sonication and incubated at the indicated concentrations with the depleted enzymes (0.015 mg of protein/ml) for measurement of ouabain-sensitive phosphatase activity. Values from a single experiment are shown: similar, but non-identical, experiments gave the same pattern of effects. ●, Phosphatidylserine; ○, phosphatidylglycerol; ▲, phosphatidylinositol; Δ, phosphatidylethanolamine; ■, diphenylglycerol; □, phosphatidylcholine.

phospholipids. The mean values ($\pm$ S.E.M.) for initial, residual and restored activities were 0.62 ± 0.09 , 0.04 ± 0.01 and 0.68 ± 0.09 μmol of P_i /min per mg of protein respectively for the ATPase, compared with 0.17 ± 0.02 , 0.11 ± 0.02 and 0.15 ± 0.03 μmol of p -nitrophenol/min per mg of protein for the phosphatase.

After extraction with deoxycholate. Provided the incubation medium contained some chelator, such as EGTA or EDTA, the phosphatase activities of the kidney enzymes that had been depleted of lipid by deoxycholate treatment were never increased by addition of exogenous phospholipids. This finding is in agreement with that of Towle & Copenhaver (1970).

Effects of chelators

In general, if neither EGTA nor EDTA was present during phosphatase assays, then variable and inconsistent effects of added phospholipids were observed with both microsomal fractions and lipid-depleted preparations. Since these effects were similar to those

reported earlier for the ATPase activity (Wheeler, 1971) and since we wish to discriminate between such effects and those apparently related to lipid-protein interactions, the requirements described here have been restricted to those in which enzymic activities were measured in the presence of 0.5 mM-EGTA, as described in the Experimental section.

Discussion

The results presented above confirm the isolated report by Tanaka (1969) that extraction of membrane ($\text{Na}^+ + \text{K}^+$)-dependent ATPases with deoxycholate tends to decrease the ATPase activity much more than the K^+ -dependent phosphatase activity. In addition they show, first, that extraction with the non-ionic detergent Lubrol has a similar effect and, secondly, that although the relative amounts of the two residual activities are quantitatively different, they are correlated. Since differential losses of phosphatase and ATPase activities (Taniguchi & Tonomura, 1971) as well as proportional ones (Stahl, 1973; Goldman & Albers, 1973) have been reported to result from phospholipase digestion, it seems reasonable to conclude

Fig. 4. Maximal reactivation of phosphatase activity

ATPase preparations were made from rabbit kidney, partly depleted of lipid by extraction with Lubrol, and 'restored' by incubation with sonicated dispersions of phosphatidylserine, or equimolar mixtures of phosphatidylserine plus phosphatidylcholine (0.02–0.20 mM), as described in the text. Ouabain-sensitive phosphatase activity was measured and both the residual activity (after lipid depletion) and the maximal 'restored' activity (in the presence of added phospholipid) expressed as percentages of the activity of the original preparation. Symbols have the same meaning as in Figs. 1 and 2.

that the phosphatase activity is lipid-dependent, but not in exactly the same way that the ATPase reaction is. This difference between the two activities can be revealed by lipid depletion provided the extraction procedure is not too severe, and is also apparent from the different responses of the lipid-depleted preparations to exogenous phospholipids. Reactivation of the ATPase by added phospholipids has now been demonstrated very convincingly by several groups, with the use of different enzyme preparations and different ways of removing lipid. But careful examination of the literature suggests that the evidence for reactivation of the phosphatase is less well founded. These are two main questions: has the possibility of chelation been excluded and why is reactivation dependent on such a narrow range of phospholipid concentration?

Taniguchi & Tonomura (1971) showed activation of their phospholipase-treated enzyme by exogenous phospholipids in the presence of 0.1 mM-EDTA, with proportional effects on the ATPase and phosphatase, but none of the other six studies in the literature excluded the possibility that added phospholipid was acting as a chelator (Tanaka & Mitsumata, 1969; Tanaka, 1969; Tanaka & Sakamoto, 1969; Wheeler & Whittam, 1970; Stahl, 1973; Goldman & Albers, 1973). Stahl (1973) did check activation by EGTA as well as by phospholipids, but he did not show whether or not the effects were additive, which seems to be an essential requirement if the two reagents have different actions. Since all the evidence in the literature (Ting-Beall & Wells, 1971; Wheeler, 1971; Specht & Robinson, 1973; Wallick *et al.*, 1973) shows that the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase system is particularly sensitive to activation by low concentrations of chelators, any effects of added acidic phospholipids measured without excluding the possibility of chelation must be treated with caution.

The other question concerns the common finding that when added phospholipids have apparently reactivated the phosphatase, they have done so only over limited concentration ranges, such that higher concentrations have failed to activate, or actually inhibited. This is the kind of effect we found (Fig. 3) and it not only contrasts markedly with our findings for the ATPase, but also seems to have no obvious explanation at present.

Somewhat similar discrimination between ouabain-sensitive ATPase and phosphatase activities have been reported for a variety of treatments, including exposure to glycerol (Mayer & Avi-Dor, 1970), dimethyl sulphoxide (Mayer & Avi-Dor, 1970;

Robinson, 1972) and digitonin (Winter, 1972). It is possible that some, or all, of those effects are related to the differential dependence on phospholipids described above, which would be in keeping with Winter's (1972) conclusion that 'the partial reactions catalyzed by the sodium pump have less stringent organizational requirements than the NaK-ATPase '. That conclusion rests on the assumption that the phosphatase activity does indeed represent the terminal dephosphorylation step of the complete ATPase reaction; and it follows that the main effect of partial lipid depletion with Lubrol should be on one or more of the intermediate steps preceding dephosphorylation. This prediction can be readily tested.

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