Short Communications

Phospholipase C-Induced Changes in Intestinal Adenosine Triphosphatase Prepared from Goldfish Acclimatized to Different Temperatures

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(Received 9 July 1969)

The specific activation of membrane ATPase* by $\text{Na}^+ + \text{K}^+$, first described by Skou (1957), can be eliminated by procedures designed to disorganize membrane lipids. This requirement for lipids is fully recognized in the present definition of the enzyme as an $\text{Na}^+ + \text{K}^+$ -activated ATPase system (Skou, 1965). The fatty acid composition of the two major phospholipids, choline phosphoglyceride and ethanolamine phosphoglyceride, extracted from the intestinal membranes of goldfish, changes with the environmental temperature of the fish (P. Kemp & M. W. Smith, unpublished work), and the membrane $\text{Na}^+ + \text{K}^+$ -activated ATPase system also changes (Smith, 1967). Phospholipase C hydrolyses choline phosphoglyceride and ethanolamine phosphoglyceride to phosphorylcholine and phosphoryl-ethanolamine respectively, diglycerides being produced in each case (Bangham & Dawson, 1962). The present paper shows that incubation of goldfish membranes with phospholipase C inhibits the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity and enhances the Mg^{2+} -stimulated ATPase activity of membranes from cold-adapted fish. The Mg^{2+} -stimulated ATPase activity of membranes from warm-adapted fish is not changed during incubation with phospholipase C. An attempt was made to relate these findings to the normal operation of the ATPase system in goldfish intestinal membranes.

Methods and results. Experiments were carried out over a 5-week period during late autumn with groups of fish adapted previously to 8° or 30° for periods of 3–5 weeks. The conditions under which fish were kept and the methods used to prepare membrane fractions from the intestinal mucosa were as described by Smith, Colombo & Munn (1968). Assays of ATPase activity were carried out in the presence of (final concentrations) 40 mM-NaCl, 20 mM-KCl, 6 mM-MgCl₂ and 3 mM-Na₂ATP in 10 mM-histidine-HCl buffer, pH 7.1, with or without 0.1 mM-ouabain. The ouabain-sensitive hydrolysis of ATP has been found to equal the $\text{Na}^+ + \text{K}^+$ -sensitive hydrolysis (Smith, 1967). Phospholipase C was a gift from Dr R. O. Thompson, Wellcome Research Laboratories, Beckenham, Kent. It consisted of a freeze-dried powder obtained

from a dialysed $(\text{NH}_4)_2\text{SO}_4$ precipitate of a *Clostridium perfringens* culture filtrate.

Various concentrations of Ca^{2+} and different buffers have been used to activate phospholipase C incubated with membranes. Initial experiments were designed to establish which medium to use. Eight μmoles of o-oleicithin (phosphatidylcholine) were incubated at 37° with 100 $\mu\text{g.}$ of phospholipase C in 4 ml. of medium containing: (i) 25 μmoles of Ca^{2+} in 25 mM-tris-HCl buffer, pH 7.5; (ii) 15 μmoles of Ca^{2+} in an imidazole-HCl buffer, pH 7.1, containing MgCl_2 , NaCl and KCl (Schatzmann, 1962); (iii) 0.4 μmole of Ca^{2+} in 25 mM-tris-HCl buffer, pH 7.5 (cf. Finean & Martinosi, 1965). Water-soluble phosphate liberated by phospholipase C was measured by the method of King (1932). Lecithin hydrolysis was complete within 10 min. in medium (i) and half complete in 20 min. in medium (ii); there was only 1% hydrolysis after 45 min. in medium (iii). The incubation procedure of Schatzmann (1962) was used to test whether the method applied equally well to preparations of intestinal membranes.

Membranes prepared from the pooled mucosal scrapings of four fish, adapted to 8° or 30°, were suspended in 10 mM-histidine-HCl buffer, pH 7.1, and the phospholipids were extracted from 1 ml. of this suspension by addition of 20 ml. of chloroform-methanol (2:1, v/v). Two further membrane samples were extracted after a preliminary 50 min. incubation at 30° in 12.5 mM-imidazole-HCl buffer, pH 7.1, containing (final concentrations) 3.7 mM- CaCl_2 , 64 mM-NaCl, 16 mM-KCl, 4.8 mM-MgCl₂ and 0.8 mg. of membrane protein/ml., with or without 0.2 mg. of phospholipase C/ml. All extracts were washed once in 0.9% NaCl and the lower phases evaporated to dryness. Chloroform extracts of these residues, together with known phospholipids used for reference, were placed on silica gel H t.l.c. plates. Development was in chloroform-methanol-water (19:7:1, by vol.), individual phospholipids being located by spraying first with ninhydrin and then with the spray of Dittmer & Lester (1964). Choline phosphoglyceride in membranes from 8°- and 30°-adapted fish was stable during control incubation at 30° and completely hydrolysed in the

* Abbreviation: ATPase, adenosine triphosphatase.

presence of phospholipase C. The ethanolamine phosphoglyceride in membranes from cold-adapted fish was also stable at 30° and largely hydrolysed when phospholipase C was present. Membranes prepared from warm-adapted fish lost substantial amounts of this phospholipid during incubation under control conditions and phospholipase C did not noticeably increase the amount lost.

Goldfish acclimatized to 8° or 30°, killed in groups of three, provided 1.5–2.0 g. of intestinal scrapings, sufficient to prepare about 7 mg. of membrane protein; this protein was suspended in the imidazole medium described above (Schatzmann, 1962), the suspension divided in two and phospholipase C added to one half in the proportion 1 part of phospholipase C to 4 parts of membrane protein.

Fig. 1. Phospholipase C-dependent changes in goldfish intestinal ATPase activities. Each point is the mean of five determinations, each determination being derived from the pooled mucosa of three fish. ● and ▲, Membranes prepared from 30°-adapted fish; ○ and △, membranes prepared from 8°-adapted fish. ● and ○, Mg²⁺-activated ATPase; ▲ and △, Na⁺+K⁺-activated ATPase. The values for Mg²⁺-activated ATPase of membranes from 8°-adapted fish, measured in the presence and absence of phospholipase C, were subjected to a statistical analysis of variance and the standard errors of the differences between means derived from the residual mean square used in *t*-test comparisons between treatment means. There was no significant difference between the zero-time samples. The difference was significant after 10 min. incubation ($P < 0.05$) and differences thereafter were significant at the 0.01 level of probability.

Samples were taken from control and phospholipase C-containing suspensions immediately after the enzyme was added to the latter and at 10 min. intervals for the next 50 min. Samples taken were placed in the incubation mixture described above for assay of ATPase activity but without MgCl₂ or ATP and containing in addition 3.75 μmoles of ethylenedioxybis(ethylamine)tetra-acetic acid to chelate Ca²⁺. Tubes were kept in ice, and MgCl₂ was added immediately before assay. All incubations were carried out in duplicate at 30° for 15 min.

Considering first the stability of ATPase under control conditions, the Mg²⁺-stimulated ATPase activities at zero time (37.1 and 68.9 μmoles of P_i/hr./mg. of protein for membranes from 8°- and 30°-adapted fish respectively) fell to 35.2 and 64.5 μmoles of P_i/hr./mg. of protein after 50 min. incubation at 30°, in each case about a 6% decrease. The losses of Na⁺+K⁺-stimulated ATPase activity were more pronounced, namely from 14.2 to 8.0 and from 12.1 to 7.4 μmoles of P_i/hr./mg. of protein for membranes from 8°- and 30°-adapted fish respectively. The phospholipase C-dependent changes in Mg²⁺- and Na⁺+K⁺-stimulated ATPase activities are plotted in Fig. 1. All values were corrected for control losses in these enzymes. Phospholipase C caused a small but significant increase in Mg²⁺-stimulated ATPase activity measured in membranes from 8°-adapted fish. This effect was not seen with membranes prepared from 30°-adapted fish. The fall in Na⁺+K⁺-stimulated ATPase activity was of similar magnitude for membranes from both 8°- and 30°-adapted fish. This inhibition was accompanied either by a fall in total ATPase activity, the Mg²⁺-stimulated ATPase activity remaining unchanged (30°-adapted fish), or by no change in the total ATPase activity, the Mg²⁺-stimulated ATPase activity increasing (8°-adapted fish). Also, there were differences in enzyme activities measured at the start of incubation. The membranes from 30°-adapted fish contained more Mg²⁺- and less Na⁺+K⁺-activated ATPase/mg. of protein than did membranes prepared from 8°-adapted fish. This verified previous results (Smith, 1967, 1968; Smith *et al.* 1968).

Discussion. Phospholipase C increased the Mg²⁺-stimulated ATPase activity in membranes from cold-adapted fish and this, though a small effect, might be of significance in explaining how the proportion of Mg²⁺- to Na⁺+K⁺-stimulated ATPase activity can change with the environmental temperature of the fish. The percentages of both C_{20:4} and C_{22:6} fatty acids in choline phosphoglyceride and and ethanolamine phosphoglyceride increase considerably when the fish have been adapted to 8°. If these long-chain fatty acids were to inhibit the Mg²⁺-activated ATPase their removal might be expected to enhance the activity of the enzyme. If

again such a constraint could also be removed by the combined operation of $\text{Na}^+ + \text{K}^+$, then the action of phospholipase C would be to inhibit the $\text{Na}^+ + \text{K}^+$ -activated ATPase by partial stimulation of the Mg^{2+} -activated enzyme. This assumes that both the Mg^{2+} -activated and the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -activated ATPases are different forms of the same enzyme system.

It is perhaps remarkable that the almost complete removal of choline phosphoglyceride and ethanolamine phosphoglyceride from mucosal membranes should cause such relatively small changes in ATPase activities. These phospholipids together account for about 75% of the membrane phospholipids, yet their removal left the Mg^{2+} -stimulated ATPase activity either unchanged or slightly stimulated and about half of the $\text{Na}^+ + \text{K}^+$ -activated ATPase still active. The requirement shown by treated preparations of brain and erythrocyte $\text{Na}^+ + \text{K}^+$ -activated ATPases for lecithin and phosphatidylethanolamine respectively (Tanaka & Strickland, 1965; Ohnishi & Kawamura, 1964) seems not to apply to the mucosal membranes of goldfish intestine. Possibly cholesterol, which forms a high proportion of total lipids in mammalian mucosa (Forstner, Tanaka & Isselbacher, 1968), or

phosphatidylserine, which selectively reactivates the $\text{Na}^+ + \text{K}^+$ -activated ATPases of solubilized rat kidney and brain microsomes (Fenster & Copenhaver, 1967), plays some part in stabilizing the phospholipase C-treated mucosal membranes of the goldfish intestine.

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