1,2-Diacylglycerol kinase of human erythrocyte membranes

Assay with endogenously generated substrate

David ALLAN, Paul THOMAS and Shimon GATT*

Department of Experimental Pathology, University College Hospital Medical School,
University Street, London WC1 6JJ, U.K.

(Received 1 August 1980/Accepted 13 August 1980)

1,2-Diacylglycerol kinase activity was measured in human erythrocyte membranes using an assay procedure in which the substrate was generated endogenously, either by treatment with a bacterial phospholipase C or by incubation with Ca^{2+} , which activates a membrane-bound polyphosphoinositide phosphodiesterase. The properties of 1,2-diacylglycerol kinase were broadly similar to those described previously, except that in the present work maximum activities were higher and there was evidence for a double pH optimum.

1,2-Diacylglycerol kinase appears to be a ubiquitous enzyme in animal cells (Hokin & Hokin, 1963; Sastry & Hokin, 1966; Prottey & Hawthorne, 1967; Lapetina & Hawthorne, 1971; Call & Rubert, 1973) and is also present in at least one prokaryote (Pieringer & Kunnes, 1965). There are many more tissues where 1,2-diacylglycerol kinase has not been investigated specifically but where the activity is presumed to exist as part of the enzyme system that catalyses turnover of phosphatidylinositol (Hawthorne & White, 1975; Michell, 1975). Breakdown and resynthesis of this minor phospholipid is enhanced in certain physiological situations in which the intracellular concentration of Ca^{2+} rises in response to an extracellular stimulus (Michell, 1975) and thus 1,2-diacylglycerol kinase may have a particular importance as part of a regulatory system associated with mobilization of cell Ca^{2+} (Allan & Michell, 1977). The potential significance of 1,2-diacylglycerol kinase in the regulation of $[\text{Ca}^{2+}]$ within cells is more directly indicated by recent work, which suggests that phosphatidate (the product of 1,2-diacylglycerol kinase activity) can actually function as an ionophore for Ca^{2+} (Tyson *et al.*, 1976; Salmon & Honeyman, 1980; Putney *et al.*, 1980).

On the basis of the rather fragmentary evidence that exists, there is an indication that 1,2-diacylglycerol kinase may be preferentially located in the plasma membranes of cells (Lapetina & Hawthorne, 1971; Fisher & Mueller, 1971; Allen *et al.*,

1975; Mauco *et al.*, 1978). By far the best evidence for this proposal was obtained by Hokin & Hokin (1963), who showed that in the human erythrocyte most of the 1,2-diacylglycerol kinase activity was recovered in well-washed membranes. These workers made the first serious attempt to characterize this enzyme by using purified 1,2-diacylglycerol added as an emulsion. We decided to re-investigate the properties of human erythrocyte 1,2-diacylglycerol kinase by using a diacylglycerol substrate introduced directly into the membranes by phospholipase C attack, on the assumption that this procedure would provide a more natural substrate for 1,2-diacylglycerol kinase. Two methods were used to generate endogenous diacylglycerol in the membranes; the first was by treatment of the membranes with phospholipase C from *Bacillus cereus* and the second was by addition of Ca^{2+} , which has previously been shown to activate an endogenous phospholipase C that degrades polyphosphoinositide to 1,2-diacylglycerol (Allan & Michell, 1976, 1978). Although the latter procedure gives only low concentrations of diacylglycerol (commensurate with the small amounts of polyphosphoinositide present in the membrane) it seems possible that this particular pool of diacylglycerol represents the native substrate for erythrocyte 1,2-diacylglycerol kinase since no other endogenous enzyme activity is known that could generate diacylglycerol in this cell. Furthermore, experiments on intact cells with a raised intracellular Ca^{2+} concentration demonstrate the production of 1,2-diacylglycerol, which can be more or less completely converted into phosphatidate, presumably through the agency of 1,2-diacylglycerol

* Permanent address: Laboratory of Neurochemistry, The Hebrew University–Hadassah Medical School, P.O. Box 1172, Jerusalem, Israel.

kinase (Allan & Michell, 1975; Allan *et al.*, 1976; Lang *et al.*, 1977).

The results presented here show that 1,2-diacylglycerol kinase can be assayed satisfactorily by using endogenous substrate and that activities are generally greater than those measured previously with exogenous substrate.

Materials and methods

Membrane preparation

Fresh blood from volunteer donors was collected into heparin and the erythrocytes were sedimented by centrifugation at 500 *g* for 5 min. The cells were washed four times with 0.15 M-NaCl. The buffy coat and the upper layer of erythrocytes were removed each time to avoid any white-cell contamination.

The washed erythrocytes were then lysed with 20 vol. of 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate]/NaOH buffer, pH 7.5, containing 0.2 mM-EGTA. The 'ghosts' were sedimented at 16000 *g*_{av} for 20 min in a Sorvall RC5B centrifuge and washed a further three times, or until white, with 20 vol. of the lysing buffer at 4°C. They were stored frozen at -20°C.

Assay for 1,2-diacylglycerol kinase activity

The substrate was generated *in situ* by either (a) treatment of 2 ml of packed membranes with 1–10 µg of phospholipase C/ml [*B. cereus* enzyme, suspended at 2 mg/ml in 3.2 M-(NH₄)₂SO₄; Sigma Chemical Co.] for 5 min at 37°C or (b) treatment with 0.2 mM-CaCl₂ (2 µl of 100 mM-CaCl₂/ml) for 15 min at 37°C. Treatment (a) produced extensive breakdown of phospholipid (up to 90%), whereas treatment (b) never produced more than 1% breakdown. Diacylglycerol production was stopped in (a) by addition of 1 mM-*o*-phenanthroline and in (b) with 0.2 mM-EGTA.

Portions (0.1 ml) of the treated membrane suspension were then added to 0.3 ml of 50 mM-Tris/maleate buffer containing ATP, MgCl₂ and NaF. For experiments using membranes treated with *B. cereus* phospholipase C, generally the conditions were [ATP] = 2 mM, [MgCl₂] = 2 mM, [NaF] = 10 mM, pH 7.0. Incubation was at 37°C for 15 min with 0.5 µCi of [γ -³²P]ATP (The Radiochemical Centre, Amersham, Bucks., U.K.; sp. radioactivity > 2000 Ci/mmol).

For experiments using membranes treated with CaCl₂, [ATP] = 0.8 mM, [MgCl₂] = 0.8 mM, [NaF] = 10 mM, pH 7.0. Incubation was at 37°C for 15 min with 1 µCi of [γ -³²P]ATP.

Lipids were extracted, separated and quantified as described by Allan *et al.* (1980). The relative amounts of neutral lipids were determined on t.l.c. plates by spraying with 20% (NH₄)₂SO₄, heating at 180°C and scanning the charred spots in a

Joyce-Loebl Chromoscan 201 densitometer. ³²P radioactivity in phosphatidate was measured by Čerenkov counting in a Searle liquid-scintillation counter and the amount of phosphatidate synthesized was calculated from the specific radioactivity of ATP present in the incubation.

Protein determinations were carried out by the procedure of Lowry *et al.* (1951).

Results and discussion

After attack by *B. cereus* phospholipase C and addition of ATP, phosphorylation of 1,2-diacylglycerol progressed linearly with time for periods of 1 h or more, so long as the initial breakdown of phospholipid was less than 60–70%. As much as 20% of the total diacylglycerol could be phosphorylated in prolonged incubations. The maximum rate of synthesis of phosphatidate was 1–2 nmol/min per mg of protein, similar to that seen with intact erythrocytes treated with phospholipase C (Allan *et al.*, 1975, 1978) and substantially higher than the value reported by Hokin & Hokin (1963) (about 0.6 nmol/min per mg of protein). A linear progress curve was also obtained when diacylglycerol was generated by addition of Ca²⁺, but in this case, where the amount of diacylglycerol was much lower, activities were correspondingly less (about 8 pmol/min per mg of protein).

Fig. 1 shows the dependence of phosphatidate synthesis on the amount of glycerophospholipid breakdown achieved with different amounts of *B.*

Fig. 1. Relation of phosphatidate synthesis to extent of glycerophospholipid breakdown in membranes treated with *B. cereus* phospholipase C

Membranes (0.53 mg of protein) were incubated with various amounts of phospholipase C for 5 min and then exposed to [γ -³²P]ATP for 15 min as described in the Materials and methods section. ▲, Glycerophospholipid breakdown (%). Total glycerophospholipid was 290 nmol. ○, Phosphatidate synthesis (nmol). Essentially identical results were obtained in five similar experiments.

cereus phospholipase C. Phosphatidate production was proportional to the phospholipase C concentration up to about $0.3 \mu\text{g}/\text{incubation}$ and corresponded approximately to the extent of glycerophospholipid breakdown (1,2-diacylglycerol generation) up to about 60% of the maximum.

Maximum phosphatidate synthesis occurred with about $0.5 \mu\text{g}$ of phospholipase C, which produced about 90% of the maximum possible lipid breakdown. With further increases in phospholipase C concentration, which caused up to 95% glycerophospholipid breakdown, there was a steady decline in the amount of phosphatidate synthesis. This inhibition did not appear to be due to poorer recovery of phosphatidate, since addition of carrier lipid (either total erythrocyte lipid or purified phosphatidate) did not affect the results.

It is possible that 1,2-diacylglycerol kinase requires a certain amount of residual membrane phospholipid for full activity. However, attempts to restore the full activity of membranes treated with high concentrations of phospholipase C, by addition of a sonicated preparation of total erythrocyte lipid or of purified phosphatidylcholine or phosphatidylserine before incubation with ATP, were unsuccessful. Another reason for this inhibition could be that at high values of phospholipid breakdown the diacylglycerol substrate becomes inaccessible to the enzyme, perhaps because most of the lipid is then in the form of hydrophobic droplets, as described by Coleman *et al.* (1970). We have confirmed by electron microscopy of membranes treated with *B. cereus* phospholipase C that even at 60% breakdown, large numbers of lipid droplets are present in the membranes (A. R. Limbrick, personal communication), but it is not clear how a further increase in number or size of these droplets in going from 60 to 90% breakdown could inhibit enzyme activity.

With either method of diacylglycerol generation, optimal ATP concentration was 2–3 mM and half-maximum activity was seen at about 0.5 mM-ATP in the presence of 2.5 mM- Mg^{2+} . Omission of Mg^{2+} prevented any formation of phosphatidate. Optimum activities were obtained at 35°C and complete inhibition was observed after incubation of membranes at 60°C for 5 min. Contrary to a previous report (Hokin & Hokin, 1963), there was no stimulation of activity by Na^+ or K^+ ; higher concentrations of these ions ($>100 \text{ mM}$) inhibited slightly. 1,2-Diacylglycerol kinase activity showed two approximately equal peaks of activity, one at pH 7 and the other at pH 8 (Fig. 2). This behaviour was seen with both procedures for diacylglycerol generation, but we have no explanation for the observation. It is noteworthy that a dual pH optimum was reported for an analogous enzyme, phosphatidylinositol kinase (Phillips, 1973).

Fig. 2. *pH-dependence of phosphatidate synthesis*
The basic assay system was used as described in the Materials and method section, but with variation of the pH of the Tris/maleate buffer for the incubation of membranes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Membranes (0.75 mg of protein) were treated with $0.2 \mu\text{g}$ of phospholipase C for 5 min before 15 min incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (O). Results are expressed as means $\pm$ s.d. for four experiments. Activity at pH 7.4 was significantly less than at pH 7.0 or 8.0 ($P \sim 0.05$).

No evidence was found for a difference in the enzyme reaction occurring at each pH optimum. The product of *B. cereus* phospholipase C or of Ca^{2+} treatment was more than 90% diacylglycerol, and less than 10% of this was degraded to monoacylglycerol during a 15 min incubation with ATP at pH 7 or 8. Furthermore, ^{32}P radioactivity was almost exclusively found in phosphatidate, with only about 5–10% of the total in lysophosphatidate, which was well separated from phosphatidate by the chromatographic procedure used (Skipski *et al.*, 1964).

This work was supported by an M.R.C. grant to D. A. and in part by an N.I.H. grant to S. G.

References

- Allan, D. & Michell, R. H. (1975) *Nature (London)* **258**, 348–349
- Allan, D. & Michell, R. H. (1976) *Biochim. Biophys. Acta* **455**, 824–830
- Allan, D. & Michell, R. H. (1977) *Biochem. Soc. Trans.* **5**, 55–59

- Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286
- Allan, D., Low, M. G., Finean, J. B. & Michell, R. H. (1975) *Biochim. Biophys. Acta* **413**, 309–316
- Allan, D., Watts, R. & Michell, R. H. (1976) *Biochem. J.* **156**, 225–232
- Allan, D., Thomas, P. & Michell, R. H. (1978) *Nature (London)* **276**, 289–290
- Allan, D., Thomas, P. & Limbrick, A. R. (1980) *Biochem. J.* **188**, 881–887
- Call, F. L., II & Rubert, M. (1973) *J. Lipid Res.* **14**, 466–474
- Coleman, R., Finean, J. B., Knutton, S. & Limbrick, A. R. (1970) *Biochem. Biophys. Acta* **219**, 81–92
- Fisher, D. B. & Mueller, G. C. (1971) *Biochim. Biophys. Acta* **248**, 434–448
- Hawthorne, J. N. & White, D. A. (1975) *Vitam. Horm.* **33**, 529–573
- Hokin, L. E. & Hokin, M. R. (1963) *Biochim. Biophys. Acta* **67**, 470–484
- Lang, V., Pryhitka, G. & Buckley, J. T. (1977) *Can. J. Biochem.* **55**, 1007–1012
- Lapetina, E. G. & Hawthorne, J. N. (1971) *Biochem. J.* **122**, 171–179
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mauco, G., Chap, H., Simon, M. F. & Douste-Blazy, L. (1978) *Biochimie* **60**, 653–661
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Phillips, J. H. (1973) *Biochem. J.* **136**, 579–587
- Pieringer, R. A. & Kunnes, R. S. (1965) *J. Biol. Chem.* **240**, 2833–2838
- Prottey, C. & Hawthorne, J. N. (1967) *Biochem. J.* **105**, 379–392
- Putney, J. W., Jr., Weiss, S. J., Van De Walle, C. M. & Hannas, R. A. (1980) *Nature (London)* **284**, 345–347
- Salmon, D. M. & Honeyman, T. W. (1980) *Nature (London)* **284**, 344–345
- Sastry, P. S. & Hokin, L. E. (1966) *J. Biol. Chem.* **241**, 3354–3361
- Skipski, V. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374–378
- Tyson, C. A., Zande, H. V. & Green, D. E. (1976) *J. Biol. Chem.* **251**, 1326–1332