THE FORMATION OF CYCLIC INOSITOL 1,2-MONOPHOSPHATE, INOSITOL 1-PHOSPHATE, AND GLUCOSE 6-PHOSPHATE BY BRAIN PREPARATIONS STIMULATED WITH DEOXYCHOLATE AND CALCIUM: A GAS CHROMATOGRAPHIC STUDY

Arun Lahiri Majumder and Frank Eisenberg, Jr.

National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received July 11,1974

SUMMARY: A gas chromatographic method has been developed for the separation and isolation of water-soluble phosphates as trimethylsilyl ethers. With this method cyclic inositol 1,2-monophosphate and inositol 1-phosphate, derived from endogenous phosphatidylinositol, have been shown to increase when a particulate portion of brain homogenate is stimulated with deoxycholate and Ca^{++}, confirming earlier observations of Lapetina and Michell (1). Concomitant with the appearance of inositol phosphates is the stimulated formation of glucose 6-phosphate in the whole homogenate. Although ATP can replace deoxycholate and Ca^{++} in a dialyzed homogenate, glucose 6-phosphate apparently does not arise by any known metabolic pathway but from another unidentified source.

INTRODUCTION

PI*, a minor phospholipid of cellular membranes, has been shown to undergo increased turnover in different cellular systems when stimulated by proper exogenous stimuli. Dawson et al. (2) first described a soluble enzyme which at pH 5.3 catalyzed the formation of cIP and I1-P from PI. Lapetina and Michell (1) recently reported a particulate enzyme from rat cerebral cortex, catalyzing the same reaction at pH 7. This enzyme has been localized in various membrane fractions and can be stimulated by DOC and Ca^{++} to produce increased amounts of cIP and I1-P from PI. On the basis of these observations these authors presented a model (3) for the cycle of reactions involved in PI turnover and suggested a "second messenger"-like role for cIP in stimulated systems.

In response to the need for more sensitive and specific analytical methods for further study in this area we describe a GLC method for measuring the products of PI breakdown in rat brain and other tissue preparations and confirm

*Abbreviations: PI, phosphatidylinositol; cIP, cyclic inositol 1,2-monophosphate; I1-P, inositol 1-phosphate; I2-P, inositol 2-phosphate; G6-P, glucose 6-phosphate; DOC, deoxycholate; GLC, gas-liquid chromatography; TMS, trimethylsilyl.

and extend the observations of Lapetina and Michell (1). In addition we observe a concomitant DOC, Ca^{++} stimulation of G6-P formation from an as yet unidentified source. A preliminary report of this work has been published (4).

MATERIALS AND METHODS

Chemicals - [^{14}C]PI (2 mCi/mg) was obtained from Applied Science Labs, Inc., State College, Pa. Cyclic inositol phosphate was synthesized from I2-P by the method of Pizer and Ballou (5). Glucitol 6-phosphate was prepared by borohydride reduction of G6-P.

Tissue Preparation - Rats weighing 150-200 gm were decapitated and the brains were homogenized 2 min in a glass-Teflon homogenizer in 2 vol 0.05 M Tris HCl buffer (pH 7). To obtain the total particulate fraction, the homogenate was spun in a Spinco L50 ultracentrifuge at 50,000 rpm for 60 min. Dialysis was carried out overnight against 6 l of the same buffer with constant stirring. All operations were performed at 0-4° C. Brain ischemia was induced by leaving the brain in the severed head for varying periods of time before removal.

Assay Procedure - Tissue preparations representing about 0.3 gm of brain (1 ml homogenate) were incubated with 2 mg/ml DOC and 2 mM $CaCl_2$ for 60 min at 37° with constant shaking. The tubes were placed in ice, 0.25 µmole of glucitol 6-phosphate was added as internal GLC standard, followed by 2 volumes of ethanol to terminate the reaction. The tubes were kept in a 50° water bath 15 min and the precipitated protein centrifuged off. The clear supernatant was evaporated at 30-35° and dried in high vacuum. The dried residue was freed of lipids by 2 extractions with 1 ml of $CHCl_3$/MeOH (1:1). This residue was dried to a powder in high vacuum in preparation for assay. For the assay of tissue fractions prepared in sucrose, the procedure is modified by introduction of a step in which sucrose is removed with a small column of AG11A8 (Biorad) ion retardation resin. Sucrose emerges in the void volume and is further eluted with water; phosphates are then eluted with N/100 KCl.

GLC - 0.2 - 0.5 ml TMS reagent, prepared by adding 2 ml each of trimethylchlorosilane and hexamethyldisilazane to 5 ml of anhydrous pyridine, was added to the dried residues and heated covered in a steam bath for 10 min. Samples of 5 µl were injected into an 8' x 3 mm ID glass column, packed with 3% OV-17 on 100-120 mesh GasChrom Q, in a Beckman GC-65 gas chromatograph, equipped with a H_2 flame ionization detector. N_2 was the carrier gas at 55 cc/min. Glucitol 6-phosphate-TMS gave a linear response between mass and peak area from 1.5 nanomole per injected sample up to 6 nanomole, the amount routinely injected as internal standard. Inositol butaneboronate was assayed by the method of Eisenberg (7).

Isolation of Peak Materials by Preparative GLC - Samples of 25-30 µl were injected into a 20' x 3 mm ID column at 225°. The gas effluent corresponding to respective peaks was condensed in a capillary attached to a 9:1 stream splitter. Spontaneous hydrolysis of the TMS groups enabled us to collect free sugar phosphates which were eluted from the capillaries by N/10 $NaHCO_3$ and characterized (6).

RESULTS

When rat brain homogenate was incubated with DOC and Ca^{++} three compounds were found to be increased (Fig. 1B, peaks a,c,d) 4-, 15-, and 2-fold, resp.,

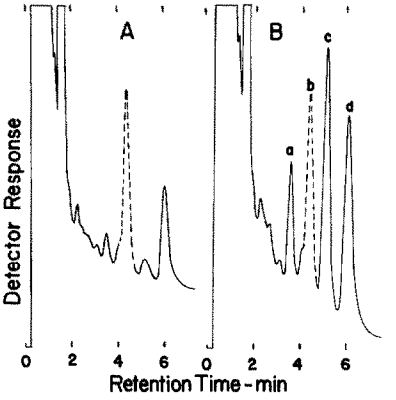

Fig. 1: GLC of TMS derivatives of water soluble phosphorylated products of brain homogenate stimulated with DOC and Ca^{++}. Rat brain ($\sim$ 1 gm) was homogenized in 2 ml buffer and divided in half. After 1 hr incubation at 37° the dried extracted residue from each sample was silylated in 0.2 ml TMS reagent. Five µl samples were injected into column, 215°. Each record represents about 15 mg tissue. A, no additions; B, DOC (2 mg/ml), Ca^{++} (2 mM). Peak a, retention time 3.6 min, cIP; b, 4.4 min, glucitol 6-phosphate, (internal standard) 6 nanomoles (10^{-9} amp.); c, 5.1 min, I1-P; d, 6.1 min, G6-P.

compared with a control without addition (Fig. 1A). Based on comparison of their retention times with authentic compounds, they were identified tentatively as cIP, I1-P, and G6-P, resp. Separately DOC increased cIP and G6-P and Ca^{++} increased I1-P.

By preparative GLC and isolation of the compounds from the gas stream, their identification was confirmed as follows:

Peak a was hydrolyzed in N/10 HCl at 100° for 20 min. GLC of the silylated products gave two peaks with retention times of I1-P and I2-P.

Peak c was incubated 30 min at 37° with Mg^{++}-dependent specific inositol 1-phosphatase (8) and the product identified as inositol by GLC.

Peak d was incubated with bacterial alkaline phosphatase (Worthington) for 45 min at 37° and part of the product was reduced with $NaBH_4$. GLC showed α- and β-glucose before reduction and glucitol after. Identification was further confirmed by incubation of Peak d with G6-P dehydrogenase (Calbiochem) and $NADP^+$ giving the characteristic increase in absorbance at 340 nm.

To determine whether I1-P was derived exclusively from PI or also from

another source, e.g., G6-P by Il-P synthase (8) (EC 5.5.1.4), brain homogenate was incubated with exogenous [^{14}C-inositol]-labeled PI and DOC, Ca^{++}. Resulting cIP (representing PI) and Il-P were isolated separately from the incubation mixture by preparative GLC and hydrolyzed to inositol for comparison of specific radioactivities. The inositols were converted into butaneboronate derivatives, dissolved in dilute Liquifluor (New England Nuclear), and assayed for radioactivity by liquid scintillation counting and for mass by GLC. The specific activities were identical showing no contribution of a non-radioactive source to Il-P and therefore its exclusive origin from PI.

The DOC, Ca^{++} stimulation of all three compounds simultaneously is seen only in the whole homogenate (Table 1, line 2); the response is absent in a boiled system. A large part of cIP- and Il-P-producing activity can be localized in the high speed pellet (line 3). G6-P stimulation is largely absent from either particulate (line 3) or supernatant (line 4). On dialysis the response of the inositol phosphates is partly retained, whereas that of G6-P is almost abolished (line 5). Addition of ATP to the dialyzed homogenate restores G6-P production (line 6).

TABLE I: Effect of DOC,Ca^{++} and ATP on the Formation of cIP, Il-P, and G6-P in Various Preparations of Rat Brain

	Preparation	μmoles/gm tissue		
		cIP	Il-P	G6-P
1.	Homogenate	0.09	0.06	0.46
2.	Homogenate + DOC,Ca^{++}	0.36	0.96	0.92
3.	Particulate + " "	0.17	0.59	0.11
4.	Supernatant + " "	0.02	0.05	0.17
5.	Dialyzed homogenate + DOC,Ca^{++}	0.09	0.38	0.07
6.	" " + ATP (2 mM)	0.18	0.06	0.85
7.	" " + " + DOC,Ca^{++}	0.20	0.44	0.79
8.	" particulate + "	0.05	0.05	0.17
9.	" supernatant + "	0	0	0

The time course for the three compounds is shown in Fig. 2. Beyond 60 min there is either cessation of production or decline.

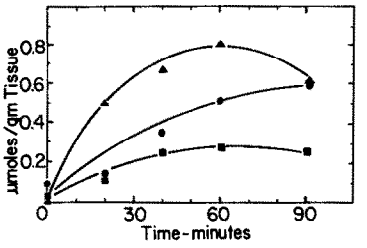

Fig. 2: Time course of formation of cIP, Il-P, and G6-P in brain homogenate stimulated with DOC and Ca^{++}. Incubation mixtures prepared as described in Fig. 1 were sampled at indicated times and assayed by GLC of TMS derivatives. Amount of each product in µmoles was calculated from the GLC response of known amounts of authentic standards with respect to internal standard. ▲, G6-P; ●, Il-P; ■, cIP. A 5 µl injected sample represents about 4 nanomoles of cIP under conditions of maximal stimulation (60 min).

The requirement for ATP in the dialyzed system suggested that G6-P might arise by one of the known pathways from glucose (hexokinase), glycogen (phosphorylase), or 3-carbon fragments (gluconeogenesis), although the prompt response to DOC, Ca^{++} (Fig. 2) argued against a long pathway. Various enzymes and inhibitors were tested for interference with the stimulation of G6-P formation either by DOC, Ca^{++} in the whole homogenate or by ATP in the dialyzed. These included 2-deoxyglucose, glucose oxidase, arsenate, fluoride, iodoacetate, iodoacetamide, and p-mercuribenzoate. Except for arsenate, which increased G6-P formation, there was no effect by any of the inhibitors tested. Prolonged ischemia, known to deplete brain glycogen in mice (9), likewise failed to diminish G6-P formation.

A study of DOC, Ca^{++} stimulation in other tissues showed no activity in liver, kidney, and testis with respect to cIP and G6-P. Testis was stimulated to produce Il-P. Since this tissue is a rich source of Il-P synthase, a soluble enzyme which catalyzes the cyclization of G6-P to Il-P, the homogenate was fractionated and both particulate and supernatant subjected to DOC, Ca^{++} stimulation. Only the particulate responded, indicating PI as the source of DOC, Ca^{++} stimulated Il-P.

DISCUSSION

Owing to the insolubility of calcium and magnesium organic phosphates in the silylating reagent, divalent cations can interfere in the assay. Crude preparations contain sufficient protein to sequester these ions but in purified systems it may be necessary to introduce a cation exchange resin step for their removal. The response of the internal standard serves to monitor such interference. The ease of separation of I1-P from G6-P by the GLC method described above commends its use also as a possible assay for I1-P synthase.

Formation of cIP and I1-P in equal quantities from PI by the total particulate fraction from rat cerebral cortex under conditions of DOC, Ca^{++} stimulation has been reported by Lapetina and Michell (1). In the present study, however, a 1:1 ratio for the two compounds is not observed, I1-P invariably exceeding cIP. In seeking an explanation for this difference, an additional source of I1-P was considered, e.g., G6-P by the I1-P synthase reaction. By comparison of specific radioactivities of cIP and I1-P produced from exogenous labeled PI, a non-radioactive source of I1-P would be detectable. Since the specific activities were identical, however, PI must be considered the sole source of I1-P.

The postulated model of Lapetina and Michell (3) can only be validated by an observed elevation in cIP catalyzed by the membrane-bound enzyme under the influence of a physiological stimulus. With the present technique attempts have been made to demonstrate stimulation of this enzyme from brain fractions by acetylcholine within a concentration range of 10^{-6} to 10^{-4} M. Our results have so far been negative, in agreement with the findings of Lapetina *et al.* (10), who could not confirm earlier positive observations (11,12). In intact avian salt gland (13) and pancreas (14), however, PI breakdown has been shown to respond to acetylcholine stimulation.

An unexpected result of this study was the appearance of G6-P with the breakdown products of PI. That it was not observed by Lapetina and Michell (1) is undoubtedly due to their use of a particulate enzyme in examining breakdown products of PI; as seen in Table I, G6-P formation requires the whole homogenate.

The failure of the various inhibitors to reduce the amount of G6-P formed is presumptive evidence that neither phosphorylation of glucose, phosphorolysis of glycogen, nor gluconeogenesis can account for the appearance of this compound. Although ATP replaces DOC, Ca^{++} in a dialyzed homogenate, there is no evidence that ATP serves a phosphorylating function under these conditions, since free glucose can not be detected in the dialyzed system. The time course (Fig. 2) indicates a prompt release of G6-P, suggesting a simple hydrolytic breakdown of a precursor, as yet unidentified. Whether stimulation of G6-P formation is a concomitant of PI breakdown under physiological conditions is unknown. Since G6-P is the only known de novo source of free inositol, which in turn is the precursor of PI, it is reasonable to speculate that the formation of G6-P concomitant with PI breakdown is not fortuitous, but is an essential component of the turnover of PI.

REFERENCES

1. Lapetina, E. G., and Michell, R. H. (1973) Biochem. J. 131, 433-442.
2. Dawson, R. M. C., Freinkel, N., Jungalwala, F. B., and Clarke, N. (1971) Biochem. J. 122, 605-607.
3. Lapetina, E. G., and Michell, R. H. (1973) Fed. Eur. Biochem. Soc. Lett. 31, 1-10.
4. Majumder, A. L., and Eisenberg, F., Jr. (1974) Fed. Proc. 33, 1551.
5. Pizer, F. L., and Ballou, C. E. (1959) J. Amer. Chem. Soc. 81, 915-921.
6. Eisenberg, F., Jr., and Bolden, A. H. (1969) Anal. Biochem. 29, 284-292.
7. Eisenberg, F., Jr. (1972) Methods in Enzymology (V. Ginsburg, ed.) XXVIII, pp. 168-178, Academic Press, New York.
8. Eisenberg, F., Jr. (1967) J. Biol. Chem. 242, 1375-1382.
9. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W. (1964) J. Biol. Chem. 239, 18-30.
10. Lapetina, E. G., and Michell, R. H. (1974) J. Neurochem., in press.
11. Durell, J., and Garland, J. T. (1969) Ann. N. Y. Acad. Sci. 165, 743-754.
12. Canessa de Scarnati, O., and Rodriguez de Lores Arnaiz, G. (1972) Biochim. Biophys. Acta 270, 218-225.
13. Hokin, M. R., and Hokin, L. E. (1964) Metabolism and Significance of Lipids (R.M.C. Dawson and D. N. Rhodes, eds), pp. 423-434, John Wiley & Sons, Ltd. London.
14. Hokin-Neaverson, M. (1974) Biochem. Biophys. Res. Comm. 58, 763-768.