POLYPHOSPHOINOSITIDE LEVELS AND BIOSYNTHESIS IN QUAKING MOUSE BRAIN

George Hauser, Joseph Eichberg and Stephen Jacobs

Research Laboratory, McLean Hospital, Belmont, MA 02178 and Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115

Received April 20, 1971

Summary - Levels of polyphosphoinositides are considerably lower in brains of quaking mutant mice, which are characterized by inadequate myelination, than in normal mouse brains. The deficiency of triphosphoinositide is greater than that of diphosphoinositide, but less than the lack of cerebrosides, indicating that, although the major portion of polyphosphoinositides is located in myelin, extra-myelin pools of these lipids exist. The ability to incorporate $^{32}P_i$ *in vivo* into polyphosphoinositides is substantially reduced in the quaking animals, probably as the result of an as yet unknown biochemical lesion related to normal myelination.

Polyphosphoinositides are largely concentrated in myelin or associated membranes. This conclusion rests primarily on experiments demonstrating the accretion of di- and triphosphoinositides in parallel with the deposition of myelin (1, 2) and on their substantial enrichment in isolated myelin compared to the tissue from which it was derived (3). However, it has not been unequivocally established that the bulk of polyphosphoinositides is in myelin as such, rather than in closely associated structures, such as the axolemma, which are difficult to separate completely from myelin. There are, furthermore, a number of indications that all polyphosphoinositides of brain are not localized in myelin. For instance, small but significant levels of di- and triphosphoinositide are present in rat brain at birth (1, 2) and in non-myelinated nerves (4). Also, non-neural tissues, especially kidney, contain small quantities of these lipids (5-7). Moreover, recent experiments have suggested that the portion of brain polyphosphoinositide which disappears rapidly after death, unless the tissue is frozen immediately (1, 2, 5), is extra-myelin in location, whereas the more stable pool is likely to be in myelin (8).

Neurologically deficient, mutant mice, which fail to form adequate amounts of myelin,(9) offer a unique system for the study some of the factors involved in the normal process of myelination. In view of our interest in the role of polyphosphoinositides in this phenomenon and in order to obtain further information on their location within the nervous system, we have determined the levels of di- and triphosphoinositides in normal and quaking mouse brains and the ability of the animals to incorporate radioactive P into these lipids. A preliminary report of this work has been presented (10).

Materials - Quaking mice (Qk/Qk) and apparently normal littermate controls (+/Qk or +/+) were obtained from the Jackson Laboratory, Bar Harbor, Me. They were reared locally to an age of at least 12 weeks. In one experiment animals obtained from Dr. N. A. Baumann through the courtesy of Dr. Richard Sidman were used.

$H_3{}^{32}PO_4$, free of HCl, was obtained from ICN-Tracerlab, Waltham, Mass. and suitably diluted in isotonic saline.

Methods - Mice were injected intraperitoneally with 1 or 5 μCi of $^{32}P_i$/g body weight. They were decapitated after 0.5 or 3 h and the brains were removed and frozen in liquid N_2 as quickly as possible (less than 1 min). Extraction of lipids with neutral and acidified solvents and washing of the lipid extracts were performed as described (8). The upper phase from the washing of the acidified $CHCl_3$-CH_3OH extract with 1N HCl was saved for the determination of the specific activity of a fraction (acid upper phase-P) analogous to acid-soluble P. The rationale for using this value to normalize specific activities of lipids has been discussed (8, 11).

Polyphosphoinositides were separated by thin-layer chromatography on precoated plates of silica-gel H (Analtech, Inc., Wilmington, Del.) as described elsewhere (8, 12). The plates were radioautographed on Kodak No-screen medical X-ray film (NS-54T) and the bands of di- and triphosphoinositides also visualized with iodine vapors before being scraped from the plates.

Aliquots of thoroughly mixed silica gel samples were analyzed for P by a method adapted from that of Chen et al. (13) and for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer, Model 3214, after gelling in toluene scintillation fluid (14).

Determinations of P in the lipid extracts and acid upper phases were done by the method of Bartlett (15). Radioactivity of lipids was measured in toluene containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-2-(5-phenyl oxazolyl)-benzene, that of water-containing samples in a dioxane based scintillation fluid (16).

Galactolipids were assayed by a micro-modification of the method of Sørenson and Haugaard (17) using cerebrosides as standards.

Results and Discussion - The general characteristics of the mutant mice corresponded to previous descriptions (9, 18). Both body and brain weights were significantly lower than in the controls (Table I). In younger quaking mice, brain weights have been reported to be decreased slightly (19) or not at all

TABLE I

BODY -, BRAIN - AND LIPID WEIGHTS IN NORMAL AND QUAKING MICE

	Normal	Quaking	Q/N x 100
Body weight g	25.6 ± 4.6	19.2 ± 3.3	75.1
Brain weight mg	443 ± 23	389 ± 17	87.8
Total lipids mg/g	87.9 ± 4.8	54.9 ± 5.2	62.5
Phospholipids mg/g	58.3 ± 7.0	44.5 ± 2.0	76.4
% Phospholipids	66.3	81.1	

Means ± SD are given. Number of samples = 7-15.

(20). Presumably the continued deposition of myelin in the normals causes a small difference to become accentuated. The reduction in total lipids and phospholipids is essentially in quantitative agreement with the deficit reported by Baumann et al. (19, 21) and Hogan and Joseph (20), although the absolute levels were higher in our older animals.

Cerebrosides are considered to be the most characteristic lipid marker for myelin and by this criterion only 20% of the normal amount of myelin was present in the brains of the quaking mice (Table II). Some disagreement ex-

TABLE II

GALACTOLIPID AND POLYPHOSPHOINOSITIDE LEVELS

IN NORMAL AND QUAKING MOUSE BRAIN

	Galactolipids	TPI	DPI
	mg/g	nmoles/g	
Normal	13.0 ± 1.5	303 ± 28	200 ± 23
Quaking	2.57 ± 0.63	82 ± 18	122 ± 22
Q/Nx100	19.8	26.9	61.0

Rat tissues	age			
	days			
Whole brain	34	12.4 ± 1.2	318 ± 50	163 ± 29
Gray matter	34	3.59 ± 1.15	103 ± 22	113 ± 26
%		29.0	32.4	69.3

TPI = triphosphoinositide; DPI = diphosphoinositide

Means ± SD are given. Number of samples: mouse, 7-9; rat, 13-15.

ists in the literature about the extent of the lack of myelin, as indicated by cerebroside levels (20-22), although we have consistently found values comparable to those reported here (23). It is conceivable that variations in the severity of the abnormality occur.

Both di- and triphosphoinositides were considerably reduced in the mutant brains, but not to the same extent (Table II). This indicates that they are not involved to the same degree in the molecular architecture of myelin, but rather that there seem to be about three molecules of triphosphoinositide for every molecule of diphosphoinositide. Insofar as their myelin content is concerned, quaking brains resemble normal cortical gray matter. Some data, which illustrate this and which we have recently obtained in a study on the regional distribution, disappearance post mortem and labeling of polyphosphoinositides in young rat brains (8), are given for comparison. The levels of both galactolipids and polyphosphoinositides in whole brain of 34 day-old rats were very similar to the normal mouse values. Since only myelin and the oligodendroglial cells from which it arises, but no other structures appear to be affected in the mutation (18, 24), this seems quite conclusive evidence that the major portion of both inositides is indeed in myelin per se. However, the fact that the remaining concentrations in quaking mouse brain, like those in rat gray matter, represent a higher percentage of normal whole brain values than is the case for cerebrosides suggests that a small pool of triphosphoinositide and a considerably higher one of diphosphoinositide are not associated with myelin. The localization of these putative extra-myelin pools is the objective of current experiments in our laboratory.

In order to establish whether the decreased levels of polyphosphoinositides are accompanied by a decreased potential for the biosynthesis of these compounds, we studied the incorporation of intraperitoneally injected inorganic ^{32}P. In every instance, the relative specific activity of the lipids from quaking brains was higher than that of the lipids from normals. Polyphosphoinositide-P approached equilibrium with the precursor pool more rapidly in the mutants (Table III).

TABLE III

SPECIFIC ACTIVITIES OF LIPIDS IN NORMAL AND QUAKING MOUSE BRAIN

Length of incorporation		Total phospholipids	DPI	TPI
h		Relative specific activity (mean ± SD)		
0.5	Normal (4)	0.010 ± 0.002	0.34 ± 0.03	0.27 ± 0.08
	Quaking (4)	0.011 ± 0.002	0.45 ± 0.02	0.80 ± 0.22
3.0	Normal (5)	0.049 ± 0.002	0.77 ± 0.03	0.82 ± 0.08
	Quaking (5)	0.059 ± 0.004	0.79 ± 0.17	1.20 ± 0.25

$$\text{Relative specific activity} = \frac{\text{cpm/}\mu\text{g lipid-P}}{\text{cpm/}\mu\text{g acid upper phase-P}}$$

The number of animals is given in parentheses.

Since, as shown above, marked differences in the levels of polyphosphoinositides exist between the brains of normal and quaking mice, the biosynthetic capability of the tissue is more realistically assessed by comparing total activities (Table IV). For total phospholipids the incorporation in 3 h was about five times as great as in 0.5 h, but at both time points there was no significant difference between normal and quaking brains. This was also true for phosphatidylcholine and phosphatidylinositol in the limited number of cases where we have separated the water-soluble products of mild alkaline hydrolysis of the lipids (25, 26). This essentially unimpaired ability of quaking mouse tissues to biosynthesize phospholipids extracted with neutral solvents is in striking contrast to the marked depression of the biosynthesis of polyphosphoinositides which was noted at the two times. The ability to form diphosphoinositide was better maintained just as its concentration was not reduced as much as that of triphosphoinositide. The

TABLE IV

BIOSYNTHESIS OF LIPIDS IN NORMAL AND QUAKING MOUSE BRAIN

Length of incorporation		Total phospholipids	PC*	PI*	DPI	TPI
h		Relative total activity (mean ± SD)				
0.5	Normal (4)	23.3 ± 1.9	-	6.75 ± 0.97	4.83 ± 0.97	9.69 ± 2.25
	Quaking (4)	20.7 ± 3.3	-	6.88 ± 0.77	3.41 ± 0.24	5.11 ± 0.81
	Q/N	0.89	-	1.05	0.71**	0.53
3.0	Normal (5)	109.8 ± 3.6	31.2 ± 0.4	23.8 ± 0.2	9.36 ± 1.08	23.2 ± 2.3
	Quaking (5)	104.2 ± 8.7	35.8 ± 5.0	29.9 ± 7.5	5.82 ± 1.77	8.64 ± 2.07
	Q/N	0.95	1.15	1.26	0.62	0.37

Relative total activity = Relative specific activity x μg lipid P/g

The number of animals is given in parentheses. * Two samples of phosphatidylcholine (PC) and phosphatidylinositol (PI) were analyzed.

Significance of differences between normal and quaking values:

total phospholipids $p > 0.2$

polyphosphoinositides $p < 0.01$ (** $p < 0.05$)

decreased biosynthetic potential for triphosphoinositide is consistent with a general disturbance of the formation and deposition of myelin constituents.

Efforts are being made in a number of laboratories to demonstrate a single biochemical lesion to which the numerous abnormalities that have been reported in quaking mice might be attributed. Since the chief characteristic of the disorder is a failure to produce normal amounts of myelin, the levels and biosynthesis of its constituents have been primarily investigated. Thus, the finding that the activity of sphingosine-UDP-galactose galactosyltransferase, which may be indicative of the ability to form cerebrosides, is markedly depressed in the mutants (27, 28) is consistent with the decreased concentration of these lipids. Furthermore, long chain fatty acids (normal and 2-OH 24:0 and 24:1), typical of myelin cerebrosides, are greatly reduced, possibly because of a block in their formation (29, 30). Among other myelin constituents cholesterol (21), plasmalogens (20, 21), sulfatides (22), sphingomyelin (20, 22), proteolipids and basic protein (31) and 2'3'cyclic nucleotide 3'phosphohydrolase (32) are markedly affected. On the other hand, gangliosides (except for G_{M1} which is also found in myelin), indicative of neuronal structures, were normal in quaking mutants (22,33), as was the enzyme responsible for the formation of their common precursor, lactosylceramide (28). Changes in the lipid pattern of peripheral tissues which cannot be related to myelination have also been noted (34).

Clearly the quaking mutation has a much more complicated etiology than was suspected initially and the failure to form normal amounts of polyphosphoinositides is merely one of its manifestations. If there is a single, genetically determined biochemical lesion responsible for the effects of this mutation, it may lie at an earlier, common, rate-limiting step along the biosynthetic pathways of the myelin components which are reduced in quantity or, more likely, at some control point in the complex series of cellular events which normally result in adequate myelination.

Acknowledgements - The authors thank Mrs. Hsi-Mei Chiang for her competent technical assistance. This work was supported by U.S.P.H.S. grants NS 00130, NS 06399 and FR 05484. Dr. Eichberg is an Established Investigator of the American Heart Association.

REFERENCES

1. J. Eichberg and G. Hauser, Biochim. Biophys. Acta, 144 (1967) 415.
2. A. Sheltawy and R.M.C. Dawson, Biochem. J., 111 (1969) 147.
3. J. Eichberg and R.M.C. Dawson, Biochem. J., 96 (1965) 644.
4. A. Sheltawy and R.M.C. Dawson, Biochem. J., 100 (1966) 12.
5. R.M.C. Dawson and J. Eichberg, Biochem. J., 96 (1965) 634.
6. J.N. Hawthorne and R.H. Michell, in H. Kindl, Editor, Cyclitols and Phosphoinositides, Pergamon, 1966, p. 49.
7. A. A. Wieneke and A. M. Woodin, Biochem. J., 105 (1967) 1039.
8. G. Hauser, J. Eichberg and F. Gonzalez-Sastre, in preparation.
9. R. L. Sidman, M.M. Dickie and S.H. Appel, Science, 144 (1964) 309.
10. G. Hauser, S. Jacobs and J. Eichberg. Transact. Am. Soc. Neurochem., 2 (1971) 79.
11. F. N. LeBaron, G. Hauser and E. E. Ruiz, Biochim. Biophys. Acta, 60 (1962) 338.
12. F. Gonzalez-Sastre and J. Folch-Pi, J. Lipid Res., 9 (1968) 532.
13. P. S. Chen, Jr., T. Y. Toribara and H. Warner, Anal. Chem., 28 (1956) 1756.
14. J. N. Bollinger, W. A. Mallow, J. W. Register, Jr., and D. E. Johnson, Anal. Chem. 39 (1967) 1508.
15. G. R. Bartlett, J. Biol. Chem., 234 (1959) 466.
16. J. E. Silbert, J. Biol. Chem., 239 (1964) 1310.
17. M. Sørensen and G. Haugaard, Biochem. Z., 260 (1933) 247.
18. T. Samorajski, R. L. Friede and P. R. Reimer, J. Neuropath. Exper. Neurol., 29 (1970) 507.
19. N. A. Baumann, C. Jacque, S. Pollet and M. L. Harpin, C. R. Acad. Sci., Paris, Ser. D., 264 (1967) 2953.
20. E. L. Hogan and K. C. Joseph, J. Neurochem., 17 (1970) 1209.
21. N. A. Baumann, C. M. Jacque, S. A. Pollet and M. L. Harpin, European J. Biochem., 4 (1968) 340.
22. M. J. Reasor and J. N. Kanfer, Life Sci., 8 (1969) 1055.
23. V. L. Friedrich, Jr. and G. Hauser, in preparation.
24. B. Berger, Brain Res., 25 (1971) 35.
25. R.M.C. Dawson, N. Hemington and J. B. Davenport, Biochem. J., 84 (1962) 497.
26. M. A. Wells and J. C. Dittmer, Biochemistry, 5 (1966) 3405.
27. N. Neskovic, J. L. Nussbaum and P. Mandel. C. R. Acad. Sci., Paris, Ser. D., 269 (1969) 1125.
28. V. L. Friedrich, Jr. and G. Hauser, Federation Proc., 29 (1970) 410 Abs.
29. C. M. Jacque, M. L. Harpin and N. A. Baumann, European J. Biochem., 11 (1969) 218.
30. N. A. Baumann, M. L. Harpin and J. M. Bourré, Nature, 227 (1970) 960.
31. S. Greenfield, W. T. Norton and P. Morell, Transact. Am. Soc. Neurochem., 2 (1971) 76.
32. T. Kurihara, J. L. Nussbaum and P. Mandel, J. Neurochem., 17 (1970) 993.
33. D. Kostic, J. L. Nussbaum and N. Neskovic, Abstr. 2nd Internat. Meeting, Internat. Soc. Neurochem., Milan, (1969) 254.
34. Y. Kishimoto, Federation Proc., 29 (1970) 894 Abs.