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Incorporation *in vitro* of [^{14}C]choline into phosphatidylcholine of rat brain synaptosomes and the effect of calcium ions*

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WE FOUND earlier that, after administration of [Me-^{14}C]choline into rat brain, the half-life of phosphatidylcholine from synaptosomes was only slightly higher than that from microsomes. Bosmann and Hemsworth[2] observed that isolated synaptosomes from guinea pig brain homogenates can incorporate labelled choline into total lipids when incubated either in a medium with an external energy source or in one containing only Tris buffer.

The present investigation reports on the incorporation of [^{14}C]choline into phosphatidylcholine of isolated rat brain synaptosomes and the influence of calcium ions and other agents on the course of this incorporation.

Rats at ages ranging from 12 to 20 days were used throughout this work. In previous studies,[3] it was observed that maximal choline incorporation into rat brain synaptosomes occurred during this stage of development. The synaptosomes were obtained by subfractionating the crude mitochondrial fraction by means of discontinuous density gradient centrifugation in a sucrose–Ficoll medium as described previously.[4,5] Incubations were carried out aerobically in Erlenmeyer flasks (25 ml capacity) for 2 hr with shaking in a water bath shaker at 37°. In general, synaptosomes, suspended in 1 ml of 0·45 M sucrose and corresponding to 3–4 mg protein, were added to an incubation medium which consisted of: 33·3 mM Tris or potassium phosphate buffer, pH 7·4; 20 mM sodium pyruvate; 1·2 mM sodium malate; 5 mM $MgCl_2$ or 1 mM $CaCl_2$ as indicated; and 2 μc [Me-^{14}C]choline (525 mμmoles) in a final volume of 3·0 ml. Other conditions and additions were as indicated. The reaction mixture was homogenized, then centrifuged at 198,000 *g* for 30 min, and the precipitate was washed three times with deionized water. The precipitate was homogenized in 8 ml of chloroform–methanol–HCl (200 : 100 : 1) containing 0·05% α-tocopherol, then placed in a water bath shaker at 50° for 15 min and left overnight at 4°. The extracts were centrifuged at 1000 *g* for 30 min and the supernatants extracted three times with 0·2 vol. saline. The organic layer was filtered, concentrated *in vacuo* and the lipid concentrate taken up in 0·5 ml chloroform–methanol (2 : 1). Phosphatidylcholine, lysophosphatidylcholine and sphingomyelin were isolated from the lipid extract on Silica gel G by means of two-dimensional thin-layer chromatography and the specific radioactivity of phosphatidylcholine was determined as described previously.[1]

The incubation medium for choline transport was essentially similar to that described by Diamond and Kennedy[6] for [^{14}C]choline uptake into synaptosomes, except that 2 mM Mg^{2+} and 100 mM Na^{+} were added to the uptake medium in a final volume of 0·5 ml.

[Me-^{14}C]choline (3·8 mc/m-mole), and choline-1,2-^{14}C (2·0 mc/m-mole) were purchased from New England Nuclear Corp. Dopamine and hemicholinium-3-bromide were obtained from Sigma Chemical Company and Mann respectively. Ficoll was purchased from Pharmacia Fine Chemicals, Inc., and purified by dialysis against deionized water.

Preliminary experiments showed that when synaptosomes were placed in an isotonic sucrose solution containing 33·3 mM Tris or phosphate buffer (pH 7·4) and 2 μc of either [Me-^{14}C]choline or choline-1,2-^{14}C, only the choline-containing lipids were found to be radioactive. About 95 per cent of the radioactivity was found in phosphatidylcholine, 4·5 per cent in lysophosphatidylcholine, and 0·5 per cent in sphingomyelin. The fact that both [Me-^{14}C]choline and choline-1,2-^{14}C were incorporated at the same rate indicates that the choline molecule as a whole, rather than the methyl group through transmethylation, is incorporated into phosphatidylcholine. Furthermore these findings (Table 1) are in agreement with the work of Bosmann and Hemsworth,[2] who found that in intact synaptosomes choline is incorporated into the lipid fraction in the presence of buffer only. The problem arises as to how choline is incorporated into phosphatidylcholine of intact brain synaptosomes-

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TABLE 1. EFFECT OF Ca^{2+}, Mg^{2+} AND OTHER AGENTS ON THE INCORPORATION *in vitro* OF [Me-^{14}C]CHOLINE INTO PHOSPHATIDYLCHOLINE OF RAT BRAIN SYNAPTOSOMES

Expt.	No. of expt.	Additions	Specific radioactivity (counts/min/μmole Pi)
1	4	None*	1522 ± 73
2	3	Medium used for *de novo* phospholipid synthesis in liver microsomes†	1470 ± 171
2	3	Medium used for *de novo* phospholipid synthesis in brain microsomes‡	1323 ± 89
3	3	1 + Mg^{2+}	3645 ± 110
4	4	3 + Malate (1·2 mM) + pyruvate (20 mM)	6352 ± 130
5	4	4 + CTP (1 mM)§	6250 ± 145
6	4	4 + D- or L-serine (1 mM)	5880 ± 138
7	4	4 + 2,4-Dinitrophenol (0·2 mM)	1395 ± 33
8‖	3	1 + Ca^{2+}	13,800 ± 235
9	3	8 + Malate + pyruvate	14,300 ± 323
10	3	9 + CTP (1 mM)	13,700 ± 423
11	3	9 + D- or L-serine (1 mM)	4312 ± 193
12	3	9 + 2,4-Dinitrophenol (0·2 mM)	14,250 ± 178
13	2	Control, enzyme boiled for 10 min at 100°	8

* Reaction mixture consisted of 33·3 mM phosphate buffer (pH 7·4), 2 μc [Me-^{14}C]-choline and 3 mg synaptosomes in 0·45 M sucrose in a final volume of 3·0 ml.
† Ref. 7.
‡ Ref. 8.
§ Similar effect was observed with ATP- and CDP-choline.
‖ Tris buffer, pH 9·0, was used in these reactions.

Choline can be incorporated into phosphatidylcholine through the following pathways: (1) *the CDP-choline pathway*,[9] which was also demonstrated *in vivo* by Ansell and Spanner[10] after they injected [Me-^{14}C]choline intracerebrally into adult brain. The finding that intact synaptosomes, when incubated in media similar to those used by McMurray and Dawson[7] in their studies *in vitro* on the *de novo* labelling of phospholipids in liver microsomes or by Porcellati *et al.*[8] in brain microsomes, had little effect on the course of choline incorporation (Table 1) argues against this pathway under our conditions of incubation. This was substantiated by the finding that the addition of CTP, ATP or CDP-choline had a negligible effect on the choline incorporation. (2) The finding in the present work that both [Me-^{14}C]choline and choline-1,2-^{14}C were incorporated into phosphatidylcholine at the same rate argues against *the transmethylation pathway*. (3) Free choline could enter phosphatidylcholine by *base exchange*. Base exchange of choline into phosphatidylcholine has been demonstrated to occur *in vitro* in microsomes[11] and it was stimulated by Ca^{2+}. Treble *et al.*[12] demonstrated this exchange after they administered [Me-^{3}H]choline intravenously to rats and observed it in both bile and liver. Our present findings on choline incorporation into phosphatidylcholine in intact rat brain synaptosomes meet all the requirements for a calcium-stimulated exchange reaction.[13] Thus, the incorporation is stimulated by three to four times in the presence of Ca^{2+} than in the presence of Mg^{2+} (Table 1). In the presence of Ca^{2+}, it is inhibited by D- or L-serine, it is not stimulated by an energy source, and 2,4-dinitrophenol exerted no effect on the labelling. In contrast, in the presence of Mg^{2+}, there was an almost two times stimulation upon the addition of malate and pyruvate, and the incorporation was inhibited when 2,4-dinitrophenol was added. It is interesting to note that Artom and Lofland[14] have shown a Ca^{2+}-activated incorporation of labelled ethanolamine into the phospholipids of various liver preparations, and this incorporation was energy dependent. Rat brain synaptosomes were also

found to incorporate [^{14}C]ethanolamine into phosphatidyl ethanolamine, which was stimulated up to five times by Ca^{2+}. Furthermore, [^{3}H]myoinositol was incorporated into phosphatidyl inositol; however, in contrast to choline and ethanolamine, it was activated by Mg^{2+} but not by Ca^{2+}, and in the presence of the former, it was stimulated up to nine times by CTP (A. Abdel-Latif and M. Roberts, unpublished work). Inositol is known to be inactive as substrate for Ca^{2+}-stimulated base exchange reactions.[13] The exchange reaction is also distinguished from the choline phosphotransferase by virtue of a different optimal pH (9·0 vs. 7·4–7·8).[13] Studies on the effect of pH on the specific radioactivity of phosphatidylcholine showed a pH optimum of about 7·5 in the presence of Mg^{2+} and of 9·0–9·5 in the presence of Ca^{2+}. The specific radioactivity of phosphatidylcholine increased with increasing concentrations of Ca^{2+} up to 2–3 mM (Fig. 1), then decreased at higher concentrations (over 6 mM), and complete inhibition was observed in the presence of 20 mM.

To show whether the calcium ion effect can be interpreted in terms of the recently described choline transport system,[6,15] its action on this system was investigated (Table 2). Calcium ions had a negligible effect on the transport system, but exerted up to four times stimulation on choline incorporation into phosphatidylcholine. In contrast, hemicholinium exerted up to 50 per cent inhibition on both the

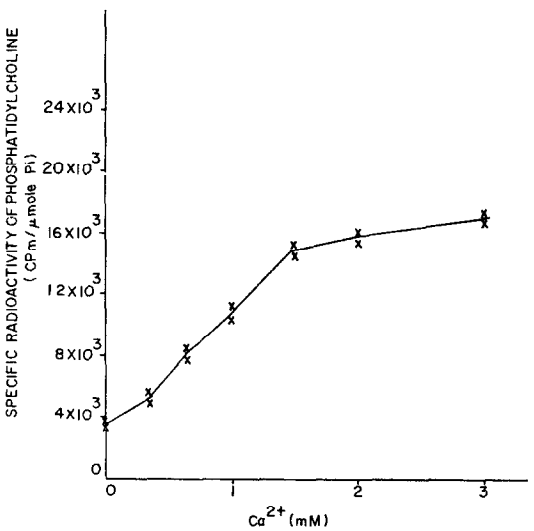

FIG. 1. Effect of different concentrations of calcium chloride on the incorporation of [^{14}C]choline into phosphatidylcholine.

TABLE 2. EFFECT OF CALCIUM IONS AND HEMICHOLINIUM ON UPTAKE OF [Me-^{14}C]CHOLINE BY RAT BRAIN SYNAPTOSOMES*

Expt.	No. of expt.	Additions	Total radioactivity in supernatant after lysing of synaptosomes (counts/min)	Total radioactivity in phosphatidylcholine (counts/min)
1	4	None	11,900 ± 223	900 ± 73
2	4	Ca^{2+} (1 mM)	11,100 ± 179	3600 ± 105
3	4	Hemicholinium-3-bromide (0·15 mM)	5900 ± 123	457 ± 89

* The uptake experiments were run according to Diamond and Kennedy,[6] with a slight modification as described previously.[3]

transport of choline and its subsequent incorporation into phosphatidylcholine. The transport studies show that the stimulatory effect of calcium ions on choline incorporation observed in the present work is not simply due to an increase in the influx of [Me-^{14}C]choline into the synaptosomes.

The results described above show that calcium ions exert a considerable stimulatory effect on the incorporation of labelled choline into phosphatidylcholine. This incorporation is not dependent on cofactors, including CoA, ATP, CTP and Mg^{2+}, which are required for *de novo* synthesis of phospholipids in microsomes. Furthermore, no increase in the level of phosphatidylcholine was observed in the reaction mixtures after incubation for 2 hr. The pH optimum for this incorporation is about 9, while that for *de novo* synthesis of phospholipids is 7·4–7·8. Incorporation of labelled choline into phosphatidylcholine is inhibited by either L- or D-serine. In contrast to ethanolamine, which was incorporated at a rapid rate into phosphatidylethanolamine in the presence of Ca^{2+}, inositol was inactive as substrate for this reaction. These findings suggest that Ca^{2+} is activating an exchange rather than a biosynthetic reaction. Dils and Hubscher[11] have suggested that the incorporation of choline or ethanolamine into lipids in this manner is due to the presence of phospholipase D. Yang *et al.*[16] and Dawson[17] showed that phospholipase D of plant tissue is capable of catalyzing transphosphatidylation with a variety of alcohols, in addition to hydrolyzing lecithin. Thus, it is possible that the Ca^{2+}-stimulated incorporation of labelled choline into phosphatidylcholine represents a transphosphatidylase activity of phospholipase D. In contrast to Ca^{2+} in intact rat brain synaptosomes, Mg^{2+} exerted a stimulatory effect on this activity and, interestingly, this activity was stimulated by an energy source and had a pH optimum around 7·5. Whether these activities represent two different enzymes or the same enzyme is not clear at the present time.

Since the neurotransmitter, acetylcholine, is hydrolyzed at the synapse by acetylcholinesterase during nerve activity,[18] the released choline could be reutilized, not only in forming acetylcholine, but also by being incorporated into the phospholipids of the synaptosomes, as was shown in the present work. Further studies on phospholipids in the synaptosomes could shed more light on their relationship to neural activity at the synapse.

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