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COMPARISON OF BASAL AND NORADRENALINE STIMULATED METHYLATION OF CHLOROFORM-EXTRACTABLE PRODUCTS IN SYNAPTOSOMAL PREPARATIONS FROM THE RAT BRAIN*

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Abstract—Noradrenaline addition increases (*S*-adenosyl-L-methionine)-methylation of chloroform extractable products in the rat brain synaptosomal preparation. The characteristics of this methylation ($MgCl_2$ dependence, *S*-adenosyl-L-methionine concentration dependence) are different from those of phosphatidylethanolamine methylase. The greatest increase was induced by noradrenaline in the 10^{-4} M concentration range; preincubation of noradrenaline with the membranes, before *S*-adenosyl-L-methionine addition, was necessary. The TLC analysis of chloroform extractable products showed that: methylation of phosphatidylethanolamine derivatives was increased and the production of another unknown product (corresponding to that described by Wazer *et al.*, *Life Sci.* 32, 2535, 1983) was more enhanced.

Phosphatidylethanolamine methylation by *S*-adenosyl-L-methionine is an alternative pathway for the synthesis of phosphatidylcholine [1]. A few years ago, Axelrod and coworkers reported the activation of this methylation by β adrenergic agonists [2]. Several other workers reported results which confirmed this fact; especially, Leprohon and coworkers showed that dopamine and noradrenaline increased methylation of phosphatidylethanolamine in rat brain neurons [3]. More recently, Wazer *et al.* [4] showed that most of the chloroform extractable methylated products were not phosphatidylethanolamine derivatives. Although they were unable to identify the exact nature of these products, the authors did show that they were formed via an enzymatically mediated reaction.

The nature of the putative methyl acceptor candidates seems to us difficult to elucidate especially after the study of Wazer *et al.* (which failed to identify this product, even after comparing it to 18 putative candidates) and we think that testing further products will be unsuccessful; so, in the present study, we chose to investigate the influence of experimental conditions on the noradrenaline induced stimulation of *S*-adenosyl-L-methionine methylation of the chloroform extractable products.

MATERIALS AND METHODS

Membrane preparation. Membranes from OFA rat (IFFA Merieux, France) brain were prepared as described previously [6]. Briefly, the P_2 pellet was prepared and then the cell structures were destroyed in hypotonic Tris–HCl buffer. The membranes were washed and resuspended in 50 mM Tris–HCl buffer, pH = 7.4. Proteins were determined according to Lowry *et al.* [5].

Methylation measurement [7, 8]. The incubate contained 0.4 ml of membrane suspension (from 50 to 100 mg fresh tissue, i.e. 1–2 mg protein); *S*-adenosyl-L-methionine concentration, ions concentration, temperature and incubation time were as indicated in each experiment. The chloroform extractable products were isolated after incubation as described by Hirata *et al.* [1]. The incubates were transferred in 3 ml of a chloroform/methanol/HCl mixture (200/100/1) and 2 ml of a methanol/water (50%) mixture containing 0.1 M KCl were added. The chloroform phase was washed once with 2 ml of the methanol/water mixture; 1 ml of the resultant chloroform phase was evaporated in a scintillation vial and the radioactivity counted.

Products. ^{3}H-*S*-adenosyl-L-methionine (methyl ^{3}H-*S*-adenosyl-L-methionine, specific activity 15 Ci/mmol) was purchased from Amersham France and purified by thin layer chromatography before use (we verified that up to 1 month of storage of the labelled product, this purification was not essential).

RESULTS

Methylation of chloroform extractable products as a function of different incubation times with noradrenaline

The increase in methylation was dependent on the preincubation time (Fig. 1) at 37° with noradrenaline; if preincubated at 0°, only a slight increase appeared after 2 hr of preincubation. Noradrenaline addition must occur before *S*-adenosyl-L-methionine addition because simultaneous addition of ^{3}H-*S*-adenosyl-L-

* This work has been reported in the international symposium "The Biochemistry of *s*-adenosyl-L-methionine as a Basis for Drug Design" in Bergen, Norway, 30 June–4 July 1985.

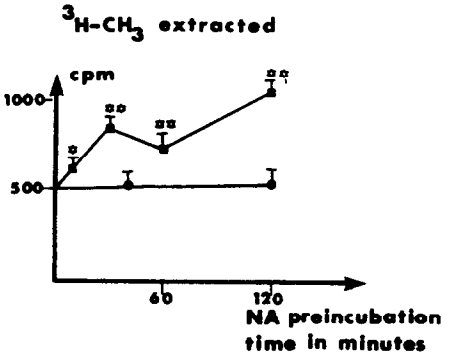

Fig. 1. Methylation of chloroform extractable products after different incubation times in presence of noradrenaline. Membranes were preincubated at either 0° (●) or 37° (■) with noradrenaline in 50 mM Tris buffer, pH 7.4 containing 10 mM $MgCl_2$; ^{3}H-*S*-adenosyl-L-methionine (5. 10^{-8} M, 30,000 cpm/assay) was then added and incubated during 20 min at 37°. Extraction was performed as described in the Materials and Methods. Each point is the mean ± SD of two experiments consisting of four determinations. * Different from the control (incubation without noradrenaline) (* α risk lower than 0.05, ** α risk lower than 0.01).

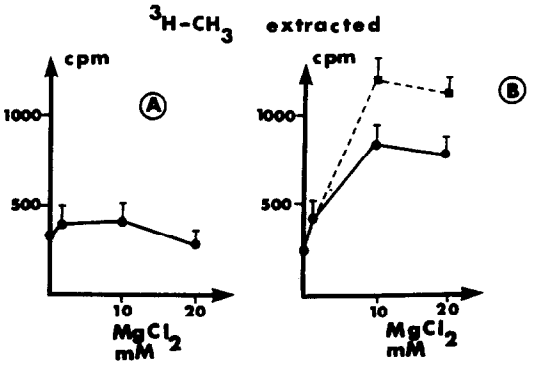

Fig. 2. Effect of $MgCl_2$ on basal methylation and noradrenaline induced increase in methylation. Preincubation was performed 30 min at 37° with noradrenaline 10^{-4} M (B) or without noradrenaline (A); the incubation with ^{3}H-*S*-adenosyl-L-methionine and chloroform extraction was then made as described in Fig. 1. (A) Basal methylation without noradrenaline; (B) methylation after preincubation with noradrenaline; ●, $MgCl_2$ added before noradrenaline preincubation, ■, $MgCl_2$ added after noradrenaline preincubation. Each point is the mean ± SD of four determinations.

methionine and noradrenaline did not show any increase, even after 80 min incubation (^{3}H-*S*-adenosyl-L-methionine alone 728 ± 53 cpm; ^{3}H-*S*-adenosyl-L-methionine + 10^{-3} M noradrenaline 653 ± 64 cpm).

Finally, we have centrifuged and washed the membranes after 10^{-4} M noradrenaline incubation (30 min at 37°) before incubation with ^{3}H-*S*-adenosyl-L-methionine: increase in methylation still remained (control membranes: 1694 ± 193; noradrenaline preincubation as described in Fig. 1: 4921 ± 209; noradrenaline preincubation as described in Fig. 1 but membranes were centrifuged and washed before ^{3}H-*S*-adenosyl-L-methionine incubation: 4729 ± 435). This result suggests that membrane modification induced by preincubation with noradrenaline cannot be reverse.

Effect of $MgCl_2$ *on basal methylation and noradrenaline induced increase in methylation*

Different results concerning the effects of $MgCl_2$ on phosphatidylethanolamine methylase have been reported; Hirata *et al.* [1] showed that $MgCl_2$ was essential to the above enzyme while Percy *et al.* [9] showed it was not; in our laboratory, we showed that $MgCl_2$ was not required for the phosphatidylethanolamine methylase activity but that the enzyme was inhibited by EDTA [7]. In Fig. 2, we reported the effect of $MgCl_2$ on basal methylation and noradrenaline induced methylation. Basal methylation was not greatly affected by $MgCl_2$ (Fig. 2A) whereas noradrenaline induced increase in methylation was strictly dependent on $MgCl_2$ (Fig. 2B). No significant differences were observed between the addition of $MgCl_2$ during preincubation with noradrenaline (Fig. 2B (●) or incubation with ^{3}H-*S*-adenosyl-L-methionine (Fig. 2B, ■). On the other hand, 5 mM EDTA was required to partially inhibit basal methylation whereas 1 mM EDTA completely abolished noradrenaline induced increase in methylation (result not shown).

Methylation of chloroform extractable products after preincubation with various noradrenaline concentrations

The effect of noradrenaline incubation with the membrane preparations was maximum in the 10^{-3}–10^{-4} range (Fig. 3).

Methylation for different S*-adenosyl-*L*-methionine concentration after preincubation with noradrenaline*

We have observed [14], that for lower *S*-adenosyl-L-methionine concentrations (10^{-8}–10^{-9} M), the apparent affinity of the phosphatidylethanolamine methylase was greater (K_m in the 10 nM range) than previously described [1]. It was therefore important

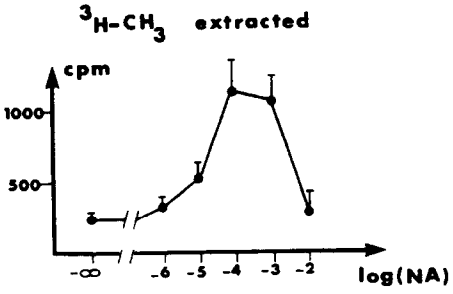

Fig. 3. Methylation of chloroform extractable products after preincubation which various noradrenaline concentrations. Membranes were incubated with various noradrenaline concentrations in 50 mM Tris buffer, pH 7.4 containing 10 mM $MgCl_2$ during 30 min. Incubation with ^{3}H-*S*-adenosyl-L-methionine and extraction were performed as described in Fig. 1. Noradrenaline concentrations were expressed as mol/l. Each point was the mean ± SD of 2 experiments consisting of four determinations.

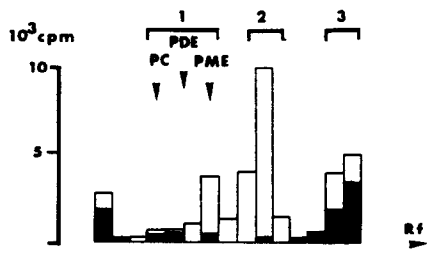

Fig. 4. TLC analysis of ^{3}H-methylated chloroform extractable product. Methylation was performed as described in Fig. 1 in the presence of 0.1 μM ^{3}H-*S*-adenosyl-L-methionine. The chloroform extract was loaded on silica gel plate (PLC Merck 13794) and the chromatogram was developed in chloroform : propionic acid : *n*-propranol : water (2/1/3/5). Compounds were visualized with iodine and the silica was scraped in scintillation vials: dark lines, analysis of ^{3}H-methylated chloroform extractable product (basal methylation); empty lines, analysis of ^{3}H-methylated chloroform extractable product after preincubation with 10^{-4} M noradrenaline. Arrows localize the migration of phosphatidylcholine (PC), phosphatidyl-*N*-monomethylethanolamine (PME), phosphatidyl-*N,N*-dimethylethanolamine (PDE) standards. Three areas are delimited and called 1, 2, 3.

to evaluate the noradrenaline induced increase in methylation for different *S*-adenosyl-L-methionine concentrations: the noradrenaline induced increase in methylation was lowered when *S*-adenosyl-L-methionine concentration was increased (results not shown). The noradrenaline induced increase in methylation exhibited an apparent K_m for *S*-adenosyl-L-methionine between 8 and 15 nM which is close to the low K_m described in ref. 4 and one to two orders of magnitude lower than this exhibited by phosphatidylethanolamine methylase [1].

Nature of the methylated products

In TLC studies, we used the solvent described in ref. 4: chloroform/propionic acid/*n*-propanol/water (2/1/3/5). In the absence of noradrenaline, the analysis pointed out three preponderant radioactivity spots (Fig. 4, dark lines) with R_f 1 : 0.65 : 0.23–0.40. Radioactivity in the area no. 1 corresponded to methylated derivatives of phosphatidylethanolamine and radioactivity in the area no. 2 corresponded to the unknown product described by Wazer *et al.* [4]. In the presence of noradrenaline (Fig. 4, empty lines) radioactivity in the area no. 1 was one to twofold increased whereas in the area no. 2, it was 10–50-fold increased.

DISCUSSION

Paradoxically, our results confirmed both those of Wazer *et al.* [4] and those of Leprohon *et al.* [3] which seemed contradictory: we observed a one to twofold increase of phosphatidylethanolamine derivatives methylation (as Leprohon *et al.* which measured the radioactivity of purified phosphatidylethanolamine derivatives) and a greater increase of the unknown methylated product described by Wazer *et al.*

Concerning the nature of this unknown product, we can assume that it is not an artefactual occurrence as its production proceeds by an enzymatic reaction with *S*-adenosyl-L-methionine affinity in the 10^{-8} M range.

This fact raises the problem of the significance of noradrenaline induced methylation; our study, in addition to other works, showed that noradrenaline enhanced phosphatidylethanolamine methylation but enhanced also some other methylations; the next question that could be asked is which of these methylations is in relation with the adrenergic receptors. The importance of the above question is emphasized by the physiological implication of noradrenaline induced methylation [10]: phosphatidylethanolamine or chloroform extractable methylated products are reported to have an effect on other noradrenaline functions [11, 12] and the synthesis of chloroform extractable products seems to regulate other neurotransmitter functions which in turn regulate the methylation [13].

In all the previous studies, from different laboratories, as well as ours, the experimental factors (such as $MgCl_2$, remaining neurotransmitters in the preparation, *S*-adenosyl-L-methionine concentration were not considered. This can help in explaining the discrepancy between the results obtained in the different reports. Therefore, for future work on this subject, special precautions should be taken.

Finally, our work is a contribution in demonstrating an enzymatically catalyzed methylation of a chloroform extractable product, methylation which is induced by noradrenaline. The enzyme, a methylase, exhibits kinetic parameters and characteristics which are different from those of basal phosphatidylethanolamine methylase.

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