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THE UNIQUELY DISTRIBUTED ISOPRENYLATED PROTEIN METHYLTRANSFERASE ACTIVITY IN THE RAT BRAIN IS HIGHLY EXPRESSED IN THE CEREBELLUM

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Isoprenylated protein methyltransferase, the enzyme which catalyzes the reversible methylation of signal transducing G-proteins was studied in nine brain regions of the rat brain using S-farnesyl cysteine analogs as substrates. Enzyme activity, as determined with N-acetyl-S-farnesyl-L-cysteine (AFC) was found in the nuclear, synaptosomal and microsomal fractions of all brain regions but not in the cytosol. The enzyme is a unique methyltransferase with respect to its brain distribution. The rank order of activity of the enzyme is cerebellum >> midbrain > medulla > forebrain regions, where activities in cerebellar synaptosomal and nuclear fractions (28-32 pmol AFC [methyl-^{3}H]ester formed/min/mg prot) are 20 to 30 times higher than those of the corresponding fraction of the forebrain regions. This distribution is reminiscent of that of neurotransmitter receptors and signal transduction molecules and suggests a regulatory role for the enzyme, particularly in the cerebellum. © 1993 Academic Press, Inc.

Isoprenylated proteins are found in all eukaryotic cell lines and tissues thus far studied and are enriched in mammalian brains (1-5). The thioether linked C-terminal cysteine in these proteins is modified by the

Abbreviations used: AdoMet, S-adenosyl-L-methionine;
AFC, N-acetyl-S-farnesyl cysteine;
FTA, S-farnesyl thioacetic acid;
FTP, S-farnesyl thiopropionic acid;
EDTA, ethylenediaminetetraacetic acid;
Tris, Tris[hydroxymethyl]amino methane;
DMSO, dimethylsulfoxide;
SDS, sodium dodecyl sulfate.

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isoprenylated protein carboxylmethyltransferase. This enzyme was first recognized as the ras methyltransferase (6) and subsequently found to be a membrane bound methyltransferase that methylates small GTP-binding proteins (1, 4, 5) and other proteins that have a prenylated C-terminal cysteine (7, 8).

Methylation is the only reversible modification (9) in a sequence of biochemical reactions that lead to maturation of these proteins (10-12), thus suggesting a regulatory role for the isoprenylated protein methyltransferase. Recent studies demonstrated that the enzyme recognizes hexapeptides with a C-terminal farensylated cysteine (3), and simple substrates such as N-acetyl-S-farnesyl-L-cysteine (AFC) (13) and S-farnesyl thiopropionic acid (FTP) (13). Use of such substrates enabled quantitative determination of the isoprenylated protein methyltransferase in rat liver (3) and brain (2) and in bovine rod outer segments (13). We have previously characterized the enzyme and its endogenous GTP-binding protein substrates in neuroblastoma N1E-115 and in pheochromocytoma PC-12 cells (4, 5, 14-16). Neurite-like outgrowth in both cell lines was found to coincide with a marked and persistent increase in methylation of GTP-binding proteins. This and the enrichment of whole rat brain in isoprenylated proteins and in the methyltransferase, prompted us to examine the regional distribution of the enzyme in the rat brain.

Materials and Methods

[Methyl-^{3}H]-S-adenosyl-L-methionine ([methyl-^{3}H]AdoMet, 15 Ci/mmole) was purchased from New England Nuclear. AdoMet was from Sigma Chemical Co. AFC, FTP and FTA (S-farnesyl thioacetic acid) were prepared, purified and tested for purity by NMR analysis as detailed elsewhere (13). Charles River adult male rats were grown as detailed previously (17). Brain regions were removed after decapitation, and homogenized in 50 mM Tris HCl pH=7.4 0.32 M sucrose containing 3 mM EDTA, 1 mM EGTA, 5 unit/ml Aprotinin and 5 µg/ml pepstatin to yield 10% (w/v) homogenates. Crude nuclear fractions were obtained by 10 min 800 x g centrifugation, crude synaptosomal fractions by 15 min 14,500 x g centrifugation and microsomal fractions by 30 min 100,000 x g centrifugation steps. Pellets were resuspended in the homogenization buffer and stored at -70°C. Methyltransferase assays were performed at 37°C in 50 mM Tris HCl buffer, pH 7.4, using 75-125 µg protein, 25 µM (methyl-^{3}H]AdoMet (300,000 cpm/nmol) and 150 µM AFC or FTP (prepared as a stock solution in DMSO) in a total volume of 50 µl. DMSO concentration in all assays was 4%. Various AFC, or FTP concentrations were used in several experiments as indicated in the text. Reactions were terminated after 10 min by adding of 500 µl chloroform methanol (2:1) and a subsequent addition of 250 µl H_2O, mixing and phase separation. A portion of 125 µl of the chloroform phase was dried down in an Appendorf tube at 40°C and 100 µl of 1 N NaOH/1% SDS solution were then added. The open tubes were placed in vials containing 5 ml scintillation liquid (Opti Fluor, Packard) and the vials closed. The [^{3}H]methanol thus formed was counted following 24 hr of vapor phase equilibration at room temperature with 90% efficiency (4, 5). Assays were routinely performed in duplicates and background counts (no AFC or FTP added) were subtracted. All experiments were repeated 3 times and data obtained averaged.

Results

Isoprenylated protein methyltransferase activity was determined in subcellular fractions of adult male rats. Enzyme assays used AFC, a specific methyl acceptor substrate (2, 13) and [methyl-^{3}H]AdoMet, the general methyl donor. Conditions were set up so as to obtain reliable measurements of enzyme activity with respect to background, linearity with time and protein concentration, and saturablility. Use of 25-150 µg of protein (depending on brain region and subcellular fraction), 200 µM AFC, 25 µM [methyl-^{3}H]AdoMet and incubation times of 10-15 min at 37°C yielded the appropriate conditions. In typical experiments background counts (no AFC added) were 50-80 cpm while methylated AFC counts were 400 to 6000 cpm. Similar results were also obtained when FTP, another specific methyl acceptor (13), was used. Figure 1 demonstrates enzyme curves with AFC and FTP as substrates and cerebellar nuclear fraction as the enzyme source. The respective estimated K_M values were 36±5 and 19±7 µM. Maximal reaction velocity (Vmax) was 38 pmol AFC [methyl-^{3}H]ester formed/min/ mg protein and 31 pmol FTP [methyl-^{3}H]ester

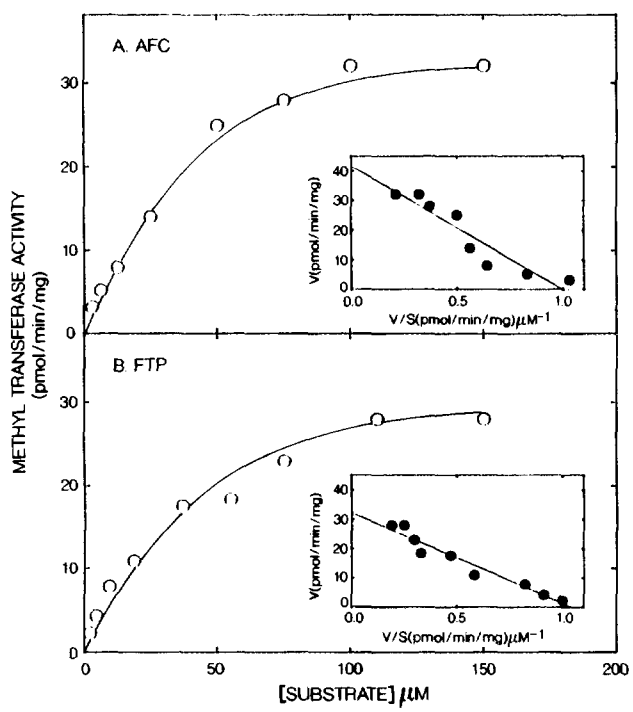

Figure 1.
Methylation of AFC and of FTP by the isoprenylated protein methyltransferase of the cerebellar nuclear raction. Assays were performed as detailed in Methods using 75 µg protein, 25 µM [methyl-^{3}H]AdoMet and the indicated AFC or FTP concentrations. Data are expressed in terms of pmol AFC[methyl-^{3}H]ester (A) or FTP [methyl-^{3}H]ester formed (B). Inset: Eadi-Hoftee plots.

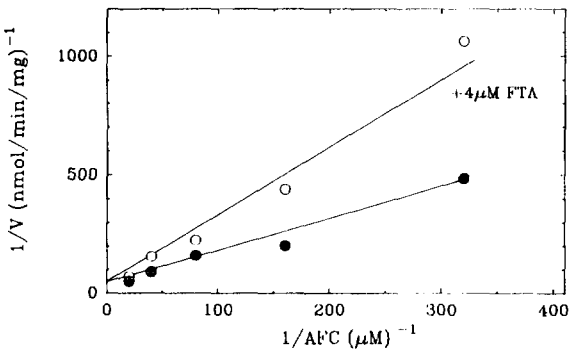

Figure 2.
Competitive inhibition of AFC methylation by FTA. Assays were performed as detailed in Figure 1. Double reciprocal plots of the data obtained for methylation of AFC in the presence (o) and in the absence (•) of 4 μM FTA are shown.

formed/min/mg protein. The reason for the observed lower Vmax for FTP is not yet known. This phenomenon was observed consistently in all of the brain preparations used here. Perhaps it reflects a higher susceptibility of FTP to an endogenous methylesterase, or product inhibition by FTP-methyl ester.

Nonetheless AFC, though presenting about 2 times higher K_M values as compared with FTP, proved to be a suitable substrate for the isoprenylated protein methyltransferase. As shown in Figure 2, methylation of AFC was competitively inhibited by S-farensyl thioacetic acid (Ki=2.8 μM), a prenylated analog known as a competitive inhibitor of the bovine rod outer segment enzyme (13).

Following these experiments, isoprenylated protein methyltransferase activity was determined in subcellular fractions obtained from 9 brain regions. In none of the brain regions tested could we detect a cytosolic enzyme that methylates either AFC or FTP. Enzyme activity was however apparent in the nuclear, synaptosomal and microsomal fractions of all brain regions studied (Table 1). In most cases specific activities of the enzyme in the three fractions were comparable with 2-3 fold differences at most. The only exception was the hypothalamus, where specific activities in the synaptosomal and microsomal fractions were significantly higher than that observed in the nuclear fraction. In terms of fractional activity (percentage of total in homogenate), estimated for each fraction in the various brain regions, the nuclear fractions presented 50-80% of activity except in the cerebral cortex, where the estimated value was 25%. The synaptosomal fractions presented 25-35% and the microsomal fractions 5-15% of total homogenate activity. The cortical synaptosomes were rich in enzyme, containing 50% of the activity found in cerebral cortex homogenates.

Table 1: Regional distribution of isoprenylated protein methyltransferase in the rat brain

	Methyltransferase activity (pmol/min/mg protein)					
	Crude nuclear		Crude synaptosomal		Microsomal	
Cerebellum	32.5±6.6	(26.6)	28.4±2.6	(24.5)	20.9±4.1	(16.7)
Midbrain	2.9±0.2		4.1±0.9		6.4±1.6	
Medulla pons	1.2±0.2	(0.90)	2.7±0.5	(2.0)	4.5±0.9	(4.2)
Hypothalamus	0.45±0.06	(0.35)	2.1±0.1	(1.5)	5.2±1.1	(3.3)
Thalamus	2.1±0.1		1.7±0.3		1.5±0.2	
Cerebral cortex	1.1±0.1		2.7±0.3		2.1±0.3	
Olfactory bulb	1.0±0.08		1.7±0.2		2.3±0.1	
Caudate putamen	1.8±0.3		1.4±0.4		0.8±0.2	
Hippocampus	2.1±0.1	(1.6)	1.0±0.05	(0.77)	1.9±0.4	(1.1)

Methyltransferase assays were performed as detailed in Methods and data are expressed in terms of AFC-[methyl-^{3}H]esters or FTP[methyl-^{3}H]esters formed (numbers in parentheses). Data represent the mean values (n=3) of three separate experiments.

The slight variations in methyltransferase observed among the subcellular fractions of the various brain regions did not, however, mask the clear picture of its distribution in the rat brain (Table 1). As seen, specific activity of the enzyme was highest in the cerebellum, followed by the midbrain and the medulla pons, thalamus, hypothalamus, cerebral cortex, hippocampus, caudate and olfactory bulbs. Data thus obtained with AFC as a substrate for the methyltransferase were confirmed with FTP, by assaying fractions of selected brain regions (see data in parentheses, Table 1).

Discussion

Previous studies have shown that rat and mouse brains contain appreciable activity of the isoprenylated protein methyltransferase (1, 2, 18). The availability of selective substrates such as AFC (2, 13) and FTP (13), and specific inhibitors such as FTA (13) made it possible to perform quantitative determinations of enzymes' levels with no, or little, interference of endogenous methyl acceptors. Such an analysis was performed in the present study which demonstrates that isoprenylated protein methyltransferase activity of the rat brain is expressed in a unique manner. The rank order of activity of the enzyme is: cerebellum >> midbrain > medulla pons > thalamus > hypothalamus > cerebral cortex $\geq$ hippocampus = caudate > olfactory bulb (Table 1). Thus, while forebrain regions express relatively low activity and the midbrain a moderate activity, the cerebellum is by far more enriched, presenting activity of about 20-30 fold higher than that found in the forebrain. The distribution of the isoprenylated protein methyltransferase is reminiscent of the distribution of neurotransmitter receptors and of enzymes involved in signal transduction mechanisms. This supports the notion

that the enzyme may have an important role in the regulation of GTP-binding proteins, which constitute a major class of its natural substrates (1, 2, 5, 12, 15).

To the best of our knowledge, none of the mammalian protein methyltransferases has a distinctive brain distribution, such as that presented by the isoprenylated protein methyltransferase. Since the isoprenylated protein methyltransferase uses prenylated proteins as substrates, we speculate that the protein prenyltransfrases (19) and perhaps also proteases that use prenylated protein as substrates (20) would be enriched in the cerebellum. In this regard it is important to note that geranylgeranylated proteins are more abundant in mammalian brains than farnesylated proteins (21). It is not yet known however, whether the two groups of prenylated proteins are methylated by distinct methyltransferases. The precedence of the rat liver (3) suggest one enzyme or at least one common catalytic subunit. Future studies with S-geranylgeranylated cysteine analogs as substrates for the brain enzyme will provide an answer to this question.

Since the rat cerebellum is by far more enriched in isoprenylated protein methyltransferase as compared to other brain regions, it is tempting to suggest that the enzyme has a unique function in this brain region, regulating activity of one or more proteins containing a prenyl cysteine C-terminus. Recent studies have shown that not only G-proteins are methylated by the cerebellar enzyme (18, 22) but other yet unidentified proteins as well, which appear to be found in the cerebellum only (22). Also, developmental studies demonstrated that the highest levels of the isoprenylated protein methyltransferase activity are expressed in the cerebellum during the period of granule cell migration and synaptogenesis (18). Thus, the rat cerebellum appears to be an interesting brain region for studying the role of the isoprenylated protein methyltransferase in neuronal development and plasticity, a role already inferred by previous experiments with cultured cell lines (15, 16).

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