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Inhibition of Calf Thymus and Rat Hypothalamic Synaptosomal Protein Carboxymethyltransferase by Analogues of *S*-Adenosylhomocysteine

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SUMMARY

In order to define the structure-activity relationships for the binding of *S*-adenosyl-L-homocysteine (AdoHcy) to protein carboxy-*O*-methyltransferse (PCM; EC 2.1.1.24), a series of synthetic and natural analogues of AdoHcy has been evaluated as inhibitors of the purified calf thymus enzyme. Of the amino acid-modified analogues of AdoHcy evaluated, only those with modification at the sulfur atom, e.g., A9145c, Sinefungin, AdoHcy sulfone, and AdoHcy sulfoxide, showed appreciable inhibitory activity of the calf thymus PCM. These data suggest that the structural features of primary importance in binding the amino acid portion of AdoHcy to PCM include (a) the chirality of the amino acid asymmetric carbon; (b) the terminal amino group; (c) the terminal carboxy group; and (d) the three-carbon atom distance between the sulfur atom and the terminal amino and carboxyl groups. Of the base-modified analogues, only TubHcy, 8-aza-AdoHcy, 2-aza-AdoHcy, and N^6-methyl-AdoHcy exhibited significant inhibitory effects. In general, these base-modified analogues were substantially less active than AdoHcy itself, suggesting that minor changes in the adenine ring of AdoHcy produce a significant reduction in enzyme affinity. Several of the PCM inhibitors identified using the calf thymus enzyme were then evaluated for their ability to inhibit methylation of endogenous methyl acceptor proteins (MAPs) by endogenous PCM in rat hypothalamic synaptosomes. All of the synthetic analogues evaluated, e.g., AdoHcy sulfoxide, AdoHcy sulfone, and 2-aza-AdoHcy, produced significant inhibition of protein carboxymethylation in this test system. Interestingly, the natural analogues, e.g., A9145c and Sinefungin, were inactive as PCM inhibitors in intact synaptosomes, but active as PCM inhibitors in lysed synaptosomes, suggesting problems with transport of these inhibitors.

INTRODUCTION

PCM[1] (EC 2.1.1.24) catalyzes the transfer of a methyl group from AdoMet to the carboxyl group of a glutamyl or aspartyl residue on a protein to yield the corresponding methyl ester (1). Protein carboxymethylation has been implicated in both leukocyte (2, 3) and bacterial (4, 5) chemotaxis, as well as neurosecretory events in the parotid gland (6), the posterior pituitary gland (7), the adrenal gland (8), and hypothalamic synaptosomes (9). Similar to other AdoMet-dependent methyltransferases (10), PCM is sensitive to inhibition by AdoHcy (1), a product of the reaction (11). This observation suggests that analogues of AdoHcy, which selectively inhibit PCM, might be used as chemical probes to elucidate further the physiological role of this macromolecular methylation.

Several laboratories have reported the synthesis of base-, amino acid-, and sugar-modified analogues of AdoHcy (10, 12–14). The *in vitro* and *in vivo* inhibitory

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[1] The abbreviations used are: PCM, protein carboxy-*O*-methyltransferase; 2′,3′-acyclic AdoHcy, 2′-[*O*-[(R)-hydroxymethyl(adenin-9-yl)methyl]]-3′-[*S*-(R)-homocysteinyl]-3′deoxy-(*S*)-glycerol; AdoCys, *S*-adenosyl-L-cysteine; AdoHcy-dialdehyde,2′-*O*-[(R)-formyl(adenin-9-yl)methyl]-3′-*S*-(R)-homocysteinyl-3′-deoxy-*S*-glyceraldehyde; AdoHcy sulfone, *S*-adenosyl-L-homocysteine sulfone; AdoHcy sulfoxide, *S*-adenosyl-L-homocysteine sulfoxide; AdoMet, *S*-adenosyl-methionine; AdoTba, *S*-adenosyl-4-thiobutyric acid; AdoTpa, *S*-adenosyl-3-thiopropylamine; 2-aza-AdoHcy, S-2-azaadenosyl-L-homocysteine; 8-aza-AdoHcy, *S*-8-azaadenosyl-L-homocysteine; CytHcy, *S*-cytidyl-L-homocysteine; D-AdoHcy, *S*-adenosyl-D-homocysteine; 3-deaza-AdoHcy, *S*-3-deazaadenosyl-L-homocysteine; InoHcy, *S*-inosyl-L-homocysteine; L-AdoHcy, *S*-adenosyl-L-homocysteine; N^6-ethyl-AdoHcy, *S*-N^6-ethyladenosyl-L-homocysteine; N^6-methyl-AdoHcy, *S*-N^6-methyladenosyl-L-homocysteine; TubHcy, *S*-tubercidinyl-L-homocysteine; UriHcy, *S*-uridyl-L-homocysteine. MAP, methyl-accepting protein; SAH, *S*-adenosylhomocysteine.

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activities of these analogues toward various small-molecule methylases (e.g., catechol-*O*-methyltransferase, histamine-*N*-methyltransferase, etc.) (12, 13, 15) and macromolecular methylases (e.g., tRNA methylases, mRNA methylases, etc.) (16–21) have also been reported. To date, little information is available concerning the structure-activity relationships for binding of AdoHcy analogues to PCM. Borchardt *et al.* (22) have reported that two naturally occurring, amino acid-modified analogues of AdoHcy, e.g., Sinefungin and A9145c (Table 1) are potent inhibitors of calf thymus and bovine adrenal PCMs. To define further the specificity of the AdoHcy-binding site on PCM and their potential utility in studying the role of PCM *in vivo*, we report here the inhibitory activity of a series of synthetic base-, amino acid-, and sugar-modified analogues of AdoHcy toward the purified calf thymus enzyme and toward protein carboxymethylation in hypothalamic synaptosomes.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (58 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.), and [*methyl*-^{3}H]L-methionine (13 Ci/mmole) was purchased from ICN Pharmaceuticals, Inc. (Plainview, N. Y.). Ovalbumin and L-AdoHcy were purchased from Sigma Chemical Company (St. Louis, Mo.). Sinefungin and A9145c were a generous gift from Dr. R. Nagarajan (Lilly Research Laboratories (Indianapolis, Ind.). The AdoHcy analogues used in the study were synthesized according to previously published procedures as cited below: D-AdoHcy, AdoHcy sulfoxide, AdoHcy sulfone, AdoTpa, and AdoTba (23); 3-deaza-AdoHcy, InoHcy, UriHcy, CytHcy, and N^6-methyl-AdoHcy (24); AdoCys, TubHcy, and 8-aza-AdoHcy (25); AdoHcy dialdehyde and 2′,3-acyclic AdoHcy (26).

2-aza-AdoHcy. 2-Azaadenosine was prepared according to the procedure of Montgomery and Thomas (27). Adenosine was oxidized with 30% hydrogen peroxide in glacial acetic acid to yield adenosine-1-*N*-oxide in 83% yield. The reaction of adenosine-1-*N*-oxide with benzyl bromide afforded 1-benzyloxyadenosine hydrobromide in 72% yield, which was converted to 5-amino-4-(*N*-benzyloxy)carboxamidine-1-β-D-ribofuranosylimidazole by treatment with absolute methanol saturated with ammonia. Reduction of the *N*-benzyloxy compound with Raney nickel (Grace #28) afforded the desired 5-amino-4-carboxamidine-1-β-D-ribofuranosylimidazole, which was cyclized to 2-azadenosine in 45% yield by treatment with sodium nitrite in glacial acetic acid.

2-Azaadenosine (280 mg, 1.04 mmoles) was allowed to react with thionyl chloride (0.45 ml) in 2.8 ml of hexamethylphosphoramide for 20 hr under N_2 using the general conditions previously described (28). The reaction was quenched with approximately 25 ml of water, concentrated *in vacuo*, and applied to a Dowex 50 W-X4 column (20 ml, H^+ form). The column was eluted with water to remove undesired impurities and then eluted with a gradient of NH_4OH (10^{-3}–10^{-1} N).

The desired 5′-chloro-5′-deoxy-2-azaadenosine eluted with 0.1 N NH_4OH. The eluted was concentrated *in vacuo* and the product crystallized to yield 268 mg (90%), m.p. 175–176°, NMR (D_2O) δ 9.00 (s, 1H, H-8), 6.65 (d, J = 5 Hz, 1H, H-1′), 5.35 (t, J = 4 Hz, H-4′), 5.20–4.80 (m, 2H, H-2′, H-3′), 4.35 (d, J = 4 Hz, 2H, H-5′). UV_{max} (95% EtOH) 257 nm (ϵ = 8800), 297 nm (ϵ = 6080).

$C_9H_{11}N_6O_3Cl$
Calculated: C 37.70, H 3.86, N 29.32
Found: C 37.27, H 3.88, N 29.50

The 5′-chloro-5′-deoxy-2-azaadenosine (406 mg, 1.42 mmoles) was allowed to react with L-homocysteine (286 mg, 1.5 Eq.) in sodium and liquid NH_3 according to the general procedure described earlier by our laboratory (28). The 2-aza-SAH was purified first by chromatography of the neutralized reaction mixture on a cellulose column (approximately 20 g) eluting with an ethanol-water gradient from 100% to 75% ethanol. The fractions containing the desired product were concentrated and then applied to an ion exchange column (Dowex 50-X4, NH_4^+ form). The column was first washed with water, followed by 0.1 N NH_4OH, which eluted the desired 2-aza-SAH. Crystallization from water afforded 69 mg (12.6%): m.p. 220–221°; NMR (D_2O) δ 8.70 (s, 1H, H-8), 6.60 (d, J = 5 Hz, 1H, H-1′), 5.5–4.5 (m, 3H, H-2′, 3′, 4′), 4.18 (t, J = 7 Hz, 1H, H-α), 3.38 (d, J = 5 Hz, 2H, H-5′), 3.00 (br t, J = 8 Hz, 2H, H-γ) and 2.50 (br t, J = 8 Hz, 2H, H-β). UV_{max} (H_2O) 257 nm (ϵ = 6,700), 297 (ϵ = 4,800).

$C_{13}H_{19}N_{17}O_5S \cdot H_2O$:
Calculated: C 38.70, H 5.24, N 24.30
Found: C 39.11, H 4.92, N 24.41

N^6-ethyl AdoHcy. 5′-Chloro-5′-deoxy-N^6-ethyladenosine was prepared by reaction of N^6-ethyladenosine with thionyl chloride in hexamethylphosphoramide using the general conditions previously described (28). N^6-Ethyl-AdoHcy was prepared by a modification of the method of Legraverend and Michelot (29). 5′-Chloro-5′-deoxy-N^6-ethyladenosine (315 mg, 1.0 mmole) was added to a solution of L-homocysteine thiolactone (372 mg, 2.4 mmole) in 20 ml of 2.0 N NaOH. The mixture was heated at 80° for 90 min, cooled to room temperature, and neutralized to pH 7.4 with 2.0 N HCl. The mixture was evaporated to dryness *in vacuo*, 20 ml of toluene were added, and the mixture was again taken to dryness. The residue was dissolved in a minimum of water and chromatographed on cellulose eluting with EtOH-H_2O (7:3). This afforded pure N^6-ethyl-AdoHcy, m.p. 194–196° [lit. (29) 193–195°], *m/e* 369 (MH^+-CO_2), 354 (MN^+-CO_2-NH_2), 153 (N^6-ethyl adenine).

Purification and assay of calf thymus PCM. PCM was purified from calf thymus (Pel-Freez Biologicals, Rogers, Ark.) through the DEAE-Sephadex and Sephadex G-100 chromatographic steps according to the procedure of Kim (30). This enzyme preparation was purified 5400-fold as compared with the crude homogenate and exhibited a specific activity of 24.18 nmoles of methylated ovalbumin/min/mg of protein. Protein carboxymethylation activity was assayed using *S*-adenosyl-L-[*methyl*-^{14}C]methionine and ovalbumin as substrates. The assay mixture (0.25 ml) contained 62 mM sodium phosphate-18.8 mM sodium citrate buffer (pH 6.0), 6 mM dithiothreitol, 2 mM EDTA, 750 μg of ovalbumin and 0.5–8.0 μM *S*-adenosyl-L-[*methyl*-^{14}C]-methionine. The reaction mixture was incubated at 37° for 20 min. The reaction was

terminated by adding 0.5 ml of 0.5 M sodium borate (pH 10.0) containing 1% methanol and 3 ml of toluene-isoamyl alcohol (3:2).

Each assay tube was capped and allowed to stand at room temperature for 30 min to complete hydrolysis of the protein methyl ester. Radioactivity in an aliquot (1 ml) of the organic supernatant was determined directly by liquid scintillation spectrometry. The inhibition constants for the AdoHcy analogues were calculated as previously described (18).

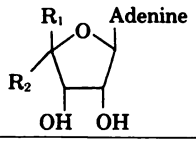
Assay of PCM activity in rat hypothalamic synaptosomes. Synaptosomes were prepared from hypothalami dissected from three or four Sprague-Dawley rats. Tissue was weighed, pooled, and homogenized in 10 ml of 0.32 M sucrose. The homogenate was centrifuged at 11,000 × *g* for 20 min to yield the P_2 or crude synaptosomal pellet. The P_2 pellet was suspended in 3 ml of physiological Buffer A (126 mM NaCl, 2.4 mM KCl, 27.6 mM $NaHCO_3$, 5.9 mM dextrose, 0.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.83 mM $MgCl_2$, and 0.5 mM Na_2SO_4; brought to pH 7.4 by equilibration with 95% O_2-5% CO_2), which contained 26.6 μM [^{3}H-*methyl*]methionine (1.25 mCi). The mixture was incubated at 37° for 10 min, diluted 10-fold with 0.32 M sucrose, and centrifuged at 17,750 × *g* for 20 min. The pellet was suspended in physiological Buffer A and distributed (200 μl) to assay tubes. This [^{3}H-*methyl*]methionine loading procedure provides a means of labeling the synaptosomal pool of AdoMet so that transmethylations can be studied using intact synaptosomes (9, 31).

The effects of AdoHcy analogues on protein carboxymethylation in these [^{3}H-*methyl*]methionine-loaded synaptosomes were determined as follows: To 200 μl of the resuspended synaptosomes previously loaded with [^{3}H-*methyl*]methionine were added either 50 μl of Buffer A (control) or a 0.5 mM solution of the inhibitor in 50 μl of Buffer A. The tubes were then filled with 95% O_2-5% CO_2,

TABLE 1

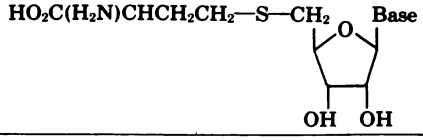
Structural analogues of AdoHcy

Amino acid modifications



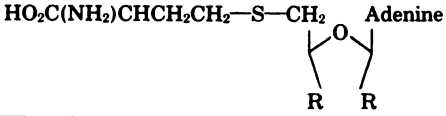
No.	Compound abbreviation	R_1	R_2
1	L-AdoHcy	—CH_2—S—$CH_2CH_2CH(NH_2)CO_2H$(L)	H
2	Sinefungin	—CH_2—$CH(NH_2)CH_2CH_2CH(NH_2)CO_2H$	H
3	A9145c	=CH—$CH(NH_2)CH_2CH_2CH(NH_2)CO_2H$	—
4	D-AdoHcy	—CH_2—S—$CH_2CH_2CH(NH_2)CO_2H$(D)	H
5	AdoHcy sulfoxide	—$CH_2S(O)CH_2CH_2CH(NH_2)CO_2H$(L)	H
6	AdoHcy sulfone	—$CH_2S(O)_2CH_2CH_2CH(NH_2)CO_2H$(L)	H
7	AdoTpa	—CH_2—S—$CH_2CH_2CH_2NH_2$	H
8	AdoTba	—CH_2—S—$CH_2CH_2CH_2CO_2H$	H
9	AdoCys	—CH_2—S—$CH_2CH(NH_2)CO_2H$	H

Base modifications



No.	Compound abbreviation	Base
10	3-Deaza-AdoHcy	3-Deazadenine
11	TubHcy	7-Deazaadenosine
12	8-Aza-AdoHcy	8-Azaadenosine
13	2-Aza-AdoHcy	2-Azaadenosine
14	N^6-Methyl-AdoHcy	N^6-Methyladenine
15	N^6-Ethyl-AdoHcy	N^6-Ethyladenine
16	InoHcy	Hypoxanthine
17	UriHcy	Uracil
18	CytHcy	Cytosine

Sugar modifications



No.	Compound abbreviation	R=
18	AdoHcy dialdehyde	—CHO
19	2′,3′-acyclic AdoHcy	—CH_2OH

capped, and incubated at 37° for 30 min. Zero-time blanks were used. The reactions were terminated by the addition of 10 ml of ice-cold 10% trichloroacetic acid. The tubes were placed in ice for 5 min and the tissue was pelleted by centrifugation. The supernatant was decanted and the pellet was resuspended in 750 μl of 0.5 M sodium borate containing 1% carrier MeOH. The tubes were capped and allowed to stand at room temperature for 30 min and then extracted with 3 ml of toluene-isoamyl alcohol (3:2). An aliquot of the organic phase was counted for radioactivity. Radioactivity was determined in duplicate aliquots of the organic phase directly or after sample evaporation at 90° for 1–2 hr under reduced pressure. The difference between total radioactivity and radioactivity present in the sample after evaporation was taken as a measure of [^{3}H-methyl]MAP formed during the incubation period.

RESULTS AND DISCUSSION

Inhibitory activity of synthetic and natural analogues of AdoHcy toward calf thymus PCM. In order to define the structure-activity relationships for the binding of AdoHcy to PCM, we have evaluated the synthetic and natural analogues of AdoHcy shown in Table 1 as inhibitors of the purified calf enzyme. These synthetic and natural analogues of AdoHcy contained single point modifications where specific functional groups on the base, amino acid or sugar moieties were altered, leaving intact the remaining structural features of the molecule. Evaluation of these analogues as enzyme inhibitors would therefore permit an assessment of the relative importance of specific functional groups on AdoHcy for PCM binding. Each compound was initially screened for its inhibitory activity toward calf thymus PCM at inhibitor concentrations of 0.2 mM and 2.0 mM and AdoMet concentrations of 8 μM (data not shown). Kinetic inhibition constants were determined only for those analogues which exhibited significant activity in these preliminary studies. Similar to other AdoMet-dependent methyltransferases (10), PCM is inhibited by AdoHcy analogues in a reversible, competitive manner when AdoMet is the variable substrate (data not shown). The calculated inhibition constants for the active AdoHcy analogues are listed in Table 2. Of the amino acid modified analogues of AdoHcy, only those with modifications of the sulfur atom showed appreciable inhibitory activity of calf thymus PCM. Both AdoHcy sulfoxide and AdoHcy sulfone exhibited inhibitory activity similar to that of AdoHcy itself. These results are consistent with our earlier observations (22) that the Sinefungin and A9145c, which are naturally occurring, sulfur-modified AdoHcy analogues, are potent inhibitors of this enzyme. For comparative purposes, the data for Sinefungin and A9145c are listed in Table 2. The other synthetic, amino acid-modified analogues of AdoHcy exhibited either weak inhibitory activity (e.g., D-AdoHcy) or were totally inactive (e.g., AdoTpa, AdoTba, AdoCys; data not shown). These data suggest that the structural features of primary importance in binding the amino acid portion of AdoHcy to PCM include (a) the chirality of the amino acid assymetrical carbon atom; (b) the terminal amino group; (c) the terminal carboxy group; and (d) the three-carbon distance between the sulfur atom and the terminal amino and carboxyl groups. The potent inhibitory activity of sulfur-modified analogues, e.g., AdoHcy sulfoxide, AdoHcy sulfone, Sinefungin, and A9145c, suggest that the enzyme can accommodate significant structural changes around the sulfur atom of AdoHcy without dramatically affecting enzymatic binding. The only AdoMet-dependent system which exhibited a similar degree of sensitivity to the sulfur-modified analogues of AdoHcy are virion mRNA (guanine-7)-methyltransferase and mRNA (nucleoside-2′)-methyltransferase (17, 18, 32). The inhibitory potency of Sinefungin toward PCM is substantially greater than that reported toward protein (arginine) methyltransferase (EC 2.1.1.23) [K_i = 3.5 μM (12); 11.1 μM (33)] and protein (lysine) methyltransferase (EC 2.1.1.25) [K_i = 4.9 μM (12); 7.1 μM (33)]. The inhibitory potencies of A9145c, AdoHcy sulfoxide, and AdoHcy sulfone toward the arginine and lysine protein methyltransferases have not been reported to our knowledge.

Of the base modified analogues, only TubHcy, 8-aza-AdoHcy, 2-aza-AdoHcy, and N^6-methyl-AdoHcy (Table 2) exhibited significant inhibitory effects of calf thymus PCM. However, in general, these base-modified analogues were substantially less active than AdoHcy itself, or than the sulfur-modified AdoHcy analogues. The inhibitory activity of TubHcy, 8-aza-AdoHcy, and 2-aza-AdoHcy suggest that minor changes in the adenine ring of AdoHcy produce a significant reduction in enzyme affinity. In addition, the binding site appears to tolerate a small alkyl substituent at the 6-amino group (e.g., N^6-methyl-AdoHcy), but incorporation of a larger substituent at N^6, such as an ethyl group (e.g., N^6-ethyl-AdoHcy), results in total loss of enzyme inhibitory activity. When more drastic structural changes are made in the base moiety, such as replacement of the adenine moiety with a pyrimidine base (e.g., UriHcy, CytHcy) or a purine base, which lacks the 6-amino group (e.g., InoHcy), complete loss of inhibitory activity results (data not shown).

Several of the base-modified analogues of AdoHcy,

TABLE 2

Inhibition of calf thymus protein PCM by analogues of AdoHcy

Purified calf thymus PCM was assayed using AdoMet-$^{14}CH_3$ and ovalbumin as described under Materials and Methods. AdoMet concentrations were varied between 0.5 and 8.0 μM. Each inhibitor showed linear competitive kinetics when plots of the reciprocal velocity versus reciprocal concentrations were made. Assays were performed in duplicate. The inhibition constants were calculated as previously described (20).

Compound	Inhibition constant, K_i ± SE
	μM
L-AdoHcy (1)	1.03 ± 0.01
Sinefungin (2)[a]	0.22 ± 0.03
A9145c (3)[a]	0.024 ± 0.013
D-AdoHcy (4)	34.1 ± 7.34
AdoHcy sulfoxide (5)	1.55 ± 0.149
AdoHcy sulfone (6)	1.39 ± 0.133
TubHcy (11)	14.5 ± 0.98
8-Aza-AdoHcy (12)	93.9 ± 2.8
2-Aza-AdoHcy (13)	42.5 ± 8.5
N^6-Methyl-AdoHcy (14)	19.9 ± 2.6

[a] Data were taken from ref. 22.

e.g., TubHcy and N^6-methyl-AdoHcy, have also shown inhibitory effects toward virion mRNA (guanine-7)-methyltransferase and mRNA (nucleoside-2′)-methyltransferase (18, 32). However, there are several interesting differences between the inhibitor specificities of these macromolecule methyltransferases, including the fact that 3-deaza-AdoHcy, which is a fairly potent inhibitor of the virion mRNA methyltransferases (18, 32), was shown in this study to be inactive as an inhibitor of PCM.

AdoHcy dialdehyde, which we had previously shown to be an affinity-labeling reagent for histamine-*N*-methyltransferase (26), was found to be inactive as an inhibitor of calf thymus PCM. In addition, 2′,3′-acyclic AdoHcy was totally inactive as an inhibitor of this enzyme, suggesting that the rigidity of the ribose ring of AdoHcy contributes significantly to its enzymatic binding. This rigid framework is perhaps necessary to orient properly the adenine and thioether residues for maximal interaction with the protein.

In general, it would appear that calf thymus PCM has rather strict structural requirements for binding the AdoHcy molecule. With the exception of modification around the sulfur atom of AdoHcy, other structural changes appear to diminish greatly the affinity of the enzyme for the AdoHcy analogue.

Effects of AdoHcy analogues on protein carboxymethylation in rat hypothalamic synaptosomes. To evaluate the feasibility of using AdoHcy analogues to inhibit methylation of endogenous MAPs by endogenous PCM, we have used as a test system synaptosomes prepared from rat hypothalamus. In earlier studies (9, 31), we have used this preparation to study the role of protein carboxymethylation in neurosecretory processes. Incubation of synaptosomes with [^{3}H-*methyl*]-L-methionine results in a rapid labeling of the endogenous AdoMet pool and a less rapid formation of [^{3}H-methyl]MAPs (31). By labeling the endogenous AdoMet pool in a short preincubation step with [^{3}H-*methyl*]-L-methionine, it is possible to then follow the formation of [^{3}H-*methyl*]MAPs in synaptsomes with time up to 30 min of additional incubation. However, it should be noted that this is a crude synaptosomal fraction and therefore it is possible that the carboxymethylation observed may result in part from contaminating cellular elements other than synaptosomes.

Using this test system, we determined the effects of inhibitors which were active toward the calf thymus PCM (Table 2) on protein carboxymethylation in hypothalamic synaptosomes. To our surprise we found that the two most active calf thymus PCM inhibitors, Sinefungin and A9145c, were ineffective in inhibiting protein carboxymethylation in intact synaptosomes. In contrast, L-AdoHcy, AdoHcy sulfoxide, AdoHcy sulfone, and 2-aza-AdoHcy produced significant inhibition of protein carboxymethylation in this test system (Table 3).

To rule out the possibility that hypothalamic synaptosomal PCM exhibited a different inhibition specificity from the calf thymus enzyme, these AdoHcy analogues were tested for their ability to inhibit protein carboxymethylation in a lysed hypothalamic synaptosomal preparation using [^{3}H-*methyl*]AdoMet as the methyl donor. In this lysed system, L-AdoHcy, AdoHcy sulfoxide, AdoHcy sulfone, and 2-aza-AdoHcy as well as Sinefungin and A9145c exhibited potent inhibitory effects (Table 3). Since we could not detect metabolism of A9145c or Sinefungin in intact hypothalamic synaptosomes under the assay conditions (data not shown), the only explanation for the differences in the effects seen between intact and lysed synaptosomes must be transport of these natural AdoHcy analogues. In a related study[2] using mouse L-cells, both Sinefungin and A9145c were inactive in inhibiting protein carboxymethylation. However, in lysed mouse L-cells, these AdoHcy analogues produced potent inhibition of AdoMet-dependent macromolecule methylations, suggesting a similar transport problem for A9145c and Sinefungin into this cellular system.

TABLE 3

Effects of AdoHcy analogues on protein carboxymethylation in intact and lysed hypothalamic synaptosomes

For intact synaptosomes, a synaptosome-enriched (P_2) fraction from rat hypothalamic tissue was labeled with [^{3}H-*methyl*]methionine, pelleted, and resuspended in either physiological buffer or physiological buffer containing AdoHcy or AdoHcy analogues (final concentration 100 μM). The samples were incubated for 30 min at 37° and reactions terminated by addition of 10% trichloroacetic acid. [^{3}H-*methyl*]MAP was hydrolyzed with borate buffer and the [^{3}H-*methyl*]methanol determined by liquid scintillation counting. The protein carboxymethylation activity in control samples was 52 ± 6 fmoles of [^{3}H-*methyl*]methyl acceptor protein per milligram of original wet weight of hypothalamic tissue.

For lysed synaptosomes, synaptosome suspensions in buffer were lysed by three cycles of freezing and thawing. Protein carboxymethylation was then followed by incubation of the lysed synaptosomes with 1.4 μM [^{3}H-*methyl*]AdoMet, and [^{3}H-*methyl*]MAP formed was measured as described under Materials and Methods. The protein carboxymethylation activity in control samples was 124 ± 21 fmoles of [^{3}H-*methyl*]methyl acceptor protein per milligram of original wet weight of hypothalamic tissue.

Inhibitor	% Inhibition of protein carboxymethylation[a]	
	Intact synaptosomes	Lysed synaptosomes
L-AdoHcy (1)	71	85
Sinefungin (2)	2	90
A9145c (3)	0	97
AdoHcy sulfoxide (5)	54	82
AdoHcy sulfone (6)	49	85
2-Aza-AdoHcy (13)	42	71

[a] Expressed as percentage inhibition of controls which contained no inhibitor. The indicated values are averages of triplicate determinations.

These results using hypothalamic synaptosomes and mouse L-cells raise an important problem relative to the use of AdoHcy analogues to inhibit endogenous AdoMet-dependent transmethylation. This problem deals with transport of the inhibitor to subcellular compartments containing the target enzyme. In earlier studies, Crooks *et al.* (15) were concerned with the effect of metabolism of AdoHcy analogues on their effectiveness as transmethylation inhibitors. The results reported in this study suggest that an equally important factor determining a transmethylation inhibitor's effectiveness *in vivo* will be its transport and distribution properties.

[2] R. T. Borchardt and S. Thakker, unpublished data.

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