Pharmacology Biochemistry and Behavior, Vol. 66, No. 4, pp. 841–849, 2000

0091-3057/00/$–see front matter

PII S0091-3057(00)00274-4

Farnesyl-L-Cysteine Analogs Block SAM-Induced Parkinson's Disease-Like Symptoms in Rats

NAZARIUS S. LAMANGO AND CLIVEL G. CHARLTON

College of Pharmacy, Florida A& M University, Tallahassee, FL

Received 8 October 1999; Revised 4 February 2000; Accepted 9 March 2000

LAMANGO, N. S. AND C. G. CHARLTON. *Farnesyl-L-cysteine analogs block SAM-induced Parkinson's disease-like symptoms in rats*. PHARMACOL BIOCHEM BEHAV **66**(4) 841–849, 2000.—Injection of the endogenous methyl donor, S-adenosyl methionine (SAM), into rat brain induces Parkinson's disease (PD)-like symptoms possibly by stimulating deleterious protein methylation. Gel-filtration chromatography of rat brain extracts treated with [^{3}H-methyl]-SAM revealed the presence of radioactive peaks with apparent molecular weights of about 5 kDa. Treatment with guanidine HCl altered the elution volumes of the labeled peaks. Lyophilized peak fractions released volatile ^{3}H-methanol on incubation with NaOH, indicating the presence of carboxyl methyl esters. Because prenylated proteins are avid methyl acceptors at the terminal carboxylic acid groups, 1 μmol S-farnesylcysteine (FC) analogs blocked the SAM-induced tremors in the experimental rats. FC analogs did not only reverse the associated rigidity, abnormal posture, and hypokinesia, but stimulated hyperactivity in the animals. This amphetamine-like effect was monitored for 20 min in an animal activity monitor and movement times between 400 ± 100 and 560 ± 125 s covering distances between 78 ± 29 to 125 ± 35 m were recorded for rats treated with FC analogs with or without SAM. Control animals moved only for 60 ± 13 s covering about 6 ± 1 m, indicating a 7–9-fold and 13–21-fold increase in duration of movement and distance covered, respectively. *N*-Acetyl-S-farnesylcysteine (AFC) potentiated amphetamine-induced ipsiversive rotation of 6-hydroxydopamine-lesioned rats from 390 ± 130 to 830 ± 110, with AFC alone having no significant effect on net rotation compared to controls. These data indicate that intracerebroventricular injection of SAM may induce PD symptoms by interfering with the methylation/demethylation homeostasis of prenylated proteins that function in the dopaminergic and other signaling pathways, and that the FC analogs may counteract the SAM effects by acting synergistically on events subsequent to neurotransmitter release. © 2000 Elsevier Science Inc.

Parkinson's disease S-adenosylmethionine Farnesylcysteine 6-Hydroxydopamine Amphetamine Tremor Hypokinesia Rigidity Methylation Abnormal posture

PARKINSON'S disease (PD) is a neurological disorder that is characterized by tremors, rigidity, hypokinesia, and postural abnormalities. Studies indicate that the degeneration of dopaminergic neurons in the substantia nigra of the brain is the main pathological feature. Consequently, low levels of dopamine, the principal transmitter in these neurons, has been reported (13). Replenishing the depleted dopamine has been the main focus of most treatment efforts. Damage to dopaminergic neurons has been replicated in experimental animals through intrastriatal infusion of the neurotoxic agent, 6-hydroxydopamine (6-OHDA). When lesioned on one side, a unilateral impairment of movement in such animals is marked by a unidirectional rotation when injected with amphetamine, due to differential synthesis, storage, and amphetamine-induced release of dopamine (17).

Tremor, the most visible symptom of PD, is not a common feature in either the 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models, suggesting some significant limitations. Because many studies have concentrated on events leading to the synthesis and release of dopamine, defects in such important phenomena as the transmission of signals from the postsynaptic receptors following dopamine receptor binding is largely unexplored for possible links to Parkinsonism. Following release, dopamine initiates a chain

Requests for reprints should be addressed to Dr. C. G. Charlton, College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307.

of events by binding to the postsynaptic dopamine receptor. The dopamine receptors belong to a class of membrane-spanning receptors that interact with the GTP-binding proteins known as heterotrimeric G-proteins (12). Multiple forms of the respective G-protein monomers exist with varying degrees of amino acid sequence similarity. The γ-subunit undergoes the posttranslational modification with either a C15 or a C20 isoprene unit. A consensus amino acid sequence directs the S-prenylation of a cysteine residue that is the fourth amino acid away from the C-terminal. This is followed by the proteolytic removal of the terminal tripeptide (14) and exposing a C-terminal prenylated protein that is capable of undergoing reversible SAM-dependent methylation (22). The γ-subunits, which vary in size from 5 to 7.5 kDa (2), exist in a stable heterodimer complex with the β-subunit, the βγ-complex only transiently interacting with the α-subunit (7). The α-subunit and the βγ-complex interact with various cellular effector enzymes and ion channels (7). For example, the βγ-complex was found to inhibit the activity of Ca^{2+}/calmodulin-stimulated type-I adenylylcyclase, but increased that of stimulatory recombinant Gα-stimulated type-II adenylyl cyclase (16). These effects on the enzyme activities were found to be dependent on the C-terminal prenylation (16). The βγ-complex also binds to and influences the activity of voltage-dependent calcium channels (9) and binds to immobilized Raf-1 protein kinase with a nanomolar dissociation constant (24). It is not clear what role, if any, prenylation and methylation may separately play in these processes.

Prenylcysteine (PC) analogs that mimic the C-terminal portion of the G-protein γ-subunits influence a variety of cellular processes. For example, farnesylthiosalicylate (FTS) increased intracellular calcium concentrations of differentiated HL60 cells, and stimulated superoxide release by HL60 cells and polymorphonuclear leukocytes (30). In neutrophils, farnesylcysteine (FC) analogs either initiated or inhibited superoxide release (10). FC analogs inhibited [^{35}S]GTP[S] binding to washed membranes of myeloid-differentiated HL60 cells (26,27) and capacitative calcium entry into cells (32). Although FC analogs are avid methyl acceptors and/or competitively inhibit the SAM-dependent methylation of prenylated proteins (21), the cellular phenomena outlined above are believed to be independent of methylation (10,26,27).

Our laboratory has previously reported that injection of SAM into the lateral ventricles of rats caused severe PD-like symptoms (4,5,18). Because SAM is an endogenous methyl donor, the belief has been that it may enhance the depletion of dopamine through SAM-dependent enzymatic methylation by cathecol-*O*-methyl transferase. The possibility that other molecules involved with dopamine signaling such as the G-protein γ-subunit may indeed be methylated and account for the observed effects has not been investigated. Methylation has been shown to have a strong influence on some physiological phenomena. A strong dependence on methylation for the activation of phosphatidylinositol-specific phospholipase C has been reported (20). Methylation inhibitors were found to modulate nutrient-induced insulin secretion from rat islets (19), permeabilized HIT-T15 cells (25) and amylase secretion by pancreatic acini (3). Strains of *Schizosaccharomyces pombe* with a defective mam4 gene that encodes the prenylated protein methyl transferase produce nonmethylated, inactive M-factor (15). Demethylation of *S. cerevisiae* a-factor resulted in loss of biological activity (1).

In this article, we report that SAM-induced PD-like symptoms in rats are associated with the in vivo methylation of molecules of about 5 kDa in size. Lyophilized aliquots of the chromatographic fractions containing the methylated products released volatile radioactive groups on incubation with NaOH. The SAM-induced PD tremors were blocked by the injection of FC analogs prior to SAM injection. The FC analogs did not only reverse the hypokinesia, rigidity, and postural abnormalities, but caused most of the injected animals to embark on persistent hyperactivity. Furthermore, the FC analogs significantly increased the amphetamine-induced rotation of 6-OHDA–lesioned rats, thus suggesting that they could be acting on a signaling pathway by synergistically influencing a process triggered by the presence of dopamine in the synapse.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague–Dawley rats, weighing 200–300 g, were obtained from Harlam Laboratories, OH, and maintained under laboratory conditions as previously described (6). SAM (chloride salt), *N*-acetyl-l-cysteine, 3-mercaptopropionic acid, thiosalicylic acid, *trans,trans*-farnesyl bromide, Sephacryl S-100-HR (Pharmacia Biotech, Upssala, Sweden), gel-filtration molecular weight standards, *d*-amphetamine sulfate, and 6-hydroxydopamine were purchased from Sigma Chemical Co., St. Louis, MO. S-[methyl-^{3}H]-SAM was supplied by DuPont NEN Research Products, Boston, MA.

N-acetyl-S-farnesyl-L-cystine (AFC), S-farnesylthiopropionic acid (FTP), and farnesylthiosalicylic acid (FTS) were synthesized as previously described (29). These were purified by silica gel chromatography, and shown by TLC to be over 95% pure. Structures were confirmed by proton and ^{13}C-NMR.

Monitoring of SAM-Induced Tremors

Rats were cannulated for subsequent injection into the lateral ventricle as previously described (6). Briefly, each rat was anesthetized with 400 mg/kg chloral hydrate. A stainless steel guide cannula was stereotaxically placed 1.5 mm lateral and 0.6 mm caudal to the bregma, with the tip extended to the inner surface of the cranium, above the dura mater. At least 2 days following cannulation, injections were made through the cannula into the lateral ventricle. Tremors were measured over a 20-min period on a Tremor-Scan Monitor (AccuScan Electronics, Inc., Columbus, OH) as described previously (18).

Labeling and Extraction of Methylated Products

At least 2 days following cannulation, rats were injected with 10 μCi of [methyl-^{3}H]-SAM in PBS over a 20-min period. This was followed by an immediate injection of SAM (2 μmol) dissolved in PBS to induce tremors. The rats were sacrificed, and the brains removed over ice and quickly homogenized in ice-cold PBS (2 ml/g of tissue, Polysciences Model X520). These were centrifuged at 25,000 × *g* (Beckman J2-MC Centrifuge, JA-20 rotor) for 30 min. The supernatant was kept frozen for subsequent analysis while the pellet was resuspended in PBS and centrifuged as before. The supernatant was removed, and the pellet resuspended in PBS containing 1% Triton X-100, agitated on ice for 1 h and centrifuged as before. The soluble fraction (Triton X-100 extract) was frozen for subsequent analysis.

Protein Determination

The bicinchoninic acid method (Pierce, Rockford, IL) was used according to the manufacturer's instructions.

Gel-Filtration Analysis

Aliquots (5 ml) of the supernatant (130 mg of protein) and the Triton X-100 extract (180 mg of protein) containing labeled molecules were separated on Sephacryl S-100-HR (Pharmacia Biotech, Upssala, Sweden) packed in an XK $^{16}/_{100}$ FPLC column (Pharmacia, 1.6 cm i.d. by 100 cm, 195 ml) that had been equilibrated with 20 mM Na_3PO_4, pH 7.4 containing 0.1% Triton X-100 and 0.2 M NaCl (buffer A). Identical amounts of the supernatant and Triton X-100 extracts were treated with 6 M guanidine HCl prior to analysis by gel-filtration chromatography. Elution was achieved with buffer A pumped at a flow rate of 2 ml/min by an LKB 2150 HPLC pump. Aliquots (100 μl) of the 4-ml fractions obtained from each chromatographic run were analyzed for radioactivity by scintillation counting.

Determination of Carboxyl Methyl Esters

The procedure was an adaptation from Philips et al. (23). Essentially, 100-μl aliquots of fractions 16, 38, and 42 of the native and denatured runs of both the supernatant and the membrane extracts were lyophilized in microcentrifuge tubes. NaOH (50 μl, 1 M) was than added and the open tubes placed in scintillation vials containing scintillation fluid. The scintillation vials were the capped and incubated at 37°C overnight so that any labile radiolabeled methyl esters could be hydrolyzed to yield tritiated methanol that evaporated into the scintillation fluid. This was then measured by scintillation counting.

Methylation of FC Analogs

AFC, FTP, and FTS (200 μM) were each incubated for 60 min with 40,000 × *g* rat brain membranes (240 μg of protein) at 37°C in 100 mM Tris-HCl, pH 7.4 containing SAM (1 μM, 1.3 μCi [^{3}H-methyl]-SAM), 5 mM $MgSO_4$ and 0.1 M. NaCl in a total incubation volume of 400 μl. The reactions were stopped by the addition of 5% TFA and chilled on ice. Hexane (400 μl) was added and the contents of the tubes mixed thoroughly. This was followed by centrifugation at 20,000 × *g* for 2 min. The upper hexane layer was removed and 250 μl analyzed by reversed-phase HPLC on a Whatman column (C18, 4.6 × 150 mm). Methylated FC analogs were eluted from the column at a flow rate of 1 ml/min with a 0 to 100% gradient of acetonitrile in 0.1% TFA from the 3rd to the 15th min of each run. This composition was maintained for a further 2 min before reequilibration of the column for the next sample. Detection was conducted with an online radiochromatographic detection system (INUS β-ram, Tampa, FL).

Effect of FC Analogs on SAM-Induced Tremors

AFC, FTP, and FTS were each suspended in PBS and dissolved by adjusting the pH to 7.4 with NaOH followed by the addition of more PBS to make 0.2 mmol/ml solutions. PBS or each FC analog, dissolved in 5 μl of solution, was injected into the lateral ventricle of cannulated rats 5 min prior to SAM injections. SAM (1 μmol in 5 μl of PBS) was injected, and the movements recorded over a 20-min period as described above.

Effect of FC Analogs on the Movement of Rats

Cannulated rats were injected icv with AFC or FTP (1 μmol, 5 μl) in combination with PBS or 1 μmol of SAM, injected similarly 5 min after injection of the FC analogs. Their movements were measured in an animal activity monitor (AccuScan Electronics, Inc., Columbus, OH) and compared to those of control animals that received either PBS alone or PBS and SAM.

Effect of FC Analogs on Amphetamine-Induced Rotation of 6-Hydroxydopamine-Lesioned Rats

Lesions were induced on the left striatum by injecting with 6-OHDA (32 μg, 10 μg/μl) 0.7 mm anterior and 2.8 mm lateral relative to bregma and 5 mm into the cranium at a rate of

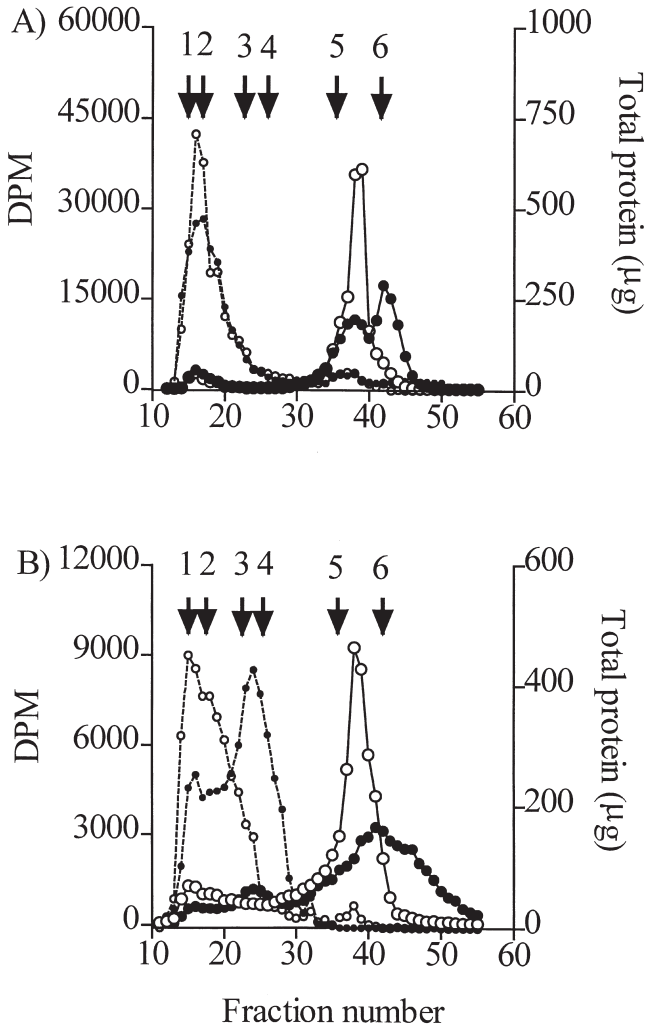

FIG. 1. In vivo methylation of cellular components. Rats were cannulated for subsequent injection into the lateral ventricle. In vivo labeling with [^{3}H-methyl]-SAM (10 μCi in PBS) followed by the preparation of brain supernatant and membrane extracts was as described in the Method section. Aliquots of the supernatant (130 mg protein, A) and Triton X-100 membrane extract (180 mg of protein, B) were separated by gel-filtration chromatography. Chromatography was conducted either on the native samples (●) or aliquots that had been denatured by treatment with 6 M guanidine HCl (○). The radioactivity content (solid lines) and the total protein (dashed lines) of the eluted fractions was determined by scintillation counting and Bradford protein assay, respectively. The elution points of the molecular weight standards are denoted by the numbers 1: blue dextran, 2: BSA (66 kDa), 3: carbonic anhydrase (29 kDa), 4: cytochrome C (12.4 kDa), 5: aprotinin (6.5 kDa) and 6: tyrosine (0.18 kDa).

1 μl/min. The rats were than cannulated for subsequent injections into either the left or right lateral ventricle of the brain. One week after intrastriatal injections of 6-OHDA, the animals received an intraperitoneal (IP) injection of either PBS or amphetamine (5 mg/ml, 5 mg/kg). The same animals also received PBS or AFC (1 μmol in 5 μl solution) delivered either into the left or the right lateral ventricle. The rotation of each animal was determined using the RotoScan Rotometer System (AccuScan Instruments, Inc., Columbus, OH).

Statistical Analysis

Tremor, movement, and rotational behavior data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparisons using the GraphPad Prism computer software program (GraphPad Software, Inc., San Diego, CA). A p-value of less than 0.05 was considered to be significant.

RESULTS

In Vivo SAM-Dependent Methylation

We have previously reported that SAM induced PD-like symptoms within about 2 min after injection of micromole amounts into the lateral ventricles of rats (18). To understand whether these behavioral changes could be due to biochemical alterations of macromolecular structures, cannulated rats were injected with radiolabeled SAM followed by extraction and analysis by gel-filtration chromatography as described in the Method section. As shown in Fig. 1A, scintillation counting of aliquots of the fractions obtained by gel-filtration of a portion of the brain supernatant revealed two main peaks of radioactivity. One of these peaks eluted with an apparent molecular weight of 5 kDa, while the second eluted with the total volume. Only one main peak that eluted with an apparent molecular weight of 5 kDa was observed when an equal amount of the brain supernatant was first treated with 6 M guanidine HCl before gel-filtration analysis. Analysis of the native Triton X-100 extract of the membranes revealed the presence of a single broad peak that extended beyond the total volume but centered around 5 kDa. However, denaturing with guanidine HCl before gel-filtration analysis resulted in a sharper peak with an apparent molecular weight value of around 5 kDa (Fig. 1B). Other minor methylation products with smaller elution volumes and, therefore, of higher apparent molecular masses were observed both in the supernatant and membrane fractions.

Detection of Carboxyl Methyl Esters in the Radioactive Peak Fractions

SAM-dependent methylation of carboxylic acid groups as occurs in proteins results in carboxyl methyl esters. These are base-labile, and are hydrolyzed under basic conditions (23,28). To determine whether the peaks of radioactivity that eluted from the chromatographic column contained such esters, 100-μl aliquots of fractions 16, 38, and 42 of the native and denatured runs of both the supernatant and the membrane extracts were lyophilized in microcentrifuge tubes. Treatment of the lyophilized samples as described in the Method section followed by scintillation counting revealed that a significant portion of the labeled products were indeed labile methyl esters (Fig. 2).

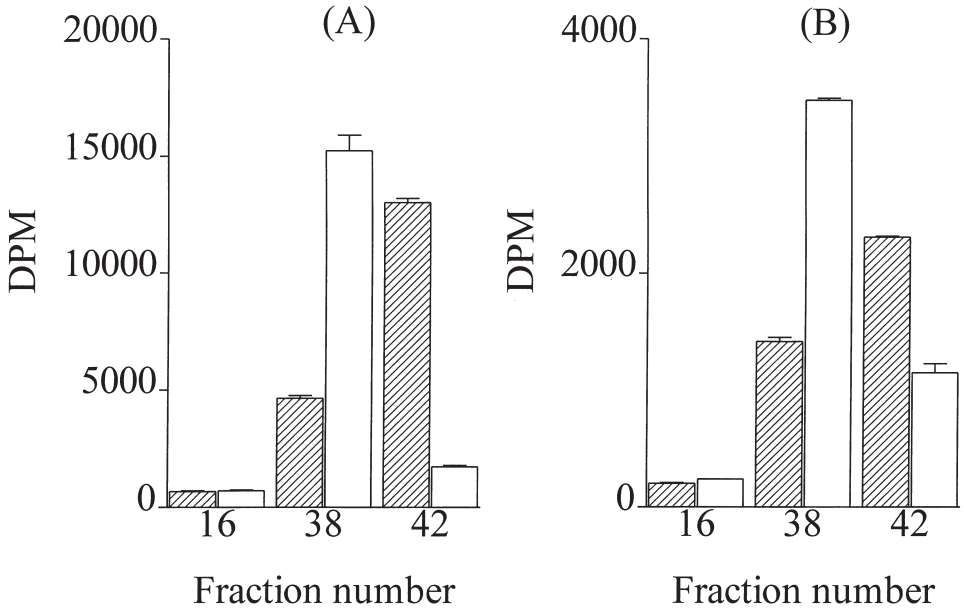

FIG. 2. Carboxyl methyl esters in radioactive peak fractions. Aliquots (100 μl) of radioactivity peak fractions from the different chromatographic runs (Fig. 1) were lyophilized in microcentrifuge tubes. NaOH (1 M, 50 μl) was added and each uncapped tube was placed into a scintillation vial containing scintillation fluid. The vials were capped and incubated overnight at 37°C so that volatile [^{3}H]-methanol could evaporate into the scintillation fluid. The volatilized radioactivity from the supernatant (A) and membrane extract (B) was determined by scintillation counting. Hatched and open bars represent aliquots from the indicated fractions from the chromatographic runs using native and denatured samples, respectively (± SEM, $n = 3$).

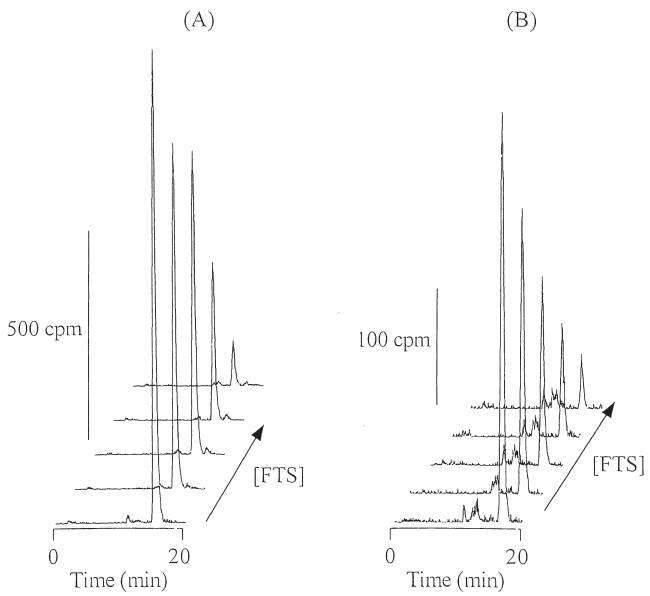

FIG. 3. SAM-dependent methylation of AFC and FTP by rat brain membranes: inhibition by FTS. AFC (A) and FTP (B) were incubated with [^{3}H-methyl]SAM, rat brain membranes (240 μg) and increasing concentrations of FTS (front to back; 0, 25, 50, 100, and 200 μM) for 60 min under the conditions described in the Methods section. Reactions were stopped with 5% TFA, the methylated FC analogs were extracted with hexane and analyzed by reversed-phase HPLC with online radiochromatographic detection.

Methylation of FC Analogs

Based on the above data, it was deemed possible that the SAM-induced symptoms could be as a result of excessive methylation of prenylated proteins. DA neurons are affected in PD and L-DOPA, a DA precursor, is used to remedy the effects of PD. DA neurotransmission is through DA receptors that function in association with trimeric G-proteins, the γ-subunits of which are prenylated, range in size between 5 and 7 kDa (2), and are capable of undergoing reversible C-terminal carboxylmethylation. The fact that some of the in vivo methylated peaks were around 5 kDa and gave off volatile radioactive groups in basic medium suggested the presence of carboxyl methyl esters. For this reason, AFC, FTP, and FTS, which chemically and functionally mimic the C-terminal prenylated portion of the Gγ-subunit, were synthesized. AFC and FTP were shown to be avid methyl acceptors when incubated with rat nerve cell membranes in the presence of [^{3}H-methyl]SAM. Although FTS was never methylated, it dose dependently inhibited the methylation process (Fig. 3).

FC Analogs Block SAM-Induced PD-Like Symptoms

When SAM was injected into the lateral ventricles of cannulated rats, tremors, hypokinesia, rigidity, and abnormal posture were routinely observed soon after injection. On the contrary, PBS-treated rats showed no behavioral changes and activity recordings on the tremor monitor revealed patterns characteristic of random movement and grooming. As shown in Fig. 4A, mainly low-amplitude signals were recorded. The onset of tremors in the SAM-treated animals was often preceded by rigidity, spreading of the limbs, and assumption of a posture in which the rats rested their ventral surface on the floor of the cage. Tremor monitor recordings of this activity showed characteristic patterns marked by periods of low-intermediate amplitude punctuated by high-amplitude signals (Fig. 4B). The low-amplitude signals were background signals characteristic of the periods without tremors, with the animal displaying severe rigidity, hypokinesia, and abnormal posture, while the high amplitudes were associated with tremor epi-

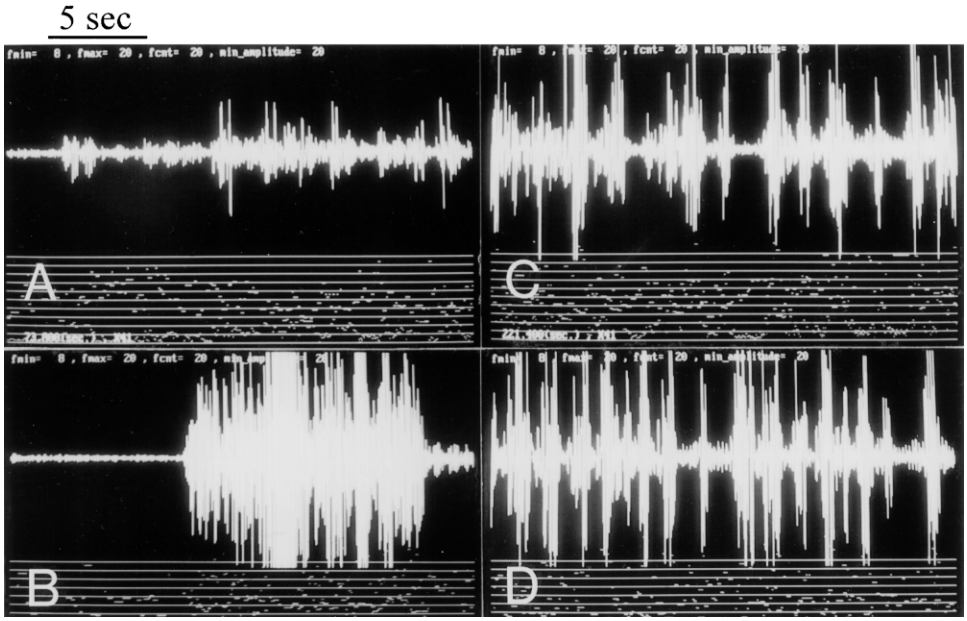

FIG. 4. Sections of recorded movements of treated rats. Rats were cannulated as described in the Method section. At least 2 days after cannulation, they were either treated with PBS alone (A), 1 μmol of SAM preceded 5 min earlier by PBS (B), 1 μmol of AFC (C), or 1 μmol of FTP (D).

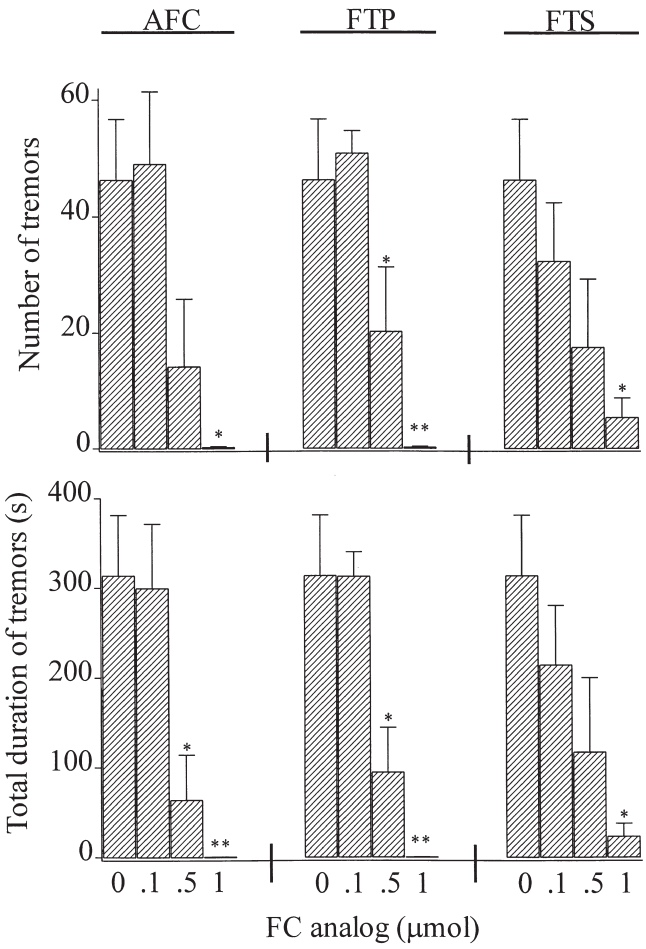

FIG. 5. FC analogs inhibit SAM-induced tremors. Cannulated rats were injected ICV with the indicated doses of the respective FC analogs. Five minutes later, 1 μmol of SAM was administered, and the movements of each animal recorded on the tremor monitor for 20 min. All animals were observed for tremors, and the recorded data for those observed to have suffered from tremors episodes were analyzed for the number (A) and duration (B) of the tremors using previously established parameters (18), $*p < 0.05$, $**p < 0.01$ when compared to animals that received no FC analog prior to 1 μmol SAM by one-way ANOVA followed by Newman–Keuls multiple comparisons. The results are the means ± SEM, $n = 6$.

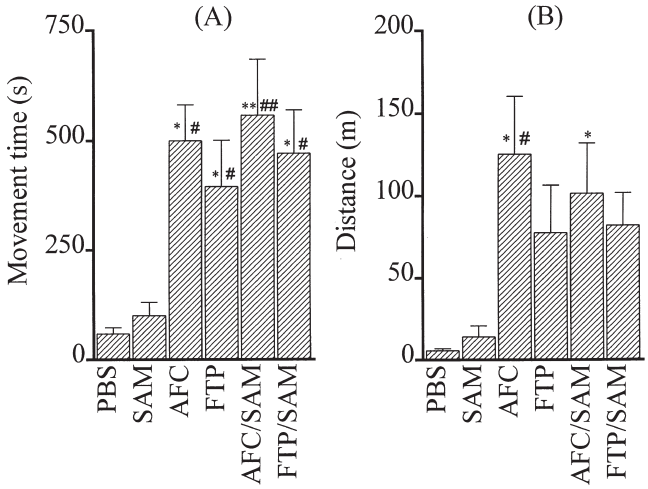

FIG. 6. FC analogs increase the mobility of PBS and SAM-treated animals. Cannulated rats were injected icv either with PBS alone, PBS and 1 μmol of AFC or FTP, 1 μmol of AFC, or FTP, followed 5 min later by 1 μmol of SAM. The mobility of each animal as indicated by the movement time (A) and the distance covered (B) was monitored over a period of 20 min in an animal activity monitor, $*\#p < 0.05$, $**\#\#p < 0.01$ when compared to PBS-only and SAM-only controls, respectively, by one-way ANOVA followed by Newman–Keuls multiple comparisons.

sodes. This behavior observed in SAM-treated animals was completely abolished if the animals were injected with 1 μmol each of FC analog 5 min before the injection of SAM. In this case, most of the animals embarked on an unusually high level of activity, rushing from one side of the cage to the other. Activity recordings of the animals whose treatment with SAM was preceded by treatment with AFC (Fig. 4C) or FTP (Fig. 4D) displayed a unique characteristic that resembled neither that of the PBS-only nor the SAM-only–treated rats. The repeated cycles of high-amplitude alternating with low-amplitude signals, respectively, depict running movements as the animals rushed from one side of the cage to the other. This kind of activity was observed in about 70% of the animals studied. In the remaining animals, the FC analogs blocked the PD-like symptoms without causing hyperactivity. This indicates that suitable dosages of FC analogs that will block PD symptoms without causing unwanted potential side effects can be obtained by titration.

The tremor activity of rats treated with 0, 0.1, 0.5, and 1 μmol of AFC, FTP, or FTS followed by 1 μmol of SAM was quantified over 20 min on the tremor monitor. As shown in Fig. 5, control rats (0 μmol FC analog) suffered on average 46 ± 10 tremors lasting 310 ± 70 s. On the contrary, no tremors were observed when rats were first treated with 1 μmol of either AFC or FTP and significantly less tremors when FTS was administered. Reduced doses of FC analogs were less effective with significant reduction of tremors only afforded by 0.5 μmol of AFC and FTP but not FTS (Fig. 5).

FC Analogs Induce Hyperactivity in Rats

One characteristic feature of PD is hypokinesia, which is marked by a decreased ability to initiate and continue movement. This and previous studies (8) revealed that this is also a routine feature of SAM-treated rats. However, this was never observed in rats that received 1 μmol of FC analog prior to treatment with SAM. Instead, an opposite effect marked by a significantly higher degree of movement was observed in rats injected with either AFC or FTP alone or in combination with 1 μmol of SAM. When these movements were measured in an animal activity monitor for a period of 20 min, it was found that rats that received FC analogs with or without SAM spent between 400 ± 100 s (FTP only) and 560 ± 125 s (AFC plus SAM) moving around the cage covering distances between 78 ± 28 and 125 ± 35 m. These values were 7–9-fold and 13–21-fold higher than those registered for the control animals that spent only about 60 ± 13 s in motion covering a distance of only 6 ± 1 m (Fig. 6).

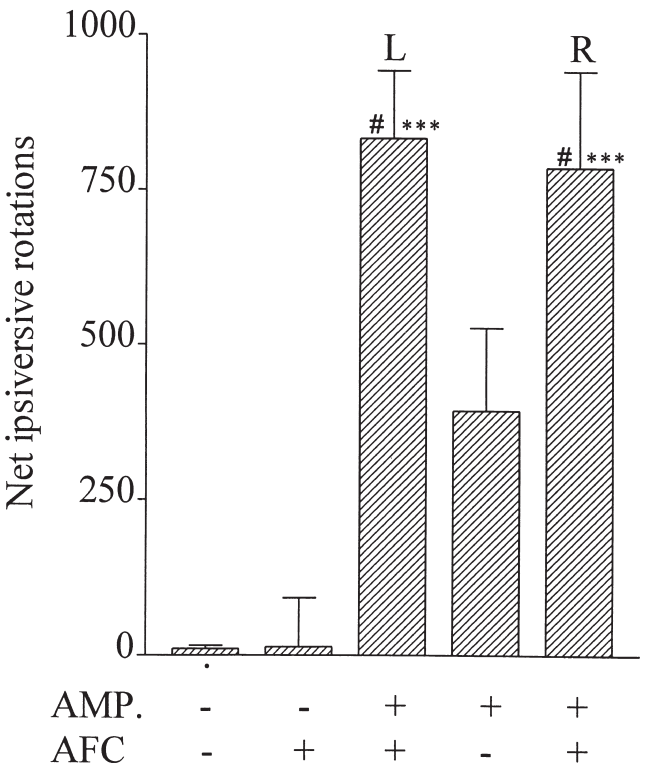

FIG. 7. FC analogs enhance the amphetamine-induced ipsiversive rotation of 6-hydroxydopamine–lesioned rats. Rats were lesioned by an acute injection of 6-OHDA into the left striatum as described in the Methods section. They were then cannulated for subsequent injections into ether the left (L) or the right (R) lateral ventricle of the brain following the same coordinates as described in the Methods section. A week later, intraperitoneal injection of either PBS or amphetamine (AMP) followed immediately by an ICV injection of 1 μmol AFC were made. The net ipsiversive rotations (360° turns to the lesion side) were determined in a RotoScan Rotometer System, $\#p < 0.05$, $***p < 0.001$ when compared to animals that received amphetamine alone, or PBS or AFC controls, respectively, by one-way ANOVA followed by Newman–Keuls multiple comparisons. The results are the means ± SEM, $n = 6$.

FC Analogs Potentiate the Amphetamine-Induced Ipsiversive Rotation of 6-Hydroxydopamine–Lesioned Rats

Intrastriatal injections of 6-OHDA causes the destruction of dopaminergic neurons in the striatum, resulting in a reduction of dopamine synthesis in the lesioned side. Differential release of dopamine between the intact and lesioned sides following IP administration of amphetamine results in rotational behavior towards the side of the lesion. Such ipsiversive rotation was observed when rats were injected with amphetamine 7 days after intrastriatal infusion with 6-OHDA. Rats injected IP with amphetamine displayed 390 ± 130 ipsiversive rotations over 90 min. The rotation increased over twofold to 790 ± 160 (203%) and 830 ± 110 (213%) when rats received amphetamine and injections of AFC (1 μmol in 5 μl of PBS) into either the right or left lateral ventricle, respectively. PBS or AFC alone induced only few net rotations (Fig. 7). It should be noted, however, that AFC alone did cause significant rotational movements on individual animals. Because these rotations were either ipsiversive or contraversive, the combined results for replicate animals resulted in net rotation values that were insignificant from the controls (Fig. 7).

DISCUSSION

Functional deficiencies in the dopaminergic system are believed to be responsible for the motor impairments observed in PD. Previous work from this laboratory and the present study shows that SAM induces symptoms that resemble those of PD (4,8,18). Because SAM is a methyl donor in several cellular processes, the observed symptoms could be due to dopamine depletion through SAM-dependent methylation by catechol-*O*-methyl transferase. In the present study, we reveal that both soluble and membrane-associated macromolecules are methylated following in vivo exposure to SAM. Although these molecules have not yet been identified, protein carboxylmethylation may be involved, because lyophilized aliquots of the radioactive peak fractions gave off [^{3}H]methanol on incubation in sodium hydroxide solution; protein carboxyl methyl esters are readily hydrolyzed in basic solutions (28). Also the chromatographic mobility on the gel-filtration column indicates that the methylated molecules involved are of sufficiently high molecular weight to elute within the 1 and 100 kDa fractionation range of the column. The fact that some of the methylated products eluted beyond the total volume, which is the point at which all molecules should have eluted indicates possible interaction between the molecules and the solid medium. The βγ-complex of trimeric G-proteins was shown to interact with a gel-filtration column in this manner (11). Because this interaction with the column packing was reversed by treatment with guanidine HCl, it is possible that the interaction between the methylated products and the chromatographic medium might have been through a complex with another molecule. The effect on the elution of the methylated molecules by 6 M guanidine HCl could also imply that sufficiently large molecules with complex three-dimensional structures may be involved. Intricate intra- and/or intermolecular interactions would have been disrupted by treatment with guanidine HCl. The resulting alterations in size and/or shape of the molecules would have altered the elution volumes of the methylated compounds as observed.

Proteins that are prenylated at their carboxyl terminals are capable of accepting methyl groups, and play significant roles in the signalling pathways of transmitters and hormones such as dopamine, adrenaline, and serotonin. The γ-subunit of this complex is isoprenylated, exists in a dimeric complex with the β-subunit, and is capable of undergoing reversible carboxyl methylation (22). The prenylation also accounts for the activation or inhibition of various adenylyl cyclases (16), thus indicating that the modified C-terminal end may contain significant functional information. Based on this knowledge and the fact that a molecule of about 5 kDa that possibly exists in a complex was carboxylmethylated following in vivo treatment with SAM, we sought to understand whether FC analogs would have any effect on the SAM induced PD-like symptoms. FC analogs have been shown to influence various cellular phenomena including protein methylation (21), ion transport (32), and superoxide production (10). If the methylation of prenylated proteins was involved, FC analogs may inhibit the process and reverse the SAM-induced effects. Because the FC analogs reversed the PD-like symptoms, this suggests that excessive methylation of prenylated proteins may alter the pattern of isoprene-mediated protein–protein interactions, thus precipitating the observed PD-like symptoms. The fact that the FC analogs could reverse the hypokinesia to the

point of inducing hyperactivity indicates that normal behavioral movements may require a carefully regulated balance between methylated and unmethylated prenylated proteins.

The potentiation of amphetamine-induced rotation of 6-OHDA–lesioned rats, the lack of significant net rotational behavior attributable to AFC alone points to the fact that AFC could only be influencing a process triggered by the binding of dopamine to the receptor. This is further supported by the fact that the FC analogs stimulated dopaminergic-like hyperactivity in rats. Released dopamine binds to its postsynaptic receptors, inducing a chain of intracellular events that involve the trimeric G-proteins, of which the γ-subunit is prenylated. Although it is unclear at this point where the FC analogs may be exerting the observed physiological effects, the fact that they had a significant rotational effect only after amphetamine-induced dopamine release suggests that it could be affecting a process downstream from the receptor. This is further supported by the fact that no significant difference was observed between 6-OHDA–lesioned animals that were injected with AFC either into the left or the right lateral ventricle; suggesting that the damaged presynaptic neurons were not the primary site for FC analog action. Postsynaptic events could be at the level of second-messenger/effector sites where the G-protein βγ-complex interacts with effector enzymes and ion channels as well as receptors. These results are in agreement with previously published work showing that FC analogs and compounds that influence methylation also affect physiological processes triggered through G-protein anchored receptor systems (3,25–27).

The G-protein γ-subunits are modified by geranylgeranylation, which are C20 isoprene groups. Enzyme studies on the methylation of prenylcysteine analogs reveal about a fourfold higher affinity by the prenylated protein methyltransferase (PPMTase) for the C20 geranylgeraylated over the C15 farnesylated substrates (31). If the effects of the FC analogs employed in this study are due to interference with the brain G-protein system, this may imply that the doses required to have similar effects could be significantly reduced if the C20 prenyl analogs were used to exploit their higher affinity for the effector sites. The C20 analogs were found to be more effective at inducing insulin release by permeabilized HIT-T15 cells than the C15 analogs while the C10 derivatives were ineffective (25).

In summary, we have shown that SAM induces symptoms that resemble those found in PD. Use of [^{3}H-methyl]SAM revealed the methylation of molecules of about 5 kDa that released radioactive methyl groups on incubation in a basic medium. The PD-like symptoms were blocked by FC analogs, which are modeled on the C-terminal prenylated portion of the Gγ-subunits of trimeric G-proteins that are involved in the signaling by such molecules as dopamine, which is a neurotransmitter whose activity is largely implicated with the symptoms of PD. The FC analogs did not only reverse the hypokinesia of the SAM-injected rats but had significant amphetamine-like hyperactivity over and PBS-only–treated rats. The behavioral effects induced by SAM and those caused by the overwhelming presence of FC analogs suggests the importance of a careful balance between the methylated and unmethylated moieties in maintaining normal behavioral movements. The potentiation of net ipsiversive rotation by 6-OHDA–lesioned rats by AFC only in the presence of amphetamine indicates that these compounds may be influencing events preceded by the release of dopamine and/or other neurotransmitters into the synapse. SAM-dependent methylation may adversely affect these events thereby precipitating the PD-like symptoms as observed. This points to the fact that excessive methylation or a progressive inability to regulate the levels of methylated products such as by the downregulation of demethylating enzymes may be involved in human PD.

ACKNOWLEDGEMENTS

This work was supported by NIH Grants NS28432, NS31177 and G12RR03020.

REFERENCES

1. Anderegg, R. J.; Betz, R.; Carr, S. A.; Crabb, J. W.; Duntze, W.: Structure of *Saccharomyces cerevisiae* mating hormone a-factor. J. Biol. Chem. 263:18236–18240; 1988.
2. Cali, J. J.; Balcueva, E. A.; Rybalkin, I.; Robishaw, J. D.: Selective tissue distribution of G protein γ subunits, including a new form of the γ subunits identified by cDNA cloning. J. Biol. Chem. 267:24023–24027; 1992.
3. Capdevila, A.; Decha-Umphai, W.; Song, K.-H.; Borchardt, R. T.; Wagner, C.: Pancreatic exocrine secretion is blocked by inhibitors of methylation. Arch. Biochem. Biophys. 345:47–55; 1997.
4. Charlton, C. G.; Crowell, B., Jr.: Parkinson's Disease-like effects of S-adenosyl-L-methionine: Effects of L-dopa. Pharmacol. Biochem. Behav. 43:423–431; 1992.
5. Charlton, C. G.; Crowell, B., Jr.: Striatal dopamine depletion, tremors and hypokinesia following the intracranial injection of S-adenosylmethionine, a possible role of hypermethylation in Parkinsonism. Mol. Chem. Neuropathol. 26:269–283; 1995.
6. Charlton, C. G.; Mack, J.: Substantia nigra degeneration and tyrosine hydroxylase depletion caused by excess S-adenosylmethionine in the rat brain. Mol. Neurobiol. 9:149–161; 1994.
7. Clapham, D. E.; Neer, E. J.: G protein βγ subunits. Annu. Rev. Pharmacol. Toxicol. 37:167–203; 1997.
8. Crowell, B. G., Jr.; Benson, R.; Schockley, D.; Charlton, C. G.: S-adenosyl-L-methionine decreases motor activity in the rat: Similarity to Parkinson's disease-like symptoms. Behav. Neural. Biol. 59:186–193; 1993.
9. De Waard, M.; Liu, H.; Walker, D.; Scott, V. E. S.; Gurnett, C. A.; Campbell, K. P.: Direct binding of G-protein βγ complex to voltage-dependent calcium channels. Nature 385:446–450; 1997.
10. Ding, J.; Lu, D. J.; Perez-Sala, D.; Ma, Y. T.; Maddox, J. F.; Gilbert, B. A.; Badwey, J. A.; Rando, R. R.: Farnesyl-L-cysteine analogs can inhibit or initiate superoxide release by human neutrophils. J. Biol. Chem. 269:16837–16844; 1994.
11. Fukada, Y.; Matsuda, T.; Kokame, K.; Takao, T.; Shimonishi, Y.; Akino, T.; Yoshizawa, T.: Effects of carboxyl methylation of photoreceptor G protein γ-subunit in visual transduction. J. Biol. Chem. 269:5163–5170; 1994.
12. Gudermann, T.; Kalkbrenner, F.; Schultz, G.: Diversity and selectivity of receptor-G protein interaction. Annu. Rev. Pharmacol. Toxicol. 36:429–459; 1996.
13. Honykiewicz, O.: Dopamine (3-hydroxytyramine) and function. Pharmacol. Rev. 18:925–964; 1966.
14. Hrycyna, C. A.; Clarke, S.: Maturation of isoprenylated proteins in *Saccharomyces cerevisiae*. J. Biol. Chem. 267:10457–10464; 1992.
15. Imai, Y.; Davey, J.; Kawagishi-Kobayashi, M.; Yamamoto, M.: Genes encoding farnesyl cysteine carboxyl methyltransferase in *Schizosaccharomyces pombe* and *Xenopus laevis*. Mol. Cell. Biol. 17:1543–1551; 1997.
16. Iniguez-Lluhi, J. A.; Simon, M. I.; Robishaw, J. D.; Gilman, A. F.: G protein βγ subunits synthesized in SF9 cells: Functional characterization and significance of prenylation of γ. J. Biol. Chem. 267:23409–23417; 1992.

17. Jones, B. E.; Boylan, C. B.; Fritsche, M.; Juhasz, M.; Jackson, C.; Wiegand, S. J.; Hyman, C.; Lindsay, R. M.; Altar, C. A.: A continuous striatal infusion of 6-hydroxydopamine produces a terminal axotomy and delayed behavioral effects. Brain Res. 709:275–284; 1996.
18. Lamango, N. S.; Nesby, R. A.; Charlton, C. G.: Quantitative determination of S-adenosylmethionine-induced tremors: A possible tremor model for Parkinson's disease. Pharmacol. Biochem. Behav. (in press).
19. Metz, S. A.; Rabaglia, M. E.; Stock, J. B.; Kowluru, A.: Modulation of insulin secretion from normal rat islets by inhibitors of the post-translocational modifications of GTP-binding proteins. Biochem. J. 295:31–40; 1993.
20. Parish, C. A.; Smrcka, A. V.; Rando, R. R.: Functional significance of βγ-subunit carboxymethylation for the activation of phospholipase C and phosphoinositide 3-kinase. Biochemistry 34:7722–7727; 1995.
21. Perez-Sala, D.; Gilbert, B. A.; Tan, E. W.; Rando, R. R.: Prenylated protein methyltransferases do not distinguish between farnesylated and geranylgeranylated substrates. Biochem. J. 284:835–840; 1992.
22. Perez-Sala, D.; Tan, E. W.; Canada, F. J.; Rando, R. R.: Methylation and demethylation reactions of guanine nucleotide-binding proteins of retinal rod outer segments. Proc. Natl. Acad. Sci. USA 88:3043–3046; 1991.
23. Philips, M. R.; Pillinger, M. H.; Staud, R.; Volker, C.; Rosenfeld, M. G. Weissmann, G.; Stock, J. B.: Carboxyl methylation of ras-related proteins during signal transduction in neutrophils. Science 259:977–980; 1993.
24. Pumiglia, K. M.; Levine, H.; Haske, T.; Habib, T.; Jove, R.; Decker, S. J.: A direct interaction between G-protein βγ subunits and the Raf-1 protein kinase. J. Biol. Chem. 270:14251–14254; 1995.
25. Regazzi, R.; Sasaki, T.; Takahashi, K.; Jonas, J.-C.; Volker, C.; Stock, J. B.; Takai, Y.; Wollheim, C. B.: Prenylcysteine analogs mimicking the C-terminus of GTP-binding proteins stimulate exocytosis from permeabilized HIT-T15 cells: Comparison with the effect of Rab3AL peptide. Biochim. Biophys. Acta 1268:269–278; 1995.
26. Scheer, A.; Gierschik, P.: Farnesylcysteine analogues inhibit chemotactic peptide receptor-mediated G-protein activation in human HL-60 granulocyte membranes. FEBS 319:110–114; 1993.
27. Scheer, A.; Gierschik, P.: S-prenylated cysteine analogues inhibit receptor-mediated G protein activation in native granulocyte and reconstituted bovine retinal rod outer segment membranes. Biochemistry 34:4952–4961; 1995.
28. Stock, J. B.; Clarke, S.; Koshland, D. E., Jr.: The protein carboxylmethyltransferase involved in *Eschericia coli* and *Salmonella tymphimurium* chemotaxis. Methods Enzymol. 106:310–321; 1984.
29. Tan, E. W.; Perez-Sala, D.; Canada, F. J.; Rando, R. R.: Identifying the recognition unit for protein methylation. J. Biol. Chem. 266:10719–10722; 1991.
30. Tisch, D.; Halpern, M.; Marciano, D.; Kloog, Y.; Aviram, I.: Activation of signaling pathways in HL60 cells and human neutrophils by farnesylthiosalicylate. Eur. J. Biochem. 242:529–536; 1996.
31. Volker, C.; Lane, P.; Kwee, C.; Johnson, M.; Stock, J.: A single activity carboxyl methylates both farnesyl and geranylgeranyl cysteine residues. FEBS 295:189–194; 1991.
32. Xu, Y.; Gilbert, B. A.; Rando, R. R.; Longchuan, C.; Tashjian, A. H., Jr.: Inhibition of capacitative Ca^{2+} entry into calls by farnesylcyesteine analogs. Mol. Pharmacol. 50:1495–1501; 1996.