Adenylyl cyclase types I and VI but not II and V are selectively inhibited by nitric oxide

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Abstract

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Adenylyl cyclase (AC) isoforms catalyze the synthesis of 3',5'-cyclic AMP from ATP. These isoforms are critically involved in the regulation of gene transcription, metabolism, and ion channel activity among others. Nitric oxide (NO) is a gaseous product whose synthesis from L-arginine is catalyzed by the enzyme NO synthase. It has been well established that NO activates the enzyme guanylyl cyclase, but little has been reported on the effects of NO on other important second messengers, such as AC. In the present study, the effects of sodium nitroprusside (SNP), a nitric oxide-releasing compound, on COS-7 cells transfected with plasmids containing AC types I, II, V and VI were evaluated. Total inhibition (~98.5%) of cAMP production was observed in COS-7 cells transfected with the AC I isoform and previously treated with SNP (10 mM) for 30 min, when stimulated with ionomycin. A high inhibition (~76%) of cAMP production was also observed in COS-7 cells transfected with the AC VI isoform and previously treated with SNP (10 mM) for 30 min, when stimulated with forskolin. No effect on cAMP production was observed in cells transfected with AC isoforms II and V.

Introduction

Adenylyl cyclase (AC) (ATP pyrophosphate-lyase, cycling; EC 4.6.1.1) catalyzes the synthesis of 3',5'-cyclic AMP (cAMP) from ATP. Several mammalian AC isoforms have been cloned (1-3). These isoforms are involved critically in mammalian signal transduction pathways where they regulate gene transcription, metabolism, and ion channel activity (4,5). ACs are induced or inhibited by a system of three components associated with the plasma membrane: G protein-coupled receptors, stimulatory and inhibito-

ry heterotrimeric G proteins, and the AC catalytic entity itself (6). AC types I and III are stimulated by Ca^{2+} and calmodulin (7), whereas types II and IV-VI are not (3,8). Furthermore, forskolin binds to the AC catalytic core (9,10) by joining the two domains of the core using a combination of hydrophobic and hydrogen-bonding interactions that are distributed equally between the two domains (11). It has been shown that all mammalian AC isoforms are activated by both forskolin and the GTP-bound α subunit of the stimulatory G protein G_s (12).

Nitric oxide (NO) is a gas whose synthe-

sis is catalyzed by the enzyme NO synthase (NOS; EC 1.14.13.39). NOS activation is NADPH dependent by oxidation of the guanidine nitrogen of its substrate L-arginine in the presence of molecular oxygen and several other cofactors (FMN, FAD, tetrahydrobiopterin, heme moiety). Three NOS isoforms have been identified so far: NOS I (brain or neuronal NOS), NOS II (macrophage or hepatocyte NOS) and NOS III (endothelial NOS) (13). The diverse activities of NO include blood vessel relaxation and modulation of immune responses, pathogen killing, and inhibition of platelet aggregation and adhesion. Moreover, NO controls the activity of enzymes and ion channels, and serves as a neuromodulator and neurotransmitter in the central and peripheral nervous systems (14). There is also evidence that NO may be a physiological intracellular regulator of mitochondrial respiration and gene activity, and can interact with oxygen-derived radicals to produce other highly reactive substances (15,16). In addition, NO is involved in the development of tolerance to and withdrawal from morphine (17,18). Overproduction of NO in mammalian systems may contribute to cell damage or cell death (19). Recently, a number of new actions of NO have been proposed: among them protein modification by S-nitrosylation via the transfer of NO^+ from nitrosothiols (20).

Although the actions of the enzymes AC and NOS in promoting two signal transduction pathways have been already characterized, little information is available about the relation between these two pathways. It has been well established that NO activates the second messenger guanylyl cyclase, but little has been reported on the effects of NO on other important second messengers such as AC. It has been previously demonstrated that the inhibition of the endogenous neuroblastoma cell line AC by NO is related to isoform type VI (21), but little information is available on the effects of NO on other mammalian AC isotypes, as in AC genes transfected to mammalian cultured cell lines.

In the present study we evaluated the effects of NO on the activity of mammalian AC isoforms I, II, V and VI in transfected mammalian cultured kidney COS-7 cells using the NO-releasing compound sodium nitroprusside (SNP) as measured by cAMP production.

Material and Methods

SNP was purchased from Alexis Biochemicals (San Diego, CA, USA). [^{3}H-2]-Adenine (18.0 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA), ionomycin and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (IBMX) and RO-20-1724 were from Calbiochem (La Jolla, CA, USA). Forskolin, cAMP, and thyroid-stimulating hormone (TSH) were from Sigma (St. Louis, MO, USA). Finally, tissue culture reagents were from Life Technologies (Gaithersburg, MD, USA).

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 0.2 mg/ml G418 and 100 IU/ml penicillin, in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37ºC. Plasmids containing AC cDNAs (pXMD1-AC-I, pXMD1-AC-II, pXMD1-AC-V and pXMD1-AC-VI) have been described (22). Transient transfection of COS-7 cells was performed as described (22,23). Briefly, COS-7 cells on 10-cm plates were transfected using the DEAE-dextran chloroquine method with 2 µg/plate of one of the AC isozyme cDNAs. Twenty-four hours later, the cells from each 10-cm plate were trypsinized and recultured on a 24-well plate, and after an additional 24 h, cells were assayed for cAMP content (as a measure of AC activity) as described below. Transfection efficiency was normally in the range of

60-80%, as determined by staining for ß-galactosidase activity (24).

Sodium nitroprusside treatment and cAMP assay

The assay was performed in triplicate as described previously (22,25,26). Briefly, cells cultured on 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml of [^{3}H]-adenine; during the last 30 min SNP at the indicated concentrations and phosphodiesterase inhibitors (described below) were added to the incubation media, and then washed with 0.5 ml/well of DMEM containing 20 mM HEPES, pH 7.4, and 0.1 mg/ml BSA. This medium was replaced with 0.25 ml/well of DMEM containing 20 mM HEPES, pH 7.4, 0.1 mg/ml BSA, and the phosphodiesterase inhibitors IBMX (0.5 mM) and RO-20-1724 (0.5 mM). AC activity was stimulated in the presence or absence of the indicated concentrations of SNP by the addition of either 1 μM forskolin, for stimulation of AC V and VI, 1 μM ionomycin for stimulation of AC I, or 0.1 mM TSH for stimulation of AC II (in this last case, cells were transfected together with the TSH receptor). After 10 min of incubation at room temperature, the medium was removed and the reaction terminated by the addition of perchloric acid containing 0.1 mM unlabeled cAMP, followed by neutralization with KOH, and the amount of [^{3}H]-cAMP was determined by a two-step column separation procedure, as previously described (23,27).

Statistical analysis

Results are reported as the mean ± SEM of triplicate determinations of a representative experiment. P<0.05 *vs* control was considered to be significant.

Results

Four AC isozyme types were studied according to their properties and sequence homologies: AC I which is stimulated by Ca^{2+}/calmodulin; AC V and AC VI, stimulated by forskolin, and AC II, stimulated by TSH (28,29), to determine whether SNP affected cAMP production by the different isoforms. For this purpose, the COS-7 cell system, which readily allows transient transfection of the desired AC isozymes, was employed.

Effects of sodium nitroprusside on the AC I isotype

Pretreatment of the transfected cells with increasing concentrations of SNP resulted in a distinct attenuation of cAMP production by AC isoform I (Figure 1A). A 38% attenuation of cAMP production was observed in the presence of 0.1 mM SNP, the NO donor. In the concentration range between 0.1 and 1 mM SNP, however, no significant difference was observed, while a strong attenuation of cAMP production was obtained in the presence of 10 mM SNP (Figure 1A).

Effects of sodium nitroprusside on the AC VI isotype

Similar results were obtained with the forskolin-stimulated AC VI isozyme. cAMP inhibition by NO donor SNP resulted in a distinct attenuation of cAMP production: 0.1 mM SNP resulted in 55% attenuation of cAMP production, while 24% attenuation was obtained using 10 mM SNP (Figure 1B).

Effects of sodium nitroprusside on the AC II and V isozymes

In contrast, no significant variations in cAMP production were detected in the TSH-stimulated AC II (Figure 1C) and forskolin-stimulated AC V isozymes (Figure 1D) when both isozymes were incubated with SNP under the same conditions as detailed above. Furthermore, in all the experiments per-

formed, the basal accumulation of cAMP, a measure of cAMP generated in the absence of hormonal stimulation, was unaffected by treatment with SNP (data not shown).

Discussion

The present results suggest a selective inhibition by the NO donor SNP in the transfected AC isoforms. Both the AC VI and AC V isozymes are stimulated by the hormone forskolin, but only AC VI was inhibited by SNP. The results observed for the AC I and VI isotypes indicate that SNP inhibition occurs at the level of the enzyme, and thus is independent of the receptor/G protein-mediated pathway. The results also suggest that the inhibitory effect of SNP is independent of forskolin binding in these isoforms, resulting in a differential effect of SNP on the various AC isotypes. It is also interesting to

mention that the inhibitory effect of SNP was on the AC I and AC VI isoforms, which are not closely related to each other either in terms of enzymatic stimulation or of genetic homology.

The complete coding sequence of AC type I is known, and there is some information concerning functional domains of the enzyme. An examination of the amino acid sequence of AC type I reveals several sequences which exhibit the general properties of calmodulin-binding domains. It has been reported that a synthetic peptide, corresponding to amino acids 495-522 from AC I, binds with high affinity and inhibits calmodulin stimulation of the enzyme, suggesting that this sequence may correspond to the calmodulin-binding domain of the enzyme (30). Furthermore, a point mutation within this domain abolished Ca^{2+} and calmodulin sensitivity of the enzyme (31). Interestingly, this

Figure 1. Effects of sodium nitroprusside (SNP) on the activity of different adenylyl cyclase (AC) isozymes transfected into cultured COS-7 cells. The figure shows the relative rate of cAMP accumulation versus control values (0 mM SNP). A, AC I was incubated with different concentrations of SNP and stimulated with ionomycin, with a maximum at 10 mM SNP treatment. B, AC VI was incubated with different concentrations of SNP and stimulated with forskolin. The figure shows the relative rate of cAMP accumulation versus control, which was maximum at 10 mM SNP treatment. C, The AC II isozyme in COS-7 cells was exposed to different concentrations of SNP and stimulated with TSH. D, The AC V isozyme was incubated with different concentrations of SNP and stimulated with forskolin. One hundred percent represents the control AC activity observed in stimulated transfected COS-7

cells. A: 1366 ± 430 cpm, B: 8574 ± 210 cpm, C: 40796 ± 2729, D: 5880 ± 215 of [^{3}H]-cAMP. All experiments were performed on intact cells and data are reported as the mean ± SEM of triplicate determinations of a representative experiment. *P<0.05 vs control (Student t-test). Iono: ionomycin; FS: forskolin; TSH: thyroid-stimulated hormone.
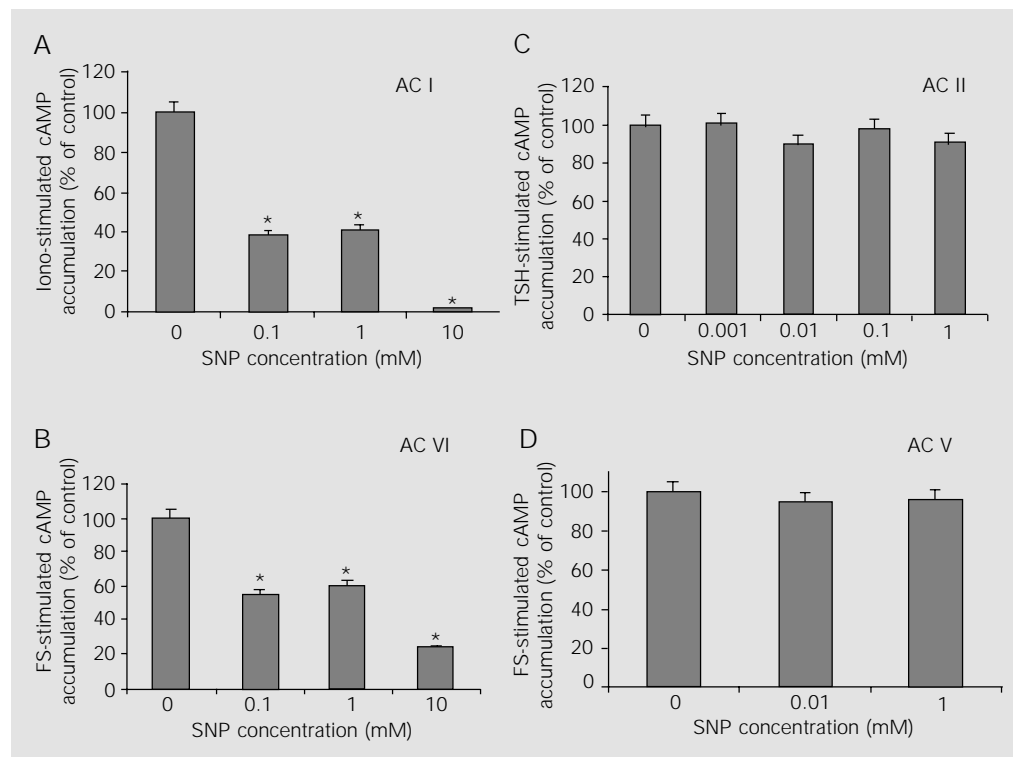
sequence contains two cysteine residues, uncommon for calmodulin-binding domains. Pretreatment of recombinant COS-7 cells with SNP inhibits catalytic activity of the transfected AC I isozyme (Figure 1A). One possible explanation for this inhibition could be the oxidation of sulfhydryl groups of neighboring cysteines in the calmodulin-binding domain of the enzyme. The site of the NO effect on the enzyme is intriguing; since the basal enzyme activity of the different AC isozymes did not vary, it is unlikely that NO action could be targeted to the catalytic site of the enzyme. In agreement with these results, inhibition of the forskolin-stimulated AC VI isotype by pretreatment with SNP of transfected COS-7 cells was also detected (Figure 1B).

The mechanism by which we propose selective NO blockage of the AC I and AC VI isoforms is in agreement with recent reports suggesting that the conformation of the enzymes AC I and AC VI determines their sensitivity (21,32,33). The basal activities of type I and VI recombinant enzymes are not altered by NO, suggesting that, in their basal states, these isoforms share a conformation that precludes susceptibility to NO. On this basis, we speculate that this inhibition is due to a change in V_{max}, with no change in the K_m of the substrate. Future experiments will be performed in order to analyze the kinetic effects of NO on cAMP production in stimulated, transfected COS-7 cells incubated with SNP and assayed at increasing [3H]-adenine concentrations.

Although it has been reported that NO did not inhibit cAMP production by the AC I isotype (32), those experiments were performed on insect cell membranes. In other previous studies (21) it was observed that SNP inhibited endogenous AC type VI activation in cultured neuroblastoma N18TG2 cells.

The present study provides evidence for the selective inhibition of cAMP production by AC enzyme types I and VI, but not types II and V, in AC-transfected cultured mammalian cell lines treated with the NO donor SNP. It is interesting to observe that both enzymes, AC I and AC VI, share a conformation that precludes any susceptibility to NO (33), suggesting that SNP might be nitrosylating two cysteine residues, thus modifying their catalytic activity. A possible means of testing this hypothesis is to induce a mutation of the residues in genetically modified AC isoforms. These results would be interesting since differential expressions of AC isotypes occur in different organs and tissues in mammals. All AC isoforms appear to be expressed in the brain, apparently in region-specific patterns; the differential expression of AC isoforms in the central nervous system has been particularly well studied. In addition, there is a predominant expression of AC I in area CA1 of the hippocampus (34). NO plays a primary role in learning and memory in the hippocampus; advanced studies on the regulation of AC by NO would shed more light on the understanding of this field. Although the inhibition observed in cultured COS-7 cells transfected with plasmids containing AC I and AC VI probably would not reflect the same results performed *in vivo* due to numerous active pathways interacting in concert, as is the case for specific regions of the brain, transfection experiments with engineered cyclase isoforms should provide powerful new tools to determine *in vivo* how a particular regulator controls a particular AC isoform in the context of many simultaneous active pathways. Recently, it has been reported that NO inhibits AC activity in brain striatal membranes (35), in agreement with the results obtained here for cultured cells. Hudson et al. (35) found that the AC I isoform was present in neurons of the striatum, together with the AC V and AC VI isoforms. Thus NO could modulate the dopaminergic regulation of cAMP along with the effects of Ca^{2+}, modifying intracellular events in striatal neurons (36). NO has been implicated in

the destruction of dopaminergic neurons and their projecting nerve fibers in the striatum that characterizes Parkinson's disease (36). Therefore, vectors containing AC isoforms transfected to striatal neurons would provide an interesting approach for a treatment aimed at preventing Parkinson's disease.

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