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Original Contribution

Dual contradictory roles of cAMP signaling pathways in hydroxyl radical production in the rat striatum

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ABSTRACT

Studies have suggested that cAMP signaling pathways may be associated with the production of reactive oxygen species. In this study, we examined how modifications in cAMP signaling affected the production of hydroxyl radicals in rat striatum using microdialysis to measure extracellular 2,3-dihydroxybenzoic acid (2,3-DHBA), which is a hydroxyl radical adduct of salicylate. Up to 50 nmol of the cell-permeative cAMP mimetic 8-bromo-cAMP (8-Br-cAMP) increased 2,3-DHBA in a dose-dependent manner (there was no additional increase in 2,3-DHBA at 100 nmol). Another cAMP mimetic, dibutyryl cAMP (db-cAMP), caused a nonsignificant increase in 2,3-DHBA at 50 nmol and a significant decrease at 100 nmol. Up to 20 nmol of forskolin, which is a direct activator of adenylyl cyclase, increased 2,3-DHBA, similar to the effect of 8-Br-cAMP; however, forskolin resulted in a much greater increase in 2,3-DHBA. A potent inhibitor of protein kinase A (PKA), H89 (500 μM), potentiated the 8-Br-cAMP- and forskolin-induced increases in 2,3-DHBA and antagonized the inhibitory effect of 100 nmol of db-cAMP. Interestingly, the administration of 100 nmol of 8-bromo-cGMP alone or in combination with H89 had no significant effect on 2,3-DHBA levels. Doses of 100 nmol of a preferential PKA activator (6-phenyl-cAMP) or a preferential PKA inhibitor (8-bromoadenosine-3′,5′-cyclic monophosphorothionate, Rp-isomer; Rp-8-Br-cAMPS), which also inhibits the cAMP-mediated activation of Epac (the exchange protein directly activated by cAMP), suppressed or enhanced, respectively, the formation of 2,3-DHBA. Up to 100 nmol of 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-cAMP, which is a selective activator of Epac, dose-dependently stimulated the formation of 2,3-DHBA. These findings suggest that cAMP signaling plays contradictory roles (stimulation and inhibition) in the production of hydroxyl radicals in rat striatum by differential actions of Epac and PKA. These roles might contribute to the production of hydroxyl radicals concomitant with cAMP in carbon monoxide poisoning, because the formation of 2,3-DHBA was potentiated by the PKA inhibitor H89 and suppressed by Rp-8-Br-cAMPS, which inhibits PKA and Epac.

Introduction

cAMP is an intracellular second messenger that is produced by adenylyl cyclases coupled with a stimulatory guanine nucleotide-binding protein in response to various extracellular signals [1,2]. To date, intracellular cAMP is known to directly bind to three proteins: protein kinase A (PKA), the exchange protein directly activated by cAMP (Epac), and cyclic nucleotide-gated ion channels [1-3], the last of which is also a target of cGMP [3,4]. The activation of these proteins triggers distinct cascades that lead to multiple physiological responses [1-3].

Many studies have shown that the production of cAMP in the brain is enhanced by brain ischemia and hypoxia/anoxia [5]. Tanaka [6] demonstrated that decreases in adenylyl cyclase activity and the binding activity of PKA to cAMP are correlated with an increase in the severity of brain damage in a brain-ischemia rat model, which suggests a neuroprotective role of the cAMP/PKA signaling pathway. This finding is supported by reports that the direct activation of adenylyl cyclase with forskolin [7] or the inhibition of the phosphodiesterase-mediated hydrolysis of cAMP with cilostazol [8,9] and rolipram [7,10] increases intracellular cAMP and results in a neuroprotective effect against brain damage due to brain ischemia. In addition, reports have shown that the inhibition of PKA with *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) aggravates ischemia-induced brain damage [9]. In addition, in vitro studies have demonstrated that dibutyryl-cAMP (db-cAMP) and 8-bromo-cAMP (8-Br-cAMP), which penetrate the cell membrane and mimic intracellular cAMP [3], and forskolin attenuate neuronal death induced by 6-hydroxydopamine [11] or lipopolysaccharide [12]. However, SCH23390, a dopamine receptor antagonist, suppressed the ischemia-induced increase in cAMP [13] and PKA-dependent phosphorylation [14], which resulted in an attenuation of brain damage due to brain ischemia [14]. Amadio et al. [15] reported that the cAMP analogue 8-(4-chlorophenylthio)-cAMP, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, and

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forskolin were all neurotoxic and caused death in cultured cerebellar granule neurons. Moreover, the inhibition of phosphodiesterase by rolipram exacerbated brain damage due to ischemia/hypoxia in rat pups [16]. Therefore, it is likely that increased cAMP in response to brain ischemia/hypoxia differentially activates the cAMP signaling pathways, which might have contradictory functions depending on environmental conditions and result in the protection or aggravation of brain injury. Interestingly, studies have suggested that the production of reactive oxygen species (ROS) might be involved in both of the outcomes of cAMP signaling [8,9,15].

Microdialysis has been widely used in in vivo studies to explore the roles of ROS that are produced by various brain insults, including brain ischemia [17,18], neurotoxins [19,20], and excitatory amino acids [21,22]. In these studies, the production of hydroxyl radicals, which are the most toxic ROS, is used as a marker of the ROS production based on extracellular 2,3-dihydroxybenzoic acid (2,3-DHBA), which is formed by a nonenzymatic reaction of hydroxyl radicals with salicylic acid [23]. We previously found that carbon monoxide (CO) poisoning stimulated the production of hydroxyl radicals in terms of extracellular 2,3-DHBA in rat striatum in vivo [24] and that the stimulation occurred in parallel with an increase in cAMP [25]. In the present study, we explored the role of cAMP signaling pathways in the production of hydroxyl radicals by in vivo modification of the pathways with cell-permeative derivatives of cAMP or forskolin in rat striatum. We also investigated the involvement of cAMP signaling pathways in the production of hydroxyl radicals in CO poisoning.

Material and methods

Animals

Male Sprague–Dawley rats weighing from 235 to 265 g were purchased from Charles River Laboratories Japan (Kanagawa, Japan). The rats were acclimated with free access to food and water in a facility with a controlled temperature (22–24 °C) on a 12-h/12-h light/dark cycle (lights on between 0800 and 2000 hours), for at least 1 week before all of the experiments.

The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of Tokyo Medical University and all of the experiments were performed in accordance with the *Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions* under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Chemicals

8-Br-cAMP, db-cAMP, 8-bromo-cGMP (8-Br-cGMP), and H89 were purchased from Sigma–Aldrich (St. Louis, MO, USA). A water-soluble derivative of forskolin [26], 7-deacetyl-7-(*O*-*N*-methylpiperazino)-γ-butyrylforskolin, was purchased from Calbiochem (San Diego, CA, USA). 8-Bromoadenosine-3′,5′-cyclic monophosphorothionate, Rp-isomer (Rp-8-Br-cAMPS); 6-phenyl-cAMP; and 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP (8-cCPT-2′-O-Me-cAMP) were purchased from BIOLOG (Bremen, Germany).

Stereotaxic surgery

Stereotaxic surgery was performed under isoflurane anesthesia. The rats were individually mounted in a stereotaxic head holder, which was equipped with a mask (David Kopf, Tujunga, CA, USA) connected to an inhalation apparatus (Univentor, Malta). A guide cannula (MI-AG-8 or AG-8; Eicom, Kyoto, Japan) was unilaterally (left) implanted in the brain at the coordinates 0.2 mm AP, 3.0 mm L, and 3.5 mm DV, according to the rat brain atlas [27]. The guide cannula was secured to the calvarium with miniature stainless steel screws and acrylic dental cement and was plugged with a solid dummy cannula (MI-AD-8 or AD-8; Eicom). The rats were allowed at least 5 days to recover from the surgery.

Determination of hydroxyl radical production using microdialysis

The rats were individually placed in a plastic chamber (26.5 cm in diameter and 28.5 cm in height) and brain microdialysis was performed according to a previously reported method [24,28]. We used two types of microdialysis probes (MI-A-I-8-03 or A-I-8-03; Eicom) with cellulose membranes (3 mm long and 0.22 mm in diameter). The MI-A-I-8-03 probe was equipped with a thin fused-silica needle (0.15 mm in diameter) for drug administration. Each probe was inserted into the striatum through the corresponding guide cannula and perfused with a modified Ringer's solution (147 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$) containing 5 mM sodium salicylate at a flow rate of 2 μL/min. The locations of the dialysis probes were verified after each experiment.

The production of hydroxyl radicals was estimated by measuring the extracellular levels of 2,3-DHBA that was formed through the nonenzymatic hydroxylation of salicylic acid [23] according to the protocol of Teismann and Ferger [22] with modifications [24,28]. The dialysate was collected in an autoinjector (EAS-20; Eicom) in 40-μL fractions every 20 min and injected into an inert HPLC system (Eicom) that was equipped with an electrochemical detector (ECD-300; Eicom) consisting of a graphite working electrode at +500 mV vs an Ag/AgCl reference electrode. Separation was carried out on an Eicompac SC-5ODS column (2.1×150 mm) at 25 °C with a mobile phase that consisted of 100 mM sodium phosphate buffer (pH 6.0) containing 13.4 μM EDTA and 2% (v/v) methanol at a flow rate of 230 μL/min.

Drug administration

Various cAMP derivatives, 8-Br-cGMP, and forskolin were dissolved in sterilized physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) and directly administered to the striatum through the thin needle of the MI-A-I-8-03 probe using an ESP-32 microsyringe pump (Eicom). The flow rate was 0.1 μL/min, and the total volume was 2 μL. H89 was dissolved in the perfused solution and administered to the striatum through the probe during the experimental period.

CO exposure

CO exposure was performed according to a previously described method [24,28]. The plastic chamber containing the rat was enclosed with a cover that had a hole for the perfusion tubes. Room air was mixed with CO (>99.9%) and introduced into the chamber at a flow rate of 8 L/min using a gas flow regulator (Koflok, Osaka, Japan). The concentration of CO in the chamber was adjusted to 3000 ppm based on the readings from a CO monitor (CM-525HB; Gastec, Kanagawa, Japan). The CO exposure lasted for 40 min and resulted in CO poisoning with a blood carboxylhemoglobin level greater than 70% [24].

Statistics

The 2,3-DHBA levels were expressed as percentages of the basal level that was determined by averaging three consecutive dialysate samples in individual rats before administration of the cAMP derivatives, 8-Br-cGMP, or forskolin. The data were expressed as the mean ± SEM (n = 5–9) and were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple-group comparisons or Student's t test for two-group comparisons.