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Short Communication

INHIBITION OF ADENYLYL CYCLASE IN RAT STRIATAL HOMOGENATES BY COMBINATIONS OF DOPAMINE AND FERRIC IRON COMPOUNDS*

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Abstract—The adenylyl cyclase activity of homogenates of striatal tissue from rat brain has been used as a model to test the hypothesis that the products of the reaction of polyphenols with ferric iron compounds are toxic. Dopamine (DA), at levels that stimulate adenylyl cyclase, inhibited the activity in the presence of 2 mol of potassium ferricyanide (FC), methemoglobin or ferricytochrome *c* per mol of DA. Combinations of potassium ferrocyanide and DA were not inhibitory. Neither pyrocatechol nor hydroquinone stimulated the activity, but these polyphenols were inhibitory in the presence of FC. Tyramine, phosphorylated DA or phosphorylated pyrocatechol had no effect on the activity of the enzyme in the presence or absence of FC. Forskolin was unable to stimulate the adenylyl cyclase once the latter was inhibited by DA plus FC, and dithiothreitol could not reverse inhibition by DA plus FC. Incubation of DA with FC, in the absence of the homogenate, resulted in substances that were not inhibitory. These findings suggest that the polyphenols plus FC react to yield substances that inhibit the adenylyl cyclase by affecting the catalytic unit of the enzyme complex.

Key words: adenylyl cyclase; dopamine; ferricyanide; dopamine-3 or 4-monophosphate ester; methemoglobin; forskolin

The oxidation of polyhydroxy aromatic compounds, including the catecholamines, yields semiquinones, quinones, and reactive oxygen compounds. Furthermore, the generation of these chemically reactive compounds is correlated with some of the adverse effects of the oxidized substances [1–5].

Iron compounds are required for life. However, iron overload is associated with toxic effects [6, 7], and dislocation of iron, due to bleeding into the parenchyma of the brain, is associated with the development of post-brain-injury epilepsy [8]. The above observations and the rapid, nonenzymatic oxidation of the catecholamines by soluble compounds of ferric iron have led to the hypothesis that the products of the oxidation of catecholamines by ferric iron compounds are associated with some of the toxic effects of both iron and the catecholamines. The present research tested this hypothesis with the dopamine-stimulated adenylyl cyclase activity of homogenates of striatal tissue from rat brain [9, 10] as a model. The results demonstrated that DA‡, L-DOPA or NE, at levels that stimulate the enzyme in a standard assay, are inhibitory in the presence of FC. In addition, DA plus methemoglobin or ferricytochrome *c* inhibited the enzyme, suggesting that ferric-iron proteins may interact with DA to yield inhibitors of the enzyme. The results are consistent with the conclusion that the combination of ferric iron and a 1,2- or 1,4-dihydroxybenzene derivative inhibits adenylyl cyclase due to effects at the catalytic subunit of the enzyme complex. The results also showed that the inhibitory products generated by the reaction of FC with DA exhibit a relatively short half-life in the assay and that the inhibition is not readily reversible by DTT.

Materials and Methods

Dopamine-3 or 4-monophosphate ester [11] and pyrocatechol monophosphate ester [12] were prepared by published methods. All other materials were purchased from the Sigma Chemical Co., St. Louis, MO, or Fisher Scientific, Pittsburgh, PA. Protein in homogenates was determined by the biuret method [13] using 3.3 mM sodium deoxycholate to clarify the assay reaction mixtures and bovine serum albumin as the standard protein. cAMP was determined by a competitive binding assay [14] with a kit from DPC of Los Angeles, CA. Striatal homogenates were prepared and adenylyl cyclase was assayed using modifications of published methods [9, 10]. Male Sprague–Dawley rats (200–300 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and exsanguinated. Whole brains were immediately removed and placed in 0° isotonic saline. Striatal tissue was dissected from the brains and homogenized with 50 vol. of 80 mM Tris–maleate, pH 7.5, at 0°. Homogenates were stored on ice and assayed within 2 hr. The standard adenylyl cyclase assay contained the following in a volume of 250 μL: 80 mM Tris–maleate, pH 7.4; 4.00 mM $MgSO_4$; 100 μM GTP; 0.60 mM sodium EDTA; 0.50 mM 3-isobutyl-1-methylxanthine; 1.00 mM sodium ATP; 50 μL of striatal homogenate (100–125 μg protein); and the indicated additions. The components of

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‡ Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; DA, dopamine; L-DOPA, L-3,4-dihydroxyphenylalanine; DTT, dithiothreitol; FC, potassium ferricyanide; and NE, *dl*-norepinephrine.

Table 1. Inhibition of striatal adenylate cyclase by DA plus FC

Additions (μM)	cAMP (pmol · mg protein^{-1} · min^{-1}) 2.5 min	5.0 min
None	168 ± 2	325 ± 5
DA (50)	361 ± 36*	634 ± 54*
FC (100)	159 ± 14	313 ± 8
DA (50), FC (100)	57 ± 4*	104 ± 30*

The standard assay described in Materials and Methods was used with the indicated additions. Means ± SEM for three replications are reported.

* Significantly different from the no addition control, $P < 0.05$.

Table 2. Effects of forskolin and forskolin plus DA and FC on adenylyl cyclase activity

Additions (μM)	cAMP (nmol · mg protein^{-1} · min^{-1})
Forskolin (6)	9 ± 2
DA (50), forskolin (6)	12 ± 3
DA (50), FC (100), forskolin (6)	0.3 ± 0.11*
FC (100), forskolin (6)	7 ± 2

The standard assay was used except that 0.6% DMSO (v/v) was added to permit addition of forskolin. The control rate was 63 ± 15 pmol cAMP · mg protein^{-1} · min^{-1}. Means ± SEM for three replications are reported.

* Significantly different from the forskolin only assay, $P < 0.05$.

the assay, except for ATP, were mixed at 0°, with the homogenate and FC or another iron compound added next to last and last. The mixtures were incubated on ice for 20 min and then transferred to a 30° water bath. After 5 min in the 30° bath, the assays were initiated with the addition of ATP. Incubation was terminated 2.5 or 5.0 min after the addition of ATP by addition of 250 μL of 6% perchloric acid. The assays were titrated to pH 6.5–7.0 with 30% (w/v) aqueous $KHCO_3$, incubated on ice for 0.5 hr, and then centrifuged for 3 min at 2000 *g*. cAMP was then determined in the supernatant fractions. The results are corrected for cAMP in assays incubated without ATP. The experiments were replicated three times, and statistical analysis was performed using Student's two-tailed *t*-test, with $P < 0.05$ indicating significance.

Results and Discussion

As seen in Table 1, 50 μM DA stimulated the adenylyl cyclase activity of striatal homogenates, as reported [9, 10], and 100 μM FC had no effect, whereas 50 μM DA plus 100 μM FC was strongly inhibitory. At the levels examined, DA or the other test compounds, as well as the tested combinations, had no effect on the determination of cAMP by the binding assay. Testing various levels of FC with 50 μM DA in the standard assay showed that 2 mol of FC/mol of DA were required to produce maximal inhibition of the enzyme. Varying DA concentrations with 100 μM FC in the assay showed that 25 μM DA produced inhibition, whereas 5.0 μM DA was not inhibitory. In the absence of FC, 25 μM DA, but not 5.0 μM DA, stimulated the activity. A 200 μM concentration of potassium ferrocyanide in the standard assay had no effect on the yield of cAMP with or without 50 μM DA. Thus, 2 mol of FC/mol of DA, with DA at a level that stimulates the activity, inhibits cAMP synthesis in the standard activity.

Several compounds were tested in the standard assay, with and without FC, to determine if ability to stimulate adenylyl cyclase activity was required for inhibitory action in the presence of FC. Fifty micromolar L-DOPA or 50 μM NE, without and with 100 μM FC in the assays, affected the enzyme in a manner similar to DA, as in Table 1. On the other hand, a 50 μM concentration of either pyrocatechol or hydroquinone had no effect on the activity of the enzyme but inhibited the activity in the presence of 100 μM FC. Tested compounds that had no effect on the enzyme with or without 100 μM FC in the assay included: 50 μM dopamine 3- or 4-monophosphate ester; 50 μM pyrocatechol monophosphate ester; and 50 μM tyramine. Thus, ability to inhibit the enzyme in the presence of FC is not correlated with ability of the test substance to stimulate the activity in the absence of FC. The inhibitory action of test compounds in the presence of FC, however, does correlate with the expected generation of quinones and other substances via oxidation by FC [1–5]. Finally, the phosphate esters of DA, which are postulated to have biological roles [11], had no role, other than inactivation of DA, in the stimulation of adenylyl cyclase by DA.

Forskolin, which acts on the catalytic subunit of adenylyl cyclase to stimulate activity [15], could not fully stimulate the enzyme complex once inhibition by DA plus FC was established (Table 2). In addition, exogenous GTP was not required for 50 μM DA plus 100 μM FC to cause inhibition in the standard assay. These results, plus the observation that pyrocatechol or hydroquinone inhibited the enzyme in the presence of FC, but did not stimulate the activity alone, indicate that the inhibition caused by DA plus FC is due to effects on the catalytic subunit of the adenylyl cyclase complex.

The reaction of DA with FC would be expected to yield the semiquinone and quinone of DA as initial products, with other products accumulating with time. It was of interest to determine if the inhibitory products were among the early products of the oxidation. To do this, DA and FC were incubated (at levels that would give 50 μM DA and 100 μM FC in the final assay) in the assay buffer for 20 min at room temperature before the otherwise standard assay was completed. Under these conditions, DA plus FC caused no inhibition of the adenylyl cyclase, showing that the initial products of the reaction of DA plus FC are inhibitory, whereas the products that appear later are not active inhibitors.

DTT, which reduces disulfide groups, rapidly bleached FC in the assay buffer due to the reduction of FC to ferrocyanide. Therefore, the effects of DTT added to the standard assay before and after FC were determined (Table 3). When added to the assays before FC, DTT protected the enzyme from inhibition; however, when added after FC, DTT was not protective. The results indicate that DTT protects by rapidly reducing FC and that the inhibition of the enzyme complex by DA plus FC is probably not due to the formation of disulfide bonds in the catalytic unit of the enzyme. DTT alone increased the activity of the enzyme with and without 50 μM DA. This accounts for the inclusion of DTT in adenylyl cyclase assays [16, 17].

Since iron is transported, stored and functions as iron-proteins in biological systems, it was of interest to determine if ferric iron-proteins could react with DA to inhibit adenylyl cyclase as FC does. Methemoglobin plus DA inhibited the enzyme (Table 4), although the preincubation period at 30° had to be extended from 5 to 20 min before starting the otherwise standard assay with ATP. Hemoglobin was not inhibitory in the presence of DA. Both hemoglobin

Table 3. Effects of DTT on the inhibition of adenylyl cyclase by DA plus FC

Additions (μM)	cAMP (pmol·mg protein^{-1}·min^{-1})	
	DTT added before any other additions	DTT added 2 min before ATP
DTT (2)	88 ± 10	147 ± 4
DTT (2), DA (0.05)	360 ± 20*	381 ± 21*
DTT (2), DA (0.05), FC (0.10)	370 ± 42*	13 ± 3*
DTT (2), FC (0.10)	143 ± 37	184 ± 35

The standard assay was used with the DTT added as indicated. Means ± SEM for three replications are reported.

* Significantly different from the DTT only assay, $P < 0.05$.

Table 4. Inhibition of adenylyl cyclase by DA plus methemoglobin

Additions (μM)	cAMP (pmol·mg protein^{-1}·min^{-1})
None	40 ± 4
DA (50)	156 ± 32*
Hemoglobin (100)	86 ± 22*
DA (50), hemoglobin (100)	230 ± 26*
Methemoglobin (100)	73 ± 11*
DA (50), methemoglobin (100)	25 ± 2*

The standard assay was used except that the assays, complete except for ATP, were incubated for 20 min at 30° before ATP was added. The rates are calculated for homogenate protein in the assays. Means ± SEM for three replications are reported.

* Significantly different from the no addition control, $P < 0.05$.

and methemoglobin alone protected the enzyme during the 30° incubation, possibly due to the added protein in the assays. With the modified assay used in Table 4, 100 μM ferricytochrome *c* plus 50 μM DA resulted in inhibition equal to that reported for methemoglobin plus DA, whereas 100 μM ferricytochrome *c* was not inhibitory (results not shown). Since proteins containing ferric iron occur *in vivo*, these results may be relevant to the toxic effects of catecholamines and similar compounds that can be oxidized by ferric iron.

In summary, DA, or several polyphenols including L-DOPA or NE, plus FC inhibited the DA-stimulated adenylyl cyclase activity of homogenates of striatal tissue. Polyphenols active in this regard did not need to be capable of stimulating the enzyme alone, and forskolin could not fully stimulate the enzyme inhibited by DA plus FC, suggesting that inhibition is at the catalytic unit of the adenylyl cyclase complex. Preincubation of DA with FC produced colored products that did not inhibit the enzyme, suggesting that the inhibitory products, generated by the reaction of DA plus FC, have relatively short half-lives in the assay buffer. DTT protected the enzyme from inhibition by DA plus FC when added to the assay before FC, but DTT was not capable of reversing inhibition established by DA plus FC, indicating that inhibition is not due to oxidation of sulfhydryl groups of the catalytic unit of the complex. The literature [1–5], as well as the results reported here, suggests that the observed inhibition is due to the generation of semiquinones, quinones, or reactive compounds of oxygen by the reaction of a polyphenol plus a soluble compound of ferric iron. The ferric iron proteins methemoglobin or ferricytochrome *c* plus DA inhibited the complex, indicating that ferric iron proteins plus catecholamines, or other polyphenols, subject to oxidation by ferric iron, may react to give inhibitory substances *in vivo*. The results support the hypothesis that catecholamines react with ferric iron compounds to yield toxic substances.

REFERENCES

1. Webb JL, Quinones. *Enzyme and Metabolic Inhibitors*, Vol. 3, pp. 421–594. Academic Press, New York, 1966.
2. O'Brien PJ, Molecular mechanism in quinone cytotoxicity. *Chem Biol Interact* **80**: 1–41, 1991.
3. Ito S, Kato T and Fujita K, Covalent binding of catechols to proteins through the sulphydryl group. *Biochem Pharmacol* **37**: 1707–1710, 1988.
4. Bhat RV, Subrahmanyam VV, Sadler A and Ross D, Bioactivation of catechol in rat and human bone marrow cells. *Toxicol Appl Pharmacol* **94**: 297–304, 1988.
5. Kalyanaraman B, Premovic PI and Sealy RC, Semiquinone anion radicals from addition of amino acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines. *J Biol Chem* **262**: 11080–11087, 1987.
6. Beard JL, Connor JR and Jones BC, Iron in the brain. *Nutr Rev* **51**: 157–170, 1993.
7. Aust SD, Chignell CF, Bray TM, Kalyanaraman B and Mason RP, Free radicals in toxicology. *Toxicol Appl Pharmacol* **120**: 168–178, 1993.
8. Hauser WA, Prevention of post-traumatic epilepsy. *N Engl J Med* **323**: 540–542, 1990.
9. Kebabian JW, Petzold GL and Greengard P, Dopamine-sensitive adenylate cyclase in caudate nucleus of rat

brain, and its similarity to the "dopamine receptor." *Proc Natl Acad Sci USA* **69**: 2145–2149, 1972.

10. Clemet-Cormier YC, Parrish RG, Petzold GL, Kebabian JA and Greengard P, Characterization of a dopamine-sensitive adenylate cyclase in the rat caudate nucleus. *J Neurochem* **25**: 143–149, 1975.
11. Byington KH, Evidence for the phosphorylation of dopamine by striatal tissue from rat brain. *Life Sci* **53**: 749–754, 1993.
12. Cherbulieux E, Schwarz M and Leber JP, Sur l'acide *O*-phenylene cyclophosphorique et le mecanisme de sa formation. *Helv Chim Acta* **34**: 841–843, 1951.
13. Gornall AG, Bardawill CJ and David MM, Determination of serum proteins by means of the biuret reaction. *J Biol Chem* **177**: 751–766, 1949.
14. Gilman AG, A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc Natl Acad Sci USA* **67**: 305–312, 1970.
15. Seamon K and Daly JW, Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *J Biol Chem* **256**: 9799–9801, 1981.
16. Barnett JV and Kuczenski R, Desensitization of rat striatal dopamine-stimulated adenylate cyclase after acute amphetamine administration. *J Pharmacol Exp Ther* **237**: 820–825, 1986.
17. Colvin RA, Oibo JA and Allen RA, Calcium inhibition of cardiac adenylyl cyclase. Evidence for two distinct sites of inhibition. *Cell Calcium* **12**: 19–27, 1991.