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REGULATION OF A PRESYNAPTIC ADENYLATE CYCLASE FROM BOVINE CEREBELLUM BY β-ADRENERGIC RECEPTORS

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Summary

Intact crude synaptosomes from bovine cerebellum contain, in addition to an externally accessible (postsynaptic) adenylate cyclase, an enzyme with its catalytic center oriented towards the inside of the synaptosome (presynaptic adenylate cyclase). This is demonstrated by the unmasking of latent adenylate cyclase activity by Triton X-100. Furthermore, intact crude synaptosomes can synthesize cyclic AMP from adenine. This synthesis takes place inside the synaptosome as the postsynaptic adenylate cyclase is inactive in the Krebs-Ringer buffer. Presynaptic adenylate cyclase activity is not influenced by depolarization, as shown by [^{3}H]adenine pulse-labeling, but is stimulated by (—)-norepinephrine and (—)-isoproterenol. (±)-Propranolol inhibits this stimulation whereas phentolamine has no effect, suggesting the presence of a β-adrenergic receptor-coupled presynaptic adenylate cyclase.

Introduction

Presynaptic effects of cyclic AMP have been reported in monoaminergic neuronal systems, the best known being the stimulation of tyrosine hydroxylation [1—4], which is the rate-limiting step of catecholamine synthesis. On the other hand, studies on the release of catecholamines from nerve terminals have led to the concept that presynaptic α- and β-receptors are involved in the feedback control of catecholamine release [5]. However, there is as yet little direct

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecyl sulphate.

evidence for the existence of a presynaptically located adenylate cyclase and it remains open to question whether or not stimulation of presynaptic receptors is coupled to changes in presynaptic adenylate cyclase activity.

Studies on tissue slices and occasionally on homogenates have demonstrated a β-adrenergic receptor-mediated cyclic AMP synthesis in cerebellum [6,7]. No attempt has yet been made to relate changes in cyclic AMP formation to a presynaptic adenylate cyclase. The presence of such an enzyme in cerebellar synaptosomes has therefore been investigated and a regulation by adrenergic receptors has been demonstrated here.

Increase in enzyme activity upon lysis of synaptosomes in hypotonic medium has been presented as evidence for a presynaptic adenylate cyclase in rat cortex by Weller [8]. The present report shows that a treatment of intact synaptosomes with small amounts of Triton X-100 also reveals latent adenylate cyclase activity in synaptosomal preparations. Moreover, intra-synaptosomal cyclic AMP synthesis from the ATP precursor, adenine, which can permeate the membranes of intact cells [9], provides further evidence for the presence of a presynaptic adenylate cyclase. Two hypotheses for the regulation of this cyclase were tested: the influence of depolarization-induced Ca^{2+} influx and the role of α- and β-adrenergic receptors. The results show that presynaptic cyclic AMP formation is not affected by depolarization but is stimulated by catecholamines, via β-adrenergic receptors.

Materials and Methods

[2-^{3}H]Adenosine 5′-triphosphate (ATP) and [2-^{3}H]adenine were from the Radiochemical Center, Amersham, U.K. (—)-Norepinephrine bitartrate, (—)-isoproterenol hydrochloride, (±)-propranolol and protoveratrine were from Sigma Chemical Co., St. Louis, MO. Phentolamine was a gift from Dr. Schorderet (Department of Pharmacology, University of Geneva).

Bovine brains were obtained from the local slaughterhouse, chilled to 4°C shortly upon removal from the animal and the cerebellum processed without delay.

Preparation of crude synaptosomal fractions P_2 [10]

For the adenylate cyclase assay, one cerebellum was homogenized in a Braun omnimixer in 9 vols. of 0.32 M sucrose, 1 mM potassium phosphate, 0.1 mM EDTA, pH 7.5 (buffer A). For pulse-labeling experiments with [^{3}H]adenine, homogenization was performed in Krebs-Ringer buffer: 117 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose, adjusted to pH 7.4 with NaOH. Either homogenate was filtered through four layers of cheesecloth and centrifuged for 5 min at 1000 × *g*. The supernatant was centrifuged for 20 min at 12 000 × *g* and the pellets (P_2) were suspended in buffer A or Krebs-Ringer buffer. All operations were performed at 4°C. Protein was determined according to the method of Lowry et al. [11] using bovine serum albumin as standard.

Adenylate cyclase assay

Intact synaptosomes: P_2 was suspended in 0.32 M sucrose; 5 mM Tris-HCl,

pH 7.5; 3 mM $MgCl_2$; 3 mM 1,4-dithio-DL-threitol (buffer B). Membranes: P_2 suspended in buffer B was lysed at pH 8 in 10 vols. of 5 mM Tris-HCl/2 mM EGTA for 1 h at 4°C. Then it was centrifuged for 20 min at 12 000 × *g* and the pellet was suspended in buffer B. The assay medium (150 µl) contained 50 mM glycylglycine, pH 7.5; 20 mM KCl; 6 mM $MgCl_2$; 0.2 mM ATP; 2 mM cyclic AMP; 5 mM phosphoenolpyruvate; 3 mM 1,4-dithio-DL-threitol; 6 mM caffeine; 10 µg pyruvate kinase and 1 µCi [^{3}H]ATP (25—50 cpm/pmol). For intact synaptosomes, 0.32 M sucrose was included in this assay medium in order to maintain isotonicity. Controls showed that sucrose does not affect cyclase activity of membranes. The assay was carried out with 25 µl (50—100 µg) of protein for 10 min at 37°C and was stopped by the addition of 150 µl of 2% SDS containing 12 mM cyclic AMP and 40 mM ATP. Cyclic [^{3}H]AMP was isolated according to the method of Krishna et al. [12].

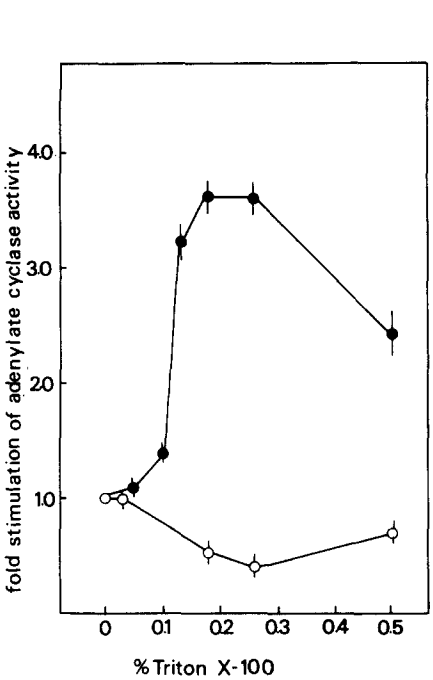
[^{3}H]Adenine labeling

Crude synaptosomes from one cerebellum were suspended in 25 ml Krebs-Ringer buffer (approx. 4 mg protein/ml), 2—4 µCi [^{3}H]adenine (15 Ci/mmol) were added and the suspension was gently stirred at 37°C for 60 min. 1-ml samples were further incubated at 37°C after addition of 1 mM 1-methyl-3-isobutylxanthine and of the compounds to be tested. Incubations with catecholamines were carried out in the presence of 0.1% ascorbic acid. The reactions were stopped by the addition of 0.5 ml of 25% perchloric acid (w/w) containing 2 mM carrier cyclic AMP. Blanks were samples to which perchloric acid was added at the same time as [^{3}H]adenine. Cyclic [^{3}H]AMP was isolated from the acid extracts according to the method of Krishna et al. [12]. Results (2—4 times higher values than those of blanks) were expressed in cpm/mg protein taking into account the recovery of the cyclic AMP isolation (76%). Phosphodiesterase digestion and thin-layer chromatography on silica gel confirmed that the isolated product was cyclic AMP [13].

Results

Unmasking of latent adenylate cyclase activity in crude synaptosomal preparations by Triton X-100

Addition of 0.1% Triton X-100 to suspensions of intact synaptosomes causes lysis, as indicated by a sharp increase in lactate dehydrogenase activity. In order to determine whether lysis affects the activity of adenylate cyclase, crude synaptosomes were assayed in the presence of small amounts of Triton X-100. Fig. 1 shows that the cyclase activity of intact synaptosomes is increased approx. 3.5-fold by 0.1—0.2% Triton (w/v), whereas no activation, or rather the opposite, takes place in the instance of the cyclase of synaptosomal membranes. An endogenous Ca^{2+}-binding protein activator of adenylate cyclase, calmodulin, has been shown to be present in cerebellar synaptosomes [14]. It was of interest to find out whether the activation by Triton is due solely to the release of calmodulin and to the subsequent stimulation of an external (postsynaptic) cyclase, or also to the unmasking of an internal (presynaptic) cyclase. Lysis by Triton was therefore repeated in the presence of saturating amounts of calmodulin, which prevents any further stimulation by the endogenous



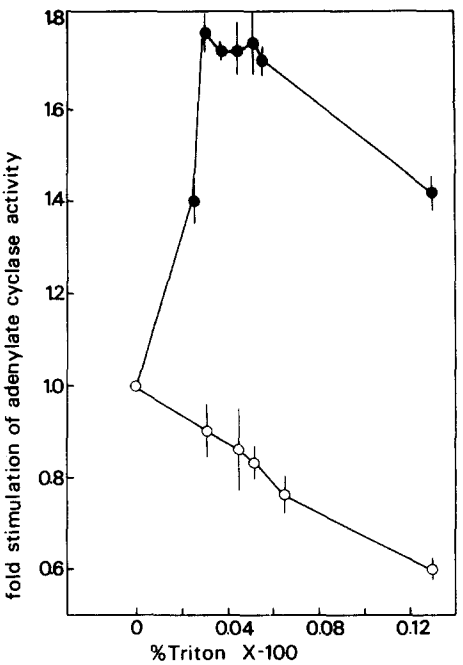


Fig. 1. Activation of adenylate cyclase activity of intact synaptosomes (•) and synaptosomal membranes (○) by Triton X-100. 120 μg of protein were used in the cyclase assay. The basal activity (i.e., 1-fold activation) was 131 ± 4 pmol · min^{-1} · mg^{-1} for intact synaptosomes and 194 ± 48 pmol · min^{-1} · mg^{-1} for membranes. Results are means of triplicate determinations ± S.E.

Fig. 2. Activation of adenylate cyclase activity of intact synaptosomes (•) and synaptosomal membranes (○) by Triton X-100. Adenylate cyclase (120 μg of protein) was assayed in the presence of 3 μg of calmodulin. Basal activity (1-fold activation) corresponded to 95 ± 3 pmol · min^{-1} · mg^{-1} for intact synaptosomes and 168 ± 7 pmol · min^{-1} · mg^{-1} for membranes. Results are means of triplicate determinations ± S.E.

activator. Fig. 2 shows that, under these conditions, addition of Triton to intact synaptosomes still activates adenylate cyclase. Thus, a substantial part of the Triton-induced activation stems from a presynaptic adenylate cyclase. Optimal concentrations of Triton for the activation of cyclase from intact synaptosomes were observed to be lower in the presence of, rather than in the absence of added calmodulin.

[^{3}H]Adenine-labeling of crude synaptosomes from bovine cerebellum

Fig. 3 shows cyclic AMP formation in crude preparations of intact synaptosomes. After addition of [^{3}H]adenine, the levels of newly synthesized cyclic AMP increase until a plateau is reached after 60—75 min. Incubation in the presence of the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine, leads to significantly higher levels of newly synthesized cyclic AMP. To determine whether the cyclic AMP formed by incubation with [^{3}H]adenine was synthesized inside or outside the synaptosomes, adenylate cyclase activity of intact synaptosomes was assayed with [^{3}H]adenine as substrate or with [^{3}H]ATP. When Krebs-Ringer buffer was used as buffer and ATP as substrate, little or no activity was observed (less than 5% of that obtained in the standard medium

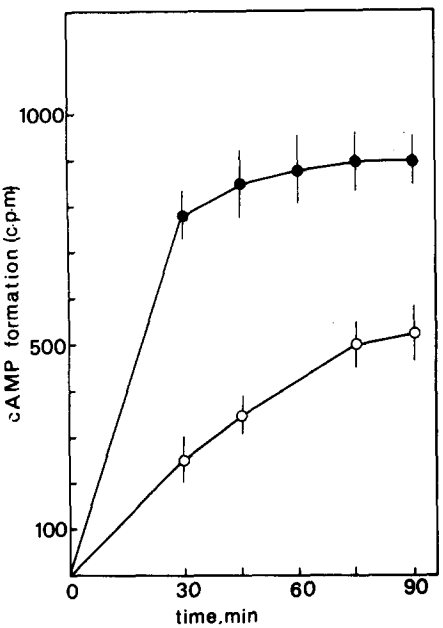


Fig. 3. Time course of cyclic AMP formation from [^{3}H]adenine in intact synaptosomes in the presence (●) and absence (○) of 1 mM 1-methyl-3-isobutylxanthine. Results are means of triplicate determinations ± S.E.

with 0.32 M sucrose). Indeed, the $CaCl_2$ concentration of Krebs-Ringer buffer (1.2 mM) is known to inhibit adenylate cyclase whereas the $MgCl_2$ concentration (1.3 mM) is too low for optimal activity [15]. When the standard assay system containing 0.32 M sucrose was used with ATP as substrate, adenylate cyclase was active and could be stimulated by NaF and 5′-guanylylimidodiphosphate as can be seen in Table IA. When the precursor, [^{3}H]adenine, was used

TABLE I

EFFECT OF NaF AND 5′-GUANYLYLIMIDODIPHOSPHATE ON CYCLIC AMP FORMATION IN CRUDE SYNAPTOSOMAL FRACTIONS

(A) P_2 prepared in buffer A (see Methods), adenylate cyclase assay in the standard medium with 0.32 M sucrose and [^{3}H]ATP as substrate. Results are means ± S.E. of triplicate determinations. Cyclic AMP formation expressed as pmol/min per mg protein. (B) P_2 prepared and assayed in Krebs-Ringer buffer with [^{3}H]adenine as substrate. P_2 was prelabeled for 60 min with [^{3}H]adenine and reactions were performed for 15 min in the presence of 1 mM 1-methyl-3-isobutylxanthine. Cyclic AMP formation expressed as % of control value ± S.E.

Additive	Cyclic AMP formed	% of control
A		
none	131 ± 4	100
NaF (10 mM)	300 ± 9	229
5′-guanylylimidodiphosphate	169 ± 11	128
B		
NaF (10 mM)	93 ± 12 (n = 3)	
5′guanylylimidodiphosphate	84 ± 26 (n = 3)	

TABLE II

EFFECTS OF DEPOLARIZING AGENTS AND CATECHOLAMINES ON CYCLIC AMP FORMATION IN [^{3}H]ADENINE-PRELABELED CRUDE SYNAPTOSOMES

Synaptosomes were prelabeled for 60 min with [^{3}H]adenine, then 1 mM 1-methyl-3-isobutylxanthine was added; 2 min were allowed for depolarization or 15 min for the incubation with the catecholamines. Control values without additives were 546 ± 40 cpm/mg protein (n = 6). Stimulation of cyclic AMP formation is expressed at % relative to control. Results are means ± S.E. and the number of separate experiments is given in brackets.

Additives	Stimulation of cyclic AMP formation
K^+ (50 mM)	94.3 ± 1.7 (3)
Protoveratrine (10^{-4} M)	106 ± 11 (3)
(—)-Norepinephrine (10^{-4} M)	137 ± 3 * (6)
(—)-Norepinephrine (10^{-4} M) + (±)-propranolol (10^{-4} M)	99 ± 2.1 (3)
(—)-Isoproterenol (10^{-5} M)	148 ± 9 * (5)
(—)-Isoproterenol (10^{-5} M) + (±)-propranolol (10^{-4} M)	95.7 ± 4.5 (5)
(—)-Isoproterenol (10^{-5} M) + phentolamine (10^{-5} M)	129 ± 7.5 * (3)
(±)-Propranolol (10^{-4} M)	98.4 ± 3.7 (3)
Phentolamine (10^{-5} M)	98.2 ± 2.3 (3)

* $P < 0.01$ according to paired Student's t-test.

with Krebs-Ringer buffer, no stimulation of cyclic AMP synthesis could be detected (Table IB), presumably because NaF and 5'-guanylylimidodiphosphate cannot penetrate into the synaptosome.

Effects of depolarizing agents and catecholamines on cyclic AMP synthesis in [^{3}H]adenine-prelabeled crude synaptosomes

Crude synaptosomes were prelabeled for 60 min with [^{3}H]adenine and incubations were then continued in the presence of 1 mM 1-methyl-3-isobutylxanthine with depolarizing agents or catecholamines. The amounts of cyclic AMP newly synthesized from [^{3}H]adenine were determined by isolating cyclic AMP and measuring its radioactivity. Table II shows that high K^+ (50 mM) or protoveratrine (100 μM), which were employed as depolarizing agents, do not affect cyclic AMP synthesis. In contrast, (—)-norepinephrine (100 μM) or (—)-isoproterenol (10 μM) stimulate cyclic AMP formation. This stimulation is blocked by the β-adrenergic receptor antagonist, propranolol, but it is not influenced by the α-adrenergic receptor antagonist, phentolamine.

Discussion

The unmasking of a latent adenylate cyclase activity in synaptosomes by hypotonic lysis [8] or by Triton X-100, as shown in this work, reveals the existence of a presynaptic adenylate cyclase with its catalytic center oriented towards the inside of the synaptosome, although a cyclase of another cellular origin (e.g., glia) cannot be ruled out completely. The 2.6-fold activation of adenylate cyclase activity upon hypotonic lysis as found by Weller [8] had led to the consideration that approx. 60% of the adenylate cyclase from rat cortex is located presynaptically. This is probably overrated, because activation of the externally located cyclase through release of calmodulin upon lysis has not

been taken into account. A presynaptically located dopamine-sensitive adenylate cyclase has also been found in the rat, *Substantia nigra*, by direct methods involving in vivo treatment with 6-hydroxydopamine, kainic acid, or by surgical lesions [16]. Harris [17,18] has described a 'membrane-enclosed' catecholamine-sensitive adenylate cyclase in the rat, *Corpus striatum*. Moreover, tyrosine hydroxylase, which is localized within synaptosomes, is known to be stimulated by cyclic AMP [2,3] via cyclic AMP-dependent phosphorylation of the enzyme [4]. These findings raise the question of how presynaptic cyclic AMP production is regulated. Weller [8] proposed that Ca^{2+} influx during depolarization might stimulate presynaptic adenylate cyclase. However, Krueger et al. [19] have shown that depolarization does not influence cyclic AMP levels in crude synaptosomal preparations but leads to Ca^{2+}-dependent phosphorylation. Determination of newly synthesized cyclic AMP by the more sensitive adenine pulse-labeling technique as performed in this work confirms that no stimulation of cyclic AMP synthesis occurs in crude synaptosomes from bovine cerebellum upon depolarization. Similarly, in other systems such as neuroblastoma [20] or pheochromocytoma [21] cells, depolarization does not alter cyclic AMP levels. In contrast, synthesis is stimulated by (—)-norepinephrine and (—)-isoproterenol as shown here. This stimulation is blocked by propranolol but not affected by phentolamine, indicative of a β-adrenergic receptor-linked adenylate cyclase. Indeed, control experiments confirm that extrasynaptosomal cyclic AMP synthesis from externally formed ATP can be ruled out under the conditions of adenine labeling. Thus, in bovine cerebellar synaptosomes, catecholamines stimulate the activity of a presynaptic adenylate cyclase. Work is in progress to determine whether this activation is instrumental to the regulation of tyrosine hydroxylase and presynaptic phosphorylations.

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References

1 Harris, J.E., Morgenroth, V.H., Roth, R.H. and Baldessarini, R.J. (1974) Nature 252, 156—158
2 Patrick, R.L., Snyder, T.E. and Brachas, J.O. (1975) Mol. Pharmacol. 11, 621—631
3 Goldstein, M., Bronaugh, R.L., Ebstein, B. and Roberge, C. (1976) Brain Res. 109, 563—574
4 Joh, T.H., Park, D.H. and Reis, D.J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4744—4748
5 Shenoy, K. and Ziance, R.J. (1979) Life Sci. 24, 255—264
6 Chasin, M., Rivkin, I., Mamrak, F., Samaniego, S.G. and Hess, S.M. (1971) J. Biol. Chem. 246, 3037—3041
7 Nishikori, K. and Maeno, H.M. (1979) Arch. Biochem. Biophys. 195, 505—517
8 Weller, M. (1977) Biochim. Biophys. Acta 469, 350—354
9 Kebabian, J.W., Kuo, J.F. and Greengard, P. (1972) Adv. Cyclic Nucl. Res. 2, 131—137
10 Gray, F.G. and Whittaker, V.P. (1962) J. Anat. 96, 79—88
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Krishna, G., Weiss, B. and Brodie, B.B. (1968) J. Pharmacol. Exp. Therm. 163, 379—385
13 Ryan, J., Rogers, G.N., Toscana, D.G. and Storm, D.R. (1977) J. Biol. Chem. 252, 1719—1722
14 Novak-Hofer, I., Malnoë, A., Cox, J.A. and Stein, E.A. (1979) Experientia 35, 973
15 Brostrom, M.A., Brostrom, C.O., Beckenridge, B.M. and Wolff, D.J. (1978) Adv. Cyclic Nucl. Res. 9, 85—99

16 Quick, M., Emson, P.C. and Joyce, E. (1979) Brain Res. 167, 355—365
17 Harris, J.E. (1975) Mol. Pharmacol. 12, 546—558
18 Harris, J.E. (1978) Biochem. Pharmacol. 27, 2919—2925
19 Krueger, B., Forn, J. and Greengard, P. (1977) J. Biol. Chem. 252, 2764—2773
20 Study, R.E., Breakfield, X.O., Bartfai, T. and Greengard, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 75, 6295—6299
21 Chalfie, M., Settipani, L. and Perlman, R.L. (1979) Mol. Pharmacol. 15, 263—270