Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 286-292

REGULATION OF CYCLIC AMP AND CYCLIC GMP LEVELS BY ADRENOCORTICOTROPIC HORMONE IN CULTURED NEURONS

Patrick Anglard, Jean Zwiller, Guy Vincendon and Jean-Claude Louis

Centre de Neurochimie du CNRS, 5 rue Blaise Pascal
67084 STRASBOURG Cedex, France

Received October 23, 1985

SUMMARY. The effect of adrenocorticotropic hormone (ACTH) on the intracellular concentration of cyclic nucleotides was studied in cultures of neurons from embryonic chick cerebral hemispheres. Incubation of neurons with ACTH(1-24) in the presence of phosphodiesterase inhibitor isobutylmethylxanthine resulted in a sustained increase in cyclic AMP while rise in cyclic GMP level was transient. The values obtained for half-maximal stimulation were 0.5 μM and 0.03 nM for cyclic AMP and cyclic GMP respectively. Concomitantly, ACTH(1-24) stimulated guanylate cyclase activity (half-maximal stimulation at 0.02nM). These results suggest the existence of two distinct populations of ACTH receptors in neurons and provide the first evidence that cyclic GMP does mediate the action of ACTH in neurons.

It is taken for granted that adrenocorticotropic hormone (ACTH) is produced and occurs widely within the central nervous system (1,2), affecting both metabolism and behaviour (3). ACTH is involved in phosphorylation of phosphatidylinositol, cyclic AMP metabolism, phosphorylation of synaptic membrane proteins and in regulation of RNA and protein synthesis (reviewed in 3). We recently demonstrated neurotropic effects of ACTH on pure cultures of neurons. ACTH was shown to promote survival and maturation of neurons (4) and to regulate glucose uptake (5). In spite of all these studies, the underlying molecular mechanism of ACTH action on nerve cells remain to be elucidated. Cyclic AMP has been proposed as the second messenger of ACTH action in rat subcortical tissue (6) and recently, evidence for the effect of ACTH on cyclic AMP metabolism in cultured rat cortical neurons (7) and astroglial cells (8) have been reported.

It is reported in the present paper that ACTH increases the levels of cyclic AMP as well as of cyclic GMP in cultured chick embryo cortical

0006-291X/85 $1.50

neurons, the hormone being 10,000-fold more potent in increasing cyclic GMP level.

MATERIALS AND METHODS

Peptides and chemicals. Synthetic ACTH(1-24) was generously provided by Drs R. Andreatta and K. Scheibli (Ciba-Geigy, Basle, Switzerland). ACTH(1-10), ACTH(4-10), ACTH(4-11), ACTH(1-13), α-melanocyte stimulating hormone (α-MSH) and isobutylmethylxanthine were purchased from Sigma (St Louis, Mo, USA). [γ-^{32}P]-guanosine triphosphate, cyclic AMP assay kits and cyclic GMP RIA kits were from the Radiochemical Centre, Amersham (Les Ulis, France). Culture media and sera were from Flow Laboratories (Asnières, France). Falcon plastic culture dishes were used.

Cell culture. Cultures of neurons from embryonic chick cerebral hemispheres were maintained as described (4,5). Cultures were initiated and grown for 3 days in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum, penicillin (25 U/ml) and streptomycin (25 µg/ml). From the fourth day, cultures were grown in serum-free DMEM containing glucose (33 mM), transferrin (100 µg/ml), insulin (5 µg/ml), putrescin (0.1 mM), progesterone (10 nM), selenium (30 nM) and antibiotics as described (5). Cultures were grown for 7 days in 60 mm plastic dishes. Media were renewed at alternate days.

Measurement of ACTH-induced changes in cyclic nucleotides levels. Seven-day-old neuronal cultures were preincubated for 20 min in the absence of peptide, followed by incubation with various concentrations of ACTH(1-24) or its related peptides. Incubations were carried out in 2 ml of Hepes-buffered Krebs-Ringer solution (KRH: NaCl 125 mM, KCl 4.8 mM, $MgSO_4$ 1.3 mM, KH_2PO_4 1.2 mM, glucose 1 g/l, Hepes 25 mM, pH 7.4) containing 1 mM isobutylmethylxanthine (IBMX). The reaction was terminated by aspiration of the media and addition of 350 µl of ice-cold 1 M perchloric acid. Cellular proteins were centrifuged at 5,000 g for 15 min. The supernatants were neutralized with 3 M K_2CO_3 and cyclic nucleotide content was determined with the cyclic AMP assay kit and the cyclic GMP RIA kit from Amersham. Protein was determined on the resuspended pellet by the method of Lowry.

Guanylate cyclase assay. Cultures were incubated for 5 min with various concentrations of ACTH(1-24) in complete serum-free DMEM. Cells were then homogenized in 250 µl of ice-cold 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 10 mM β-mercaptoethanol. Guanylate cyclase activity was measured as previously reported (9).

RESULTS

Incubation of chick cortical neurons with ACTH(1-24) in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine produced a rapid increase of the intracellular cyclic AMP and cyclic GMP concentrations (figure 1). Maximal stimulations were achieved after 6-8 min. The cyclic AMP response was only slightly decreased with longer (30 min) incubations, while a decline in the cyclic GMP response was more rapid.

Accumulation of cyclic nucleotides in neurons at various doses of ACTH(1-24) treatment is represented in figure 2. The concentrations

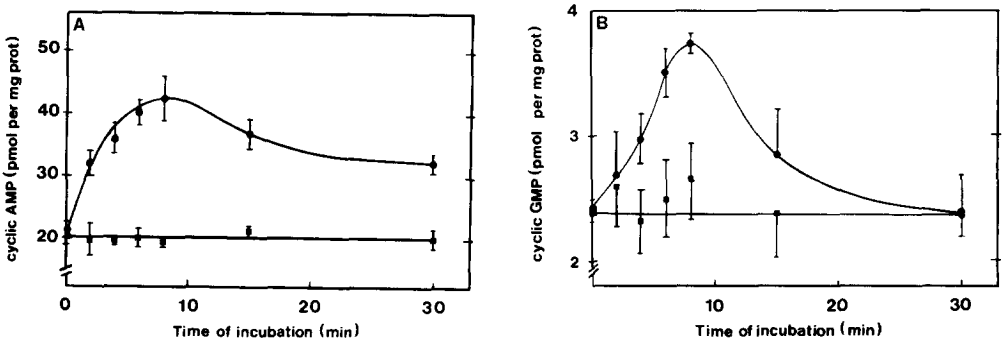
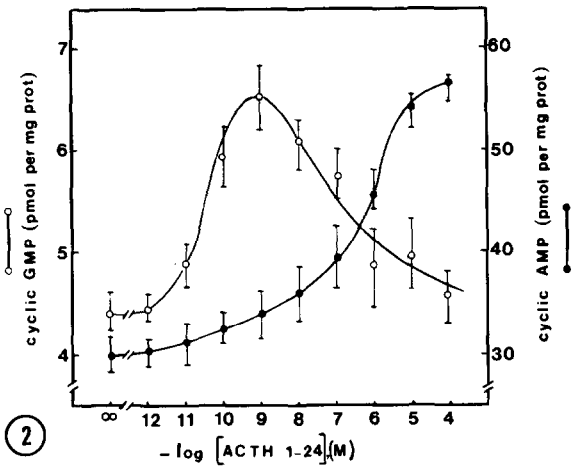


Figure 1. Time-course of ACTH-induced increase of cyclic AMP (A) and cyclic GMP (B) levels in cultured neurons.
Seven-day-old cultures were incubated in Hepes-buffered KRH, pH 7.4, in the presence (●) or in the absence (■) of ACTH(1-24) (10 μM in A and 10 nM in B). All incubations were in the presence of 1 mM IBMX. After the indicated times, intracellular cyclic AMP and cyclic GMP contents were determined with the Amersham kits. Each point is the mean ± S.D. of 6-10 determinations.

of ACTH(1-24) most efficacious were 100 μM and 1 nM for cyclic AMP and cyclic GMP respectively. The cyclic GMP response declined at higher concentrations of the peptide. The value for half-maximal stimulation was much higher for cyclic AMP (EC_{50}: 0.5 μM) than for cyclic GMP (EC_{50}: 0.03 nM). The dose-response effects of ACTH(1-24) on guanylate cyclase activity are shown in figure 3. The potency (EC_{50}: 0.02 nM) and maximal efficiency (at 1 nM) were essentially the same for the observed guanylate cyclase activation as found for the cyclic GMP rise.

A number of ACTH-related peptides were tested for their ability to affect cyclic nucleotides levels in neurons. Table 1 shows that all compounds containing the sequence (4-10) were effective in increasing cyclic AMP, but less than ACTH(1-24). This suggests that the former peptides were not able to fully activate ACTH receptors. The potency of the ACTH-peptides for the cyclic AMP response decreased in the sequence: ACTH(1-24) (EC_{50}: 0.5 μM) > ACTH(1-13), α-MSH (EC_{50}: 1 μM) > ACTH(1-10), ACTH(4-10), ACTH(4-11) (EC_{50}: 5-10 μM). By contrast, ACTH fragments (1-10), (4-10) and (4-11) were twenty-fold less potent (EC_{50}: ≃ 10 nM) and five-fold less efficient than ACTH(1-24) to induce the cyclic GMP response. Furthermore, ACTH(1-13) and α-MSH, while affecting cyclic AMP level, were unable to affect cyclic GMP production.



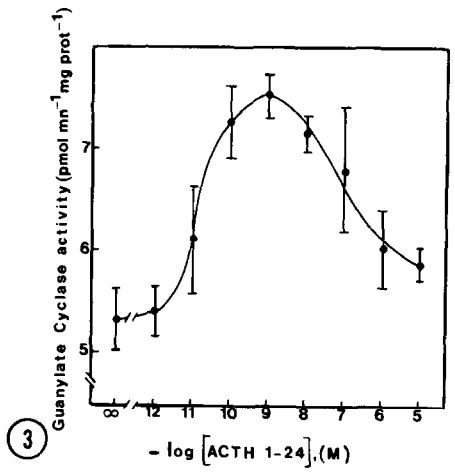


Figure 2. Concentration-response curves for the increase in the levels of cyclic AMP (●) and cyclic GMP (○) induced by ACTH(1-24) in cultured neurons. Cultures were incubated in KRH with the indicated concentrations of ACTH(1-24) in the presence of 1 mM IBMX. The intracellular contents of cyclic nucleotides were measured after incubation for 7.5 min. Each point is the mean ± S.D. of 6 different cultures.

Figure 3. Concentration-response curve for the activation of guanylate cyclase in cultured neurons. Cultures were incubated with the indicated concentrations of ACTH(1-24) in serum-free DMEM for 5 min at 37°C. Homogenates in 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 10 mM β-mercaptoethanol were used for determination of guanylate cyclase activity (9). Each point is the mean ± S.D. of duplicate determinations of 4-6 different cultures.

TABLE 1

Structure-activity relationship for various ACTH-related peptides on cyclic nucleotide production in cultured neurons

Peptide	Percentage increase in:	
	cyclic AMP	cyclic GMP
ACTH(1-24)	95	102
ACTH(1-10)	58	20
ACTH(4-10)	49	18
ACTH(4-11)	57	20
ACTH(1-13)	63	NS
α-MSH	80	NS
ACTH(18-39)	NS	NS

Cultured neurons were incubated in the presence of the different peptides listed (concentration range: 1 nM-10 μM). The intracellular cyclic AMP and cyclic GMP contents were measured after incubation for 7.5 min at 37°C in the presence of IBMX. Values are the mean of ACTH-induced increases of cyclic nucleotide levels from 3-6 independent experiments.
NS: not significantly different from control values.

DISCUSSION

The present results demonstrate the stimulation of cyclic AMP and cyclic GMP formation by ACTH in cultured neurons from embryonic chick cerebral hemispheres. Whereas several reports have suggested a role for ACTH in the regulation of cyclic AMP levels in nerve tissues (6,7,8), this is the first report to provide evidence that cyclic GMP mediates the effect of ACTH in neurons. ACTH(1-24) caused a rapid and transient accumulation of cyclic GMP and stimulated guanylate cyclase activity. These two responses were achieved at 1 nM ACTH(1-24) concentration and decreased at higher concentrations, thereby suggesting the involvement of receptor-mediated desensitization. Effect of ACTH on cyclic GMP formation (10,11) and guanylate cyclase activity (12) were already observed in adrenal cortex, although the role of cyclic AMP as a second messenger for ACTH in this gland is fully comprehended (reviewed in 13). There is a difference in the response elicited on the two cyclic nucleotides, ACTH(1-24) showing much higher potency for cyclic GMP than for cyclic AMP. The estimated EC_{50} value (0.5 μM) for the cyclic AMP response in our neuronal cultures was found almost similar to that reported in rat brain subcortical tissue (6). In cultured rat cortical neurons (7) and rat astroglial cells (8), ACTH(1-24) was, however, more potent (EC_{50}: 10 nM) for inducing cyclic AMP response than in our system. This may be attributed to differences in animal species (7,8), in cell types (8) or to the use of forskolin in the estimation of the ACTH effect (7).

On the basis of these data, it is tempting to argue that there may exist two sets of ACTH receptor populations in neurons. One set of receptors may be coupled to adenylate cyclase responsible for cyclic AMP production. The second type of receptors seems to operate by interacting with ACTH leading to guanylate cyclase activation, probably via increase of intracellular Ca^{++} concentration. Further arguments for the existence of two types of receptors come from the structure-activity stu-

dies. ACTH(1-24) was the most potent peptide in our system on both cyclic AMP and cyclic GMP. Whereas ACTH(1-13) and α-MSH, its N-terminal acetylated and C-terminal amidated form, stimulated cyclic AMP production, they were unable to induce cyclic GMP response. Whether these two types of receptors are functional on the same neuron, or if they are expressed by two distinct populations of neurons in the culture cannot be concluded presently.

ACTH has been shown to regulate polyphosphoinositide metabolism in rat brain synaptic membranes (14) and in the adrenal cortex (15) and protein kinase C activity in the adrenal cortex (16). Phosphoinositide response is known to be accompanied in many cell types by elevation of cyclic GMP (17). Recent demonstration that rat brain guanylate cyclase is a substrate of protein kinase C (18) suggests that the intracellular cyclic GMP elevation is achieved by ACTH via phosphoinositide response and protein kinase C activation. How cyclic GMP, once accumulated, is involved in the cellular functions regulated by ACTH remains to be elucidated.

ACKNOWLEDGEMENTS

We thank Mrs Marie-Odile Revel for help with guanylate cyclase assays and Dr Anant N. Malviya for critically reading the manuscript. This work was supported in part by a research grant from Ciba-Geigy (Rueil-Malmaison, France).

REFERENCES

1. Brownstein, M.J. (1980) Neural peptides and neuronal communication, pp. 93-99, Raven Press, New York.
2. Krieger, D.T. (1983) Science 222, 975-985.
3. de Wied, D. and Jolles, J. (1982) Physiol. Rev. 62, 977-1059.
4. Daval, J.L., Louis, J.C., Gérard, M.J. and Vincendon, G. (1983) Neurosci. Lett. 36, 299-304.
5. Daval, J.L., Anglard, P., Gérard, M.J., Vincendon, G. and Louis, J.C. (1985) J. Cell. Physiol. 124, 75-80.
6. Wiegant, V.M., Dunn, A.J., Schotman, P. and Gispen, W.H. (1979) Brain Res. 168, 565-584.
7. Weiss, S., Sebben, M. and Bockaert, J. (1985) J. Neurochem. 45, 869-874.
8. Van Calker, D., Löffler, F. and Hamprecht, B. (1983) J. Neurochem. 40, 418-427.
9. Zwiller, J., Basset, P. and Mandel, P. (1981) Biochim. Biophys. Acta 658, 64-75.
10. Perchellet, J.P. and Sharma, R.K. (1979) Science 203, 1259-1260.

11. Hayashi, K., Sala, G., Catt, K. and Dufau, M.L. (1979) J. Biol. Chem. 254, 6678-6683.
12. Nambi, P., Aiyar, N.V. and Sharma, R.K. (1982) Arch. Biochem. Biophys. 217, 638-646.
13. Saez, J.M., Morera, A.M. and Dazord, A. (1981) Advances in cyclic nucleotide research, vol. 14, pp. 563-579, Raven Press, New York.
14. Jolles, J., Zwiers, H., van Dongen, C., Schotman, P., Wirtz, K.W.A. and Gispen, W.H. (1980) Nature 286, 623-625.
15. Farese, R., Sabir, M.A. and Larson, R.E. (1980) J. Biol. Chem. 255, 7232-7237.
16. Vilgrain, I., Cochet, C. and Chambaz, E. (1984) J. Biol. Chem. 259, 3403-3405.
17. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.
18. Zwiller, J., Revel, M.O. and Malviya, A.N. (1985) J. Biol. Chem. 260, 1350-1353.