Vol. 120, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 30, 1984 Pages 540-547

EFFECTS OF FORSKOLIN AND CHOLERA TOXIN ON CYCLIC AMP RELEASE IN A NEUROTENSIN-SECRETING RAT C-CELL LINE.

Fusun Zeytin, Christiane Mougin, Paul Brazeau and Nicholas Ling

Laboratories for Neuroendocrinology
The Salk Institute for Biological Studies
10010 North Torrey Pines Road, La Jolla CA 92037

Received March 20, 1984

Summary. The effects of forskolin and cholera toxin on the regulation of cAMP release were studied in a neurotensin-secreting rat C-cell line. The interaction of these agents with norepinephrine, a potent neurotensin secretagogue, was also investigated. Forskolin stimulated cAMP release 10^2-10^3 fold while it increased neurotensin release 2-3 fold. Cholera toxin caused a 10^2-10^3 fold increase in cAMP release and had no effect on neurotensin release. We conclude that the 44-2 C-cells provide a new model for studying the regulation of the concomitant (via forskolin) or independent (via cholera toxin) secretion of cyclic AMP and/or neurotensin.

We established in culture mammalian C-cell lines producing calcitonin (CT) and neurotensin (NT). We developed colony clones of rMTC 44-2, 44-2C (1). We used these cells (44-2C) in which the molar amount of NT secreted is 20-30 times greater than the molar amount of CT to study the regulation of cyclic AMP and NT release in response to forskolin and cholera toxin. These two agents were chosen for the following reasons. First, studies of the relationship between cAMP accumulation (or efflux) and peptide release have been particularly difficult to carry out in intact cells mainly because of the lack of availability of general activators of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1]. The diterpene, forskolin, has been shown to be a potent and reversible activator of adenylate cyclase in intact cells (2-4). This agent has proven to be extremely useful for investigating the relationship between cAMP levels and normal physiologic functions such as neurosecretion. Second, cholera toxin (as other

ABBREVIATIONS: NT, neurotensin; CT, calcitonin; KRBG, Krebs-Ringr-bicarbonate buffer with 90 mgs% glucose, pH 7.4; DMEM, Dulbecco's Modified Eagle's medium supplemented with 15% horse serum; NE, norepinephrine; RIA, radioimmunoassay.

Address Requests for reprints to: Dr. Fusun Zeytin, Laboratories for Neuroendocrinology, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92138.

0006-291X/84 $1.50

enterotoxins) has been shown to activate adenylate cyclase both in membranes and intact cells (5). The mechanism of activation of adenylate cyclase by cholera toxin differs from that of forskolin; cholera toxin activates the enzyme irreversibly and requires interaction with a cell-surface ganglioside (6). Forskolin has been shown to activate the enzyme reversibly (3).

In order to study the effects of forskolin and cholera toxin on cAMP and NT release, we used an experimental technique in which 44-2 C-cells were incubated for 1-180 min with these agents and the accumulation of cAMP and NT into the medium was quantitated by RIA.

MATERIALS AND METHODS

Cell culture: The establishment of the 44-2 C-cell line was described in detail (1). Culture flasks are seeded with 3-5 X 10^6 cells and stocks are maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% horse serum in the absence of antibiotics, in a humidified atmosphere of 93% air and 7% CO_2 at 37° C. Stocks of rMTC 44-2 cells are maintained in liquid nitrogen as insurance against loss of the cell lines, using a method previously described (8). Subcultures are made as described (1).

Short-term secretion experiments: Cells are plated in replicate 35 mm culture dishes at a density of 1.5 x 10^6 cells/dish. Medium is changed at 48 hr intervals. For secretion experiments, each dish is washed twice with KRBG containing 0.5 mM Ca^{++}. 1 ml of fresh KRBG with 1.0 mM Ca^{++} is added to each dish with or without test substances and the incubation is carried out for the indicated time periods at 37°C in a humidified atmosphere of 7% CO_2 and 93% air. At the end of the incubation, buffer is removed and immediately stored at -20°C for radioimmunoassay (RIA) of cAMP and NT. In each experiment, six replicate dishes are washed three times with sterile saline and the protein concentration in these dishes is estimated with the fluorescamine protein determination method (9).

Radioimmunoassays. Quantitation of cAMP and NT secretion is carried out by RIA as described (1).

Materials. Culture medium is obtained from Microbiological Associates, Walkersville, Maryland. Horse serum is purchased from Sterile Systems, Inc., Logan Utah. Forskolin and cholera toxin are from Calbiochem-Behring Co., La Jolla, California. Norepinephrine and all other reagents were from Sigma, St. Louis, Missouri.

RESULTS AND DISCUSSION

Effect of forskolin on cAMP and NT release. As shown in Figure 1, right panel, forskolin stimulates cAMP release; and at 100 μM forskolin, release of cAMP was greater than 10,000 fold as compared to the levels of cAMP measured following incubation with Ca^{++} or NE alone. The left panel shows cAMP release in control (1.0 mM Ca^{++}), high Ca^{++} (4.0 mM) and NE (0.1 and 1.0 μM)-treated cells. Incubation of cells with forskolin and NE results in greater release of cAMP than that measured following stimulation with either agent alone.

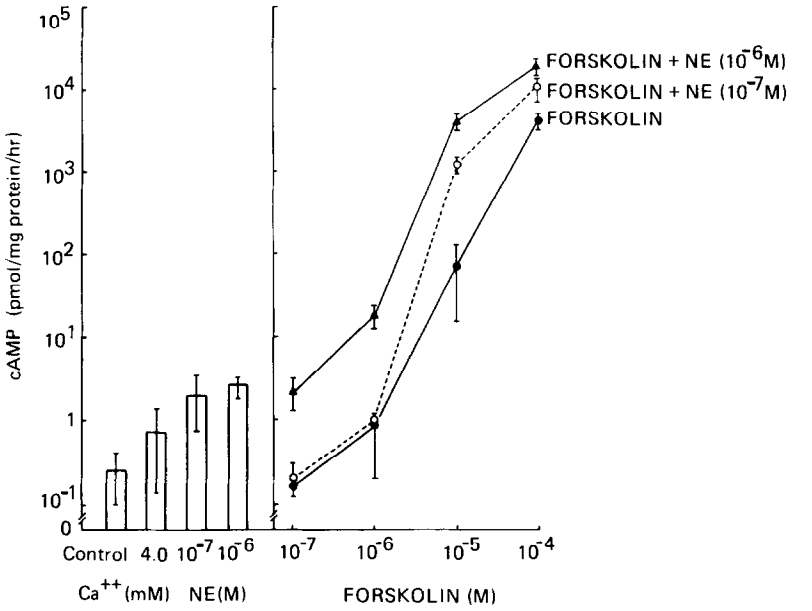

Fig. 1. Dose response of rMTC cells to forskolin: Release of cAMP. rMTC 44-2 C cells were incubated for 1 hr at 37°C in KRBG supplemented with 1.0 or 4.0 mM Ca^{++}, or 1.0 mM Ca^{++} plus 10^{-7} M or 10^{-6} M NE (left panel, clear bars), or they were incubated with increasing concentrations of forskolin with or without NE at 10^{-7} M or 10^{-6}M. The concentration of cAMP in the medium was measured by radioimmunoassay. Each bar (left panel) and each point (right panel) represents the mean and the brackets give the S.E. for six dishes.

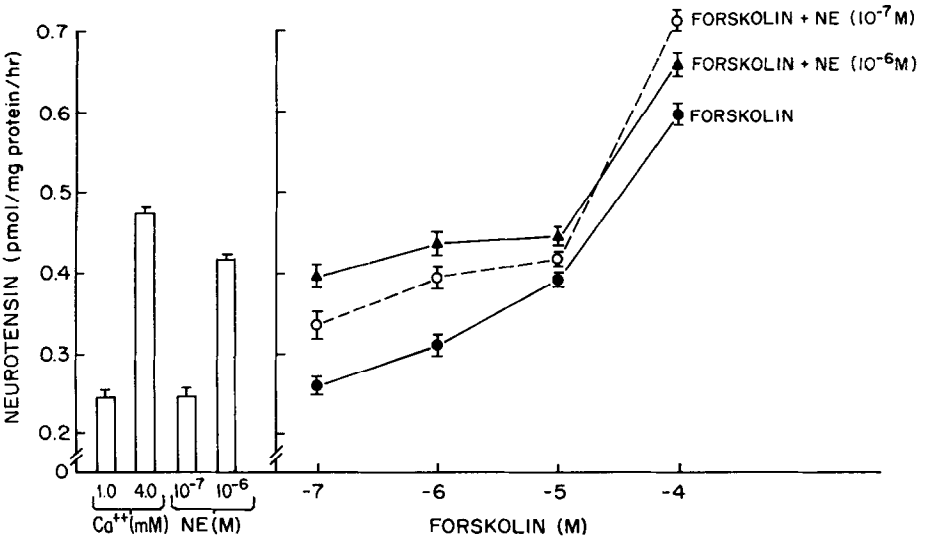

Fig. 2 Dose response of rMTC cells to forskolin: release of NT. rMTC 44-2 C cells were incubated for 1 hr at 37°C in KRBG supplemented with 1.0 or 4.0 mM Ca^{++}, or 1.0 mM Ca^{++} plus 10^{-7} M or 10^{-6} M NE (left panel, clear bars); or they were incubated with increasing concentrations of forskolin with or without NE at 10^{-7} M or 10^{-6} M. The concentration of NT in the buffer was measured by radioimmunoassay. Each bar (left panel) and each point (right panel) represent the mean and the brackets give the S.E. for six dishes.

Forskolin stimulates NT release in a dose-dependent manner. The secretion of NT in the presence of forskolin at 100 μM is greater than that observed with maximally effective dose of NE (10^{-6} M) or Ca^{++}(4.0 mM) (Fig 2).

The activation of adenylate cyclase by forskolin in rat cerebral cortical slides, rat liver and heart slices (2-4) has been shown to occur in the same concentration range of forskolin (0.1-100 μM) shown by us to stimulate cAMP efflux and NT release in 44-2 C-cells.

Time course of action of forskolin on cAMP and NT release. The time-course of action of forskolin on cAMP and NT release are shown in Figs 3 and 4 respectively. Incubation of 44-2 C-cells with forskolin (Fig 3) causes a marked increase in cAMP release as compared to the control treatment group (inset). In the presence of 1.0 mM Ca^{++} the maximum release of cAMP occurs at 120-180 min and the cAMP levels range between 12-18 pmol/ng protein. In remarkable contrast, in the forskolin treatment group, the maximum cAMP release is in the range of 100-500 nmol/mg protein and the highest levels of

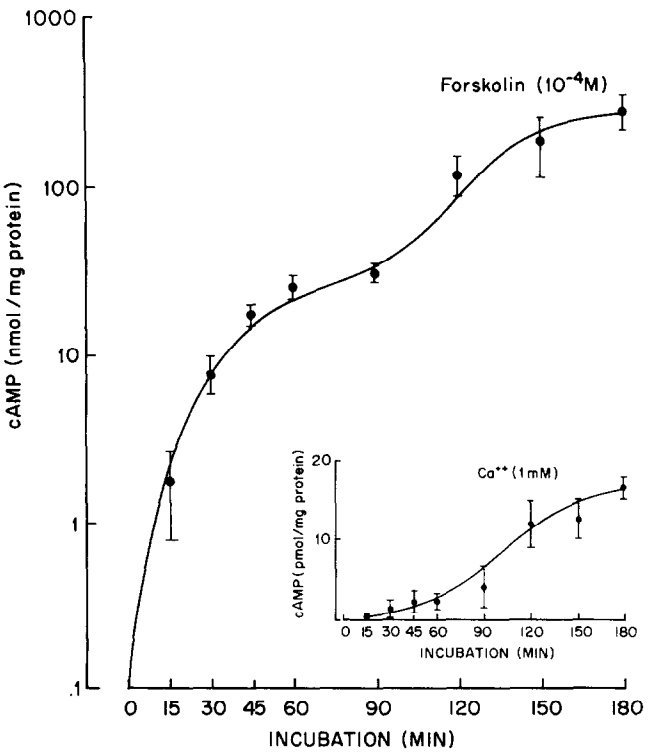

Fig. 3. Time course of forskolin-stimulated cAMP release. Cells were incubated in KRBG supplemented with 1.0 mM Ca^{++} alone, or with 10^{-4} M forskolin for the indicated times and cAMP accumulation in the medium was measured by radioimmunoassay. Each point represents the mean and the brackets give the S.E. for six dishes.

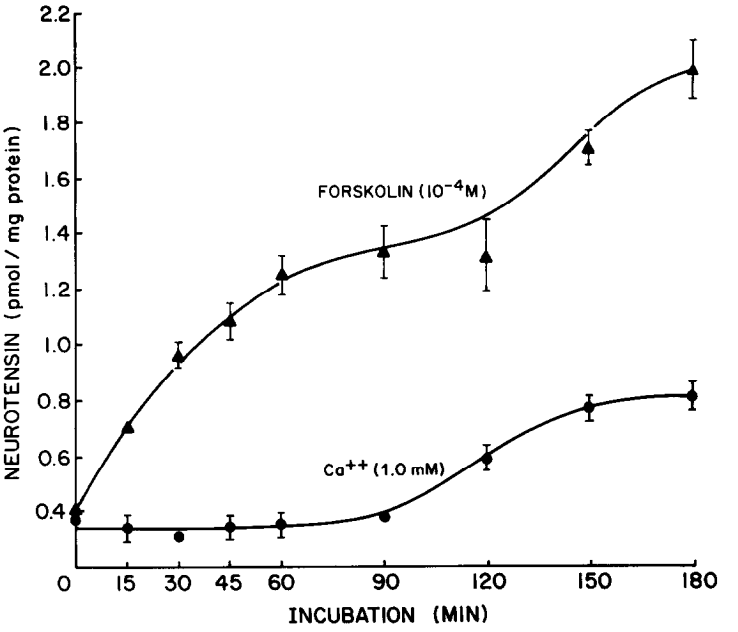

Fig. 4. Time course of forskolin-stimulated NT release. Cells were incubated in KRBG supplemented with 1.0 mM Ca++ alone or with 10^{-4} M forskolin for the indicated times and NT accumulation in the incubation buffer was measured by radioimmunoassay. Each point represents the mean and the brackets give the S.E. for six dishes.

cAMP release occur at 150-180 min incubation. As shown in Fig 4, forskolin at 100 μM causes a brisk increase in NT release over control (1.0 mM Ca^{++}) values within 15 min and NT release remains elevated and does not reach a plateau during the course of the 180 min incubation.

Effect of repeated additions of forskolin on cAMP and NT release. The top panel of Fig 5 shows release of cAMP following repetitive treatment of 44-2 C-cells with forskolin (10^{-4} M) or Ca^{++} (4.0 mM). cAMP release was maximal in control cultures at 0-10 min and declined progressively thereafter (top left panel). In forskolin-treated cells a maximal accumulation of cAMP in the medium was observed at 10-20 min and this level did not fluctuate significantly in the two subsequent 10 min pulses (i.e. 20-30 and 30-40 min; top right panel).

The mechanism whereby forskolin stimulates cAMP efflux and NT release by 44-2 C-cells is not known. In other intact cell systems, such as rat brain cortical slices (2), the activation of adenylate cyclase by forskolin is purported not to be directly related to interaction with cell surface receptors for biogenic amines because alpha and beta adrenergic blockers do not block

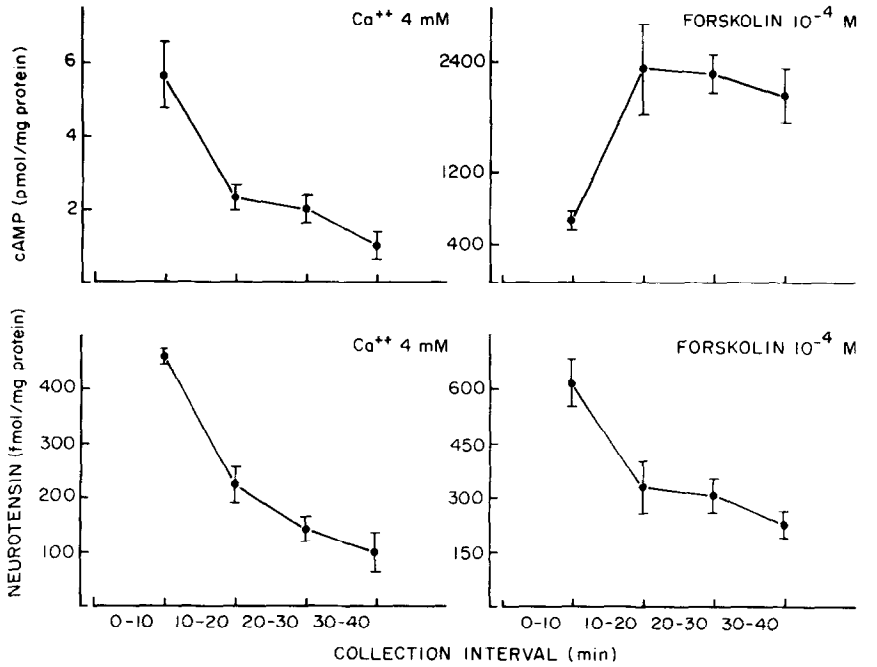

Fig. 5. Effect of repetitive pulses with Ca++ or forskolin on NT and cAMP release. Two groups of six dishes were treated as follows: The control group (left panels top and bottom) were incubated in KRBG containing 4.0 mM Ca^{++}. The treatment group was incubated in KRBG containing 4.0 mM Ca^{++} plus 10^{-5} M forskolin (right panels, top and bottom). At 10 min intervals, during a 40 min incubation period, KRBG supplemented with 4.0 mM Ca^{++} alone or with forskolin (10^{-5} M) was added. The concentrations of cAMP and NT in the buffer were measured by radioimmunoassay. Each point represents the mean and the brackets give the S.E. for six dishes.

the response of these cells to forskolin. In their initial report on forskolin action, Seaman and Daly (3) reported that forskolin does not require a functional guanine-nucleotide binding subunit for activation of adenylate cyclase. Darfler et al. (10) have data suggesting that in S49 lymphoma cells the stimulation of adenylate cyclase and expression of hormone (isoproterenol)-mediated responses do require an intact nucleotide regulatory protein of adenylate cyclase.

Effect of cholera toxin. Incubation of 44-2 C-cells with a maximally effective concentration of cholera toxin (10^{-9} M) for up to 180 min, in KRBG stimulated cAMP release (Fig. 6). There is a 30 min lag between the stimulation of 44-2 C-cells with cholera toxin and the accumulation of cAMP in the medium, cAMP release continued to rise from 30-150 min and a maximal effect was observed at 150 min. In the same experiment, cholera toxin did not increase NT release by 44-2 C-cells (data not shown). The results we have

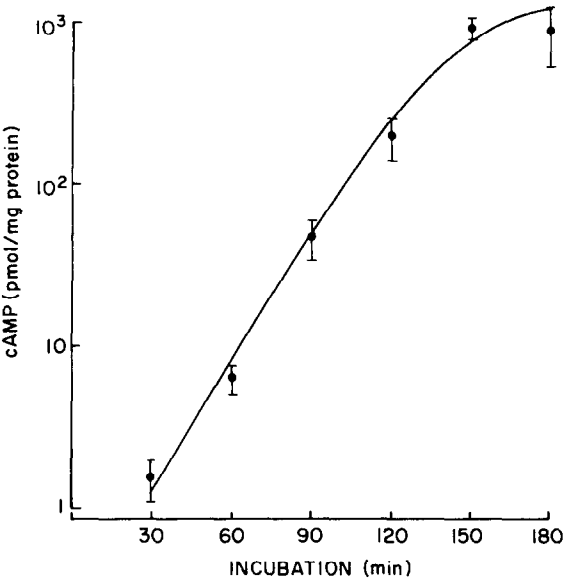

Fig. 6 Time course of cholera toxin-stimulated cAMP release. Cells were incubated for the indicated times in KRBG containing 1.0 mM Ca^{++} plus 10^{-9} M cholera toxin. cAMP accumulation in the medium was measured by radioimmunoassay. Each point represents the mean and the brackets give the S.E. for six dishes.

obtained with 44-2 C-cells are similar to those obtained with the wild type S49 lymphoma cells; a 45 min delay was observed in the accumulation of cAMP in the medium following treatment of the lymphoma cells with cholera toxin.

In conclusion, the effect of forskolin upon cAMP release in 44-2 C-cells is remarkable, and ranges between 10^2-10^5 fold stimulation. Forskolin stimulates NT release 2-3 fold. Cholera toxin stimulates cAMP release but does not affect NT secretion by these cells. We have shown previously that in their morphologic characteristics (60) and their secretory response (1,7), the rat C-cell line cells behave in a manner analogous to neuroendocrine cells. The present study also indicates that these cell lines are useful in carrying out detailed investigations of the relationship between the activation of adenylate cyclase and the regulation of cAMP and peptide release.

ACKNOWLEDGEMENTS:

The authors wish to thank Barbara Alford for expert technical assistance, and Bernice Gayer and Weda Collins for preparation of the manuscript.

REFERENCES

1. Zeytinoglu, F.N., Brazeau, P., Culkin, M.K. (1983) Reg. Peptides 6:155-162.
2. Seamon, K.B., Padget, W., Daly, J.W. (1981) Pro. Natl. Acad. Sci. USA 781:3363-3367.

3. Seamon, K.B., Daly, J.W. (1981) J. Biol. Chem. 256:9799-9801.
4. Seamon, K.B., Daly, J.W. (1981). J. Cyclic Nucleotid. Res. 7:201-224.
5. Kassis, K., Hagman, J., Fishman, P.H., Chang, P.P., Moss, J. (1982) J. Biol. Chem. 257:12148-12152.
6. Zeytin, F.N., Gagle, R.F., DeLellis, R.A., Wolfe, H.J., Tashjian, A.H., Jr., Hammer, R.A., Leeman, S.E. (1983) Lab. Invest. 49:453-459.
7. Zeytinoglu, F.N., Brazeau, P., Mougin, C. (1983) Reg. Peptides 6:147-154.
8. Zeytinoglu, F.N., DeLellis, R.A., Gagel, R.F., Wolfe, H.J., Tashjian, A.H. Jr (1980) Endocrinology 107:509-515.
9. Böhlen, P., Stein, S., Dairman, W., Udenfriend, S. (1973) Arch. Biochem. Biophys. 155:213-217.
10. Darfler, F.J., Mahan, L.C., Koachman, A.M., Insel, P.A. (1982) J. Biol. Chem. 257:11901-11907.
11. Insel, P.A., Darfler, F.J., Kennedy, M.S. (1981) FEBS Lett. 128:59-62.