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α-Melanocyte-stimulating hormone secretion from permeabilized intermediate lobe cells of rat pituitary gland

The role of guanine nucleotides

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The non-hydrolyzable GTP analogue, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and cyclic AMP potentiated the Ca^{2+}-evoked secretion of α-melanocyte-stimulating hormone (α-MSH) from permeabilized neurointermediate lobe (IL) cells of rat pituitary gland. The enhancement by Mg-GTPγS (100 μM) and cyclic AMP (1 μM) depended on the intracellular Ca^{2+} concentration ($EC_{50} = 4.8 \pm 1.8$ and 4.6 ± 1.7 μM; mean ± SE, with and without Mg-GTPγS and cyclic AMP, respectively). A similar effect was observed with guanine nucleotide triphosphate (GTP and GppNHp). Mg was absolutely required for this event. Neither Mg-GTPγS nor cyclic AMP alone was effective in potentiating α-MSH secretion. GDPβS blocked the Mg-GTPγS (100 μM) and cyclic AMP augmented secretion of α-MSH. Neither neomycin (which affects the process of inositol 1,4,5-triphosphate-mediated Ca^{2+} mobilization) or colchicine (which influences microtubule assembly) had an effect on the cyclic AMP and Mg-GTPγS potentiation of α-MSH secretion. These data suggest that the GTP-binding protein may be involved in the regulation of α-MSH secretion after Ca^{2+} entry into the cells, since the intracellular environment is controlled in the permeabilized cells.

GTP; Permeabilization; cyclic AMP; Exocytosis; (Pituitary intermediate cell)

1. INTRODUCTION

Many cellular responses to a hormonal stimulus appear to involve guanine nucleotide exchange reactions, including visual reception (transducin) [1,2], the hormone receptor-adenylate cyclase system for signal transduction (G_s, G_i) [3–7], microtubular assembly (tubulin) [8] and the initiation of protein synthesis in eukaryotic cells (eIF-2) [9]. In general, there is agreement regarding the need and importance of GTP-binding proteins for many biochemical processes [10,11].

Recently, Cockcroft and Gomperts [12] proposed a novel GTP-binding protein (G_p) which might be involved in coupling to polyphosphoinositide (PPI) phosphodiesterase. In the previous paper we described for the first time that cyclic AMP and Mg-GTPγS potentiates Ca^{2+}-evoked α-melanocyte-stimulating hormone (α-MSH) secretion from permeabilized rat pituitary intermediate lobe (IL) cells [13]. The intracellular environment was controlled experimentally by Ca^{2+}-EGTA buffers after permeabilization of melanotrophs by brief exposure to intense electrical fields.

Here, we investigated further the effect of GTP analogues and evaluated the possible involvement of a GTP-binding protein in the secretion of α-MSH from rat melanotrophs. Our data suggest that a novel GTP-binding protein might participate in exocytotic events from permeabilized IL cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Culture medium was obtained from Gibco (Grand Island, NY), ATP, cyclic AMP, CTP, ITP, GTP, GMP, cyclic GMP, colchicine and neomycin from Sigma (St. Louis, MO), and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) from Boehringer Mannheim. All reagents were of analytical grade.

2.2. *Methods*

A previously described procedure was used to disperse enzymatically IL cells from the neurointermediate lobe of 250–300 g Sprague-Dawley rats [14] and to quantify the secretion of α-MSH from these cells [15] with several modifications [16].

The solution used for cell permeabilization contained: 146 mM sodium acetate, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM Hepes (pH 7.4), 1.5 mM $CaCl_2$ and 7.8 mM glucose. Solutions free of ionized calcium were prepared by omitting the $CaCl_2$ and adding 1 mM EGTA.

For some experiments, Ca^{2+}-EGTA-buffered solutions were prepared by varying the concentration of calcium and then adjusting the pH to 7.4 by addition of NaOH [17]. The concentration of Ca^{2+} was determined with a calcium-sensitive electrode (Orion Research) [18]. All solutions were gassed with a mixture of 95% O_2 and 5% CO_2 before use.

Cells were permeabilized using the method of Knight and Baker [19] with several modifications [13]. In brief, the dispersed cells were mixed with 3 vols (5.0×10^6 cells/ml) of Ca^{2+}-free permeabilizing solution and washed once by centrifugation. The cells were resuspended in 1 ml of the same solution ($8–10 \times 10^6$ cells/ml) and permeabilized by double exposure to an intense electric field (each exposure to a field strength of 2.0 kV/cm; time constant 200 μs). The suspension of cell was diluted 10-fold with Ca^{2+}-free permeabilizing solution, centrifuged and resuspended in the same solution at 2×10^6 cells/ml. Induction of secretion was started immediately following permeabilization by transferring 0.05 ml of the cell suspension to 0.95 ml permeabilizing solution containing test substances. Routinely, the cells were incubated for 5 min at 30°C. Incubation was terminated by centrifugation in a Beckman microfuge, and removal of an aliquot of the supernatant solution for assay of IR α-MSH. Zero-time samples were taken by centrifuging the complete assay system without the 5 min incubation. All assays were performed in triplicate. Statistical significance was determined with Student's *t*-test.

3. RESULTS

In permeabilized IL cells, α-MSH secretion was stimulated by free Ca^{2+} in a concentration-dependent manner (fig.1). Half-maximal stimulation of α-MSH by Ca^{2+} was reached at 4.8 ± 1.8 M (mean ± SE, $n = 4$) with the use of Ca^{2+}-EGTA-buffered medium. Cyclic AMP (1 μM) and GTPγS (100 μM) augmented the amount of α-MSH secreted in response to Ca^{2+} without altering the molar potency of the ion (EC_{50} = 4.6 ± 1.7 μM). Fig.1 summarizes the results of a series of experiments in which the concentration of Ca^{2+} was varied with Ca^{2+}-EGTA buffers (as described in section 2). Although the half-maximal effect of Ca^{2+} in the presence and absence of GTPγS and cyclic AMP was not significantly changed, more pronounced secretion of α-MSH

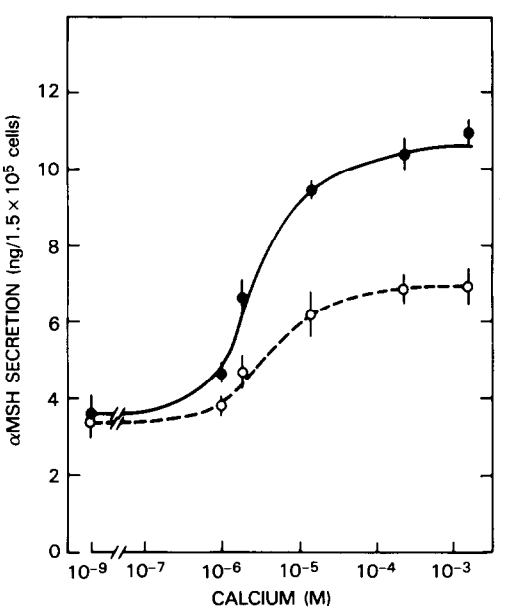

Fig.1. Ca^{2+} dependence of α-MSH secretion from permeabilized IL cells. The concentrations of free Ca^{2+} in the Ca^{2+}-EGTA buffers were measured as described in section 2. (○) None, (●) 1 μM cyclic AMP and 100 μM GTPγS.

was seen in the presence of Mg-GTPγS and cyclic AMP. The magnitude of cyclic AMP- and Mg-GTPγS-enhanced α-MSH secretion was 2-fold greater than that of Ca^{2+}-evoked secretion within 5 min of incubation. Mg was also absolutely required for this event.

In the absence of Ca^{2+}, Mg-GTPγS and cyclic AMP were virtually ineffective in stimulating α-MSH secretion. Mg-GTPγS-augmented hormonal secretion was concentration-dependent; half-maximal potentiation of secretion occurred at approx. 5 μM Mg-GTPγS (fig.2). As may be seen from this figure, GTP as well as its non-hydrolyzable analogues, GTPγS and GppNHp, in the presence of cyclic AMP (1 μM) caused concentration-dependent potentiation of α-MSH secretion from permeabilized cells. Other guanine nucleotides, GDPβS, GMP and cyclic GMP (each at 100 μM), however, were without effect. GDP was the only substance that affected the secretion of α-MSH (25% of maximal GTPγS effect). None of the other nucleotides tested (ATP, CTP and ITP), at concentrations up to 100 μM, had any effect on cyclic AMP potentiation of α-MSH secretion (table 1).

Table 1

Effect of nucleotides on the secretion of α-MSH potentiated by cyclic AMP (1 μM) in the presence of 1.5 mM $CaCl_2$

	% of maximum potentiation by GTPγS and cyclic AMP
GTPγS	100
GTP	97 ± 7
GppNHp	83 ± 7
GDP	39 ± 4
GMP	17 ± 5
Cyclic GMP	18 ± 4
ATP	33 ± 5
CTP	11 ± 2
ITP	18 ± 4

The concentration of each nucleotide was 100 μM, the control value 2.86 ± 0.18 ng α-MSH/10^5 cells and the value of potentiation by cyclic AMP (1 μM) and GTPγS (100 μM) 5.97 ± 0.35 ng α-MSH/10^5 cells. Each value is the mean ± SE for three determinations

The augmented secretion of α-MSH by Mg-GTPγS and cyclic AMP was significantly inhibited by the concomitant presence of GDPβS (fig.3).

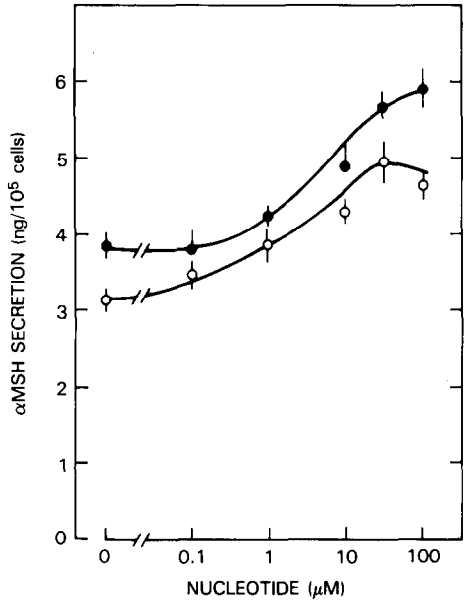

Fig.2. Effects of GTP analogues on α-MSH secretion. Permeabilized cells were incubated for 5 min at 30°C, in the presence of 1 μM cyclic AMP and various concentrations of GTPγS (●) and GppNHp (○). Each value is the mean ± SE for three determinations.

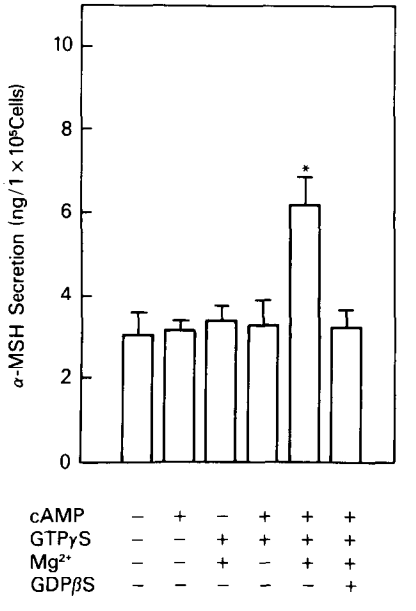

Fig.3. Effect of Mg on the potentiated secretion of α-MSH by cyclic AMP (1 μM) and GTPγS (100 μM) in the presence of 1.5 mM $CaCl_2$. Each value is the mean ± SE for three determinations. * Significantly different from control. $p < 0.05$ by Student's t-test.

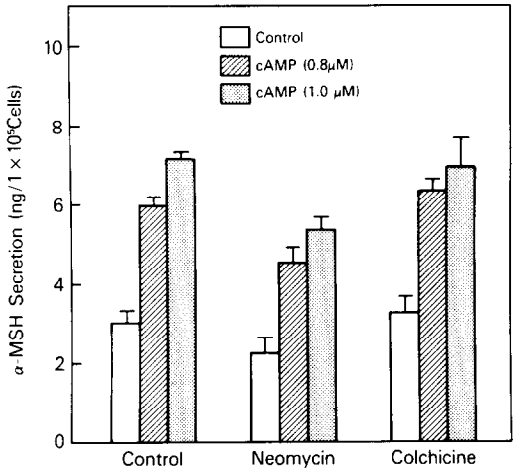

Fig.4. Effect of neomycin and colchicine on the potentiation of α-MSH secretion by cyclic AMP and GTPγS (100 μM) in the presence of 1.5 mM $CaCl_2$. Each value is the mean ± SE for three determinations.

Mg-GDPβS, at 100 μM, virtually abolished the Mg-GTPγS-induced stimulation of α-MSH secretion.

Neomycin (100 μM), an inhibitor of phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate, did not block GTPγS-mediated potentiation of α-MSH secretion by cyclic AMP (fig.4). Colchicine (1 mM) had no effect on the secretion of α-MSH from permeabilized cells.

4. DISCUSSION

As reported before, the technique of Baker and Knight [19] was applied in order to investigate the mechanisms of exocytosis in the intermediate lobe of the pituitary gland [13]. The results obtained from these studies showed that permeabilized IL cells retain functional secretory potency and that Ca^{2+}-evoked α-MSH secretion can be potentiated by cyclic AMP in the presence of Mg-GTPγS. Both guanine nucleotide triphosphate and Mg^{2+} are absolutely required for potentiation of Ca^{2+}-evoked α-MSH secretion by cyclic AMP.

The results clearly indicate that only GTP and its analogues (GTPγS and GppNHp) potentiate α-MSH secretion, other nucleotides being ineffective. Mg-ATP by itself potentiates Ca^{2+}-evoked α-MSH secretion at higher concentration (over 300 μM, maximum at 1 mM) as reported in [13].

It is of great interest that the cyclic AMP effect on hormonal secretion is most pronounced in the presence of a non-hydrolyzable guanine nucleotide. Generally, it is known that GTP-binding proteins regulate activities of hormone-sensitive adenylate cyclase [3–7,20], voltage-dependent ion channels [21–23] and polyphosphoinositide (PPI) phosphodiesterase [12,24,25]. Guanine nucleotides have already been shown to be involved in the biochemical physiology of the melanotroph: these nucleotides are essential for the β-adrenergic enhancement and D-2 dopaminergic inhibition of adenylate cyclase activity [26–28]. The guanine nucleotide regulatory proteins N_s and N_i presumably mediate the stimulation and inhibition of melanotroph adenylate cyclase activity, respectively. Thus, it is feasible that GTP analogues act on N_s and not on N_i leading to elevation of the level of cyclic AMP, since exogenous cyclic AMP is required for stimulation. However, cyclic AMP added exogenously, by itself, does not potentiate Ca^{2+}-evoked α-MSH secretion at concentrations up to 100 μM (not shown). Furthermore, Mg-GTP analogues alone are also inactive and the slight stimulation of adenylate cyclase activity by GTP analogues appears after 6 min of incubation in homogenates of IL tissues as reported by Cote et al. [26]. Our results showing potentiation of secretion by cyclic AMP and Mg-GTP analogues within 5 min (not shown), are not consistent with possible stimulation of N_s by GTP analogues [26,29,30]. Therefore, it is unlikely that GTP analogues interact with the GTP-binding protein coupled to adenylate cyclase.

It is also unlikely that the potentiation of secretion by cyclic AMP and Mg-GTP analogues results from a change of some ion channels (i.e. Ca^{2+} channel, K^+ channel, etc.) [22,23], since low-M_r substances are controlled in the permeabilized cells and neomycin is ineffective.

Moreover, we can exclude the possibility of GTP analogues interacting with GTP-binding protein coupled to PPI-phosphodiesterase (N_p) [12], since neomycin did not inhibit the potentiated secretion of α-MSH by cyclic AMP and Mg-GTP analogues.

It is also known that tubulin requires GTP for polymerization and that GTP is hydrolyzed during microtubular assembly [8,9,24]. Therefore, we examined the possibility of cyclic AMP- and Mg-GTP analogue-augmented α-MSH secretion in

relation to microtubules with the use of colchicine. However, this agent did not affect α-MSH secretion either with or without the presence of cyclic AMP and Mg-GTP analogues. Moreover, both membrane fusion [31] and microtubule assembly are also sensitive to guanine nucleotides at relatively higher concentrations (5–10 mM), but these sites are unlikely to be included in cyclic AMP potentiation of α-MSH secretion, since the specificity and efficacy for nucleotides are quite different in the microtubular system [8].

Finally, the involvement of nucleotide regulatory protein (defined N_o), coupled to other unknown systems as a family with N_s and N_i [32–34], should be considered as part of the processes of hormonal secretion, however it is not likely because both basal and β-agonist-stimulated hormone secretion from intact dispersed IL cells remain unchanged after pretreatment with or without pertussis toxin as reported by Cote et al. [35]. Even though this possibility cannot be excluded completely, the present results strongly suggest that the cyclic AMP potentiation of Ca^{2+}-evoked α-MSH secretion may be regulated by GTP binding to a novel GTP-binding protein. Thus, these findings suggest that a unique GTP-binding protein may participate in the hormonal secretory process associated with modification of cyclic AMP-dependent events in the membrane.

REFERENCES

[1] Fung, B.K.K., Hurley, J.B. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152–156.

[2] Yamazaki, A., Stein, P.J., Chernoff, N. and Bitensky, M.W. (1983) J. Biol. Chem. 258, 8188–8194.

[3] Rodbell, M. (1980) Nature 284, 17–22.

[4] Northup, J.K., Smigel, M.D., Sternweis, P.C. and Gilman, A.G. (1983) J. Biol. Chem. 257, 11369–11376.

[5] Gilman, A.G. (1984) Cell 36, 577–579.

[6] Kurose, H., Katada, T., Amano, T. and Ui, M. (1983) J. Biol. Chem. 258, 4870–4875.

[7] Bokoho, G.M., Katada, T., Northup, J.K., Hewlett, E.L. and Gilman, A.G. (1983) J. Biol. Chem. 258, 2072–2075.

[8] Maccioni, R.B. and Seeds, N.W. (1977) Proc. Natl. Acad. Sci. USA 74, 462–466.

[9] March, P.E. and Inouye, M. (1985) Proc. Natl. Acad. Sci. USA 82, 7500–7504.

[10] Jacobson, M.A. and Colman, R.F. (1982) Biochemistry 21, 2177–2186.

[11] McGrath, J.P., Capon, D.J., Goeddel, D.V. and Levinson, A.D. (1984) Nature 310, 644–649.

[12] Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.

[13] Yamamoto, T., Furuki, Y., Guild, S. and Kebabian, J.W. (1987) Biochem. Biophys. Res. Commun., in press.

[14] Goldman, M.E., Beaulieu, M., Kebabian, J.W. and Eskay, R.L. (1983) Endocrinology 112, 435–441.

[15] Eskay, R.L., Giraud, P., Oliver, C. and Brownstein, M. (1979) Brain Res. 178, 55–67.

[16] Miyazaki, K., Goldman, M.E. and Kebabian, J.W. (1984) Endocrinology 114, 761–766.

[17] Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. 10, 219–242.

[18] Affolter, H. and Sigel, E. (1979) Anal. Biochem. 97, 315–319.

[19] Knight, D.E. and Baker, P.F. (1982) J. Membrane Biol. 68, 107–140.

[20] Ross, E.M. and Gilman, A.G. (1980) Annu. Rev. Biochem. 49, 533–564.

[21] Holz, G.G. iv, Rane, S.G. and Dunlap, K. (1986) Nature 319, 670–672.

[22] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.

[23] Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M. and Bertil, H. (1985) Nature 317, 536–538.

[24] Gomperts, B.D. (1983) Nature 306, 64–66.

[25] Litosh, I., Wallis, C. and Fain, J.N. (1985) J. Biol. Chem. 260, 5464–5471.

[26] Cote, T.E., Grewe, C.W. and Kebabian, J.W. (1982) Endocrinology 110, 805–811.

[27] Cote, T.E., Chen, T.C. and Kebabian, J.W. (1980) Brain Res. 181, 127–138.

[28] Cote, T.E., Grewe, C.W., Tsuruta, K., Stoof, J.C., Eskay, R.L. and Kebabian, J.W. (1982) Endocrinology 110, 812–819.

[29] Tolkovsky, A.M. and Levitzki, A. (1978) Biochemistry 17, 3795–3810.

[30] Hanski, E., Rimon, G. and Levitzki, A. (1979) Biochemistry 18, 846–853.

[31] Paiement, J. (1984) Biochim. Biophys. Acta 777, 274–282.

[32] Sternweis, D.C. and Robishow, J.D. (1984) J. Biol. Chem. 259, 13806–13813.

[33] Florio, V.A. and Sternweis, P.C. (1985) J. Biol. Chem. 260, 3477–3483.

[34] Neer, E.J., Lok, J.M. and Wolf, L.G. (1984) J. Biol. Chem. 259, 14222–14229.

[35] Cote, T.E., Frey, E.A. and Sekura, R.D. (1984) J. Biol. Chem. 259, 8693–8698.