Involvement of Calcium in the Release of Immunoreactive β -Endorphin-Like Peptide from Dispersed Cells of the Neurointermediate Lobe of the Rat Pituitary Gland

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Abstract—The presence of Ca^{2+} in the incubation medium was required for stimulation of the release of the immunoreactive β -endorphin-like peptide (IR- β -EP) from the dispersed cells of the neurointermediate lobe of rat pituitary gland by adenosine 3',5'-monophosphate (cAMP) analogs, a phosphodiesterase inhibitor, L-isoproterenol, cholera toxin and forskolin. The basal release observed in the absence of the stimulants was also dependent on the addition of Ca^{2+} . A calcium antagonist (verapamil) inhibited the effects of the stimulants. A calcium ionophore (A23187) enhanced the release of IR- β -EP, but did not stimulate the formation of cAMP. These findings suggest that Ca^{2+} has the essential role in the release of β -endorphin from the neurointermediate lobe of rat pituitary gland.

β -Endorphin, an endogeneous opioid peptide, is known to be localized in the intermediate and anterior lobes of the rat pituitary gland (1). The intermediate lobe of the rat pituitary gland consists of a homogeneous population of cells synthesizing and secreting various peptides related to adrenocorticotrophic hormone (ACTH) and lipotropin (LPH) derived from a common glycoprotein precursor, proopiomelanocortin (2–4). In fact, the spontaneous and L-isoproterenol-stimulated release of such peptides was shown during the in vitro superfusion of the rat anterior and neurointermediate lobes with Krebs-Ringer medium (5).

We have shown that the release of α -MSH from the dispersed cells of the intermediate lobe of the rat pituitary gland is stimulated by cAMP and catecholamines (6). We have also reported that the release of the immunoreactive- β -endorphin-like peptide (IR- β -EP) is regulated by cAMP analogs and several agents which stimulate the intracellular formation of cAMP (7). On the other hand, Ca^{2+} plays an important role in the

stimulus-secretion coupling of neurotransmitters and hormones (8). The secretion of hormones from the anterior pituitary gland requires extracellular Ca^{2+} (9). Incubation of isolated anterior pituitary gland in Ca^{2+} -free medium greatly diminishes the secretion of thyroid stimulating hormone (10), luteinizing hormone (11), ACTH (12, 13), prolactin (14) and growth hormone (15). Furthermore, it was reported that Ca^{2+} is involved in the release of α -MSH from the intermediate lobe of the pituitary gland (16) and that Ca^{2+} has a coordinated action with cAMP for the release of α -MSH from the tissue (17).

In the present study, we attempted to elucidate the role of Ca^{2+} and its interaction with cAMP for the release of β -endorphin from the dispersed cells of the neurointermediate lobe of rat pituitary gland.

Materials and Methods

Materials: The chemicals used were obtained from the following sources: L-isoproterenol, dibutyryl cAMP and cholera toxin, Sigma Chemical Co. (St. Louis, MO);

A23187 and forskolin, Calbiochem (La Jolla, CA); 8-bromo cAMP, P-L Biochemicals, Inc. (Milwaukee, MA); and Earle's balanced salt solution (complete or without Ca^{2+} and Mg^{2+}), Gibco (New York NY); verapamil, Eisai Co. Ltd. (Tokyo); ethylene glycol bis (β -aminoethylether)-N, N,N',N'-tetraacetic acid (EGTA), Nakarai Chemicals Ltd. (Kyoto); and synthetic human β -endorphin, Protein Research Foundation (Minoo, Japan). Male rats of the Wistar strain, weighing between 250 g and 300 g, were used in all experiments.

Preparation and incubation of dispersed cells from the neurointermediate lobe: The procedures used to obtain dispersed cells from the neurointermediate lobe as well as the standard incubation procedures were as described previously (6). Incubation was initiated by the addition of the dispersed cells to the sample tube.

A23187 was dissolved in a mixture of equal volumes of dimethylsulfoxide and ethanol to obtain a final concentration of 10 mM and subsequently diluted in saline (containing 50 $\mu\text{g}/\text{ml}$ ascorbic acid). Control samples contained vehicles. Ca^{2+} -free Earle's balanced salt solution (EBSS) was prepared by adding MgSO_4 to Ca^{2+} and Mg^{2+} -free EBSS.

Determination of cAMP from dispersed cells: cAMP was determined by radioimmunoassay following succinylation according to the procedure of Honma et al. (18), utilizing kits supplied by Yamasa Co., Ltd., Japan.

Radioimmunoassay of β -endorphin: The determination of β -endorphin was performed by the methods of Matsumura et al. (19, 20), using synthetic human β -endorphin as a standard. Radioiodination of the β -endorphin was performed with ^{125}I by the method of Hunter and Greenwood (21). The labeled peptide was subjected to gel filtration column chromatography on Sephadex G-25 (medium, 0.9×18 cm column), and it was eluted with 0.02 M phosphate buffer, pH 7.4, containing 0.15M NaCl, 25 mM EDTA, 0.1% NaN_3 and 500 KIU/ml Trasylol. Anti- β -endorphin serum was a generous gift from Dr. M. Matsumura, the Department of Internal Medicine, Tokushima

University Medical School, Tokushima, Japan (22). The antibody showed only 4.5% cross-reactivity with human β -LPH on a mass basis, and it did not react with other peptides, including Leu-enkephalin, Met-enkephalin, endorphins (α , γ and δ), γ -LPH, $^{1-39}\text{ACTH}$, TRH, LH-RH and somatostatin.

The final volumes of the standards (2–512 pg/tube) and samples were adjusted to 0.3 ml by the addition of the assay medium (0.02M phosphate buffer, pH 7.4, containing 0.15M NaCl, 25 mM EDTA, 0.1% NaN_3 and 500 KIU/ml Trasylol). Anti- β -endorphin serum (100 μl) which was diluted to 1:5,000 with the assay buffer was added, and the incubation was carried out for 24 hr at 4°C .

^{125}I - β -Endorphin (10,000 cpm/100 μl) was then added, and the mixture was incubated for 24 hr at 4°C . The antibody-bound antigen was precipitated by the addition of anti-rabbit γ -globulin goat serum (diluted to 1:10) and normal rabbit serum (diluted 1:100), followed by incubation at 4°C for 24 hr. Then the supernatant was decanted, and the radioactivity of the precipitate was counted. The average rate of binding was 42% in the sample without β -endorphin. A dose-related response in the radioimmunoassay was observed in the range of 4 to 256 pg of β -endorphin/tube.

Statistics: The data were expressed as the mean $\pm$ S.E. ($n=3$ to 6). Student's t -test (two-tailed) was employed to determine statistically significant difference between experimental groups of data. The procedures for calculation of the S.E. between two groups were as described previously (6).

Results

Effect of Ca^{2+} on the release of IR- β -EP stimulated by cAMP: A close relationship between the increase of cAMP formation and the stimulative effect of IR- β -EP release was previously reported (7). In the previous study, Ca^{2+} was originally included in the incubation medium, therefore the effect of Ca^{2+} on the release of IR- β -EP was examined in the present study. Ca^{2+} was absolutely required for the stimulative effects of 8-bromo cAMP (10 mM), dibutyryl cAMP (10 mM) and 3-isobutyl-1-methyl xanthine (IBMX, 1 mM), although these

agents had substantial effects in the presence of 1.8 mM Ca^{2+} (Fig. 1). The removal of Ca^{2+} from the incubation medium resulted in the abolishment of the effect of the agents. The basal release of IR- β -EP observed without the addition of cAMP analogs or phosphodiesterase inhibitor was also influenced by the addition of Ca^{2+} .

The requirement for Ca^{2+} was observed for the effects of the agents which stimulate

cAMP formation (Table 1). L-Isoproterenol, cholera toxin and forskolin stimulated the release of IR- β -EP in the presence of Ca^{2+} . However, the effects of these agents decreased in the absence of Ca^{2+} . To further test the effect of Ca^{2+} , 1 mM EGTA was added to the incubation medium with or without cholera toxin. The values of IR- β -EP released were 5.08 ± 0.37 and 3.10 ± 0.12 ng/ 3×10^4 cells/3 hr in the absence and

Fig. 1. Effect of Ca^{2+} on the release of IR- β -EP stimulated by cAMP analogs and phosphodiesterase inhibitor. Incubation was carried out for 3 hr with the indicated drugs in the absence or presence of 1.8 mM CaCl_2 in EBSS-BSA, which had been equilibrated with a mixture of 95% O_2 /5% CO_2 under standard conditions. The zero-time samples contained 2.47 ± 0.13 and 2.00 ± 0.12 ng of IR- β -EP in the absence or presence of 1.8 mM CaCl_2 , respectively. The values represent the extracellular amount of IR- β -EP released during 3 hr incubation, are corrected for those of the zero-time samples and are expressed as the mean $\pm$ S.E. ($n=3$ to 6).

Table 1. Effect of Ca^{2+} on the release of IR- β -EP stimulated by L-isoproterenol, cholera toxin and forskolin

Addition	IR- β -EP released (ng/ 3×10^4 cells/3 hr)	
	No calcium	1.8 mM CaCl_2
None	3.85 ± 1.12	$5.97 \pm 0.07^*$
L-Isoproterenol (100 nM)	4.51 ± 0.24	$8.44 \pm 0.10^*$
Cholera toxin (30 nM)	4.97 ± 0.57	$9.31 \pm 0.24^\#$
Forskolin (10 μM)	4.44 ± 0.20	$9.51 \pm 0.35^*$

* $P < 0.001$ vs. no calcium, $^\#P < 0.005$ vs. no calcium. Dispersed cells were incubated for 3 hr with the indicated drugs in the absence or presence of 1.8 mM CaCl_2 under standard conditions. The values are expressed as the mean $\pm$ S.E. ($n=3$ to 6).

presence of EGTA, respectively, and 5.21 ± 0.32 and 9.41 ± 0.23 ng/ 3×10^4 cells/3 hr in the absence and presence of cholera toxin, respectively. These findings again show that the release of IR- β -EP requires the presence of extracellular Ca^{2+} .

The addition of 1 mM EGTA to the Ca^{2+} -free incubation medium was considered to completely remove the extracellular Ca^{2+} , although a minute amount of contaminating Ca^{2+} may be present in only the Ca^{2+} -free incubation medium without EGTA. The amounts of IR- β -EP released were 2.75 ± 0.07 and 2.71 ± 0.27 ng/ 3×10^4 cells/3 hr in the Ca^{2+} -free incubation medium in the absence and presence of EGTA, respectively. This indicates that the abolishment of the extracellular Ca^{2+} does not bring about the complete inhibition of the IR- β -EP release.

Effect of Ca^{2+} concentration on the release of IR- β -EP: The effect of Ca^{2+} concentration on the release of IR- β -EP was examined (Fig. 2). The release of IR- β -EP in the

presence and absence of L-isoproterenol both increased by the addition of Ca^{2+} in a dose-dependent manner. It was still increasing at 2.5 mM Ca^{2+} which was the highest concentration tested.

Effect of verapamil on the release of IR- β -EP: It was examined whether or not verapamil, a calcium antagonist, counteracts the effect of Ca^{2+} on the release of IR- β -EP. Verapamil significantly had an inhibitory effect at 10^{-7} M and decreased the release of IR- β -EP with the increase of the concentration in the presence of L-isoproterenol (Fig. 3). Meantime, verapamil had no effect on the release in the absence of L-isoproterenol. These inhibitory effects of verapamil on the release of IR- β -EP were also observed for the stimulative effects of 8-bromo cAMP, dibutyryl cAMP and IBMX (Table 2A) and cholera toxin and forskolin (Table 2B).

Effect of A23187 on the release of IR- β -EP: The effect of A23187, a calcium

Fig. 2. Effect of Ca^{2+} concentration on the release of IR- β -EP in the absence and presence of L-isoproterenol. Incubation was carried out for 3 hr with the indicated concentrations of CaCl_2 in the absence ($\circ$) or presence ($\bullet$) of 100 nM L-isoproterenol in EBSS-BSA under standard conditions. The values are expressed as the mean $\pm$ S.E. ($n=3$ to 6).

Fig. 3. Effect of verapamil on the release of IR- β -EP in the absence and presence of L-isoproterenol. Dispersed cells were incubated for 3 hr with the indicated concentrations of verapamil in the absence ($\circ$) or presence ($\bullet$) of 100 nM L-isoproterenol in EBSS-BSA containing 1.8 mM CaCl_2 under standard conditions. The zero-time sample contained 2.33 ± 0.19 ng of IR- β -EP. The values are corrected for that of the zero-time sample and expressed as the mean $\pm$ S.E. ($n=3$ to 6).

Table 2. Effect of verapamil on the release of IR- β -EP stimulated by cAMP analogs, phosphodiesterase inhibitor, cholera toxin and forskolin

Addition	IR- β -EP released (ng/ 3×10^4 cells/3 hr)	
	No verapamil	10 μ M verapamil
(A) None	5.77 $\pm$ 0.31	5.17 $\pm$ 0.31
8-Bromo cAMP (10 mM)	10.27 $\pm$ 0.56	6.60 $\pm$ 0.67
Dibutyryl cAMP (10 mM)	10.50 $\pm$ 0.52	6.57 $\pm$ 0.10*
3-Isobutyl-1-methyl xanthine (1 mM)	9.30 $\pm$ 0.13	6.83 $\pm$ 0.41*
(B) None	5.90 $\pm$ 0.07	5.77 $\pm$ 0.18 [#]
Cholera toxin (30 nM)	10.30 $\pm$ 0.47	6.23 $\pm$ 0.35*
Forskolin (10 μ M)	10.03 $\pm$ 0.53	5.23 $\pm$ 0.42*

[#]P, not significant. *P<0.005 vs. no verapamil. Dispersed cells were incubated for 3 hr with the indicated drugs in the absence or presence of 10 μ M verapamil in EBSS-BSA containing 1.8 mM CaCl₂ under standard conditions. The values are expressed as the mean $\pm$ S.E. (n=3 to 6).

Fig. 4. Effect of A23187 on the release of IR- β -EP during the time course. Dispersed cells were incubated for the indicated times in the absence ($\circ$) or presence ($\bullet$) of 10 μ M A23187 in EBSS-BSA containing 1.8 mM CaCl₂ under standard conditions. The zero-time sample contained 4.40 $\pm$ 0.12 ng of IR- β -EP. The values are corrected for that of the zero-time sample and expressed as the mean $\pm$ S.E. (n=3 to 6).

ionophore, on the release of IR- β -EP was examined (Fig. 4). A23187 increased the release of IR- β -EP higher than the control (without A23187) during the time course,

Fig. 5. Effect of A23187 concentration on the release of IR- β -EP. Dispersed cells were incubated for 1 hr with the indicated concentrations of A23187 in EBSS-BSA containing 1.8 mM CaCl₂ under standard conditions. The values are expressed as the mean $\pm$ S.E. (n=3 to 6).

although the control value increased in the course of time. The dose-response relationship between the concentration of A23187 and the release of IR- β -EP is shown in Fig. 5. The concentration of A23187 required to give the half-maximal release was 1.0 μ M.

Fig. 6. Effect of A23187 on cAMP accumulation. A) Dispersed cells were incubated for 10 min in the absence (control) and presence of 100 nM L-isoproterenol or 10 μM A23187 under standard conditions. B) Dispersed cells were incubated for 60 min in the absence (control) and presence of 30 nM cholera toxin or 10 μM A23187 under standard conditions. Incubation was carried out in EBSS-BSA containing 10 mM theophylline and 1.8 mM CaCl₂ under standard conditions. The amount of cAMP measured represents the sum of cAMP in the cells and incubation medium. The zero-time sample contained 200±29 fmole per 3×10⁴ cells. The values are corrected for that of the zero-time sample and expressed as the mean±S.E. (n=3 to 6).

The maximal release was attained at 10 μM of A23187.

Effect of A23187 on cAMP accumulation:

As shown in the previous study (7), L-isoproterenol and cholera toxin stimulated the formation of cAMP in the dispersed cells of the intermediate lobe of the rat pituitary gland (Fig. 6). The addition of 10 μM A23187 which stimulates the release of IR-β-EP (Fig. 5) had no effect on the formation of cAMP in the dispersed cells incubated for 10 min (Fig. 6A) and 60 min (Fig. 6B). Under the conditions, L-isoproterenol (Fig. 6A) and cholera toxin (Fig. 6B) each stimulated the accumulation of cAMP.

Discussion

A series of biologically active peptides related to ACTH and lipotropin (LPH) is formed in the corticotrophs of the anterior lobe and the melanotrophs of the intermediate lobe of the pituitary gland by proteolytic cleavage of a common precursor

molecule (2–4). β-Endorphin, an endogenous opioid, has the same amino acid sequence as residues 61–91 of β-LPH. Immunocytofluorescence techniques with well-characterized antisera to β-endorphin indicate the presence of this peptide in all cell elements of the pars intermedia of the rat hypophysis and discrete cells of the pars distalis free from the neurohypophysis (1). It is considered that the results obtained from the preparation of dispersed cells derived from the neurointermediate lobe accurately reflect the function of this tissue (6, 7).

β-Adrenoceptor which enhances the release of α-MSH is known to occur in the parenchymal cells of the intermediate lobe (23). Previous studies have characterized the β-adrenergic receptors which are present on the intermediate lobe cells of rat pituitary gland. Based on biochemical criteria, these β-adrenergic receptors belong to the β₂-subcategory. Evidence has been presented to support the view that β-adrenergic stimu-

lation of α -MSH release involves an increase in adenylate cyclase activity (6, 24).

The effect of cholera toxin on the release of α -MSH from the dispersed cells of the intermediate lobe was also investigated. Thus it proved that cholera toxin enhances the accumulation of cAMP and stimulates the release of α -MSH (17).

Furthermore, forskolin has been reported to stimulate adenylate cyclase activity, cAMP production and α -MSH release from the intermediate lobe of the rat pituitary gland (25). The effect of forskolin is probably mediated through interaction with the catalytic subunit of the associated protein of adenylate cyclase (26). Similarly, stimulation of β -adrenergic receptor or administration of cAMP analogs and consequent accumulation of cAMP in the dispersed cells of the intermediate lobe resulted in the release of IR- β -EP (7). Our present findings demonstrate that although cAMP stimulates the release of IR- β -EP from the dispersed cells of the neurointermediate lobe, Ca^{2+} is essentially required. Similar results on the effects of Ca^{2+} and cAMP were reported for the release of α -MSH from the dispersed cells of the neurointermediate lobe (17).

Verapamil, a calcium antagonist, has an inhibitory effect on the voltage-dependent calcium channel (27). Verapamil actually inhibited the release of GH, prolactin, TSH and LH from the anterior pituitary gland (9, 10, 15, 28, 29). In the present study, verapamil also inhibited the effects of L-isoproterenol, cAMP analogs, the phosphodiesterase inhibitor, cholera toxin and forskolin on the release of IR- β -EP. The removal of the extracellular Ca^{2+} and the higher concentration of verapamil did not completely inhibit the release of IR- β -EP from the dispersed cells. The basal level of the IR- β -EP release were observed in both cases. The results were consistent with those reported previously (10, 14, 17). At present, there is no proper explanation for these findings.

However, it may be likely that the Ca^{2+} release from the intracellular Ca^{2+} storage sites continues at a low concentration even in the absence of the extracellular Ca^{2+} and that this may maintain the basal level of the IR- β -EP release.

A23187, a calcium ionophore, stimulated the release of IR- β -EP from the dispersed cells of the neurointermediate lobe in a dose-dependent manner ranging from 0.1 μM to 20 μM . The accumulation of cAMP was not stimulated by A23187. It has been reported that A23187 stimulates hormone secretion in the anterior pituitary gland and many other cells (30) and increases the uptake of ^{45}Ca and the release of α -MSH from the neurointermediate lobe of rat pituitary gland (17). In contrast to our results, Shettini et al. (31) reported that A23187 increased the formation of cAMP and the release of prolactin in the anterior pituitary cells. The difference between the results is unclear at present.

These results suggest that the effects of verapamil and A23187 may be exerted by the inhibition and enhancement, respectively, of Ca^{2+} influx through the membrane. In view of the fact that A23187 stimulates the release of IR- β -EP without enhancing the accumulation of cAMP in the present study, Ca^{2+} may have an essential role in the release of the hormone in the neurointermediate lobe. On the other hand, cAMP may modulate the degree of the release of the hormone in the presence of Ca^{2+} . The possibility is not ruled out that cAMP may affect the mobilization of Ca^{2+} into the cells.

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