HELODERMIN STIMULATES INTRACELLULAR ACCUMULATION OF CYCLIC AMP AND *N*-ACETYLTRANSFERASE ACTIVITY IN RAT PINEAL GLAND

KOHEI KAKU¹, YUMIKO HARADA¹, MASASHI OKUBO¹, KEN YAGA¹, NOBORU YANAIHARA² and TOSHIO KANEKO¹

¹Third Department of Medicine, Yamaguchi University School of Medicine, Kogushi, Ube 755, and ²Laboratory of Bioorganic Chemistry, Department of Pharmacy, Shizuoka Prefectural University, Shizuoka 422, Japan

ABSTRACT

The effects of helodermin (HD) on the intracellular cyclic AMP (cAMP) accumulation and *N*-acetyltransferase (NAT) activity were examined using rat pineal glands. HD stimulated the pineal cAMP accumulation and NAT activity in a dose-dependent manner. The stimulatory potency of HD was nearly equal with that of vasoactive intestinal polypeptide (VIP). HD stimulation of pineal cAMP accumulation was further potentiated by an addition of 4- β -phorbol 12-myristate 13-acetate (PMA). Our results suggest that HD is one of neurotransmitter candidates of the pineal gland, and support a hypothesis that neuropeptides play a role in regulating pineal function as well as norepinephrine.

Helodermin (HD) is a peptide consisting of 35 amino acid residues and originally isolated from the venom of the Gila monster lizard (4). HD is included in the secretin family, and is structurally similar to vasoactive intestinal polypeptide (VIP) and peptide histidine isoleucine (PHI) (14). Several studies have revealed that HD activates adenylate cyclase in many tissues as efficiently as VIP and PHI (13, 15, 16, 20).

Recent studies have demonstrated that VIP is a neurotransmitter which regulates the pineal function characterized by the activation of adenylate cyclase and serotonin *N*-acetyltransferase (NAT) through its specific binding sites (5, 8, 21). The potency of VIP stimulation of pineal cyclic AMP (cAMP) accumulation oscillates with the lighting condition or daily cycles (6, 7, 22). VIP action is independent of β -adrenergic receptor-adenylate cyclase which is known to be a major transmitter system in the pineal. These observations suggest the possibility that HD also acts as a transmitter in the pineal gland although evidence that HD colocalizes with VIP in the gland has not yet been obtained.

In the present study, we examined the effects of

HD on the pineal cAMP accumulation and the activity of NAT, a rate limiting enzyme of melatonin synthesis. Furthermore, the action potency of HD was compared with that of VIP. This is the first demonstration that HD, as well as VIP, stimulates the pineal function.

MATERIALS AND METHODS

HD was prepared by solid phase methodology using a peptide synthesizer (Beckman model 990B). The synthetic HD was identified by HPLC with a natural HD preparation as standard. The synthetic HD used in this study includes a full length of a natural HD (1-35). Porcine VIP was purchased from Peptide Institute (Osaka, Japan). Trypsin was from Difco (Detroit, MI). Dulbecco's Eagle's Modified Medium (DMEM) was from Flow Laboratories (McLean, VA), fetal calf serum (FCS) from MA Bioproducts (Walkersville, MD), and penicillin (10,000 U/ml)-streptomycin (10,000 mg/dl) solution from Grand Island Biological (Grand Island, NY). cAMP RIA kits were from Yamasa Shoyu (Choshi, Japan). All other reagents were of the purest grade available.

Fig. 1 Intracellular accumulation of cAMP induced by various concentrations of HD (●) and VIP (○). Each value indicates the mean $\pm$ SD (in 5 separate experiments). * $P < 0.05$ and ** $P < 0.01$ vs control (agonist-free), † $P < 0.05$ vs value in HD treatment

Preparation of Rat Pineal Glands and Dispersed Pinealocytes

Male Wistar rats weighing 80–100 g were kept under diurnal lighting conditions with the light on from 06 00 to 18 00. The pineal glands were quickly removed and preincubated at 37°C for 30 min in HEPES-Krebs-Henseleit bicarbonate-Ringer buffer, pH 7.4, containing 5 mM glucose and 0.1% BSA, and gassed with 95% O₂-5% CO₂. Pinealocytes were prepared by the method of Buda and Klein (1), with minor modifications. Dispersed cells prepared by trypsinization and mechanical dissociation of pineal glands were suspended in DMEM, pH 7.4, containing 10% FCS, penicillin (100 U/ml) and streptomycin (100 μ g/ml), and cultured for 24 h in a gas mixture of 95% air-5% CO₂ before use. Viability of pinealocytes after 24 h of culture was more than 90%, as judged from trypan blue exclusion.

Assay of Pineal cAMP Content

After a 24 h culture, pinealocytes were incubated in DMEM-10% FCS for 30 min at 37°C in water bath. Pinealocytes (10⁵ cells/500 μ l) were resuspended in 10 mM HEPES-Krebs-Henseleit bicarbonate-Ringer buffer, pH 7.4, containing 5 mM glucose and 0.1% BSA, with or without effectors. In the experiments using the pineal body, two pineal glands were suspended in the same buffer with or without effectors after a 30 min preincubation. After incubation for 10 min at 37°C, the same

Fig. 2 Effects of HD (10⁻⁷ M) and VIP (10⁻⁷ M) on cAMP accumulation in pineal gland obtained under the lighted condition (at 12 00) and the dark (at 24 00). Each value represents the mean $\pm$ SD (in 4 separate experiments). * $P < 0.01$ vs control (agonist-free)

volume of 0.2 N HCl was added to the mixture to stop the reaction, and the mixture was boiled for 3 min. The samples were lyophilized, and stored at -20°C until use. cAMP contents were determined by RIA as described previously (6). The assay was done in duplicate.

Assay of Serotonin NAT Activity

To determine pineal NAT activity, the pineal organ culture was carried out. After preincubation for 30 min, pineal glands were placed in a sterile plastic dish (five glands for each culture dish) with 2.5 ml of DMEM-10% FCS containing 2 mM glutamine, ascorbic acid (0.1 mg/ml), penicillin (100 U/ml), and streptomycin (100 μ g/ml). The pineals were cultured for 5 h at 37°C under 95% air-5% CO₂. NAT activity was assayed by the method of Deguchi and Axelrod (2) using [1-¹⁴C]-acetyl coenzyme A.

Statistical Analysis

A statistical significance was analyzed by Student's *t*-test.

RESULTS

To assess a response of pinealocytes to agonists, the effect of isoproterenol, a β -receptor agonist, on the

Fig. 3 Effect of PMA on HD-induced intracellular cAMP accumulation. Each value represents the mean $\pm$ SD (in 4 separate experiments).

intracellular cAMP accumulation was first examined. Isoproterenol, at a concentration of 10^{-5} M, stimulated the intracellular cAMP accumulation by 40-fold (data not shown). As shown in Fig. 1, HD and VIP increased the intracellular cAMP accumulation in a dose-dependent manner. HD, at a concentration of 10^{-7} M, increased the intracellular cAMP content by 10-fold. A concentration giving a half-maximal effect (EC_{50}) of HD on pineal cAMP production was rather lower than that of VIP (10^{-10} M for HD, and 5×10^{-10} M for VIP), while the maximal potency of HD observed at 10^{-6} M was slightly lower than that of VIP at 10^{-6} M ($P < 0.05$).

In separate experiments, cAMP accumulation in response to HD and VIP was investigated using the pineal glands obtained under the light condition (12 00) and under the dark (24 00). As shown in Fig. 2, the stimulatory effect of HD on pineal cAMP accumulation was significantly greater in the lighting condition than in the dark; cAMP accumulation at 12 00 was stimulated by HD 5-fold above the basal level, whereas the level at 24 00 was increased twice as much as the basal level by the dose of HD that produced the maximum effect, 10^{-7} M. VIP also showed the same effect as HD.

4- β -Phorbol 12-myristate 13-acetate (PMA) is known to activate protein kinase C (PKC). The stimulatory effect of PMA on the intracellular cAMP accumulation was examined using the dispersed pinealocytes. PMA alone had no effect on the intracellular cAMP accumulation. PMA, however, further potentiated HD-induced cAMP

Fig. 4 Effects of HD, VIP and isoproterenol on NAT activity in the pineal gland obtained under the lighted condition (at 12 00) and the dark (at 24 00). Each value indicates the mean $\pm$ SD (in 4 separate experiments). * $P < 0.01$ vs control (agonist-free), † $P < 0.05$ vs value in HD treatment

accumulation in a dose-dependent manner (Fig. 3). VIP effect on the pineal cAMP accumulation was also potentiated by PMA (data not shown).

As shown in Fig. 4, the basal activity of NAT in the dark (24 00) was significantly higher than that in the light (12 00). Isoproterenol, HD and VIP significantly stimulated the NAT activity in the pineal gland obtained at 12 00. The potency of HD on the NAT activation was not different from that of VIP although its potency was lower than that of isoproterenol (Fig. 4). On the other hand, response of the NAT activity to isoproterenol, HD and VIP was not significant in the pineal glands obtained at 24 00.

DISCUSSION

HD stimulation of adenylate cyclase activity has been demonstrated in many tissues such as rat and guinea-pig pancreatic membranes, rat liver membranes, rat brain, rat heart membranes, and human lymphoid cells (13, 15, 16, 20). HD is likely to interact with VIP- or secretin-receptors; HD, at low concentration, stimulated pancreatic amylase secretion via VIP-preferring receptors coupled to adenylate cyclase, and, at a high concentration, HD stimulated secretion via secretin-preferring receptors (3). We previously demonstrated the existence of specific VIP and PHI receptors on rat pinealocytes (5, 19). These pieces of evidence may support that, in the pineal tissue, HD interacts with VIP or PHI receptors coupled to adenylate cyclase, although

the existence of specific HD receptors has not yet been definitively excluded. Since EC_{50} of HD on pineal cAMP accumulation was rather lower than that of VIP, the existence of specific binding sites on pinealocytes is suggested.

The pineal gland is known to be an excellent model for the study of adrenergic control systems. Norepinephrine, released from sympathetic nerve terminals in the gland, causes a large increase in melatonin synthesis by stimulating the NAT activity (10, 12). An essential step in the induction of the pineal NAT activity is an increase in intracellular cAMP (11, 17). In the previous studies, we demonstrated that VIP, independently of β -adrenergic receptor-adenylate cyclase system, stimulated the pineal NAT activity through its specific receptor coupled to adenylate cyclase (5, 8). Response of cAMP accumulation to VIP varied with a periodicity inversely related to the daily light and dark cycle of VIP content; cAMP accumulation in response to VIP increased in daylight and decreased with the onset of darkness (7). The stimulatory potency of VIP to cAMP accumulation is dependent on the number of available VIP receptors which is altered by the lighting condition or agonist (6).

In this study, response of pineal NAT activity to VIP, HD and isoproterenol was significant in the lighted condition, whereas the stimulatory effects of these hormones on the NAT activity were not significant in the dark. HD stimulates pineal cAMP accumulation and NAT activity with the same potency of VIP. The EC_{50} of HD on the intracellular cAMP accumulation was rather lower than that of VIP, suggesting the possibility that HD is a neurotransmitter candidate to modulate the pineal function, like VIP. The fact that responses of pineal cAMP accumulation and NAT activity to HD were significantly less in the dark than in the light implies that the environmental light changes the number of available receptors and the magnitude of cAMP accumulation produced by HD. The potency of HD may be altered in the daily light/dark cycle in the same manner as that of VIP. Thus our results support a hypothesis that neuropeptides regulate pineal adenylate cyclase and NAT activities with circadian cycles, as seen with norepinephrine.

A synergistic interaction between PKC and β -adrenergic agonist-dependent stimulation of cAMP was observed (9, 18). Klein *et al.* demonstrated that activation of PKC is involved in the α_1 -adrenergic potentiation of β -adrenergic agonist-

stimulated cAMP accumulation in pinealocytes (9, 18). In this study, PMA, an activator of PKC, potentiated HD stimulation of pineal cAMP accumulation. We also observed that VIP-induced cAMP accumulation was amplified by PMA (data not shown). The present results suggest an interaction between α_1 -adrenergic stimulation and the peptidergic component of the cAMP response in pinealocytes.

In conclusion, this is the first demonstration of HD action on the pineal function. Our results suggest the possibility that HD also plays a role as a neurotransmitter in the pineal gland. In order to confirm this possibility, it is required to demonstrate HD release from peptidergic nerve endings innervating the pineal gland. In addition, the intracellular mechanisms through which peptides (e.g. VIP and HD) stimulate pineal functions, including NAT induction and melatonin biosynthesis, are not fully known. To assess a physiological role of HD in the pineal gland more profoundly, we plan to investigate HD release in the pineal gland and to analyze HD-specific receptors-adenylate cyclase system in the light/dark cycle.

Received 3 April 1992; and accepted 13 April 1992

REFERENCES

1. BUDA M. and KLEIN D. C. (1978) A suspension culture of pinealocytes: regulation of *N*-acetyltransferase activity. *Endocrinology* **103**, 1483-1493
2. DEGUCHI T. and AXELROD J. (1972) Sensitive assay for serotonin *N*-acetyltransferase activity in rat pineal. *Anal. Biochem.* **50**, 174-179
3. DEHAYE J. P., WINAND J., DAMIEN C., GOMEZ F., POLOCZEK P., ROBBERECHT P., VANDERMEERS A., VANDERMEERS-PIRET M.-C., STIÉVENART M. and CHRISTOPHE J. (1986) Receptors involved in helodermin action on rat pancreatic acini. *Amer. J. Physiol.* **251**, G602-G610
4. HOSHINO M., YANAIHARA C., HONG Y.-M., KISHIDA S., KATSUMARU Y., VANDERMEERS A., VANDERMEERS-PIRET M.-C., ROBBERECHT P., CHRISTOPHE J. and YANAIHARA N. (1984) Primary structure of helodermin, a VIP-secretin-like peptide isolated from Gila monster venom. *FEBS Lett.* **178**, 233-239
5. KAKU K., INOUE Y., MATSUTANI A., OKUBO M., HATAO K., KANEKO T. and YANAIHARA N. (1983) Receptors for vasoactive intestinal polypeptide on rat dispersed pineal cells. *Bio-medical Res.* **4**, 321-328
6. KAKU K., TSUCHIYA M., MATSUDA M., INOUE Y., KANEKO T. and YANAIHARA N. (1985) Light and agonist alter vasoactive intestinal peptide binding and intracellular accumulation of adenosine 3',5'-monophosphate in the rat pineal gland. *Endocrinology* **117**, 2371-2375
7. KAKU K., TSUCHIYA M., TANIZAWA Y., OKUYA S., INOUE Y., KANEKO T. and YANAIHARA N. (1986) Circadian cycles in VIP content and VIP stimulation of cyclic AMP accumula-

- tion in the rat pineal gland. *Peptides* 7, Suppl. 1, 193-195
8. KANEKO T., CHENG P.-Y., OKA H., ODA T., YANAIHARA N. and YANAIHARA C. (1980) Vasoactive intestinal polypeptide stimulates adenylate cyclase and serotonin *N*-acetyltransferase activities in rat pineal *in vitro*. *Biomedical Res.* 1, 84-87
 9. KLEIN D. C., SUGDEN D. and WELLER J. L. (1983) Postsynaptic α -adrenergic receptors potentiate the β -adrenergic stimulation of pineal serotonin *N*-acetyltransferase. *Proc. Natl. Acad. Sci. USA* 80, 599-603
 10. KLEIN D. C. and WELLER J. L. (1972) Rapid light-induced decrease in pineal serotonin *N*-acetyltransferase activity. *Science* 177, 532-533
 11. KLEIN D. C. and WELLER J. L. (1973) Adrenergic-adenosine 3',5'-monophosphate regulation of serotonin *N*-acetyltransferase activity and the temporal relationship of serotonin *N*-acetyltransferase activity to synthesis of ^3H -*N*-acetylserotonin and ^3H -melatonin in the cultured rat pineal gland. *J. Pharm. Exp. Ther.* 186, 516-527
 12. KLEIN D. C., WELLER J. L. and MOORE R. Y. (1971) Melatonin metabolism: Neural regulation of pineal serotonin: Acetyl coenzyme A *N*-acetyltransferase activity. *Proc. Natl. Acad. Sci. USA* 68, 3107-3110
 13. ROBBERECHT P., DE NEEF P., GOURLET P., CAUVIN A., COY D. H. and CHRISTOPHE J. (1989) Pharmacological characterization of the novel helodermin/VIP receptor present in human SUP-T₁ lymphoma cell membrane. *Regul. Peptides* 26, 117-126
 14. ROBBERECHT P., VANDERMEERS A., VANDERMEERS-PIRET M.-C., GOURLET P., CAUVIN A., DE NEEF P. and CHRISTOPHE J. (1988) Helodermin-like peptide. *Ann. N. Y. Acad. Sci.* 527, 186-203
 15. ROBBERECHT P., WAELEBROECK M., DEHAYE J.-P., WINAND J., VANDERMEERS A., VANDERMEERS-PIRET M.-C. and CHRISTOPHE J. (1984) Evidence that helodermin, a newly extracted peptide from Gila monster venom, is a member of the secretin/VIP/PHI family of peptides with an original pattern of biological properties. *FEBS Lett.* 166, 277-282
 16. ROBBERECHT P., YANAIHARA N., YANAIHARA C., DE NEEF P., CAMUS J.-C., VANDERMEERS A., VANDERMEERS-PIRET M.-C. and CHRISTOPHE J. (1986) Interaction of synthetic N- and C-terminal fragments of helodermin with rat liver VIP receptors. *Peptides* 7, Suppl. 1, 79-82
 17. ROMERO J. A. and AXELROD J. (1975) Regulation of sensitivity to β -adrenergic stimulation in induction of pineal *N*-acetyltransferase. *Proc. Natl. Acad. Sci. USA* 72, 1661-1665
 18. SUGDEN D., VANECEK J., KLEIN D. C., THOMAS T. P. and ANDERSON W. B. (1985) Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. *Nature* 314, 359-361
 19. TSUCHIYA M., KAKU K., MATSUDA M., KANEKO T. and YANAIHARA N. (1987) Demonstration of receptors specific for peptide N-terminal histidine and C-terminal isoleucine (PHI) using rat PHI and rat dispersed pineal cells. *Biomedical Res.* 8, 45-51
 20. VANDERMEERS A., VANDERMEERS-PIRET M.-C., ROBBERECHT P., WAELEBROECK M., DEHAYE J.-P., WINAND J. and CHRISTOPHE J. (1984) Purification of a novel pancreatic secretory factor (PSF) and a novel peptide with VIP- and secretin-like properties (helodermin) from Gila monster venom. *FEBS Lett.* 166, 273-276
 21. YUWILER A. (1983) Vasoactive intestinal peptide stimulation of pineal serotonin-*N*-acetyltransferase activity: general characteristics. *J. Neurochem.* 41, 146-153
 22. YUWILER A. (1983) Light and agonists alter pineal *N*-acetyltransferase induction by vasoactive intestinal polypeptide. *Science* 220, 1082-1083