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Adrenergic and Peptidergic Control of the Regulation of cAMP Efflux and Melatonin Secretion from Perifused Rat Pineal Gland

Zoltan Rekasi, Norbert Sule, Valer Csernus, and Bela Mess

Department of Human Anatomy, University Medical School of Pecs, Hungary

Mammalian pineal gland receives peptidergic (e.g., vasoactive intestinal peptide [VIP]; peptide histidine isoleucine [PHI]; neuropeptide Y, NPY; substance P, calcitonin gene-related peptide [CGRP], arginine vasopressin [AVP] and oxytocin [OXT]) fibers in addition to sympathetic innervation. The dynamics of cAMP efflux and melatonin (MT) secretion were compared during the infusion of these peptides in our long-term perifusion system. VIP and PHI enhanced both pineal cAMP efflux and MT secretion in a dose-dependent manner (10 n*M* to 10 μ*M*). However, the potency of PHI was slightly less. The peak of cAMP release always precedes that of MT production. The possible interactions between adrenergic and peptidergic compounds in the regulation of pineal cAMP efflux and MT secretion were also studied. VIP acts on specific peptidergic receptors, since its stimulatory effect could only be reduced by a VIP receptor antagonist. VIP has an additive effect at a lower (100 n*M*) concentration combined with norepinephrine (NE). NPY (100 nM) can completely block NE-induced MT secretion, but the decrease in cAMP efflux is less. However, NPY does not significantly influence VIP-stimulated cAMP efflux or MT secretion. These data suggest that NE, VIP, and NPY are differently involved in the cAMP and calcium signaling. The other neuropeptides are ineffective.

Key Words: Melatonin; cyclic AMP; VIP; NPY; norepinephrine; neuropeptides.

Introduction

In addition to the sympathetic innervation *(1)*, an increasing number of peptidergic neurons have been reported to play modulating role in pineal function *(2–4)*. Vasoactive intestinal peptide (VIP) and peptide histidine isoleucine- (PHI) immunoreactive nerve fibers have been demonstrated in the mammalian pineal gland by Uddman et al. *(5)* and Moller and Mikkelsen *(6)*, which probably originate from the pterygopalatine ganglion *(7)*. Specific binding sites for both VIP and PHI have been identified on rat pinealocytes *(8,9)*. The pineal VIP content similarly to that of norepinephrine (NE) oscillates over a 24-h period *(10)*. Functional studies have demonstrated that VIP also stimulates adenylate cyclase activity *(11)*, cAMP accumulation *(12,13)*, *N*-acetyltransferase (NAT) activity *(11,14)*, and melatonin (MT) secretion *(15–17)*. PHI also stimulates cAMP production *(18)*, NAT activity, and MT secretion in rat pineal gland, but its potency is slightly less *(19)*.

Neuropeptide Y (NPY) is another neuropeptide candidate for modulating pineal function colocalized in noradrenergic fibers originating mainly from the superior cervical ganglia *(4,7,20,21)*. NPY has been reported to stimulate pinealocyte indole metabolism in vitro *(22)* and during the daytime in vivo, but to inhibit the metabolism at night *(23)*. NPY has been shown to inhibit NE-stimulated cAMP accumulation via the activation of NPY-Y1 receptors *(24)*, thus resulting in reduced MT secretion *(25,26)*. The circadian variations of NPY-like immunoreactivity in the pineal have also been described *(27)*.

Substance P-(SP) and calcitonin gene-related peptide-(CGRP) like immunoreactive fibers have also been found in the pineal gland arising from the trigeminal ganglia *(7,28,29)*. Though high-affinity binding sites for SP have been demonstrated in bovine pineal gland *(30)*, nothing is known about the direct effects of these peptides on cAMP accumulation and MT secretion.

Arginine vasopressin- (AVP) and oxytocin- (OXT) containing central fibers have also been demonstrated in the rat pineal gland *(31)* in addition to peripheral pinealopetal nerve fibers. These peptidergic axons originate from the hypothalamic magnocellular nuclei and probably reach the pineal gland via the subcommissural organ or habenular commissure. The circadian variation of AVP level in the rat pineal gland has also been described *(32)*. In one report, AVP and OXT decreased the basal MT secretion, whereas high concentration of these peptides potentiated the isoproterenol-induced MT secretion *(33)*.

Until recently, little has been known about the dynamics of simultaneously secreted cAMP (the key second messen-

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Author to whom all correspondence and reprint requests should be addressed: Dr. Zoltan Rekasi, Department of Human Anatomy, University Medical School of Pecs, 7643 Pecs, Hungary. E-mail address: rekasiz@apacs.pote.hu

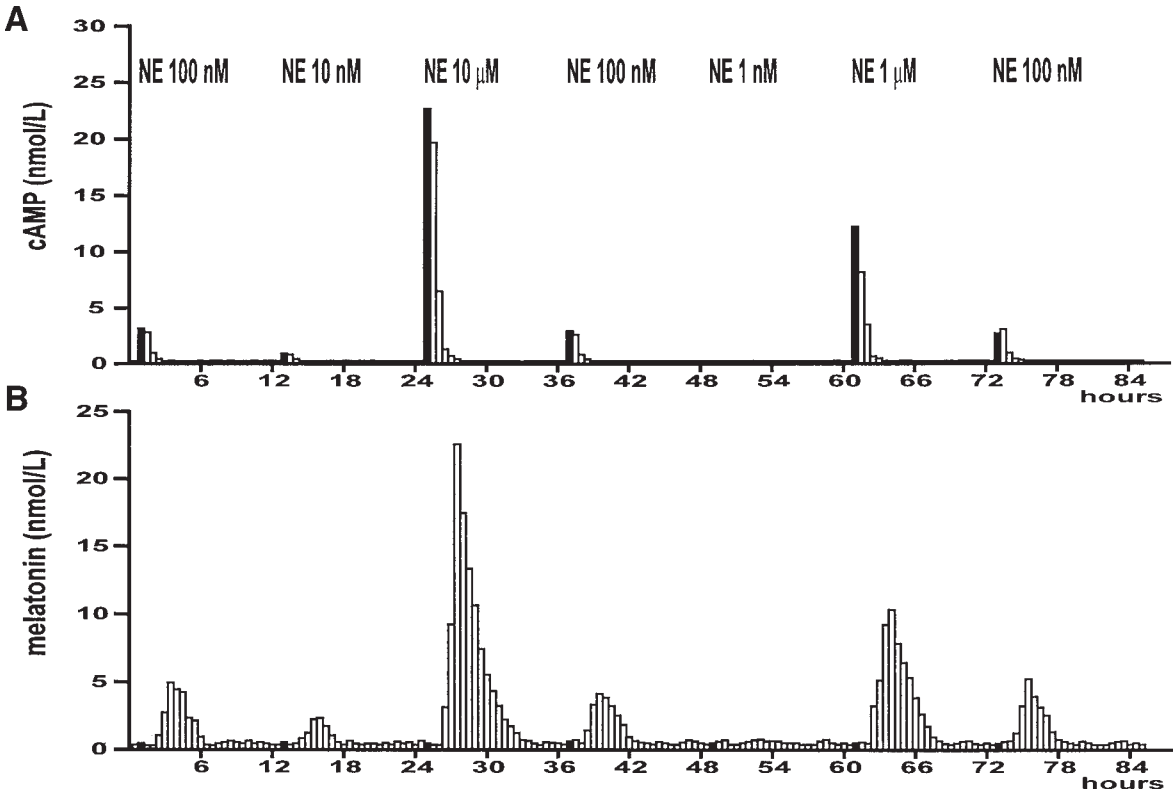

Fig. 1. Dose–response relationship between NE application and cAMP efflux **(A)** or MT secretion **(B)** from the same pinealocytes in a long-term perifusion system. Fragments of four male rat pineal glands were stimulated for 30 min with different concentrations of NE (1 n*M* to 10 μ*M*) as indicated above. Each net integral value of responses is compared to that of first NE infusion (100 n*M*). Fractions were collected every 30 min. Each black column represents the fraction in which the infused compound was present in the perifusion chamber. A representative curve of five experiments is shown.

ger) and MT (the end product) from pinealocytes following peptide stimuli. We have developed a long-term perifusion system for measuring the kinetics of MT secretion from pinealocytes during and following multiple stimulation *(34)*. This approach is useful to examine the kinetics of cAMP efflux and MT secretion simultaneously. This system also permits studying interactions between adrenergic and peptidergic compounds on the production of cAMP and MT.

The aims of the present work were:

1. To compare the dynamics of cAMP efflux and the time-course of MT production from the same pinealocytes after NE, VIP, PHI, NPY, SP, CGRP, AVP, and OXT exposures.
2. To test the possible modulating influence of these peptides on NE-induced MT secretion.
3. To examine the interactions between VIP and other neuropeptides in the regulation of cAMP efflux and MT secretion in the long-term perifusion system.

Results

Effect of NE on the Kinetics of cAMP Efflux and MT Secretion from the Same Pinealocytes

NE (10 n*M* to 10 m*M*) caused a transient sharp increase in cAMP efflux from pinealocytes in dose dependent way (Fig. 1A, Table 1). The release of cyclic nucleotide started to increase immediately after the beginning of NE exposure reaching the maximal value in the first 20.0 ± 12.2 min and then declined to the basal values in 1.75 ± 0.5 h. MT secretion from the same cells was also enhanced by these doses of NE in dose-dependent manner, but the MT responses revealed significantly different kinetics (Fig. 1B, Table 1). The MT production increased only after the NE exposure had been stopped and even after cAMP release had started to return to basal values. Following this relatively long lag (1.42 ± 0.2 h) MT response reaches its peak value in the next 1.67 ± 0.2 h and then slowly declines to baseline in 2.58 ± 1.36 h.

Effect of Neuropeptides on the Kinetics of cAMP Efflux and MT Secretion from the Same Pinealocytes

Similarly to NE, both VIP (10 n*M* to 10 m*M*) and PHI (100 n*M* to 10 m*M*) promptly stimulated the efflux of cAMP from pinealocytes in a dose-dependent manner, but with lower potency, PHI proved to be less effective (Figs. 2A and 3A, Table 1). The culmination of cAMP release into the medium was observed in a short interval (22.5 ± 15 min) after the beginning of VIP exposures, and the nucleotide level returned to basal values in 1.0 ± 0.7 h following stimulation. Both neuropeptides were able to induce MT synthe-

Table 1
Effect of NE, VIP, and PHI on cAMP Efflux and MT Secretion NET INT Values[a]

	NE		VIP		PHI	
Drug concentration	cAMP nmol/L	MT nmol/L	cAMP nmol/L	MT nmol/L	cAMP nmol/L	MT nmol/L
1 n*M*	ND	ND	ND	ND	ND	ND
10 n*M*	2.3 ± 0.4	6.5 ± 1.2	0.9 ± 0.1	1.8 ± 0.2	ND	ND
100 n*M*	7.8 ± 0.9	25.3 ± 4.8	1.4 ± 0.3	7.5 ± 1.8	1.0 ± 0.2	1.7 ± 0.3
1 μ*M*	21.2 ± 3.4	47.0 ± 6.4	3.2 ± 0.5	17.1 ± 3.2	2.0 ± 0.3	7.7 ± 1.1
10 μ*M*	50.4 ± 4.4	118.0 ± 32.3	10.8 ± 1.2	45.2 ± 5.1	5.4 ± 0.9	36.6 ± 4.2

[a]ND, Not significantly different from baseline. At other concentrations tested, drugs induced a significant increase (at least $p < 0.05$) in cAMP efflux or MT secretion.

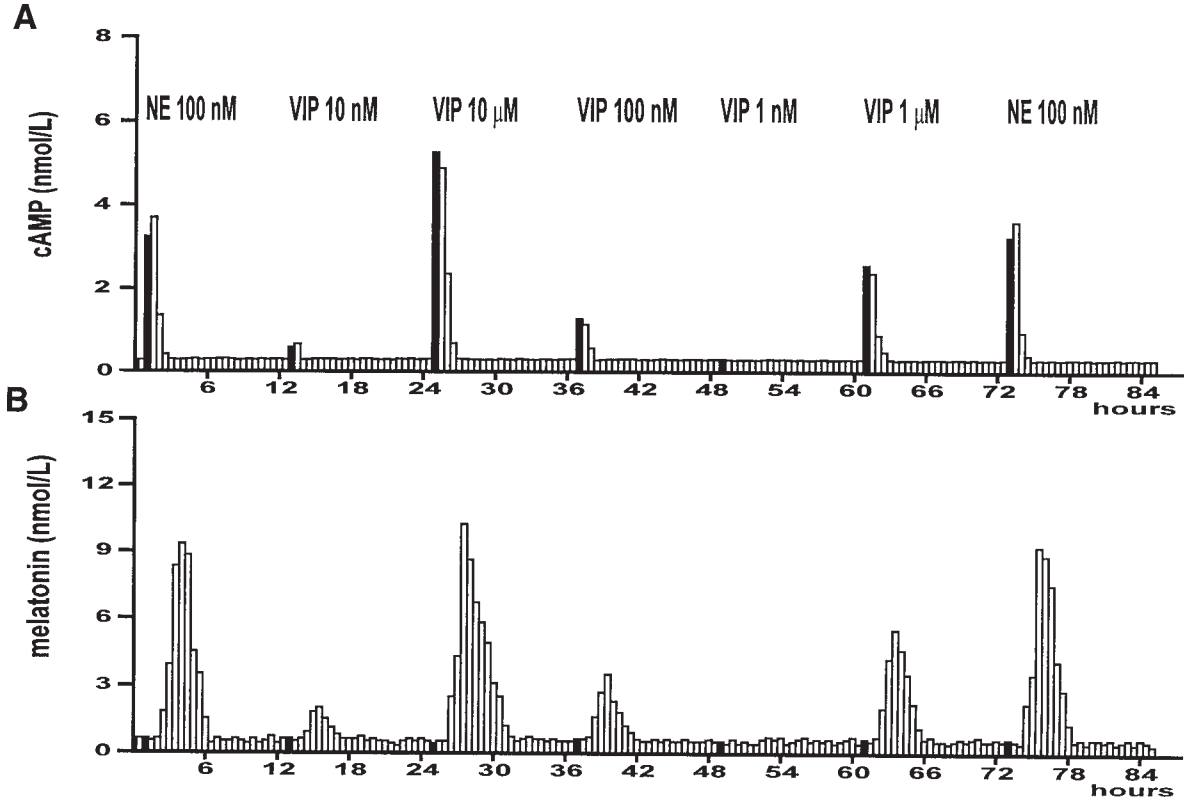

Fig. 2. Dose–response relationship between VIP application and cAMP efflux **(A)** or MT secretion **(B)** from the same pinealocytes in a long-term perifusion system. Fragments of four male rat pineal glands were stimulated for 30 min with different concentrations of VIP (1 n*M* to 10 μ*M*) as indicated above. Further details were described in the legend to Fig. 1. A representative curve of four experiments is shown.

sis alone, but they proved to be less effective than NE. VIP/PHI enhanced MT secretion from the same pinealocytes in a dose-dependent way, but the elevated hormonal response started to be manifested with a 1.38 ± 0.3 h delay, after cAMP release having declined (close) to baseline (Figs. 2B and 3B, Table 1). MT level reached peak values only in 1.63 ± 0.3 h following delay and gradually returned to basal values in an additional 2.25 ± 0.9 h. PHI influenced MT production similarly to VIP, but its potency was slightly less.

NPY, SP, CGRP, OXT, and AVP (1 n*M* to 10 m*M*) infused alone did not influence either cAMP efflux or MT secretion from pinealocytes (data not shown).

Effect of β-adrenergic and VIP Receptor Antagonists on NE- or VIP-Stimulated Melatonin Secretion

To study the target site of VIP, the most effective peptide regarding MT secretion, we compared the effect of an adrenergic and VIP-ergic receptor antagonist combined with NE or VIP. Propranolol (100 n*M*) a potent β-adrenergic antagonist, completely inhibited NE-evoked (100 n*M*) cAMP efflux and MT response, but it did not influence the effect of VIP (100 n*M*). In contrast, a selective VIP receptor antagonist (100 n*M*) decreased the VIP-stimulated cAMP efflux and MT secretion by 70% (from net integral values [NET INT]: 1.8 ± 0.2 nmol/L to NET INT: 0.54 ± 0.1 nmol/L) and 64% (from NET INT:

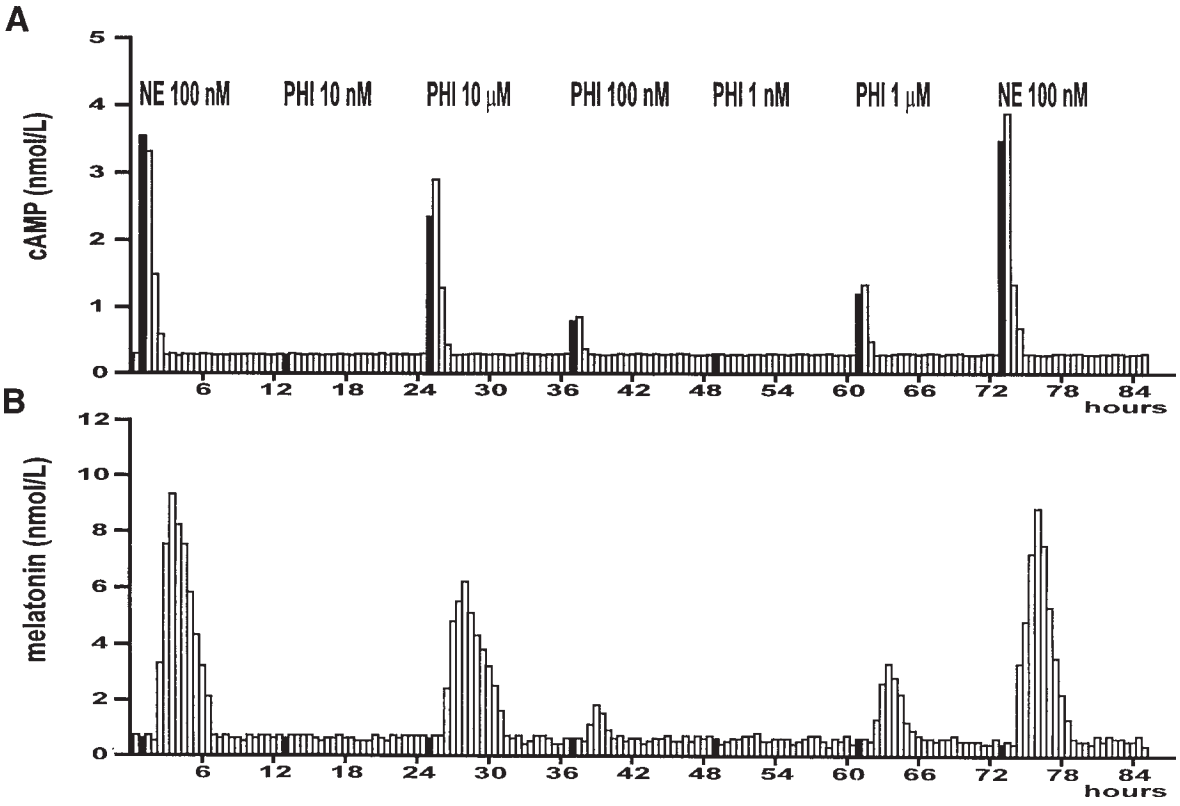

Fig. 3. Dose–response relationship between PHI application and cAMP efflux **(A)** or MT secretion **(B)** from the same pinealocytes in a long-term perifusion system. Fragments of four male rat pineal glands were stimulated for 30 min with different concentrations of PHI (1 n*M* to 10 μ*M*) as indicated above. Further details were described in the legend to Fig. 1. A representative curve of three experiments is shown.

14.0 ± 2.5 nmol/L to NET INT: 5.1 ± 1.2 nmol/L), respectively, but the NE-induced elevation of MT production remained unchanged (Fig. 4).

Effect of Combined Treatment with NE and Neuropeptides on cAMP Efflux and MT Secretion from Pinealocytes

The effects of a low dose (100 n*M*) of VIP and NE on cAMP release and MT secretion from pinealocytes were additive (cAMP—from NET INT/NE/: 8.4 ± 0.9 nmol/L and NET INT/VIP/: 3.2 ± 0.5 nmol/L to NET INT/NE + VIP/: 13.7 ± 1.8 nmol/L; MT – from NET INT/NE/: 38.0 ± 5.2 nmol/L and NET INT/VIP/: 11.7 ± 2.6 nmol/L to NET INT /NE + VIP/: 76.1 ± 9.9 nmol/L), but the combined treatment with maximally effective concentrations of these drugs (10 μ*M*) did not produce further enhanced responses (Fig. 5). NPY (10–100 n*M*) significantly reduced the NE- (100 n*M*) stimulated MT secretion (total blockade was observed in case of a 100-n*M* dose) (Fig. 6B), but the decrease in cAMP efflux was subtler (from NET INT: 7.2 ± 0.8 nmol/L to NET INT: 5.0 ± 0.4 nmol/L/70%/ and NET INT: 4.7 ± 0.6 nmol/L/65%/, respectively) (Fig. 6A). In contrast, NPY (10–100 n*M*) did not influence the VIP-(100-n*M*) stimulated cAMP efflux and MT secretion at all (Fig. 6). SP, CGRP, OXT, and AVP (10–100 n*M*) proved to be ineffective regarding either NE- or VIP-stimulated cAMP efflux and MT secretion (data not shown).

Discussion

In the mammalian pineal, norepinephrine released from postganglionic sympathetic nerve terminals at night *(35)* activates both β- and α_1-adrenergic receptors to control adenylate cyclase activity stimulating cAMP accumulation *(36,37)*. In turn, cAMP regulates the activity of serotonin *N*-acetyltransferase (EC 2.3.1.87; NAT), the rate-limiting enzyme of melatonin synthesis and secretion *(36)*, via cAMP response element-binding protein (CREB) phosphorylation *(38)*. Our data on the dynamics of cAMP efflux and MT secretion simultaneously from the same pinealocytes also support this mechanism of action. Using our long-term perifusion system *(34)* NE increased both cAMP efflux and MT secretion in a dose-dependent way. Adrenergic stimulation caused a transient sharp increase in cAMP efflux, which was followed later by a long-lasting MT response. The increase in cyclic nucleotide level always precedes the MT elevation, but the areas under the curves of NE-induced cAMP and MT responses enhance parallel in a dose-dependent way indicating the essential role of this messenger signal in the process of NE-stimulated MT secretion. The changes in efflux of cAMP correlate well with the alterations in intracellular level of nucleotide, since approx 30–50% of the intracellular content is secreted into the medium under both nonstimulated and stimulated conditions *(39)*. The time necessary to reach the highest level of

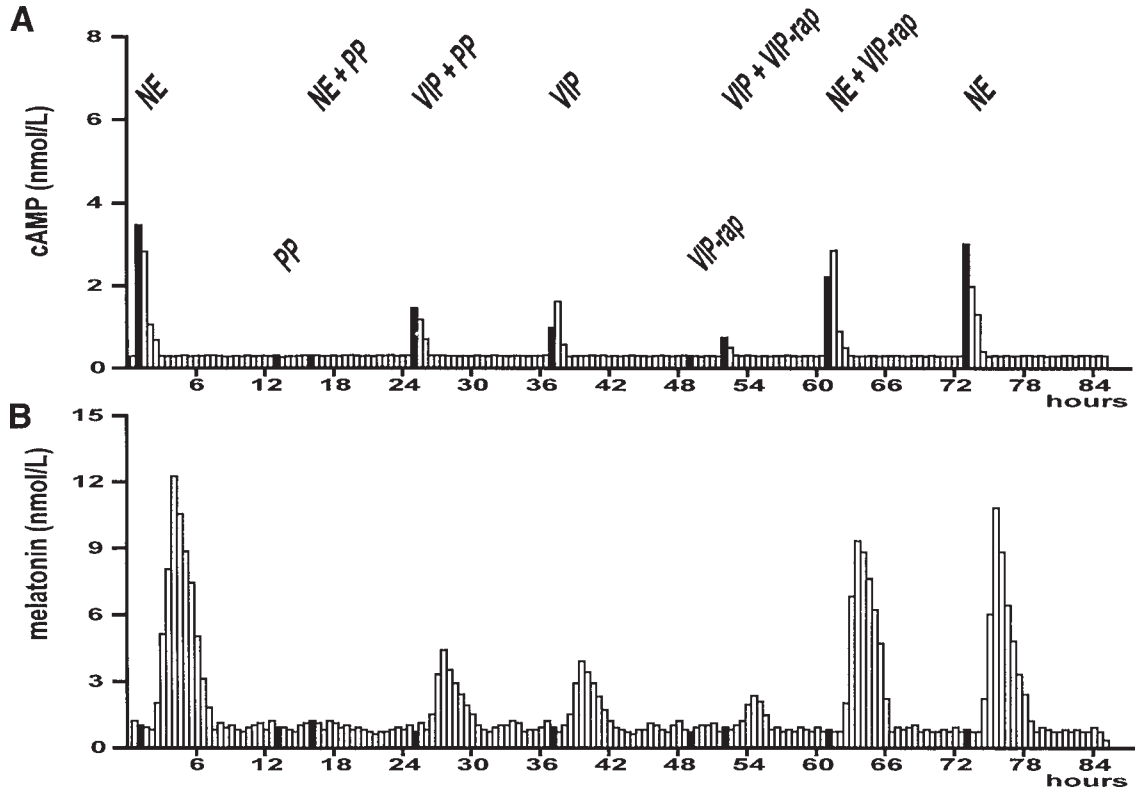

Fig. 4. Effect of propranolol (PP) or a VIP receptor antagonist peptide (VIP-rap) on NE- or VIP-induced cAMP efflux **(A)** or MT secretion **(B)** from the same pinealocytes. Fragments of four male rat pineal glands were stimulated for 30 min with NE or VIP and PP or VIP-rap as indicated above (all compounds were infused at 100-n*M* concentration). Further details were described in the legend to Fig. 1. A representative curve of three experiments is shown.

nucleotide release was 20.0 ± 12.2 min following the onset of stimulation, whereas the peak value of MT secretion was reached after a relatively long delay (3.09 ± 0.2 h) similar to the time-course of NAT induction *(40)*. This delayed onset of MT synthesis supports the recent findings that NE-induced cAMP accumulation triggers gene expression of NAT *(41–43)* and/or any regulatory factors through a cascade.

According to our results among neuropeptides, VIP and PHI were able to elevate cAMP efflux and MT secretion alone from the same pinealocytes in a dose-dependent manner, but with lower potency compared to that of NE. The weaker effect of PHI is consistent with the result of Kaneko and Kaku *(18)* and Moujir et al. *(19)*. The increase in cAMP efflux induced by peptide infusions always precedes the elevation of MT secretion similarly to the kinetics of NE-induced responses. These data support the fundamental role of this intracellular messenger molecule in the action of VIP/PHI-regulated melatonin synthesis as well. However, the duration and the peak value of MT responses to peptides are smaller when comparing to those to NE probably because of the lack of involvement of α_1-adrenergic receptor stimulation. Recently VIP has been shown to be less effective than NE in stimulating NAT gene expression *(43)*.

In these experiments, we have also demonstrated that VIP acts on its own specific postsynaptic receptor, which is independent from β-adrenergic receptor supporting the earlier findings *(8,15,17)*. A potent VIP receptor antagonist could block only the VIP-stimulated MT response, whereas the NE-evoked MT secretion remained unchanged.

We provided further evidence of the interaction between adrenergic and VIP-ergic systems sharing the same limiting second messenger (cAMP) *(2)*. The effect of combined administration of NE and VIP regarding cAMP efflux and MT secretion was additive when concentrations were low (100 n*M*). Using higher concentrations, we could not achieve a further increase either in cyclic nucleotide efflux or in MT secretion.

NPY in one study increased MT secretion alone and enhanced NE-stimulated MT production *(22)*. Our preliminary observation was partly consistent with these data *(16)*. Recently, by eliminating the remnant of NE from nerve endings in our perifusion system (i.e., 40–48 h recovery period was maintained between column preparation and administration of first compound instead of 16 h, which we had used earlier; *see* Methods), we could study the stand-alone postsynaptic effect of NPY, and we could lower the effective concentrations of applied compounds. In this series of experiments a low dose of NPY (100 n*M*) decreased the NE-induced cAMP and MT release. However, it had no effect on cyclic nucleotide and hormone secretion by itself. This result supports the recent data on the postsynaptic inhibitory role of NPY *(24–26)*. The stimu-

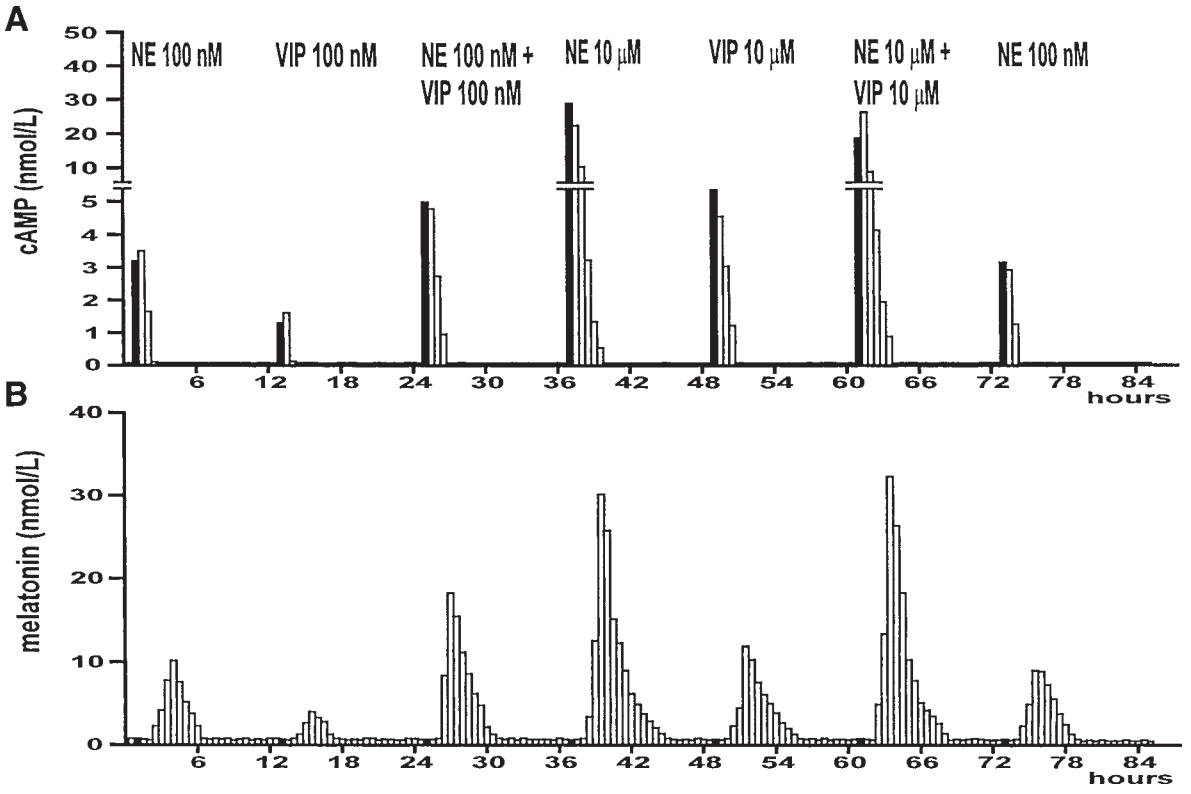

Fig. 5. Interaction between NE and VIP in the regulation of cAMP efflux **(A)** or MT secretion **(B)** from the same pinealocytes. Fragments of four male rat pineal glands were stimulated for 30 min with different concentrations of NE and VIP (100 n*M* or 10 μ*M*) as indicated above. Further details were described in the legend to Fig. 1. A representative curve of three experiments is shown.

latory effect of NPY alone reported by Vacas et al. *(22)* can be explained by the presence and influence of noradrenergic nerve endings, which could not degenerate in the organ culture owing to the short interval after preparation *(40)*.

According to our data, NPY caused a relatively subtle decrease in NE-induced cAMP efflux in contrast to its complete blockade in stimulated MT secretion. This result may exclude the principal role of cAMP in the inhibitory action of NPY, suggesting the role of another intracellular messenger molecule (i.e., Ca^{2+}).

Regarding VIP–NPY peptidergic interaction, in our perifusion system, NPY was not able to influence the VIP-induced increase in cAMP efflux and MT secretion at all. This discrepancy in the NPY-caused inhibition regarding NE- and VIP-induced increase in cAMP efflux and MT secretion may be explained by the different routes of intracellular Ca^{2+} elevation following adrenergic or VIP-ergic stimulation *(44)*. Another explanation could be that the intracellular Ca^{2+} does not play a principal role in the mechanism of action of VIP at all (45).

Since both VIP *(10)* and NPY *(27)* contents show circadian rhythm in the pineal gland with the highest levels in darkness, these peptide compounds may play some role in transmission of photic information. VIP activating simultaneously with NE may serve as a "turn-on" signal helping the enhancement of MT synthesis stimulated by NE in the early phase of darkness, whereas NPY may decease the NE-induced MT synthesis as a "turn-off" signal.

In our long-term perifusion system, SP or CGRP did not influence either basal or NE-induced cAMP efflux/MT secretion from the same pinealocytes. These peptides may serve as neurotransmitters of sensory neurons, the perikarya of which are located in the trigeminal ganglion *(29)*.

The data on direct in vitro effect of OXT and AVP on MT production are controversial. OXT and AVP could decrease the basal MT level and potentiate the isoproterenol-induced increase of MT secretion at high concentrations *(33)*. In our long-term perifusion system, even high doses of OXT or AVP (100 n*M* and 10 μ*M*) could not influence either basal or NE-induced cAMP efflux/MT secretion. According to our findings, cAMP does not seem to be involved in the mechanism of action of AVP/OXT. The results of the inhibitory effect of these peptides on basal MT secretion reported earlier (33) are not fully convincing because of the lack of dose dependency of the effect as it has been discussed by the same authors. Furthermore, the potentiating effect of high concentrations of peptides (10 μ*M*) is probably not physiological. Thus, the exact role of AVP/OXT in pineal function remains to be elucidated.

In conclusion, using the long-term perifusion system, we tested the effects of the morphologically identified peptidergic and adrenergic neuronal systems and their

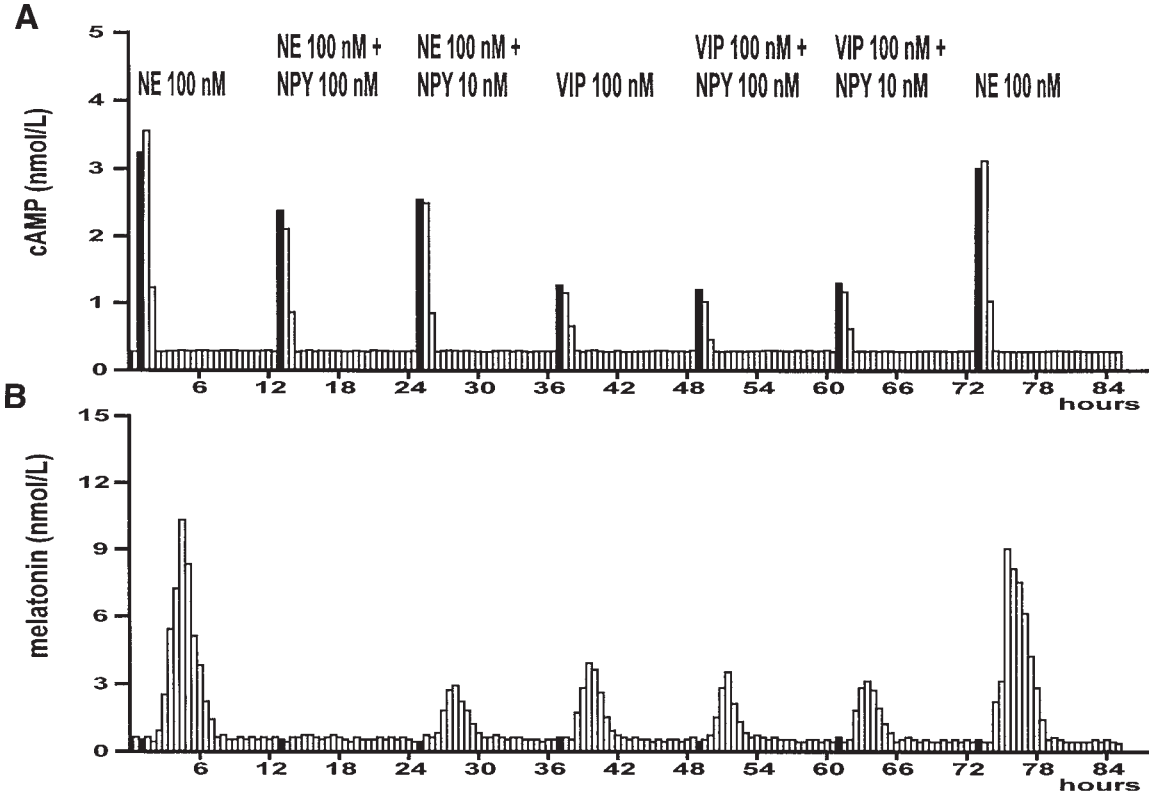

Fig. 6. Interaction between NE or VIP and NPY in the regulation of cAMP efflux **(A)** or MT secretion **(B)** from the same pinealocytes. Fragments of four male rat pineal glands were stimulated for 30 min with different concentrations of NE (100 n*M*), VIP (100 n*M*) and NPY (10–100 n*M*) as indicated above. Further details were described in the legend to Fig. 1. A representative curve of three experiments is shown.

interactions in the regulation of pineal indole metabolism. Among these, VIP-ergic, NPY-ergic, and sympathetic pathways may converge in pineal cells to control MT biosynthesis in which cAMP plays a fundamental, but not absolute role as a second messenger.

Materials and Methods

Chemicals

NE, VIP, PHI, NPY, SP, CGRP, AVP, OXT, propranolol, VIP receptor antagonist peptide, cyclic AMP, MT, Sephadex G-10, Medium 199, bovine serum albumin (BSA), and gentamicin sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). The inorganic substances were obtained from Reanal Chemical Co. (Budapest, Hungary).

Animals

Adult male rats (Wistar R, Amsterdam, The Netherlands) were kept under a 14-h light/10-h dark photoperiod (lights on at 5:00 AM) and were given food and water ad libitum. Animals were sacrificed by decapitation at around 2:00 PM. The experimental protocols were approved by the appropriate institutional review committee and meet the guidelines of their responsible governmental agency.

Perifusion System

A long-term pineal gland perifusion was performed as described earlier *(34)*. Briefly, for each experiment, fragments of four pineal glands were transferred onto each perifusion chamber and allowed to sediment simultaneously with 0.8 mL of Sephadex G-10. Medium 199 containing BSA (2.5 g/L), $NaHCO_3$ (2.2 g/L), and gentamicin sulfate (85 mg/mL) was equilibrated with a mixture of 95% air/5% CO_2 and used as the culture medium. The medium was pumped at a flow rate of 0.1 mL/min. After a 40- to 48-h recovery period, in which the baseline of cAMP and MT output became stabilized and the cells regained their full responsiveness, the samples were infused through a four-way valve. First, the system was standardized with a 30-min exposure to 100 n*M* NE. The test materials were infused at various concentrations for 30 min (one fraction) usually at 12-h intervals.

Radioimmunoassay (RIA)

Samples from collected fractions were diluted as required with acetic acid (5 m*M*), cyclic AMP were acetylated *(46)*. The samples or the standards, the antibody (DK 388/020889, 1:7,500) and the iodinated cyclic nucleotide (generously provided by David C. Klein, NIH) were mixed with 50 m*M* sodium acetate buffer, pH 6.2, containing 2.5% normal rabbit serum and 0.1% gelatin. After incubation (4°C, 16–18 h), protein was precipitated by the addition of ethanol (1 mL, 4°C). Tubes were centrifuged (3000*g*, 30 min, 4°C), and radioactivity in pellet was determined. Each sample was analyzed in duplicate. The limit of detection of cAMP was 2.0 fmol/tube.

MT content from collected perifusion fluid was assayed by RIA developed in our laboratory *(34)*. The sensitivity limit of the assay was 15 fmol/tube.

Statistical Analysis

The results of RIA were analyzed with a computer program developed in our institute *(47)* involving analysis of variance and Student's *T*-test. The NET INTs of responses exposed to drugs were calculated (expressed as mean ± SD) and compared. The NET INT is the difference between the total area under the peak and the area under the baseline along the peak, representing the net amount of nucleotide or hormone secreted in response to stimulus. The NET INT of cAMP/MT response after the first 100 n*M* NE infusion was used as an intra-assay standard in each experiment. The results in the figures were representative values from three to five experiments. The variances in these in vitro experiments were negligible and, therefore, are not indicated on the graphs.

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