Accumulation of rat pineal serotonin N-acetyltransferase mRNA induced by pituitary adenylate cyclase activating polypeptide and vasoactive intestinal peptide *in vitro*

Z Rekasi and T Czompoly

Department of Human Anatomy, Medical Faculty, University of Pecs, Pecs, 7624 Hungary

(Requests for offprints should be addressed to Z Rekasi, Department of Human Anatomy, Medical Faculty, University of Pecs, Szegedi ut 12. Pecs, 7624 Hungary; Email: zoltan.rekasi@aok.pte.hu)

Abstract

In mammals, pineal melatonin secretion is under the control of adrenergic and peptidergic inputs regulating serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase; AA-NAT) activity. In this study, the accumulation of AA-NAT mRNA induced by norepinephrine (NE) and peptides of the secretin superfamily (pituitary adenylate cyclase activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), growth hormone releasing factor (GRF), secretin) was investigated by a new quantitative reverse transcription-PCR (RT-PCR) assay. We demonstrated that PACAP was the most potent peptide to increase the expression of AA-NAT mRNA and to induce cAMP production in rat pinealocytes. VIP was also able to elevate the AA-NAT mRNA level and cAMP efflux in a dose-dependent manner; however, it was six- and threefold, respectively, less potent than PACAP. The maximal values of AA-NAT mRNA level after PACAP and VIP exposures were similar (523.1 ± 52.5 amol to 640.7 ± 68.8 amol vs 461.5 ± 54.3 amol to 579.2 ± 72.4 amol). These saturable peak values were approximately five- to eightfold less than that after NE (3.0 ± 0.3 fmol to 3.6 ± 0.4 fmol). GRF and secretin were less potent than VIP in inducing AA-NAT gene expression and cAMP efflux. These data suggest that the peptides act mostly on VIP₁/PACAP (VPAC₁) receptors of pinealocytes with different affinity. The peak cAMP efflux always preceded the elevation of AA-NAT gene expression during the 3-h infusion of VIP or NE. The cAMP efflux had declined by the time of onset of maximal AA-NAT gene expression, but remained significantly higher than its basal values. Our data indicate that even a submaximal level of cAMP is sufficient for maintaining the maximal AA-NAT mRNA accumulation. These findings show that, in addition to NE, PACAP and VIP may have an important role in the regulation of AA-NAT mRNA levels in rat pinealocytes.

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Introduction

In mammals, the pineal gland serves as a neuroendocrine transducer (Axelrod 1974), transmitting the circadian and seasonal photic information into a hormonal signal (melatonin, MT). The connection between retina and pineal gland consists of a multisynaptic pathway, finally resulting in sympathetic innervation of the pineal gland from the superior cervical ganglion (Klein & Moore 1979). Norepinephrine (NE) released from the sympathetic nerve terminals during the dark phase of the photoperiod (Drijfhout *et al.* 1996) evokes a

nocturnal increase in MT production of pinealocytes (Klein 1985), which is a substantial hormonal message for timing several circadian and seasonal functions (Reiter 1993).

Norepinephrine controls the activation of serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, EC 2.3.1.87; AA-NAT), the rate-limiting enzyme of MT synthesis, by transcriptional and posttranscriptional regulatory mechanisms (Klein *et al.* 1997). In rats, NE induces AA-NAT transcription through activation of protein kinase A (PKA) and phosphorylation of cAMP response element (CRE)-binding protein

(CREB) (Roseboom & Klein 1995, Tamotsu *et al.* 1995, Maronde *et al.* 1999a, b). Phosphorylated CREB activates AA-NAT gene expression via CREs in the AA-NAT promoter (Borjigin *et al.* 1995, Coon *et al.* 1995, Roseboom *et al.* 1996, Baler *et al.* 1997, Burke *et al.* 1999, Maronde *et al.* 1999a). NE also results in accumulation of AA-NAT protein by the inhibition of proteosomal proteolysis (Gastel *et al.* 1998). This dual transcriptional/proteolysis mechanism is cAMP-dependent, and their relative importance in AA-NAT activation varies on a species-to-species basis among vertebrates (Klein *et al.* 1997, Schomerus *et al.* 2000).

In addition to the sympathetic innervation (Kappers 1960) an increasing number of peptidergic neurons have been reported to play a modulating role in pineal function (Simonneaux 1995, Yuwiler *et al.* 1995, Moller *et al.* 1996, Rekasi *et al.* 1998). Among these, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) seem to play the most important role in mammalian pineal indole metabolism. VIP and PACAP immunoreactive nerve fibers have been demonstrated in the mammalian pineal gland (Moller *et al.* 1996, 1999). Binding sites for both VIP (Kaku *et al.* 1983) and PACAP (Masuo *et al.* 1992) on rat pinealocytes have been identified as VIP₁/PACAP (VPAC₁) and PACAP (PAC₁) receptors (Olcese *et al.* 1996, Simonneaux *et al.* 1998). The pineal VIP content, like that of NE, oscillates over a 24-h period (Kaku *et al.* 1986). Functional studies have demonstrated that VIP also stimulates adenylate cyclase activity (Kaneko *et al.* 1980a), cAMP production (Kaneko *et al.* 1980b, Chik *et al.* 1988, Rekasi *et al.* 1998), CREB phosphorylation (Schomerus *et al.* 1996), AA-NAT activity (Kaneko *et al.* 1980a, Yuwiler 1983) and MT secretion (Simonneaux *et al.* 1990, Rekasi *et al.* 1994, 1998) with, however, much less potency than NE. PACAP also stimulates cAMP-induced AA-NAT activity and MT secretion (Simonneaux *et al.* 1993, Chik & Ho 1995, Yuwiler *et al.* 1995) in rat pineal gland in a similar manner to VIP.

Until recently, little has been known about the AA-NAT mRNA accumulation induced by VIP, PACAP and the other peptides of the secretin superfamily. Therefore, we studied the effect of VIP, PACAP, growth hormone releasing factor (GRF) and secretin on AA-NAT mRNA levels, in addition to a detailed study on the time- and

dose-dependent effect of NE. For reliable quantitation of AA-NAT expression we developed a sensitive competitive reverse transcription-PCR (RT-PCR) assay. The kinetics of AA-NAT gene expression were compared with the dynamics of cAMP efflux from the same cells in a dispersed pinealocyte superfusion system.

Materials and methods

Animals

Adult male Sprague–Dawley rats were housed in a controlled lighting environment (12 h light:12 h darkness cycle, lights on at 0600 h) and food and water were available *ad libitum*. Animals were killed by decapitation at around 1400 h. The experiments on animals conformed with the Guidelines of the UFAW Handbook on the Care and Management of Laboratory Animals and with the FRAME's guidelines involving the use of laboratory animals.

Superfusion system

The dispersed pinealocyte superfusion system was developed by modifying earlier methods (Rekasi *et al.* 1991, Rekasi & Schally 1993) to increase the responsiveness of pinealocytes. Briefly, for each superfusion column, pineal glands of 4 animals were digested with 0.75% collagenase CLS 2 (Worthington Biochemical Corp., Freehold, NJ, USA) for 20 min. After incubation, the fragments were dispersed into clusters (5–40 cells) by mechanical dispersion, then transferred onto three columns, and allowed to sediment simultaneously with 0.8 ml Sephadex G-10 (Sigma Chemical Co., St Louis, MO, USA). The dead volume of the system was set to 1 ml. Medium 199 (Sigma) containing bovine serum albumin (1 g/l), NaHCO₃ (2.2 g/l), penicillin G (50 mg/l) (Sigma), and gentamicin sulfate (87 mg/l) (Sigma) was equilibrated with a mixture of 95% air/5% CO₂ and used as the culture medium. The medium was pumped at a flow rate of 1 ml/3 min. After an overnight recovery period in which the baseline of cAMP efflux was stabilized and the cells regained their full responsiveness, the samples were infused through a four-way valve. The test materials (NE, VIP, PACAP-38, GRF(1–29)NH₂ and secretin) (Sigma) were infused at various concentrations for between 10 min and 3 h. Each experiment was performed in three identical superfusion columns

simultaneously. Immediately after collection of fractions (2 ml/6 min), 25 µl of a freshly prepared mixture of triethylamine and acetic anhydride (2:1) were added to 500 µl ice-cold aliquots of the medium fractions and kept frozen at -20°C until radioimmunoassay (RIA).

Cyclic AMP RIA

Rat cAMP levels in aliquots of acetylated medium were determined by double-antibody RIA to measure the basal and stimulated efflux. The antibody (anti-cAMP-NIDDK CV-27) for cAMP RIA was provided by the National Hormone and Pituitary Program (NIDDK, NIH, USA). Duplicate samples of 100 µl acetylated medium, 200 µl PBS (0.05 M, pH=7.4), 100 µl antibody solution (at 1:166 000 final dilution) and 100 µl tracer (15 000 c.p.m. in PBS) were incubated in borosilicate tubes. The ^{125}I -labeled tracer was prepared from tyrosyl-methyl ester cAMP (Sigma) using the lactoperoxidase method, followed by HPLC purification on a Vydac C-18 column with a linear gradient of acetonitrile and aqueous 0.1% trifluoroacetic acid. Standards in the range 0.65–658 pg/tube were dissolved in superfusion medium and were acetylated together with the samples. After an overnight incubation at 4°C , the bound fraction was separated using a second antibody–polyethylene glycol method. The sensitivity of the assay was 1.2 pg/tube. Interassay and intra-assay coefficients of variation were 8.5% and 4.5% respectively.

RNA extraction

Total RNA of dispersed pinealocytes was extracted using the TRI REAGENT (Sigma) procedure, an improvement of the single-step method reported by Chomczynski & Sacchi (1987). At the end of the experiments, the infusion of different test materials was immediately followed by the administration of 1 ml TRI REAGENT into the superfusion column. After the columns had been completely filled with this solution, the flow was temporarily stopped for 5 min. During that time the columns were opened and the cells were lysed in TRI REAGENT by repeated pipetting. Restarting the flow, the homogenous lysate was washed out from the column and collected into sterile Eppendorf tubes. The procedure of isolation of total RNA was then

continued according to the TRI REAGENT protocol (phenol/1-bromo-3-chloropropane extraction prior to isopropanol precipitation) and the concentration was calculated from the optical density determined at 260 nm.

Generation of a competitor RNA for AA-NAT

Four primers derived from the rat complete AA-NAT cDNA sequence (Roseboom *et al.* 1996) were designed for the generation of AA-NAT competitor RNA and for quantitative RT-PCR. Primer sequences were as follows: P1-sense primer (212–231): 5'-AGT GAG TTC CGC TGC CTC AC-3'; P2-antisense primer (718–737): 5'-TGG GTC AGC AGC CAC TGT TC-3'; P3-sense 'deletion' primer (GC clamp, T7 promoter, 212–231 and 303–322): 5'-GCG TAA TAC GAC TCA CTA TAG GGA GAG GAG AGT GAG TTC CGC TGC CTC ACT CCA CTT GGA TGA GAT CCG G-3'; P4-antisense primer (784–817): 5'-AGA GAA GGA TGT GAC CTG GGA AGT GGA CAG GAG C-3'.

To prepare a template DNA for AA-NAT competitor RNA a 71-nt deletion was incorporated into AA-NAT cDNA by PCR amplification using P3 and P4 primers. P3 'deletion' primer is 70 nt in length and its sequence includes a GC clamp, a T7 promoter, and two 20 nt sequences corresponding to bases 212–231 (P1 primer) and 303–322 of the rat AA-NAT cDNA. P4 is a conventional PCR primer, 34 nt in length, that corresponds to the opposite strand of the AA-NAT cDNA sequence 47 nt upstream from P2 primer. Thus, the generated competitor DNA template includes primer sites for P1 and P2 primers, a T7 promoter sequence for the subsequent *in vitro* transcription, and a deletion within the region being amplified. This deletion can be used to readily distinguish the competitor PCR product from the amplification product of the endogenous target. The amplification was carried out in a reaction volume of 25 µl containing 5 µl cDNA, a master mixture (0.2 mM of each dNTP, $1 \times$ PCR buffer II, 2 mM MgCl_2 and 0.625 U AmpliTaq DNA Polymerase) (Perkin Elmer, Norwalk, CT, USA) and 0.15 µM of both P3 and P4 primers. The PCR conditions were: 105 s at 95°C , then 28 cycles of 95°C , 15 s; 60°C , 30 s followed by 7 min at 72°C in a GeneAmp PCR System 2400 (Perkin Elmer). Five microliters of this competitor template DNA were

reamplified in a 100- μ l reaction mixture during 33 cycles with the same PCR conditions mentioned above.

DNA templates produced by PCR were purified using the High Pure PCR Product Purification kit (Boehringer Mannheim, Indianapolis, IN, USA) and transcribed into RNA with T7 RNA polymerase according to the manufacturer's protocol for the RT-PCR Competitor Construction kit (Ambion, Austin, TX, USA). Briefly, the reaction mixture of 20 μ l contained 1 $\times$ transcription buffer, 0.5 mM of each dNTP, 0.3 μ g of the PCR-generated DNA template, 40 U T7 RNA polymerase and 3 mM MnCl_2 . The reactions were incubated overnight (16 h) at 37 °C and were stopped by the addition of 40 μ l probe elution buffer prior to ethanol precipitation. The competitor RNA was subsequently treated with 10 U RNase-free DNase I at 37 °C for 1 h to remove the DNA template, and extracted using the TRI REAGENT procedure, then quantitated by absorbance at 260 nm.

Quantitative competitive RT-PCR

In the competitive RT-PCR the AA-NAT competitor RNA was reverse transcribed together with total RNA of pinealocytes containing the AA-NAT target mRNA. Regarding the ratio of competitor RNA and total RNA, different series of RT-PCR were performed to set the conditions of competitive RT-PCR used for quantitation of AA-NAT target mRNA in different experimental samples. (i) Ten-fold dilutions (5 pmol to 5×10^{-4} amol) of the competitor RNA and a constant amount (0.25 μ g) of total RNA (mixture of total RNA derived from stimulated and control pinealocytes) – to obtain an initial estimation of dilution of competitor that yields a similar amount of PCR product as the experimental samples; (ii) approximately equal amounts (2 fmol) of competitor RNA (calculated from the previous RT-PCR) and target RNA – for comparing their amplification efficiencies up to 34 cycles; (iii) twofold dilutions (12 fmol to 11 amol) of target RNA and a constant amount (0.5 fmol) of competitor RNA estimated from step (i) – to have a standard (calibration) curve for quantitative analysis of the changes in AA-NAT target mRNA levels after various treatments in the subsequent RT-PCR reactions; and (iv) a constant amount (0.5 fmol) of AA-NAT competitor RNA

and 0.1 μ g total RNA – for quantitation of AA-NAT target mRNA in different experimental samples.

Each reverse transcription reaction was performed in a final volume of 20 μ l containing different mixtures of target and competitor RNA described above, and 5 mM MgCl_2 , 1 $\times$ PCR buffer II, 1 mM of each dNTP, 1 U RNase inhibitor, 2.5 U murine leukemia virus (MuLV) reverse transcriptase and 2.5 μ M random hexamers (GeneAmp RNA PCR Core kit, Perkin Elmer) in a GeneAmp PCR System 2400 (Perkin Elmer). The samples were incubated at 42 °C for 15 min and the reverse transcriptase was inactivated by heating to 99 °C for 5 min.

The co-amplification of AA-NAT competitor and target cDNA was performed by PCR using P1 and P2 primers as conventional PCR primers both 20 nt in length. To 5 μ l of these cDNA samples a master mixture (0.2 mM of each dNTP, 1 $\times$ PCR buffer II, 2 mM MgCl_2 and 0.625 U AmpliTaq DNA polymerase) (Perkin Elmer) and 0.15 μ M of both P1 and P2 primers were added to bring the final volume to 25 μ l. The PCR conditions were: 105 s at 95 °C, then 28 cycles of 95 °C, 15 s; 60 °C, 30 s followed by 7 min at 72 °C in a GeneAmp PCR System 2400 (Perkin Elmer). The primers amplified a 526-bp PCR product from AA-NAT target cDNA and a 455-bp PCR product from AA-NAT competitor cDNA. This endogenous target PCR product has been reported previously as rnp8 by Coon *et al.* (1995); however, they used ovine primers to generate this product from rat AA-NAT cDNA. Two negative control reactions were also performed in the RT-PCR: a no-template negative control reaction and a no-reverse transcriptase RT reaction to check PCR or genomic and competitor template DNA contamination.

To check the integrity of total RNA isolated from pinealocytes in the superfusion system and to normalize the sample-to-sample variations in the amount of total RNA, rat β -actin was also amplified from 5 μ l of the same cDNA through 25 cycles under the conditions mentioned above. Primer sequences for rat β -actin (sense primer: 5'-GTC ACC CAC ACT GTG CCC ATC T-3', bp 2267–2288; antisense primer: 5'-ACA GAG TAC TTG CGC TCA GGA G-3', bp 2999–3020) amplifying a 542-bp PCR product have been reported previously by Murata *et al.* (1997).

All the PCR products were electrophoresed on a 2% agarose gel (Sigma) and visualized under ultraviolet illumination by ethidium bromide (EtBr) staining followed by scanning and quantification of the gel (Kodak Digital Science Electrophoresis Documentation and Analysis System 120, Eastman Kodak, New Haven, CT, USA). The relative level of competitor RNA/target RNA was adjusted by β -actin [$\times 1/\text{BA}$]. The target and competitor AA-NAT PCR products were purified from the gel by using a NucleoTrap Gel Extraction kit (CLONTECH, Palo Alto, CA, USA), and the sequence of both strands was determined at least three times by cycle sequencing using AmpliTaq DNA polymerase FS with an ABI Prism model 373 fluorescent sequencer (Applied Biosystems, Foster City, CA, USA) with appropriate oligonucleotide primers by the Biological Research Center (Szeged, Hungary).

Statistical analysis

The results of cAMP RIA were analyzed with a computer program developed in our Institute (Csernus & Schally 1991) involving analysis of variance (ANOVA) and Duncan's test. The net integral values (NET INT) of responses exposed to drugs were calculated (expressed as means $\pm$ S.E.M.) 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 released in response to stimulus. The results in the figures are representative values from three to five experiments.

Significance of differences in AA-NAT mRNA levels was analyzed using ANOVA followed by Tukey's test. Results are expressed as means $\pm$ S.E.M. $P < 0.05$ was considered significant.

Results

Validation of the competitive RT-PCR

To determine the appropriate amount of competitor RNA that yields a similar amount of PCR product as the experimental samples, tenfold serial dilutions of competitor RNA were reverse transcribed with a constant amount of total RNA and amplified in PCR (data not shown). Competitive RT-PCR was then performed with this dilution of AA-NAT competitor RNA (2 fmol) and

Figure 1 Kinetics of amplification of the AA-NAT target cDNA and AA-NAT competitor cDNA following RT reaction. Approximately equal molar quantities (2 fmol) of a target mRNA and competitor RNA for AA-NAT were added to a single RT reaction followed by PCR. Aliquots (10% portions of the reaction) were removed after each second cycle for a total of 12 cycles (between cycles 22 and 34) and resolved on a 2.0% EtBr-agarose gel. The intensity of bands was quantitated by a gel documentation and analysis system. The sizes of target (tAA-NAT) and competitor (cAA-NAT) PCR products were 526 and 455 bp respectively (A). The optical density (O.D.) of RT-PCR products was plotted against cycle number (B). Regressions were computed for the exponential phase and the plateau phase after transforming the equation $Y_n = A \times (1+E)^n$ (Chelly *et al.* 1988) (C). L, PCR ladder; solid circles, AA-NAT target PCR product; solid triangles, AA-NAT competitor PCR product.

with an approximately equal molar quantity of the endogenous target RNA to compare their amplification efficiency (Fig. 1). Our results indicate that the target and competitor cDNAs shared very similar amplification kinetics in PCR. There was an exponential increase in the amount of both

Figure 2 Calibration curve for quantitative analysis of AA-NAT target mRNA concentrations. Competitive RT-PCR was performed with twofold serial dilutions of known amount of target mRNA (12 fmol to 11 amol) and a constant amount (0.5 fmol) of competitor RNA. After 28 cycles of amplification, the products were resolved on a 2.0% EtBr-agarose gel. The intensity of bands was quantitated by a gel documentation and analysis system. The sizes of target (tAA-NAT) and competitor (cAA-NAT) PCR products were 526 and 455 bp respectively (A). The ratio of the optical density (O.D.) of competitor to target amplification product was plotted against the reciprocal of the attomolar amount of AA-NAT target mRNA (B). L, PCR ladder; NT, no-template negative control; NR, no reverse transcriptase RT reaction.

products up to 30 cycles followed by the plateau phase.

To have a standard (calibration) curve for quantitative analysis of the changes in AA-NAT target mRNA levels after various treatments, we performed competitive RT-PCR with twofold serial dilutions of known amounts of target RNA (12 fmol to 11 amol) and a constant amount (0.5 fmol) of competitor RNA (Fig. 2). The ratio of the optical density of AA-NAT competitor to that of AA-NAT target was plotted against the reciprocal of the molar amount of AA-NAT target. The amount of AA-NAT target mRNA in different experimental samples was then calculated by extrapo-

lating from the intersection of the curve down to the x -axis with the ratio mentioned above.

The reproducibility of the RT-PCR assay was also tested ($n=5$) with samples containing 20 amol, 0.2 fmol, and 2 fmol target RNA (and 0.5 fmol competitor RNA). The coefficients of inter-assay variation were 17.5%, 11.2% and 13.8% respectively.

Quantitative analysis of changes in AA-NAT mRNA levels and cAMP efflux from the same pinealocytes

Dose- and time-response relationship between NE and AA-NAT gene expression or cAMP efflux

The basal AA-NAT mRNA content of pinealocytes and the cAMP efflux were 18.1 ± 3.3 amol and 17.3 ± 0.3 pg/ml respectively. These are referred to as untreated control values. NE treatment increased AA-NAT mRNA levels and cAMP efflux in a dose- and time-dependent manner (Figs 3 and 4). Treatment with NE (1 nM–1 μ M) for 3 h produced a significant increase in AA-NAT mRNA which was saturated at 100 nM, resulting in an approximately 166.5-fold elevation (3.0 ± 0.3 fmol) (Fig. 3). In contrast, the induction of cAMP efflux could not be saturated at the concentrations tested (Fig. 3B).

The release of cyclic nucleotide started to increase immediately after the beginning of 100 nM NE exposure (within 3 min) reaching the maximal value (28.7 ± 3.3 ng/ml) in the first 15 min and then it started to decline but remained significantly higher than the basal values during the 3-h infusion (17.3 ± 0.3 pg/ml vs 10.3 ± 0.6 ng/ml, $P < 0.001$) (Fig. 4A). In contrast, the NE-induced increase in AA-NAT gene expression was delayed (10 min), then started to increase rapidly (30 min) after cAMP release had started to decline (Fig. 4B,C). The maximal expression (3.1 ± 0.3 fmol), a ≈ 171.4 -fold increase in AA-NAT mRNA levels was achieved only after 1 h of treatment and was maintained during the 3-h infusion of 100 nM NE even with a cAMP level much lower than its peak value.

Dose- and time-response relationship between VIP and AA-NAT gene expression or cAMP efflux

Similarly to NE, VIP stimulated AA-NAT gene expression and cAMP efflux from pinealocytes in a

Figure 3 Dose-dependent effects of NE (1 nM to 1000 nM) on AA-NAT mRNA expression and cAMP efflux. Competitive RT-PCR for AA-NAT mRNA was performed with 0.1 µg total RNA extracted from dispersed pinealocytes at the end of the 3-h treatment with various concentrations of NE. After 28 cycles of amplification, the 526-bp target PCR products (tAA-NAT) and the 455-bp competitor PCR products (cAA-NAT) were resolved on a 2.0% EtBr-agarose gel. The relative level of competitor/target amplification products was adjusted by the 542-bp β-actin [$\times 1/BA$]. RNA preparation and competitive RT-PCR are described in Materials and Methods (A). cAMP efflux into the medium was also monitored during the NE exposures (3 h) by RIA as described in Materials and Methods. The ratio of treated to control values (T/C) was plotted against dose. Data represent the mean $\pm$ S.E.M. cAMP and AA-NAT mRNA levels (B). L, PCR ladder; C, untreated control; * $P < 0.001$ compared with control; + $P < 0.001$ compared with treatment with 10-fold lower concentration of the same compound.

dose- and time-dependent manner, although to a lesser extent (Figs 5 and 6). Infusion of VIP (1 nM–1 µM) for 3 h caused a significant increase in AA-NAT mRNA level with a maximal effect at 10 nM concentration (≈ 25.5 -fold elevation, 461.5 ± 54.3 amol) (Fig. 5). In contrast to NE, the effect of VIP on cAMP efflux was apparently

Figure 4 Time-dependent effects of NE (100 nM) on cAMP efflux and AA-NAT mRNA expression. Kinetics of cAMP efflux into the medium were monitored by RIA during the NE exposure (3 h) as described in Materials and Methods (A). Competitive RT-PCR for AA-NAT mRNA was performed with 0.1 µg total RNA extracted from dispersed pinealocytes at the time-points indicated by arrows (0, 10, 30, 60 and 180 min). After 28 cycles of amplification, the 526-bp target PCR products (tAA-NAT) and the 455-bp competitor PCR products (cAA-NAT) were resolved on a 2.0% EtBr-agarose gel. The relative level of competitor/target amplification products was adjusted by the 542-bp β-actin [$\times 1/BA$]. RNA preparation and competitive RT-PCR are described in Materials and Methods (B). AA-NAT mRNA level after various durations of 100 nM NE exposure. The ratio of treated to control values (T/C) was plotted against time. Data represent the mean $\pm$ S.E.M. AA-NAT mRNA levels (C). L, PCR ladder; NT, no-template negative control; NR, no-reverse transcriptase RT reaction; * $P < 0.001$ compared with control; + $P < 0.001$ compared with AA-NAT mRNA extracted at the preceding time-point.

saturable at 10 nM; however, a further increase was observed at 1 µM VIP (Fig. 5B).

Following a prompt increase in cAMP efflux after the start of the 10 nM VIP exposure, the

Figure 5 Dose-dependent effects of VIP (1 nM to 1000 nM) on AA-NAT mRNA expression and cAMP efflux. Competitive RT-PCR for AA-NAT mRNA was performed with 0.1 µg total RNA extracted from dispersed pinealocytes at the end of the 3-h treatment with various concentrations of VIP. After 28 cycles of amplification, the 526-bp target PCR products (tAA-NAT) and the 455-bp competitor PCR products (cAA-NAT) were resolved on a 2.0% EtBr-agarose gel. The relative level of competitor/target amplification products was adjusted by the 542-bp β-actin [$\times 1/BA$]. RNA preparation and competitive RT-PCR are described in Materials and Methods (A). cAMP efflux into the medium was also monitored during the VIP exposures (3 h) by RIA as described in Materials and Methods. The ratio of treated to control values (T/C) was plotted against dose. Data represent the mean $\pm$ S.E.M. cAMP and AA-NAT mRNA levels (B). L, PCR ladder; C, untreated control; * $P < 0.001$ compared with control; + $P < 0.001$ compared with treatment with 10-fold lower concentration of the same compound.

cyclic nucleotide level culminated at 9 min (857.3 ± 99.2 pg/ml), then it started to decline without returning to basal values during the 3-h infusion (17.3 ± 0.3 pg/ml vs 68.5 ± 0.8 pg/ml, $P < 0.001$) (Fig. 6A). In contrast, VIP-induced AA-NAT gene expression started to be manifested with only a 30-min delay, after cAMP release had

Figure 6 Time-dependent effects of VIP (10 nM) on cAMP efflux and AA-NAT mRNA expression. Kinetics of cAMP efflux into the medium were monitored by RIA during the VIP exposure (3 h) as described in Materials and Methods (A). Competitive RT-PCR for AA-NAT mRNA was performed with 0.1 µg total RNA extracted from dispersed pinealocytes at the time-points indicated by arrows (0, 10, 30, 60 and 180 min). After 28 cycles of amplification, the 526-bp target PCR products (tAA-NAT) and the 455-bp competitor PCR products (cAA-NAT) were resolved on a 2.0% EtBr-agarose gel. The relative level of competitor/target amplification products was adjusted by the 542-bp β-actin [$\times 1/BA$]. RNA preparation and competitive RT-PCR are described in Materials and Methods (B). AA-NAT mRNA level after various durations of 10 nM VIP exposure. The ratio of treated to control values (T/C) was plotted against time. Data represent the mean $\pm$ S.E.M. AA-NAT mRNA levels (C). L, PCR ladder; NT, no-template negative control; NR, no-reverse transcriptase RT reaction; * $P < 0.05$, ** $P < 0.001$ compared with control; + $P < 0.05$ compared with treatment with the same compound for a shorter time; ++ $P < 0.001$ compared with AA-NAT mRNA extracted at the preceding time-point.

started to decline (Fig. 6B,C). The increase in AA-NAT mRNA induced by 10 nM VIP was

saturated at 1 h (481.5 ± 50.7 amol) showing a ≈ 26.6 -fold elevation (Fig. 6).

Effects of other peptides of the secretin superfamily on AA-NAT expression and cAMP efflux

PACAP (1 nM) was found to be approximately six- and threefold more potent than VIP in elevating AA-NAT mRNA expression: 640.7 ± 68.8 amol vs 107.7 ± 16.3 amol, $P < 0.001$ (≈ 35.4 -fold elevation vs ≈ 5.9 -fold elevation related to untreated control) and cAMP efflux: 83.0 ± 8.6 pg/ml vs 27.7 ± 3.5 pg/ml, $P < 0.001$ (≈ 4.8 -fold elevation vs ≈ 1.6 -fold elevation related to untreated control) respectively (Figs 5 and 7). No significant difference was observed in the AA-NAT mRNA levels after treatment with PACAP or VIP at higher concentrations (10 nM): 523.1 ± 52.5 amol vs 461.5 ± 54.3 amol (≈ 28.9 -fold elevation vs ≈ 25.5 -fold elevation related to untreated control). The maximal values of AA-NAT mRNA levels after 3 h of PACAP or VIP exposure were similar (523.1 ± 52.5 amol to 640.7 ± 68.8 amol vs 461.5 ± 54.3 amol to 579.2 ± 72.4 amol, $P > 0.05$), showing approximately 28.9- to 35.4-fold and 25.5- to 32.0-fold elevations in AA-NAT gene expression respectively. The cAMP efflux after 10 nM PACAP infusion was approximately twice as high as after VIP: 185.1 ± 20.8 pg/ml vs 85.2 ± 10.4 pg/ml (≈ 10.7 -fold elevation vs ≈ 4.9 -fold elevation related to untreated control) (Fig. 7B). GRF and secretin were at least 10-fold and 100-fold respectively less effective than VIP (Figs 5B, 7B).

Discussion

In our present *in vitro* study we compared the kinetics of AA-NAT gene expression to that of the cAMP efflux after treatments with NE or various neuropeptides. Our perfusion system (Rekasi *et al.* 1991) applied successfully for studying cAMP efflux and MT secretion from pinealocytes (Rekasi *et al.* 1998) was slightly modified for this purpose. A higher sensitivity for detecting changes in cAMP efflux could be achieved with the replacement of pieces of pineal glands with dispersed pinealocytes. However, the extractable amount of total RNA from dispersed pinealocytes was much less than the RNA extracted from pieces of pineal glands (data not shown). This required the use of RT-PCR for

Figure 7 Effects of PACAP (1 nM and 10 nM), GRF (100 nM) and secretin (100 nM) on AA-NAT mRNA expression and cAMP efflux. Competitive RT-PCR for AA-NAT mRNA was performed with 0.1 µg total RNA extracted from dispersed pinealocytes treated with the indicated concentrations of peptides for 3 h. After 28 cycles of amplification, the 526-bp target PCR products (tAA-NAT) and the 455-bp competitor PCR products (cAA-NAT) were resolved on a 2.0% EtBr-agarose gel. The relative level of competitor/target amplification products was adjusted by the 542 bp β-actin [$\times 1/BA$]. RNA preparation and competitive RT-PCR are described in Materials and Methods (A). Comparison of the dynamics of cAMP productions and AA-NAT mRNA levels in the same pinealocytes exposed to various concentrations of peptides for 3 h. The ratio of treated to control values (T/C) was plotted against dose. Data represent the mean $\pm$ S.E.M. cAMP and AA-NAT mRNA levels (B). L, PCR ladder; C, untreated control; NT, no-template negative control; NR, no-reverse transcriptase RT reaction; * $P < 0.001$ compared with control; + $P < 0.001$ compared with treatment with 10-fold lower concentration of PACAP.

detection of AA-NAT mRNA expressed in low copy number in untreated control pinealocytes.

For the accurate quantification of AA-NAT mRNA molecules with a sufficiently high sensitivity, the development of a reliable competitive RT-PCR was required using internal standard RNA. Therefore, we generated a AA-NAT competitor RNA based on the sequence of rat AA-NAT target

mRNA (Roseboom *et al.* 1996) using a special deletion oligonucleotide primer. Although the competitor PCR product containing the same primer sites of the target cDNA was 13.5% (71 nt) shorter than the target PCR product, the efficiency of amplification of both PCR products was similar not only in the exponential phase but also in the plateau phase (Fig. 1). Thus, the ratio between the target and competitor PCR products remained constant throughout the amplification.

Our *in vitro* studies indicate that the stimulation of AA-NAT mRNA in rat pinealocytes appears primarily to involve a β -adrenergic-cAMP mechanism. The activation of β - and α_1 -adrenergic receptors with NE controls adenylate cyclase activity stimulating cAMP accumulation (Klein 1985, Vanecek *et al.* 1985). In turn, cAMP increases the activity of AA-NAT (Klein 1985) as a result of enhanced transcription via activation of PKA and CREB phosphorylation (Roseboom & Klein 1995, Tamotsu *et al.* 1995, Maronde *et al.* 1999a,b). Our data on the dynamics of cAMP efflux and AA-NAT mRNA levels also support this mechanism of action. Using our superfusion system, NE increased both cAMP efflux and AA-NAT gene expression in a time- and dose-dependent manner. Adrenergic stimulation caused a sharp increase in cAMP efflux which was followed by a long-lasting elevation of AA-NAT mRNA levels similar to the kinetics of MT secretion (Rekasi *et al.* 1998). The increase in cyclic nucleotide level always preceded the increase in AA-NAT gene expression suggesting the essential role of this signal in the process of NE-stimulated induction of AA-NAT gene expression. cAMP efflux increased promptly in the first 3 min (second half of the first fraction due to a 3 min/1 ml dead volume of the system) while AA-NAT mRNA levels started to increase only between 10 and 30 min, reaching the peak value after a relatively long delay (1 h). The maximal gene expression was then maintained during the 3-h infusion of 100 nM NE even with a cAMP level much lower than its peak value. The declining level of cAMP efflux appears to be sufficient for the long-term accumulation of AA-NAT mRNA. A similar long-lasting increase in the production of an inhibitory transcription factor of AA-NAT gene (inducible cyclic AMP early repressor, ICER) has been found after the start of NE stimulation in rat pinealocytes (Pfeffer *et al.* 1999). However, ICER protein levels peak only several hours after the

peak of AA-NAT mRNA accumulation (8 h vs 1 h). The accumulating ICER protein displaces phosphorylated CREB, an activating transcription factor from the AA-NAT-CRE, causing the cessation of AA-NAT transcription (Pfeffer *et al.* 1999).

Regarding the dose-response relationship between NE and AA-NAT gene expression, AA-NAT mRNA levels peaked at approximately 100 nM NE, similar to an earlier report (Roseboom *et al.* 1996). In contrast, cAMP efflux could not be saturated at the concentrations tested, indicating an excess of this signal molecule in the accumulation of AA-NAT mRNA; however, a relatively low amount of cAMP seemed to be sufficient to induce maximal AA-NAT gene expression. These findings are consistent with the observation that sub-maximal increases in cAMP accumulation are sufficient to produce maximal phosphorylation of CREB (Roseboom & Klein 1995).

In addition to the adrenergic regulation of MT secretion, an increasing number of peptidergic neurons have been reported to play a modulating role in pineal function (Simonneaux 1995, Yuwiler *et al.* 1995, Moller *et al.* 1996, Rekasi *et al.* 1998, Pfeffer *et al.* 1999). Among the peptides of the secretin superfamily, VIP and PACAP proved to be the most potent in stimulating MT synthesis and release (Simonneaux *et al.* 1990, 1993, Rekasi *et al.* 1994, 1998, Pfeffer *et al.* 1999).

However, little was known about the accumulation of AA-NAT mRNA induced by these peptides. VIP was chosen for testing the time- and dose-response relationship between a peptide and AA-NAT gene expression or cAMP efflux in detail, because in a preliminary study VIP had been shown to stimulate AA-NAT gene expression (Roseboom *et al.* 1996).

In this study, we demonstrated that PACAP was the most potent peptide to increase the expression of AA-NAT mRNA and to induce cAMP production; however, it was less effective than NE. VIP was also able to elevate AA-NAT mRNA levels and cAMP efflux in a dose-dependent manner; however, it was sixfold and threefold, respectively, less potent than PACAP. The increase in cAMP efflux induced by VIP always preceded the elevation of AA-NAT expression similar to the kinetics of NE-induced responses. These data support the fundamental role of this intracellular messenger molecule in the action of VIP-regulated

AA-NAT gene expression. GRF and secretin were less potent than VIP in inducing cAMP efflux and AA-NAT gene expression. The order of potencies of the peptides was as follows: PACAP $\geq$ VIP $\gg$ GRF $\geq$ secretin. The maximal values of AA-NAT mRNA levels after PACAP and VIP exposures were similar (523.1 ± 52.5 amol to 640.7 ± 68.8 amol vs 461.5 ± 54.3 amol to 579.2 ± 72.4 amol). These data suggest that the peptides act mostly on VPAC₁ receptors of pinealocytes with different affinity which is consistent with other published data (Vertongen *et al.* 1997, Simonneaux *et al.* 1998). The fact, however, that PACAP exerts the strongest effect on AA-NAT gene expression indicates the possible role of PAC₁ receptors in pineal function which has also been proposed previously (Masuo *et al.* 1992, Olcese *et al.* 1996).

The saturable peak values of AA-NAT gene expression after PACAP and VIP infusions were approximately five- to eightfold smaller than that of NE (≈ 0.46 to 0.64 fmol vs ≈ 3.0 to 3.6 fmol). Similarly, the peak values in ICER levels observed for VIP and PACAP were less than half-maximum of the ICER increase after NE stimulation (Pfeffer *et al.* 1999). This might be explained by the finding that only 30–60% of the pinealocytes respond to VIP or PACAP whereas 95% of the pinealocytes respond to NE with CREB phosphorylation (Schomerus *et al.* 1996). VIP (10 nM) resulted in a significantly lower elevation in AA-NAT gene expression than NE at the same concentration (≈ 25.5 -fold vs ≈ 111.5 -fold elevation), whereas the increase in cAMP efflux was similar (≈ 4.9 -fold vs ≈ 4.3 -fold increase) (Figs 3B, 5B). These data suggest the possible involvement of signaling system(s) other than cAMP in the NE-regulated AA-NAT gene expression. NE-stimulated α_1 -adrenergic receptor regulation of AA-NAT activity has been shown to involve an increase in intracellular Ca^{2+} (Sugden *et al.* 1986, Yu *et al.* 1993) and protein kinase C activity (Sugden *et al.* 1985). However, their role in the regulation of mRNA and protein encoding AA-NAT has not been established (Klein *et al.* 1997).

The submaximal activation of the cAMP-signaling pathway by VIP or PACAP allows for an α -adrenergic potentiation of cAMP accumulation (Chik *et al.* 1988, Chik & Ho 1995), CREB phosphorylation (Pfeffer *et al.* 1999), AA-NAT enzymatic activity (Yuwiler 1983, Yuwiler *et al.* 1995), MT

synthesis (Simonneaux *et al.* 1993, Chik & Ho 1995, Yuwiler *et al.* 1995) and ICER protein levels (Pfeffer *et al.* 1999). However, the α -adrenergic potentiation of AA-NAT mRNA has to be elucidated.

A moderate increase in AA-NAT mRNA has been observed after the activation of the α_1 -adrenergic receptors by themselves (Roseboom *et al.* 1996); however, previous studies have shown that activation of the α_1 -adrenergic receptor by itself does not result in CREB phosphorylation (Roseboom & Klein 1995). This indicates that AA-NAT mRNA expression might also be controlled by a mechanism other than PKA-dependent phosphorylation of CREB.

In this paper, we conclude that, in addition to NE, PACAP and VIP are able to activate AA-NAT gene expression in rat pinealocytes. Therefore, these peptides may have an important role in the regulation of AA-NAT activity and MT secretion, and consequently, in the control of biological rhythms. The combination of the superfusion system and competitive RT-PCR assay reported here could be a useful tool for the simultaneous study of different gene expressions, signaling systems and hormonal secretions in other endocrine tissues.

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