cAMP Regulation of Arylalkylamine N-Acetyltransferase (AANAT, EC 2.3.1.87)

A NEW CELL LINE (1E7) PROVIDES EVIDENCE OF INTRACELLULAR AANAT ACTIVATION*

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Arylalkylamine N-acetyltransferase (serotonin N-acetyltransferase, AANAT, EC 2.3.1.87) is the penultimate enzyme in melatonin synthesis. As described here, a cell line (1E7) expressing human AANAT (hAANAT) has been developed to study the human enzyme. 1E7 hAANAT is detectable in immunoblots as a 23-kDa band and is immunocytochemically visualized in the cytoplasm. The specific concentration of hAANAT in homogenates is comparable to that of the night rat pineal gland. Kinetics of AANAT extracted from 1E7 cells are the same as those of bacterially expressed hAANAT; both preparations of hAANAT are equally sensitive to the inhibitor CoA-S-acetyltransferase; and, that forskolin, isobutylmethylxanthine, or isoproterenol elevate cellular hAANAT within intact 1E7 cells ~8-fold without markedly increasing the abundance of AANAT protein or the activity of AANAT in broken cell preparations; and, that forskolin, isobutylmethylxanthine and isoproterenol elevate cyclic AMP production. These observations extend our understanding of cAMP regulation of AANAT activity, because it is currently thought that this only involves changes in the steady-state levels of AANAT protein. This previously unrecognized switching mechanism could function physiologically to control melatonin production without changing AANAT protein levels.

Melatonin has important endocrine and paracrine roles in vertebrate physiology (1). It is synthesized in the pineal gland and acts as “the hormone of the night.” Hormonal melatonin has been implicated in slow and circadian clock function in vertebrates in general, and melatonin has been used as a drug to reduce jet lag. In many species it plays an essential role in seasonal reproduction, because seasonal changes in the length of the night are transduced as changes in the duration of the melatonin signal. Retinal melatonin is thought to play a paracrine role in some species by mediating dark-adaptive retinal processes (2).

Melatonin is a highly lipophilic compound and is not stored to a significant degree at the sites of synthesis. Hence, the release and circulating levels of melatonin reflect the rate at which melatonin is synthesized (serotonin (5-HT) → N-acetylsertotonin (NAS) → melatonin (5-methoxy-N-acetyltryptamine)). The mechanisms that regulate dynamic changes in melatonin synthesis are thought to converge on the penultimate enzyme in melatonin synthesis, arylalkylamine N-acetyltransferase (serotonin N-acetyltransferase, AANAT, EC 2.3.1.87). Accordingly, this enzyme plays a unique dual regulatory/synthetic role in vertebrate photochemical transduction (3, 4).

Large changes in the abundance of this enzyme, as indicated by measurements of activity in homogenates and immunoreactive protein, are thought to be responsible for the large day/night rhythms in melatonin synthesis in the pineal gland and retina; and, for the rapid light-induced suppression of melatonin production at night (1-4).

AANAT regulatory mechanisms operate at several levels. One is transcription: in some, but not all, species, changes in AANAT transcription occur on a circadian basis. This is most evident in rodents, in which the night/day difference in pineal AANAT mRNA is >100-fold (5). As a result, AANAT synthesis is essentially limited to the period of the night when AANAT mRNA is elevated. A similar, albeit less dynamic, situation exists in the chicken and pike (6, 7), in which the night/day difference in AANAT mRNA is smaller because of higher day time levels. In ungulates, however, AANAT mRNA is continually elevated and does not exhibit a marked night/day difference, enabling AANAT protein to be synthesized continuously (8, 9).

A second regulatory mechanism involves cAMP control of AANAT protein through inhibition of proteasomal proteolysis (10). This appears to be important in all species and explains the increase in AANAT protein seen in ungulate pinealocyes upon noradrenergic stimulation and the disappearance of AANAT protein that occurs when animals or photosensitive

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1 The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); 5-MT, 5-methoxytryptamine; AANAT, arylalkylamine N-acetyltransferase; DBcAMP, dibutylryl cAMP; FSK, forskolin; IBMX, isobutylmethylxanthine; ISO, isoproterenol; h, human; o, ovine; r, rat; ORF, open reading frame; RIA, radioimmunoassay; NAS, N-acetylserotonin; HTOH, hydroxytryptophol; HIAA, hydroxyindole acetic acid; MTOH, methoxytryptophol; MIAA, methoxyindole acetic acid; PBS, phosphate-buffered saline; DTT, dithiothreitol; CoA, coenzyme A; AcCoA, acetyl CoA; PKA, protein kinase A; BSA, bovine serum albumin; CAPS, 3-cychoexyaminol-1-propanesulfonic acid; ICC, immunocytochemistry; BSI, bisubstrate inhibitor.
pinealocytes are exposed to light during the middle of the night (9–12). These two mechanisms contribute to steady-state levels of AANAT protein; it is now generally thought that a change in the steady-state levels of AANAT protein is the primary factor regulating changes in AANAT activity and melatonin production.

Although most data are consistent with the view that AANAT activity is regulated by controlling the steady-state levels of AANAT protein, limited observations in the literature point to the existence of another regulatory mechanism through which melatonin production might change without altering AANAT protein and AANAT activity as estimated in broken cell preparations (13–15). This hypothetical activation/inactivation mechanism is of special interest, because it may explain the very rapid physiological increase in circulating melatonin seen at the onset of the dark period in humans as well as other species (1, 16–18).

Analysis of the regulation of the human form of AANAT (hAANAT) has been difficult for several reasons. In the case of the pineal gland and retina, tissue availability is limited; in the case of the human retinoblastoma-derived Y79 cell line, AANAT expression is very low (19). In addition, analysis of the regulation of the intracellular activity of AANAT in tissue or primary cell preparations from any species is confounded, because the rate of production of acetylated products, either NAS or melatonin, may reflect changes in the synthesis of serotonin (tryptophan → hydroxytryptophan → 5-HT) or in factors involved in the conversion of 5-HT to melatonin other than AANAT.

To overcome these limitations, we established a clonal hAANAT-expressing cell line (1E7) derived from COS7 cells, as described in this report. Expression is comparable to that in homogenates of rat pineal glands obtained at night. It was possible to use this system to monitor the activity of AANAT in intact cells, by providing cells with 5-methoxytryptamine, which was converted by AANAT to melatonin. The highly lipophilic nature of melatonin resulted in it being released, providing an estimate of the intracellular activity of AANAT.

Using this approach, it was found that AANAT can be activated in the intact cell without significant changes in AANAT protein or broken cell activity. This advance may have important physiological implications.

**EXPERIMENTAL PROCEDURES**

**Cells and Animals**

COS7 cells were obtained from T. Kim, National Institutes of Health, Bethesda, MD. Cells were maintained at 37 °C, 95% air, 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD). Other details are provided in the figure legends.

Sheep pineal glands were obtained from male Ile de France lambs (12–16 weeks old) that had been housed for 2 weeks in a light:dark 14:10 lighting cycle for 2 weeks prior to killing. Animals were killed by exposure to CO₂, and the pineal glands were immediately (4 °C, 16,000 × g) centrifuged immediately (5 min, 4 °C, 16,000 × g), and the supernatant fraction was assayed for AANAT activity as described below.

**Bacterial Expression and Purification of Human and Ovine AANAT**

Cloning and expression of the recombinant hAANAT and ovine AANAT (oAANAT) in bacteria were done essentially as described previously (20), with modifications detailed here. After centrifugation, bacterial cells from 4 liters of culture were suspended in 100 ml of lysis buffer (100 mM Pbs with 10 mM DTT) and stored at –80 °C. After thawing, cells were lysed by sonication at 4 °C in the presence of Complete™ protease inhibitor mixture, and supernatant was prepared by centrifugation at 48,400 × g for 45 min. Twenty milliliters of glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ), previously equilibrated in the lysis buffer, was added to the supernatant, and the slurry was mixed at 4 °C for 2 h. The resin was then poured into a 100-ml column (Bio-Rad, Hercules, CA) and washed with 10 column volumes of the lysis buffer followed by 10 column volumes of Tris-HCl (50 mM, pH 8.0) containing 0.1 mM sodium citrate, 10 mM DTT, and 10% glycerol (Life Technologies). The protein was eluted using 50 ml of 10 mM glutathione (Sigma) dissolved in the Tris buffer used for washing. The eluate was concentrated to about 10 ml and dialyzed against 20 mM Tris-HCl, pH 8.0, containing 0.1 mM sodium citrate, 2 mM DTT, and 10% glycerol.

The dialyzed preparation was incubated with thrombin (1 unit/ml, Roche Molecular Biochemicals) at 4 °C for 12 h. The digested sample was mixed with a suspension of glutathione-Sepharose and benzamidine-Sepharose (Amersham Pharmacia Biotech) and incubated for 2 h with gentle mixing. The gel/enzyme mixture was poured into an empty column of aANAT, the concentrated sample was further purified by fractionation on a size-exclusion column (TSK 3000, Tosoh, Japan) equilibrated with the buffer used for dialysis. The fractions containing the enzyme activity were pooled and concentrated. Both enzyme preparations were stored in 0.05-ml aliquots at –80 °C. SDS-polyacrylamide gel electrophoresis analysis (see below) revealed that the oAANAT preparation was homogenous and the hAANAT preparation was a mixture of free hAANAT and of a contaminating chaperonin (~90%). The specific activity based on total protein of three independent preparations of oAANAT and hAANAT were 0.81 ± 0.2 (n = 5) and 2.7 ± 0.7 (n = 5) mmol/mg of protein/h, respectively, as measured with 10 mM tryptamine and 1 mM CoA.

**Production of the 1E7 Cell Line**

An hAANAT CDNA clone, derived from a gt11 library (21), was cut with MscI and PvuII to release the open reading frame (ORF). This fragment was subcloned into the Smal site of pGEX-4T-3 (Invitrogen, Carlsbad, CA). The ORF was then shuttled into the mammalian expression vector pCDNA3.1 (Invitrogen) by excising and ligating into EcoRI and XhoI sites, so that it would be constitutively expressed under...
the control of the cytomembrane grammar. The resulting clone was termed HNATN3.

For development of a stable cell line expressing hAANAT, clone HNATN3 was transfected into COS7 cells using LipofectAMINE (Life Technologies) following the manufacturer’s protocol. After 48 h, the cells were trypsinized, collected, and diluted into a 3-mm-diameter cloning medium with 600 µg/ml G418 (MediTech, Herndon, VA). After 10 days, isolated colonies were selected using 3-mm-diameter cloning discs (Labcor, Fredrick, MD) saturated with trypsin. After another 10 days, the largest colonies were tested for their capacity to convert exogenous 5-methoxytryptamine (5-MT) to melatonin, as described in detail. Colony NCS30 had the highest rate of melatonin production and was used as a source of cells for producing the clonal cell line. This was done by serial dilution into 96-well plates (22). Clonal lines were assessed for growth characteristics, for conversion of exogenous 5-MT to melatonin by intact cells, and for AANAT activity in broken cell homogenates, as detailed below. 1E7 was one of several clonal lines selected this way.

**Immunocytochemical Detection of AANAT**

Cells for ICC were grown on two-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) and were either treated with FSK for 3 h prior to the end of culture or left untreated. They were fixed with 2.5% glutaraldehyde (10 min, room temperature) and then washed with phosphate-buffered saline (PBS). Immunocytochemical detection was done using the ABC method and 3,3’-diaminobenzidine as a chromogen (for details, see Ref. 9). The primary antiserum (3236, anti-hAANAT; bleeding: 8/24/98) was immunopurified as described below. In addition, the immunopurified antisemir was pre-absorbed for 6 h at room temperature by incubation with COS7 cells (200,000 cells) that were fixed with 2.5% glutaraldehyde and washed twice in PBS; the resulting preparation was used for ICC analysis at a dilution of 1:200. Photographs were taken using a Vidas 2.1 (Kontron) on a Zeiss photomicroscope II or a Zeiss LSM 510.

**Analysis of Intact Cell (Cellular) AANAT Activity**

Intact cell AANAT activitya was estimated from the conversion of 5-MT to melatonin (N-acetyl-5-MT) and from the conversion of [3H]5-HT to [3H]5-NAS; details follow.

**Cellular Conversion of 5-MT to Melatonin—Intracellular AANAT activity in intact cells was assayed by determining the capacity of cells to produce melatonin from exogenously supplied 5-MT (0.1 mM, Research Biochemicals International, Natick, MA). 5-MT added to the medium is taken up by cells and converted to melatonin by acetylation; melatonin is highly lipophilic and diffuses through the cell membrane out of the cell. Samples of medium were removed at selected times and frozen at -80 °C until assayed.

In experiments we determined that AANAT was not secreted and that 5-MT was not acetylated in the medium (data not presented). This was done by determining whether 5-MT was acetylated by cell-free conditioned medium obtained from 3-h control or FSK-treated cultures; and, from 24-h control cultures. In some experiments FSK was added to the conditioned medium but did not result in detectable acetylation of 5-MT. This is consistent with the absence of acetyl CoA (AcCoA) from the culture medium and requirement of AcCoA for arylalkylamine acetylation. It was also found that AANAT activity was not detectable in conditioned culture media using a broken cell assay containing saturating concentrations of [3H]AcCoA and tryptamine (see below), which indicates that AANAT is not secreted in this system. These findings established that 5-MT is not being acetylated in the culture medium; rather, it appears that it is taken up by cells and converted to melatonin by acetylation and that the resulting melatonin diffuses through the cell membrane into the medium, where it accumulates.

Melatonin in the culture medium was assayed by radioimmunoassay (RIA (18)). Radiolabeled ligand was prepared by adding 10 µl of a dioxane solution containing 1 µmol of 5-MT and 1 µmol of tri-n-butylammonium to 250 µCi of (0.1 mM) drierol[125I]Bolton-Hunter Reagent (PerkinElmer Life Sciences). The reaction was allowed to proceed for 1 h before purifying the product by disc gel electrophoresis. Duplicate aliquots of 2 µl of each sample and a 2-fold serial dilution of standard melatonin (RBI) concentrations (1–500 pg/2 µl) was incubated with 50,000 cpm of radiolabeled ligand and a 1,250,000 dilution of antisemir (R1055, bleed: 9/6/74) in 400 µl of PBS, pH 7.4, containing 0.1% gelatin. At the end of the 48-h incubation period, a 3-mM solution of ethanol (4 °C, 95%) was added to each assay tube, and the bound radioactivity precipitated by centrifugation (30 min, 2000 × g). The supernatant was decanted, and the radioactivity in the precipitate was determined. The amount of immunoreactive melatonin in the samples was calculated by computer (23). All solutions were maintained at 4 °C throughout the RIA procedure. In the course of these experiments it was determined that chloroform extraction of samples increased the background attributable to 5-MT; therefore, the samples were not extracted. In the absence of extraction, the background immunoreactivity due to 5-MT was equivalent to 3 pmol/m1 untreated 1E7 cells converted 5-MT to melatonin at a rate of 300 pmol/106 cells/h.

**Conversion of [3H]5-HT to [3H]NAS**—Cells were incubated with 0.1 mM [3H]5-HT (specific activity (S.A.) 84 Ci/mmol, Amersham Pharmacia Biotech). Following incubation, 40 µl of the culture medium was combined with 10 µl of ethanol containing 10 mM each of melatonin, 5-MT, methoxytryptophol (MTOH, Regis, Chicago, IL), and hydroxytryptophol (HTOH, Regis Chemical Co., Chicago, IL), and hydroxyindole acetic acid (HIAA, Calbiochem). A 20-µl sample was transferred to an aluminum foil-backed silica gel thin layer chromatography sheet (Whatman No. 420 222, Maidstone, Kent, United Kingdom) and dried under N2. The chromatogram was developed twice in the first dimension (chloroform:methanol:glacial acetic acid, 9:3:1, v/v) and once in the second dimension (ethyl acetate). The carriers were located by fluorescent quenching using ultraviolet light (300 nm) and marked; the region of interest was cut out and transferred to a scintillation vial. Compounds were extracted by agitation with 0.5 ml of either ethanol (NAS, HTOH, and HIAA) or water (5-HT). Radioactivity was measured by liquid scintillation.

**Analysis of Melatonin Stability**

The stability of melatonin was studied in two ways. Melatonin stability in the medium was estimated by incubating labeled melatonin with cells. COS7 or 1E7 cells (106 cells) were incubated (4 or 24 h) in a 96-well plate with 50 µl of culture medium containing 100 nM [3H]melatonin (S.A. = 84 Ci/mmol, Amersham Pharmacia Biotech). Following incubation, 40 µl of the culture medium was combined with 10 µl of ethanol containing 10 mM each of melatonin, 5-MT, methoxytryptophol (MTOH, Regis), and methoxyindole acetic acid (MIAA, Regis). A sample (25 µl) of this ethanol solution was applied to an aluminum foil-backed silica gel TLC sheet (Whatman #420 222, dried under heat and separated by one-dimensional TLC using chloroform: methanol:glacial acetic acid, 97:3:1 (24, 25). Regions containing melatonin, MTOH, MIAA, and 5-MT were identified by fluorescent quenching by the carriers. Each sample lane was cut transversely into 5-mm sections from the origin to the solvent front (16.5 cm), each section was transferred to a scintillation vial, and compounds were extracted into 0.5 ml of ethanol; scintillation fluid was then added and radioactivity was determined. Similar experiments were performed with 1 µM (S.A. = 10 Ci/mmol) and 10 µM (S.A. = 1.0 Ci/mmol) of [3H]melatonin.

The second examination of melatonin metabolism was designed to detect deacetylation of melatonin in lysates. Cell lysates were incubated with [3H]melatonin (26). Trout retinas were used as a positive control for deacetylase activity (26, 27). Homogenates of 1E7 and COS7 cells and retinas were prepared in 50 mM sodium phosphate (pH 6.5) containing 100 µM ascorbate and 0.5% Triton X-100. [3H]Melatonin was added to 100 mM final concentration, and the reactions were incubated at 37 °C for 45 min. The reactions were stopped by addition of ethanol (final concentration = 20%) containing 10 mM each of melatonin, 5-MT, MTOH, and MIAA. The samples were analyzed by one-dimensional TLC as described above.

**Analysis of hAANAT Activity in Broken Cell Homogenates and Enzyme Preparations**

**Standard Assay**—Unless otherwise indicated, a 10-µl sample of homogenate or extract was incubated (20 min, 37 °C) in 0.1 M sodium phosphate, pH 6.8 (final volume = 40 µl), containing tryptamine-HCI (final concentration = 10 mM, Research Biochemicals International) and [3H]AcCoA (S.A. = 4 mol/Ci/mmol, PerkinElmer Life Sciences, final concentration = 0.5 mM); the specific activity of [3H]AcCoA was adjusted by addition of unlabeled AcCoA (Calbiochem). The reaction was a
ended by addition of 1 ml of chloroform. N-[3H]Acetyltryptamine was extracted into chloroform, which was then washed (2×) with 0.2 ml of 0.1 M sodium phosphate, pH 6.8. A 0.5-ml sample of chloroform was taken to dryness, and the radioactivity was determined.

**Kinetic Analysis**—To determine the *Km* for arylalkylamine substrates, enzyme activity was determined using a radiochemical method similar to that used above. Samples of the enzyme preparations in buffer containing 1 mg/ml bovine serum albumin (BSA, Sigma) were incubated (100 or 50 μl, 15 min, 30 °C) with a range of concentrations (7-27) of the arylalkylamine substrate and 1 μM [3H]AcCoA (S.A. = 2 M Ci/mmol). Appropriate enzyme concentrations were chosen so that less than 10% of the substrate was consumed. Enzyme activity was estimated by determining the amount of *H*-acetylated product formed. The N-[3H]-acetylated product was extracted into chloroform in the case of tryptamine-HCl and phenylethylamine-HCl (Sigma); in the case of 5-HT and tyramine-HCl (Sigma), the labeled products were resolved by TLC (24) because NAS and N-acetytyramine are only partially soluble in chloroform. The *Km* values were determined using nonlinear regression analysis (Kaleidagraph, Synergy Software, Reading, PA), and the Lineweaver-Burk method of *Km* determination (1/V versus 1/S) yielded similar *Km* values (28).

**cAMP Measurement**

cAMP in media samples was measured using an RIA (Covance Laboratories, Vienna, VA).

**Protein Measurement**

Protein was measured by a dye binding method using a commercial reagent (Bio-Rad Protein Assay) with BSA as the standard.

**SDS-Polyacrylamide Electrophoresis**

Proteins were resolved on pre-formed 14% Tris/glycine (1 mm) gels using the manufacturer’s protocol (Novex, San Diego, CA (29)). Rainbow standards (Amersham Pharmacia Biotech) were used to determine the molecular mass of the proteins. Gels were either stained for proteins (Coomassie Blue) or used for Western blot, as described below.

**Western Blot Analysis of hAANAT**

**Electrobotts**—The proteins were electroblotted onto an Immobilon-P (0.45 μm) transfer membrane in a semi-dry blotting system (Investigator Graphite Electroblotter System, Genomic Solutions, Chelmsford, MA) according to the procedure described in the manufacturer’s protocol. The proteins were equilibrated (5 min) and transferred using 10 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer, pH 11, containing 20% methanol and 0.1% SDS. The conditions for electrical transfer were 400 μA/cm² (20 min), 600 μA/cm² (20 min), 800 μA/cm² (20 min), followed by 1200 μA/cm² (45 min).

**Immunodetection**—Membranes were air-dried and then blocked for 2 h (100 μl HBT containing 10% non-fat dry milk (Bio-Rad), 0.2% Tween-20 (Bio-Rad), and 0.05% thimerosal (Sigma)). The primary antibody (antisemur 3236, an anti-hAANAT1–26 serum or antiserum 2500, an anti-phosphorylated rAANAT23-27 serum; see “Experimental Procedures” below for details) was prepared in PBS containing 1 mg/ml BSA fraction V and 0.05% thimerosal. The membranes were incubated (18 h, room temperature) with either immunopurified antiserum, 3236 (1:125), or antiserum 2500 (1:25,000) and washed (2 × 5 min) in TBPS (PBS containing 0.05% Tween-20) followed by 2 × 5 min in PBS. The washed membranes were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase (0.0090 μg/ml, 1 h, room temperature, Kirkegaard and Perry, Gaithersburg, MD) prepared in TBPS containing 0.05% thimerosal and 2.5 μl/ml normal goat serum (Pierce). The membranes were then washed 3 × 10 min in TBPS followed by 3 × 5 min washes in PBS. The secondary antibody was detected by enhanced chemiluminescence using Lumiglo (Kirkegaard and Perry). The blots were exposed to BIO-MAX MR or X-Omat AR film (Eastman Kodak Company, Rochester, NY).

**Northern Blot Analysis of hAANAT mRNA**

Total RNA was extracted using the guanidine-HCl/phenol procedure (TRizol Reagent, Life Technologies). RNA was separated on a 1.5% agarose/0.7 M formaldehyde gel, transferred to a charged nylon membrane (Nytran, Schleicher and Schuell, Keene, NH) by passive capillary transfer and cross-linked to the membrane using ultraviolet light. The hybridization probe consisted of the hAANAT ORF and was 32P-labeled by random priming. Blots were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase to ensure the quality of the RNA and equal loading. Blots were hybridized at 68 °C for 1.5 h in QuikHyb buffer (Stratagene, La Jolla, CA). The final wash was in 0.1× SSC/0.1% SDS at 60 °C for 20 min. Blots were visualized and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Materials**

In cases where the sources of materials used are not given, the purest grade available was commercially obtained. The bisubstrate inhibitor CoA-S-N'-acetyltryptamine was obtained from Research Biochemicals International (Natick, MA) through the National Institute of Mental Health’s Drug Synthesis Program.

**Preparation of Antiserum**

Antiserum 3236 and 2500 were raised in rabbits (Covance Laboratories) against synthetic peptides (Notre Dame Biosciences Core Facility, Notre Dame, IN). Antiserum 3236 was raised against a human peptide hAANAT1–26 (MSTQSTHLPKEAPRLFPFGEPSPC; GenBank accession number U40347) conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce). This antiserum was immunopurified using sections of membrane containing synthetic peptide hAANAT1–26. Immunopurified antibodies were eluted with 0.1 M acetic acid, pH 2.65, containing 0.1% BSA (30). When used for immunocytochemistry, this preparation was pre-absorbed by incubation (6 h, room temperature) with 2.5% glutaraldehyde-fixed COS7 cells.

Antiserum 2500 was raised against a rat peptide rAANAT23–27 (LGCQRHRHLPASEFRC; GenBank accession number U38306) that was phosphorylated using the catalytic subunit of cAMP-dependent protein kinase as described below and conjugated to cationized BSA (Pierce). The conserved PKA site (Thr-29) of peptide rAANAT23–27, was phosphorylated in 20 mM sodium citrate, pH 7.2, containing 2 mM peptide rAANAT23–27, 15 mM MgCl₂, 2 mM ATP, 3.8 mM DTT, and 250 units of the catalytic subunit of PKA (Sigma) in a reaction volume of 1 ml. The reaction was incubated at 30 °C for 5 h; another 250 units of the catalytic subunit of PKA was added, and the reaction was incubated for an additional 12 h. After stopping the reaction by boiling 5 min, the phosphorylated peptide was conjugated to cationized BSA (Pierce) and desalted using Fresto Desalting Columns (Pierce). In preliminary studies it was determined that antiserum 3236 (bleed: 8/24/98) and 2500 (bleed: 4/7/97) immuno detected a 23-KDa band in 1E7 cells and human pineal glands, corresponding to bacterially expressed hAANAT. Antiserum 2500 does not detect the unphosphorylated parent peptide, unphosphorylated bacterially expressed hAANAT, unphosphorylated bacterially expressed rAANAT, or the phosphorylated C-terminal PKA site.3

**Statistics**

Data are presented as means of replicates within an experiment; error bars represent the standard error of the mean. Statistical significance is based on the results of the Student’s t-test.

**RESULTS**

**1E7 Cells Stably Express hAANAT**—Clonal selection was done using intact cells and was based on detecting the uptake and acetylation of 0.1 mM 5-MT in the culture medium. The acetylated product, melatonin (N-acetyl-5-MT) was measured by radioimmunoassay. 5-MT was acetylated at relatively low levels by untransformed COS7 cells (Fig. 1), presumably reflecting the action of arylamine N-acetyltransferase (EC 2.3.1.5), a broadly distributed enzyme known to acetylate 5-MT (31). Cellular acetylation of 5-MT was >25-fold higher in colony NC30, and in clonal cell line 1E7 (Fig. 1A), which was selected from the NC30 colony.

The intracellular distribution of AANAT protein in 1E7 cells was examined using a pre-absorbed preparation of immunopurified antiserum 3236 that selectively identifies hAANAT1–26. This generated an intense staining in 1E7 cells; there was no difference in the labeling intensities between untreated and FSK-treated 1E7 cells. Untransfected COS7 cells were not labeled whether they were stimulated with FSK or not. The immunoreactivity had a granular appearance and was concen-
measured by a radiochemical procedure. Data are means during a 6-h period. AANAT activity in broken cell preparations was estimated by measurement (RIA) of the conversion of 5-MT to melatonin in the medium (200,000/well, 2.0 ml of medium). Intact cell AANAT activity was estimated by measurement (RIA) of the conversion of 5-MT to melatonin in the medium (200,000/well, 2.0 ml of medium). Intact cell AANAT activity was estimated by measurement (RIA) of the conversion of 5-MT to melatonin in the medium (200,000/well, 2.0 ml of medium). AANAT activity in broken cell preparations was measured by a radiochemical procedure. Data are means ± S.E. (n = 3). The results were confirmed in >5 independent experiments. For details see “Experimental Procedures.”

**Fig. 1.** AANAT in COS7 and 1E7 cells detected by intact cell assay, broken cell assay, and immunocytochemistry (ICC). COS7 and 1E7 cells were cultured as described under “Experimental Procedures.” A, AANAT activity estimated by intact cell and broken cell assays. Cells were incubated in 6-well culture plates (200,000/well, 2.0 ml of medium). Intact cell AANAT activity was estimated by measurement (RIA) of the conversion of 5-MT to melatonin in the medium during a 6-h period. AANAT activity in broken cell preparations was measured by a radiochemical procedure. Data are means ± S.E. (n = 3). The results were confirmed in >5 independent experiments. For details see “Experimental Procedures.” B, ICC detection of AANAT. Cells were grown on two-well chamber slides and fixed with 2.5% glutaraldehyde; ICC was done using a pre-absorbed immunopurified serum (3236). The granular appearance of the AANAT immunoreactivity (Fig. 1B) is consistent with a perinuclear localization of AANAT in both COS7 and 1E7 cells. The perinuclear localization of AANAT may reflect its role in mediating the release of melatonin from the pineal gland.

**Fig. 2.** Effects of forskolin on cellular acetylation of exogenous [14C]serotonin by 1E7 cells. Cells (50,000/well) were incubated in 24-well culture plates with and without 0.01 mM forskolin (FSK) in 0.3 ml of medium containing 0.1 mM [14C]serotonin for 24 h. [14C]Serotonin oxidation products [14C]hydroxyindole acetic acid (HIAA) and [14C]hydroxytryptophol (HITOP), and N-acetyl-[14C]serotonin (NAS) present in the medium were resolved by TLC. The amounts of [14C]serotonin metabolites remaining in the cells was less than 1% of that in the medium and exhibited the same general profile. Data are means ± S.E. (n = 4). The results were confirmed in three independent experiments. For details see “Experimental Procedures.”

**hAANAT Activity in Broken Cell Preparations of 1E7 Cells Is Comparable to That in Night Pineal Glands—**Broken cell AANAT activity of 1E7 cells was compared with that of other preparations of AANAT, including day and night pineal glands from several species, and bacterially expressed hAANAT and oAANAT. Broken cell AANAT activity in the cell line was in the range of values typically found in rat pineal glands obtained at night, as measured using saturating substrate concentrations (Table I).

**Inhibition of 1E7 AANAT by CoA-S-N-acetyltryptamine—**Broken cell AANAT activity was inhibited by the potent and selective bisubstrate inhibitor of AANAT CoA-S-N-acetyltryptamine (32) to the same degree (IC50 ~ 0.2–0.5 μM) as detected with AANAT from night pineal glands and bacterially expressed hAANAT and oAANAT (Table I). The failure of this inhibitor to markedly inhibit the low level of apparent activity in day rat pineal homogenates probably reflects the action of arylamine N-acetyltransferase, which is only weakly inhibited by CoA-S-N-acetyltryptamine (32).

**Melatonin Is Not Metabolized by 1E7 or COS7 Cells—**When 1E7 or COS7 cells were incubated with [3H]melatonin for 4 h (or 24 h), there was no evidence of metabolism of melatonin or nonspecific degradation of melatonin (Table II). In addition, when [3H]melatonin was incubated with homogenates, there was no detectable metabolism of melatonin; in the same experiment, trout retinal homogenates metabolized a significant fraction of [3H]melatonin. Thus, it appears likely that 1E7 cells do not metabolize melatonin and that measurement of melatonin accumulation in the medium is an accurate estimate of melatonin synthesized and released by these cells.

**Kinetic Characteristics of 1E7 and Bacterially Expressed hAANATs Are Similar—**Western blot analysis of samples of homogenates containing similar levels of hAANAT activity indicated that 1E7 and bacterially expressed hAANAT had the same mass and similar specific activity, i.e. homogenates with similar amounts of cell-free activity generated similar immunospecific signals in Western blot analysis. In addition, their masses were similar to that of AANAT from the human pineal gland (Fig. 3). The similar mass of hAANAT in the 1E7 cell line supports the interpretation that the enzyme is physically similar to that expressed in bacteria and in human pineal tissue and that the enzyme is not altered in Escherichia coli or 1E7 cells, as might occur as a result of clipping of N- or C-terminal regions.

The K_m values for each of four arylalkylamines were deter-
The values given were obtained using the radiometric assay described under “Experimental Procedures” with saturating concentrations of substrates. 1E7 cells were assayed the day after plating at 200,000 cells/well in a 6-well plate. Individual ovine pineal glands were prepared from times of approximate minimum (1200 h) and maximum (0300 h) AANAT activity. Pools of 11–50 rat pineal glands were prepared from times of approximate minimum (1200 h) and maximum (2400 h) AANAT activity. Mean values of bacterially expressed enzymes are based on independent preparations. Inhibition of AANAT activity was determined using the specific AANAT bisubstrate inhibitor CoA-S-N-acetyltryptamine (BSI) at five concentrations ranging from 0.1 to 10 μM. The IC₅₀ values were calculated using the Kaleidascop-progam. Values given are the mean ± S.E. of at least three replicates (the number of replicates is given in parentheses); where three replicates are not available, duplicate values are given. Further details are given under “Experimental Procedures.”

| Source       | AANAT activity (nmol/mg protein/h | BSI IC₅₀ (μM) |
|--------------|-----------------------------------|--------------|
| 1E7 cells    |                                   |              |
| (hAANAT)     | 169 ± 39 (8)                      | 0.21 ± 0.08  |
| Ovine pineal |                                    |              |
| gland, day   | 114 ± 4.1 (4)                     | 0.29 ± 0.04  |
| Ovine pineal |                                    |              |
| gland, night | 384 ± 26.4 (4)                    | 0.49 ± 0.09  |
| Rat pineal gland, day | 1.5 ± 0.7 (3) | 26.3, 18.1 (2) |
| Rat pineal gland, night | 175 ± 5.1 (3) | 0.36, 0.57 (2) |
| E. coli (hAANAT, purified) | 810,000 ± 200,000 (3) | 0.16 ± 0.02 (3) |
| E. coli (oAANAT, purified) | 2,700,000 ± 700,000 (3) | 0.28 ± 0.02 (3) |

TABLE II

*COS7 and 1E7 cells do not degrade melatonin*

Cells were incubated with 100 nM [³H]melatonin in either intact or broken cell assays. The presence of metabolic products was investigated using one-dimensional TLC. An entire TLC lane was analyzed, and the percentage of the total counts recovered as melatonin is given. Approximately 5% of the total applied radioactivity in blanks (No cells) was not recoverable by the methods used. Values are means of duplicate determinations that were within 3% of each other. Details of assays used to study the metabolism of melatonin are provided under “Experimental Procedures.”

| Sample             | [³H]Melatonin recovered [% of total originally present] |
|--------------------|------------------------------------------------------|
| No cells           | Trout retina | COS7 cells | 1E7 cells |
| Intact cell        | 95.5         | Not tested | 94.6     | 95.0 |
| Broken cell        | 95.0         | 19.1       | 95.0     | Not tested |

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**TABLE IV**

*Specific activity of AANAT and effects of BSI on AANAT activity as measured using broken cell preparations and bacterially expressed AANAT*

The values given were obtained using the radiometric assay described under “Experimental Procedures” with saturating concentrations of substrates. 1E7 cells were assayed the day after plating at 200,000 cells/well in a 6-well plate. Individual ovine pineal glands were prepared from times of approximate minimum (1200 h) and maximum (0300 h) AANAT activity. Pools of 11–50 rat pineal glands were prepared from times of approximate minimum (1200 h) and maximum (2400 h) AANAT activity. Mean values of bacterially expressed enzymes are based on independent preparations. Inhibition of AANAT activity was determined using the specific AANAT bisubstrate inhibitor CoA-S-N-acetyltryptamine (BSI) at five concentrations ranging from 0.1 to 10 μM. The IC₅₀ values were calculated using the Kaleidascop-program. Values given are the mean ± S.E. of at least three replicates (the number of replicates is given in parentheses); where three replicates are not available, duplicate values are given. Further details are given under “Experimental Procedures.”

**Fig. 3. Western blot analysis of hAANAT.** Samples of 1E7 homogenates and bacterially expressed hAANAT were loaded, which contained similar levels of AANAT activity (~3.5 nmol of tryptamine acetylated per hour) in 65 μg and 2.6 ng of protein, respectively. A sample of a homogenized human pineal gland containing 33.5 μg of protein was loaded on a separate gel. These three samples were analyzed together on a Western blot immunodetected using immunopurified antiserum 3236, which was raised against hAANAT, and 326 fore, cellular mass markers are given on the bottom. The source of hAANAT is indicated at the bottom. The results were confirmed at least four times. For details see “Experimental Procedures.”

**TABLE III**

*cAMP Enhances Intact Cell AANAT Activity—Treatment with the cAMP protagonist FSK increased intact cell AANAT activity 4- to 10-fold over control values, whereas broken cell AANAT activity increased only about 2-fold (Figs. 5–7). This pattern of a large increase in intact cell AANAT activity with a ~2-fold increase in cell-free AANAT activity was seen consistently: The increase in intact cell AANAT activity due to FSK treatment was 8.1 ± 0.7 (n = 21 experiments) and that for broken cell AANAT activity was 1.6 ± 0.1 (n = 24 experiments). Similar effects were seen at FSK concentrations from 1 to 100 μM (Table IV). FSK treatment did not markedly alter the abundance of AANAT mRNA (Figs. 5 and 6). The effect of FSK on total immunoreactive AANAT protein detected by Western blot was also small, as measured using antiserum (3236) that was raised against hAANAT, and 326 fore, cellular mass markers are given on the bottom. The source of hAANAT is indicated at the bottom. The results were confirmed at least four times. For details see “Experimental Procedures.”

FSK was found to elevate cAMP production by these cells, according to measurements of cAMP in culture medium (Table V). To confirm that the effects of FSK on intracellular AANAT activity were due to the elevation of cAMP, the effects of DBcAMP and two additional cAMP protagonists, IBMX and ISO (36), were examined. All enhanced intact cell AANAT activity ~4-fold but had little effect on activity measured in broken cell preparations (Figs. 6 and 7). DBcAMP, like FSK, induced this effect without increasing total AANAT immunoreactive protein or AANAT mRNA (Fig. 6). The degree of stimulation of intracellular AANAT activity by IBMX and ISO was not greater than by FSK, even though IBMX and ISO increased cAMP to a higher level (Fig. 7). FSK increased intact cell AANAT activity within the first hour of exposure (Table VI).

One explanation of the effects of cAMP is that it increased the concentration of intracellular AcCoA. This does not seem likely, because FSK treatment did not increase acetylation of 5-MT by COS7 cells not expressing AANAT (Fig. 5). 5-MT is acetylated by these cells by another enzyme, as indicated above, and an increase in AcCoA in these cells would have
5-HT and 5-MT might be due to a decrease in cellular acetylation of 5-HT by another mechanism. Rather, it would appear that cAMP selectively enhances formation of these oxidation products would also increase. That cAMP does not appear to act by increasing the intracellular concentration of tryptamine, tyramine, phenylethylamine, and 5-hydroxytryptamine (serotonin).

The FSK-induced increases in intracellular acetylation of 5-HT is presented. Each point is the mean of duplicate determinations; one of three independent experiments used to generate the corresponding data. The Km values of tryptamine, tyramine, and phenylethylamine were also examined with another arylalkylamine, [14C]5-HT (Fig. 3). Forskolin treatment increased the production of [14C]HIAA and [14C]HTOH, was not elevated. This indicates that the Km for 5-HT was altered by FSK treatment; this was confirmed in an extensive kinetic analysis of tryptamine acetylation using [3H]AcCoA (data not shown).

The possibility that phosphorylation of AANAT alone alters the kinetics of the protein was investigated using expressed oAANAT and hAANAT; no evidence to support this was obtained, which is consistent with previous observations based on studies with expressed rAANAT (37).

**DISCUSSION**

These studies establish that the 1E7 cell line expresses hAANAT stably, at levels that are similar to those in night rat pineal glands. Accordingly, this cell line will be of special value for analysis of hAANAT, because it provides an inexpensive source of material to study the enzyme in a cellular setting at levels that approximate those found in the night pineal gland. One additional advantage is that the host cell is of primate origin. Expression of a primate protein in a primate cell increases the likelihood that the natural biology of the protein is reproduced and that hAANAT could interact with other primate proteins as it would in the human pineal gland.

The 1E7 cell line might also be a useful alternative source of hAANAT for biochemical studies, because purification of hAANAT from bacterial preparations is difficult due to containing chaperonins, which tenaciously bind to the enzyme (35).

Studies presented here show that hAANAT expressed in 1E7 cells has the same general physical and kinetic characteristics as E. coli–expressed hAANAT. This suggests that the enzyme does not undergo significant post-translational modification in 1E7 cells that result in alteration of substrate specificity or sensitivity to inhibitors.

It should be noted that a 6-fold discrepancy exists between the apparent Km value for tryptamine reported in the literature (35) for E. coli hAANAT (0.9 mM) and those observed here (0.15 mM); the 6-fold higher value diminishes the apparent selectivity of AANAT for 5-HT and tryptamine when Km values are compared, weakening the argument that hAANAT can differentiate between these two substrates. For example, in the current study, the 5-HT Km/tryptamine Km ratio is 10-fold for both E. coli–expressed hAANAT and 1E7 hAANAT (Table III).
the parental cell line NC30, and COS7 cells, were incubated in 75-cm² tissue culture flasks with vehicle (0.1% ethanol) or with the indicated concentration of forskolin in 2 ml of medium for 6 h; 0.1 mM 5-MT was present in all dishes. The culture medium was assayed for the amount of melatonin produced by intact cells from exogenous 5-MT. Broken cell AANAT activity was measured in cell homogenates. Data are the mean of duplicate determinations. Numbers in parentheses are the -fold increase over control values.

![Graph of AANAT mRNA and hAANAT expression](image)

**Effects of FSK concentration on intact and broken cell AANAT activity in 1E7 cells**

| Forskolin µM | Intact cell nmol/mg prot/h | Broken cell nmol/mg prot/h |
|-------------|---------------------------|---------------------------|
| Control     | 1.0                       | 36.1                      |
| 1           | 7.0 (7.0)                 | 62.5 (1.7)                |
| 3           | 6.6 (6.8)                 | 74.5 (2.1)                |
| 10          | 7.8 (7.8)                 | 77.8 (2.2)                |
| 30          | 8.6 (8.6)                 | 85.6 (2.4)                |
| 100         | 11.3 (11.3)               | 79.0 (2.2)                |

Cells (200,000/well) were incubated in 6-well culture plates with vehicle (0.1% ethanol) or with the indicated concentration of forskolin. The culture medium was assayed for the amount of melatonin produced by intact cells from exogenous 5-MT. Broken cell AANAT activity was measured in cell homogenates. Data are the mean of duplicate determinations. Numbers in parentheses are the -fold increase over control values.

**TABLE IV**

| Forskolin µM | Intact cell nmol/mg prot/h | Broken cell nmol/mg prot/h |
|-------------|---------------------------|---------------------------|
| Control     | 1.0                       | 36.1                      |
| 1           | 7.0 (7.0)                 | 62.5 (1.7)                |
| 3           | 6.6 (6.8)                 | 74.5 (2.1)                |
| 10          | 7.8 (7.8)                 | 77.8 (2.2)                |
| 30          | 8.6 (8.6)                 | 85.6 (2.4)                |
| 100         | 11.3 (11.3)               | 79.0 (2.2)                |

Whereas that in the literature for E. coli-expressed hAANAT is ~1.5-fold ($K_m$ 5-HT = 1.2 ± 0.08 mM versus $K_m$ tryptamine = 0.91 ± 0.04 mM, mean ± S.E.). The 10-fold difference in $K_m$ values found in the present study for 5-HT versus tryptamine (Table III), with both 1E7 and bacterially expressed hAANAT, is consistent with the >20-fold difference in $K_m$ values for tryptamine (hydroxyphenylethylamine) versus phenylethylamine found in both studies. The basis for the inconsistency in the published data on the 5-HT/tryptamine selectivity, as compared with the results in this report, is unclear. With this one inconsistent tryptamine $K_m$ value set aside, however, the data indicate that hAANAT strongly (~10-fold) prefers non-hydroxylated arylalkylamine substrates over their hydroxylated analogs, in contrast to oAANAT, which does not exhibit this selectivity.

The basis for the capacity of hAANAT to differentiate between hydroxylated versus non-hydroxylated arylalkylamines may lie in the residues lining the binding pocket of the enzyme, as discussed previously (20, 33, 35). However, it is not unreasonable that differences in primary sequence distant to the binding pocket might also contribute to this.

As indicated in the introduction, previous reports (13, 14) document circumstances in which changes in melatonin production in vivo appear to be distinctly greater than those in oAANAT activity measured in pineal homogenates. These results have been generally ignored, in part because melatonin production in vivo can be influenced by many factors. The observation that a similar difference occurs with 1E7 cells, i.e. changes in intact cell AANAT activity but not in broken cell activity, provides reason to give serious consideration to the assessment that measurement of broken cell AANAT activity is not a reliable indication of intact cell AANAT activity under all conditions. The data are in agreement with the interpretation that cellular AANAT activity can be inhibited and that this inhibition is reversed when tissue is homogenized and assayed.

The existence of a rapidly reversible activation/inactivation mechanism is supported by observations made in the sheep, where there are ~10-fold changes in NAS and melatonin but only 3- to 5-fold changes in broken cell AANAT activity (13, 14). Similarly, a pulse of light at night causes a rapid decrease in both compounds by more than 85% within 30 min but does not significantly alter broken cell AANAT activity (13, 14). Changes in the levels of these compounds could reflect the experiments it has been observed that neither immunoreactive total AANAT nor phosphorylated AANAT increase, or increase only to a small degree, as a function of FSK treatment.
inhibition of the cellular activity of AANAT; and, the small change in broken cell AANAT activity preparations may indicate that this inhibition can be reversed when the cell is homogenized. A similar situation may exist with control versus FSK-treated 1E7 cells. Data on the pineal gland in culture are also in agreement with this hypothesis, because it has been found the DBcAMP treatment increases melatonin production >10-fold, without markedly increasing AANAT activity measured in broken cell preparations (15).

The observations of the current investigation indicate that the phosphorylation state of AANAT alone may not be a reliable indicator of intracellular activity of AANAT, because FSK-induced changes in intracellular activity were observed without large changes occurring in the N-terminal phosphorylation site. The stimulatory effects of FSK might be due primarily to effects on another protein, and the lack of an effect on the N-terminal PKA site of AANAT might be explained by tight binding of AANAT to another protein that protects against dephosphorylation. It is reasonable to suspect that there might be a two-tiered AANAT regulatory system, involving two dynamic ranges of cAMP, i.e., a lower range, in which AANAT is phosphorylated at the N-terminal site and accumulates because proteolysis is blocked, and a second and higher range within which AANAT protein is activated.

The mechanism through which AANAT activity is inhibited or activated by cAMP requires further investigation; there are several interesting possibilities worthy of brief discussion. One involves the PKA phosphorylation sites on AANAT, which occur at both N- and C-terminal sites (3, 8). Activation could reflect cAMP-dependent phosphorylation of AANAT, which leads to interactions with other proteins, which in turn alter activity. For example, it is thought that binding of 5-HT to AANAT requires prior occupancy of the enzyme by AcCoA, which in turn relocates a mobile element of the enzyme Loop 1 (33). A protein-protein interaction could also modulate movement of this element. It should be added that the two PKA sites within which AANAT protein is activated.

An important implication of the discrepancy between cellular versus homogenate AANAT activity is that measurement of AANAT activity in homogenates may not provide an accurate estimate of intracellular AANAT activity. Whereas it can be assumed that undetectable AANAT activity in homogenates reflects low levels of intracellular AANAT activity, it cannot be assumed that an elevated level of broken cell AANAT is a valid indication of elevated cellular AANAT activity. A better esti-
were incubated in 6-well plates with vehicle (0.1% ethanol), forskolin (FSK, 0.01 mM), isobutylmethylxanthine (IBMX, 1 mM), FSK + IBMX (0.01 and 1 mM, respectively), or isoproterenol (ISO, 0.001 mM) for 3 h; 0.1 mM 5-MT was present in all wells. At the end of 3 h, the culture medium was removed and cells were then harvested. **Middle panel.** AANAT activity in broken cell homogenates. **Bottom panel,** the amount of cAMP produced by intact cells from exogenous 0.1 mM 5-MT during 3 h. **Bottom panel,** the amount of cAMP released by cell into the medium. For details see "Experimental Procedures." Data are means of duplicate wells each analyzed in duplicate; duplicates did not differ from the mean by more than 11%. Results for IBMX and ISO have been confirmed in one additional experiment; the effects of FSK were replicated as given in Fig. 5, except that the effect on cAMP has been confirmed in three additional experiments.

**Fig. 7.** Effects of cAMP antagonists on intact and broken cell activity and cAMP production by 1E7 cells. Cells (200,000/well) were incubated in 6-well plates with vehicle (0.1% ethanol), forskolin (FSK, 0.01 mM), isobutylmethylxanthine (IBMX, 1 mM), FSK + IBMX (0.01 and 1 mM, respectively), or isoproterenol (ISO, 0.001 mM) for 3 h; 0.1 mM 5-MT was present in all wells. At the end of 3 h, the culture medium was removed and cells were then harvested. **Top panel,** AANAT activity in broken cell homogenates. **Middle panel,** the amount of melatonin produced by intact cells from exogenous 0.1 mM 5-MT during 3 h. **Bottom panel,** the amount of cAMP released by cell into the medium. For details see "Experimental Procedures." Data are means of duplicate wells each analyzed in duplicate; duplicates did not differ from the mean by more than 11%. Results for IBMX and ISO have been confirmed in one additional experiment; the effects of FSK were replicated as given in Fig. 5, except that the effect on cAMP has been confirmed in three additional experiments.

In addition to the possible role of inhibition/activation mechanisms in the light-induced "turn off" of melatonin production, it is obvious that the opposite, a "turn on," would be important. As indicated above, there are striking similarities in the nocturnal pattern of the increase in melatonin in sheep and primates. In both, melatonin increases immediately after lights off, without a lag (1, 16–18). This suggests that melatonin synthesis in the human pineal gland might also be regulated by an activation/inhibition mechanism in vivo. The 1E7 cell line might prove to be a useful tool to use to examine the issue of how cellular AANAT activity is controlled.

In summary, the findings of this report together with previous studies provide impetus to consider that 1) melatonin production may be controlled in the pinealocyte and retinal photoreceptors by inactivation/activation of AANAT without requiring a change in AANAT protein and 2) this would be of special importance in mediating immediate and rapid changes in cellular activity of pineal AANAT in sheep, primates, and other vertebrates.

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| Time interval | Control | Forskolin |
|---------------|---------|-----------|
| Hour 0–1      | 1.26    | 6.44      |
| Hour 1–2      | 0.76    | 6.05      |
| Hours 2–4     | 0.98    | 9.34      |

**Table VI.** Table VI. Time course of the forskolin-induced increase in intact cell AANAT activity

**Cells (200,000/well) were incubated in 6-well culture plates with vehicle (0.1% ethanol) or with forskolin (0.01 mM) in 2 ml of medium. Aliquots of medium were sampled at the indicated times and assayed for melatonin by RIA as described under "Experimental Procedures." Data are calculated as the nanomoles of melatonin produced in the medium per mg of cellular protein from exogenous 5-MT during each interval of the experiment. Data are the mean of duplicate determinations. Similar results were seen in two additional experiments.
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