Role of a pineal cAMP-operated arylalkylamine *N*-acetyltransferase/14-3-3-binding switch in melatonin synthesis

Surajit Ganguly*†, Jonathan A. Gastel*†, Joan L. Weller*, Christian Schwartz*, Howard Jaffe‡, M. A. A. Namboodiri§, Steven L. Coon*, Alison B. Hickman¶, Mark Rollag§, Tomas Obsil¶, Philippe Beauverger‖, Gilles Ferry‖, Jean A. Boutin‖, and David C. Klein*§**

*Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-4480; ‡Protein Sequencing Facility, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892; §Circadian Research Center, Department of Anatomy, Physiology, and Genetics, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; and ¶Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; and ‖Institut de Recherches Servier, 125, Chemin de Ronde, 78290 Croissy-sur Seine, France

Edited by Martha Vaughan, National Institutes of Health, Rockville, MD, and approved May 4, 2001 (received for review March 9, 2001)

The daily rhythm in melatonin levels is controlled by cAMP through actions on the penultimate enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT; serotonin *N*-acetyltransferase, EC 2.3.1.87). Results presented here describe a regulatory/binding sequence in AANAT that encodes a cAMP-operated binding switch through which cAMP-regulated protein kinase-catalyzed phosphorylation [RRHTLPAN → RRHpTLPAN] promotes formation of a complex with 14-3-3 proteins. Formation of this AANAT/14-3-3 complex enhances melatonin production by shielding AANAT from dephosphorylation and/or proteolysis and by decreasing the K_m for 5-hydroxytryptamine (serotonin). Similar switches could play a role in cAMP signal transduction in other biological systems.

Circulating melatonin exhibits a ≈10-fold rhythm in vertebrates, with highest values occurring at night; this highly conserved signal is important for biological timekeeping (1). The melatonin rhythm is regulated in mammals by a complex photoneural circuit that includes the Mind's Clock—the suprachiasmatic nucleus. At the level of the pinealocyte, norepinephrine is released and acts through cAMP to control the steady-state levels and the activation state of the penultimate enzyme in the melatonin pathway, arylalkylamine *N*-acetyltransferase (AANAT; serotonin *N*-acetyltransferase, EC 2.3.1.87; refs. 1–4). Steady-state levels are influenced by cAMP in some species through activation of transcription, and are influenced in all species through inhibition of proteasomal proteolysis (3, 4).

We propose that cAMP regulates AANAT protein in part through the two evolutionarily conserved protein kinase A (PK-A) phosphorylation sites located at either end of the molecule (4). This hypothesis was pursued and as described here, we now have obtained evidence that cAMP-dependent phosphorylation triggers the binding of AANAT to 14-3-3 proteins (30 and 33 kDa), a family of highly conserved isoforms that exist as dimers (5–7). Our results also reveal that this association has functional importance.

Materials and Methods

Affinity Chromatographic Preparation of AANAT-Binding Proteins. A 100 μg sample of glutathione *S*-transferase (GST)-ovine AANAT (oAANAT; ref. 8) was mixed with 100 μl (50 μl of dry volume) of Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) (22°C for 20 min). The beads were centrifuged (1,000 × *g*; 4°C; 1 min) and washed two times with 1 ml of PK-A buffer (100 mM NaCl/50 mM Tris, pH 7.5/10 mM $MgCl_2$/2 mM DTT)]. PK-A buffer (250 μl) containing 10 mM ATP (Sigma) was added to both (+) and (−) PK-A samples; PK-A (120 units in10 μl; Promega) was added only to the (+) tube. The beads were incubated (22°C; 20 min), centrifuged, and washed two times with binding buffer (50 mM sodium citrate, pH 6.5/10 mM DTT/150 mM NaF).

C6 cells were labeled by incubation (12 h) in methionine-free DMEM (Life Technologies, Rockville, MD) plus 5% (vol/vol) full DMEM [containing 10% (vol/vol) FCS] and 1 mCi ^{35}S-labeled methionine/cysteine (EasyTag, New England Nuclear) per 150-mm plate (12 ml of medium). Cells were washed, scraped, and collected by centrifugation (15,000 × *g*; 4°C; 10 min). The pellet was disrupted, a 15,000 × *g* supernatant was prepared, and samples of this were loaded onto the columns [0.5 × 2.5 cm; 0.05 ml of GST-oAANAT or GST-phospho-oAANAT (GST-p-oAANAT)]. The bound protein was eluted (10 mM glutathione/50 mM Tris·HCl, pH 8.0), concentrated 100-fold (10 kDa; Amicon), and resolved by SDS/PAGE (NOVEX, San Diego; ref. 4). The gel was dried and exposed overnight on BioMAX film (Kodak).

Mass Spectrometric Analysis of the Two ^{35}S-Labeled Protein Bands that Selectively Bind to p-oAANAT. ^{35}S-labeled 30- and 33-kDa proteins that bound preferentially to GST-p-oAANAT matrix as compared with GST-oAANAT and were resolved and identified by using SDS/PAGE (14%, 250-ng protein load from 25 100-mm plates of cells). Gel containing the bands was excised and treated with trypsin (9); tryptic peptides were subjected to liquid chromatography (LC)-MS analysis (10). Mass spectrometric analysis was done by using 250-ng protein samples (25 100-mm plates of cells). MS/MS spectra were analyzed by using the BIOEXPLORE software package (Finnigan-MAT, San Jose, CA). Individual MS/MS spectra were searched against the OWL database by using the SEQUEST program.

Phosphorylation of oAANAT. oAANAT (50 μg) was phosphorylated [15 mM $MgCl_2$, 2 mM ATP, 5 mM DTT, and catalytic subunit of PK-A (8 units) in 0.2 ml of 0.1 M Tris·HCl, pH 7.2, buffer (4°C; 18 h)]. The mixture was dialyzed against 20 mM Tris·HCl, pH 7.5, containing 50 mM NaCl and 1 mM DTT.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AANAT, arylalkylamine *N*-acetyltransferase; oAANAT, ovine AANAT; hAANAT, human AANAT; rAANAT, rat AANAT; p-AANAT, phospho-AANAT; 5-HT, 5-hydroxytryptamine (serotonin); HA, hemagglutinin; GST, glutathione *S*-transferase; PK-A, protein kinase A; IP, immunopurified polyclonal.

†S.G. and J.A.G. contributed equally to this work.

**To whom reprint requests should be addressed. E-mail: klein@helix.nih.gov.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

NEUROBIOLOGY

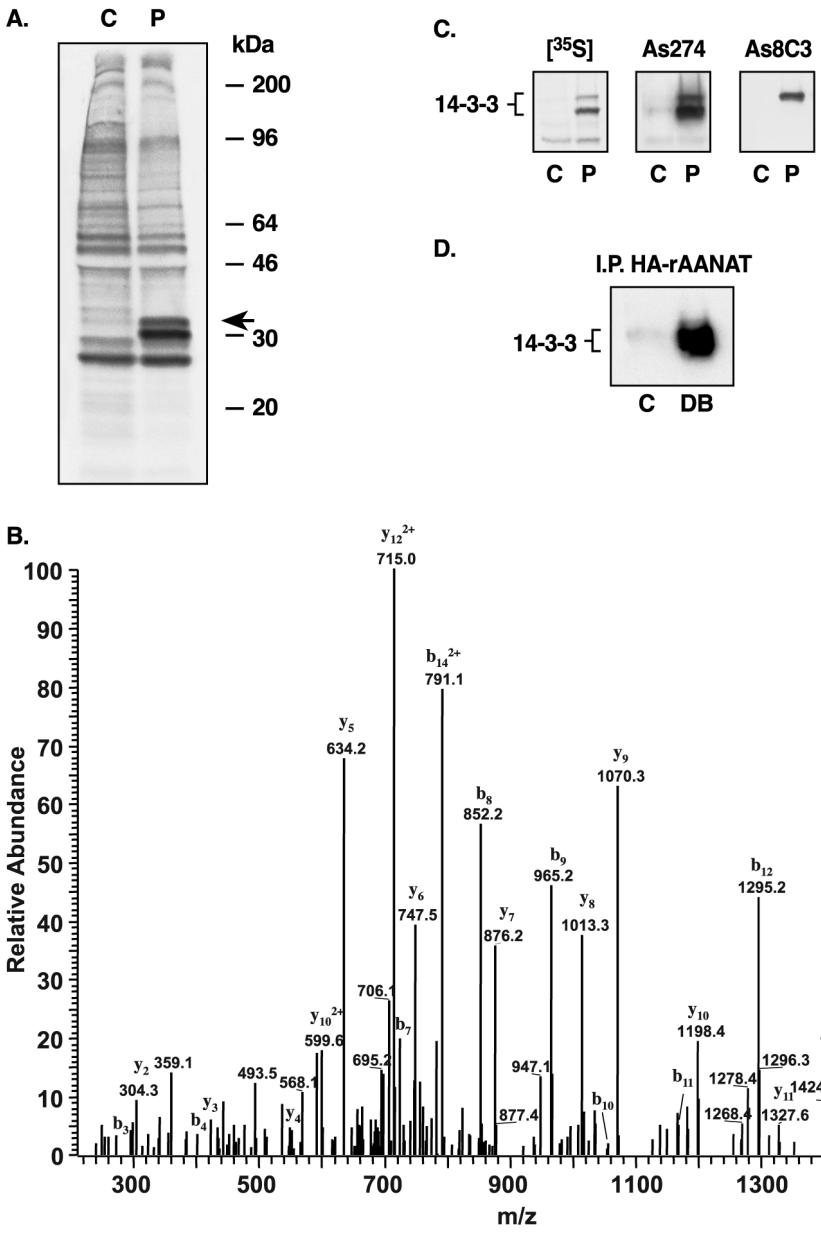

Fig. 1. 14-3-3 proteins bind to phosphorylated AANAT. (*A*) Two ^{35}S-labeled proteins bind to p-AANAT. ^{35}S-labeled cytosolic proteins were applied on GST-oAANAT (C) and GST-p-oAANAT (P) as described in *Materials and Methods*. The 30- and 33-kDa bands (arrow) bound preferentially to p-AANAT. (*B*) Mass spectrometric analysis of the two ^{35}S-labeled proteins that selectively bind to p-oAANAT. A representative full-scale MS/MS spectrum of the doubly charged ion at m/z 800.2 is presented, which was determined to result from the rat 14-3-3-β tryptic peptide, (K)AVTEQGHELSNEER. Observed y and b ions, including a continuous y_2-y_{12} and b_7-b_{13} series, consistent with those predicted for the peptide are labeled. Eleven other 14-3-3-derived peptides were identified, (K)AASDIAMTELPPTHPIR, (R)YLAEFATGNDRK, ε; (R)YLAEVAAGDDKK, (R)VVSSIEQK, (K)SVTEQGALELSNEER, ζ; (R)ATVVESSEK, (K)AYSEAHEISK, γ; (K)NVVGAR, β, γ, or ζ; (K)LAEQAER, (R)NLLSVAYK, β, γ, θ, or ζ; and (K)EMQPTHPIR, β, θ, or ζ. (*C*) Immunochemical identification of the bound proteins as 14-3-3 proteins. ^{35}S-labeled proteins were detected by autoradiograph (24 h) (*Left*) and immunodetected with antiserum As 274 (*Center*), which detects multiple 14-3-3 isoforms. Monoclonal antiserum As 8C3 (*Right*) detects 14-3-3-ε (33 kDa). The P/C ratios for radioactivity and immunoreactivity are >10. (*D*) HA-rAANAT coimmunoprecipitation of 14-3-3 in dibutyryl cAMP-treated C6 cells transfected with HA-rAANAT. Anti-HA was used to coimmunoprecipitate 14-3-3 from HA-rAANAT-transfected C6 cells, as described in *Materials and Methods*. IP antiserum As 274 was used to detect 14-3-3 proteins, as described in *C*.

Phosphorylation was monitored in a parallel reaction by using [γ-^{32}P]ATP. The control oAANAT sample was prepared identically with the omission of ATP.

Coimmunoprecipitation of 14-3-3 by Hemagglutinin (HA)-Tagged AANAT. Rat AANAT (rAANAT$_{1–205}$) cDNA was amplified by PCR from clone rLL13 (3) and ligated into pHB6 (Roche Molecular Biochemicals) producing HA-rAANAT. C6 cells were transfected with HA-rAANAT and 24 h later were treated with dibutyryl cAMP (1 mM), collected, centrifuged, resuspended (500 μl of 100 mM NaF, 20 mM sodium citrate, pH 6.5, 10 mM DTT), and lysed. The supernatant (15,000 $\times$ g; 4°C; 10 min) was mixed with a 2 μl of rat monoclonal anti-HA (Roche Molecular Biochemicals). Protein G Sepharose beads (Amersham Pharmacia Biotech) [blocked with 5% (vol/vol) fish gelatin; Sigma] were added. After incubation (4°C; 60 min), the beads were recovered, washed (six times in lysis buffer), and boiled in SDS/PAGE sample buffer. Then solubilized proteins were separated by SDS/PAGE in 14% gel and transferred to poly(vinylidene difluoride) membrane. The 14-3-3 proteins were immunodetected by using the immunopurified polyclonal (IP) antiserum As 274 (7).

Preparation of Recombinant Proteins and Chromatographic Analysis. cDNA encoding 14-3-3-ζ was generated from human fetal brain cDNA pool by PCR and ligated to *Eco*RI and *Bam*HI sites of expression vector pGEX-4T-1 (Amersham Pharmacia Biotech). The selected clone Z20 was transformed into BL21(DE3)pLysS (Novagen) for expression as GST-14-3-3-ζ fusion protein (11). After purification (Glutathione Sepharose 4B; Amersham Pharmacia Biotech), the fusion product was cleaved (thrombin; 1 unit/mg protein; Roche Molecular Biochemicals), and GST was removed by using Glutathione Sepharose, resulting in pure 14-3-3-ζ. AANAT was phosphorylated as described above. Samples (50 μl) were chromatographed on a Superdex 75 PC 3.2/30 column (SMART System, Amersham Pharmacia Biotech), using 20 mM Tris·HCl, pH 7.5, containing 50 mM NaCl and 1 mM DTT (buffer A) at 6–10°C (50 μl/min; 50 μl of fractions). The

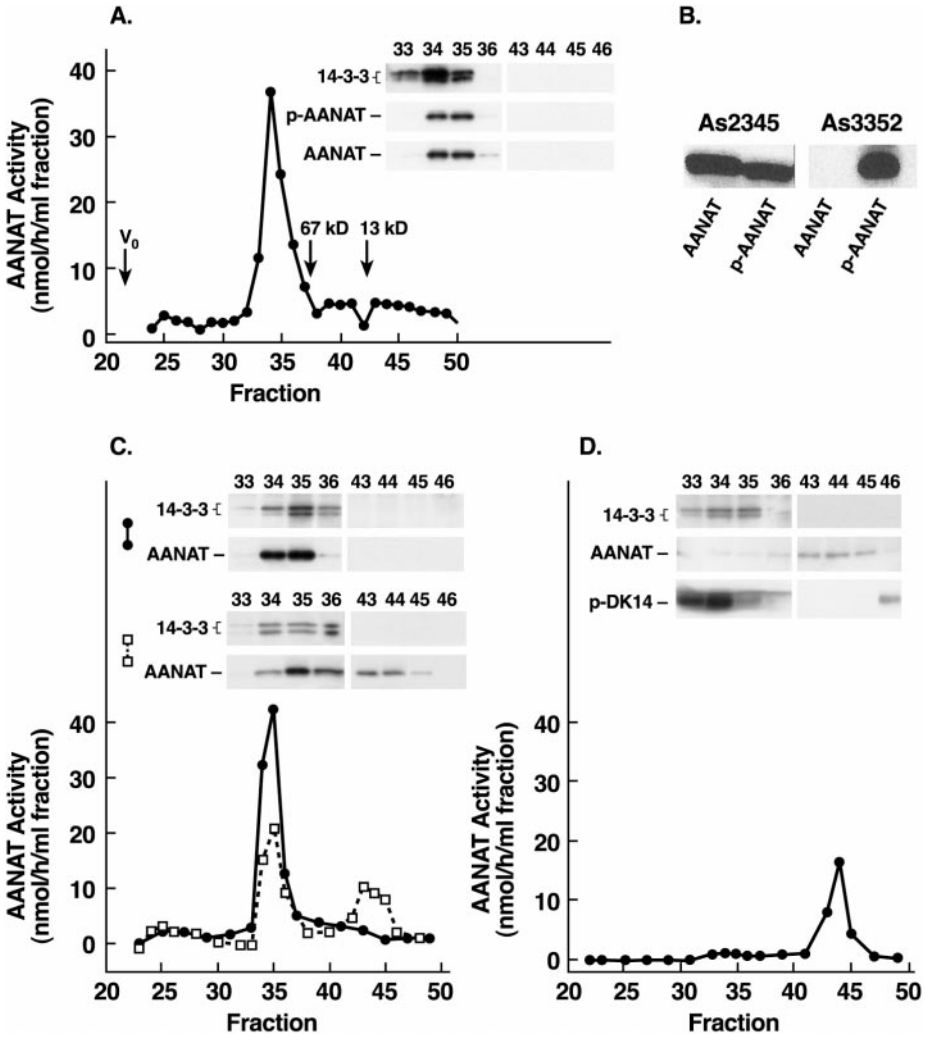

Fig. 2. N-terminal PK-A/14-3-3 site in AANAT is involved in 14-3-3 binding. (*A*) AANAT from ovine pineal gland coeluting with 14-3-3 is phosphorylated. Ovine pineal tissue (300 mg; ref. 4) was homogenized in 3 ml of 0.1 M ammonium acetate buffer (pH 6.8; 4°C) and centrifuged (14,000 × *g*; 10 min). Proteins in 2.5 ml of the supernatant were fractionated by gel filtration [200 ml of TSK3000 column, Toya Soda, Tokyo; 0.1 M ammonium acetate buffer, pH 6.8, containing 10 mM DTT and 10% (vol/vol) glycerol; 1 ml/min flow rate; 4-ml fractions; 4°C]. AANAT activity was measured with 50 μl each 4-ml fraction (4). oAANAT was detected by Western blot with IP antisera As 2345 (1:125; raised against $oAANAT_{24–39}$) and with As 3352 (1:125; raised against p-rAANAT $_{22–37}$; ref. 4). The 14-3-3 proteins were detected with antiserum As 274. (*B*) Selectivity of antisera used. Western blot analysis of p-oAANAT and unphosphorylated oAANAT, using IP antisera As 2345 and 3352. IP antiserum As 2345 detects both phosphorylated and unphosphorylated forms; antiserum As 3352 detects only phosphorylated AANAT. (*C*) Coelution of hAANAT and 14-3-3 proteins in forskolin-treated cells expressing hAANAT. HA6 cells (see *Materials and Methods*) were either untreated (□—□) or treated (●—●) with forskolin (10 μM; 6 h), which elevated cAMP production >20-fold (data not presented). Chromatography and enzyme assays were as described in *A*. The 14-3-3 proteins were detected on a Western blot as in *A* and hAANAT was detected by using IP antiserum As 3236 raised against $hAANAT_{1–26}$. (*D*) p-DK14 ($p\text{-}oAANAT_{24–39}$) displaces hAANAT. Conditions were as described in *C* with the exception that the cell homogenate was incubated with p-DK14 (0.5 mM; 4°C; 30 min) before chromatography. The sequence of DK14 peptide is given in the text. The 14-3-3 and hAANAT proteins were detected as in *C* and p-DK14 was detected with antiserum As 2345.

fractions were analyzed by SDS/PAGE and Coomassie brilliant blue staining. Chromatography was done by using recombinant oAANAT (8), p-oAANAT, and 14-3-3-ζ. To test for complex formation, samples of p-AANAT or AANAT were incubated before chromatography with the indicated amounts of 14-3-3 (4°C; 30 min; 50 μl of buffer A).

Preparation of Human AANAT (hAANAT)-Expressing Cell Line. Chinese hamster ovary (CHO)-K1 cells were seeded (5×10^6 per 75 cm^2 culture flask) and 24 h later were transfected (10 μg of a pcDNA3.1-hAANAT plasmid; ref. 12) by using Lipofectamine (GIBCO/BRL). After geneticin (0.8 mg/ml) selection, colonies were selected for expansion based on AANAT activity (13); the HA6 cell line was established after two rounds of subcloning. AANAT activity in HA6 cells was ~7 nmol per h per 10^6 cells (4).

Results

14-3-3 Preferentially Binds to the Phosphorylated Form of AANAT. The cAMP-dependent nature of the 14-3-3/AANAT association (5, 7) was discovered by using a differential (AANAT vs. p-AANAT) affinity chromatography screen of [^{35}S]methionine/cysteine-labeled proteins in C6 cell extracts (Fig. 1*A*). Differential analysis of the ^{35}S-labeled proteins bound to the respective columns reveal that the p-AANAT column eluate (P in Fig. 1*A*) was enriched markedly with 30- and 33-kDa proteins. To identify these proteins, the gel containing these 30- and 33-kDa bands was excised and trypsinized. Mass spectrometric analysis of the tryptic peptides identified 14-3-3 isoform-derived peptides (Fig. 1*B*). This identification was confirmed by Western blot analysis (Fig. 1*C*), using anti-14-3-3 sera (7). These findings provided the first indication that PK-A-dependent phosphorylation of AANAT promotes association with 14-3-3 proteins.

Cellular Formation of p-AANAT/14-3-3 Complex. To determine whether AANAT is phosphorylated in the naturally occurring AANAT/14-3-3 complex, night-killed sheep pineal homogenate was resolved by gel permeation chromatography (Fig. 2*A*). Analysis of the fractions revealed that AANAT coeluted with 14-3-3 in a 90-kDa fraction (Fig. 2*A*). Free AANAT was not detected in the lower molecular mass fractions by IP antiserum As 2345, which recognizes both p-AANAT and AANAT (Fig. 2*B*). Further analysis of the AANAT fractions with a p-AANAT-specific IP antiserum, As 3352, confirmed that AANAT in the 90-kDa 14-3-3 complex was phosphorylated at the N-terminal PK-A site (Fig. 2 *A* and *B*).

Formation of the AANAT/14-3-3 complex also could be induced in an AANAT-expressing HA6 cell line by forskolin treatment (Fig. 2*C*); free AANAT was not detected. AANAT/

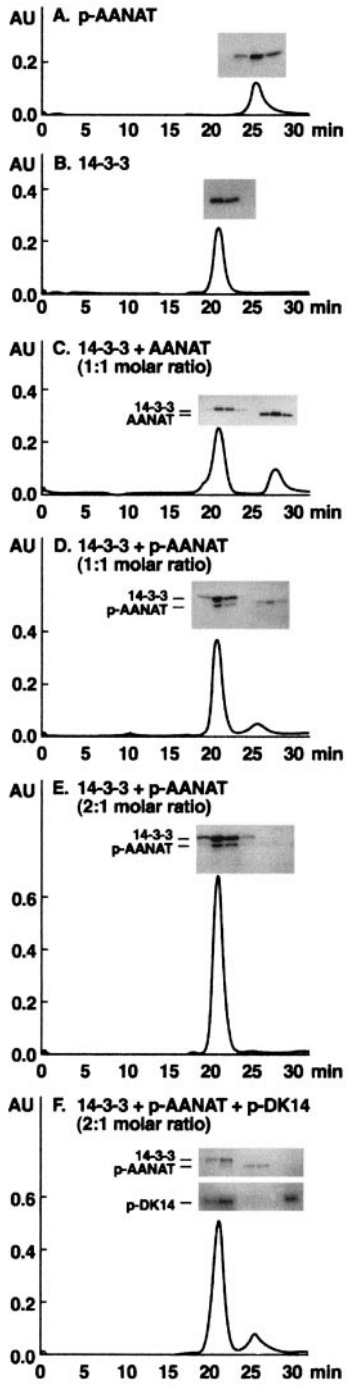

Fig. 3. Complex formation by purified p-AANAT and 14-3-3-ζ proteins. (*A*) Elution profile of p-AANAT (23 μg). (*B*) Elution profile of 14-3-3-ζ (30 μ g). (*C*) Elution profile of a 1:1 molar mixture of 14-3-3-ζ monomer (30 μg) and AANAT (23 μg). (*D*) Elution profile of a 1:1 molar mixture of 14-3-3-ζ (30 μg) monomer and p-AANAT (23 μg) (0.5:1 molar ratio of 14-3-3-ζ dimer and p-AANAT). p-AANAT coelutes with 14-3-3-ζ, representing a complex, and, in a later fraction, representing unbound p-AANAT. (*E*) Elution profile of a 2:1 molar mixture of 14-3-3-ζ monomer (60 μg) and p-AANAT (23 μg; 1:1 molar ratio of 14-3-3-ζ dimer and p-AANAT). p-AANAT coelutes with 14-3-3-ζ; unbound p-AANAT is not detected. (*F*) Role of PK-A/14-3-3 motif on elution of 14-3-3-ζ/AANAT complex. Incubation (4°C; 30 min; 50 μl) of a preformed p-AANAT/14-3-3 complex (see *E*) with a 25-fold excess of p-DK14 (0.5 mM) causes p-AANAT to elute as unbound protein. A portion of the p-DK14 coelutes with 14-3-3-ζ and also in later fractions as unbound peptide (a representative fraction is shown). In the same type of competition experiment, unphosphorylated DK14 neither coelutes with 14-3-3 nor displaces p-AANAT (data not shown).

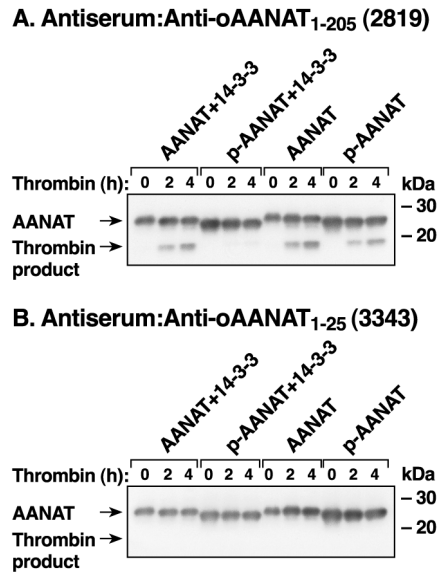

Fig. 4. 14-3-3-ζ protects p-AANAT against clipping by thrombin. oAANAT or p-oAANAT (1 μg) was incubated with 0.1 units of thrombin (Roche Molecular Biochemicals) in the presence or absence of 14-3-3-ζ protein (5 μg) at 4°C for 0, 2, or 4 h (h) in a 50-μl reaction mixture containing 0.1 M Tris·HCl, pH 8.0. Proteins in a 5-μl volume of each sample were detected by Western blot analysis. (*A*) Immunodetection with an antiserum raised against oAANAT$_{1–205}$ (2819) detects a ~18-kDa product. N-terminal sequencing of the ~18-kDa fragment, using Edman degradation (protein sequencer model 477A, Applied Biosystems), indicated the cleavage site was at residue Arg^{26}. (*B*) Immunodetection with an IP antiserum raised against oAANAT$_{1–25}$ (3343). The thrombin degradation product generated in *A* is not detected, indicating that the N-terminal AANAT epitope has been removed.

14-3-3 association was detected in a pull-down experiment also, in which cells were transfected with HA-tagged AANAT; 14-3-3 was recovered in the HA immunoprecipitate if the host cells were treated with an activator of adenylyl cyclase, dibutyryl cAMP (Fig. 1*D*). This set of observations is of interest because it provides evidence that complex formation in a cellular environment does not require endogenous factors specific to the pineal gland.

The N-terminal PK-A site (RRHT) of AANAT is embedded in a sequence (AANAT$_{28–33}$, RRHpTLP) that resembles motifs (RSX**pS**XP; RXY/FX**pS**XP) which bind to the amphipathic binding groove of 14-3-3 (14, 15). Here we have found that a phosphorylated peptide containing the PK-A/14-3-3[††] motif of AANAT (PGRQRRHpTLPANEFRC, p-DK14; corresponding to oAANAT$_{24–39}$) disrupts the AANAT/14-3-3-ζ complex (Fig. 2*D*). In contrast, the unphosphorylated peptide did not have this effect, suggesting that phosphorylation of the PK-A/14-3-3-binding motif is required for complex formation.

Recombinant p-AANAT and 14-3-3 32 ζ Form a Complex. The above studies raise the possibility that p-AANAT and 14-3-3 may interact directly. This possibility was supported by the finding that recombinant p-AANAT formed a complex with 14-3-3 ζ (Fig. 3 *A–D*), whereas unphosphorylated AANAT did not. Related results using recombinant proteins provided insight into the issue of whether one or two molecules of p-AANAT bound to the 14-3-3 dimer. As indicated above, the use of a 1:1 molar ratio of 14-3-3 and p-AANAT resulted in the formation of a

[††]The PK-A/14-3-3 site (RRHpTLPAN → RRHpTLPAN) containing a consensus protein kinase A phosphorylation motif (RRHT → RRHpT) embedded within a sequence that resembles 14-3-3-binding motifs (RSXpSXP; RXY/FXpSXP).

Table 1. 14-3-3-ζ alters the kinetics of p-oAANAT

Additions	Tryptamine		5-HT	
	K_m, mM	k_{cat}, s^{-1}	K_m, mM	k_{cat}, s^{-1}
AANAT + 14-3-3-ζ	0.170 ± 0.032	25.5 ± 0.5	0.150 ± 0.020	18.5 ± 0.3
p-AANAT	0.190 ± 0.026	34.8 ± 1.3	0.170 ± 0.035	21.4 ± 2.0
p-AANAT + 14-3-3-ζ	0.014 ± 0.003	9.9 ± 0.4	0.020 ± 0.004	4.6 ± 0.2

Effects on K_m and k_{cat}. K_m and k_{cat} values of oAANAT/p-AANAT (~2 ng of AANAT enzyme/100 μl of assay mixture) for the indicated indoleamine substrate by using saturated AcCoA concentration (0.5 mM) in presence or absence of 14-3-3-ζ isoform (1:5 molar ratio p-AANAT/AANAT:14-3-3-ζ monomer) were determined as described (12). Ten concentrations (1 μM to 4 mM) of indoleamines were used. Less than 10% of the AcCoA was consumed at the highest amine concentration. The results presented are the mean (± standard error of the mean) of the results of four experiments, in which each data point was done in duplicate. The specific activity of the preparations of oAANAT used range from 5 to 10 nmol/ng of protein per h (10 mM trytpamine, 0.5 mM AcCoA). Radiochemical assays were done (30°C; 15 min) following a published procedure (4).

complex that involved only a fraction of the added p-AANAT. However, the unbound p-AANAT peak became almost negligible when the 14-3-3:p-AANAT molar ratio was 2:1 (1 mole of 14-3-3 dimer and 1 mole of p-AANAT; Fig. 3*E*). This result suggests that only one molecule of p-AANAT binds to a 14-3-3 dimer. It also was found that complex formation by the recombinant proteins was blocked by p-oAANAT$_{24–39}$, indicating that association involves the N-terminal PK-A/14-3-3 motif (Fig. 3*F*).

14-3-3-ζ Complex Formation Protects AANAT *In Vitro*. We determined that formation of the 14-3-3 complex shielded the N-terminal region of AANAT by taking advantage of the serendipitous finding that thrombin cleaves AANAT and p-AANAT at Arg^{26}, resulting in a ~18-kDa fragment (Fig. 4). Cleavage of p-AANAT was reduced in the presence of 14-3-3. Although thrombin cleavage of AANAT has little physiological significance, because thrombin is an extracellular enzyme, these observations indicate that 14-3-3 blocks access to the N-terminal region of complexed p-AANAT. This indication was supported by the finding that dephosphorylation of p-AANAT by phosphatase PP1 is inhibited when complexed to 14-3-3 (data not shown), which is of interest because dephosphorylation may precede proteasomal destruction (J.A.G. and D.C.K., unpublished data).

14-3-3-ζ Complex Formation Activates AANAT. The functional significance of complex formation was investigated by determining whether 14-3-3 binding alters AANAT activity. Kinetic analysis revealed that 14-3-3 binding decreases the arylalkylamine K_m of p-AANAT 10-fold (Table 1), which is below the physiological concentration of 5-hydroxytryptamine (serotonin) (5-HT; ref. 16). As predicted from the above observations, the effects of 14-3-3 on p-AANAT kinetics seem to involve the PK-A/14-3-3-binding motif of AANAT for two reasons. First, the phosphorylation of the N-terminal portion of AANAT was found to be necessary for 14-3-3 to increase the AANAT activity (~3-fold) as measured with low substrate concentrations (Table 2). The level of 5-HT selected (1 μM) approximates that of free cytoplasmic 5-HT in the nighttime ovine pineal gland and of AcCoA (25 μM) in tissues, assuming uniform distribution (16). Second, the PK-A/14-3-3-binding motif seems to be involved because this activation effect was prevented by p-oAANAT$_{24–39}$ (Table 2; Fig. 2*B*). Accordingly, these studies indicate that the N-terminal PK-A/14-3-3 site is required for 14-3-3 to activate AANAT.

Table 2. Effects on activity at low concentrations of substrates

Additions	AANAT activity, nmol/ng of protein per h
AANAT	0.8
p-AANAT	0.9
AANAT + 14-3-3-ζ	0.8
p-AANAT + 14-3-3-ζ	3.2
p-AANAT + p-oAANAT$_{24–39}$ + 14-3-3-ζ	1.1

Assays (30°C; 15 min) were done with full-length oAANAT. p-oAANAT$_{24–39}$ was used where indicated to disrupt 14-3-3/AANAT complexes. The concentration of 5-HT was 1 μM and AcCoA was 25 μM (4 Ci/mmol; 5 μCi/40 μl of assay).

Role of the PK-A/14-3-3 Site in Cellular Activation of AANAT. Treatment of AANAT-transfected cells with an activator of adenylyl cyclase increases AANAT activity in intact cells, with relatively little increase in the amount of AANAT protein (12). To investigate the involvement of PK-A/14-3-3 site in this activation of AANAT, COS7 cells were transfected either with wild-type hAANAT or with a form in which the PK-A/14-3-3 site was mutated (Thr^{31} → Ala). After 48 h, cells were treated with forskolin and incubated with 5-methoxytryptamine (100 μM). Intracellular AANAT activity, i.e., activity in intact cells, was estimated from the amount of melatonin in the media (12). Forskolin treatment increased intracellular AANAT activity Î0-fold in cells transfected with wild-type hAANAT but not in cells with the Thr^{31} → Ala mutant (Table 3). In contrast, forskolin treatment did not induce immunodetectable AANAT, indicating that the increase reflects activation of AANAT. AANAT activity in broken cell preparations was not increased by forskolin. The difference between intact cell and broken cell activity in response to forskolin treatment has been reported (12). The failure to observe forskolin-mediated activation with the Thr^{31} → Ala mutant indicates that the PK-A/14-3-3 site is important for cAMP regulation of the activation state of AANAT in intact cells.

Discussion

This study establishes that AANAT exists *in vivo* in a phosphorylated state complexed to 14-3-3, and moreover that cAMP controls complex formation through a binding switch (RRH**T**LPAN → RRH**pT**LPAN).

The finding that recombinant p-AANAT and 14-3-3 form a complex was extended in crystallographic studies of a p-AANAT$_{1–201}$:14-3-3-ζ complex (17). This investigation identified contacts between the PK-A/14-3-3 AANAT site and the amphipathic grove of 14-3-3, which is in agreement with the indications of this study and the 14-3-3 literature (14, 15). It also should be added that crystallographic analysis has revealed extensive interactions between p-AANAT and 14-3-3 outside this region, which contribute to the strength and specificity of binding. These interactions also seem to explain how 14-3-3 influences p-AANAT kinetics—by restricting movement of a

NEUROBIOLOGY

Table 3. The PK-A/14-3-3 motif in AANAT mediates cAMP activation of AANAT in intact cells

	Intact cell AANAT activity, pmol/mg of protein per h			Broken cell AANAT activity, nmol/mg of protein per h			ir-hAANAT
Vector	Cont	FSK	FSK/Cont	Cont	FSK	FSK/Cont	FSK/Cont
hAANAT	328 ± 38	3173 ± 850	9.6 ± 2.8	82 ± 23	200 ± 56	2.5 ± 0.1	2.8 ± 2.3
hAANAT T31A	166 ± 4	279 ± 26	1.7 ± 0.2	91 ± 14	106 ± 5	1.1 ± 0.1	1.2 ± 0.3

COS7 cells were transfected transiently with pcDNA3.1 (Invitrogen) containing either wild-type hAANAT or a mutant form (hAANAT T31A). Mutant hAANAT T31A was constructed from wild-type hAANAT cloned in pcDNA3.1 (Invitrogen), using the QuikChange kit (Stratagene). The AANAT activity in intact cells was estimated from the amount of melatonin in the media formed from 5-MT (12). AANAT activity in broken cells was assayed by using tryptamine (1 mM) as substrate (4). ir-hAANAT (immunoreactive-hAANAT) was immunodetected by antiserum As 3236. The intensity of the immunoreaction was quantitated after scanning the autoradiographs (IMAGEQUANT 5.1). The data presented represent the mean (± range) of the results of two experiments, in which each treatment was done in duplicate. FSK, forskolin; Cont, control.

flexible element (Loop 1) of AANAT (17, 18)—thereby optimizing the organization of the substrate-binding domain. This constraint also may lower the V_{max} by restricting flux through the active site domain.

It is likely that the p-AANAT/14-3-3 regulatory complex plays a pivotal role in melatonin production (1–3) in two ways. First, regulation of the activation state of AANAT in the intact cell may be the dominant mechanism that mediates very rapid changes in melatonin levels induced by light-to-dark and dark-to-light transitions, as seen in human, Rhesus monkeys, and sheep (1, 3). The demonstration that the $Thr^{31} \rightarrow Ala$ mutant can abrogate cAMP regulation of the activation state suggests that formation of a p-AANAT/14-3-3 regulatory complex may explain the difference between the intact and broken cell activity measurements as described by Coon *et al.* (12). Reversible formation of the 14-3-3/ANNAT complex also might explain the cAMP elevation of melatonin production in cultured fish pineal glands, which is not accompanied by a similar increase in activity of AANAT measured in homogenates (19). It should be added that the 14-3-3-induced reduction in K_m may be essential for AANAT to acetylate the low levels of 5-HT that occur at night, which fall to 1/10 or 1/20 day values (16). Second, in addition to this activation effect, the complex formation could play a central role in controlling steady-state levels of pineal AANAT by mediating the protective effects of cAMP against proteasomal proteolysis (3, 4).

A role of 14-3-3 as a scaffold that mediates protein–protein interactions has been discussed (17, 20–23). It is possible that one or more other proteins might be involved in regulating AANAT through formation of trimeric complexes (X/14-3-3/AANAT). The 14-3-3 dimer could bind p-AANAT via the amphipathic groove on one monomer and another protein through the amphipathic groove on the second monomer.

The PK-A/14-3-3 motif in vertebrate AANATs is absent from AANATs present in fungi (3), which contain 14-3-3 proteins. It is of interest to speculate that during evolution, acquisition of this regulatory motif was influenced by 14-3-3. Addition of this motif may have been essential for the evolution of AANAT as the transducer for cAMP regulation of melatonin production and for melatonin to have become a vertebrate photic signal.

The AANAT/14-3-3 regulatory mechanism described here provides another example of how protein phosphorylation can have rapid and profound physiological effects without changing protein levels. It is likely that cAMP regulation of AANAT activity by means of phosphorylation of the PK-A/14-3-3 site is conserved highly, because this motif is found in all vertebrate AANATs. In contrast, regulation of transcription by means of cAMP and cAMP-response elements is not (3, 24). cAMP-dependent activation of AANAT seems to be of profound importance in regulating rapid increases in melatonin production seen in all vertebrates.

It should be noted that cAMP-operated binding switches, similar to that described here, may be essential elements of cAMP-mediated signal transduction in many cells. This hypothesis is based on the ubiquitous presence of 14-3-3 proteins and the existence of PK-A/14-3-3-like binding motifs in many proteins in the databases.

M.A.A.N. is supported by National Institutes of Health Grant RO1 NS39387. M.R. is supported by Uniformed Services University of the Health Sciences Grant RO70HY.

1. Arendt, J. (1995) *Melatonin and the Mammalian Pineal Gland* (Chapman & Hall, London), pp. 1–273.
2. Klein, D. C. & Weller, J. L. (1970) *Science* **169,** 1093–1095.
3. Klein, D. C., Coon, S. L., Roseboom, P. H., Weller, J. L., Bernard, M., Gastel, J. A., Zatz, M., Iuvone, P. M., Rodriguez, I. R., Begay, V., *et al.* (1997) *Recent Prog. Horm. Res.* **52,** 307–358.
4. Gastel, J. A., Roseboom, P. H., Rinaldi, P. A., Weller, J. L. & Klein D. C. (1998) *Science* **279,** 1358–1360.
5. Aitken, A. (1996) *Trends Cell Biol.* **6,** 341–347.
6. Fu, H., Subramanian, R. R. & Masters, S. C. (2000) *Annu. Rev. Pharmacol. Toxicol.* **40,** 617–647.
7. Roseboom, P. H., Weller, J. L, Babila, T., Aitken, A., Sellers, L. A., Moffett, J. R., Namboodiri, M. A. & Klein, D. C. (1994) *DNA Cell Biol.* **13,** 629–640.
8. DeAngelis, J., Gastel, J., Klein, D. C. & Cole, P. A. (1998) *J. Biol. Chem.* **273,** 3045–3050.
9. Moritz, R. L., Eddes, J., Hong, J., Reid, G. E. & Simpson, R. H. (1995) in *Techniques in Protein Chemistry*, ed. Crabb, J. W. (Academic, San Diego), Vol. VI, pp. 311–319.
10. Jaffe, H., Veeranna, R., Shetty, K. T. & Pant, H. C. (1998) *Biochemistry* **37,** 3931–3940.
11. Jones, D. H., Martin, H., Madrazo, J., Robinson, K. A., Nielsen, P., Roseboom, P. H., Patel, Y., Howell, S. A. & Aitken, A. (1995) *J. Mol. Biol.* **245,** 375–384.
12. Coon, S. L., Weller, J. L., Korf, H. W., Namboodiri, M. A., Rollag, M. & Klein, D. C. (2001) *J. Biol. Chem*. **275,** in press.
13. Ferry, G., Loynel, A., Kucharczyk, N., Bertin, S., Rodriguez, M., Delagrange, P. Galizzi, J. P., Jacoby, E., Volland, J. P., Lesieur, D., *et al.* (2000) *J. Biol. Chem.* **275,** 8794–8805.
14. Muslin, A. J., Tanner, J. W., Allen, P. M. & Shaw, A. S. (1996) *Cell* **84,** 889–897.
15. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J. & Cantley, L. C. (1997) *Cell* **91,** 961–971.
16. Namboodiri, M. A., Sugden, D., Klein, D. C., Tamarkin, L. & Mefford, I. N. (1985) *Comp. Biochem. Physiol.* **80,** 731–736.
17. Obsil, T., Ghirlando, R., Klein, D. C., Ganguly, S. & Dyda, F. (2001) *Cell* **105,** 257–267.
18. Dyda, F., Klein, D. C. & Hickman, A. B. (2000) *Annu. Rev. Biophys. Biomol. Struct.* **29,** 81–103.
19. Thibault, C., Falcon, J., Greenhouse, S. S., Lowery, C. A., Gern, W. A. & Collin, J. P. (1993) *J. Neurochem.* **61,** 332–339.
20. Xing, H., Kornfeld, K. & Muslin, A. J. (1997) *Curr. Biol.* **7,** 294–300.
21. Braselmann, S. & McCormick, F. (1995) *EMBO J.* **14,** 4839–4848.
22. Yaffe, M. B. & Cantley, L. C. (1999) *Nature (London)* **402,** 30–31.
23. Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., Gamblin, S. J. & Yaffe, M. B. (1999) *Mol. Cell* **4,** 153–166.
24. Baler, R. & Klein, D. C. (1997) *J. Biol. Chem.* **272,** 6979–6985.