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Regulation of Pineal α_{1B}-Adrenergic Receptor mRNA: Day/Night Rhythm and β-Adrenergic Receptor/Cyclic AMP Control

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SUMMARY

Mammalian pineal function is regulated by norepinephrine acting through α_{1B}- and β_1-adrenergic receptors (ARs). Noradrenergic stimulation of α_{1B}-ARs potentiates the β_1-AR-driven increase in cAMP, serotonin *N*-acetyltransferase, and melatonin production. In the present study, we describe a 3-fold daily rhythm in mRNA-encoding α_{1B}-ARs in the pineal gland, with a peak at midnight. Pharmacological studies indicate that this increase in α_{1B}-AR mRNA is due to activation of β-ARs. Second messenger studies indicate that α_{1B}-AR mRNA is increased by agents that increase cAMP, including dibutyryl cAMP, cholera toxin, forskolin, or vasoactive intestinal peptide. These observations indicate that α_{1B}-AR mRNA can be physiologically regulated by a β-AR-dependent enhancement of cAMP. It also was observed that *in vivo* and *in vitro* changes in α_{1B}-AR mRNA are not accompanied by similar changes in α_{1B}-AR binding, indicating that turnover of α_{1B}-AR protein is significantly slower than that of α_{1B}-AR mRNA and that post-transcriptional mechanisms play an important role in regulating α_{1B}-AR binding.

The pineal gland contains a high abundance of both α_1- and β_1-ARs, which play an important role in regulating circadian pineal function, including melatonin synthesis (1–3). Adrenergic stimulation of the gland increases at night in response to central stimulation of the release of norepinephrine from sympathetic nerve processes, terminating in pineal extracellular space.

α_1- and β_1-ARs interact in the pineal gland through biochemical "AND" gates (4–6). The receptor-receptor interaction occurs at postreceptor sites and involves α_1-AR potentiation of the effects of β_1-ARs. Potentiation is evident from analysis of cAMP, cGMP, serotonin *N*-acetyltransferase, and melatonin (7, 8). β_1-ARs positively regulate the activity of both adenylyl and guanylyl cyclases through mechanisms involving G proteins (9, 10). The specific functions of α_1-ARs are mediation of adrenergically stimulated increases in $[Ca^{2+}]_i$ and activation of PKC and phospholipases (9, 11–13). Pharmacological studies suggest that the α_1-ARs in the pineal gland are predominantly of the α_{1B} subtype (14), which is consistent with evidence of a high abundance of α_{1B}-AR mRNA (15).

Because of the importance of α_{1B}-AR to pineal physiology, it is important to understand the mechanisms regulating α_{1B}-ARs in this neuroendocrine gland. Previously, rat pineal glands have been shown not to exhibit a daily rhythm in α_{1B}-AR binding *in vivo* despite the large nocturnal increase in NE (16). Additionally, neither acute nor chronic administration of phenylephrine or isoproterenol had any effect on α_{1B}-AR binding (16). In contrast, pineal β_1-AR binding has been shown to fluctuate on a daily basis (17). Furthermore, pineal β_1-AR mRNA can be up-regulated through β-AR, an effect that can be mimicked by forskolin (18). Because α_{1B}-AR binding and mRNA can be up-regulated in DDT_1 MF-2 smooth muscle cells by β_2-AR acting through cAMP (19), we investigated whether α_{1B}-AR mRNA might be regulated in the rat pineal gland by an adrenergic → cAMP mechanism.

Materials and Methods

***In vivo* experiments.** The animals used in this study were 150–200 g male Sprague-Dawley rats that had been in our facilities for at least 1 week (light:dark 14:10, lights on at 5:00 a.m.). In some cases, animals were kept in light at night to block stimulation of the pineal gland by the SCN (5). Animals were killed by decapitation at the times indicated. Tissues were removed immediately and placed on dry ice. Handling and maintenance of rats were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

***In vitro* experiments.** Adult female Sprague-Dawley rats were decapitated and the pineal glands were placed immediately in Dulbecco's modified Eagle's medium containing 50 units/ml penicillin,

ABBREVIATIONS: AR, adrenergic receptor; PKC, protein kinase C; NE, norepinephrine; SCN, suprachiasmatic nuclei; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, standard saline citrate; HEAT, iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone; VIP, vasoactive intestinal peptide.

50 μg/ml streptomycin, 2 mM glutamine, and 20 mM HEPES, pH 7.4 (4°). After cleaning, the glands were cultured in 200 μl of Biggers-Gwatkin-Judah medium with Fitton-Jackson modification (no serum) supplemented with 0.5 mM ascorbic acid as an antioxidant in 24-well cluster dishes as described previously (2). For treatments, glands were transferred into fresh medium containing freshly prepared ascorbic acid (0.5 mM) and the indicated drugs or vehicle. Drug treatments were performed in 24-well cluster dishes in a top-loading incubator with a humidified atmosphere of 5% CO_2/95% O_2 at 37° for the indicated times (2).

RNA preparation and Northern blot analysis. Total RNA was extracted using the guanidine HCl/phenol procedure (20) and poly(A)$^+$ RNA was subsequently purified using oligo-dT latex beads (Qiagen, Santa Clarita, CA). RNA was separated on a 1.5% agarose/0.7 M formaldehyde gel for 5 hr at 2.5 V/cm. Electrophoresed RNA was transferred to a charged nylon membrane by passive capillary transfer and cross-linked to the membrane using UV. The hybridization probe for α_{1B}-AR mRNA was ^{32}P-labeled by random-priming a full-length cDNA for rat α_{1B}-AR (21). Blots were also probed for either 18 S ribosomal RNA (22) or G3PDH (Clontech, Palo Alto, CA) mRNA to normalize data for variations in RNA loading; G3PDH mRNA did not vary on a 24-hr basis or as a result of drug treatments. Blots were hybridized at 68° for 1 hr in QuikHyb buffer (Stratagene, LaJolla, CA). The final wash was in 0.1 × SSC (1× = 150 mM NaCl, 15 mM sodium citrate)/0.1% sodium dodecyl sulfate at 60° for 15 min. Blots were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). α_{1B}-AR mRNA was present as three bands of approximately 2.0, 2.3, and 2.5 kb in the pineal gland and the brain regions examined; transcripts of approximately 2.5 and 3.0 kb were detected in the liver. For quantitation, the abundance of α_{1B}-AR mRNA in all bands has been summed and normalized to 18 S ribosomal RNA or G3PDH mRNA expression. Transcript sizes were estimated by comparison with standard RNA markers (BRL RNA ladder; GIBCO BRL, Gaithersburg, MD).

***In situ* hybridization.** *In situ* hybridization was performed as described previously (23). Briefly, brains were dissected from adult male Sprague-Dawley rats immediately after decapitation, frozen, and stored at −80° until used. Frozen sections (20 μm) were mounted on slides and fixed with 4% formaldehyde. After dehydration, the sections were hybridized overnight at 37° in 120 μl of 50% formamide, 4× SSC, 1× Denhardt's solution, and 10% dextran sulfate with 500 μg/ml salmon sperm DNA and 100 mM fresh dithiothreitol containing 1–3 × 10^6 cpm of ^{35}S-labeled probe. Oligonucleotide probes for α_{1B}-AR mRNA were 3′ end-labeled using terminal deoxynucleotidyl transferase and ^{35}S-dATP to a specific activity of 5–15 × 10^8 cpm/μg. After overnight hybridization, the slides were washed in 2× SSC with 50% formamide at 40° for 1 hr with three changes, followed by a 1-hr wash at room temperature in 1× SSC with two changes. The sections were exposed to Hyperfilm-β max film (Amersham Life Science, Clearbrook, IL) for 11–17 days at room temperature.

Estimation of α_{1B}-AR density. α_{1B}-AR density was estimated using radioligand binding methodology (16). Pineal glands were removed from the culture plates after treatment, frozen immediately on dry ice, and then stored frozen (−70°). Individual pineal glands were sonicated (3 × 10 sec) in 100 μl of buffer A (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). The homogenate was diluted to 1.4 ml and centrifuged (100,000 × *g*, 20 min at 4°). The pellet was dispersed by sonication in 720 μl of buffer A. Protein was measured using a Bradford dye-binding procedure. Duplicate membrane aliquots (150 μl, 3–5 μg protein) were incubated (25°, 20 min) with [^{125}I]HEAT (50 or 200 pM; DuPont-New England Nuclear, Boston, MA) in the presence or absence of phentolamine (10 μM) to define nonspecific binding (16). In saturation experiments, the K_d was determined to be 82 ± 13 pM and the B_{max} was found to be 273 ± 19 fmol/mg protein (mean ± standard error, n = 4). α_{1B}-AR density also was measured by a similar method in which pairs of glands were homogenized together and duplicate aliquots of membrane were incubated (31°, 50 min) with [^{3}H]prazosin (DuPont-New England Nuclear) in the presence or absence of 10 μM phentolamine (24).

Results

α_{1B}-AR mRNA is highly abundant in the pineal gland compared with the brain and liver. *In situ* hybridization and Northern blot analysis were used to estimate the relative abundance of α_{1B}-AR mRNA in the pineal gland and other tissues (Fig. 1). *In situ* hybridization indicated that the abundance of mRNA encoding this receptor was higher than in any other brain area examined, including the thalamus, which is believed to contain the highest brain level of α_{1B}-AR mRNA (15, 23, 25). Northern blot analysis confirmed this and also indicated that the pineal gland contained a higher abundance of α_{1B}-AR mRNA as compared with liver (Fig. 1, B and C), which is known to express high levels (25, 26).

Pineal α_{1B}-AR mRNA exhibits a 3-fold day/night rhythm that is under photoneural regulation. The abundance of pineal α_{1B}-AR mRNA varies on a 24-hr basis (Fig. 2). The lowest levels occur at the beginning of the night and the highest values occur approximately 6 hr after lights are turned off (Fig. 2). The increase at night is approximately 3-fold, according to the results of three independent experiments. The nocturnal increase in α_{1B}-AR mRNA was blocked by continued exposure to light at night (Fig. 2).

β-Adrenergic and VIP regulation of pineal α_{1B}-AR mRNA.. Sympathetic stimulation of the pineal gland is believed to be mediated primarily by the release of NE. To determine whether NE regulates pineal α_{1B}-AR mRNA, organ culture was used. NE treatment increased the abundance of α_{1B}-AR mRNA by 3- to 5-fold in a dose-dependent manner (Fig. 3A). The maximum stimulation was observed at approximately 9 hr, and α_{1B}-AR mRNA levels remained elevated above basal levels for as long as 24 hr (Fig. 3B). The abundance of α_{1B}-AR mRNA also was increased by the β-adrenergic agonist isoproterenol but not by the α-adrenergic agonist phenylephrine (Fig. 4A), suggesting that NE is acting through pineal β_1-ARs. This conclusion was supported by the finding that the β-adrenergic antagonist propranolol blocked the action of NE and that the α-adrenergic antagonist prazosin did not (Fig. 4B). Propranolol alone had a small positive effect on α_{1B}-AR mRNA; the basis of this effect was not pursued.

cAMP regulates pineal α_{1B}-AR mRNA. β_1-AR activation of the pineal gland and many other tissues increases intracellular cAMP concentrations. This and a report that cAMP elevates α_{1B}-AR mRNA in a transformed cell line [DDT_1 MF-2 vas deferens cells (19)] point to the possible involvement of cAMP in the regulation of pineal α_{1B}-AR mRNA. This was examined by treating glands with cAMP protagonists, including dibutyryl cAMP, cholera toxin, and forskolin or with VIP, which has been shown to elevate cAMP in this tissue (27). These agents elevated α_{1B}-AR mRNA, suggesting the existence of a β_1-AR → cAMP → α_{1B}-AR mRNA regulatory link (Fig. 5).

Adrenergic agonists do not alter α_{1B}-AR binding. Because the conditions under which we observed a daily rhythm in α_{1B}-AR mRNA *in vivo* (see above) had been shown previously not to effect α_{1B}-AR binding (16), we investigated *in vitro* whether changes in α_{1B}-AR mRNA could be correlated

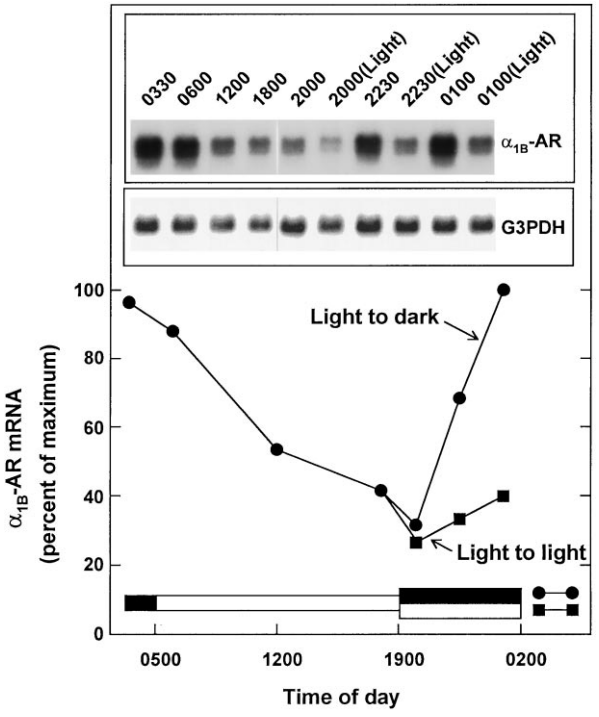

Fig. 2. Daily rhythm in pineal α_{1B}-AR mRNA. Northern blot analysis of α_{1B}-AR mRNA in pineal glands taken from rats at the indicated times of the day. Each *lane* contained 20 μg of total RNA from a pool of seven pineal glands. Three groups of rats remained in light during the final dark period and were killed at the same time as their lights-off counterparts. *Shaded bar*, lights off. Similar results were obtained in three additional experiments. RNA preparation and Northern blot analysis are as described in the text. *Graph,* derived from the Northern blots shown in the *inset*. The abundances of the three α_{1B}-AR transcripts have been summed and normalized to G3PDH mRNA expression.

to changes in α_{1B}-AR binding. Stimulation with NE for up to 24 hr did not affect α_{1B}-AR binding, whereas direct stimulation of adenylyl cyclase with forskolin resulted in a 70–90% increase in α_{1B}-AR binding after long term exposure (Fig. 6, Table 1). Elevation of cAMP using VIP (Table 1) or cholera toxin (data not shown) for 12 hr did not effect α_{1B}-AR binding. Similarly, treatment with selective adrenergic agonists phenylephrine or isoproterenol had no effect on binding (Table 1).

Discussion

Data in this report demonstrate that the abundance of pineal α_{1B}-AR mRNA exhibits a physiological daily rhythm that is abolished by continued light at night. These charac-

Fig. 1. α_{1B}-AR mRNA is expressed at high levels in the rat pineal gland. A, Coronal sections from the brain of an adult rat killed during the day were hybridized with a suite of three α_{1B}-AR-specific oligonucleotides. B, Northern blot analysis of α_{1B}-AR mRNA in the pineal gland compared with total brain and liver showing the transcripts detected. The blot contained poly(A)$^+$ RNA from day pineal glands (1 μg), total brain (6 μg), and liver (3 μg). Molecular size standards are indicated to the right and the calculated sizes of the three α_{1B}-AR pineal transcripts are indicated to the left. C, Northern blot analysis comparing the abundance of α_{1B}-AR mRNA in various brain regions and liver. Each *lane* contained 20 μg of total RNA. RNA preparation and Northern blot analysis are as described in the text. The *histogram* is derived from the Northern blots shown in the *inset*. The abundances of the three α_{1B}-AR transcripts have been summed and normalized to 18 S ribosomal RNA expression.

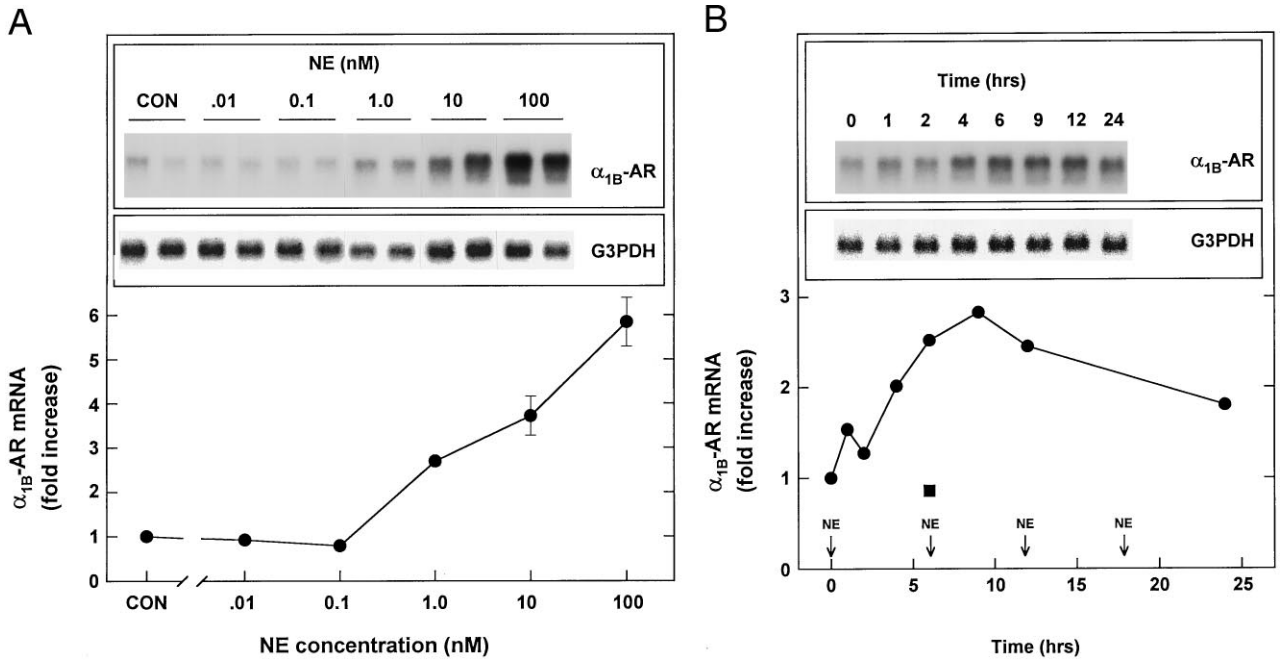

Fig. 3. Norepinephrine increases the abundance of α_{1B}-AR mRNA. A, Northern blot analysis of the effect of NE concentration on accumulation of α_{1B}-AR mRNA. Cultured pineal glands were exposed to the indicated doses for 6 hr. Each *lane* contained 3 μg of total RNA from a pool of 4 pineal glands. Data are means $\pm$ range of duplicate determinations. Similar results were obtained in three other experiments. B, Northern blot analysis of α_{1B}-AR mRNA from pineal glands exposed to NE (1 μM) for the indicated times; glands were transferred every 6 hr to fresh media containing NE (*arrows*). Each *lane* contained 10 μg of total RNA from a pool of six pineal glands. ■, Level of α_{1B}-AR mRNA after 6 hr under control conditions. Similar results were obtained in two other experiments. RNA preparation and Northern blot analysis were as described for Fig. 2.

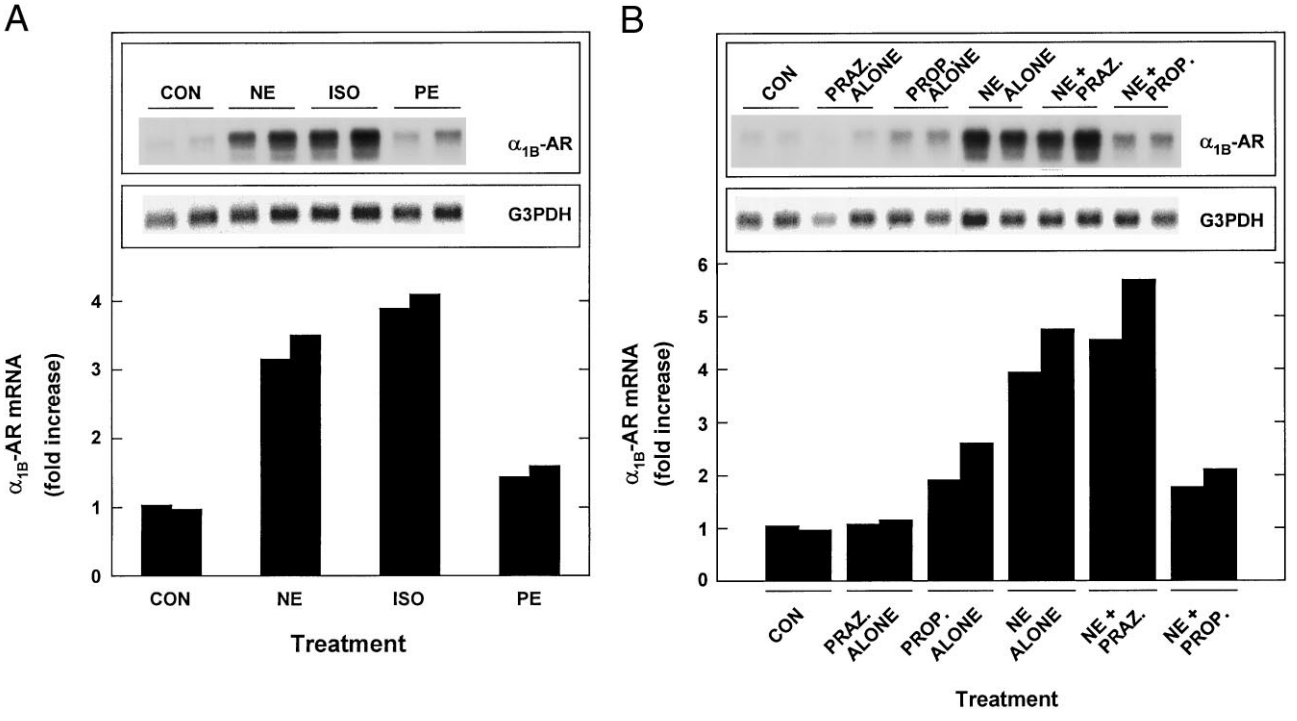

Fig. 4. Regulation of α_{1B}-AR mRNA by β-ARs. A, Northern blot analysis of α_{1B}-AR mRNA from pineal glands exposed to adrenergic agonists. Pineal glands were cultured for 6 hr with NE (1 μM), isoproterenol (*ISO*; 0.1 μM), or phenylephrine (*PE*; 1 μM). Each *lane* contained 5 μg of total RNA from a pool of three pineal glands. Duplicate lanes are independent samples from one experiment that was representative of three experiments. B, Northern blot analysis of α_{1B}-AR mRNA from pineal glands exposed to adrenergic antagonists. Glands were cultured with: prazosin alone (5 μM, 6.5 hr); propranolol alone (10 μM, 6.5 hr); NE alone (0.1 μM, 6 hr); prazosin (0.5 hr) then NE + prazosin (6 hr); propranolol (0.5 hr) then NE + propranolol (6 hr). Each *lane* contained 3 μg of total RNA from a pool of four pineal glands. Duplicate lanes are independent samples from one experiment. RNA preparation and Northern blot analysis were as described for Fig. 2.

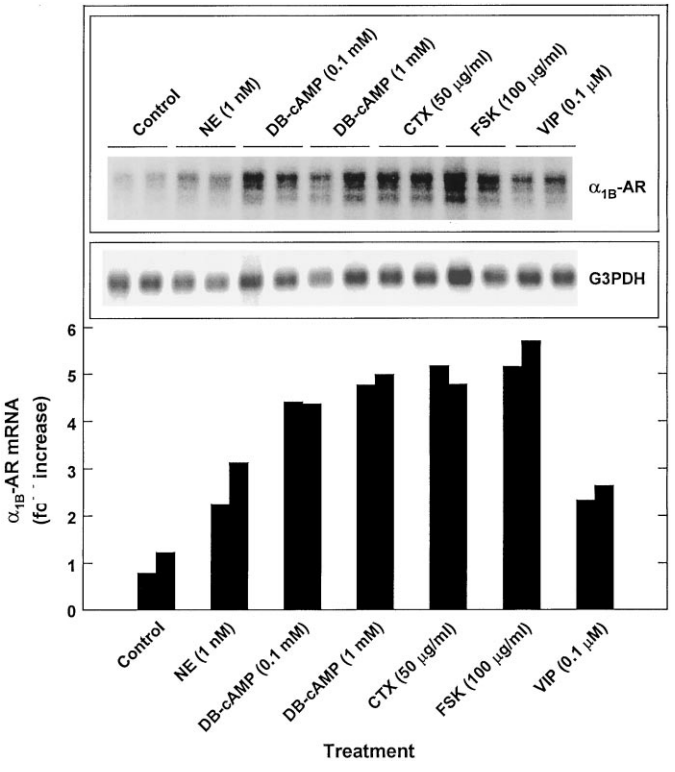

Fig. 5. α_{1B}-AR mRNA is regulated via cAMP. Northern blot analysis of α_{1B}-AR mRNA from pineal glands exposed to cAMP protagonists or VIP. Pineal glands were cultured for 6 hr with NE (1 nM), dibutyryl cAMP (*DB-cAMP*; 0.1 mM or 1.0 mM), cholera toxin (*CTX*; 50 μg/ml), forskolin (*FSK*; 100 μg/ml), or VIP (0.1 μM). Each *lane* contained 5 μg of total RNA from a pool of two pineal glands. Duplicate lanes are independent samples from one experiment that was representative of four experiments. RNA preparation and Northern blot analysis were as described for Fig. 2.

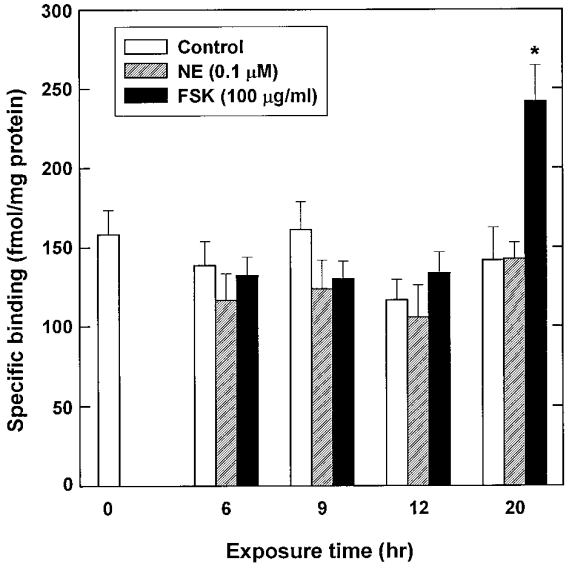

Fig. 6. Forskolin (*FSK*) increases α_{1B}-AR binding but NE has no effect. Measurement of α_{1B}-AR binding in membranes from cultured pineal glands following exposure to NE (0.1 μM) or FSK (100 μg/ml). Binding data were generated using 200 pM [^{125}I]HEAT as described in the text. Data are mean ± standard error of determinations on seven to eight individual glands.

TABLE 1

Effects of treatments with adrenergic agonists and cAMP protagonists on pineal α_{1B}-AR density

Pineal glands were treated in culture for the indicated times with the drugs. *Experiment I:* Treatment for 12 hr, binding determined on seven to eight individual glands using 50 pM ^{125}I-HEAT. *Experiment II:* Treatment for 24 hr, binding determined on two to three pairs of glands using 1 nM [^{3}H]prazosin. *Experiment III:* Treatment for 24 hr, binding determined on four to five pairs of glands using 1 nM [^{3}H]prazosin. Data are presented as the mean ± standard error, and statistical significance was tested using one-way ANOVA within each experiment.

Treatment	Specific binding		
	Experiment I	*Experiment II*	*Experiment III*
		fmol/mg protein	
Control	88.3 ± 2.6	203 ± 11	590 ± 111
NE (0.1 μM)	96.0 ± 6.2	258 ± 48	656 ± 105
Isoproterenol (0.1 μM)	n.d.	181 ± 102	595 ± 124
Phenylephrine (1 μM)	n.d.	150 ± 67	453 ± 104
Forskolin (100 μg/ml)	118 ± 11[a]	389 ± 26[a]	n.d.
VIP (0.1 μM)	92.0 ± 8.4	n.d.	n.d.

[a] $p < 0.05$ compared with control; n.d., not determined.

teristics are typical for many aspects of pineal biochemistry that are regulated by the circadian stimulation of the pineal gland via a neural circuit that includes the SCN, other central neural structures, and the superior cervical ganglia (5, 6). Light acts through the retina and a retina-to-SCN neural connection to block SCN stimulation of the pineal gland, which probably explains why pineal α_{1B}-AR mRNA does not increase in animals exposed to light at night. A prominent feature of this system is that NE is released at night from the sympathetic fibers innervating the pineal gland.

The role of NE in regulating α_{1B}-AR mRNA was demonstrated in organ culture, in which NE induced a sustained increase in α_{1B}-AR transcripts. This increase seems to be mediated by a β-AR → cAMP mechanism, because the effect could be mimicked by the β-AR agonist isoproterenol and cAMP protagonists, including dibutyryl cAMP, forskolin, cholera toxin, and VIP.

Regulation of steady state levels of α_{1B}-AR mRNA has been shown previously to be tissue- and agonist-specific and may involve multiple second messenger and transcriptional mechanisms. For example, in thyroid cells, thyrotropin, and cAMP induce a long term increase in α_{1B}-AR mRNA like that seen here in the pineal gland (28). In contrast, in vascular smooth muscle cells, NE and bradykinin transiently decrease α_{1B}-AR mRNA through a mechanism involving PKC (29). In DDT$_1$ MF-2 cells, exposure to NE results in a transient rise in α_{1B}-AR mRNA followed by a long term decrease; the initial increase is mimicked by cAMP and β-AR stimulation (19). In the same cells, long term activation of PKC, or stimulation by glucocorticoids, can cause a prolonged increase in α_{1B}-AR mRNA (30, 31). Thyroid state may either increase or decrease α_{1B}-AR mRNA in rats, depending on the tissue (32). The diversity of regulatory mechanisms may be explained partially by the presence in the α_{1B}-AR promoter of putative cAMP response elements, glucocorticoid response elements, and thyroid response elements (33–35). Additional sequences have been identified for AP-2, SP-1, and other transcription factor binding sites (33–35). The effect of cAMP in up-regulating the abundance of α_{1B}-AR mRNA could be mediated by phosphorylation of the cAMP response element binding protein, which has been shown to occur in the pineal gland (36).

It was observed that the severalfold changes in α_{1B}-AR

mRNA seen in both *in vivo* and *in vitro* studies are not accompanied by similar changes in α_{1B}-AR binding (16, this study). The fact that forskolin could increase α_{1B}-AR binding provides evidence that changes in mRNA can, under limited circumstances, be translated into changes in binding. However, the absence of a day/night rhythm in binding and of the lack of an effect of isoproterenol, cholera toxin, and VIP suggests that α_{1B}-AR protein turns over slower than α_{1B}-AR mRNA, thereby masking the mRNA rhythm. The ability of forskolin to induce changes, whereas other cAMP protagonists did not, may reflect temporal differences in their actions on cAMP.

The possibility that the constant level of receptor binding observed when glands were stimulated with NE was due to a balance between α_{1B}-AR down-regulation induced by α_{1B}-AR occupancy and α_{1B}-AR mRNA up-regulation by β-AR can be discounted because neither phenylephrine nor isoproterenol changed α_{1B}-AR binding. A lack of an agonist-induced decrease in total adrenergic receptor number also has been reported previously (37, 38). The possibility exists that increased receptor synthesis is compensated for by an unknown mechanism that is not receptor mediated. Our findings are consistent with previous evidence that a complex relationship between mRNA and receptor binding exists for many adrenergic receptors (39, 40).

These studies indicate that the pineal gland is a useful model for studying regulation of α_{1B}-AR mRNA. The discovery that large changes in mRNA are not paralleled by similar changes in binding is of special interest because it indicates that mechanisms may exist which damp out hour-to-hour changes in mRNA, thereby maintaining a relatively constant level of receptors. Future studies on regulation of α_{1B}-AR protein in this system may reveal more about the post-transcriptional processes that are involved in regulating the abundance of this receptor.

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