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Modulation of nicotinic receptor channels by adrenergic stimulation in rat pinealocytes

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**Yoon JY, Jung SR, Hille B, Koh DS.** Modulation of nicotinic receptor channels by adrenergic stimulation in rat pinealocytes. *Am J Physiol Cell Physiol* 306: C726–C735, 2014. First published February 19, 2014; doi:10.1152/ajpcell.00354.2013.—Melatonin secretion from the pineal gland is triggered by noradrenergic release from sympathetic terminals at night. In contrast, cholinergic and parasympathetic inputs, by activating nicotinic cholinergic receptors (nAChRs), have been suggested to counterbalance the noradrenergic input. Here we investigated whether adrenergic signaling regulates nAChR channels in rat pinealocytes. Acetylcholine or the selective nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) activated large nAChR currents in whole cell patch-clamp experiments. Norepinephrine (NE) reduced the nAChR currents, an effect partially mimicked by a β-adrenergic receptor agonist, isoproterenol, and blocked by a β-adrenergic receptor antagonist, propranolol. Increasing intracellular cAMP levels using membrane-permeable 8-bromoadenosine (8-Br)-cAMP or 5,6-dichlorobenzimidazole riboside-3′,5′-cyclic monophosphothioate (cBIMPS) also reduced nAChR activity, mimicking the effects of NE and isoproterenol. Further, removal of ATP from the intracellular pipette solution blocked the reduction of nAChR currents, suggesting involvement of protein kinases. Indeed protein kinase A inhibitors, H-89 and Rp-cAMPS, blocked the modulation of nAChR by adrenergic stimulation. After the downmodulation by NE, nAChR channels mediated a smaller Ca2+ influx and less membrane depolarization from the resting potential. Together these results suggest that NE released from sympathetic terminals at night attenuates nicotinic cholinergic signaling.

AT NIGHT, THE PINEAL GLAND secretes melatonin, a neuroendocrine hormone that helps to regulate activity and the sleep-wake cycle (21, 46). The pineal gland is composed of melatonin-secreting pinealocytes and small numbers of other cell types including glial-cell-like interstitial cells (31). In mammals, melatonin production is tightly regulated by norepinephrine (NE) released from sympathetic nerve fibers with parasympathetic origin in the superior cervical ganglia. NE release to pinealocytes occurs exclusively at night. Synthesis of melatonin is powerfully upregulated by Gs-coupled β-adrenergic receptors and Gq-coupled α2-adrenergic in pinealocytes (3, 49). Coincident activation of both receptors by NE increases cAMP by ~100-fold (52). In turn, cAMP induces the expression and activation of arylalkylamine N-acetyltransferase (AANAT), a rate-limiting enzyme in melatonin production (22). Morphological and immunohistochemical evidence supports parasympathetic innervation of mammalian pineal glands with perikaryal origin in the sphenopalatine and otic ganglia (25; for reviews, see Refs. 32, 35). However, in contrast to the well-characterized adrenergic sympathetic inputs, roles for cholinergic innervation and when it might be active are not yet fully understood. Acetylcholine can act on both nicotinic and muscarinic acetylcholine receptors (nAChRs and mAChRs) in pinealocytes (34, 38, 47, 53). Nicotinic cholinergic signals from the parasympathetic or the central nervous system have been suggested to have inhibitory effects on pineal melatonin synthesis (56, 58). In many cell types including central neurons, nAChRs are modulated via protein phosphorylation by second messenger-dependent protein kinases (15, 51). Protein kinase A (PKA) and protein kinase C can phosphorylate nAChRs, resulting in faster desensitization (36, 41). Physiological modulation of pineal nicotinic receptors has not been reported so far.

Considering that sympathetic inputs counter parasympathetic actions in various organs and that pineal adrenergic signals are coupled to PKA and protein kinase C (46), we hypothesized that adrenergic input would inhibit nAChRs in the rat pineal gland. Here we demonstrate that NE downregulates nAChRs via cAMP/PKA-dependent pathways and reduces the current, Ca2+ influx, and membrane depolarization mediated by nAChRs.

**MATERIALS AND METHODS**

**Preparation of rat pinealocytes.** Pinealocytes were isolated from pineal glands of male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) at postnatal weeks 5–6 as described previously (20). Animals were on a 14:10 day-night cycle and killed in the mid-morning during the light period. All procedures followed the animal use guidelines of the University of Washington and the protocol was approved by Institutional Animal Care and Use Committee. Rats were euthanized using carbon dioxide and subsequently decapitated. After removal of the brain, the pineal gland was washed with ice-cold Eagle’s balanced salt solution (Invitrogen, Carlsbad, CA). The gland was cut into a few pieces with scissors and treated with 4 mg/ml collagenase (type I; Sigma-Aldrich, St. Louis, MO) in HBSS (Invitrogen) containing 5 mM d-glucose, 20 mM HEPES, and 1 mg/ml bovine serum albumin for 35–40 min at 37°C with gentle shaking. Cell clusters were centrifuged at 180 g for 5 min and dispersed into single cells by trituration in Ca2+-free HBSS containing 1 mM EGTA, 20 mM HEPES, and 10 mg/ml bovine serum albumin. Isolated cells were resuspended in DMEM (Invitrogen) supplemented with 10 mM glucose, 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin.
100 IU/ml penicillin. The cells were plated on glass coverslips coated with 2 μg/ml of poly-o-lysine (Invitrogen) and cultured in supplemented DMEM for 1–3 days before experiments. The pinealocytes were visually identified by tiny spots on the cell surface (20).

**Patch-clamp recording and analysis.** To measure nAChR currents and the membrane potential, we used standard patch-clamp technique in the whole cell configuration (12). For most voltage-clamp experiments, pipettes were filled with an internal solution containing the following (in mM): 145 Cs-aspartate, 20 creatine phosphate, 10 EGTA, 5 HEPES, 3 Na₂ATP, 0.1 Na₃GTP, and 0.1 BAPTA pH 7.4. For some current-clamp experiments the internal solution was K⁺-based and contained the following (in mM): 137 K aspartate, 10 HEPES, 3 Na₃ATP, 0.1 Na₃GTP, and 0.1 BAPTA pH 7.4. Currents were recorded using an EPC-9 patch-clamp amplifier (HEKA Instruments; Lambrecht/Pfalz, Germany), low-pass filtered at 100 Hz, and digitized at 200 Hz using Pulse software (HEKA Instruments). Data were analyzed using Igor Pro software (WaveMetrics). As reported previously, some properties of neuronal nAChR change during the first few minutes of patch-clamp recordings, probably due to dialysis of the cytoplasm (17, 27). Therefore, we waited 3–5 min after formation of whole cell configuration before making records.

**Fast agonist application.** To avoid fast desensitization of nAChRs, agonists had to be applied quickly to measure peak current amplitudes (19). A double-barreled pipette was fabricated from theta-glass tubing (cat. no. BT-150-10; Sutter Instruments, Novata, CA) and manipulated by a piezo-electric device (Burleigh LSS-3200; Thorlabs, Newton, NJ) under computer control. The pipette was positioned close (~100 μm) to the patched pinealocyte. Solution exchange time (20–80%) was estimated as ~3 ms when measured as the junction potential change with an open patch pipette during solution changes between 10 and 100% saline solutions.

**Ca²⁺ imaging.** Intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was measured using the Ca²⁺-sensitive fluorescent dye fura-2. The cells were preincubated with 2 μM membrane-permeable fura-2 AM (Molecular Probes, Life Technologies, Grand Island, NY) in saline solution for 45 min at room temperature and further incubated without the dye for ~30 min for deesterification of the dye by cellular esterase. Single pinealocytes were imaged using an inverted microscope (TE2000-U; Nikon) equipped with a Polychrome monochromator (TILL Photonics, Graefelfingen, Germany) and an Evolve CCD camera (Photometrics, Tucson, AZ). The excitation wavelengths were 340 and 380 nm, and fluorescence images were recorded at 510 nm using Metaflour software (Molecular Devices, Sunnyvale, CA) every 2 s. Background fluorescence determined at cell-free areas was subtracted from the signal in single cells. We calculated the ratio of fluorescence at two excitation wavelengths, F₃₄₀/F₃₈₀, in regions of interest and report Ca²⁺ transients as the change of this ratio, ΔF₃₄₀/F₃₈₀, without absolute calibration. For Ca²⁺ imaging experiments, solutions were exchanged by a local perfusion system that allowed complete exchange of extracellular solutions within 0.5 s (23). These cells were not patch clamped, and therefore intracellular calcium transients were not attenuated by EGTA. All electrophysiological and Ca²⁺ measurements were made at room temperature (22–24°C).

**Chemicals.** 8-Bromoadenosine (8-Br)-cAMP and Rp-adenosine cyclic 3′,5′-phosphorothioate (Rp-cAMPS) were from Biolog Life Science Institute (Bremen, Germany), 5,6-dichlorobenzimidazole riboside-3′,5′-cyclic monophosphorothioate (cBIMPS) from Enzo Life Sciences (Farmingdale, NY), and H-89 from Calbiochem (now EMD Millipore, Billerica, MA), and all other chemicals were obtained from Sigma-Aldrich.

**Statistics.** All data are expressed as means ± SE, and n values refer to the number of analyzed cells or measurements. The statistical difference between two groups was evaluated by Student’s t-test. Probabilities of P ≤ 0.05 were considered significant.

**RESULTS**

Adrenergic signaling inhibits cholinergic receptor current in pinealocytes. Application of ACh evoked an inward ion current in every rat pinealocyte we tested with patch-clamp recording. The mean current density was large (97 ± 12 pA/pF at −60 mV; n = 22). Figure 1A shows a typical response to rapid application of 50 μM ACh at a holding potential of −60 mV. The induced inward current activated quickly, reaching a peak within 30 ms, and then, in 21 of 22 cells, desensitized gradually during 15-s ACh stimulation (n = 22). The speed of onset and the agonist and antagonist selectivity given in the next paragraph show that this response is from nAChRs. The time course of desensitization was quite variable and partial with an average time constant of desensitization of 2.5 ± 1.3 s (n = 22). There was also a slow and variable rundown of current...
during the experiment, perhaps due to dialysis into the patch pipette of components critical for maintenance of the channel activity.

We tested whether this cholinergic response could be modulated by adrenergic signaling in pinealocytes. Activation of adrenergic receptors with the physiological agonist NE gradually and substantially reduced both the peak and steady-state cholinergic currents (Fig. 1, A and B). After 2 min in NE (1 μM), the peak amplitude and the current measured at the end of 15 s ACh (I_{15s}) had decreased by 29 ± 6 and 36 ± 6%, respectively (Fig. 1C). Currents did not recover from down-regulation within 5 min.

**NE-induced modulation of nAChRs has little voltage dependence.** Next, we studied whether the modulation occurs at all membrane potentials. Since rat pinealocytes express both nicotinic and muscarinic acetylcholine receptors (46), for this and the following experiments we switched from ACh to a selective agonist dimethylphenylpiperazinium (DMPP) to activate only nAChRs. In the experiments shown in Fig. 2A, we applied 50 μM DMPP at different holding potentials. Currents reversed sign near +5 mV after junction potential correction, suggesting little selectivity between Cs⁺ and Na⁺ (PNa/Pcs = 1.2), a nonselective cation channel. The channel conducted large inward currents at negative membrane potentials and almost no outward current at corresponding positive potentials (Fig. 2B). Such strong inward rectification, typical of neuronal nAChRs, is likely due to voltage-dependent block by cytoplasmic polyamines (11). Block of the currents by a neuronal-nAChR-specific antagonist mecamylamine (Meca) confirmed that DMPP activates nAChRs selectively (33). When cells were preincubated with NE, both peak and steady-state (I_{2s}) nAChR currents were reduced at all negative membrane potentials. There was no statistically significant difference in NE-mediated nAChR inhibition measured at −80 and −40 mV (Fig. 2C). If there is any voltage dependence of this modulation, it is weak. Likewise, desensitization time constants measured by fitting a single exponential were not significantly modified by NE at −80, −60, −40, and −20 mV (n = 5).

**β-Adrenergic signaling inhibits nAChRs via cAMP-dependent phosphorylation.** Pinealocytes express two classes of adrenergic receptor (AR), α₁-AR and β-AR (3). α-ARs couple to G_s and phospholipase C, elevate intracellular Ca²⁺, and activate protein kinase C and K(Ca) channels, whereas the β-ARs elevate cAMP and cGMP (26, 46, 49, 59). We identified the adrenergic signal pathway to nAChRs using selective agonists and antagonists.

The β-AR agonist isoproterenol partially mimicked the NE effect. A 2-min application of 1 μM isoproterenol reduced the DMPP-evoked nAChR current (I_{peak} by 19 ± 5% and I_{2s} by 31 ± 6%; Fig. 3, A and B). By comparison, 1 μM NE was somewhat more effective, reducing the currents by 38 ± 5 and 46 ± 5% respectively (Fig. 3B). We tested the involvement of cAMP signaling using membrane-permeable 8-Br-cAMP and the phosphodiesterase-resistant cAMP analog cBiMPS to activate PKA. Each analog applied alone decreased the nAChR current (Fig. 3, Ab, Ac, and B). Thus a 2-min application of

**Fig. 2. Voltage-independent modulation of nAChR currents by NE: A: currents activated by 50 μM dimethylphenylpiperazinium (DMPP) without (control), with 1 μM NE, or with 10 μM mecamylamine (Meca) at membrane potentials between −80 and +60 mV in 20-mV steps. All records were from the same cell. A: current-voltage (I-V) relationships of I_{max} (left) and I_{2s} (right) in control (circles), with NE (squares), or with Meca (triangles). Currents were normalized to the control peak current at −80 mV. Each point is the average of 4–6 cells. C: average inhibition of nAChR currents at −80 and −40 mV. N.S.: not significantly different between recordings at −80 mV and −40 mV (α = 5; P > 0.05).**
8-Br-cAMP reduced $I_{\text{peak}}$ by $26 \pm 2\%$ and $I_{2s}$ by $30 \pm 6\%$, and a pretreatment with 8-Br-cAMP occluded further inhibition of nAChRs by isoproterenol (Fig. 3B). In addition, the isoproterenol-induced nAChR inhibition could be prevented by incubating cells with PKA-inhibitors Rp-cAMPS and H-89 (Fig. 3B). Similarly, internal perfusion of cells with an ATP-free pipette solution containing nonhydrolyzable AMP-PCP prevented nAChR modulation (Fig. 3Ad). Overall, these results suggest that adrenergic inhibition of nAChRs largely uses the β-AR pathway, cAMP, and PKA.

An earlier study (52) found that high concentrations of isoproterenol may act on α1-AR as well as on β-AR in pinealocytes. Therefore, it was important for us to guard against this possible artifact as follows. We always used a lower isoproterenol concentration (1 µM) that was reported not to affect α1-ARs (52). Control experiments showed that in the presence of the β-AR antagonist propranolol, isoproterenol was prevented from inhibiting nACh current (Fig. 3B), whereas the α1-AR antagonist doxazosin (10 nM) had no effect on the inhibition. Tests on intact pinealocytes (no pipette) revealed no Ca$^{2+}$ elevations during application of isoproterenol, and all whole cell experiments included 10 mM EGTA in the pipette solution to depress any Ca$^{2+}$ signaling. Thus we attribute actions of isoproterenol seen here only to its agonist actions at β-ARs.

**Downregulation of nAChRs reduces Ca$^{2+}$ influx into pinealocytes.** It was previously reported that ACh and NE increase the intracellular Ca$^{2+}$ concentration in pinealocytes (28, 44). The adrenergic response was attributed to α1-AR stimulation of Ca$^{2+}$ release from internal stores followed by additional store-operated calcium entry (26, 49). Using Ca$^{2+}$ imaging with AM-loaded fura-2, we confirmed the Ca$^{2+}$ rises. In these experiments there was no whole cell pipette or dialysis with EGTA. The transient increments of fluorescence ratio $F_{340}/F_{380}$ with 50 µM ACh and with 1 µM NE were $3.7 \pm 0.8$ and $8 \pm 0.2$, respectively (Fig. 4). Here we focus on the cholinergic response and ask whether Ca$^{2+}$ entry is mediated by nAChR channels and whether it is modulated by β-adrenergic signals.

In the first set of Ca$^{2+}$ experiments, we activated nAChRs specifically with DMPP (Fig. 5A, top and left trace). Repeated applications of the agonist evoked repeated Ca$^{2+}$ transients.
The ΔF340/F380 of the first Ca²⁺ peak was 3.1 ± 0.3 (n = 11). The peak amplitude was slightly reduced in successive applications (by 9 ± 2 and 14 ± 5% for the 2nd and 3rd DMPP application; Fig. 5B). Application of isoproterenol to activate β-ARs significantly attenuated the subsequent DMPP-induced Ca²⁺ increase (Fig. 5A, bottom trace, and Fig. 5B for summary). The inhibitory effect was slightly but significantly reversed by 5 min of washing (P < 0.01), but after the isoproterenol treatment the third response remained significantly different from that without isoproterenol treatment (P < 0.05) due to incomplete recovery.

Open nAChR channels might initiate Ca²⁺ entry in two ways. They should pass a small Ca²⁺ influx and would also depolarize the membrane potential, potentially activating voltage-gated Ca²⁺ channels (28, 57). β-AR stimulation may also have multiple effects on Ca²⁺ signaling. In addition to downregulating nAChR channels, it can modulate several types of voltage-gated Ca²⁺ channels (7, 8), mACHR-mediated Ca²⁺...
release (29), and store-operated calcium entry (26). To study the modulatory effect of β-AR on the nAChR-mediated Ca²⁺ influx in isolation, we repeated the same experiments with a cocktail of 10 μM nifedipine (to block L-type voltage-gated Ca²⁺ channels) and 1 μM atropine (to block muscarinic receptors that might evoke Ca²⁺ release from the stores and activate store-operated calcium entry; Fig. 5, C and D). As expected, in a control experiment, nifedipine completely blocked the Ca²⁺ transient evoked by direct membrane depolarization with 50 mM extracellular K⁺ (n = 4, data not shown). Ca²⁺ transients mediated by DMPP were also reduced with nifedipine and atropine (ΔF₃₄₀/F₃₈₀ = 1.1 ± 0.1; n = 9; Fig. 5C), but pretreatment with isoproterenol still reduced the Ca²⁺ transients further (by 63%, n = 9 compared with control, n = 28; Fig. 5C, bottom trace, and Fig. 5D). Thus, like the inward current, direct Ca²⁺ influx through nAChR channels is significantly reduced by β-AR activation in rat pinealocytes.

Similarly we tested whether voltage-gated Ca²⁺ channels (VGCC) are modulated by isoproterenol (Fig. 5, E and F). We measured Ca²⁺ transients evoked directly by KCl-depolarization. The Ca²⁺ rise induced by 20 mM KCl was 1.9 ± 0.3 (ΔF₃₄₀/F₃₈₀, n = 12) and did not show significant run-down during subsequent stimulation. The second Ca²⁺ signal was 97 ± 7% of the first. Pretreatment with 1 μM isoproterenol decreased the Ca²⁺ rise significantly (49 ± 5% compared with the first Ca²⁺ rise and significantly different from the second KCl of control cells; P < 0.01; n = 19). Thus VGCC also can be negatively modulated by cAMP in pinealocytes.

Adrenergic modulation of nAChRs reduces membrane voltage responses of pinealocytes. Activating the nAChR nonselective cation channels depolarizes pinealocytes (28). We tested whether NE attenuates the nicotinic membrane depolarization. The resting membrane potential (Vₑ) measured with the current-clamp configuration of the patch-clamp technique and a K⁺-based pipette solution (Fig. 6A) was −49 ± 3 mV (n = 15). This value becomes about −59 mV after correction for the electrode potential, more negative than in previous reports (1, 6, 20, 28, 59) for two reasons. First, most authors did not correct for junction potentials, and second, unlike the others, our internal solution contained no chloride and thus could induce membrane hyperpolarization if pinealocytes have a resting chloride conductance. When cells were treated with 10 μM DMPP, Vₑ depolarized as expected (by 20 ± 3 mV; n = 3). Addition of NE did not change Vₑ by itself, but it did attenuate the DMPP-induced depolarization. This attenuation...
was large and statistically significant for low concentrations of DMPP (5 μM) but less clear for high concentrations (50 μM; Fig. 6B).

To understand the voltage responses to DMPP and the effect of NE better, we had to explore the electrical properties of pineocytes further by injecting small hyperpolarizing and depolarizing test currents (Fig. 6C). Injecting hyperpolarizing current gave large negative changes of \( V_{m} \) in accordance with the high resting membrane resistance (∼1.8 GΩ in Fig. 6C; 1–2 GΩ in Refs. 1, 20) and reflecting the absence of many open ion channels at rest. Injecting small depolarizing currents depolarized \( V_{m} \), but larger currents failed to depolarize \( V_{m} \) beyond −20 mV (Fig. 6D, symbols; after correction for electrode junction potential, this would become closer to −30 mV). It is apparent that other ion channels become active that oppose further membrane depolarization. As shown before with voltage-clamp experiments, depolarization to −20 mV activates several voltage-gated K+ channels that are highly expressed in pineocytes (1, 6, 20). Their large outward current would counteract the applied depolarizing current, establishing a ceiling on the evoked depolarization. Quite likely, these potassium channels limit the normal physiological operating voltage range of pineocytes to values negative of −20 mV (59). The voltage-current relations of Fig. 6D (symbols) also show that by opening nAChR channels, DMPP decreases the resting resistance (slope of the \( V_{m} \) curve), and again the depolarization it induces is limited by potassium channels at −20 mV. Thus, as we saw in Fig. 6B, at high DMPP concentrations, where many nAChR channels are opened and the membrane potential is near its “ceiling,” a fractional reduction of functional nAChR channels by adrenergic modulation would have only a small impact on the DMPP-induced depolarization. This ceiling effect was absent when K currents were precluded by replacing all K+ in the patch pipette with Cs+ (dashed and solid lines).

DISCUSSION

We have described functional properties of the nAChRs of rat pineocytes and their downregulation by adrenergic signaling. The nAChRs of pineocytes have functional properties typical of neuronal nAChR subtypes including strong inward rectification, desensitization (5, 37), block by Meca, a blocker of ganglionic and brain nAChRs (33), and block by α-tubocurarine but not α-bungarotoxin (13, 28). In agreement, published immunoprecipitation, RNAase protection assays, and gene-chip analysis have identified the pineal nAChRs as the neuronal α3β2-subunit subtype (3, 13). The gene chips show that these subunits are expressed >16-fold higher in pineal than in several other brain regions (3). Accordingly, the current density we see from nAChRs in rat pineocytes is remarkably high, especially considering the high resting resistance of these cells. Therefore, ACh released from cholinergic nerve terminals could evoke enough inward current through nAChRs to depolarize the membrane and activate voltage-gated Ca2+ channels. Since nAChRs themselves are also appreciably Ca2+-permeable (28, 56, and Figs. 4 and 5), this cholinergic system could initiate robust Ca2+ signal generation in pineocytes. However, depolarizing membrane excitation by nAChRs would be capped at membrane potentials approaching −20 mV (or −30 mV) by the activation of counteracting K+ conductances (Fig. 6). This ceiling would restrict firing of action potentials in pineocytes. In agreement, we observed no action potentials in our current-clamp experiments even with strong current injection. There are repeated older reports of action potentials seen with extracellular recording in pineal glands of anesthetized animals (39, 40, 48). We favor the hypothesis that these originate from nerve fibers terminating in the pineal rather than from the pineocytes themselves. Indeed, some of the single units recorded were reported to be driven directly by shocks to the habenula (40). Nevertheless, the opposite view is common, and more clarification may still be needed.

We implicate an adrenergic pathway involving cAMP and PKA in downregulation of nAChRs. nAChRs can be phosphorylated by PKA, protein kinase C, and protein tyrosine kinases (16, 54). Such phosphorylation is likely to underlie the modulation we report here. In published experiments on neuronal nAChRs, indirect evidence suggests a speeding of receptor desensitization after stimulation of PKC (16) and more direct evidence suggests a slowing of desensitization with chronic increase of cAMP (9). In our experiments, cAMP and PKA signals evoked by NE decrease the evoked current in nAChRs. They also may accelerate receptor desensitization slightly during several-second DMPP treatment since the inhibition of current is more prominent on \( I_{2} \), than \( I_{peak} \) (Figs. 2 and 3). This effect is small. The direction of change may be affected by other accessory proteins, such as 14-3-3 proteins, which are known to interact with some nAChR α subunits in a PKA-dependent manner and to control membrane targeting of the α subunit.
subunit proteins. Tonic activation of cAMP-PKA signaling may change the stoichiometry of α- and β-subunit expression and channel properties (24). This may explain why Di Angelantonio et al. (9) saw slowing of receptor desensitization with chronic treatments whereas we saw slight speeding with an acute treatment.

The key biochemical and physiological roles of sympathetic input to the pineal are well understood (Fig. 7). NE released as a night signal from varicosities and endings of superior cervical ganglion sympathetic neurons initiates new gene expression and synthesis and release of melatonin (46). Thus nocturnal NE governs the nocturnal endocrine secretion of the pineal. We have considered possible antagonism between adrenergic and cholinergic signaling in pinealocytes. Here we showed that NE reduces the depolarization and calcium elevation responses to nicotinic agonists in the rat pineal. Previous work also showed that ACh reduces the release of NE from the nerve terminals of sympathetic ganglion neurons (10, 23; reviewed by Ref. 4) and the synthesis of melatonin (see below). Such reciprocal interactions between sympathetic and cholinergic inputs could fine tune nocturnal melatonin synthesis. In our experiments, the pinealocytes were initially in what we call a “neutral” condition that is probably most related to the daylight portion of the diurnal cycle since they had been incubated without NE for more than 24 h (3). The very brief exposure to NE could be like the very first minutes of a night signal. These few minutes would be quite insufficient to accomplish the 1–2 h program of new gene expression that marks a full transition to night state (46).

The findings here would be of greatest physiological interest if we knew more about cholinergic input into the pineal gland and its possible circadian activity profiles. Previous work shows that the pineal glands of several mammals contain acetylcholine (by HPLC), choline acetyl transferase (by immunoreactivity), vesicular acetylcholine transporter (by immunoreactivity) (35, 42, 55) with some reported exceptions (e.g., Ref. 45), and nerve fibers staining for cholinergic markers (31, 35, 50). Further, as already discussed, pinealocytes express functional nAChRs abundantly and mAChRs. The amounts of ACh and of vesicular ACh transporter show 10-fold diurnal cycling in rat pineal (55). Cholinergic nerve fibers in the pineal seem to originate both peripherally (parasympathetic) and centrally (e.g., habenula). Many peptides that are candidate cotransmitters in parasympathetic fibers are present in the pineal, and the nerve terminals and varicosities remaining after lesioning the superior cervical ganglia show a vesicular morphology that is parasympathetic like (31, 35). However, we cannot yet point to specific fiber pathways and describe when they might be active.

Figure 7 summarizes some acute signaling pathways in the pineal gland. Cholinergic signals from the parasympathetic or central nervous systems (31) may have inhibitory effects on pineal melatonin synthesis. An inhibitory action of ACh through nAChRs has been described (18, 56, 58). In rat pinealocytes, acetylcholine-activated nAChRs depolarize the membrane, open L-type voltage-gated Ca2+ channels, and ultimately trigger glutamate release. The paracrine secreted glutamate is postulated to decrease cAMP via inhibitory G,-coupled type-3 metabotropic glutamate receptors on nearby pinealocytes and to suppress NE-induced melatonin biosynthesis (for review, see Ref. 43). In addition, acetylcholine acting on muscarinic receptors also inhibits melatonin synthesis both through presynaptic inhibition of NE release from sympathetic terminals and by inhibiting the enzyme arylalkylamine N-acetyltransferase in rat pinealocytes (10, 34). Thus ACh is a candidate “day signal” or a signal to terminate the “night” actions of NE, sharpening the night-to-dawn transition. NE, acting as a night signal, not only stimulates melatonin synthesis but also antagonizes cholinergic actions, perhaps thus sharpening the transition from dusk-to-night.

Convergence and antagonism of adrenergic and cholinergic inputs to several areas of the central and peripheral nervous systems have been postulated based on anatomical and functional assays (2, 14, 30). In this study, we clearly demonstrate potential cross talk between sympathetic and cholinergic inputs to rat pinealocytes by studying modulation of nAChR by β-adrenergic receptors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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