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ALPHA-ADRENERGIC POTENTIATION OF BETA-ADRENERGIC STIMULATION OF RAT PINEAL *N*-ACETYLTRANSFERASE

STUDIES USING CIRAZOLINE AND FLUORINE ANALOGS OF NOREPINEPHRINE

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Abstract—Recent evidence indicates that melatonin production is controlled by norepinephrine acting via alpha$_1$-and beta$_1$-adrenoceptors on pinealocytes; activation of alpha$_1$-adrenoceptors appears to potentiate the effects of beta$_1$-adrenoceptor activation. However, alpha-adrenergic potentiation of beta$_1$-adrenergic activation has been demonstrated with only one alpha-adrenergic agonist. For this reason, this issue was reinvestigated using two other alpha-adrenergic agonists, 6-fluoronorepinephrine and cirazoline. Both compounds, which were found to have a high affinity for pineal alpha$_1$-adrenoceptors, potentiated the stimulatory effects of isoproterenol on pineal *N*-acetyltransferase. 6-Fluoronorepinephrine also potentiated the stimulation of *N*-acetyltransferase activity produced by another beta-adrenergic agonist, 2-fluoronorepinephrine. These findings support the hypothesis that pineal *N*-acetyltransferase activity is regulated by norepinephrine acting through both alpha$_1$- and beta$_1$-adrenoceptors.

Norepinephrine (NE§) released from sympathetic nerve terminals produces a 30- to 70-fold nocturnal increase in the activity of rat pineal serotonin *N*-acetyltransferase (NAT) [1]. This increase causes the large nocturnal increase in pineal and circulating melatonin [2].

The mechanism through which NE acts has been thought to be purely beta-adrenergic [1, 3]. However, pineal alpha$_1$-adrenoceptors have been identified [4], and recent evidence from studies using adrenergic agonists and antagonists indicates that NE probably acts through both alpha$_1$- and beta$_1$-adrenergic receptors [5]. Occupation of alpha$_1$-adrenoceptors by adrenergic agonists appears to potentiate, by a yet undescribed mechanism, the beta-adrenergic stimulation of NAT. However, in the original studies potentiation was demonstrated with only a single alpha-adrenergic agonist, phenylephrine (PE).

In the present study we have reinvestigated the alpha$_1$- and beta$_1$-adrenergic regulation of pineal *N*-acetyltransferase using three drugs not used previously: two fluorine analogs of NE, 6-fluoronorepinephrine (6-FNE) and 2-fluoronorepinephrine (2-FNE), and cirazoline (CIR). Fluorine substitution of NE at the 6-position reduces beta-adrenergic activity of NE, without reducing the alpha-adrenergic activity; 2-fluoro substitution has the opposite effect, resulting in a relatively pure beta-adrenergic agonist [6]. CIR is a strong alpha$_1$-adrenergic agonist in other tissues and has high affinity for the pineal alpha$_1$-adrenoceptor [4, 7]. The results from these studies confirm that NE regulates NAT activity through synergistic actions on alpha$_1$- and beta$_1$-adrenoceptors.

MATERIALS AND METHODS

The animals used in this study were 200 g male Sprague–Dawley rats (Zivic–Miller, Allison Park, PA), which had been in our facilities (L:D 14:10, lights on at 5:00 a.m.) for 2 weeks. Pineal glands were cultured for 48 hr before testing as described previously [8]. Agonists were dissolved in sterile water immediately before use and diluted in media to give the required concentrations. Glands were transferred to dishes containing diluted agonists and cultured for 6 hr. *N*-Acetyltransferase activity was measured using a radiometric method [9]. Alpha$_1$-adrenoceptors on pineal membranes were analyzed using the alpha$_1$-adrenoceptor radioligand [^{125}I]-iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]

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§ Abbreviations: NE, norepinephrine; 6-FNE, 6-fluoronorepinephrine; 2-FNE, 2-fluoronorepinephrine; [^{125}I]HEAT, [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone; CIR, cirazoline; NAT, serotonin *N*-acetyltransferase; PE, phenylephrine; and ISO, isoproterenol.

tetralone ([^{125}I]HEAT) [4]. Membranes were prepared from glands that had been cultured for 48 hr in control medium. IC_{50} Values in competition experiments were determined from log-logit plots, and K_i values were calculated using the Cheng–Prusoff equation [10]. The synthesis of fluoro analogs of NE and the sources of the drugs used have been reported [5, 11]. The *dl*-isomers of all asymmetric compounds were used, except for (−)-norepinephrine and (−)-isoproterenol.

RESULTS

Inhibition of [^{125}I]*HEAT binding to pineal alpha$_1$-adrenoceptors* (Fig. 1). 6-FNE, 2-FNE, NE and CIR competitively inhibited the binding of [^{125}I]HEAT to pineal membranes. The relative potency was CIR > 6-FNE > NE ≫ 2-FNE. 6-FNE was almost 100-fold more potent a competitor than was 2-FNE for the alpha$_1$-adrenoceptor. In contrast, prior experiments using a beta$_1$-adrenergic radioligand established that 2-FNE was about 20-fold more potent a competitor than was 6-FNE for the pineal beta$_1$-adrenoceptor [6].

Stimulation of N-acetyltransferase activity by adrenergic agonists. The dose–response curves for the stimulation of pineal *N*-acetyltransferase activity by NE and fluoro derivatives of NE are presented in Fig. 2. 6-FNE was markedly less potent than NE, and 2-FNE was intermediate in potency. At concentrations of 10^{-6} and 10^{-7} M, a 1:1 combination of 2-FNE and 6-FNE resulted in a greater response than that produced by either compound alone, or by the sum of their individual effects.

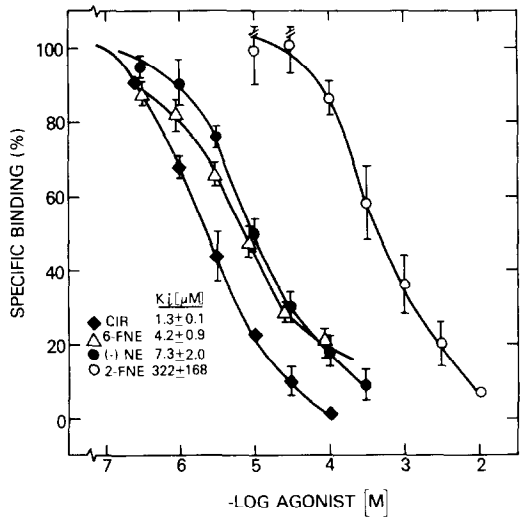

Fig. 1. Competition for [^{125}I]HEAT binding sites by adrenoceptor agonists. Rat pineal membranes (10 μg) were incubated (30°; 15 min) with [^{125}I]HEAT (100 pM) and increasing concentrations of the adrenergic agonists indicated. Specific binding was determined as described [4] and expressed as a percentage of the specific binding determined in the absence of added drug. Each point represents the mean ± S.E.M. from three or four determinations, each of which was run in duplicate. K_i values were calculated using the Cheng–Prusoff equation [10].

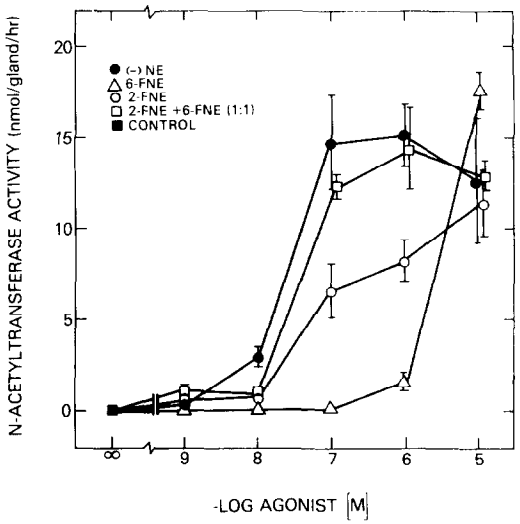

Fig. 2. Dose–response curves for the *in vitro* stimulation of pineal NAT activity by NE, 6-FNE, 2-FNE or 6-FNE + 2-FNE. Pineal glands were cultured for 48 hr prior to treatment and then transferred to fresh medium containing the agonist(s) at the indicated concentration. Glands were treated for 6 hr and then frozen in tubes placed on solid CO_2. Each value is the mean ± S.E.M. of four pineal glands. The absence of an error bar indicates that the S.E.M. fell within the area covered by the symbol.

In a similar experiment the effect of 6-FNE on isoproterenol (ISO) stimulation of NAT was determined (Fig. 3). 6-FNE (10^{-7} M and 3×10^{-7}) potentiated ISO (10^{-9} M) stimulation of NAT. However, 6-FNE at a lower concentration (10^{-8} M) did not potentiate ISO stimulation, and a higher concentration (10^{-6} M) gave a stimulation which was not significantly greater than 6-FNE alone. The effect of this high concentration (10^{-6} M) was probably due to beta-adrenergic activity retained by 6-FNE because (−)-propranolol (10^{-5} M) blocked the stimulation of NAT (data not shown).

The effect of CIR on ISO stimulation of NAT was also investigated (Fig. 4). Treatment with CIR (3×10^{-7} M) and ISO (10^{-9} M) produced an NAT response which was about 3-fold greater than the sum of the responses produced by each compound individually. Evidence of potentiation was also apparent at a higher (10^{-6} M) concentration.

DISCUSSION

These results provide additional evidence that pineal NAT activity can be stimulated through a combined action on alpha$_1$- and beta$_1$-adrenoceptors. Previous studies with the alpha- and beta-adrenoceptor agonists, PE and ISO, indicated that alpha-adrenergic stimulation potentiates beta-adrenergic stimulation of NAT activity [5]. Similarly, the present results show that the stimulatory effect of a beta-adrenergic agonist, 2-FNE, on NAT activity was potentiated by 6-FNE, an alpha-adrenergic agonist. Notably, 6-FNE was effective at concentrations which by themselves produced only a small change in enzyme activity. 6-FNE also potentiated the

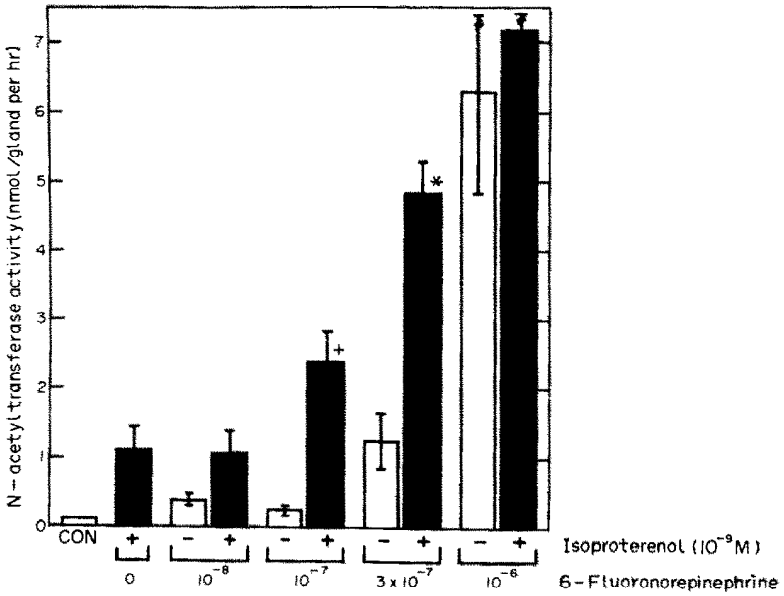

Fig. 3. Effect of 6-FNE on the *in vitro* stimulation of pineal NAT activity by ISO (10^{-9} M). For details see the legend to Fig. 2. Each value represents the mean ± S.E.M. of four to six pineal glands, except the control group (N = 3). Key: (+) significantly different from the sum of isoproterenol (10^{-9} M) plus 6-FNE (10^{-7} M), ($P < 0.05$); and (*) significantly different from the sum of isoproterenol (10^{-9} M) plus 6-FNE (3×10^{-7} M), ($P < 0.05$).

effects of ISO on NAT activity, as did the potent alpha$_1$-adrenergic agonist, CIR. Thus, from this and our previous study it is clear that alpha-adrenergic agonists can be shown to potentiate the effects of beta-adrenergic agonists on pineal NAT.

Interestingly, when the alpha-adrenergic agonists were used alone at a high concentration, each was able to elevate NAT activity. This probably reflects a weak beta-adrenergic activity of these compounds [6, 12], which restricts the dose range over which the potentiation phenomenon can be observed. High doses of these alpha-adrenergic agonists which have beta-adrenergic actions are simply additive with effects of a beta-adrenergic agonist. This also makes it difficult to correlate the alpha$_1$-adrenoceptor affinity of these agonists and their potency in potentiating beta-adrenergic induction of NAT activity.

The occurrence of alpha + beta potentiation is of

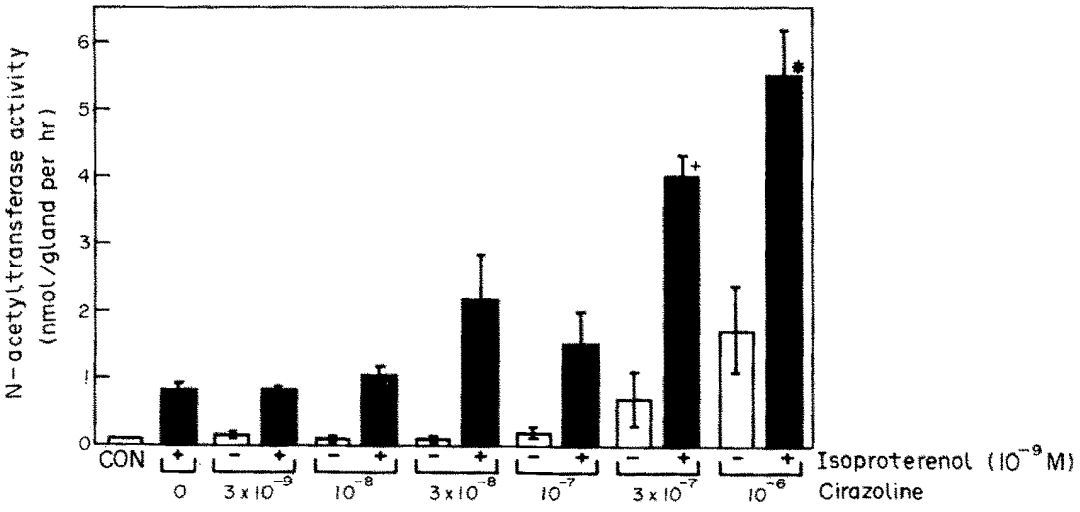

Fig. 4. Dose–response curve for the effect of CIR on the *in vitro* stimulation of NAT activity by ISO (10^{-9} M). For details see the legend to Fig. 2. Each value represents the mean ± S.E.M. of four pineal glands. Key: (+) significantly different from the sum of isoproterenol (10^{-9} M) plus cirazoline (3×10^{-7} M) ($P < 0.05$), and (*) significantly different from the sum of isoproterenol (10^{-9} M) plus cirazoline (10^{-6} M) ($P < 0.05$).

obvious physiological relevance if one considers that NE, the neurotransmiter controlling the daily rhythm in NAT, has both alpha- and beta-adrenergic agonist activity. Previously we showed, using $alpha_1$- and $beta_1$-adrenoceptor blockers, that NE induction of NAT in pineals maintained in culture involved both alpha-and beta-adrenoceptor components. The new evidence presented here using 2-FNE and 6-FNE leaves little doubt that, in the rat, pineal melatonin production is regulated by NE acting through $alpha_1$- and $beta_1$-adrenoceptors. As previously proposed, it appears that an $alpha_1$-adrenoceptor mediates the potentiation of $beta_1$-adrenergic stimulation of NAT.

The mechanism through which potentiation occurs is an issue of central importance to understanding cellular regulation of melatonin synthesis. Cyclic AMP is generally thought to control NAT activity; combined treatment of pineal glands with an alpha- and a beta-adrenergic agonist (PE and ISO) produces a higher elevation of cyclic AMP than either agonist alone (unpublished observation). Beta-adrenoceptors are known to control pineal adenylate cyclase [13]; perhaps alpha-adrenoceptor stimulation potentiates beta-adrenergic activation of pineal adenylate cyclase activity or inhibits cyclic nucleotide phosphodiesterase activity, or both. Alpha-adrenergic stimulation in the pineal gland, as in other tissues, is known to increase the turnover of membrane phospholipids [14, 15]. Perhaps this response and the probable increase in intracellular calcium which may accompany it [16] are involved in controlling the changes in cyclic AMP. This possibility gains some interest from the report that the NAT response to adrenergic stimulation is reduced *in vitro* by removing calcium from the culture medium [17].

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