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Superinduction of Serotonin N-Acetyltransferase and Supersensitivity of Adenyl Cyclase to Catecholamines in Denervated Pineal Gland

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SUMMARY

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Norepinephrine or isoproterenol injected into rats increases serotonin *N*-acetyltransferase (EC 2.3.1.5) activity many fold in the pineal gland. One or two days after denervation by bilateral ganglionectomy, norepinephrine produced a 5-10-fold higher increase in *N*-acetyltransferase activity than in intact pineals. When low doses of isoproterenol are injected, *N*-acetyltransferase activity in denervated pineals increases much more than in intact pineals, while high doses result in similar increases in *N*-acetyltransferase activity in intact and denervated pineals. Intact pineals cultured in the presence of norepinephrine show maximal increases in *N*-acetyltransferase activity at about 10 μM, whereas maximal increases are observed in denervated pineals at 20 nM norepinephrine. Isoproterenol (0.1-1 μM) causes a maximal increase in enzyme activity in intact pineals, whereas 4 nM isoproterenol increases *N*-acetyltransferase activity to the maximal level in denervated pineals. The concentration of catecholamines in culture required for the maximal increase in *N*-acetyltransferase activity differs by almost 100-fold for norepinephrine and by 20-fold for isoproterenol between intact and denervated pineals. Maximal enzyme activity, however, is the same in both intact and denervated pineals. The elevation of adenosine cyclic 3',5'-monophosphate levels in denervated pineals greatly exceeds that in intact pineals when submaximal doses of either norepinephrine or isoproterenol are injected into rats. When pineals are cultured in the presence of dibutyryladenosine cyclic 3',5'-monophosphate, there is no difference in the increase of *N*-acetyltransferase activity between intact and denervated pineals. These results indicate that denervation induces rapid supersensitivity in the postsynaptic *beta* adrenergic receptor site on the pineal cell to catecholamines, which in turn enhances the elevation of adenosine cyclic 3',5'-monophosphate and results in the superinduction of *N*-acetyltransferase in the pineal gland.

INTRODUCTION

During the past 70 years a number of studies have demonstrated that denervation of adrenergically innervated end organs (iris, nictitating membrane, vas deferens, and heart) results in supersensitivity to sympathomimetic amines. Based on a considerable number of observations, various hypotheses have been proposed to account for the mechanisms involved in the development of supersensitivity after denervation (1, 2).

Recently we have shown that injection of

β-(3,4-dihydroxyphenyl)-L-alanine (L-dopa), the precursor of catecholamines, increased the activity of serotonin *N*-acetyltransferase (EC 2.3.1.5) in rat pineal 20–30-fold *in vivo*. After surgical or chemical denervation there was a marked increase in *N*-acetyltransferase activity with L-dopa, which was completely blocked by *beta* adrenergic blocking agents or by the protein synthesis inhibitor cycloheximide (3). A greater increase in *N*-acetyltransferase activity in denervated rat pineal (superinduction) would be comparable to the denervation supersensitivity observed with contraction of smooth muscle (1, 2). It has been clearly established that *N*-acetyltransferase activity in pineal is controlled by a neurotransmitter released from sympathetic nerves originating in the superior cervical ganglion. The neurotransmitter stimulates a *beta* adrenergic receptor on the pineal cell, which in turn activates the adenylate cyclase system (3–8). Thus pineal *N*-acetyltransferase offers a productive model for studying the mechanism underlying supersensitivity in adrenergically innervated end organs.

Weiss (9) has reported that after chronic denervation adenylate cyclase in rat pineals became more sensitive to both norepinephrine and sodium fluoride, and concluded that the amount of adenylate cyclase was increased after denervation of the pineal. However, the superinduction of *N*-acetyltransferase appeared 24 hr after ganglionectomy (3), whereas the increase in adenylate cyclase activity was not manifest until 4 weeks after denervation (9). The present report describes the relationship between superinduction of *N*-acetyltransferase and enhanced elevation of adenosine cyclic 3′,5′-monophosphate after denervation of rat pineal.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 160–180 g were supplied by Hormone Assay Laboratories, Chicago, and were kept under diurnal lighting conditions with light on from 6:00 a.m. to 6:00 p.m. Drugs were dissolved in 0.9% NaCl and injected subcutaneously. Rats were killed by decapitation between 2:00 and 3:00 p.m. The rats for pineal culture were killed between 9:00 and 10:00 a.m. Bilateral ganglionectomy of the superior cervical ganglion was performed under ether anesthesia. Ptosis was taken as a measure of the success of the operation. Rats were used between 6 and 7 days after surgery unless otherwise indicated.

Pineal culture. After decapitation of the rats, the pineals were quickly and aseptically removed and cultured by a slight modification of the method of Klein and Weller (10). Four or five pineals were placed in a sterile plastic dish (5 cm in diameter) with 2.5 ml of BGJb medium (Grand Island Biological Company) containing ascorbic acid (0.1 mg/ml), glutamine (2 mM), streptomycin (100 μg/ml), and penicillin (100 units/ml). Pineals were cultured for 10 hr at 37° under 95% O_2–5% CO_2.

Assay of serotonin N-acetyltransferase activity. A pineal was quickly removed and *N*-acetyltransferase activity was assayed by the method previously described (11), but with 20 nmoles of [1-^{14}C]acetyl coenzyme A (Amersham/Searle, 5.8 mCi/mmole) instead of the 3.4 nmoles of [1-^{14}C]acetyl coenzyme A used in the original assay.

Assay of cyclic AMP. A pineal was quickly removed, frozen on Dry Ice, and homogenized in 0.7 ml of 5% trichloracetic acid with a 1-ml glass homogenizer. After the precipitate had been removed by centrifugation, the supernatant fluid was extracted four times with 5 ml of ether. The aqueous layer was evaporated to dryness under vacuum, and the residue was dissolved in 300 μl of 0.01 M sodium acetate (pH 4.0). Cyclic AMP concentrations were measured by the method of Gilman (12), using 25–200 μl of sample solution. Under the assay conditions employed here linearity was observed between 5 and 50 pmoles of cyclic AMP. The recovery of cyclic AMP during the procedure was checked by adding cyclic [^{3}H]AMP, and the results were corrected for a recovery of 80%. To study the nonspecific interference of tissue extracts in the binding assay, pineals (both untreated and drug-treated glands) were homogenized with 0.35 ml of distilled water, heated at 95° for 5 min, and centrifuged. To the supernatant fraction, 80 μg of phosphodiesterase (0.385 unit/mg, Sigma), 0.2

μmole of potassium phosphate (pH 7.4), and 0.02 μmole of $MgCl_2$ were added, and the mixture was incubated at 30° for 30 min. After incubation, 0.35 ml of 10% trichloracetic acid was added, and cyclic AMP was extracted as described above. The tissue blank thus obtained was equivalent to 3–5 pmoles/pineal, which was duly subtracted. In several experiments the tissue extract was purified on a column of Dowex 50 by the method of Krishna *et al.* (13), and the eluate was evaporated and assayed for cyclic AMP content. The level of cyclic AMP in purified samples was slightly lower than in the unpurified samples, which would correspond to the tissue blank. It was also ascertained that none of the drugs employed (catecholamines, adrenergic blockers, or protein synthesis inhibitor) interfered with the binding assay when added to the reaction mixture at very high concentrations. Although Weiss and Strada (14) showed that the cyclic AMP level increased in rat pineal after decapitation, the effect of decapitation should not affect the interpretation of the observations reported here, since the pineals were frozen within 30 sec after decapitation. There was no significant difference in the baseline level of cyclic AMP among intact, propranolol-treated, and denervated pineals (15).

RESULTS

Superinduction of N-acetyltransferase in vivo. Serotonin *N*-acetyltransferase activity was induced in rat pineals by injection of isoproterenol or L-dopa and reached the maximal level 3 hr after injection (3, 15). Norepinephrine increased *N*-acetyltransferase activity 2-fold at a dose of 1.0 mg/kg and 17-fold at a dose of 2.5 mg/kg in intact pineals (Table 1). The increase in *N*-acetyltransferase activity was much higher in denervated pineals, resulting in a 30-fold increase at 1.0 mg/kg and a 100-fold increase at 2.5 mg/kg of norepinephrine. The superinduction by norepinephrine could have been due to the absence of uptake of norepinephrine, increasing the available norepinephrine at the receptor site (1, 16), or to increased responsiveness of the postsynaptic membrane. To exclude the former possibility, isoproterenol, a synthetic catecholamine which is not taken up by nerve endings (17), was injected into intact and denervated rats (Table 2). Denervated pineals showed much higher increase in *N*-acetyltransferase activity than intact pineals at low doses of isoproterenol. At high doses the difference in enzyme activity between intact and denervated pineals disappeared. The superinduction by norepinephrine or isoproterenol appeared 1 or 2 days after denervation (Table 3), in agreement with our previous observation with L-dopa (3). The response of *N*-acetyltransferase activity to isoproterenol did

TABLE 1

Superinduction of N-acetyltransferase with norepinephrine in vivo

l-Norepinephrine was injected subcutaneously into rats, at the doses indicated, 2 hr before they were killed. Results are expressed as means ± standard errors of five rats.

Dose of norepinephrine	*N*-Acetyltransferase	
	Intact	Denervated
mg/kg	*pmoles/pineal/10 min*	
0	21 ± 3	19 ± 2
1.0	46 ± 9	583 ± 167[a]
2.5	354 ± 37	1920 ± 320[a]

[a] $p < 0.01$ in comparison with intact rats.

TABLE 2

Superinduction of N-acetyltransferase with isoproterenol in vivo

l-Isoproterenol HCl was subcutaneously injected into rats, at the doses indicated, 3 hr before they were killed. Results are expressed as means ± standard errors of six rats.

Dose of isoproterenol	*N*-Acetyltransferase	
	Intact	Denervated
mg/kg	*pmoles/pineal/10 min*	
0	35 ± 4	26 ± 7
0.2	69 ± 21	620 ± 202[a]
0.5	523 ± 97	1720 ± 190[b]
1.0	868 ± 101	1420 ± 65[b]
2.0	1350 ± 240	1840 ± 300
5.0	1880 ± 320	1980 ± 370

[a] $p < 0.05$ in comparison with intact rats.
[b] $p < 0.01$ in comparison with intact rats.

not change from the 5th to the 35th day after denervation.

Superinduction of N-acetyltransferase in cultured pineals. To study the mechanism of denervation superinduction more directly, intact and denervated pineals were cultured in the presence of norepinephrine or isoproterenol. *N*-Acetyltransferase activity increased gradually, reaching a maximum between 6 and 10 hr of culture. Intact pineals showed almost no increase in *N*-acetyltransferase activity at concentrations of norepinephrine less than 0.1 μM, and displayed a maximum increase at about 10 μM norepinephrine (Table 4). In denervated pineals the maximal increase in enzyme activity was obtained at 20 nM norepinephrine.

A comparable result was obtained with isoproterenol (Table 5). In intact pineals *N*-acetyltransferase activity reached a maximum at 0.1–1 μM isoproterenol, whereas denervated pineals showed a maximal increase in enzyme activity at 4 nM isoproterenol. Superinduction by isoproterenol was observed in cultured pineals 2 days after ganglionectomy, a pattern similar to that obtained *in vivo* (Table 3).

There was no difference in the increase of *N*-acetyltransferase activity between intact and denervated pineals when cultured in the presence of dibutyryl cyclic AMP (Table 6). Thus the denervation superinduction was probably due to changes proximal to the site of action of cyclic AMP.

Supersensitivity of adenylate cyclase in vivo after denervation. The cyclic AMP concentration in pineals was measured after the injection of norepinephrine or isoproterenol. Norepinephrine (2.5 mg/kg) caused a 2.5-fold elevation of cyclic AMP levels in intact pineals in 10 min (Fig. 1). In denervated pineals the same dose of norepinephrine in-

TABLE 3

Appearance of superinduction after denervation

Either *l*-norepinephrine (1.5 mg/kg) or *l*-isoproterenol HCl (0.25 mg/kg) was subcutaneously injected into rats 3 hr before they were killed. Results are expressed as means ± standard errors of five rats.

Time after denervation	*N*-Acetyltransferase	
	Norepinephrine	Isoproterenol
days	*pmoles/pineal/10 min*	
0	92 ± 35	415 ± 98
1	1520 ± 210[a]	
2	1960 ± 390[a]	1100 ± 210[b]
5	2600 ± 370[a]	1620 ± 260[a]
35		1880 ± 140[a]

[a] $p < 0.01$ in comparison with intact rats (on day of operation).

[b] $p < 0.05$ in comparison with intact rats.

TABLE 4

Superinduction of N-acetyltransferase with norepinephrine in cultured pineals

Either intact or denervated pineals were cultured for 10 hr in the presence of *l*-norepinephrine as indicated. Results are expressed as means ± standard errors of five rats.

Concentration of norepinephrine	*N*-Acetyltransferase	
	Intact	Denervated
M	*pmoles/pineal/10 min*	
4×10^{-9}	9 ± 1	79 ± 15[a]
2×10^{-8}	12 ± 1	1600 ± 290[a]
1×10^{-7}	45 ± 16	2980 ± 210[a]
1×10^{-6}	228 ± 38	2390 ± 150[a]
1×10^{-5}	700 ± 66	1420 ± 250[b]
1×10^{-3}	1470 ± 40	

[a] $p < 0.01$ in comparison with intact pineal.

[b] $p < 0.05$ in comparison with intact pineal.

TABLE 5

Superinduction of N-acetyltransferase with isoproterenol in cultured pineals

Intact or denervated pineals were cultured for 10 hr in the presence of *l*-isoproterenol as indicated. Results are expressed as means ± standard errors of five rats.

Concentration of isoproterenol	*N*-Acetyltransferase	
	Intact	Denervated
M	*pmoles/pineal/10 min*	
1×10^{-9}	13 ± 2	328 ± 90[a]
4×10^{-9}	143 ± 44	1830 ± 200[a]
2×10^{-8}	681 ± 155	2190 ± 330[a]
1×10^{-7}	820 ± 120	1860 ± 430[b]
1×10^{-6}	1390 ± 160	1490 ± 170
1×10^{-5}	1120 ± 130	1850 ± 240[b]
1×10^{-4}	1960 ± 330	

[a] $p < 0.01$ in comparison with intact pineal.

[b] $p < 0.05$ in comparison with intact pineal.

creased cyclic AMP levels more than 10-fold in 5 min. Injection of submaximal doses of isoproterenol also caused a much larger increase in cyclic AMP in denervated pineals than in intact pineals (Table 7).

To exclude the possibility that superinduction of *N*-acetyltransferase and supersensitivity of adenyl cyclase after denervation was due to the absence of uptake of the neurotransmitter into nerve endings, pineals were cultured for 15 min in the presence of [7-^{3}H]-*l*-norepinephrine (50 and 500 nM) or [7-^{3}H]*dl*-isoproterenol (10, 100, and 1000 nM). The uptake of norepinephrine into denervated pineals was less than 20% of that in intact pineals. There was, however, no difference between the amounts of isoproterenol taken up into intact and denervated pineals, indicating that isoproterenol is not taken up by nerve endings in the pineal gland.

TABLE 6

Induction of N-acetyltransferase with dibutyryl cyclic AMP in cultured pineals

Pineals were cultured for 10 hr in the presence of dibutyryl cyclic AMP as indicated. Results are expressed as means ± standard errors of five rats.

Concentration of dibutyryl cyclic AMP	*N*-Acetyltransferase	
	Intact	Denervated
M	*pmoles/pineal/10 min*	
0	18 ± 2	15 ± 2
1.5×10^{-4}	131 ± 29	191 ± 36
4×10^{-4}	788 ± 63	901 ± 142
1×10^{-3}	1520 ± 180	1790 ± 240

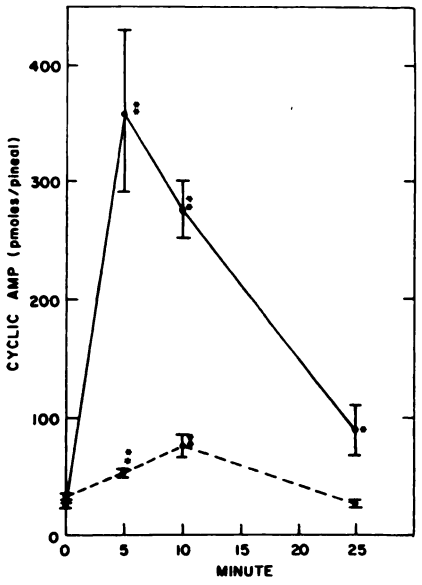

FIG. 1. *Elevation of cyclic AMP in pineal with norepinephrine*

l-Norepinephrine (2.5 mg/kg) was subcutaneously injected into rats. Rats were killed 5, 10, or 25 min after injection, and the cyclic AMP level in pineals was measured (●- - -●, intact rat; ●——●, denervated rat). Vertical bars indicate standard errors of the mean of five rats.

* $p < 0.05$ in comparison with untreated rats.

** $p < 0.01$ in comparison with untreated rats.

TABLE 7

Elevation of cyclic AMP with isoproterenol in vivo

The cyclic AMP level was measured 10 min after subcutaneous injection of *l*-isoproterenol HCl at the doses indicated. Results are expressed as means ± standard errors of six rats.

Dose of isoproterenol	Cyclic AMP	
	Intact	Denervated
mg/kg	*pmoles/pineal*	
0	21 ± 4	18 ± 3
0.25	262 ± 17	445 ± 31[a]
2.0	195 ± 9	505 ± 57[a]
5.0	403 ± 43	591 ± 84
10.0	561 ± 82	

[a] $p < 0.01$ in comparison with intact pineal.

DISCUSSION

Pineal *N*-acetyltransferase activity is increased by norepinephrine or isoproterenol *in vivo* and in organ cultures of rat pineals (3–5, 15). The increase in *N*-acetyltransferase activity was much higher in denervated than intact pineals when submaximal doses of either norepinephrine or isoproterenol were injected into rats. Induction of *N*-acetyltransferase was also much higher in denervated pineals cultured in the presence of submaximal concentrations of either norepinephrine or isoproterenol. Superinduction of *N*-acetyltransferase by denervation was more marked with norepinephrine than with isoproterenol, suggesting that the absence of uptake of norepinephrine is partially responsible for the superinduction of *N*-acetyltransferase. However, the observation that

isoproterenol, a compound that is not taken up by nerve endings (17), also causes superinduction in denervated pineals indicates that there is another change at the postsynaptic site of the pineal cell. There was no superinduction in denervated pineals cultured in the presence of dibutyryl cyclic AMP. Thus the superinduction by denervation appeared to be due to a change somewhere between the receptor and the formation of cyclic AMP. The concentration of catecholamines needed to obtain the maximal increase in *N*-acetyltransferase activity was almost 100-fold different for norepinephrine and 20-fold different for isoproterenol between intact and denervated pineals, whereas the maximal enzyme activity did not differ between the two groups.

Trendelenburg (2) proposed two mechanisms, presynaptic and postsynaptic, for denervation supersensitivity. A presynaptic mechanism would be the absence of uptake of neurotransmitter by nerve endings (15). Postsynaptic mechanisms would involve changes in sensitivity of the receptors or subsequent steps in the end organ response. Presynaptic supersensitivity can be demonstrated with norepinephrine, but not with isoproterenol (2), whereas postsynaptic supersensitivity can be demonstrated with both norepinephrine and isoproterenol (2). The presynaptic type of supersensitivity appears between the first and second postoperative days as the nerve terminals degenerate, and postsynaptic supersensitivity appears slowly over several weeks after denervation. Our observations reported here, however, demonstrate that denervation superinduction by isoproterenol appears very early, is significantly increased by the second postoperative day, and is fully developed 5 days after denervation.

Weiss (9) reported that sensitivity of adenyl cyclase to norepinephrine and sodium fluoride was increased only 4 weeks after denervation, and concluded that adenylate cyclase activity was greater after chronic denervation. We have shown that the elevation of cyclic AMP is enhanced in the denervated pineal by the sixth or seventh postoperative day. Maximal induction of *N*-acetyltransferase and maximal elevation of cyclic AMP were the same in intact and denervated pineals, while submaximal doses of catecholamines resulted in much larger increases in *N*-acetyltransferase activity and in the level of cyclic AMP in denervated pineals compared to intact pineals. The disagreement between his result and ours could be due to the difference in experimental procedures. Adenylate cyclase activity was assayed by Weiss using a pineal homogenate, whereas in the present study the elevation of the endogenous cyclic AMP level *in vivo* was measured. The response of adenylate cyclase to hormones is usually changed after homogenization of tissues. An alternative explanation is the possible existence of two types of supersensitivity of the adenylate cyclase system after denervation: the change in K_m and that of V_{max}, which might appear at different time intervals. Another possibility would be that the enhanced elevation of cyclic AMP and superinduction of *N*-acetyltransferase are due to the decrease in the cyclic AMP-degrading enzyme in pineal after denervation. Such a phenomenon, however, has not yet been reported.

The question arose as to whether the absence of nerve endings or the absence of neurotransmitter is responsible for the appearance of denervation superinduction. We have recently found that treatment of rats with reserpine, a compound that depletes norepinephrine in nerves, also caused superinduction of *N*-acetyltransferase by isoproterenol in innervated pineals. The superinduction with reserpine or by denervation was completely suppressed by administration of isoproterenol (18). It has been shown that pineal adenylate cyclase also became supersensitive to norepinephrine after continuous lighting (14), a condition which reduces the release of norepinephrine (8). Thus it seems likely that the absence or the continuous presence of neurotransmitter could change the sensitivity of the pineal receptor to neurotransmitter.

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