Rapid changes in rat pineal β-adrenergic receptor: Alterations in l-[³H]alprenolol binding and adenylate cyclase

(cell responsiveness/drug tolerance/gland subsensitivity/gland supersensitivity)

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ABSTRACT The properties of the β-adrenergic receptor which regulates adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in the pineal gland are similar to the properties of the sites which specifically bind l-[³H]alprenolol, a potent β-adrenergic antagonist. Stimulation of the β-adrenergic receptor results in a 30-fold increase in the activity of N-acetyltransferase (= arylamine acetyltransferase; acetyl CoA:arylamine N-acetyltransferase, EC 2.3.1.5), an enzyme involved in the synthesis of the pineal hormone melatonin. In the normal diurnal light-dark cycle there is greater physiological stimulation of the β-adrenergic receptor in the pineal during the night than during the day. Pineals from rats kept in constant light for 24 hr possess more hormone-sensitive adenylate cyclase and specifically bind more l-[³H]alprenolol than do pineals from rats kept in the dark overnight. When rats, exposed to light for 24 hr, are treated with the β-adrenergic agonist isoproterenol, there is a rapid loss of both hormone-sensitive adenylate cyclase activity and specific l-[³H]alprenolol binding sites. There is no change in the affinity of adenylate cyclase for isoproterenol or for its substrate, ATP. Similarly, although there are fewer binding sites, there is no change in the affinity of the remaining sites for either agonist or antagonist. Inhibition of protein synthesis with cycloheximide does not affect the loss of either adenylate cyclase activity or specific binding sites. The data suggest that stimulation of the β-adrenergic receptor causes a rapid decrease in the number of available receptors and in hormone-sensitive adenylate cyclase activity; conversely, lack of stimulation causes an increase in these parameters. It is suggested that these changes contribute to the phenomena of super- and subsensitivity in the pineal gland by regulating the capacity of the pineal to synthesize cyclic AMP in response to β-adrenergic stimulation.

The pineal gland has proven to be a useful model in the study of the interaction between sympathetic nerves and the β-adrenergic receptors of responsive cells (1). Stimulation of the pineal β-adrenergic receptor results in increased levels of intracellular cyclic adenosine 3':5'-monophosphate (cyclic AMP) as a consequence of enhanced adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity (2, 3). In addition, through its influence on cyclic AMP levels, the β-adrenergic receptor controls both the induction and maintenance of serotonin N-acetyl-transferase activity (= arylamine acetyltransferase; acetyl CoA:arylamine N-acetyltransferase, EC 2.3.1.5) (4, 5). Serotonin N-acetyltransferase forms N-acetylserotonin, a precursor for the synthesis of melatonin, the pineal hormone (1).

Recent studies have shown that both the sensitivity and the magnitude of these biochemical responses of the pineal to β-adrenergic stimulation are rapidly affected by the de-

gree of prior stimulation of the receptor (6–8). Thus, following exposure of the pineal to either the noradrenergic neurotransmitter or to synthetic β-adrenergic agonists, the subsequent responses are "subsensitive" in comparison with responses obtained from previously unstimulated glands. In contrast, after the reduction of sympathetic nerve firing by exposure of animals to light (9, 10), the responses are "supersensitive" (7, 8). These changes in the sensitivity of the pineal to β-adrenergic stimulation can occur rapidly; subsensitivity has been shown to develop within hours and supersensitivity can be demonstrated within a day.

Recently, competitive β-adrenergic antagonists of high specific activity have been used to characterize binding sites whose properties are similar to those of the β-adrenergic receptor (11–13). In the rat pineal gland the sites which specifically bind l-[³H]alprenolol‡ were found to be indistinguishable from the β-adrenergic receptor coupled to adenylate cyclase (14). The present study utilizes the binding of l-[³H]alprenolol and the activity of hormone-sensitive adenylate cyclase to characterize changes in the properties of the β-adrenergic receptor which occur following stimulation of this receptor in vivo. We find that decreased β-adrenergic stimulation of the pineal results in both an increase in the hormone-sensitive adenylate cyclase activity and an increased number of specific l-[³H]alprenolol binding sites. In contrast, increased stimulation causes a decrease in both the amount of hormone-sensitive adenylate cyclase and the number of specific binding sites.

MATERIALS AND METHODS

l-Alprenolol, tritiated by New England Nuclear Corp. (Boston, Mass.) to a specific activity of 17 Ci/mmol as previously described (11), was supplied by R. J. Lefkowitz. l-Isoproterenol D-bitartrate was supplied by Sterling Winthrop Laboratories; propranolol was supplied by Ayerst Laboratories. [³H]Adenosine 3':5'-monophosphate (25 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.). All other chemicals were reagent grade and were obtained from commercial sources.

Animals. Male Sprague-Dawley rats (150–175 g) were

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

‡ "l-[³H]Alprenolol" has been used throughout this manuscript to identify the compound resulting from catalytic reduction of l-alprenolol with tritium. l-Alprenolol contains an unsaturated bond in the aliphatic chain on the two position of the aromatic ring. This compound, therefore, might be appropriately referred to as "l-[³H]dihydroalprenolol." The nature of the labeling process, however, is such that tritium exchange might also take place, yielding l-[³H]alprenolol. The tritiated material used for these studies has biological activity identical to that of native l-alprenolol (23).

Table 1.   Effect of environmental lighting on adenylate cyclase activity and specific binding of *l*-[³H] alprenolol in rat pineal

Condition	Adenylate cyclase activity (pmol/mg of protein per min) in the presence of		Specific binding of *l*-[³H]-alprenolol (cpm/mg of protein)
	No addition	*l*-Isoproterenol (100 μM)	
12 hr in dark	23 ± 3 (8)	103 ± 2 (8)	6,500 ± 600 (16)
24 hr in light	87 ± 10 (18)*	225 ± 14 (19)*	10,500 ± 600 (18)*

Pineal glands were removed from rats at 0600 hr. Rats had either been kept in the dark overnight (12 hr in dark) or been kept in continuous light for the previous 24 hr (24 hr in light). Adenylate cyclase activity and specific *l*-[³H]alprenolol binding were determined as described in *Materials and Methods*. The data represent the mean ± SEM for determinations in the number of separate experiments indicated in parentheses.
* P < 0.001 versus 12 hr in dark.

FIG. 1.   Time course of the *l*-isoproterenol-induced decrease in adenylate cyclase activity in the rat pineal. Rats were kept in constant light for 27 hr prior to the injection of *l*-isoproterenol (5 mg/kg, subcutaneous) at 0 hr. At the indicated times, groups of three rats were killed and the adenylate cyclase activity was determined either in the absence (●) or in the presence (○) of 10 μM *l*-isoproterenol as described in *Materials and Methods*. The data represent the mean and range of three replicate samples, each assayed in duplicate.

supplied by Zivic-Miller Laboratories (Allison Park, Pa.). The animals were kept in our animal facilities under diurnal lighting conditions with the lights on from 0600 to 1800 hr for at least 5 days before each experiment. Rats exposed to constant light for 24 hr were transferred to plastic cages (six to eight animals per cage) with free access to food and water and kept in a room illuminated with fluorescent lights overnight. In experiments in which animals were killed at 0600 hr (at the end of the dark period), the animals were transferred the previous day into plastic cages and kept in the darkened animal room overnight with access to food and water. At 0600 hr, several minutes before the lights were turned on, the animals were removed from the darkened room and were killed within 3 min of being brought into the light.

***l*-[³H]Alprenolol Binding Assay.** The binding of *l*-[³H]alprenolol was performed as previously described (14). Pineals were homogenized in 25 μl per pineal of a solution containing: Tris-maleate, pH 7.4, 80 mM; MgSO₄, 6 mM; and ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 0.6 mM. The standard assay system (final volume, 0.12 ml) for measurement of *l*-[³H]alprenolol binding contained: Tris-maleate, pH 7.4, 80 mM; MgSO₄, 6 mM; EGTA, 0.6 mM; *l*-[³H]alprenolol, 0.06 μM (approximately 100,000 cpm); 0.05 ml of tissue homogenate (240 μg of protein); and indicated test substances. The concentration of *l*-[³H]alprenolol used in the assay saturated the specific binding sites (14); thus, the amount of specific binding obtained reflected the number of sites available. Incubation was for 5 min at 37°. Incubations were terminated by removing duplicate 0.05 ml aliquots; free and bound *l*-[³H]alprenolol were separated by centrifugation as previously described (11, 14). In each experiment, the amount of *l*-[³H]alprenolol bound in the presence of 5 × 10⁻⁵ M *d,l*-propranolol was also determined and was assumed to represent "nonspecific binding." The "specific binding" of *l*-[³H]alprenolol was calculated by subtracting the "nonspecifically" bound radioactivity from the total radioactivity bound in those samples incubated in the absence of propranolol. Nonspecific binding represented approximately 35% of the total radioactivity in the pellet.

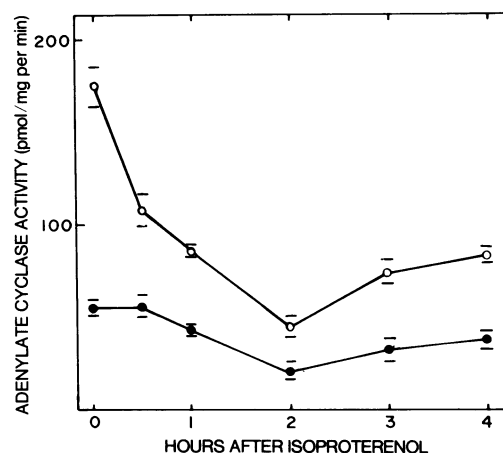**Adenylate Cyclase Assay.** The adenylate cyclase activity

was measured as described previously (14). Pineals were homogenized in 50 volumes of a buffer containing 2 mM Tris-maleate, pH 7.4, and 2 mM EGTA. The standard enzyme assay system (final volume 0.05 ml) contained Tris-maleate, pH 7.4, 80 mM; ATP, 1.5 mM; MgSO₄, 6.0 mM; theophylline, 10 mM; EGTA, 0.8 mM; 10 μl of homogenate; and the indicated test compounds. Reactions were initiated with the addition of ATP, and incubations were for 5 min at 30°. The reaction was terminated by placing the tubes in boiling water for 2 min. The cyclic AMP formed was measured by the method of Brown *et al.* (15). Protein was measured by the method of Lowry *et al.* (16) using bovine serum albumin as a standard. The statistical significance of the data was determined with Student's *t* test.

RESULTS

Environmental lighting affects the rate of firing in the postganglionic neurons which innervate the pineal (9, 10). In the dark, there is enhanced firing of these neurons and consequently the turnover of norepinephrine is increased; thus, during the night there is a continuous, tonic stimulation of the pineal β-adrenergic receptor. In contrast, light decreases the firing rate of the sympathetic fibers in the pineal; thus, while animals are exposed to light, the turnover of norepinephrine is diminished. The enhanced sympathetic activity which occurs at night decreases the sensitivity of the pineal to subsequent β-adrenergic stimulation (7, 8).

The effects of environmental lighting on the amount of adenylate cyclase activity and the number of specific alprenolol binding sites in the pineal were examined (Table 1). Animals kept either in darkness overnight or in constant light for 24 hr were killed at 0600 hr. The pineals from animals which had been in the dark had less adenylate cyclase and fewer specific alprenolol binding sites than did the pineals from animals which had been kept in constant light. Thus, following the reduction of β-adrenergic stimulation by light, the amount of adenylate cyclase activity and the number of binding sites can increase within 24 hr.
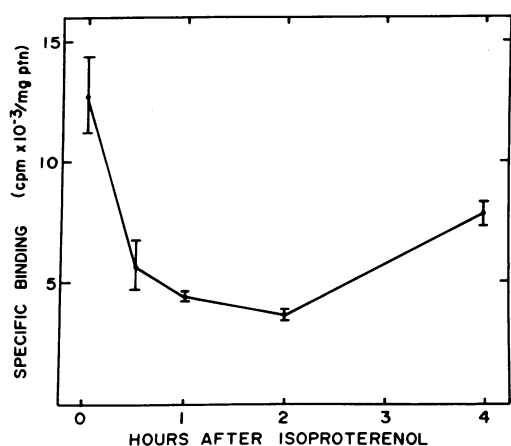
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FIG. 2. Time course of the l-isoproterenol-induced decrease in specific binding sites for l-[^{3}H]alprenolol. Rats were kept in constant light for 26 hr prior to the injection of l-isoproterenol (5 mg/ kg, subcutaneous) at 0 hr. At the indicated times, groups of seven rats were killed and the specific binding of l-[^{3}H]alprenolol in the pineal homogenate was determined as described in Materials and Methods. The data represent the mean ± SEM of five replicate samples, each assayed in duplicate.

Effects of Isoproterenol Pretreatment upon Hormone-Sensitive Adenylate Cyclase and l-[^{3}H]Alprenolol Binding.

To examine the effect of pharmacological stimulation of the β-adrenergic receptor, rats which had been kept in constant light for at least 24 hr were injected with l-isoproterenol. Following treatment with isoproterenol there was a rapid decrease in the amount of adenylate cyclase activity (Fig. 1). Thirty minutes after the injection of the catecholamine, hormone-stimulated activity was reduced while the basal activi-
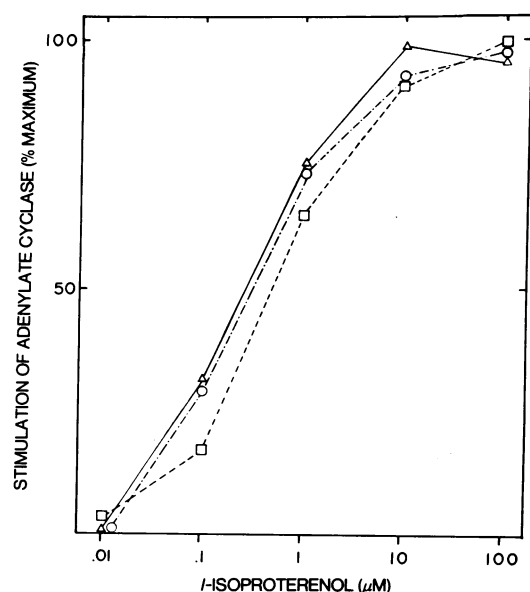

FIG. 3. Stimulation of pineal adenylate cyclase activity by l-isoproterenol. Adenylate cyclase activity in the absence or the presence of the indicated concentrations of l-isoproterenol was measured in homogenates of pineal glands from: (a) rats kept in the light for 24 hr (O); (b) rats kept in the light for 24 hr and pretreated for 2 hr with l-isoproterenol (5 mg/kg, subcutaneous) (□); and (c) rats sacrificed at 0600 hr (△). For each homogenate, the increments in enzyme activity in the presence of isoproterenol are expressed as a percentage of the maximal increment observed. The data represent the means of from three to seven individual experiments.

Table 2. Properties of specific l-[^{3}H]alprenolol binding sites in supersensitive and subsensitive rat pineal glands

Condition	Maximum binding (cpm/mg of protein)	K_a of l-[^{3}H]- alprenolol (μM)	K_i of l-isopro- terenol (μM)
12 hr in dark	7,250	0.019	0.43
24 hr in light	11,760	0.022	0.58
24 hr in light plus l-iso- proterenol pretreat- ment	6,151	0.031	0.73

For each of the three experimental conditions, specific binding was determined at 5 concentrations of l-[^{3}H]alprenolol ranging from 0.005 μM to 0.084 μM. At each concentration of alprenolol tested, specific binding was determined in four replicate samples and nonspecific binding was determined in two replicate samples. Maximum binding and the affinity constant (K_a) were determined by the method of Lineweaver and Burk. The affinity of l-isoproterenol for the binding sites (K_i) was calculated from data shown in Fig. 4 from the relationship, $K_i = I_{50}/(1 + S/K_a)$; where I_{50} is the concentration of l-isoproterenol producing 50% inhibition of l-[^{3}H]alprenolol binding, S is the concentration of l-[^{3}H]alprenolol utilized (0.06 μM) and K_a is the affinity of l-[^{3}H]alprenolol for the specific binding site (24). Pineal glands were removed from rats at 0600 hr. Supersensitive pineals were removed from rats which had been kept in continuous light for the previous 24 hr (24 hr in light); subsensitive pineals were removed either from rats kept in the dark overnight (12 hr in darkness) or from rats kept in continuous light for the previous 24 hr and injected with l-isoproterenol (5 mg/kg, subcutaneous) 2 hr prior to killing (24 hr in light plus l-isoproterenol pretreatment).

ty was unaffected. After 2 hr, both the hormone-sensitive and the basal adenylate cyclase activities had been reduced substantially (75% and 60%, respectively). The effect of injected l-isoproterenol was long lasting; even 4 hr after a single injection, basal and hormone-stimulated enzyme activity were diminished.

Similarly, isoproterenol caused a rapid decrease in the number of specific binding sites for l-[^{3}H]alprenolol. Within 30 min after treatment, the number of specific binding sites was decreased 55% (Fig. 2). The maximal loss of specific binding sites occurred after 2 hr; this represented approximately 70% fewer specific binding sites than in the pineals of untreated animals. Some recovery of the number of binding sites was observed 4 hr after a single injection of isoproterenol; however, the amount of l-[^{3}H]alprenolol specifically bound was still substantially reduced. The magnitude and the time course of both the decrease in the number of specific binding sites and the loss of hormone-sensitive adenylate cyclase activity were similar.

Pretreatment of the animals with cyclohexamide (2 mg/ kg) did not affect either the loss of hormone-sensitive adenylate cyclase or the loss of specific l-[^{3}H]alprenolol binding sites caused by isoproterenol in vivo. This indicates that the loss of enzyme and binding sites does not require new protein synthesis.

Affinities of Agonist for Supersensitive and Subsensitive β-Adrenergic Receptors.

Supersensitive and subsensitive pineal glands differ not only in the magnitude of the maximal response to β-adrenergic stimulation but also in the sensitivity of the response (5–8). To investigate the possibility that the difference in the sensitivity of the pineal might reflect an alteration in the affinity of the β-adrenergic receptor for agonist, the direct effects of isoproterenol on ade-

Table 3. Effect of ATP concentration on adenylate cyclase activity in supersensitive and subsensitive pineals

Final concentration of ATP (mM)	Isoproterenol-stimulated adenylate cyclase activity (pmol/mg of protein per min) in	
	Supersensitive pineal	Subsensitive pineal
6.0	250 ± 12	100 ± 7
2.0	246 ± 10	96 ± 3
1.5	209 ± 11	75 ± 5
1.0	183 ± 12	49 ± 1
0.6	89 ± 6	17 ± 2
0.3	25 ± 3	Not detectable

Enzyme activity was assayed as described in *Materials and Methods* with the exception that the concentrations of ATP and Mg^{++} were varied as indicated (maintaining a ratio of concentrations of $ATP:Mg^{++}::1:4$). Isoproterenol was present at a concentration of 100 μM. Supersensitive pineals were removed from animals kept in constant light for 24 hr prior to killing; subsensitive pineals were removed from animals kept in constant light for 24 hr and injected with *l*-isoproterenol (5 mg/kg, subcutaneous) 2 hr prior to killing. Data represent mean ± SEM for three replicate samples of homogenate, each assayed in duplicate for cyclic AMP content.

nylate cyclase activity and on the specific binding of *l*-[³H]alprenolol were examined. Supersensitive pineals were obtained from rats kept in constant light for at least 24 hr; subsensitive pineals were obtained either from rats killed at 0600 hr or from rats kept in constant light overnight but pretreated with isoproterenol.

Supersensitive pineals contained more adenylate cyclase activity than did the subsensitive pineals (e.g., Table 1). However, when the increments in enzyme activity due to the direct action of isoproterenol were expressed as a percentage of the maximum increase, no appreciable difference in the affinity of *l*-isoproterenol was observed (Fig. 3). Thus, half-maximal increases in enzyme activity were obtained with concentrations of *l*-isoproterenol between 0.2 μM (supersensitive) and 0.4 μM (subsensitive, isoproterenol-pretreated).

Although more *l*-[³H]alprenolol was specifically bound by homogenates of supersensitive pineals than by homogenates of subsensitive pineals, there was no difference in the affinity of the ligand for the specific binding sites (Table 2). Furthermore, there was no difference in the potency of *l*-isoproterenol as an inhibitor of the specific binding of *l*-[³H]alprenolol (Fig. 4). Utilizing these data, the affinity of *l*-isoproterenol for the specific binding sites was calculated (Table 2). The affinities for agonist in the subsensitive pineals were within 25% of the affinity calculated for the supersensitive pineals. Thus, the data demonstrate that the affinities of the β-adrenergic receptor for agonist are the same in supersensitive and subsensitive pineals.

Properties of Adenylate Cyclase Activity in Supersensitive and Subsensitive Pineals. To further characterize the nature of the apparent loss of enzyme activity induced by isoproterenol, the affinity of the enzyme for ATP and the effects of fluoride on enzyme activity were investigated. Animals were kept in light for 24 hr and some were injected with isoproterenol. The development of subsensitivity did not alter the affinity of the enzyme for its substrate ATP (Table 3). The isoproterenol-stimulated enzyme activity was measured utilizing a variety of concentrations of ATP (while maintaining a ratio of the concentrations of Mg^{++} and ATP of 4:1). With all concentrations of ATP tested, there was
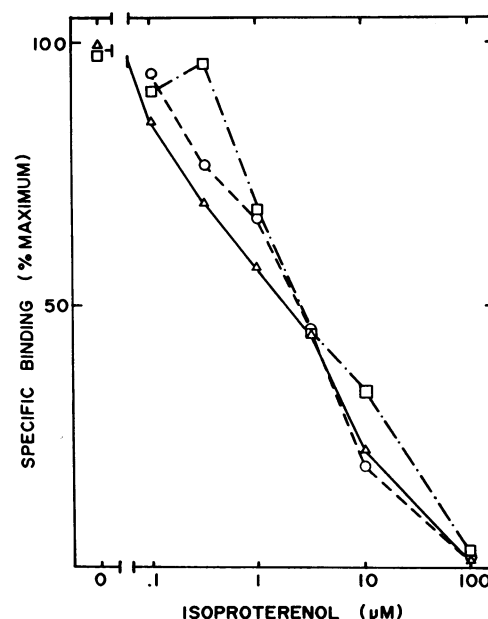

FIG. 4. Effect of *l*-isoproterenol on specific binding of *l*-[³H]alprenolol to pineal homogenate. Specific binding of *l*-[³H]alprenolol was determined as described in *Materials and Methods*. The ratio of the specific binding in the presence and absence of the indicated concentrations of *l*-isoproterenol is plotted. Pineal homogenates were obtained from the following sources: (a) rats kept in the light for 24 hr (O); (b) rats kept in the light for 24 hr and pretreated for 2 hr with *l*-isoproterenol (5 mg/kg, subcutaneous) prior to killing (□); and (c) rats sacrificed at 0600 hr (△). Values shown for each point are the means of two replicate samples each assayed in duplicate.

considerably less enzyme activity in the homogenate of subsensitive pineals. However, approximately the same concentration of ATP, 1 mM, sustained half-maximal enzyme activity in either preparation of enzyme (Table 3). The subsensitive pineals also had substantially less fluoride-stimulated adenylate cyclase activity than did untreated supersensi-

Table 4. Properties of adenylate cyclase activity in homogenates of supersensitive and subsensitive pineal glands

Condition	Adenylate cyclase activity (pmol/mg of protein per min) in the presence of		
	No addition	*l*-Isoproterenol (100 μM)	NaF (10 mM)
24 hr in light	136 ± 8	245 ± 15	316 ± 18
24 hr in light plus isoproterenol pretreatment	23 ± 1*	52 ± 3*	170 ± 10*

Supersensitive pineals were removed from animals exposed to continuous light for 24 hr prior to killing; subsensitive pineals were removed from animals exposed to continuous light for 24 hr and injected with *l*-isoproterenol (5 mg/kg, subcutaneous) 2 hr prior to killing. Pineals from two or three animals were pooled and enzyme activity was measured as described in *Materials and Methods*. The data for each class of pineal homogenate represent mean ± SEM for enzyme activity measured in four separate pineal homogenates; for each homogenate, activity under the indicated conditions was measured in three replicate samples, each assayed in duplicate for cyclic AMP. There was no significant difference in the amount of protein in aliquots of homogenates of supersensitive or subsensitive pineals.

* $P < 0.001$ versus 24 hr in light.

tive pineals (Table 4). These observations suggest that, except for the difference in the amount of enzyme activity, the hormone-sensitive adenylate cyclase activities in supersensitive and subsensitive pineal glands are similar.

DISCUSSION

Recently, it has been demonstrated that several of the biochemical responses of the pineal to β-adrenergic stimulation can be rapidly altered by previous exposure of the gland to β-adrenergic agonists (6–8, 17). For example, subsensitive pineals accumulate less cyclic AMP in response to catecholamines than do supersensitive pineals (2, 8). Our results indicate that the reduced capacity of the subsensitive pineal to accumulate cyclic AMP is accompanied by a reduction in the amount of adenylate cyclase activity. In subsensitive pineals, both the basal and the hormone-stimulated enzyme activities are substantially lower than those in the supersensitive glands. There is also a reduced number of β-adrenergic receptors (i.e., specific binding sites for l-[^{3}H]alprenolol). In addition, the decreases in adenylate cyclase activity and the number of receptors produced by isoproterenol are similar in time course and in magnitude, suggesting that these phenomena are closely coupled and causally related. Neither the decrease in enzyme activity nor the decrease in the number of binding sites requires protein synthesis. The loss or inactivation of the hormone-sensitive adenylate cyclase and the β-adrenergic receptors arises as a consequence of either physiological or pharmacological stimulation. In contrast, the increase in both enzyme activity and the number of receptors in supersensitive pineals arises as a consequence of reduced stimulation of the β-adrenergic receptor.

The rapid pharmacological "desensitization" of catecholamine-sensitive adenylate cyclase has been previously reported (18–21); however, only a diminished response to catecholamine, without a loss of basal enzyme activity, was observed. This contrasts with the results from the pineal, where the development of maximal subsensitivity was accompanied by a loss of both basal and stimulated adenylate cyclase activity. A decrease in specific receptors similar to that observed in the pineal has been reported following exposure of appropriate tissues to either insulin or catecholamines (21, 22).

The data indicate that the affinity of the β-adrenergic receptor for agonist does not change as a consequence of the development of subsensitivity; this is in accord with previous studies of receptors for insulin or catecholamines (21, 22). Yet, the potency of isoproterenol to induce N-acetyltransferase activity is approximately 10-fold greater in supersensitive pineals than in subsensitive pineals (8). However, the potency of dibutyryl cyclic AMP to induce N-acetyltransferase activity is also greater in supersensitive pineals (8); this suggested that the effectiveness of cyclic AMP to mediate the intracellular responses to β-adrenergic stimulation is increased in supersensitive pineals. Thus, the variation in the

potency of isoproterenol to induce N-acetyltransferase activity may reflect intracellular regulatory mechanisms rather than changes in the properties of the β-adrenergic receptor *per se*.

The sensitivity of the response of the pineal to β-adrenergic stimulation involves the capacity of the gland to generate cyclic AMP, as well as the intracellular mechanisms which produce the final physiological response. Stimulation of the β-adrenergic receptor can rapidly alter the capacity of the pineal to generate cyclic AMP, by decreasing both the number of β-adrenergic receptors and the amount of adenylate cyclase activity. Such mechanisms may participate in the regulation of the sensitivity of the pineal and other tissues to hormones or drugs, and may contribute to the phenomena of tachyphylaxis and drug tolerance.

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