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Postsynaptic Induction of Serotonin *N*-Acetyltransferase Activity and the Control of Cyclic Nucleotide Metabolism in Organ Cultures of the Rat Pineal

KENNETH P. MINNEMAN[1]

Medical Research Council Neurochemical Pharmacology Unit, Department of Pharmacology, Medical School, Cambridge University, Cambridge CB2 2QD, England

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SUMMARY

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After maintenance of rat pineal organs in organ culture for 12 hr, brief exposure to choleragen caused a 4-fold stimulation of adenylate cyclase activity and induction of serotonin *N*-acetyltransferase activity. Choleragen also caused a sharp, transient increase in the intracellular content of adenosine cyclic 3′,5′-monophosphate (cAMP) and a rapid reduction in guanosine cyclic 3′,5′-monophosphate (cGMP). Cyclic nucleotide phosphodiesterase activity was also increased after choleragen exposure. Addition of 100 μM norepinephrine caused an increase in cAMP and a reduction in cGMP; however, phosphodiesterase activity was not affected. The stimulation of adenylate cyclase activity by norepinephrine did not occur in pineals that had previously been exposed to 10 μM norepinephrine for 1 hr. Incubation with choleragen (50 μg/ml) and subsequent culture in the presence of 10 μM norepinephrine resulted in a larger induction of serotonin *N*-acetyltransferase activity than that caused by either treatment alone. Neither the adenylate cyclase response to norepinephrine nor the cAMP content after norepinephrine administration was markedly increased by previous choleragen exposure; however, the increase in cAMP content was maintained over a longer time period. Addition of the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM) caused induction of *N*-acetyltransferase and phosphodiesterase activity. This treatment led to a 2–3-fold increase in intracellular cAMP and a 5–6-fold increase in intracellular cGMP. The rise in *N*-acetyltransferase activity caused by choleragen was blocked by the addition of 1 μM ouabain or 80 mM K^+ to the culture medium. The results indicate that adenylate cyclase activation is the critical signal for postsynaptic enzyme induction in the cultured rat pineal. *Beta* adrenoceptor sensitivity may be affected both by the sensitivity of adenylate cyclase to stimulation by norepinephrine and by regulation of the rate of cyclic nucleotide degradation through changes in phosphodiesterase activity.

INTRODUCTION

Since the original observation that the dibutyryl derivative of adenosine cyclic 3′,5′-monophosphate is capable of increasing the conversion of [^{14}C]tryptophan to [^{14}C]melatonin in cultured rat pineal organs (1), evidence has been accumulating

[1] Graduate Fellow of the National Science Foundation.

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which suggests that cAMP[2] mediates the postsynaptic events associated with *beta* adrenoceptor stimulation in this system (2). Stimulation of *beta* adrenoceptors in the rat pineal *in vivo* is controlled by norepinephrine released from postganglionic sympathetic nerve endings originating in the superior cervical ganglion (3). Norepinephrine release is controlled in turn by environmental lighting conditions (4). Stimulation of these receptors either *in vivo* (5) or *in vitro* (6) causes a large increase in melatonin synthesis in the postsynaptic parenchymal cells, with the critical regulatory step in this response being induction of serotonin *N*-acetyltransferase (EC 2.3.1.5) (7). The rat pineal survives well in organ culture (8), although under culture conditions the adrenergic innervation degenerates. This system can therefore be used to examine the postsynaptic mechanisms translating cell surface receptor stimulation into an intracellular biochemical response.

Several lines of evidence point to the intracellular mediation of *beta* adrenoceptor stimulation by cAMP in the rat pineal organ (1, 2). Norepinephrine and other *beta* agonists stimulate adenylate cyclase activity in broken cell preparations of this organ (9), and increase the intracellular levels of cAMP after administration both *in vivo* (10) and in culture (11). Also, addition of dibutyryl cAMP or high concentrations of the phosphodiesterase inhibitor theophylline to pineal organs in culture mimics the effects of norepinephrine on this system (5, 12), and these effects are not blocked by *beta* adrenoceptor-blocking drugs. The present study examines the effects of the stimulation of adenylate cyclase by cholera toxin (choleragen), through a non-*beta* adrenoceptor-mediated mechanism, on postsynaptic enzyme induction in cultured pineal organs. The only known mechanism of action of choleragen is an irreversible stimulation of adenylate cyclase activity in intact cells following its binding to a cell surface receptor, GM1 ganglioside (13–15). This represents a useful method for dissociating adenylate cyclase activation from neurotransmitter receptor occupation.

Large variations in the sensitivity of the pineal organ to *beta* adrenoceptor stimulation have been reported (5, 16). Periods of receptor stimulation reduce the sensitivity of serotonin *N*-acetyltransferase in this organ to further stimulation, and periods of receptor inactivity increase this sensitivity (17, 18). Since cAMP appears to be involved in the translation of the drug-receptor interaction into its postsynaptic biochemical response, alterations in cyclic nucleotide metabolism may affect the *beta* adrenergic sensitivity of this organ. A preliminary report of parts of this work has appeared (19).

METHODS

Pineal organ cultures. Male albino Sprague-Dawley rats (150–250 g) were killed by decapitation, and the pineal organs were quickly and aseptically removed and placed in organ culture as described by Klein and Berg (20). Pineal organs were supported on Millipore filters on an expanded metal grid in 90% Eagle's minimal essential medium and 10% heat-inactivated (60° for 1 hr) fetal bovine serum containing penicillin and streptomycin (100 IU/ml), 2 mM glutamine, and 25 mM glucose at 37° under 95% O_2 and 5% CO_2. Pineals were cultured for 12 hr to allow nerve endings to degenerate and enzyme levels and receptor sensitivity to stabilize. In some experiments the preliminary period of maintenance in culture was extended to 60 hr, with fresh medium added after the first 36 hr.

After the initial 12-hr period, drugs (except choleragen) were added directly to the culture medium by 100-fold dilution of a concentrated solution, and pineals were maintained in culture for varying periods of time. The pineals were then removed, frozen, and stored on solid carbon dioxide prior to biochemical analysis.

Exposure to choleragen was accomplished by transferring pineals to oxygenated Krebs-Ringer-bicarbonate buffer

[2] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IBMX, isobutylmethylxanthine.

either with or without various concentrations of choleragen and incubating them in a shaking water bath at 37° for 15 min. Pineals were then returned to culture for further periods of time (calculated from time of return to culture), and cultures were terminated as described above. Addition of choleragen directly to the culture medium had no effect, presumably because the high ganglioside content of the medium effectively neutralized the toxin. Incubation for 15 min in Krebs-Ringer-bicarbonate buffer was found to give maximal rises in enzyme activity. All points represent the mean ± standard error of four to five separate pineal organs.

Assay of pineal enzymes. Frozen pineal organs were homogenized in 100 μl of distilled water, and 50 μl were taken for serotonin *N*-acetyltransferase assay, 20 μl for adenylate cyclase assay, and two 2-μl aliquots for cAMP and cGMP phosphodiesterase assay. Serotonin *N*-acetyltransferase activity was determined by the method of Deguchi and Axelrod (21) in a final volume of 70 μl containing 50 mM phosphate buffer (pH 6.5), 1.4 mM tryptamine, and 50 μM [^{14}C]acetyl coenzyme A (58 mCi/mmole). Adenylate cyclase activity was measured as described by Kebabian *et al.* (22) in a final volume of 100 μl containing 80 mM Tris-maleate (pH 7.4), 2 mM $MgSO_4$, 10 mM theophylline, 0.2 mM EGTA, 1 mM ATP, and various concentrations of norepinephrine bitartrate. Fifty microliters of the extracted supernatant were taken for cAMP determination by the method of Brown *et al.* (23). Phosphodiesterase activity was assayed by the method of Thompson and Appleman (24) in a final volume of 100 μl as previously described (25), with either [^{3}H]cAMP or [^{3}H]cGMP as substrate at a concentration of 1 μM, and enzyme activity values were corrected for nucleotide recovery by the method of Lynch and Cheung (26). All enzyme assays were linear with time and tissue concentration.

Cyclic nucleotide extraction and assay. Cyclic nucleotides were extracted as described by Deguchi and Axelrod (27). A frozen pineal was homogenized in 700 μl of 5% trichloracetic acid, and the denatured protein was removed by centrifugation. The supernatant was extracted four times with 5 ml of diethyl ether to remove the trichloracetic acid, a 500-μl sample was evaporated to dryness under vacuum, and the residue was dissolved in 125 μl of distilled water. Twenty-five microliters were taken for cAMP assay, and 100 μl for cGMP assay. Recovery of authentic added cAMP was 78%, and of cGMP, 74%.

cAMP was measured by the protein binding method of Brown *et al.* (23), and cGMP was measured by radioimmunoassay (28). cAMP did not interfere with cGMP determinations when present in up to a 10,000-fold excess, and cGMP did not interfere with cAMP determinations when present in up to a 50-fold excess.

Norepinephrine. Norepinephrine was measured by the radioenzymatic method of Cuello *et al.* (29). Frozen pineals were homogenized in 100 μl of 0.1 N perchloric acid, frozen, thawed, and microcentrifuged. Twenty-five microliters were taken for measurement of norepinephrine as described (29), except that extractions were performed with water-saturated ethyl acetate–methanol (10:1) instead of toluene–isoamyl alcohol.

Materials. [8-^{3}H]Adenosine cyclic 3′,5′-monophosphate (27.5 mCi/mmole), ammonium salt; [8-^{3}H]guanosine cyclic 3′,5′-monophosphate (21 Ci/mmole), ammonium salt; and [1-^{14}C]acetyl coenzyme A (58 mCi/mmole) were purchased from the Radiochemical Centre, Amersham. Nonradioactive cAMP, cGMP, tryptamine HCl, and ATP were obtained from Sigma, London; isobutylmethylxanthine, from Aldrich Chemical Company; anion-exchange resin AG1-X2, 200–400 mesh, chloride form, from Bio-Rad; and penicillin, streptomycin, fetal calf serum, Eagle's essential medium, and glutamine, from Flow Laboratories, Irvine, Scotland. Cholera toxin in 0.05 M Tris–0.001 M sodium EDTA–0.2 M NaCl–0.003 M NaN was kindly provided by Dr. R. Finkelstein, and cGMP antiserum was raised in rabbits and kindly provided by Dr. J. Albano.

RESULTS

Time course of neural degeneration in pineal organ culture. Norepinephrine fell

from an initial value *in vivo* (at 10 a.m.) of 1.2 ng/pineal to zero after 24 hr in culture (Fig. 1). All experiments described here were done after an initial culture period of 12 hr, when more than 50% of the norepinephrine had disappeared and pineal cyclic nucleotide enzymes had declined (20% for adenylate cyclase and 50% for phosphodiesterase) to stable plateaux (Fig. 1).

Effect of choleragen on adenylate cyclase and serotonin N-acetyltransferase activity in cultured pineals. As reported previously (19), incubation of intact pineals with choleragen (50 μg/ml) caused a 4-fold activation of adenylate cyclase activity, after a lag period of less than 1 hr (Fig. 2). This effect was still significant 24 hr after choleragen exposure. There was also a significant increase in serotonin *N*-acetyltransferase activity, beginning 4 hr after exposure to choleragen and reaching a maximum at 6 hr; the activity was still elevated at 24 hr (Fig. 2). The dose-response curves for the effect of choleragen on these two enzymes are shown in Fig. 3. Half-maximal stimulating concentrations of choleragen were 0.5 μg/ml for adenylate cyclase and 3 μg/ml for serotonin *N*-acetyltransferase. The presence of 10 μM *l*-propranolol in the incubation medium had no effect on the stimulation of adenylate cyclase activity or on the induction of serotonin *N*-acetyltransferase activity by choleragen (19); however, higher concentrations (100 μM) caused a nonspecific 50% decrease, in which the *d* and *l* isomers were equipotent. The presence of 50 μg of cycloheximide per milliliter in the culture medium blocked the induction of serotonin *N*-acetyltransferase, but not the activation of adenylate cyclase by choleragen (19). In order to demonstrate that the effects of choleragen were not mediated by release of the residual pool of norepinephrine, in some experiments pineals were first cultured for 60 hr, exposed to choleragen, and returned to culture. Serotonin *N*-acetyltransferase activity measured 6 hr later was elevated 23-fold by choleragen (1.8 ± 0.42 pmoles/10 min/pineal in control and 42.4 ± 5.2 pmoles/10 min/pineal in toxin-exposed organs).

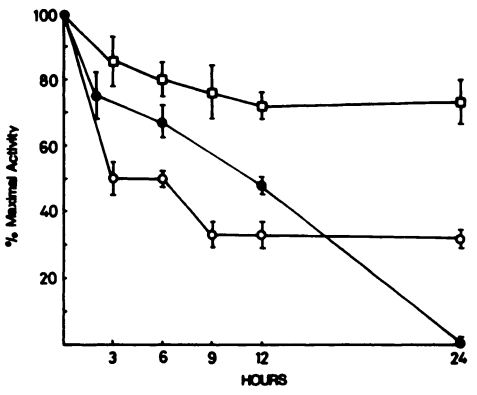

FIG. 1. *Time course of biochemical degeneration in pineal organ cultures*

Pineals were collected as described in METHODS, cultured for 0–24 hr, and frozen, and the following substances were determined as described in METHODS: ●——●, norepinephrine content (original level, 1.2 ng/pineal); □——□, adenylate cyclase activity (original level, 3.2 pmoles/min/pineal); ○——○, cAMP phosphodiesterase (original level, 2.3 nmoles/pineal/10 min).

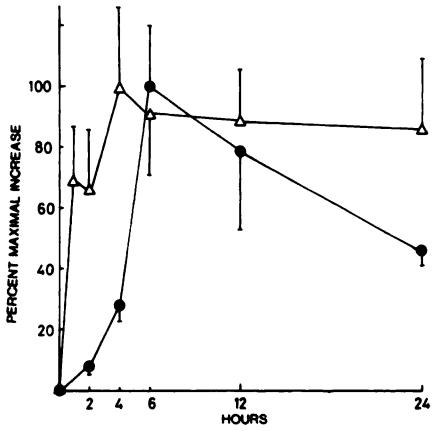

FIG. 2. *Choleragen-induced increases in enzyme activity in pineal organ cultures*

Pineals were cultured for 12 hr, incubated for 15 min at 37° in Krebs-Ringer-bicarbonate in the presence of choleragen (50 μg/ml), and returned to culture for various periods of time. At the termination of the culture period, pineals were frozen and serotonin *N*-acetyltransferase and adenylate cyclase activities were determined as described in the text. ●——●, serotonin *N*-acetyltransferase activity (control levels, 4.8 ± 1.8 pmoles/10 min/pineal; stimulated levels, 242 ± 44); △——△, adenylate cyclase activity (control levels, 3.8 ± 0.22 pmoles/min/pineal; stimulated levels, 13.7 ± 3.5).

Effect of choleragen on cyclic nucleotide content of cultured pineals. cAMP content was not changed after 30 min of exposure to choleragen; however, by 45 min the levels had increased 10-fold (Fig. 4). Thereafter they declined rapidly and at 6 hr were not significantly different from control values. cGMP was significantly reduced 15 min after choleragen exposure, to 35% of control values (Fig. 4), and showed no significant recovery up to 24 hr.

Effect of choleragen on cyclic nucleotide phosphodiesterase activity in cultured pineals. The activity of cyclic nucleotide phosphodiesterase with either cAMP or cGMP as substrate was significantly elevated 4 hr after choleragen exposure (Fig. 5), reached a maximum after 6 hr, and thereafter declined. After 24 hr, the activity of the enzyme was still slightly above control values.

Effect of cycloheximide on choleragen-induced changes in whole cell cyclic nucleotide content in cultured pineals. The presence of cycloheximide (50 μg/ml) in the culture medium prevented the increase in cAMP and cGMP phosphodiesterase activities normally seen after choleragen exposure (19). In the presence of cycloheximide (50 μg/ml), however, cAMP levels after choleragen rose normally (Fig. 4), but the subsequent decline was markedly attenuated. In the presence of cycloheximide, cAMP was still significantly elevated 5-fold 24 hr after choleragen exposure, suggesting that the decline in cAMP content normally seen might have been caused by increased hydrolysis resulting from increased phosphodiesterase activity. In contrast, cGMP levels declined still further in the presence of cycloheximide, possibly because of the toxic effects of this compound on the organ culture system.

Effect of norepinephrine on cyclic nucleotide levels and enzyme activities in cultured pineals. As described previously (11), addition of norepinephrine to a final concentra-

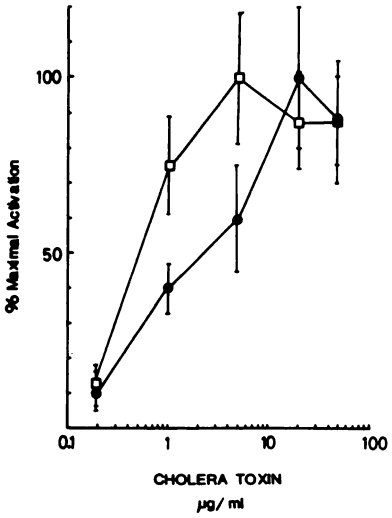

FIG. 3. *Effect of choleragen concentration on adenylate cyclase activation and serotonin N-acetyltransferase induction in cultured pineals*

Pineals were cultured for 12 hr and incubated for 15 min at 37° in Krebs-Ringer-bicarbonate with 0.2–50 μg/ml of choleragen. Pineals were returned to culture for a further 6 hr and frozen, and basal adenylate cyclase (□——□) and serotonin *N*-acetyltransferase (●——●) activities were determined as described in METHODS.

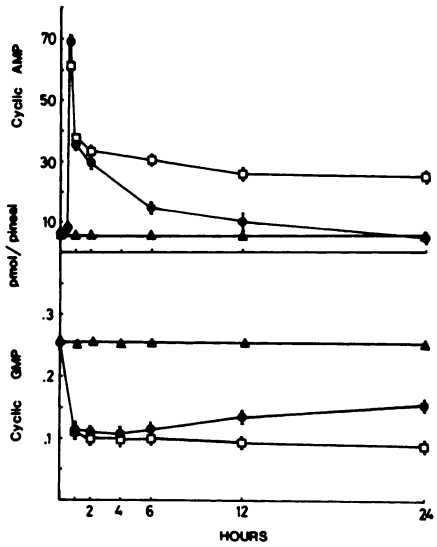

FIG. 4. *Effect of choleragen on whole cell cyclic nucleotide content in cultured pineals*

Pineals were cultured for 12 hr, incubated for 15 min at 37° in Krebs-Ringer-bicarbonate, either with (●——●) or without (▲——▲) 50 μg/ml of choleragen, and returned to culture for various times. At the termination of the culture period, pineals were frozen and cAMP (top) and cGMP (bottom) were extracted and measured as described in METHODS. Some pineals exposed to choleragen were subsequently cultured in a medium containing 50 μg/ml of cycloheximide (□——□).

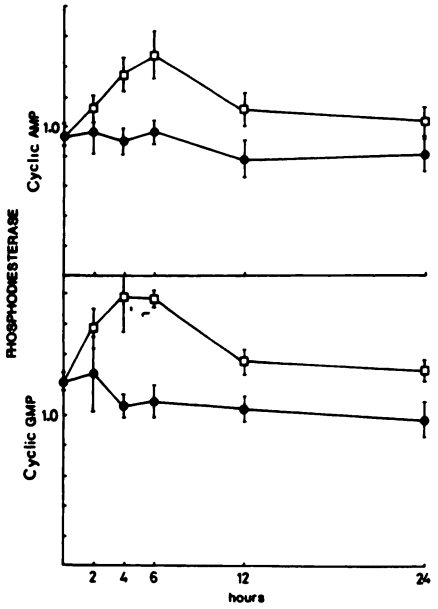

FIG. 5. *Effect of choleragen on cyclic nucleotide phosphodiesterase activity in cultured pineals*

Pineals were cultured for 12 hr, incubated for 15 min at 37° in Krebs-Ringer-bicarbonate, either with (□——□) or without (●——●) 50 μg/ml of choleragen, and returned to culture for various periods of time. At the termination of the culture period, pineals were frozen and phosphodiesterase activity toward either 1 μM cAMP (top) or 1 μM cGMP (bottom) as substrate was measured as described in METHODS.

tion of 100 μM to pineal organs in culture caused a rapid, 12-fold increase in intracellular cAMP content (Fig. 6) after 15 min, and a subsequent rapid decrease to basal levels after 30 min. cGMP levels, on the other hand, were reduced by more than 50% 30 min after addition of norepinephrine (Fig. 6) and were still reduced after 3 hr (after 12 hr cGMP levels were not significantly different from control values; data not shown). Neither cAMP nor cGMP phosphodiesterase activity was significantly affected 0.5, 1, 2, 3, 6, or 12 hr after norepinephrine exposure (data not shown).

It has previously been shown that 6 hr after 10 μM norepinephrine exposure, there is a maximal rise in serotonin *N*-acetyltransferase activity (12). Exposure for less than 30 min caused a half-maximal effect (30). In the present studies, exposure of cultured pineal organs to norepinephrine for 1 hr resulted in a 30-fold increase in serotonin *N*-acetyltransferase activity after 6 hr (data not shown). This increase could be blocked by addition of 10 μM *l*-propranolol 10 min prior to norepinephrine exposure.

Effect of exposure of pineal cultures to norepinephrine on sensitivity of broken cell adenylate cyclase to norepinephrine. The possible role of adrenoceptor desensitization in explaining the transient cAMP response to norepinephrine was examined by investigating the effects of previous norepinephrine exposure on the ability of this compound to activate the broken cell adenylate cyclase. Concentrations of norepinephrine from 0.1 to 100 μM caused a 4-

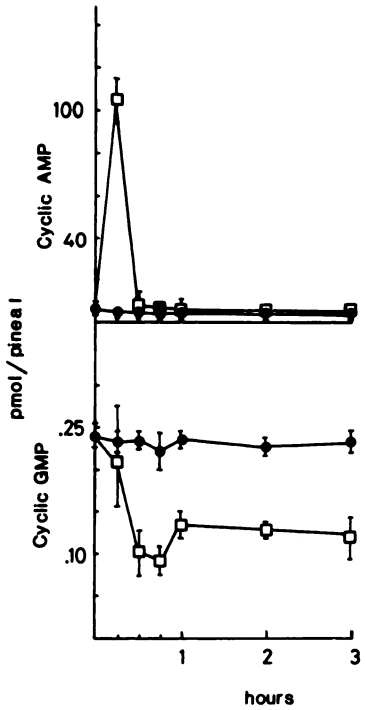

FIG. 6. *Effect of norepinephrine on cyclic nucleotide content of cultured pineals*

Pineals were cultured for 12 hr, and norepinephrine was added to the culture medium to a final concentration of 100 μM. Pineals were further cultured for various periods of time and frozen, and cAMP (top) and cGMP (bottom) were extracted and measured as described in METHODS. □——□, norepinephrine-treated; ●——●, control.

fold, dose-dependent increase in the activity of this enzyme prepared from pineals previously maintained in culture for 12 hr (Fig. 7). Addition of 100 μM norepinephrine to pineal cultures for 1 hr before preparing a homogenate significantly raised basal adenylate cyclase activity, and completely blocked the ability of norepinephrine at concentrations up to 100 μM to stimulate the enzyme (Fig. 7).

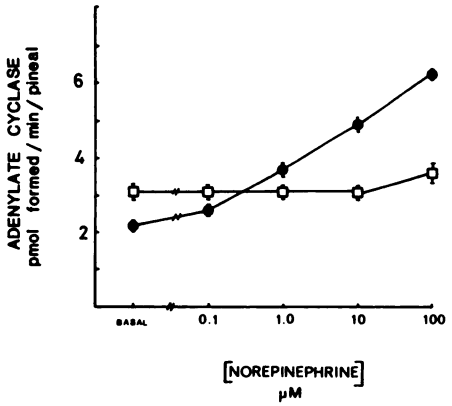

FIG. 7. *Effect of culturing in the presence of norepinephrine on sensitivity of broken cell adenylate cyclase to norepinephrine*

Pineals were cultured for 12 hr, and norepinephrine was added to some cultures (□——□), but not to others (●——●), to a final concentration of 100 μM. Pineals were further cultured for 1 hr and frozen, and adenylate cyclase activity was determined in the presence of various concentrations of norepinephrine as described in METHODS.

Combined effect of norepinephrine and choleragen on induction of serotonin N-acetyltransferase activity in cultured pineals. Incubation of cultured pineals with maximally stimulating quantities of choleragen (50 μg/ml) and subsequent exposure to maximally stimulating concentrations of norepinephrine (10 μM) caused a significantly larger (in fact, additive) increase in serotonin *N*-acetyltransferase activity after 6 hr than that seen after either treatment alone (Table 1). There was no significant change in either the maximal response or the dose-response curve to norepinephrine of the broken cell adenylate cyclase prepared immediately after incubation with norepinephrine plus choleragen. At this time, the toxin was bound to its receptors but adenylate cyclase was not yet activated. If the cell-free preparation was made 1 hr after choleragen exposure, however, when the cyclase was maximally activated by choleragen (Fig. 8), adenylate cyclase activity was not further increased by concentrations of norepinephrine up to 100 μM. Neither the increase in whole cell cAMP content nor the decrease in cGMP content observed 15 min after addition of 100 μM norepinephrine to pineal cultures was greatly affected by previous exposure to choleragen (Table 1). Simultaneous exposure of the cultured organs to maximally stimulating concentrations of both norepinephrine and choleragen caused a

TABLE 1

Effect of combined choleragen-norepinephrine treatment on serotonin N-acetyltransferase activity in cultured rat pineals

Pineals were cultured for 12 hr, then incubated with or without 50 μg/ml of choleragen, and returned to culture. Norepinephrine was added to some cultures, and pineals were cultured for a further 15 min for cyclic nucleotide measurements, or for 6 hr for serotonin *N*-acetyltransferase measurements. Norepinephrine was added to a final concentration of 10 μM for enzyme experiments and 100 μM for cyclic nucleotide experiments. Cultures were terminated by freezing, and enzymatic activity and cyclic nucleotide levels were determined as described in the text. Each value is the mean ± standard error of four separate pineals.

Treatment	Serotonin *N*-acetyltransferase activity	cAMP	cGMP
	pmoles/10 min/pineal	*pmoles/pineal*	
Control	10.2 ± 2.4	6.6 ± 1.06	0.24 ± 0.018
Norepinephrine	221 ± 12.2[a]	82.6 ± 13.52[b]	0.19 ± 0.031
Choleragen	287 ± 25.2[a]	7.8 ± 0.72	0.11 ± 0.015[b]
Norepinephrine + choleragen	524 ± 28.2[a]	122.3 ± 11.1[b]	0.16 ± 0.30[b]

[a] $P < 0.001$ compared with control.
[b] $P < 0.05$ compared with control.

virtual superimposition of the temporal changes in cAMP content seen after either substance alone (Fig. 9).

Induction of pineal enzymes by isobutylmethylxanthine. Addition of 1 mM isobutylmethylxanthine, a potent and specific phosphodiesterase inhibitor, to pineal cultures also caused a marked rise in serotonin *N*-acetyltransferase activity after 6 hr (Table 2); unlike that of theophylline (30), this effect was larger than that observed with norepinephrine alone. Addition of 1 mM IBMX also caused a significant increase in the activity of both cAMP and cGMP phosphodiesterases after 6 hr. Addition of 1 mM IBMX markedly in-

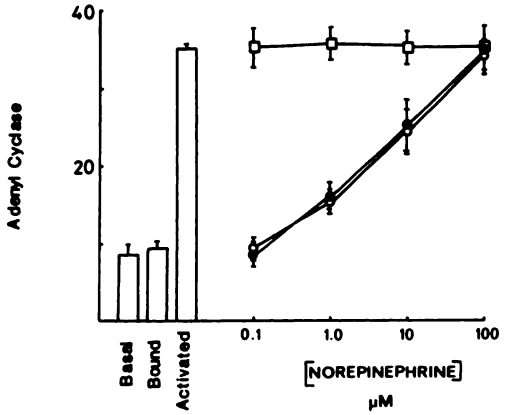

FIG. 8. *Effect of choleragen on sensitivity of broken cell adenylate cyclase to norepinephrine*

Pineals were cultured for 12 hr and incubated for 15 min at 37° either with or without 50 μg/ml of choleragen. Pineals either were frozen immediately after the choleragen incubation, when choleragen was bound but adenylate cyclase was not activated ("bound"), or were further cultured for 1 hr, at which time adenylate cyclase was activated ("activated"), and then frozen. Basal (●——●), bound (○——○), and activated (□——□) levels of adenylate cyclase were assayed as described in METHODS in the presence of various concentrations of norepinephrine. Adenylate cyclase activity is expressed as picomoles of cAMP formed per 10 min per pineal.

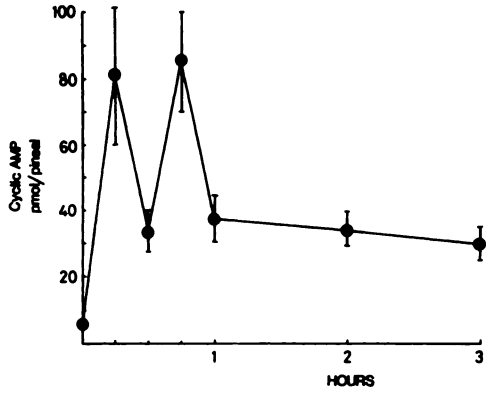

FIG. 9. *Effect of combined choleragen-norepinephrine treatment on cAMP levels*

Pineals were cultured for 12 hr and then incubated for 15 min at 37° in the presence of 50 μg/ml of choleragen as described. Pineals were returned to culture, and norepinephrine was immediately added to a final concentration of 100 μM. Cultures were terminated by freezing at various times thereafter, and cAMP content was determined as described in METHODS.

TABLE 2

Effect of isobutylmethylxanthine on enzyme activities and cyclic nucleotide levels in cultured rat pineals

Pineals were cultured for 12 hr, and IBMX was then added to a final concentration of 1 mM. After various times, pineals were removed and frozen and enzyme activity and cyclic nucleotide levels were determined as described in the text. Each value is the mean ± standard error of four separate pineals.

Time after IBMX	Serotonin *N*-acetyltransferase activity	Phosphodiesterase activity		Nucleotide levels	
		cAMP	cGMP	cAMP	cGMP
hr	*pmoles/10 min/pineal*	*nmoles/10 min/pineal*		*pmoles/pineal*	
0	5.2 ± 0.81	0.66 ± 0.05	0.77 ± 0.03	6.8 ± 0.24	0.19 ± 0.013
1				16.0 ± 0.44[a]	1.23 ± 0.246[b]
2				17.3 ± 1.60[a]	0.92 ± 0.434
3				13.7 ± 1.08[a]	0.85 ± 0.206[b]
4				15.3 ± 1.74[a]	0.69 ± 0.081[a]
6	297 ± 35.2[a]	1.02 ± 0.02[a]	1.18 ± 0.14[b]		

[a] $P < 0.001$ compared with zero-time value.

[b] $P < 0.05$ compared with zero-time value.

creased both cAMP (2–3-fold) and cGMP (4–6-fold) levels in whole pineals in culture (Table 2). The finding that IBMX increases serotonin *N*-acetyltransferase activity and yet reduces the ratio of cAMP to cGMP (from 36:1 to 13:1) (Table 2), whereas choleragen exposure also increases serotonin *N*-acetyltransferase activity but increases the ratio of cAMP to cGMP (from 32:1 to 1060:1), demonstrates that neither cGMP nor the ratio of cAMP to cGMP can be involved in determining this response.

Effect of ouabain and high potassium concentration on choleragen-induced rise in serotonin N-acetyltransferase activity. Ouabain (1 μM) and KCl (80 mM) blocked the rise in serotonin *N*-acetyltransferase activity produced by choleragen (Table 3), lending support to the hypothesis that the effects of cAMP on postsynaptic enzyme induction in the pineal may involve membrane hyperpolarization (31).

DISCUSSION

The stimulation of adenylate cyclase activity in rat pineal organ cultures by cholera toxin, through a mechanism which does not involve *beta* adrenoceptors, results in an induction of serotonin *N*-acetyltransferase activity which in its size and temporal characteristics is similar to that seen following *beta* adrenoceptor activation. The pharmacological dissociation of adenylate cyclase activation from *beta* adrenoceptor occupation allows the unequivocal conclusion that it is the activation of adenylate cyclase, and not adrenoceptor occupation per se, which is crucial for enzyme induction in this system. cAMP thus functions as a true intracellular mediator of at least one of the postsynaptic biochemical responses to norepinephrine in the rat pineal organ. cGMP is not involved in this response, since various treatments which produce opposing effects on cGMP levels, although having similar effects on cAMP levels, all result in the induction of serotonin *N*-acetyltransferase. It seems likely, as has been previously proposed (31), that the mechanism through which cAMP may effect induction of this enzyme may involve hyperpolarization of the pinealocyte membrane, since agents which prevent this phenomenon (ouabain and high external potassium) also block the induction of serotonin *N*-acetyltransferase after choleragen.

Since the *beta* adrenoceptor-stimulated postsynaptic pineal enzyme induction appears to be mediated by intracellular cAMP, it seems clear that alterations in the metabolic machinery for this nucleotide may play a major role in the well-documented alterations in *beta* adrenoceptor sensitivity in this system (17, 18, 32). Alterations both in the basal activity (33) of pineal adenylate cyclase and in the sensitivity of this enzyme to agonists (34) have been observed *in vivo* and have been demonstrated here *in vitro*, indicating that alterations in pineal sensitivity may occur via alterations in cAMP synthesis. Alterations in cAMP hydrolysis also occur *in vivo*, both naturally in response to daily lighting changes (35) and after *beta* agonists (36), and were demonstrated here *in vitro* after choleragen but not after norepinephrine. Such changes in phosphodiesterase activity can markedly affect the cAMP content of the cell. A very high, stable adenylate cyclase activity is observed after choleragen treatment, although the increase in cAMP levels is only

TABLE 3

Effects of ouabain and high potassium concentration on choleragen-induced rise in serotonin N-acetyltransferase activity in cultured pineals

Pineals were cultured for 12 hr, then incubated either with ("toxin") or without ("control") choleragen (50 μg/ml), and returned to culture for a further 6 hr. Ouabain (1 μM) or KCl (80 mM) was added to some cultures for the final 6 hr. At the end of this time, pineals were removed and frozen, and enzymatic activity was determined as described in the text. Each value is the mean ± standard error of four separate pineals.

Treatment in final 6 hr	Serotonin *N*-acetyltransferase activity	
	Control	Toxin
	pmoles/10 min/pineal	
No drug	5.3 ± 2.1	254 ± 28.2[a]
1 mM ouabain	3.2 ± 0.62	6.2 ± 2.5
80 mM KCl	2.8 ± 0.41	2.8 ± 1.21

[a] $P < 0.001$ compared with control.

transient. The decline in cAMP levels is temporally correlated with a rise in phosphodiesterase activity. When the increase in phosphodiesterase activity is blocked by addition of cycloheximide (which does not affect the choleragen *activation* of adenylate cyclase), the decline in cAMP levels is markedly attenuated. This is good evidence to suggest that minor changes (i.e., 60%) in the level of phosphodiesterase activity may have marked effects on the intracellular concentration of cAMP, and therefore that another site of control of cyclic nucleotide metabolism (and therefore *beta* adrenoceptor sensitivity) is cAMP degradation.

That alterations in both the synthesis and degradation of cAMP actually do play a part in cellular sensitivity was demonstrated by combining choleragen and norepinephrine treatment. The combination of these treatments produces a much larger induction of serotonin *N*-acetyltransferase activity than either treatment alone. Furthermore, the increase in intracellular cAMP is extended over a longer period by combined treatment than by either treatment alone. It seems, therefore, that if cAMP synthesis were not turned off by the desensitization of adenylate cyclase to norepinephrine, the resulting induction of serotonin *N*-acetyltransferase would be much larger. Similarly, if choleragen treatment did not induce phosphodiesterase activity, and therefore decrease intracellular cAMP levels, one would again expect a larger induction of serotonin *N*-acetyltransferase. It is interesting to note that while changes in adrenoceptor-adenylate cyclase sensitivity occur quickly (less than 1 hr) after receptor activation, changes in phosphodiesterase activity are slower in onset (1 to 4 hr). This may indicate a dual regulation of sensitivity, with receptor-coupled adenylate cyclase sensitivity controlling short-term changes and phosphodiesterase induction appearing after prolonged receptor stimulation. While these two mechanisms may account for the regulation of the absolute magnitude of the *beta* adrenoceptor response, studies with actinomycin D have suggested that the lag period of the response may be regulated by cellular levels of messenger RNA (37). The cycloheximide-insensitive decline in cAMP levels also suggests that cAMP extrusion may play a part in the regulation of intracellular pineal cAMP levels.

The induction of phosphodiesterase in the pineal requires new protein synthesis and appears to be regulated by intracellular levels of cAMP, as agents which increase cAMP levels beyond a certain amount (which do not include norepinephrine) also increase the activity of cAMP phosphodiesterase. The observation that cGMP phosphodiesterase is also significantly increased after choleragen, although intracellular cGMP levels are greatly reduced, suggests that this activity is also induced by increases in intracellular cAMP in the pineal. cGMP levels do appear to be controlled to a certain extent by phosphodiesterase activity, as IBMX increases the intracellular cGMP levels. However, as blockade of the choleragen-induced increase in phosphodiesterase had virtually no effect on cGMP levels, this nucleotide may not be particularly sensitive to phosphodiesterase levels. The similarity between the time course and magnitude of the induction of cAMP and cGMP phosphodiesterases raises the possibility that both nucleotides are hydrolyzed by the same enzyme in the pineal; biochemical evidence has recently been presented suggesting that this may also be the case in rat neostriatum (25). In support of this, both cAMP and cGMP phosphodiesterases vary with similar diurnal rhythms in the pineal *in vivo* (35). Although cGMP is not involved in postsynaptic serotonin *N*-acetyltransferase induction in the pineal, the large and unusually long-lasting decrease in intracellular cGMP after norepinephrine suggests that it may have some function. This observation is in good agreement with the recent observation that norepinephrine inhibits pineal guanylate cyclase (38). It has recently been reported (39) that norepinephrine causes a presynaptic, calcium-dependent increase in pineal cGMP. This apparent discrepancy can be explained by the fact that the present experiments were concerned mainly with postsynaptic changes.

Although most aspects of postsynaptic

pineal metabolism are very similar in organ culture to the properties observed *in vivo*, there are obvious differences between the two situations. The extrapolation of results obtained with pineal cultures to the functioning of this organ *in vivo* should be approached with caution. The culture system can, however, give valuable mechanistic information which is unobtainable in the more complex situation *in vivo*.

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