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Adenyl Cyclase Activity in Rat Pineal Gland: Effects of Chronic Denervation and Norepinephrine

Abstract. *Adenyl cyclase activity in the pineal gland of rats was determined by measuring the rate of formation of radioactive cyclic 3′,5′-adenosine monophosphate from ^{14}C-labeled adenosine triphosphate. Norepinephrine added in vitro to pineal homogenates enhanced this activity, while denervation of the pineal gland by superior cervical ganglionectomy did not significantly reduce it. The enzyme in these denervated glands was more responsive to the stimulatory effects of norepinephrine.*

Over the past several years there has been an accumulation of evidence which indicates that cyclic 3′,5′-adenosine monophosphate (cyclic 3′,5′-AMP) is a mediator of certain hormone-induced responses (*1*). The enzymes that catalyze the formation (adenyl cyclase) (*2*) and the hydrolysis (cyclic nucleotide phosphodiesterase) (*3*) of cyclic 3′,5′-AMP are widely distributed in mammalian and nonmammalian tissues. The evidence that catecholamines stimulate adenyl cyclase activity (*4*) suggests that in some tissues adenyl cyclase functions as a receptor for these amines. Since chronically denervated tissues are at least as responsive to catecholamines as innervated tissues are (see *5*), adenyl cyclase must be situated distally to nerve endings if it is to qualify as such a receptor. Loss of adenyl cyclase activity after denervation would indicate that the enzyme is not involved in catecholamine-induced responses at postjunctional sites. We have, therefore, investigated this activity in innervated and chronically denervated structures to determine the location of the enzyme and to examine its responsiveness to catecholamines.

The pineal gland is ideally suited for studying the changes in adenyl cyclase activity that might occur with chronic denervation. This organ is innervated exclusively by sympathetic fibers originating bilaterally from the superior cervical ganglia (*6*). Thus, superior cervical ganglionectomy causes degeneration of the pineal nerves (*6, 7*) and depletion of the catecholamines stored in its nerve terminals (*8, 9*), as shown by microscopic observation of the gland and by direct chemical analysis of norepinephrine.

Adenyl cyclase activity was determined by measuring the rate of accumulation of radioactive cyclic 3′,5′-AMP formed from its labeled precursor, ^{14}C-adenosine triphosphate (ATP). Cyclic 3′,5′-AMP is separated from ATP and other metabolites of ATP in two separate stages. The compounds are adsorbed onto ion-exchange columns [Dowex 50W-X4(H^+)] and eluted with 1-ml portions of water. This procedure eliminates approximately 99.9 percent of ATP from the cyclic 3′,5′-AMP fraction. Final purification of this fraction is achieved by precipitating trace contaminants by adding equal volumes of barium hydroxide (7.2 percent) and zinc sulfate (8 percent). Approximately 75 percent of the cyclic 3′,5′-AMP is recovered in a final volume of 3 ml, and the fraction is pure enough to enable detection of one part of the cyclic nucleotide in more than 50,000 parts of ATP. Moreover, rate of accumulation of the cyclic nucleotide in rat brain homogenates is linear with respect to both time and concentration of enzyme, if proper precautions are taken to maintain adequate substrate concentrations and to prevent the enzymatic hydrolysis of cyclic 3′,5′-AMP (*10*). An account of the present experiment has recently been reported (*11*).

In the present experiments, adenyl cyclase activity of pineal glands of male Sprague-Dawley rats (200 to 240 g) was determined as follows. Within 30 seconds after the rats were decapitated, each organ was removed, homogenized in tris-HCl buffer ($5 \times 10^{-2}M$, *p*H 7.4), and incubated individually in 100 μl of an assay mixture described in Table 1 and Fig. 1. Tubes were incubated at 30°C in a Dubnoff metabolic shaker. The reaction was stopped by immersing the tubes in boiling water for 5 minutes; carrier cyclic 3′,5′-AMP was added, and, after purification, the specific activity of the cyclic nucleotide in each sample was determined by measuring its radioactivity with a liquid scintillation spectrometer and its optical density, at 260 mμ, with a spectrophotometer.

A study of the time course of the accumulation of cyclic 3′,5′-AMP-^{14}C in pineal homogenates incubated with ATP-^{14}C indicated that cyclic 3′,5′-AMP was formed rapidly and destroyed rapidly and that the rate of formation was enhanced by norepinephrine. For

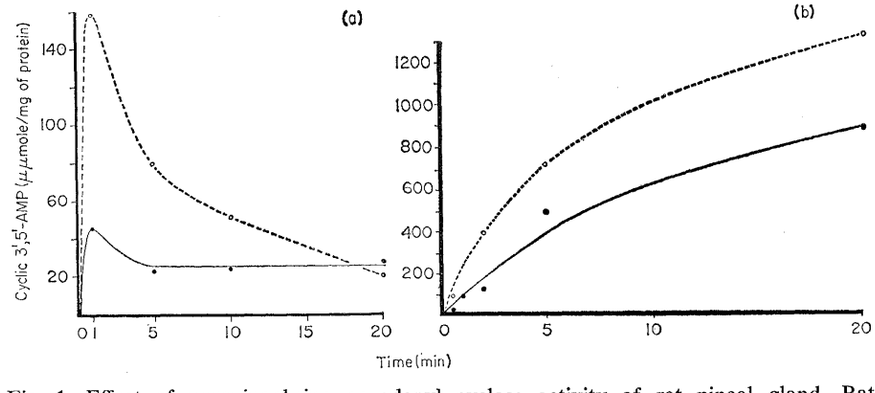

Fig. 1. Effect of norepinephrine on adenyl cyclase activity of rat pineal gland. Rat pineal homogenates were assayed for adenyl cyclase activity by measuring radioactive cyclic 3′,5′-AMP formed from ^{14}C-ATP. (a) Each incubation tube contained: 1 pineal, 1 μc of ATP-8-^{14}C ($2 \times 10^{-5}M$), and Mg^{++} ($3 \times 10^{-3}M$) in 100 μl of tris-HCl buffer ($5 \times 10^{-2}M$, *p*H 7.4). (b) Same as (a) except for the addition of theophylline ($10^{-2}M$) to prevent the enzymic hydrolysis of cyclic 3′,5′-AMP. Solid lines, without norepinephrine; dashed lines, with *l*-norepinephrine bitartrate ($1 \times 10^{-4}M$). Each point represents one determination.

example, after incubation of pineal homogenates for 1 minute in the presence of *l*-norepinephrine ($10^{-4}M$), there was a threefold increase in cyclic 3′,5′-AMP as compared with homogenates incubated in the absence of norepinephrine (Fig. 1a). However, the amount of cyclic nucleotide fell rapidly thereafter, reaching control levels after 20 minutes, which indicates its rapid destruction. Theophylline, which inhibits heart (*3*) and adipose tissue (*12*) phosphodiesterase, was therefore added to prevent the enzymic hydrolysis of the cyclic nucleotide. Figure 1b shows that, in the presence of theophylline, cyclic 3′,5′-AMP continued to accumulate for the 20-minute period of incubation. Addition of norepinephrine further enhanced the rate of formation of the cyclic nucleotide; that is, the catecholamine stimulated adenyl cyclase activity. These experiments showed that adenyl cyclase in pineal, like that in cerebellum (*4*), is stimulated by norepinephrine. Other experiments indicated that this effect of norepinephrine on pineal adenyl cyclase was specific for the *l*-isomer (relative activities: control, 1.0; *l*-norepinephrine, 1.8; and *d*-norepinephrine, 1.1).

In studies of the subcellular distribution of adenyl cyclase activity, Rodriguez de Lores Arnaiz (*13*) showed that in rat brain cortex the enzyme is localized in the crude mitochondrial fraction. Further analysis indicated that this activity is present in nerve endings and synaptic membranes. We have obtained similar results from pineal homogenates, finding more than 50 percent of the enzyme activity localized in the crude mitochondrial fraction. While the presence of adenyl cyclase in this fraction is consistent with the view that the enzyme is situated postjunctionally, the evidence is still equivocal, since the available fractionation methods fail to separate the closely attached pre- and postsynaptic membranes.

We, therefore, studied adenyl cyclase activity in rat pineals that had been chronically denervated by bilateral superior cervical ganglionectomy. This procedure, as well as sham operations, was performed under pentobarbital anesthesia. After 3 to 4 weeks, the rats were decapitated, the pineal gland was removed, and adenyl cyclase was assayed as described above. In the absence of added norepinephrine, adenyl cyclase activity in the pineal glands of ganglionectomized rats was not significantly different from that of control glands (Table 1), which suggests that in this gland the enzyme is not located in sympathetic nerve endings. However, when norepinephrine was added to the incubation medium, the increased activity of adenyl cyclase elicited by the catecholamine was significantly greater ($P < .01$) in pineals from denervated rats than it was in glands from sham-operated animals (Table 1).

Table 1. Effect of norepinephrine on pineal adenyl cyclase activity of sham-operated and ganglionectomized rats. Bilateral superior cervical ganglionectomies or sham operations were performed on male rats 21 to 28 days prior to use; there were 12 controls and 12 test animals in each group. Pineal homogenates were assayed for adenyl cyclase activity by measuring the rate of formation of radioactive cyclic 3′,5′-AMP from ^{14}C-ATP. Each incubation tube contained: 1 pineal, 1 μc of ATP-8-^{14}C ($2 \times 10^{-3}M$), theophylline ($10^{-2}M$), and Mg^{++} ($3 \times 10^{-3}M$) in the presence or absence of *l*-norepinephrine bitartrate ($1 \times 10^{-4}M$). The reaction was carried out for 5 minutes at 30°C. AMP, adenosine monophosphate; S.E., standard error.

Condition	Cyclic 3′,5′-AMP ($\mu\mu$mole/mg of protein per minute ± S.E.)		
	Control	Norepinephrine	Increase due to norepinephrine
Sham-operated	81 ± 9	96 ± 9	15
Ganglionectomized	56 ± 9	149 ± 19	93

With chronically sympathectomized tissues, not only do sympathetically denervated structures retain their responsiveness to catecholamines, but, in fact, they are also hypersensitive to the amines (*5*). To explain this, it has been suggested that nerve endings normally take up catecholamines and thereby decrease the concentration of amines at the receptor sites. Absence of nerve endings, by allowing a higher concentration of catecholamine at the receptor, could result in the increased sensitivity seen in chronically denervated tissues (*5*). However, it is difficult to explain our data on this basis. For example, our homogenization medium, being hypotonic, would be expected to disrupt nerve endings. Furthermore, dose-response studies showed that increasing the concentration of norepinephrine above $10^{-4}M$ fails to increase the response, which indicates that at this concentration the enzyme is already saturated. The possibility that the increased response of adenyl cyclase to norepinephrine in denervated tissue is due to a decreased rate of destruction of the catecholamine is unlikely in view of data that show that 21 days after bilateral removal of the superior cervical ganglia monoamine oxidase activity in denervated pineals was similar to that of innervated glands (*7*).

We suggest, therefore, that the increased response of adenyl cyclase to norepinephrine in chronic denervation is due to an alteration of the adenyl cyclase system itself. Evidence that denervation results in an increased response of adenyl cyclase to added norepinephrine, but does not significantly alter the enzyme activity in the absence of added norepinephrine, suggests a change in the sensitivity rather than in the quantity of enzyme.

Environmental lighting also affects the response of adenyl cyclase to norepinephrine. The stimulatory effect of norepinephrine on pineal adenyl cyclase activity was two to three times greater in rats exposed to constant light for 9 days than it was in animals kept in darkness or in normal diurnal lighting. Since in all cases nerve endings were intact, these results further our contention that the increased response of adenyl cyclase to norepinephrine is not due to a lack of nerve endings per se, but rather to a change at some postjunctional site. How this effect of the photoperiod on adenyl cyclase activity relates to the effect of light and darkness on other pineal enzymes (*14*) is a question for further investigation.

The major points demonstrated by these experiments are (i) norepinephrine enhanced adenyl cyclase activity in the pineal gland, and (ii) sympathetic denervation of the rat pineal gland caused little or no loss of this activity. Furthermore, pineal adenyl cyclase from ganglionectomized rats, compared with that from sham-operated animals, showed an increased responsiveness to norepinephrine. The implications of these observations are that the majority of the adenyl cyclase activity in the pineal resides at a postneuronal site, distal to the sympathetic nerve endings, and that sympathetic denervation sensitizes adenyl cyclase to the stimulatory effects of norepinephrine.

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Tetrodotoxin: Effects on Brain Metabolism in vitro

Abstract. *A 3-micromolar concentration of tetrodotoxin completely inhibits the stimulation of respiration of rat brain cortex that takes place upon application of electrical impulses. It also inhibits increase in the rate of the respiration that occurs when calcium ions are omitted from the incubation medium. No effect of tetrodotoxin on brain respiration takes place when stimulation is brought about by the addition of 100 millimolar potassium chloride. Tetrodotoxin prevents the fall in the rate of oxidation of cerebral acetate that occurs during electrical stimulation but does not affect the increased rate of the oxidation that occurs in the presence of an increased concentration of potassium chloride. The data indicate that oxidation of cerebral acetate is diminished by influx of sodium ions, which is prevented by tetrodotoxin, and is increased by influx of potassium ions, which is unaffected by tetrodotoxin.*

Tetrodotoxin (TTX), a potent neurotoxin found in the Japanese puffer fish and elsewhere (*1*), in low concentrations, such as 0.3 μM, suppresses action potentials in a variety of excitable tissues, including frog myelinated nerve fibers and lobster and squid giant axons (*2–4*). It is considered to be a specific blocker of sodium conductance, but with no effect on potassium conductance (*3–6*). It does, however, have suppressing effects on action potentials produced in media containing salts of divalent cations only (Ca^{++}, Sr^{++}, and Ba^{++}) (*7, 8*).

We have carried out experiments to observe whether tetrodotoxin affects the metabolism and transport reactions of brain cortex slices incubated aerobically in a physiological Ringer medium. It is well known that various aspects of the metabolism of isolated brain tissue are greatly influenced by changes in the cation concentration of the incubation medium or by application of electrical impulses (*9*). It seemed possible that use of tetrodotoxin might help to throw further light on the mechanisms whereby cations secure, in part, control of brain cell metabolism.

Metabolism and transport reactions in slices of brain cortex from adult hooded rats were investigated by methods already described (see *10*). Cortex slices (70 to 80 mg) were cut from the cerebral hemispheres with a Stadie-Riggs tissue slicer, care being taken to make the slices not more than 0.3 mm thick, but not so thin that they would tend to disintegrate when shaken in the Warburg manometric apparatus in which incubations were usually conducted. All incubations were normally carried out at 37°C for 1 hour in an atmosphere of oxygen in a Krebs-Ringer medium containing 128 mM NaCl, 5.0 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM Na_2HPO_4 (brought to *p*H 7.4 with 1.0N HCl), and 10 mM glucose, the final volume in all cases being 3 ml. When potassium ions were increased, potassium chloride was added to the normal medium to the desired concentration. For a calcium-free medium, calcium chloride was omitted from the normal medium. In some experiments, 2 mM sodium acetate-1-^{14}C was added to the normal medium at the commencement of the experiment and the amount of $^{14}CO_2$ evolved at the end of the incubation period was estimated by methods previously described (*11*).

Electrical stimulation of slices of brain cortex was carried out in vessels with silver grid electrodes similar to those used by Wallgren and Kulonen (*12*), with electrical impulses from the alternating frequency generator of the Teratron type, described in detail by Wallgren (*13*). The impulses had a pulse frequency of 100 cycle/sec with 1-msec duration, the peak potential being 4 volts. The impulses were displayed on a type 561A oscilloscope during the experiments.

Studies were made of the velocities of oxygen consumption in the presence or absence of TTX (Sankyo Co.) used at a concentration of 3 μM. Measurements were also made of the rates of evolution of $^{14}CO_2$ in the presence or absence of TTX in media containing 2 mM sodium acetate-1-^{14}C. The results are given in Table 1 which records, for each condition, the means of four independent observations ± standard deviation.

These results show that while TTX has no effect, within experimental error, on the rate of respiration of slices of adult rat brain cortex incubated in a physiological Ringer medium containing glucose, it completely blocks the stimulation of respiration that takes place on application of electrical impulses. An equal inhibitory effect of TTX occurs at lower concentrations (for example, 0.3 μM). Table 1 shows that TTX inhibits, almost completely, the increase in the rate of rat brain cortex respiration that occurs when calcium ions are omitted from the medium. Thus, TTX may exert an effect on brain metabolism in vitro independently of the application of electrical impulses. When the rate of brain respiration is increased by the addition of 100 mM KCl, no effect of TTX within experimental error is observed. On the basis of results given in Table 1, we conclude that, in the presence of 3 μM TTX, neither electrical stimulation nor the absence of calcium ions will cause enhancement of brain cell respiration, but that the addition of high concentrations of potassium ions is equally as effective, in stimulating brain respiration, in the presence as in the absence of TTX.

These results may be interpreted as indicating that, with electrical stimulation, there is an increased influx into the brain cell of sodium ions (*14*) which, by their accelerating effect on the activity of membrane adenosine triphosphatase, result in an increased rate of brain cell respiration controlled by adenosine diphosphate. The TTX suppresses, because of its combination with a specific membrane component, this response of the brain cell to an applied electrical impulse. Presumably, in the absence of calcium ions from the incubating medium, there is also an increased influx of sodium ions (*15*) mediated by the TTX-sensitive component of the cell membrane. It is known that removal of divalent cations from the external medium may lead to marked fluctuations of the membrane potential and to spontaneous excitation (*8*).

In accordance with the observation that TTX does not affect potassium conductance, there is no effect within experimental error of TTX on potas-