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EFFECTS OF CALCIUM-FREE MEDIUM ON THE INDUCTION OF SEROTONIN *N*-ACETYLTRANSFERASE IN THE RAT PINEAL

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Abstract—Incubation of rat pineal glands in calcium-free medium containing ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetra-acetic acid (EGTA) inhibited the induction of serotonin *N*-acetyltransferase activity caused by *l*-isoproterenol or dibutyryl cyclic AMP. Although changes in the dose–response relationship between *l*-isoproterenol and *N*-acetyltransferase activity were observed, the most prominent effect of calcium-free medium containing EGTA was to delay and prolong the increase in *N*-acetyltransferase activity. There was also a reduction in the maximal enzyme activity attained. Differences between supersensitive and subsensitive glands in the rate and extent of induction were retained in calcium-free medium containing EGTA. Other results indicate that chelation of extracellular calcium indirectly reduced the rate of the protein synthesis which is required for the induction of increased *N*-acetyltransferase activity.

The rat pineal gland responds to β-adrenergic stimulation with a 50-fold increase in the activity of serotonin *N*-acetyltransferase (EC 2.3.1.5) [1, 2]. This enzyme plays a pivotal role in the synthesis of the pineal hormone, melatonin [3]. Under physiologic conditions, enzyme activity is high at night [4], in the dark, when there is an increased release [1] of the neurotransmitter norepinephrine from the sympathetic nerve endings in the gland [5, 6].

After the nocturnal rise in *N*-acetyltransferase activity, exposure of rats to light reduces the release of norepinephrine [6] and causes the enzyme activity to drop precipitously [7, 8]. As long as the lights stay on, sympathetic stimulation is reduced, and *N*-acetyltransferase activity remains at its lowest levels [4, 8]. Pineal *N*-acetyltransferase activity, however, can be increased in animals exposed to light by the injection of isoproterenol [8, 9], which acts directly on the β-adrenergic receptors [10]. Similarly, enzyme activity can be increased by catecholamines in organ culture [3].

N-acetyltransferase appears to be an inducible enzyme. The increase in its activity shows a variable lag after stimulation [9], requires protein synthesis [3, 8], and, under ordinary conditions, requires RNA synthesis [11, 12]. There is compelling evidence that the induction of *N*-acetyltransferase activity is mediated by cyclic AMP [13–18]. The sensitivity of the pineal gland to β-adrenergic stimulation in terms of cyclic AMP accumulation and *N*-acetyltransferase induction varies in a diurnal cycle [19–21]. Glands taken from animals in the morning, after 12 hr of darkness, are relatively subsensitive, whereas glands taken from animals exposed to light for 12–24 hr are relatively supersensitive [9].

In a number of tissues there appears to be an interaction between cyclic AMP and calcium in mediating the effects of hormones [22, 23]. This appears to occur even in tissues, such as the adrenal cortex, in which the second messenger role of cyclic AMP is well established [24]. We were interested in the possibility that such an interaction might be relevant to the phenomena of supersensitivity and subsensitivity in the pineal gland. We, therefore, examined the effects of extracellular calcium on the induction of pineal *N*-acetyltransferase under several conditions. Chelation of extracellular calcium inhibited the induction by *l*-isoproterenol or dibutyryl cyclic AMP. There was, however, no differential effect on supersensitive and subsensitive glands. Rather, the results indicate that chelation of extracellular calcium indirectly reduces the rate and magnitude of induction by inhibiting protein synthesis.

MATERIALS AND METHODS

Chemicals. Acetyl-1-[^{14}C]coenzyme A (3.5 to 6.6 mCi/m-mole) was purchased from Amersham-Searle, Chicago, IL. *l*[^{14}C]Proline (261 mCi/m-mole) was purchased from New England Nuclear Corp., Boston, MA. *l*-Isoproterenol-*d*-bitartrate was provided by the Sterling-Winthrop Research Institute, Rensselaer, NY. Dibutyryl cyclic AMP was purchased from Cal-Biochem, La Jolla, CA.

Animals. Male Sprague–Dawley rats (150–175 g) were obtained from Zivic-Miller, Allison Park, PA. They were kept under diurnal lighting conditions (12 hr light, 12 hr dark) in our facilities for at least 6 days before the experiments.

Pineal explant culture. Groups of six rats were killed by decapitation. The pineal glands were rapidly removed and placed in organ culture in plastic petri dishes (Falcon, 60 mm dia.). Each dish contained 2.5 ml of modified BGJ_b Fitton–Jackson

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medium (Grand Island Biological Co., Grand Island, NY), supplemented with ascorbic acid (0.1 mg/ml), glutamine (2.0 mM), penicillin (100 units/ml,) and streptomycin (100 ßg/ml.). Medium contained 1.25 mM $CaCl_2$ and 1 mM $MgCl_2$, unless otherwise noted. Six pineals were incubated in each dish at 36° under 95% O_2–5% CO_2. Isoproterenol or dibutyryl AMP was added to the medium, after 1 hr preincubation, at the concentrations indicated.

Assays. Glands were assayed individually for serotonin *N*-acetyltransferase activity as described previously [25], using 20 nmoles [^{14}C]acetyl CoA. Units of activity are pmoles *N*[^{14}C]acetyltryptamine formed/10 min. Cyclic AMP was assayed by the method of Brown *et al.* [26]. Protein synthesis was assessed as described by Klein *et al.* [27].

RESULTS

Incubation of pineal glands in calcium-free medium containing the chelator EGTA* appeared to inhibit the induction of *N*-acetyltransferase by *l*-isoproterenol (Table 1). Inhibition was not evident in calcium-free medium in the absence of EGTA. However, the effect of EGTA was prevented by the addition of calcium but not by the addition of magnesium, implicating chelation of calcium as the basis of the inhibition.

Table 1. Effect of divalent cations and EGTA on the induction of *N*-acetyltransferase activity by *l*-isoproterenol*

Medium	*N*-acetyltransferase activity (pmoles/pineal/10 min)
Normal	845 ± 64
$-Ca^{2+}$	805 ± 127
$-C^{2+}$ + EGTA (1 mM)	424 ± 16†
$-Ca^{2+}$ + EGTA (1 mM) + Ca^{2+} (1.5 mM)	834 ± 76
$-Ca^{2+}$ + EGTA + Mg^{2+} (1.5 mM)	481 ± 51†

* Groups of six rats were killed after exposure to light for 24 hr. Pineals were removed and placed in organ culture containing 1.25 mM $CaCl_2$ and 1 mM $MgCl_2$ (normal) or modifications as indicated. After 1 hr, *l*-isoproterenol was added 0.1 μM, final concentration and the glands were further incubated for 8 hr. Pineals were assayed individually for *N*-acetyltransferase. Data represent mean ± S. E. M.

† Significantly different from normal media, $P < 0.001$.

The effects of varying extracellular calcium on the dose–response relationship between *l*-isoproterenol and *N*-acetyltransferase are shown in Fig. 1. In the absence of isoproterenol, control values were 13 ± 2.5 (n = 6) in normal medium and 7.6 ± 2.5 (n = 6) in calcium-free medium containing EGTA. Both the potency of *l*-isoproterenol and the maximal enzyme activity attained apparently increased with increasing concentrations of extracellular calcium. The dose–response curves in the presence of extracellular calcium were essentially parallel. However, the slope of the curve in the absence of calcium was less than that

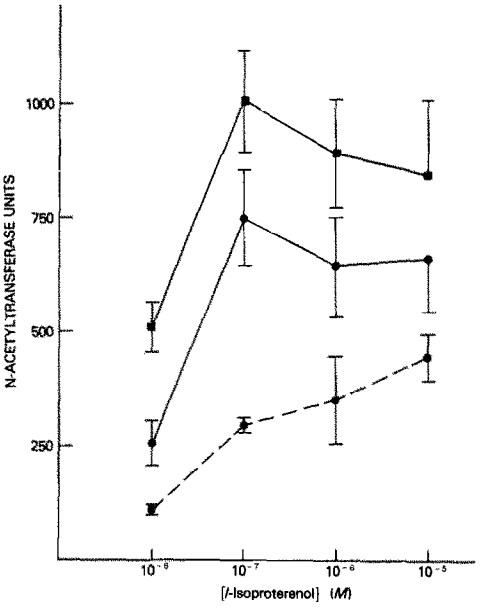

Fig. 1. Effect of calcium concentration on the induction of *N*-acetyltransferase by various concentrations of *l*-isoproterenol. Groups of six rats were killed in the morning after 3 hr of light. Pineal glands were removed and placed in organ culture containing either 7.5 mM $CaCl_2$ (■—■), 1.25 mM $CaCl_2$ (●—●), or no $CaCl_2$ and 1 mM EGTA (●----●). After 1 hr, *l*-isoproterenol was added at the final concentrations indicated. Glands were assayed for *N*-acetyltransferase activity 6 hr thereafter as described. In the absence of isoproterenol, *N*-acetyltransferase activity remained below 15 units for each group. Data shown represent mean ± S. E. M.

of the curves in the presence of the cation (Fig. 1). Incomplete induction during the 6 hr of incubation could account for such an effect.

Therefore, the effect of extracellular calcium on the time course of induction was examined. Time courses up to 12 hr, in normal medium, have been reported previously [9]. In the presence of calcium, *N*-acetyltransferase activity reached a maximum in supersensitive glands 8 hr after the addition of isoproterenol and then fell, approaching basal levels after 16 hr (Fig. 2). In contrast, supersensitive glands incubated in calcium-free medium containing EGTA did not attain their maximal *N*-acetyltransferase activity until at least 12 hr after the addition of isoproterenol. Furthermore, enzyme activity had not diminished even after 24 hr. Thus, the absence of extracellular calcium delayed and prolonged the induction of *N*-acetyltransferase.

In the subsensitive glands, maximal induction was achieved 6 hr after the addition of isoproterenol. Enzyme activity then fell, reaching basal levels after 16 hr (Fig. 2). Delayed induction in the absence of calcium is not evident in these glands. However, *N*-acetyltransferase activity in the absence of calcium remained elevated for at least 24 hr. In the subsensitive glands, maximal *N*-acetyltransferase induction was clearly greater in the presence of extracellular calcium.

Incubation in calcium-free media containing EGTA did not abolish the differences between supersensitive

* EGTA = ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetra-acetic acid.

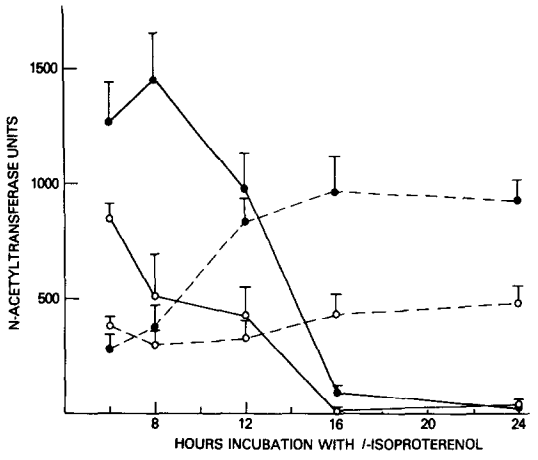

Fig. 2. Effect of calcium on the time course of induction of *N*-acetyltransferase in supersensitive and subsensitive pineal glands. Groups of six rats were killed in the morning after 24 hr of light (supersensitive) (●) or 12 hr of darkness (subsensitive) (○). Pineals were removed and placed in organ culture containing 1.25 mM $CaCl_2$ (—) or no $CaCl_2$ plus (0.1 μM final concentration) and the glands were further incubated for the times indicated. *N*-acetyltransferase activity was then assayed as described. Data shown represent mean ± S. E. M.

and subsensitive glands. In both normal and calcium-free media supersensitive glands showed a greater induction of *N*-acetyltransferase activity and took longer to reach maximal activity (Fig. 2).

The fall in *N*-acetyltransferase activity after its induction appears to be a function of the initial concentration of agonist (Table 2). When supramaximal concentrations of isoproterenol were used in normal medium, *N*-acetyltransferase activity remained maximally elevated even after 17 hr. In the calcium-free medium containing EGTA, induction was maximal after 17 hr and there was no further elevation of *N*-acetyltransferase by the higher dose. This allowed the direct comparison of maxima in the presence and absence of extracellular calcium, in supersensitive glands. As suggested by the previous data, the presence

Table 2. Effect of initial isoproterenol concentration and calcium on *N*-acetyltransferase activity after 17 hr*

	N-acetyltransferase activity (pmoles/pineal/10 min)	
l-Isoproterenol (μM)	0 $CaCl_2$, 1 mM EGTA	1.25 mM $CaCl_2$
0.1	925 ± 137 (6)	19 ± 9 (6)
10	711 ± 144 (6)	1295 ± 106 (5)

* Groups of rats were killed after exposure to light for 24 hr. Pineals were removed and placed in organ culture containing either 1.25 mM $CaCl_2$ or no $CaCl_2$ and 1 mM EGTA. After 1 hr, *l*-isoproterenol was added as indicated and the glands were further incubated for 17 hr. Pineals were assayed for *N*-acetyltransferase as described. Data represent mean ± S. E. M. for the number of glands indicated in parentheses.

Table 3. Effect of calcium on the induction of *N*-acetyltransferase activity by dibutyryl cyclic AMP*

	N-acetyltransferase activity (pmoles/pineal/10 min)	
	0 $CaCl_2$, 1 mM EGTA	1.25 mM $CaCl_2$
24 hr light	113 ± 31 (5)	827 ± 112 (5)
12 hr dark	112 ± 27 (6)	220 ± 49 (6)

* Groups of rats were killed after exposure to 24 hr of light or 12 hr of darkness. Pineals were removed and placed in organ culture containing either 1.25 mM $CaCl_2$ or no $CaCl_2$ and 1 mM EGTA. After 1 hr, 1 mM dibutyryl cyclic AMP was added and the glands were further incubated for 8 hr. Pineals were assayed for *N*-acetyltransferase activity as described. Data represent mean ± S. E. M. for the number of glands indicated in parentheses.

Table 4. Effect of calcium on the accumulation of cyclic AMP in response to isoproterenol*

	Cyclic AMP (pmoles/gland)	
	0 $CaCl_2$, 1 mM EGTA	1.25 mM $CaCl_2$
Control	8.5 ± 2	9.5 ± 1
l-Isoproterenol	41.0 ± 7	43.0 ± 8

* Groups of six rats were killed after exposure to 24 hr of light. Pineals were removed and placed in organ culture containing either 1.25 mM $CaCl_2$ or zero $CaCl_2$ and 1 mM EGTA. After 1 hr, *l*-isoproterenol was added to a final concentration of 0.1 μM, where indicated, and the glands were further incubated for 10 min. Pineals were homogenized and heated at 90° and assayed for cyclic AMP. Data represent mean ± S. E. M.

of extracellular calcium raised the maximum enzyme activity achieved.

Dibutyryl cyclic AMP, which by-passes the receptor–adenylate cyclase complex, induces *N*-acetyltransferase in cultured pineals [16]. When glands were incubated 8 hr with this compound, in calcium-free medium containing EGTA, induction of *N*-acetyltransferase was inhibited as compared to inductions in the presence of extracellular calcium (Table 3). Furthermore, the presence or absence of extracellular calcium and EGTA did not affect significantly the levels of cyclic AMP achieved 10 min after the addition of isoproterenol to preincubated glands (Table 4). As previously shown [28], cyclic AMP levels reach their maxima, under the conditions used, at this time. These data indicate that at least part of the action of extracellular calcium is indirect and that it affects steps in the induction beyond the accumulation of cyclic AMP.

Under ordinary conditions, cyclic AMP mediates the induction of *N*-acetyltransferase by affecting both RNA and protein syntheses [11, 12]. However, the reinduction of *N*-acetyltransferase activity, after it has been artificially reduced in the middle of the night, does not require RNA synthesis, although it still

Table 5. Effect of calcium on the induction of *N*-acetyltransferase activity in glands from animals exposed to light or darkness*

	N-acetyltransferase activity (pmoles/pineal/10 min)	
	0 $CaCl_2$, 1 mM EGTA	1.25 mM $CaCl_2$
18 hr light	406 ± 53	1024 ± 99
6 hr dark, 20 min light	342 ± 39	1089 ± 80

* Groups of six rats were killed after exposure to 18 hr of light or to 20 min of light after 6 hr of darkness. Pineals were removed and placed in organ culture containing either 1.25 mM $CaCl_2$ or no $CaCl_2$ and 1 mM EGTA. After 1 hr, *l*-isoproterenol was added (0.1 μM, final concentration) and the glands were further incubated for 6 hr. Glands were assayed for *N*-acetyltransferase activity as described. Data represent mean ± S. E. M.

requires protein synthesis [11]. Thus, if extracellular calcium indirectly affected RNA synthesis, its effect would not be observed during the reinduction of *N*-acetyltransferase in the middle of the night. When the effects of extracellular calcium on induction and reinduction were examined (Table 5), they were found to be similar. Reinduction in glands from animals which had been exposed briefly to light after 6 hr of darkness was inhibited to essentially the same extent in the absence of calcium as was induction in glands from animals that had remained in the light. Thus, extracellular calcium does not act predominantly through an effect on RNA synthesis.

It appears that the calcium-free medium containing EGTA has an inhibitory effect on the protein synthesis required for the induction of *N*-acetyltransferase activity (Table 6). Incorporation of radioactive proline into acid precipitable material was inhibited by more than 50 per cent when glands were incubated in calcium-free medium containing EGTA. There was no reduction in the acid-soluble radioactivity taken into the glands (data not shown). Although β-adrenergic stimulation does not increase total protein synthesis (Ref. 27, Table 6), ongoing protein synthesis is required for induction of *N*-acetyltransferase [3, 8, 11]. The general inhibition of protein synthesis observed in calcium-free medium containing EGTA presumably also affected the specific protein synthesis required for the induction of *N*-acetyltransferase.

Table 6. Effect of extracellular calcium and EGTA on pineal protein synthesis*

Medium	Incorporation of [^{14}C]proline (cpm)
Normal	2860 ± 175
$-C^{2+}$	2820 ± 130
$-Ca^{2+}$ + EGTA (1 mM)	1190 ± 155†
Normal + *l*-isoproterenol (0.1 μM)	2730 ± 190
$-Ca^{2+}$ + EGTA (1 mM) + *l*-isoproterenol (0.1 μM)	1080 ± 125†

* Groups of 6 rats were killed after exposure to light for 24 hr. Pineals were removed and placed in organ culture containing 1.25 mM $CaCl_2$ (normal) or modifications as indicated. After 1 hr, 5 μCi [^{14}C]proline was added to each incubation dish (1 ml). Where indicated, *l*-isoproterenol (0.1 μM final concentration) was also added. Glands were further incubated for 6 hr. Individual glands were washed, homogenized in trichloroacetic acid, filtered, washed with acid, chloroform-methanol, and ether, and counted as described [27]. Data represent mean ± S. E. M.

† Significantly different from normal media, normal media $-Ca^{2+}$ or normal media + *l*-isoproterenol, $P < 0.001$.

DISCUSSION

Increasing concentrations of extracellular calcium appeared to shift the dose–response relationship between *N*-acetyltransferase activity and isoproterenol to the left, as well as to increase the maximal enzyme activity achieved. These effects must be interpreted with caution, however, in view of the complex interrelationships between dose of agonist, time course of induction, and sensitivity of the glands used. The increased maximum in the presence of extracellular calcium was readily confirmed under several conditions. The apparent effect on EC_{50} could have been due to an increased rate of induction caused by extracellular calcium, particularly if the rate of induction also increased with the dose of isoproterenol.

There was a clear effect of extracellular calcium on the time course of induction. In the absence of calcium, induction was delayed and prolonged. Thus, the absence of calcium appeared to reduce induction when its effects were examined after 8 hr, but appeared to increase induction when its effects were examined after 17 hr. These data demonstrate the importance of time course in the interpretation of 'inhibitory' effects on induction.

An additional effect on the time course, that of the dose of isoproterenol, was also demonstrated. High concentrations of isoproterenol prolonged the period of maximal *N*-acetyltransferase activity. This was not due simply to the continued presence of active agonist when the higher concentration (10 μM) was used. When glands incubated in 0.1 μM isoproterenol were transferred to fresh medium every 6 hr, enzyme activity still fell after 12 hr (data not shown).

The possibility that the effects of zero calcium and EGTA might be secondary to an effect on the degradation of isoproterenol in culture was considered. This possibility seemed highly unlikely. A decreased rate of degradation should potentiate rather than inhibit the effect of isoproterenol, while an increased rate of degradation should shorten, rather than prolong, the effects of the catecholamine. Furthermore, zero calcium and EGTA had similar effects on the induction by dibutyryl cyclic AMP as on the induction by isoproterenol. This could not be due to an effect on the degradation of isoproterenol. Finally, since isoproterenol has no effect on total protein synthesis, a change in its rate of degradation could not account for the effects on proline incorporation into protein.

The effect of reduced concentrations of extracellular calcium on the dose–response relationship between *N*-acetyltransferase activity and isoproterenol concentration appeared to mimic reduced sensitivity of the glands to induction [9]. However, other data

suggested that this appearance was misleading. Subsensitive glands show a more rapid rate of induction [9], whereas glands incubated in the absence of calcium showed a reduced rate of induction. Furthermore, the differences between supersensitive and subsensitive glands in the extent of induction were retained in the absence of extracellular calcium. Supersensitive and subsensitive glands were 'inhibited' equally by the absence of extracellular calcium. Thus, although extracellular calcium concentrations affected the rate and extent of *N*-acetyltransferase induction, they did not modify the differences between supersensitive and subsensitive glands.

Experiments which measured the accumulation of cyclic AMP indicated that at least part of the effect of calcium involves intracellular sites distal to those regulating cyclic AMP levels. Cyclic AMP appears to act at several sites in the induction of *N*-acetyltransferase activity [11]. These include the stimulation of specific RNA and protein syntheses. Comparison of the effects of calcium-free media on the induction and reinduction of *N*-acetyltransferase activity indicated that extracellular calcium does not act predominantly through an effect on RNA synthesis. Rather, it appears that calcium-free medium has an indirect, inhibitory effect on the rate of synthesis of the protein required for the induction of *N*-acetyltransferase activity.

The adrenal cortex is another organ in which the synthesis and secretion of hormone are stimulated through a cyclic AMP-mediated mechanism which requires protein synthesis [24]. Extracellular calcium is also required for the complete response to hormone or dibutyryl cyclic AMP [29]. The interactions between cyclic AMP and calcium appear to be multiple and complex [23, 24]. However, one of these interactions enhances the rate of protein synthesis required for the steroidogenic effect of hormone or of cyclic AMP [30]. A similar interaction affecting the rate of protein synthesis may occur in the rat pineal gland.

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