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Calcium-Dependent Blockade of Cardiac Cyclic AMP Accumulation by Batrachotoxin and Veratridine

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SUMMARY

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Batrachotoxin and veratridine are cardiotoxic alkaloids that cause a persistent activation of the fast sodium channel of excitable cells. Murine cardiac atria exposed to these toxins *in vitro* lose the capacity to accumulate cyclic AMP in response to isoproterenol. This effect is seen at concentrations of toxin comparable to those necessary to increase sodium permeability in other systems (10-100 nM for batrachotoxin; 20-100 μM for veratridine) and is antagonized by the specific sodium channel blocking agent, tetrodotoxin. Extracellular calcium is required for inhibition of the cyclic AMP response by batrachotoxin and veratridine. Atria exposed to these agents also undergo a marked loss of intracellular ATP and there is a concomitant decrease in GTP content. ATP depletion is also antagonized by tetrodotoxin and markedly attenuated when extracellular calcium is removed. There is a significant correlation between the intracellular ATP concentration of the atrium and the ability to accumulate cyclic AMP in response to isoproterenol which suggests a causal relationship. Other possible mechanisms for batrachotoxin and veratridine inhibition of the cyclic AMP response are considered, and the possibility that calcium affects ATP pools critical for adenylate cyclase activity is discussed.

INTRODUCTION

The cyclic nucleotide concentrations of brain slices and cultured nerve cells can be increased by conditions that depolarize excitable cells, e.g., elevated extracellular K^+, veratridine, batrachotoxin, and ouabain (1-3). Enhanced cyclic AMP accumulation results, at least in part, from adenylate cyclase activation by released adenosine (1, 2). In contrast to such findings made with neuronal preparations, depolarization of cardiac muscle (4, 5) or uterine smooth muscle (6, 7) with elevated extracellular K^+ does not increase cyclic AMP but instead prevents cyclic AMP accumulation in response to catecholamines. The studies described here demonstrate that batrachotoxin and veratridine, agents that depolarize excitable cells by increasing sodium permeability through the action potential or fast sodium channel, also block cyclic AMP formation in cardiac muscle. However, in contrast to the effect of 57 mM K^+ depolarization on murine atria, which is indirectly mediated through release of myocardial acetylcholine (5, 8), the sodium-channel neurotoxins act through a unique calcium-dependent pathway to block hormone-stimulated cyclic AMP accumulation.

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METHODS

Adult male Swiss-Webster mice were killed by cervical dislocation and the right and left atrial appendages were excised from the *in situ* heart within 30 sec after death. Atria were rinsed in oxygenated Krebs-Henseleit buffer of the following composition (millimolar): NaCl, 118; KCl, 4.7; $CaCl_2$, 3.0; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; Na_2EDTA, 0.5; and glucose, 10, pH 7.4 at 37°. Individual atria were then transferred to flasks containing 5 ml of buffer, gassed constantly with 95% O_2-5% CO_2. Tissues were incubated for 40 min before the medium was replaced by experimental medium (*e.g.*, medium with elevated K^+, batrachotoxin, etc.) containing 100 μM isobutylmethylxanthine. When the concentration of KCl in the medium was increased, that of NaCl was reduced to maintain isotonicity; for calcium-free medium, $CaCl_2$ was omitted, and 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid replaced EDTA. At the end of a 20-min incubation in experimental medium, isoproterenol (±) or sodium ascorbate (100 μM final, as vehicle for isoproterenol) were added for 1.5 min. The atria were removed, blotted, and frozen in freon cooled in liquid nitrogen and stored at −70° until assayed.

For cyclic AMP determinations, frozen tissues were prepared and assayed by the protein binding assay of Gilman as previously described (5). Protein was deter-

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mined by the method of Bradford (9). ATP was determined on neutralized perchlorate (0.6 N) extracts of frozen tissue by the fluorometric method of Lowry and Passonneau (10). The concentrations of adenine nucleosides and nucleotides in the incubation medium were determined by high-performance liquid chromatography (11). GTP was determined by high-performance liquid chromatography on Partisil SAX using a linear gradient with a low concentration buffer (A) of 50 mM KH_2PO_4, pH 3.8, and a high concentration buffer (B) of 0.5 M KCl, 0.25 M KH_2PO_4, pH 3.6. The gradient was from 0–100% of B in 12 min, followed by 100% of B for an additional 25 min.

Materials. Veratridine was purchased from ICN K&K Laboratories Inc. (White Plains, N. Y.). Batrachotoxin was a gift of Dr. John Daly (National Institutes of Health, Bethesda, Md.). Tetrodotoxin (citrate-free) and the calcium ionophore A23187 were from Calbiochem (San Diego, Calif.). All other drugs and chemicals were from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Isoproterenol-stimulated cyclic AMP accumulation was markedly reduced when atria were incubated with the depolarizing agents, batrachotoxin or veratridine, for 20 min (Table 1). Batrachotoxin produced concentration-dependent inhibition which was maximal (80%) at 100 nM, whereas veratridine, although less potent, produced comparable inhibition at a concentration of 100 μM. Neither depolarizing agent significantly lowered the basal cyclic AMP concentration. Batrachotoxin did not nonspecifically depress hormone-stimulated cyclic nucleotide formation, since it was without effect on cyclic AMP accumulation in S49 lymphoma cells (data not shown).

Tetrodotoxin (10 μM)[1] abolished the effects of batrachotoxin (Table 2) and veratridine on cyclic AMP accumulation, whereas atropine, at concentrations up to 100 μM, did not (Table 2). The mechanism of inhibition by the sodium channel toxins thus differs from that previously found with 57 mM K^+, which resulted from release of acetylcholine and was unaffected by tetrodotoxin but completely antagonized by atropine (5).

When calcium was removed from (and 0.5 μM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid added to) the incubation medium, batrachotoxin and veratridine no longer inhibited isoproterenol-stimulated cyclic AMP accumulation (Table 2). The calcium-dependent effect of the toxins on cyclic AMP metabolism was not mimicked by incubation of atria in normal buffer containing the calcium ionophore A23187 (Table 2).

The inhibitory effect of batrachotoxin was evident after 5 min and intensified over a 45-min incubation. Inhibition was not reversed by washing atria in batrachotoxin-free medium for 15 min, and only partial reversal of inhibition was achieved by a 50-min wash in batrachotoxin-free medium containing 10 μM tetrodotoxin (see Fig. 1). While these observations may be explained by the highly lipophilic nature of batrachotoxin, they also suggested permanent metabolic changes consequent to exposure to the toxin.

[1] Blockade of cardiac muscle sodium channels requires higher concentrations of tetrodotoxin than are necessary in nerve and skeletal muscle (12).

TABLE 1

Depolarizing agents inhibit cyclic AMP accumulation in mouse atrium

Atria were incubated in medium containing batrachotoxin (BTX) or veratridine for 20 min and then challenged with isoproterenol (ISO) (3 μM) or ascorbate (ASC) for 1.5 min. Data are means from several experiments, with at least 10 individual atria per experimental group, and standard errors of less than 15% of the mean values.

Additions to medium	Cyclic AMP, after challenge with		% Inhibition
	ASC	ISO	
	pmoles/mg protein		
None	25	181	
BTX			
10 nM	——	129	34
100 nM	24	56	81
1 μM	28	58	79
Veratridine			
20 μM	32	100	53
100 μM	28	63	77

The concentration of ATP was determined in atria incubated for 20 min with veratridine or batrachotoxin (Table 3). Toxin concentrations that maximally inhibited cyclic AMP formation (100 nM batrachotoxin; 100 μM veratridine) produced marked depletion of intracellular ATP. Partial (30%) ATP depletion was seen with a concentration of veratridine submaximal for blockade of the cyclic AMP response (20 μM). In contrast, depolarization with 57 mM K^+ did not lower the intracellular ATP concentration.

Tetrodotoxin antagonized and calcium omission greatly attenuated ATP depletion by batrachotoxin (Table 3). There was no decrease in ATP in atria incubated for 20 min with 10 μM A23187 (not shown). Ouabain did not block the effect of batrachotoxin on ATP or on cyclic AMP metabolism, although high concentrations of

TABLE 2

Inhibition by batrachotoxin (BTX) requires calcium and is blocked by tetrodotoxin (TTX)

Atria were incubated for 20 min in control medium, in medium containing 10 μM A23187, or in medium containing 100 nM BTX and then challenged with ascorbate (basal) or with 3 μM isoproterenol (ISO) for 1.5 min. The effect of BTX was also examined in the presence of 10 μM TTX (TTX added 15 min prior to BTX), in the presence of 3 μM atropine, and in calcium-free medium. Omission of calcium prevented the action of BTX even when the divalent cation concentration was maintained by replacing calcium with magnesium. Values given are means ± standard error of the mean from 15–25 individual atria.

Condition	Cyclic AMP
	pmoles/mg protein
Basal	26 ± 4
ISO	135 ± 11
ISO, BTX	33 ± 2
ISO, BTX, TTX	127 ± 15
ISO, BTX, atropine	38 ± 3
ISO, BTX, no calcium	129 ± 15
ISO, A23187	119 ± 19

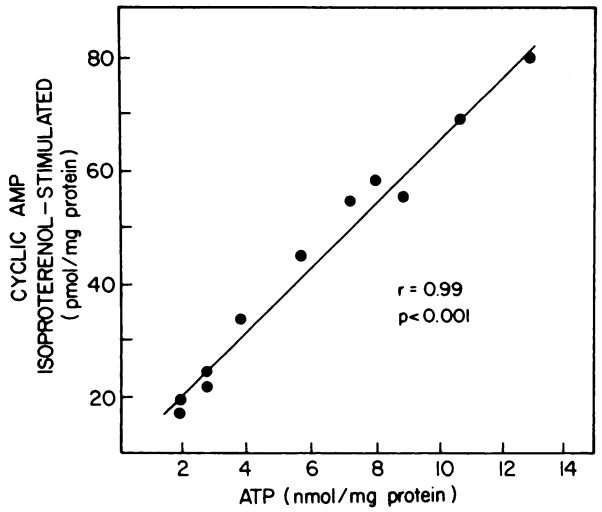

FIG. 1. *Correlation between isoproterenol-stimulated cyclic AMP concentration and intracellular ATP content*

Atria were incubated under a variety of conditions for 20 min, and frozen 1.5 min after the addition of 3 μM isoproterenol. The ATP and cyclic AMP content of individual atria were determined. Mean values for cyclic AMP and ATP for each experimental group (six atria) are plotted; standard errors (omitted for clarity) ranged from 10–20% of the mean for both ATP and cyclic AMP values. The correlation coefficient was calculated by least squares regression analysis. Incubation conditions were as follows: the lowest four points are from atria treated with batrachotoxin ± ouabain (20 or 500 μM), and the fifth point is batrachotoxin followed by a 50-min wash with tetrodotoxin; the middle point is ouabain (500 μM) alone, and the upper five points are from atria incubated under control conditions, in calcium-free medium, or with 20 μM ouabain. The basal cyclic AMP concentration (not subtracted) was 12 pmoles/mg of protein.

ouabain (500 μM) alone caused some ATP depletion (see Fig. 1).

In preliminary experiments, we found that the intracellular GTP concentration also decreased in atria incubated with 100 nM batrachotoxin for 20 min. The GTP concentration in control tissues was 0.43 ± 0.07 nmol/mg of protein ($N = 3$), whereas that in toxin-treated tissues was 0.11 ± 0.02 ($N = 5$).

To determine whether the loss of ATP was associated with the impaired cyclic AMP response, we compared hormone-stimulated cyclic AMP concentrations in groups of atria incubated under conditions that differentially affected the ATP concentration. There was a highly significant positive correlation between the intracellular ATP content and the magnitude of the cyclic AMP response to isoproterenol (Fig. 1).

The nucleotide and nucleoside concentration in media from control and batrachotoxin-treated (100 nM, 20 min) atria was also determined. Neither ATP nor ADP was recovered in medium of toxin-treated atria, but the concentrations of adenosine, inosine, and hypoxanthine were increased 5- to 10-fold. The adenosine concentration in medium from batrachotoxin-treated atria was 0.15 μM. Adenosine has been shown to inhibit isoproterenol-induced cyclic AMP accumulation in the ventricle (13). The presence of a methylxanthine phosphodiesterase inhibitor in our incubation would likely block any adenosine-mediated responses. Indeed, exogenous adenosine (10 μM) added to the mouse atrium along with isoproterenol affected neither the basal cyclic AMP level nor the response to isoproterenol (data not shown). Furthermore, adenosine did not enhance the effect of a submaximal concentration of batrachotoxin on cyclic AMP metabolism. Thus, release or accumulation of this nucleoside cannot explain toxin-induced inhibition of cyclic AMP formation.

TABLE 3

Batrachotoxin (BTX)- and veratridine-induced ATP depletion in mouse atrium

Atria were incubated in either normal, 57 mM K^+, or calcium-free medium containing the appropriate toxins, for 20 min. Tetrodotoxin (TTX) was added for 15 min prior to the addition of BTX. The toxin concentrations were: BTX, 100 nM; veratridine, 100 μM; and TTX, 10 μM. Isoproterenol (3 μM) was added for 1.5 min in some experiments and did not significantly affect the ATP concentration. Values are means ± standard error of the mean of 5–10 individual atria.

Additions to medium	ATP
	nmoles/mg protein
Control	14.8 ± 1.8
57 mM K^+	16.3 ± 2.5
BTX	3.2 ± 0.2[a]
Veratridine	2.4 ± 0.3[a]
TTX	17.3 ± 2.3[b]
TTX + BTX	17.0 ± 2.4
No calcium	12.8 ± 1.7[b]
No calcium + BTX	8.8 ± 1.2[c]

[a] Significantly different from control, $p < 0.01$.

[b] Not significantly different from control.

[c] Not significantly different from no calcium control, by Student's *t*-test.

DISCUSSION

The alkaloid neurotoxins veratridine and batrachotoxin activate the action potential or fast sodium channels of nerve and muscle cells (14–17). Sodium uptake is increased by concentrations of batrachotoxin and veratridine comparable to those found here to block isoproterenol-stimulated cyclic AMP accumulation (15, 16). Tetrodotoxin, a specific inhibitor of the fast sodium current (18), antagonizes the increase in sodium influx (14, 16, 17) and the positive inotropic responses of cardiac muscle to these agents (17, 19–21). Tetrodotoxin likewise blocked the inhibitory effects of batrachotoxin and veratridine on cyclic AMP accumulation in the atrium (Table 2). These data suggest that inhibition of cyclic AMP accumulation by batrachotoxin and veratridine is a consequence of activation of the fast sodium channel. Our finding that batrachotoxin did not alter cyclic nucleotide metabolism in the presumably nonexcitable S49 lymphoma cell further supports this notion, and argues against a nonspecific cytotoxic effect of this agent.

The primary effect of batrachotoxin and veratridine is to prolong activation of the fast sodium conductance, and these agents can actually cause sodium to accumulate in nerve and muscle cells (16, 17, 22). In the atrium, conditions that increase intracellular sodium lead to calcium influx (23), and in cultured cardiac cells veratridine has been shown to stimulate a sodium-dependent and tetrodotoxin-sensitive calcium uptake (16). The secondary increase in intracellular calcium is apparently brought

about by altered sodium-calcium exchange, a process which has been characterized in both nerve and muscle cells (23–25) and hypothesized to explain the positive inotropic effects of batrachotoxin and veratridine (19, 20). Calcium influx secondary to sodium channel activation also appears necessary for the altered cyclic AMP response produced by batrachotoxin and veratridine, which requires extracellular calcium (Table 2). Our results with the calcium ionophore suggest that calcium influx alone is not sufficient to inhibit cyclic AMP formation in the heart; however, more complete studies using A23187 under conditions where calcium concentration is also varied are necessary.

Batrachotoxin and veratridine caused marked ATP depletion in the atrium (Table 3). Similar loss of ATP has been noted in brain slices (2).The simplest explanation, that ATP supplies are exhausted through the valiant attempts of Na^+-K^+ ATPase to remove excess sodium, is unlikely since inhibition of the membrane ATPase with ouabain did not attenuate batrachotoxin-induced ATP loss. The molecular basis for ATP depletion by batrachotoxin and veratridine and the role of sodium and calcium in this response is clearly complex. It is noteworthy, however, that toxin effects on ATP and on cyclic AMP metabolism both occurred at similar threshold and maximal toxin concentrations, both required extracellular calcium, and both were antagonized by prior but not by subsequent addition of tetrodotoxin. Thus, there is an intimate association between the dual effects of the toxin on ATP and on cyclic AMP metabolism.

Although a causal relationship between ATP depletion and inhibition of cyclic AMP formation is not unequivocally demonstrated here, no conditions have been found to dissociate these events. Treatments such as ouabain, tetrodotoxin , and calcium omission affected both parameters in parallel, resulting in a striking correlation between ATP and hormone-stimulated cyclic AMP concentrations (Fig. 1). Thus, although the intracellular ATP concentration may approximate 1 mM even in toxin-treated atria, there may be critical pools of ATP which must be replete for full expression of hormone-sensitive cyclic AMP accumulation. Basal cyclic AMP may turn over more slowly, especially in the presence of a phosphodiesterase inhibitor, and thus be unaffected by lowered availability of substrate ATP over a 20-min period.

Activation of adenylate cyclase requires GTP and treatments that affect GTP metabolism in intact cells can alter hormonally induced cyclic AMP accumulation (26). Therefore, it is possible that batrachotoxin and veratridine selectively block hormonal activation of adenylate cyclase by decreasing the availability of GTP. Further studies will be necessary to critically evaluate this hypothesis. One might also speculate that calcium influx induced by the toxins alters cyclic AMP metabolism through calmodulin-mediated activation of phosphodiesterase (27). However, the calmodulin-stimulated phosphodiesterase from heart should be effectively inhibited by 100 μM isobutylmethylxanthine (28).

A number of laboratories have suggested that the cyclic AMP concentration of cardiac muscle is lowered by manipulations expected to increase intracellular calcium, for example, increased extracellular calcium (4), decreased extracellular sodium (29), and increased frequency of stimulation (30). Inhibition of isoproterenol-stimulated cyclic AMP formation in the uterus also results from interventions that increase intracellular calcium (6, 7). The general theme that calcium influx antagonizes cyclic AMP formation has thus appeared in a number of studies of cardiac and smooth muscle. The mechanism for this antagonism has not yet been elucidated. The data presented here demonstrate that batrachotoxin and veratridine cause a marked inhibition of cyclic AMP accumulation through activation of the fast sodium channel and subsequent calcium-dependent events, which include ATP depletion. The biochemical changes that underlie these responses and the possibility that more subtle changes in intracellular ATP concentration can regulate cyclic AMP formation are in need of further study.

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