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EFFECTS OF INTRACELLULAR LITHIUM ON EPINEPHRINE-INDUCED ACCUMULATION OF CYCLIC AMP IN SKELETAL MUSCLE*

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Abstract—The effects of lithium and of magnesium ions on adenosine 3′,5′-monophosphate (cyclic AMP) accumulation in isolated rat hemidiaphragms were investigated. Magnesium ions caused a small but significant increase in the basal concentration of cyclic AMP, whereas lithium ions had no effect. However, pretreatment of tissues with LiCl caused a significant dose-dependent reduction in the effect of epinephrine on the concentration of cyclic AMP. Magnesium ions enhanced the rise in tissue cyclic AMP caused by epinephrine, but this stimulatory effect of magnesium ions was reduced in tissues that had been pretreated with LiCl. It was concluded that lithium ions act at an intracellular site to inhibit hormone-induced accumulation of the cyclic nucleotide and was proposed that magnesium and lithium ions act at different cellular locations to produce their effects on hormone-induced cyclic AMP accumulation.

Many studies have shown that lithium ions can reduce hormone-induced stimulation of adenylate cyclase in several different tissues including brain [1–8]. Whether this pharmacological action of the cation is related to its therapeutic effects in affective disorders [9–12] is now unknown but is a subject for speculation and research [13]. Certain of the side effects seen during lithium treatment in man may be a consequence of an inhibition of hormone-induced stimulation of adenylate cyclase. For example, the administration of lithium carbonate to man can produce polyuria [14, 15] that is resistant to administration of antidiuretic hormone (ADH) [16, 17]. The antidiuretic effect of this hormone is thought to result from an increase in medullary adenosine 3′,5′-monophosphate (cyclic AMP) [18]. Since ADH activation of adenylate cyclase preparations from human renal medullary tissue is decreased by lithium ions [19], the diuretic effect of lithium may be, in part, a consequence of this cellular effect of the ion. Recent evidence indicates that lithium may also interfere with the effects of ADH at a site distal to the formation of cyclic AMP [17]. In a similar manner, the antithyroid effects of lithium [20, 21] may be related to an inhibition the effect of thyroid-stimulating hormone (TSH) on thyroid adenylate cyclase [5, 6], in addition to actions in the thyroid gland at sites subsequent to cyclic AMP formation [22, 23].

In the present investigation, we have demonstrated that the lithium ion decreases hormone-induced accumulation of cyclic AMP in skeletal muscle as it does in other tissues. Experiments are also reported that indicate that lithium in its effect on adenylate cyclase acts at an intracellular site.

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METHODS

Male Wistar strain rats weighing between 100 and 150 g were used. Animals were killed by decapitation, and hemidiaphragms were removed, blotted lightly on filter paper and weighed on a torsion balance.

Incubation. In most of the experiments, hemidiaphragms were preincubated for 30 min at 37° in Erlenmeyer flasks with 2 ml of solutions containing 40 mM HEPES (*N*-2-hydroxyethyl piperazine-*N*-2-ethane sulfonic acid) adjusted to pH 7·2 with NaOH, 5 mM $MgCl_2$, 10 mM KCl and 103 mM NaCl in control experiments in the absence of LiCl. When indicated, LiCl was added at concentrations of either 2·5, 7·5 or 25 mM; the LiCl was substituted for the equivalent amount of NaCl. At the end of the preincubation, the tissues were rinsed twice in 0·28 M sucrose and transferred into 2 ml of a medium containing 40 mM HEPES, 5 mM $MgCl_2$, 10 mM KCl, 97 mM NaCl and 12 mM glucose. After 5 min of incubation, 30 μM epinephrine was added to the medium in order to stimulate adenylate cyclase activity. The hemidiaphragms were immersed in liquid nitrogen 2 min later. Identical experiments were carried out in the absence of the hormone.

In some experiments, we studied the effects of magnesium ions on the stimulatory action of epinephrine on cyclic AMP accumulation in hemidiaphragms preincubated in the absence or presence of 7·5 mM LiCl. These experiments were carried out as described above except that the concentration of $MgCl_2$ was varied in both the preincubation and the incubation periods. When $MgCl_2$ was added to the media it was substituted for an isosmotic amount of NaCl. As

before, LiCl was present only during the 30-min preincubation.

In another set of experiments, hemidiaphragms were incubated at 37° for a total of 7 min in the absence or presence of 0·5 mM LiCl. The media contained HEPES, $MgCl_2$, KCl, NaCl and glucose as above. After 5 min of incubation, 30 μM epinephrine was added to the medium and the tissues were frozen 2 min later.

Cyclic AMP analysis. Cyclic AMP in the tissues was extracted as described previously [24] and measured by the protein binding method of Gilman [25]. All analyses were done in duplicate.

Lithium determinations. The lithium ion content of tissues and media was determined by atomic absorption spectrophotometry as described previously [24].

RESULTS

In preliminary experiments, it was established that the maximal stimulation of cyclic AMP accumulation by epinephrine occurred after 2 min of incubation. Therefore, this time period was used in subsequent experiments on the action of the hormone on tissue cyclic AMP.

Figure 1 presents the results of experiments in which paired hemidiaphragms were preincubated for 30 min in the absence or presence of different concentrations of LiCl and then transferred to incubation media containing no lithium ion. The tissues were then exposed to epinephrine as described in Methods and the concentration of cyclic AMP in the tissue was measured. Lithium produced a dose-related inhibition of the stimulatory effect of epinephrine on cyclic AMP accumulation. This effect became highly significant at a mean tissue lithium ion concentration of about 4 μmoles/g ($P < 0{\cdot}005$, paired t-test) (Fig. 1).

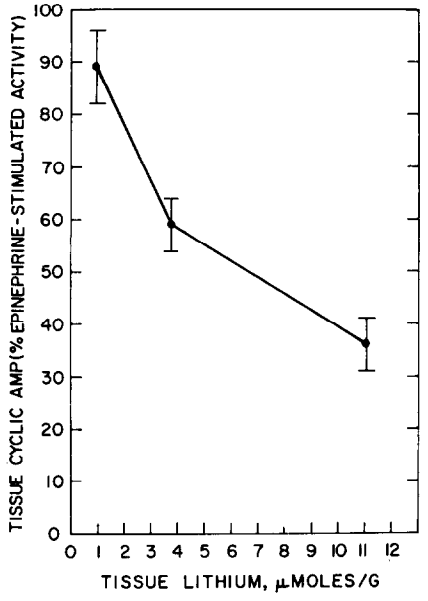

Fig. 1. Inhibition of epinephrine-stimulated accumulation of cyclic AMP by lithium ion. In paired experiments, hemidiaphragms were preincubated at 37° for 30 min in media containing either no LiCl or 2·5 (N = 8), 7·5 (N = 10) or 25 mM (N = 5) LiCl. The tissues were then transferred to media containing no added LiCl and incubated for 7 min, epinephrine (30 μM) being added for the final 2 min of incubation. The tissue content of cyclic AMP and of lithium was measured at the end of this incubation. Tissue lithium ion concentrations were, respectively, 1·0 ± 0·1, 3·8 ± 0·3 and 11·1 ± 0·3 μM/g. The mean tissue contents of cyclic AMP in the diaphragms that served as epinephrine-stimulated controls for the 2·5, 7·5 and 25 mM Li^+ experiments were, respectively, 6·8 ± 1·4, 6·5 ± 1·0 and 7·7 ± 1·1 nmole/g, wet wt. The brackets around each mean represent the S.E.M.

It should be emphasized that this inhibitory effect of lithium was seen only when epinephrine was present. As previously reported [24], in ten paired experiments the control basal tissue concentration of cyclic AMP was 0·67 ± 0·10 nmole/g ($\bar{X}$ ± S.E.M.), whereas hemidiaphragms preincubated in the presence of 25 mM LiCl contained 0·57 ± 0·06 nmole/g of cyclic AMP ($P > 0{\cdot}2$). In the experiments presented in Fig. 1, the tissue Li^+ concentrations shown on the abscissa are those measured at the end of the 7-min incubation. In separate experiments previously reported [24], we measured lithium ion content of rat hemidiaphragms incubated for 30 min in the presence of 25 mM LiCl. A mean value of 20·0 ± 0·9 μmoles Li^+/g of tissue (N = 11) was obtained. Since in tissues preincubated in 25 mM LiCl the lithium ion content at the end of the additional 7 min of incubation was 11·1 ± 0·3 μmoles/g (N = 17), approximately 9 μmoles/g of lithium was lost by the tissue during this incubation. The hemidiaphragms ranged in weight between 90 and 120 mg; therefore, approximately 0·8 to 1·1 μmoles lithium would be lost by each diaphragm to the medium. Since the volume of medium was 2 ml, the concentration of Li^+ in the medium would be expected to be 0·4 to 0·5 mM at the end of the experiment. Indeed, this value was measured directly and found to be 0·44 ± 0·03 mM (N = 6). To see whether a concentration of LiCl of this magnitude in the incubation medium itself would affect the epinephrine-induced increase in tissue cyclic AMP, the following experiments were done.

Paired hemidiaphragms were incubated with or without 0·5 mM LiCl for 7 min and epinephrine was added after 5 min of this incubation. The results of this experiment are shown in Table 1, experiment A. Lithium at this concentration and for this time period

Table 1. Effect of extracellular lithium ions on epinephrine-induced cyclic AMP accumulation in isolated rat diaphragm

Experiment	Incubation* Li^+ (mM)	Incubation* Time (min)	Cyclic AMP (nmole/g, wet wt)
A	0	7	7·7 ± 0·7†(5)‡
	0·5	7	8·9 ± 1·8 (5)
B§	0	4	2·7 ± 0·4 (4)
	7·5	4	2·8 ± 0·4 (4)

* Epinephrine, 30 μM present during the last 2 min of incubation in all experiments.

† $\bar{X}$ ± S.E.M.

‡ Number of observations.

§ Experiments done 6 months later than those in experiment A.

did not alter significantly the ability of epinephrine to stimulate cyclic AMP production. Even when the lithium ion concentration in the medium was 7·5 mM, but the total time of incubation shortened to 4 min, no inhibition of epinephrine-stimulated cyclic AMP was observed (Table 1, experiment B). These experiments were carried out about 6 months later than those in Table 1, experiment A. There is no obvious explanation for the smaller effects of epinephrine on cyclic AMP accumulation observed in these later experiments. However, it is clear that lithium ions at the relatively high concentration of 7·5 mM were without effect on the hormonal activation of adenylate cyclase in these short-term experiments.

Because of the similarity in certain physiochemical characteristics between lithium and magnesium ions (e.g. the ionic radius) [26], we carried out experiments to determine whether there was any interaction between the two ions in the regulation of the tissue content of cyclic AMP. When diaphragms were incubated for 7 min in the absence of added epinephrine, the inclusion of 10 mM $MgCl_2$ in the medium caused a small but significant increase in cyclic AMP. In five paired experiments, the mean cyclic AMP content was 0·33 ± 0·04 nmole/g in the absence of magnesium and 0·56 ± 0·06 nmole/g in the presence of magnesium ($P < 0·05$). As mentioned, lithium did not influence the basal cyclic AMP content of the diaphragm. In contrast, the epinephrine-induced accumulation of cyclic AMP was affected to a marked extent by both lithium and magnesium. The results of these experiments are reported in Fig. 2.

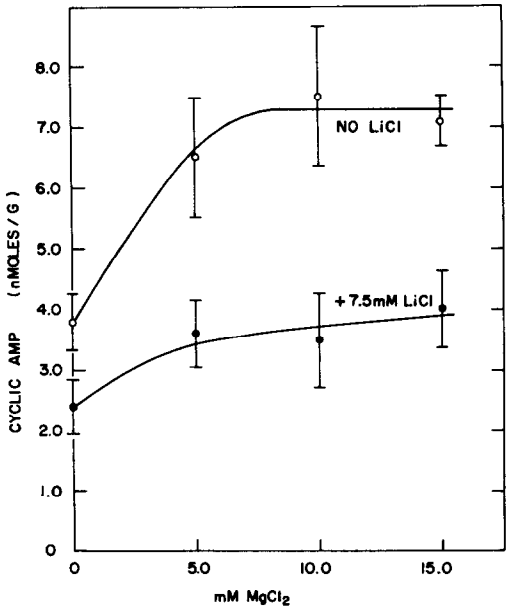

Fig. 2. Effect of magnesium ion on epinephrine-stimulated accumulation of cyclic AMP in the absence or presence of lithium. In paired experiments, hemidiaphragms were preincubated at 37° for 30 min in media containing either 0 or 7·5 mM LiCl (gas phase O_2). $MgCl_2$ was present in the media at the concentrations indicated on the abscissa. The tissues were then transferred to media containing no added LiCl, but the same concentration of $MgCl_2$ that was present during the preincubation. The tissues were incubated for 7 min, epinephrine (30 μM) being added for the final 2 min of incubation. The tissue content of cyclic AMP was measured at the end of this incubation. The number of paired determinations for magnesium concentrations of 0, 5, 10 and 15 mM were, respectively, 10, 10, 7 and 8. The brackets around each mean represent the S.E.M.

Tissue levels of cyclic AMP 2 min after addition of epinephrine are plotted as a function of the medium concentration of $MgCl_2$. These data were evaluated by analysis of variance [27]. The inhibitory effects of Li^+ were highly significant ($P < 0·001$) regardless of the presence of magnesium. On the other hand, the addition of magnesium enhanced the stimulating effect of epinephrine both in controls and in tissues pretreated with lithium ($P < 0·005$). The maximal effect of magnesium was observed at a concentration of 5 mM of this ion, and higher concentrations had no further effect. However, stimulation of cyclic AMP accumulation produced by magnesium was influenced by Li^+ present in the tissue. Since there were no significant differences among the values for tissue cyclic AMP at 5, 10 and 15 mM $MgCl_2$ (with or without Li^+), we calculated the means of the values obtained after incubation with $MgCl_2$ for each curve in Fig. 2. The increments in tissue cyclic AMP produced by magnesium ions were then determined. It was clearly established that the effect of magnesium was significantly greater in the absence of lithium than in tissues pretreated with this ion ($P < 0·005$, Student's t-test). If the results are calculated on the basis of the percentage increase caused by the magnesium ion, the increase is still significantly greater in the absence of lithium than in the tissues containing Li^+. These results show that lithium antagonizes the enhancement of epinephrine-induced net synthesis of cyclic AMP produced by magnesium ion.

DISCUSSION

The results of this investigation show that epinephrine-induced accumulation of cyclic AMP in skeletal muscle incubated *in vitro* is inhibited by lithium ion under conditions where this ion has no effect on the tissue level of cyclic AMP in the absence of hormone. Such an action of lithium could be due either to an inhibition of adenylate cyclase or to an increase in the activity of phosphodiesterase. It has been observed by several investigators that lithium ion inhibits hormone stimulation of cell-free preparations of adenylate cyclase from several tissues [2, 4, 5, 7, 8]. Therefore, it is likely that the effect of Li^+ shown here with a muscle preparation is caused by an action of the ion on adenylate cyclase.

Our experiments strongly support the view that the lithium ion exerts this action after it has entered the cell. In previous studies [24], we observed that when diaphragms were incubated in the presence of LiCl, under conditions similar to those used here, lithium did indeed accumulate intracellularly in exchange for potassium ion. After 30 min of preincubation, a considerable amount of Li^+ has entered the cell (see Fig. 1), and the effect of lithium on epinephrine-stimulated cyclic AMP accumulation is readily apparent. In experimental situations in which little lithium would be expected to enter the diaphragm, the cation had no effect on epinephrine-induced cyclic AMP accumulation. The data presented here, then, do not support

the suggestion made recently that lithium need not be taken up by the cell to inhibit hormonal effects on adenylate cyclase [28].

The problem of whether the effects of lithium ions on adenylate cyclase in skeletal muscle reported here occur in patients receiving therapy with lithium salts needs further investigation. The plasma level of lithium in such patients is about 1 mM, considerably lower than the concentrations of 3–5 mM needed to inhibit hormone activation of adenylate cyclase in broken cell preparations. However, since lithium ions are taken up by muscle [24], concentrations of the ion within the cell may be considerably higher than extracellular concentrations and sufficient to influence adenylate cyclase activity. In addition, it has been demonstrated that after administration of lithium salts to rats, the concentration of Li in several tissues including kidney [17], basal ganglia [29], pituitary [29]* and thyroid* was found to be several-fold higher than that of the simultaneously measured plasma concentration.

Studies of adenylate cyclase preparations from different tissues in the presence and absence of hormones have led to the formulation of various hypotheses to explain the unique properties of this enzyme. Robison *et al.* [30] proposed several years ago that the enzyme is composed of two subunits: (1) a regulatory subunit exposed to the extracellular space and containing the receptor to which the hormone binds; and (2) a catalytic subunit with its active center on the inner portion of the membrane and acting on MgATP. Birnbaumer *et al.* [31] have extended this model of the adenylate cyclase system to include three components. In this model, a transducer component has been interposed between the regulatory subunit (discriminator) and the catalytic part (amplifier) of the enzyme.

The precise mechanism by which bound hormone activates adenylate cyclase is unknown. Several investigators have suggested that free magnesium ion binds to a site on the enzyme different from the binding site of the substrate [4, 32, 33], and that hormones activate the enzyme by increasing the affinity of this second site for free magnesium ion [4]. However, conflicting results have been obtained [33–35] and alternative explanations have been proposed [36].

We can offer the following suggestions to explain our results with lithium and with magnesium. The finding that it is tissue lithium, and not extracellular lithium, that inhibits epinephrine-stimulated accumulation of cyclic AMP is consistent with the idea that Li^+ is acting at a site subsequent to the binding of hormone to the regulatory subunit. In other words, lithium appears to be acting directly at some site on the enzyme, either at the level of the transducer or the amplifier component. This view is strengthened by the reports that lithium can inhibit the stimulatory effect of several different hormones on adenylate cyclases from various tissues [1–8].

Because of similarities in certain physical properties between lithium and magnesium, the inhibitory effect of Li^+ may be the result of competition with magnesium for some site on the enzyme. Consistent with this view are the findings with thyroid tissue [5] and platelets [8] that the inhibitory effect of lithium is reduced by raising the magnesium concentration of the incubation medium. In contrast, in the present investigation elevation of the medium concentration of magnesium did not overcome the inhibitory effect of lithium. When added by itself, magnesium ion enhanced the stimulation of cyclic AMP produced by epinephrine. However, the inhibition produced by lithium was apparent at all magnesium concentrations tested. In fact, the inhibitory effect of lithium is most prominent when magnesium is present in the medium at a concentration high enough to produce maximal accumulation of tissue cyclic AMP in response to epinephrine (see Fig. 2).

Nevertheless, an interaction of lithium and magnesium in this system was observed. The enhancement of epinephrine-stimulated accumulation of cyclic AMP, seen when magnesium was present in the medium, was reduced significantly in tissues preincubated with lithium compared to control hemidiaphragms. The reason for an interaction of this nature is not readily apparent. It may be that the magnesium ion exerts its stimulatory effect at an external site on the plasma membrane and that little of the added magnesium enters the cell during the incubation period. Bianchi and Bolton [37] have shown that the flux of magnesium across skeletal muscle is slow, having a calculated time constant of about 40 hr. If this is so, then under our experimental conditions, magnesium ion would not penetrate to the intracellular site where lithium is acting. Lithium, however, by acting inside the cell at a site subsequent to that activated by magnesium, would be expected to antagonize the stimulatory effect of magnesium. This scheme is admittedly speculative but can be tested in future investigations. Indeed, because of its ability to inhibit hormone-induced activation of adenylate cyclases in many different tissues, lithium may be a valuable tool in studies concerned with the mechanisms by which hormones activate this enzyme.

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* S. Stern, unpublished observations.

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