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Lithium, an inhibitor of cAMP-induced inositol 1,4,5-trisphosphate accumulation in *Dictyostelium discoideum*, inhibits activation of guanine-nucleotide-binding regulatory proteins, reduces activation of adenylylcyclase, but potentiates activation of guanylyl cyclase by cAMP

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Li⁺ drastically alters pattern formation in *Dictyostelium* by inhibiting cAMP-induced prespore-gene expression and promoting cAMP-induced prestalk-gene expression. We reported previously that Li⁺ inhibits inositol monophosphatases in this organism and strongly reduces basal and cAMP-stimulated inositol 1,4,5-trisphosphate levels. We show here that Li⁺ also reduces cAMP-induced accumulation of cAMP, but promotes cAMP-induced accumulation of cGMP. This effect is not due to inhibition of cGMP hydrolysis or inhibition of adaptation and may therefore reflect stimulation of guanylyl-cyclase activation. Li⁺ does not affect the binding of cAMP to surface receptors but interferes with the interaction between receptors and guanine-nucleotide-binding regulatory (G) proteins. These effects are complex; in the absence of Mg²⁺, Li⁺ increases guanosine 5'-[γ-thio]triphosphate (GTP[S])-binding activity to similar levels as 1 mM Mg²⁺. However, while Mg²⁺ potentiates cAMP-induced stimulation of GTP[S]-binding activity, Li⁺ effectively inhibits stimulation. Li⁺ also inhibits cAMP-stimulated, but not basal high-affinity GTP-ase activity, indicating an inhibitory effect on cAMP-induced activation of G-proteins.

Our data suggest that in addition to inositolphosphate metabolism, the activation of G-proteins may be a second biochemical target for Li⁺ effects on pattern formation and signal transduction in *Dictyostelium*.

Lithium strongly affects processes as diverse as human behaviour and embryonic development, but its effects are nevertheless quite selective. Lithium can control manic depressive psychosis at serum levels around 1 mM, without major peripheral side effects. During amphibian development, lithium acts primarily at the early cleavage stages and dorso-lizes the response of animal cells to mesoderm-inducing signals (Backstrom, 1954; Breckenridge et al., 1987; Kao and Elinson, 1989).

Both therapeutic and teratogenic effects of Li⁺ may result from inhibition by Li⁺ of inositol monophosphatases

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Abbreviations. GTP[S], guanosine 5'-[γ-thio]triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate; App[NH₃], adenosine 5'-[βγ-imino]triphosphate; CAMP[S], adenosine 3',5'-[βγ-imino]phosphate, Sp-isomer; 2'deoxy-CAMP, 2'deoxy-adenosine 3',5'-monophosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetrakisphosphate; G protein, guanine-nucleotide-binding regulatory protein; G₁₅ protein, guanine-nucleotide-binding regulatory protein activating adenylylcyclase. Enzymes. Adenylylcyclase (EC 4.6.1.1); guanylyl cyclase (EC 4.6.1.2); phospholipase C (EC 3.1.4.3); cGMP phosphodiesterase (EC 3.1.4.35); creatine kinase (EC 2.7.3.2).
nucleotide-binding regulatory proteins activating adenylylcyclase (G_s proteins) in brain are more sensitive to inhibition by Li^+ than their homologues in heart, which may provide an alternative explanation for the specific sensitivity of the brain to Li^+ (Schreiber et al., 1990).

During development of the primitive eukaryote Dictyostelium discoideum, Li^+ perturbs pattern formation by inhibiting expression of prespore genes and promoting expression of a subclass of prestalk genes (Mead, 1970; Van Lookeren Campagne et al., 1988a; Peters et al., 1989). Both classes of genes are regulated by extracellular cAMP (Kay, 1982; Mehdy et al., 1983; Wang et al., 1988). cAMP-signal processing is initiated by the binding of cAMP to cell surface receptors. This interaction induces G-protein-mediated activation of adenylylcyclase and phospholipase C, as well as the processing. The most striking result is a pronounced inhibitory effect of Li^+ on CAMP-induced activation of G-proteins, suggesting a second target for Li^+ effects on gene expression and signal transduction.

MATERIALS AND METHODS

Materials

Creatine phosphate, creatine kinase, GTP[S], adenosine 5'-[β,γ-imino]triphosphate (App[NH]p), adenosine 5'-[β-thio]triphosphate (ATP[S]) and adenosine 3',5'-[thiolphosphate, Sp-isomer (cAMP[S]) were obtained from Boehringer Mannheim. [2,8-3H]cAMP, [35S]GTP[S], cGMP-radioimmunoassay kits and Ins(1,4,5)P3 assay kits were from Amersham, [2,8-3H]cAMP, [35S]GTP[S], 1 mM ATP and variable additions of GTP, LiCl and MgCl2. After 30 min incubation at 0°C, samples were centrifuged for 3 min at 10000 x g and 4°C. The supernatant was aspirated and the radioactivity of the pellet was determined. Assay controls were obtained by including 0.1 mM GTP in the incubation mixture (Snaar-Jagalska et al., 1988a).

Processes of genes are regulated by extracellular cAMP (Kay, 1982; Mehdy et al., 1983; Wang et al., 1988). cAMP-signal processing is initiated by the binding of cAMP to cell surface receptors. This interaction induces G-protein-mediated activation of adenylylcyclase and phospholipase C, as well as the activation of guanylylcyclase (Mato and Malchow, 1978; Thiebert and Devreotes, 1986; Europe-Finner and Newell, 1981) were grown in association with

CAMP-signal processing. The most striking result is a pronounced inhibitory effect of Li^+ on CAMP-induced activation of G-proteins, suggesting a second target for Li^+ effects on gene expression and signal transduction.

RESULTS

Effects of Li^+ on cAMP-receptor/G-protein interactions

cAMP-signal transduction is initiated by binding of cAMP to cell surface receptors and activation of one or more G

Culture conditions

Dictyostelium strain NC4 and stem F mutant NP368 (Ross and Newell, 1981) were grown in association with Escherichia coli 281 on glucose/peptone agar. Cells were freed from bacteria by repeated washing with 10 mM Na/K phosphate buffer (MgCl2, 0.1 mM EGTA, 0.2 mM App[NH]p, 0.1 mM ATP[S]), 10 mM dithiothreitol, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase and 2 mg/ml bovine serum albumin in 50 mM triethanolamine/1 M LiCl, pH 7.4, was incubated for 5 min at 25°C. The reaction was started by adding 30 ml membranes to 70 µl reaction mixture and terminated after 3 min by adding 0.5 ml 5% (mass/vol.) activated charcoal in 50 mM sodium phosphate, pH 2.0. Samples were centrifuged for 5 min at 10000 x g and 4°C and the amount of 32P02- in the supernatant was determined.

Low-affinity GTPase was determined in the presence of 100 µM GTP and high-affinity GTPase in the presence of 0.01 µM GTP. Stimulation of high-affinity GTPase was measured by adding 100 µM cAMP to the reaction mixture (Snaar-Jagalska et al., 1988b).

Assays for cAMP and cGMP accumulation

To measure cAMP levels, 27-µl aliquots of suspension of 4 x 107 cells/ml were stimulated with 3 µl 50 µM 2'-deoxy-cAMP, 50 mM dithiothreitol. After 0 min or 3 min of incubation at 20°C, the reaction was terminated by addition of 30 µl 3% perchloric acid. cAMP levels in the neutralized extracts were measured by means of a cAMP isotope-dilution assay.

To measure cAMP-stimulated cGMP levels 27-µl aliquots of 8 x 107 cells/ml were stimulated with 3 µl 1 µM cAMP or 2 µM cAMP[S], at 20°C. The reaction was terminated by adding 30 µl 5% perchloric acid cAMP levels in the neutralized extracts were measured by means of a cGMP radioimmunoassay.
proteins. Receptor mediated activation of G proteins results in increased GTP binding and increased GTPase activity (Cassel and Selinger, 1976, 1978; Snaar-Jagalska et al., 1988a, b). Activated G proteins, however, reduce the affinity of agonist receptors (Ross et al., 1977; Van Haastert, 1984). Mg\(^{2+}\) increases GTP\(^{3+}\) binding activity, by causing dissociation of GDP from the G protein, which makes the GTP-binding site on the \(\alpha\) subunit accessible to GTP, or its slowly hydrolysed derivative GTP\(^{3+}\) (Higashima et al., 1987).

We first measured effects of LiCl on cAMP-activated and Mg\(^{2+}\)-activated \(^{35}\)S-GTP\(^{3+}\) binding to membranes of aggregation-competent Dictyostelium cells. Fig. 1 shows that 1 mM and 10 mM Mg\(^{2+}\) cause twofold and fourfold increases of \(^{35}\)S-GTP\(^{3+}\) binding, respectively. cAMP stimulation of GTP\(^{3+}\) binding is low in the absence of Mg\(^{2+}\) and optimal at 1 mM Mg\(^{2+}\). At 10 mM Mg\(^{2+}\), further stimulation by cAMP becomes less pronounced. Remarkably, Li\(^{+}\) (2–10 mM) induces an increase in \(^{35}\)S-GTP\(^{3+}\) binding comparable to that induced by 1 mM Mg\(^{2+}\). However, in contrast to 1 mM Mg\(^{2+}\), which potentiates cAMP stimulation of \(^{35}\)S-GTP\(^{3+}\) binding, Li\(^{+}\) almost completely inhibits stimulation by cAMP.

Fig. 2 shows the effect of Li\(^{+}\) on cAMP-stimulated GTPase activity. Dictyostelium membranes exhibit both high-affinity and low-affinity GTPase activity. cAMP stimulates only high-affinity GTPase, by increasing the affinity for GTP (Snaar-Jagalska et al., 1988b). Basal low-affinity and high-affinity GTPase activities were not affected by Li\(^{+}\). However, 10 mM Li\(^{+}\) completely inhibited cAMP stimulation of high-affinity GTPase activity; half-maximal inhibition was achieved by about 1 mM Li\(^{+}\). No effects of other monovalent cations such as Na\(^{+}\) and K\(^{+}\) on GTP\(^{3+}\) binding or GTPase activity were observed (data not shown).

Fig. 3 shows the effect of LiCl on \([2,5,3\text{H}]cAMP\)-binding to membranes. LiCl concentrations up to 10 mM did not significantly affect \([2,5,3\text{H}]cAMP\)-binding activity. GTP\(^{3+}\) induced an 80% reduction of \([2,5,3\text{H}]cAMP\) binding, which was also not altered by Li\(^{+}\).

**Effects of Li\(^{+}\) on activation of second messenger systems**

We showed previously that Li\(^{+}\) inhibits cAMP-induced production of the intracellular messenger Ins(1,4,5)P\(_3\) (Peters et al., 1989). The second messengers cAMP and cGMP are also formed upon cAMP stimulation. Fig. 4 shows the effects of LiCl on cAMP accumulation induced by the receptor agonist 2'-deoxy-cAMP. Saturating agonist concentrations induce a 10-fold increase of basal cAMP levels, which reach a maximum at about 4 min. LiCl causes a 50% reduction of this increase. A half-maximal reduction was obtained by about 2 mM LiCl (Fig. 4B). No effects of LiCl on basal cAMP levels were observed.

Fig. 5 shows the effects of Li\(^{+}\) on the cAMP-induced cGMP response. In this case, Li\(^{+}\) appears to potentiate cAMP-stimulated accumulation of cGMP. Basal cGMP levels are not significantly affected by LiCl. Agonist-induced accumulation of cGMP is a complex response which consists of activation of guanylyl cyclase, rapid degradation of cGMP by
DISCUSSION

Pronounced effects of Li⁺ were observed on cAMP-induced activation of GTP-binding proteins. Li⁺ inhibits cAMP stimulation of GTP[S]-binding activity and high affinity GTPase activity. The effects of Li⁺ on GTP[S] binding are complex: basal and cAMP-stimulated GTP[S]-binding activity increase in the presence of Mg²⁺. Li⁺ induces a moderate increase in GTP[S]-binding activity, comparable to that of 1 mM Mg²⁺, but completely prevents stimulation by cAMP.

Avisser et al. (1988, 1991) reported that in rat cortex, Li⁺ inhibits stimulation of GTP binding by both cholinergic and adrenergic agonists. In their system, Li⁺ also inhibits the guanine-5'-[βγ-imino]triphosphate-induced reduction in receptor affinity, but does not inhibit agonist-stimulated GTPase activity. This is rather unexpected, since both GTP binding and GTP hydrolysis are determined by Mg²⁺-induced and agonist-induced GDP dissociation (Gillman, 1987). The effects of Li⁺ in rat cortex can be abolished by 1–5 mM Mg²⁺, which suggested that Li⁺ competes with Mg²⁺ for binding to the low-affinity Mg²⁺ site, which controls GDP/GTP exchange (Higashima et al., 1987).

In *Dictyostelium*, Li⁺ inhibits agonist stimulation of GTP[S] binding as well as GTPase activity, but does not affect receptor affinity. The effects of Li⁺ on agonist-stimulated GTP[S] binding cannot be abolished by Mg²⁺ at concentrations up to 10 mM. Higher Mg²⁺ concentrations could not be tested, because agonist stimulation of GTP[S] binding then becomes negligible. The low-affinity Mg²⁺ site may also be the target for Li⁺ in *Dictyostelium*, and a speculative explanation for both the stimulatory and inhibitory effects of this ion on GTP[S] binding, could be that it partially mimics Mg²⁺-induced GDP/GTP exchange, but prevents further activation of the G protein, for instance by blocking βγ subunit dissociation. The major conclusion, relevant to the present study is however, that G proteins are evidently a putative

cGMP-stimulated cGMP phosphodiesterase (Bulgakov and Van Haastert, 1982), sensory adaptation and inhibition of guanylyl-cyclase activity (Van Haastert and van der Heijden, 1983).

We analysed the level at which Li⁺ interferes with the cGMP response. Possible effects on cGMP degradation were determined in *stim* F mutant NP368, which lacks cGMP phosphodiesterase (Ross and Newell, 1981). Fig. 6A shows that instead of being reduced, the stimulatory effect of LiCl on the cGMP response is much more pronounced in the absence of LiCl (31 ± 12 pmol/10⁷ cells). Values are means ± SEM, n = 3.

Li⁺ could also increase the cGMP response by inhibiting sensory adaptation. To test this possibility, cells were stimulated with the slowly hydrolysable cAMP-derivative cAMP[S], at a non-saturating concentration. After 30 s, cells were re-stimulated with a saturating stimulus of 100 nM cAMP. Fig. 6B shows that the second cGMP response to 100 nM cAMP is considerably reduced, compared to the response to 100 nM cAMP which is not preceded by a non-saturating stimulus. The difference in responsiveness to a first or second stimulus is due to adaptation of the cells. Both first and second responses are higher in the presence of 2 mM or 10 mM LiCl, but when background cGMP levels (t = 30 s) are subtracted, the relative decrease is about the same in the presence or absence of LiCl. This indicates that LiCl does not increase cGMP levels by inhibiting adaptation and we conclude that Li⁺ stimulates activation of guanylyl cyclase.
target for the effects of Li⁺ on signalling and differentiation in *Dictyostelium*. It is at present not clear, which G proteins are inhibited by Li⁺. Eight different G-protein α subunits have been cloned in *Dictyostelium* (Pupillo et al., 1989; Hadwiger et al., 1991; Wu and Devreotes, 1991), but a function for only one G protein has been indicated. One of the first cloned G proteins, Gα2, probably mediates cAMP-induced phospholipase-C activation (Keszbeck et al., 1988; Snaar-Jagalska et al., 1988c; Kumagai et al., 1989). Since Li⁺ effectively inhibits cAMP-induced Ins(1,4,5)P₃ production (Peters et al., 1989), Ga2 is a possible target for Li⁺.

cAMP-stimulated GTP[S] binding and GTPase in aggregative cells are partially inhibited by pertussis toxin and may represent an inhibitory G protein controlling adenylyl-cyclase activity, since pertussis toxin abolishes GTP[S]-induced inhibition of this enzyme (Van Haastert et al., 1987; Snaar-Jagalska et al., 1988a, b). Li⁺ reduces adenylyl-cyclase activation, and it is therefore less likely that this pertussis-toxin-sensitive G protein is inhibited by Li⁺.

The Gα₅ protein for *Dictyostelium* adenylyl-cyclase has not yet been identified, but could be a putative Li⁺ target. If so, we would expect Li⁺ inhibition of adenylyl-cyclase activation to be more severe than the observed 50% reduction at 10 mM LiCl, which saturates inhibition of G-protein activation.

Involvement of G proteins in guanylyl-cyclase activation has not yet been unequivocally demonstrated (Janssens et al., 1989). Remarkably, Li⁺ stimulates cAMP-induced accumulation of cGMP. This effect is not due to inhibition of cGMP phosphodiesterase, because in *sst F* mutant NP368, which lacks cGMP phosphodiesterase, the stimulatory effect is much more pronounced than in wild-type cells. Apparently, hydrolysis of cGMP in wild-type cells largely overrules the stimulatory effect of LiCl. Since Li⁺ does also not inhibit cAMP-induced adaptation of guanylyl cyclase, it most likely stimulates activation of the enzyme.

The observation that Li⁺ inhibits Ins(1,4,5)P₃ accumulation, but stimulates guanylyl-cyclase activation contradicts an earlier hypothesis that guanylyl cyclase is activated via the Ins(1,4,5)P₃/Ca²⁺ pathway (Europe-Finner and Newell, 1985, 1986; Small et al., 1986; Snaar-Jagalska et al., 1988c). Further evidence against this mechanism are observations that submicromolar Ca²⁺ concentrations completely inhibit guanylyl-cyclase activity in vitro (Janssens et al., 1989) and that a partial CAMP antagonist, which cannot increase Ins(1,4,5)P₃ levels, nevertheless induces normal cGMP accumulation (Peters et al., 1991). In contrast to this hypothesis, it seems plausible that the cAMP-induced Ins(1,4,5)P₃/Ca²⁺ increase actually serves to switch off the guanylyl-cyclase enzyme. The stimulatory effect of LiCl on guanylyl-cyclase activation could then be due to its inhibitory effect on Ins(1,4,5)P₃/Ca²⁺ accumulation.

**Relevance for Li⁺ effects on gene expression**

Li⁺ effectively inhibits cAMP induction of prespore-gene expression, but potentiates cAMP induction of prestalk-gene and aggregative-gene expression (Van Lookeren Campagne et al., 1988b; Peters et al., 1989). cAMP-induced expression of these genes is mediated by surface cAMP receptors (Schaa and Van Driel, 1985; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Mann and Firtel, 1987) and does not require adenylyl-cyclase activation (Schaa et al., 1986; Mann and Firtel, 1987). However, intracellular messengers controlling gene regulation have not yet been identified.
Li⁺ inhibition of prespore-gene expression correlates with Li⁺ inhibition of \( \text{Ins}(1,4,5)P_3 \) accumulation, suggesting that \( \text{Ins}(1,4,5)P_3 \) may mediate prespore-gene expression. This is supported by observations that introduction of \( \text{Ins}(1,4,5)P_3 \) and diacylglycerol in permeabilized cells enhances expression of prespore genes (Ginsburg and Kimmel, 1989).

Li⁺ stimulates cAMP-induced prestalk-gene and aggregative-gene expression. The cAMP-induced production of cGMP is the only second-messenger response found so far, which is also stimulated by Li⁺. This suggests that intracellular cGMP may mediate induction of prestalk-gene and aggregative-gene expression. However, the stimulatory effect of Li⁺ on the cGMP response is small, and further evidence is required to support involvement of cGMP in gene regulation. Since Li⁺ abolishes cAMP-induced \( \text{Ins}(1,4,5)P_3 \) production, this response is most likely not involved in positive regulation of prestalk-gene and aggregative-gene expression.

It was suggested earlier that Li⁺ effects on prespore-gene expression and inositolphosphate signalling result from inhibition of inositolphosphatases by Li⁺, causing depletion of inositol (Van Lookeren Campagne et al., 1988a; Peters et al., 1989; Berridge et al., 1989). We recently found that inositol up to 0.3 M cannot abolish Li⁺ inhibition of prespore-gene expression (Brandt, R. and Schaap, P., unpublished results) and now consider it more likely that Li⁺ inhibition of G-protein activation causes its effects on pattern formation.

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