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Published in:
European Journal of Biochemistry

DOI:

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1992

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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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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(Received June 10, 1992) — EJ 92 0815

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'-[y-thio]triphosphate (GTP[S])-binding activity to similar levels as 3 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 dorsalizes 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'-[y-thio]triphosphate; ATP[S], adenosine 5'-[y-thio]triphosphate; App[NH]p, adenosine 5'-[p,y-imino]triphosphate; cAMP[S], adenosine 3',5'-([y-thio]phosphate, S- 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; GS 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 (Gs 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 prestalk genes (Maeda, 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 receptors. This interaction induces G-protein-mediated activation of cAMP-signal transduction. 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'-[thio]phosphate, Sp-isomer (cAMPS[S]) were obtained from Boehringer Mannheim.
- [2,8-3H]cAMP, [35S]GTP[S], cGMP-radioimmunoassay kits and Ins(1,4,5)P3 levels assay kits were from Amersham.
- [γ-32P]GTP was from New England Nuclear, and 2'-deoxyadenosine 3',5'-monophosphate (2'-deoxy-cAMP) and dithiothreitol were obtained from Sigma.

Culture conditions

D. discoideum strain NC4 and stm 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, pH 6.5, and subsequently resuspended in this buffer and shaken at 150 rpm at 22°C, or distributed on phosphate-buffered agar and incubated at 22°C.

Assays for [35S]GTP[S] binding, GTPase and [2,8-3H]cAMP-binding activity in cell membranes

Preparation of membranes

Cells starved for 5 h on phosphate-buffered agar were resuspended in ice-cold lysis buffer (0.5 mM EDTA, 250 mM sucrose, 40 mM Hepes, pH 7.7) to a density of 10^6 cells/ml and forced through a nucleopore filter (pore size, 3 µm). Lysates were centrifuged at 10000 × g for 5 min; pellets were washed and resuspended to a density of 2 × 10^8 cells/ml in either phosphate buffer (GTP[S] and cAMP binding) or 50 mM triethanolamine/HCl, pH 7.4 (GTPase activity).

[^35S]GTP[S] binding

Binding of [^35S]GTP[S] was measured in a total volume of 100 µl phosphate buffer containing 50 µl membranes, 0.1 nM [^35S]GTP[S], 1 mM ATP and variable additions of cAMP, LiCl and MgCl2. After 30 min incubation at 0°C, samples were centrifuged for 3 min at 10000 × 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).

[2,8-3H]cAMP binding

Binding of [2,8-3H]cAMP to membranes was measured at 20°C in a total volume of 100 µl, containing 50 µl membranes, 2 nM [2,8-3H]cAMP and variable additions of GTP[S] and LiCl. After 75 s, membranes were centrifuged through silicon oil and the radioactivity of the pellet was measured. Control values were obtained by including 1 mM cAMP in the incubation mixture.

GTPase activity

The GTPase reaction mixture, containing 37 nM [γ-32P]GTP (0.1 µCi/assay), 2 mM 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 µl 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 × g and 4°C and the amount of [32P]PO4 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 relay, 27-µl aliquots of suspension of 4 × 10^7 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 3.5% 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 × 10^7 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 3.5% perchloric acid. cGMP levels in the neutralized extracts were measured by means of a CGMP radioimmunoassay.

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 protein...
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$^{2+}$ increase GTP$^S$ binding activity, by causing dissociation of GDP from the G protein, which makes the GTP-binding site on the $z$ subunit accessible to GTP, or its slowly hydrolysed derivative GTP$^S$ (Higashima et al., 1987).

We first measured effects of LiCl on cAMP-activated and Mg$^{2+}$-activated $[^3S]$GTP$^S$ 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 $[^3S]$GTP$^S$ binding, respectively. cAMP stimulation of GTP$^S$ 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 $[^3S]$GTP$^S$ binding comparable to that induced by 1 mM Mg$^{2+}$. However, in contrast to 1 mM Mg$^{2+}$, which potentiates cAMP stimulation of $[^3S]$GTP$^S$ 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. GTPase 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$^S$ binding or GTPase activity were observed (data not shown).

Fig. 3 shows the effect of LiCl on $[2,8,3H]$cAMP-binding to membranes. LiCl concentrations up to 10 mM did not significantly affect $[2,8,3H]$cAMP-binding activity. GTP$^S$ induced an 80% reduction of $[2,8,3H]$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...
a 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 reducing the stimulatory effect of LiCl on the cGMP response, LiCl causes a decrease in the amount of cGMP accumulated after 3 min of stimulation 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 incubated with the slowly hydrolysable cAMP-derivative cAMP[S], at a non-saturating concentration. After 30 s, cells were resuspended at a low-affinity Mg⁺⁺ site, which controls GDP/GTP exchange, but prevents further receptor affinity. The effects of Li⁺ on agonist-stimulated GDP dissociation (Gilman, 1987). The effects of Li⁺ on GTP[S]-binding activity are complex; basal and agonist-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.

Avissar 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 (Gilman, 1987). The effects of Li⁺ in rat cortex can be abolished by 1-5 mM Mg²⁺, which suggests that Li⁺ competes with Mg²⁺ for binding to the low-affinity Mg²⁺ site, which controls GDP/GTP exchange (Higashima et al., 1987).

In Dicystostelium, 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 Dicystostelium, 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 fgs subunit dissociation. The major conclusion, relevant to the present study is however, that G proteins are evidently a putative

**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 activity 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.

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protein has been indicated. One of the first cloned G proteins, Wu and Devreotes, 1991), but a function for only one G
induced Ins(1,4,5)P3 production (Peters et al., 1989),
response. To measure adaptation, cells were first stimulated at
expressed as percentage of cGMP levels reached after
pertussis-toxin- Jagalska et al., 1988a, b). Lit reduces adenylylcyclase acti-
phosphodiesterase, because in
more pronounced than in wild-type cells. Apparently, hy-
drolysis of cGMP in wild-type cells largely overrules the
indicated times. Values are means 
for
CAMP-stimulated GTP[S] binding and GTPase in aggre-
gative cells are partially inhibited by pertussis toxin and may
represent an inhibitory G protein controlling adenylylcyclase
activity, since pertussis toxin abolishes GTP[S]-induced inhi-
activation (Kesbeke et al., 1988; Snaar-Jagalska et al., 1988c;
Kumagai et al., 1989). Since Li+ effectively inhibits CAMP-
produced Ins(1,4,5)P3 production (Peters et al., 1989), Gz2 is a
possible target for Li+.
cAMP-stimulated GTP[S] binding and GTPase in aggrega-
tively cells has not yet been identified, but could be a putative Li+ target. If so, we
would expect Li+ inhibition of adenylylcyclase 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 smt 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 accumu-
lation (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 (Schaap and Van Driel, 1985; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Mann and Firtel, 1987) and does not require adenylylcyclase activation (Schaap et al., 1986; Mann and Firtel, 1987). However, intracellular messengers controlling gene regulation have not yet been identified.

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**Fig. 6. Effects of Li+ on cGMP phosphodiesterase or cGMP adaptation.** (A) Aggregation-competent cells of stm F mutant NP368 were incubated for
15 min with 0 (O), 2 (A) or 10 (Q) mM LiCl and subsequently stimulated with 100 nM CAMP. cGMP levels were measured at the indicated times. Values are means ± SEM, n = 2 (100% ± 6 pmol cGMP/10⁷ cells). (B) Aggregation-competent NC4 cells were incubated for
15 min with 0, 2 or 10 mM LiCl. Cells were stimulated for 10 s with a saturating stimulus of 100 nM CAMP to induce a maximal cGMP
response. To measure adaptation, cells were first stimulated at t = 0 s with a suboptimal stimulus of 200 nM CAMP (comparable with about
2 nM CAMP) and restimulated at 30 s with 100 nM CAMP. cGMP levels were measured after 0, 10, 30 and 40 s of stimulation. Data are
expressed as percentage of cGMP levels reached after 10 s stimulation with 100 nM CAMP in the absence of LiCl. Values are means ± SEM.
Li⁺ inhibition of prespore-gene expression correlates with Li⁺ inhibition of Ins(1,4,5)P₃ accumulation, suggesting that Ins(1,4,5)P₃ may mediate prespore-gene expression. This is supported by observations that introduction of Ins(1,4,5)P₃ 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 Ins(1,4,5)P₃ 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 (BION), which is subsidized by the Netherlands Organisation for Scientific Research (NWO).

We thank Raymond Brandt for excellent technical assistance. This work was supported by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organisation for Scientific Research (NWO).

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