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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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(Received June 10, 1992) — EJR 92 0815

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nucleotide-binding regulatory proteins activating adenyllylcyclase (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 prespore genes and promoting expression of a subclass 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 3',5'-monophosphate (2'deoxy-CAMP) and dithiothreitol were obtained from Sigma. 5'-[β,γ-imino]triphosphate (App[NH]p), adenosine 5'-[γ-thio]triphosphate (ATP[S]) and adenosine 3',5'-[γ-thio]phosphate, 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 were obtained from Amersham, [γ-32P]GTP was from New England Nuclear, and 2'deoxy-adenosine 3',5'-monophosphate (2'deoxy-cAMP) and dithiothreitol were obtained from Sigma.

Culture conditions

Dictyostelium strain NC4 and 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 5h 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 10,000 x g for 5 min; pellets were washed and resuspended to a density of 2 x 10^6 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 mM [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 10,000 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).

[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 trithanolamine/11Cl, 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 10,000 x g and 4°C and the amount of 32PO4 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 x 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 x 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 proteins activating adenylylcyclase and phospholipase C, as well as the receptors. This interaction induces G-protein-mediated activation of 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.
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). Mg2+ increase GTP binding activity, by causing dissociation of GDP from the G protein, which makes the GTP-binding site on the α 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 Mg2+-activated [35S]-GTP[S] binding to membranes of aggregation-competent Dictyostelium cells. Fig. 1 shows that 1 mM and 10 mM Mg2+ cause twofold and fourfold increases of [35S]GTP[S] binding, respectively. cAMP stimulation of GTP[S] binding is low in the absence of Mg2+ and optimal at 1 mM Mg2+. At 10 mM Mg2+, further stimulation by cAMP becomes less pronounced. Remarkably, Li+ (2–10 mM) induces an increase in [35S]GTP[S] binding comparable to that induced by 1 mM Mg2+. However, in contrast to 1 mM Mg2+, which potentiates cAMP stimulation of [35S]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. 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[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)P3 (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 stn 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 stn F mutant than in wild-type cells, indicating that the effect of Li⁺ is not due to inhibition of cGMP phosphodiesterase. The comparatively small effect of Li⁺ on the cGMP response in wild-type cells is probably due to the fact that cGMP is very rapidly degraded.

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.

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.

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'-[β,γ-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 suggests 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.
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 \(\alpha\) 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, Ga\(_2\), probably mediates cAMP-induced phospholipase-C activation (Kesbeke et al., 1988; Snaar-Jagalska et al., 1988c; Kumagai et al., 1989). Since Li\(^+\) effectively inhibits cAMP-induced Ins\((1,4,5)P_3\) production (Peters et al., 1989), Ga\(_2\) is a possible target for Li\(^+\).

cAMP-stimulated GDP binding and GTPase in aggregative cells are partially inhibited by pertussis toxin and may represent an inhibitory G protein controlling adenylcyclase 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 adenylcyclase activation, and it is therefore less likely that this pertussis-toxin-sensitive G protein is inhibited by Li\(^+\).

The G\(_a\) protein for *Dictyostelium* adenylcyclase has not yet been identified, but could be a putative Li\(^+\) target. If so, we would expect Li\(^+\) inhibition of adenylcyclase 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 *stn 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\((1,4,5)P_3\) accumulation, but stimulates guanylyl-cyclase activation contradicts an earlier hypothesis that guanylyl cyclase is activated via the Ins\((1,4,5)P_3/Ca^{2+}\) 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 Ca\(^{2+}\) concentrations completely inhibit guanylyl-cyclase activity in vitro (Janssens et al., 1989) and that a partial CAMP antagonist, which cannot increase Ins\((1,4,5)P_3\) levels, nevertheless induces normal cGMP accumulation (Peters et al., 1991). In contrast to this hypothesis, it seems plausible that the cAMP-induced Ins\((1,4,5)P_3/Ca^{2+}\) 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\((1,4,5)P_3/Ca^{2+}\) 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 Looheren 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 adenylcyclase activation (Schaap 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 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 inositol (Van Looikeren Campagne et al., 1988; 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.

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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