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Lithium ions induce prestalk-associated gene expression and inhibit prespore gene expression in *Dictyostelium discoideum*

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Summary

We investigated the effect of Li^+ on two types of cyclic AMP-regulated gene expression and on basal and cyclic AMP-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) levels. Li^+ effectively inhibits cyclic AMP-induced prespore gene expression, half-maximal inhibition occurring at about 2 mM-LiCl. In contrast, Li^+ (1–3 mM) promotes the cyclic AMP-induced increase of cysteine proteinase-2 mRNA levels, and induces the expression of this prestalk-associated gene in the absence of cyclic AMP stimuli. At concentrations exceeding 4–5 mM, LiCl inhibits cysteine proteinase-2 gene expression. LiCl reduces basal Ins(1,4,5)P_3 levels and decreases the cyclic AMP-induced accumulation of Ins(1,4,5)P_3; both effects occur half-maximally at 2–3 mM-LiCl. These results indicate that the induction of the cysteine proteinase-2 gene by Li^+ is not due to elevated levels of Ins(1,4,5)P_3. It is, however, possible that inhibition of prespore gene expression by Li^+ is caused by Li^+-induced reduction of basal and/or stimulated Ins(1,4,5)P_3 levels.

Key words: lithium, gene expression, *Dictyostelium discoideum*.

Introduction

In the presence of nutrients, the cellular slime mold *Dictyostelium discoideum* lives as a unicellular organism. Upon starvation, cells start to secrete the chemoattractant cyclic AMP in a pulsatile manner. Surrounding cells detect cyclic AMP by means of cell surface receptors and the interaction of cyclic AMP with these receptors evokes a number of rapid responses, such as transient synthesis and secretion of cyclic AMP, which is responsible for relay of the original cyclic AMP pulse through the population, and transient increases in the levels of intracellular inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), Ca^{2+} and cyclic GMP, as well as increases in actin polymerization and myosin heavy-chain phosphorylation. The second group of responses are considered to result in chemotactic movement towards the cyclic AMP source (for review, see Janssens & Van Haastert, 1987). As a consequence of signal relay and chemotaxis, the cells aggregate to form a multicellular mound, which develops into a slug-shaped structure and finally culminates in a fruiting body, composed of stalk cells and spores. Besides its function as a chemoattractant, extracellular cyclic AMP also acts as a morphogen and regulates gene expression during several stages of development.

The chemotactic signal, i.e. nanomolar cyclic AMP pulses accelerate the synthesis of gene products, which are involved in the aggregation process, such as cell surface cyclic AMP receptors, cyclic AMP phosphodiesterase, and the adhesive contact sites A (Darmon *et al.* 1975; Gerisch *et al.* 1975; Franke *et al.* 1987). Steady-state concentrations of cyclic AMP in the micromolar range, which are considered to accumulate in the multicellular structures after aggregation (Wang *et al.* 1988), induce the expression of prespore-specific genes as well as the synthesis of a class of gene products, which are preferentially present in the anterior prestalk region of the slug (Kay, 1982; Schaap & Van Driel, 1985; Mehdy *et al.* 1983; Oyama & Blumberg, 1986).

Earlier studies have shown that the induction of prespore- and prestalk-associated gene expression by cyclic AMP is most likely mediated by the chemotactic cyclic AMP receptor (Schaap & Van Driel, 1985; Oyama & Blumberg, 1986; Haribabu & Dottin, 1986), but the intracellular mechanisms mediating cyclic AMP-induced gene expression are still obscure. In the case of prespore gene expression, it was demonstrated that cyclic AMP-induced adenylate and guanylate cyclase activation are not involved in transduction of the response. This type of gene expression was, however, strongly inhibited by

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Ca^{2+} channel blockers and inhibitors of the interaction of Ca^{2+} with Ca^{2+}-binding proteins (Schaap *et al.* 1986).

In higher eukaryotes, the interaction of certain growth factors and hormones with surface receptors induces hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PtdIns(4,5)P_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (Ins(1,4,5)P_3). Ins(1,4,5)P_3 is postulated to induce the release of Ca^{2+} from intracellular stores, which in turn activates Ca^{2+}-binding proteins, such as calmodulin, or in combination with DAG, protein kinase C. Tumor-promoting phorbol esters bypass the requirement for the ligand–receptor interaction and activate protein kinase C directly (Downes & Michell, 1985; Nishizuka, 1984).

In *Dictyostelium*, cyclic AMP also induces Ins(1,4,5)P_3 production (Europe-Finner & Newell, 1987; Van Haastert, unpublished), while Ins(1,4,5)P_3 was shown to induce an increase in intracellular Ca^{2+} levels (Europe-Finner & Newell, 1986). The sensitivity of prespore induction to Ca^{2+} antagonists could therefore indicate that these responses to cyclic AMP mediate the transduction of the signal to prespore gene expression. However, attempts to bypass cyclic AMP-induced $Ca^{2+}$and DAG production by treatment of permeabilized cells with Ca^{2+} and phorbol esters have hitherto not been successful (unpublished data).

In this study, we attempt another approach to investigating a possible involvement of cyclic AMP-stimulated Ins(1,4,5)P_3 accumulation in the regulation of gene expression. It was recently found that LiCl inhibits several steps in the dephosphorylation of inositol phosphates in *Dictyostelium in vitro* (Van Lookeren Campagne *et al.* 1988) and could significantly alter basal and stimulated inositol phosphate levels *in vivo*. Early reports showed that LiCl diverts *Dictyostelium* cells from the spore into the stalk differentation pathway (Maeda, 1970; Sakai, 1973). We investigated the possibility that LiCl causes this effect by interfering with the intracellular processing of cyclic AMP signals to cell-type-specific gene expression. It was found that LiCl inhibits cyclic AMP-induced prespore gene expression, while it promotes and partially bypasses cyclic AMP induction of prestalk-associated gene expression. These results are discussed in relation to the effects of LiCl on cyclic AMP-activated Ins(1,4,5)P_3 production in *Dictyostelium*.

Materials and methods

Materials

[α-^{32}P]dATP and the Ins(1,4,5)P_3 assay kit were obtained from Amersham (UK). Gene Screen was from New England Nuclear, cyclic AMP from Sigma, LiCl from Merck and beef-heart phosphodiesterase from Boehringer-Mannheim.

Culture and incubation conditions

Dictyostelium discoideum NC4 (H) was cultured on glucose–peptone agar in association with *Escherichia coli* 281 as described (Schaap & Spek, 1984). Vegetative cells were freed from bacteria, by repeated washing with 10 mM-Na/K phosphate buffer, pH 6·5 (PB) and resuspended in PB to 10^7 cells ml^{-1}. The cell suspension was shaken at 150 revs min^{-1} and 21 °C.

To obtain aggregation-competent cells, vegetative cells, freed from bacteria were distributed on non-nutrient agar plates (1·5 % agar in PB) at $2{\cdot}5\times10^6$ cells cm^{-2} for 16 h at 6 °C. The cells were subsequently incubated in PB at 5×10^6 cells ml^{-1}. Cells were pulsed with 30 nM-cyclic AMP every 6 min or cyclic AMP was added every hour as a single dose of 10^{-4} M. During incubation of both vegetative and aggregation-competent cells, the cells were collected by centrifugation at 2-h intervals and resuspended in fresh incubation mixture. This was done to avoid excessive accumulation of cellular secretion products.

RNA isolation and analysis

Total cellular RNA was isolated from 5×10^7 lysed cells as described previously, purified by phenol/chloroform extraction and ethanol precipitation (Mann & Firtel, 1987). RNA was size fractionated on 1·5 % agarose gels containing 2·2 M-formaldehyde and transferred to Gene Screen membranes. Northern transfers were hybridized in 50 % formamide and 1 % SDS in 5 × SSPE (0·9 M-NaCl and 5 mM-EDTA in 50 mM-Na_2HPO_4, pH 7·4) with 5 × Denhardt's solution (0·25 % Ficoll, 0·25 % polyvinylpyrrolidone, 0·25 % bovine serum albumin (BSA)) to cDNA probes, which were labeled with [α-^{32}P]dATP by means of nick-translation (Rigby *et al.* 1977). After hybridization, the membranes were rinsed three times with 0·5 × SSPE and washed twice during 30 min at 42 °C with 1 % SDS in 5 × SSPE and exposed to X-ray films. The amount of ^{32}P-labeled cDNA probes hybridized to specific mRNAs was semiquantitatively determined by measuring the absorbance on autoradiographs by means of an Ultrascan XL densitometer (LKB).

Determination of LiCl effects on basal and stimulated Ins(1,4,5)P_3 levels

Vegetative cells were starved in PB at 10^7 cells ml^{-1}. After 5 h the cells were washed and incubated at 10^8 cells ml^{-1} with 0 to 10 mM-LiCl for 15 min. To measure LiCl effects on basal Ins(1,4,5)P_3 levels, 100 μl of cell suspension was added to 100 μl of 3·5 % perchloric acid (PCA). To measure LiCl effects on cyclic AMP-stimulated Ins(1,4,5)P_3 levels, the cells were stimulated with 10^{-6} M-cyclic AMP and 100 μl cell suspension was added to 100 μl of 3·5 % PCA at different times after cyclic AMP stimulation. The samples were neutralized with 50 μl of 50 % saturated $KHCO_3$ and the amount of Ins(1,4,5)P_3 was assayed with a commercially available Ins(1,4,5)P_3 isotope dilution assay kit. The kit was tested for the specificity for Ins(1,4,5)P_3 by Van Haastert (1989). The assay was performed as described by the manufacturer, with the following modifications: the incubation mixture contained 20 μl of each [^{3}H]-Ins(1,4,5)P_3 (2700 cts min^{-1}), neutralized cell lysate, Ins(1,4,5)P_3-binding protein, and assay buffer. After 15 min of incubation at 0 °C, samples were centrifuged for 2 min at 10 000 ***g***. The supernatant was aspirated and the radioactivity of the pellet was measured by liquid scintillation counting. Data were converted to pmol of Ins(1,4,5)P_3 by using a standard curve with authentic Ins(1,4,5)P_3.

Results

Effects of Li^+ on cyclic AMP-induced prespore gene expression

Prespore gene expression is effectively induced by micromolar cyclic AMP concentrations in aggregation-competent cells (Schaap & Van Driel, 1985; Oyama & Blumberg, 1986). Fig. 1 shows that D19 prespore mRNA levels increase strongly after 5 h of incubation

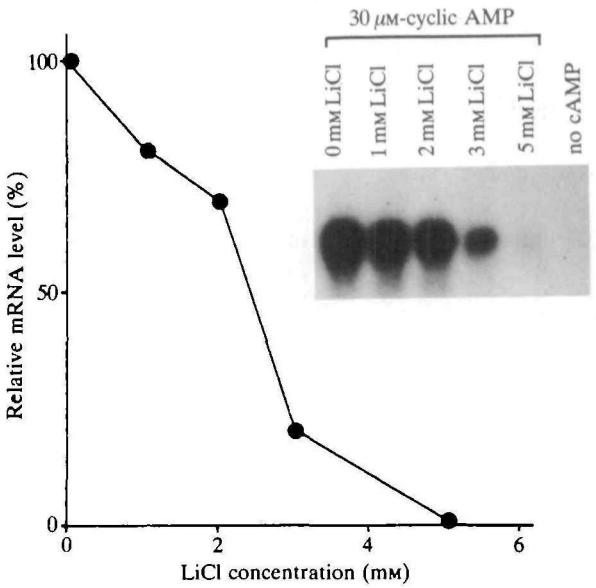

Fig. 1. Dose dependency of the effects of LiCl on the cyclic AMP-induced increase of D19 mRNA levels. Aggregation-competent cells were incubated in PB with various LiCl concentrations in the presence of 30 μM-cyclic AMP, added every hour. After 5 h of incubation, RNA was isolated, size fractionated and probed with D19 cDNA. The data represent relative O.D. values of D19 mRNA bands on autoradiograms expressed as percentage of values obtained after cyclic AMP stimulation in the absence of LiCl.

with 30 μM-cyclic AMP, added at 60-min intervals. LiCl completely inhibits the cyclic AMP-induced increase of D19 prespore mRNA at concentrations of 5 mM. Half-maximal inhibition occurs at about 2·5 mM-LiCl.

Effects of Li^+ on cysteine proteinase-2 gene expression in vegetative cells

In aggregation-competent cells, micromolar cyclic AMP concentrations also induce the expression of a class of genes whose products are preferentially, but not exclusively, present in the prestalk region of the slug, such as the prestalk cathepsin or cysteine proteinase-2 (CP-2) gene (Mehdy *et al.* 1983; Pears *et al.* 1985). We recently found that CP-2 gene expression can also be induced by nanomolar cyclic AMP pulses instead of micromolar cyclic AMP concentrations. Fig. 2 shows the effect of nanomolar cyclic AMP pulses and micromolar cyclic AMP concentrations on the levels of CP-2 mRNA in vegetative and aggregation-competent cells. In vegetative cells, CP-2 mRNA levels increase strongly after 4–6 h in response to nanomolar cyclic AMP pulses. Micromolar cyclic AMP concentrations also induce an increase in CP-2 mRNA levels, which seems to occur somewhat earlier than induction by pulses (Fig. 2A). In aggregation-competent cells, CP-2 mRNA is already present at a low level and a further increase is induced by nanomolar cyclic AMP pulses. However, in this stage, stimulation with micromolar concentrations increases CP-2 mRNA to higher levels than stimulation with nanomolar pulses (Fig. 2B).

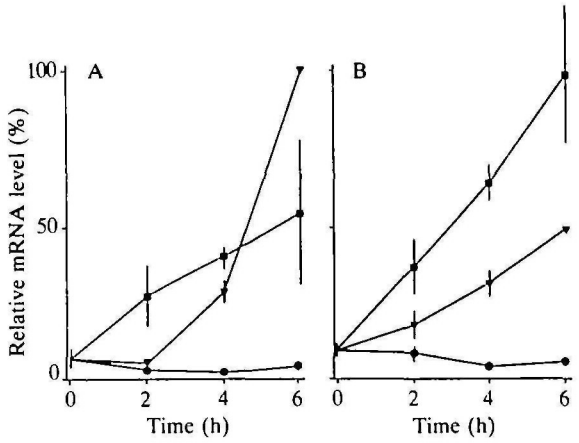

Fig. 2. The effects of different cyclic AMP stimulation regimes on CP-2 mRNA levels. Vegetative cells (A) and aggregation-competent cells (B) were incubated in PB without cyclic AMP (●), with 30 nM pulses of cyclic AMP every 6 min (▼) or with 30 μM-cyclic AMP added every hour (■). After 2, 4 and 6 h RNA was isolated, size fractionated and probed with PDd8 cDNA. Values of densitometric scans of specific RNA bands are expressed as percentage of the value of pulse-induced cells at 6 h incubation. The means and S.E.M. of data from three to six experiments are presented.

We next investigated whether Li^+ affects CP-2 induction by nanomolar cyclic AMP pulses. Vegetative cells were incubated with different Li^+ concentrations in the presence and absence of nanomolar cyclic AMP pulses. Fig. 3 shows that Li^+ at concentrations up to about 3 mM promotes the increase of CP-2 mRNA levels, which occurs in response to cyclic AMP pulses (Fig. 3A,B,D) and micromolar cyclic AMP concentrations (Fig. 3C). At concentrations above 4 mM, Li^+ starts to inhibit cyclic AMP-induced gene expression. Li^+ also induces a significant increase in CP-2 mRNA levels in the absence of cyclic AMP pulses; the Li^+-induced increase amounts to about 30% of the levels induced by nanomolar cyclic AMP pulses. At concentrations above 4 mM-Li^+, this increase is no longer observed. The Li^+ concentration at which optimal induction or stimulation of cyclic AMP-induced CP-2 expression occurred varied when individual experiments were compared. Lithium carbonate has the same effect as lithium chloride (see Fig. 3A,B), which indicates that the observed effects are caused by Li^+ rather than by the anions.

Is cyclic AMP receptor occupancy required for Li^+ induction of gene expression?

To investigate whether the effects of Li^+ represent a true bypass of the cyclic AMP receptor interaction and occur in the complete absence of cyclic AMP receptor stimulation, we incubated cells with LiCl in the presence of $2{\cdot}5\times10^{-3}$ units ml^{-1} beef-heart cyclic AMP phosphodiesterase (PDE) to hydrolyse all the extracellular cyclic AMP, which is secreted by the cells. As shown in Fig. 4, LiCl not only induces an increase in CP-2 mRNA levels in the presence of beef-heart PDE, but the inducing effect of LiCl is two- to threefold higher than in the absence of the enzyme and now approaches the induction

of CP-2 mRNA by cyclic AMP pulses. These experiments demonstrate that the inducing effects of LiCl represent a true bypass of the cyclic AMP receptor and seem to be counteracted by low background cyclic AMP levels.

Effects of LiCl on basal and stimulated Ins(1,4,5)P_3 levels

It was recently shown that LiCl effectively inhibits dephosphorylation of inositol phosphates in *D. discoideum in vitro* (Van Lookeren Campagne *et al.* 1988), which may result in altered intracellular levels of these compounds *in vivo*. We investigated the effects of Li^+ on Ins(1,4,5)P_3 levels in cyclic AMP-stimulated and non-stimulated cells. As shown in Fig. 5A, cyclic AMP induces a transient Ins(1,4,5)P_3 accumulation. Half-maximal inhibition of cyclic AMP-stimulated Ins(1,4,5)P_3 accumulation occurs at about 2–3 mM-LiCl (Fig. 5B). LiCl also causes a 30 % reduction of the basal Ins(1,4,5,)P_3 levels (Fig. 5B), with a half-maximal effect at 2·5 mM-LiCl. It thus appears that *in vivo*, LiCl acts to decrease basal and stimulated Ins(1,4,5)P_3 levels.

Discussion

Li^+ exerts divergent effects on cyclic AMP-induced cell type-specific gene expression in *D. discoideum*. It effectively inhibits cyclic AMP-induced prespore gene expression, half-maximal inhibition occurring at about 2 mM-LiCl. The effects of Li^+ on the expression of the

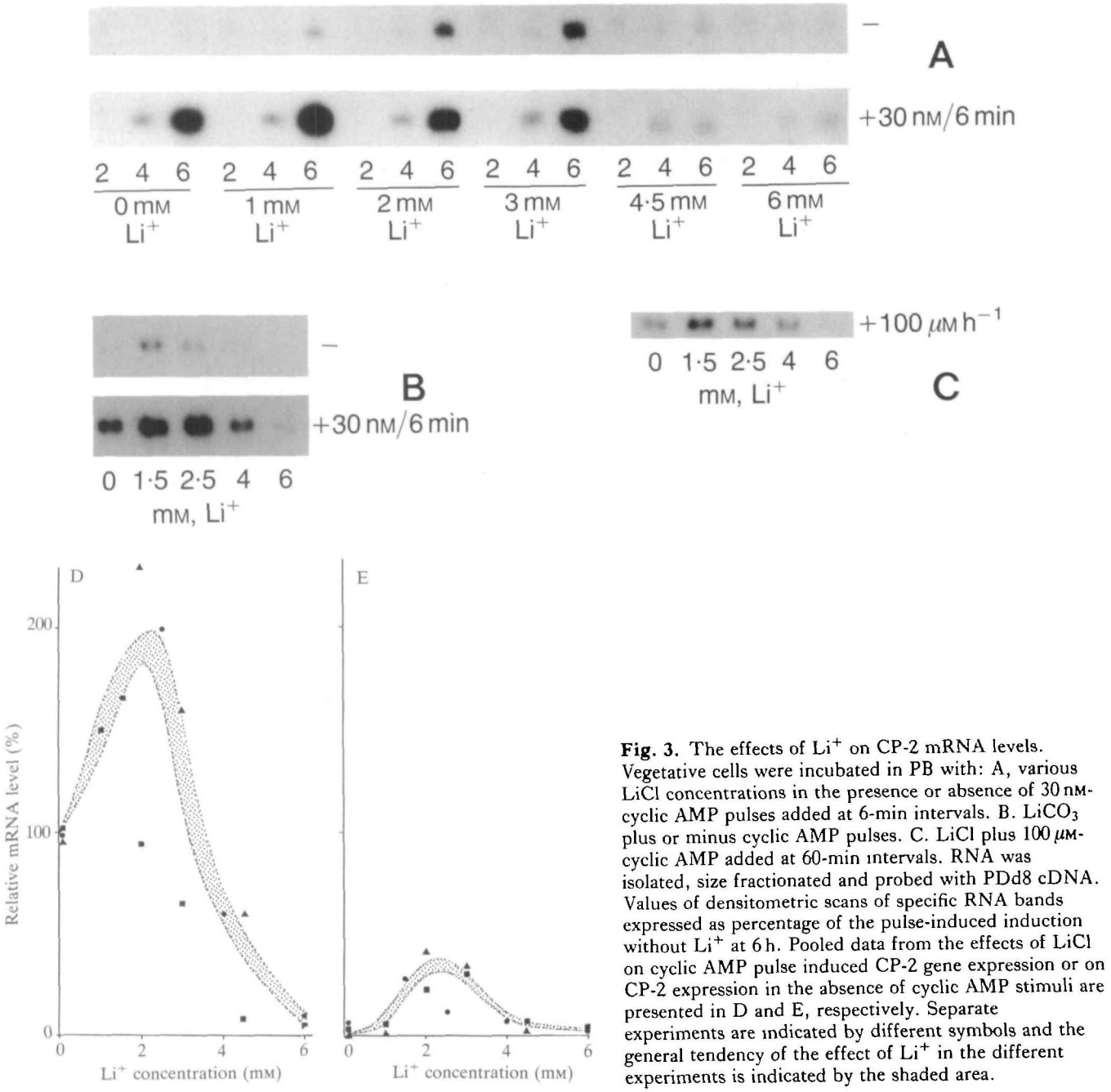

Fig. 3. The effects of Li^+ on CP-2 mRNA levels. Vegetative cells were incubated in PB with: A, various LiCl concentrations in the presence or absence of 30 nM-cyclic AMP pulses added at 6-min intervals. B. $LiCO_3$ plus or minus cyclic AMP pulses. C. LiCl plus 100 μM-cyclic AMP added at 60-min intervals. RNA was isolated, size fractionated and probed with PDd8 cDNA. Values of densitometric scans of specific RNA bands expressed as percentage of the pulse-induced induction without Li^+ at 6 h. Pooled data from the effects of LiCl on cyclic AMP pulse induced CP-2 gene expression or on CP-2 expression in the absence of cyclic AMP stimuli are presented in D and E, respectively. Separate experiments are indicated by different symbols and the general tendency of the effect of Li^+ in the different experiments is indicated by the shaded area.

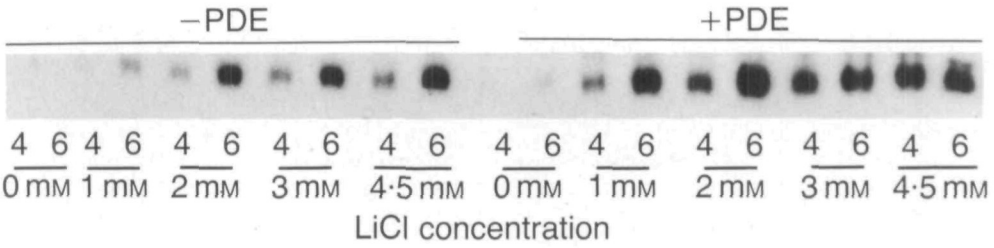

Fig. 4. Effect of beef-heart cyclic AMP phosphodiesterase on the Li^+-induced increase of CP-2 mRNA levels. Vegetative cells were incubated with various LiCl concentrations in the presence and absence of $2{\cdot}5\times10^{-3}$ units ml^{-1} beef-heart cyclic AMP phosphodiesterase (PDE). After 4 and 6 h of incubation RNA was isolated, size fractionated and probed with PDd8 mRNA. An autoradiogram of a Northern transfer of a typical experiment is shown.

CP-2 gene, which is preferentially expressed in prestalk cells, are more complex. It was previously reported that CP-2 gene expression is induced in response to constant micromolar cyclic AMP stimulation (Mehdy *et al.* 1985; Pears *et al.* 1985). We show here that in vegetative cells CP-2 expression is also effectively induced by nanomolar cyclic AMP pulses (Fig. 2A). Depending on the concentration, LiCl exerts both stimulatory and inhibitory effects on CP-2 expression (Fig. 3). At concentrations from 1 to 3 mM, LiCl enhances the induction of CP-2 expression by cyclic AMP pulses (Fig. 3A,B,D) and by constant micromolar cyclic AMP levels (Fig. 3C). Furthermore, at these concentrations LiCl can partially replace the requirement for cyclic AMP stimulation to increase CP-2 mRNA levels. The LiCl-induced increase in CP-2 mRNA levels is more pronounced when extracellular cyclic AMP levels are reduced by addition of cyclic AMP-phosphodiesterase (Fig. 4). It is possible that low background cyclic AMP levels cause partial desensitization of the cells (Theibert & Devreotes, 1983), which counteracts the stimulatory effect of LiCl. At concentrations above 4–5 mM, LiCl no longer induces an increase in CP-2 mRNA levels and inhibits the increase induced by cyclic AMP pulses (Figs 3, 4). Possibly, the stimulatory effect of LiCl would be more pronounced if this inhibitory effect was not also present to counteract it.

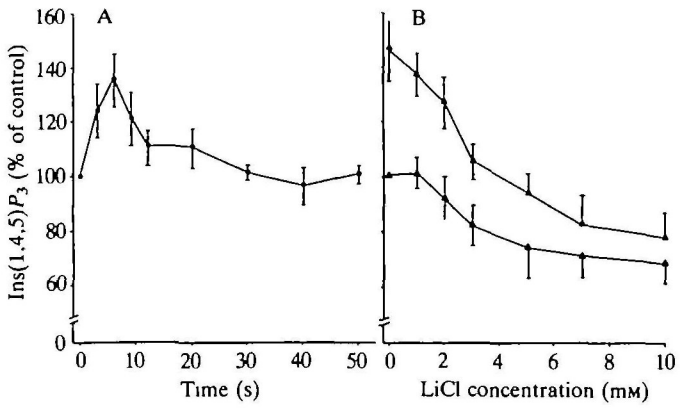

Fig. 5. The effect of LiCl on the basal and cyclic AMP-stimulated Ins(1,4,5)P_3 levels. A. Cells, starved for 5 h, were incubated 15 min at 10^8 cells ml^{-1} and then stimulated with 10^{-6} M-cyclic AMP. At the times indicated, 100 μl cell suspension was added to 100 μl PCA, and Ins(1,4,5)P_3 levels were measured in the neutralized extracts by isotope dilution assay. Results are expressed as percentage of the amount of Ins(1,4,5)P_3 in cells treated without cyclic AMP at $t = 0$. Means and S.D. of 3 experiments are shown. B. Cells, starved for 5 h, were incubated for 15 min at 10^8 cells ml^{-1} with 0 to 10 mM-LiCl; 100 μl of cell suspensions was added to 100 μl PCA and basal Ins(1,4,5)P_3 levels (△) and cyclic AMP-stimulated Ins(1,4,5)P_3 levels (▲) were measured in the neutralized extracts by an isotope dilution assay. Results are expressed as percentage of the amount of Ins(1,4,5)P_3 in cells incubated without LiCl. Means and S.D. of 3 experiments are shown.

An obvious explanation of the stimulatory effects of LiCl would be that it increases the levels of the intracellular messenger Ins(1,4,5)P_3, which in turn results in stimulation of CP-2 gene expression. In *D. discoideum* homogenates Li^+ inhibits the dephosphorylation of Ins(1,4,5)P_3 at the 1 position (Van Lookeren Campagne *et al.* 1988). Thus Ins(1,4,5)P_3 levels may increase in Li^+-treated cells. On the other hand, Li^+ also inhibits the dephosphorylation of inositol monophosphate. Since inositol monophosphate, formed from either glucose 6-phosphate or from inositol polyphosphates, is the endogenous source of inositol, this effect of Li^+ may lead to reduced levels of inositol and hence of PtdIns(4,5)P_2 and Ins(1,4,5)P_3. Using the recently introduced isotope dilution assay for Ins(1,4,5)P_3, the molar levels of this compound were determined in Li^+-treated cells (Van Haastert, 1989).

LiCl concentrations of 1 mM and higher were found to decrease basal Ins(1,4,5)P_3 levels and the cyclic AMP-stimulated Ins(1,4,5)P_3 accumulation (Fig. 5). It is therefore clear that the stimulatory effects of LiCl on CP-2 gene expression are not due to stimulation of Ins(1,4,5)P_3 production. We recently measured effects of LiCl on cyclic AMP binding and on two other cyclic AMP-activated second messenger systems. Besides inducing a small increase in the affinity of the cell surface cyclic AMP receptor, LiCl does not stimulate the cyclic AMP-induced activation of adenylate or guanylate cyclase activity, which indicates that the stimulatory effect of LiCl on CP-2 gene expression is also not due to stimulation of cyclic AMP-induced cyclic AMP or cyclic GMP production (unpublished data).

The inhibitory effects of LiCl on cyclic AMP-induced Ins(1,4,5)P_3 production argue against induction of CP-2 gene expression by Ins(1,4,5)P_3, but could be the cause of the inhibition of prespore gene expression by LiCl.

A possible involvement of Ins(1,4,5)P_3, and Ins(1,4,5)P_3-induced release of Ca^{2+} from intracellular stores, as intermediates of cyclic AMP-induced prespore

gene expression, is corroborated by earlier findings that prespore gene expression is effectively inhibited by Ca^{2+} channel blockers and by antagonists of Ca^{2+} binding proteins (Schaap *et al.* 1986).

Although the present study shows that LiCl does interfere with *Dictyostelium* inositol phosphate metabolism, this process need not be the only target for the effects of Li^+. It was shown, for instance, that in the rat cerebral cortex Li^+ also interferes with the function of G-proteins (Avissar *et al.* 1988). The observed LiCl-induced increase of cyclic AMP binding activity could reflect an effect on G-proteins, since the functional interaction between cyclic AMP receptors and G-proteins involves changes in receptor affinity (Janssens *et al.* 1986; Van Haastert *et al.* 1986). The identity of the target for the effect of Li^+ on CP-2 expression will be further investigated.

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