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Tyrosine kinase inhibitors and cycloheximide inhibit Li^+ protection of cerebellar granule neurons switched to non-depolarizing medium

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Abstract

Recently, it has been shown that Li^+ robustly enhances the survival of cerebellar granule neurons acutely switched to non-depolarizing medium after maturing in vitro, a condition which elicits massive apoptotic death in this cell type. Tyrosine protein phosphorylation is known to underlie the activity of a number of trophic factors. This prompted us to investigate whether specific tyrosine kinase inhibitors could modulate the Li^+ protection of cultured granule neurons switched to non-depolarizing medium. Genistein and herbimycin A dose dependently abolished the Li^+ effect. Furthermore, this effect was substantially prevented by the translational inhibitor cycloheximide, suggesting that it requires de novo protein synthesis. Overall, these results suggest that Li^+ protection of cerebellar granule neurons switched to non-depolarizing medium involves tyrosine kinases and transcriptional activation.

Keywords: Cerebellar granule cell; Cell death; Tyrosine kinase; Li^+

1. Introduction

Despite their narrow therapeutic index, lithium salts remain the archetypal mood stabilizing medication for the treatment of manic-depressive disease. The precise basis of their action remains however elusive (Jope and Williams, 1994). Recently, a trophic effect of Li^+ was demonstrated in cultured cerebellar granule neurons. Gao et al. (1993) showed that Li^+, added to cultures from the first to seventh day in vitro induced a significant increase in [^{3}H]ouabain binding (used as an index of cell viability) in the 1–3 mM range. D'Mello et al. (1993, 1994) used a different approach. Cerebellar granule cells were routinely cultured under depolarizing conditions (30 mM K^+); they underwent extensive cell death, with salient features of apoptosis such as DNA fragmentation and chromatin condensation, when switched to physiological (5 mM) K^+ concentrations after the fourth day in vitro. Under these conditions Li^+ was shown to exert a surprising biphasic effect: when added from the first day in vitro to cultures kept in low K^+, Li^+ induced massive cell loss; conversely, when added to 'mature' (7 days in vitro) cultures switched to non-depolarizing conditions, Li^+ protected granule neurons in a dose-dependent fashion, in the 1–5 mM range. In immature cells, the Li^+ effect was shown to be partly antagonized by excess *myo*-inositol addition (Copani et al., 1995) although this result is somewhat conflicting with those of D'Mello et al. (1993, 1994). Li^+ protection of mature neurons, however, was completely unaffected by *myo*-inositol, and this effect has so far remained unexplained.

Tyrosine-specific protein phosphorylation is known to underlie the mitotic and trophic effects of a wide array of growth factors (Malarkey et al., 1995). Moreover, Li^+ has been shown to enhance the mitotic effect of thyroid stimulating hormone in cultured rat thyroid cells through increased tyrosine protein phosphorylation (Takano et al., 1994). These findings prompted us to investigate whether tyrosine kinase inhibitors could modulate Li^+-induced survival of cultured granule neurons.

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2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, glutamine, hepes, penicillin-streptomycin were obtained from BioWhittaker. Polyornithine, glucose, potassium, insulin, cytosine arabinoside, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT), synthetic genistein, herbimycin A and cycloheximide were from Sigma.

2.2. Methods

2.2.1. Cell culture

Cerebellar granule cells were obtained according to published procedures (Grignon et al., 1993) and plated in 24-well cell culture plates (Nunc) at a density of 10^6 cells $\cdot$ ml^{-1} (0.5 ml per well) in DMEM supplemented with 10% fetal bovine serum, 25 mM KCl, 25 mM glucose, 0.5 μM insulin, 2 mM glutamine, 5 mM Hepes, 50 U $\cdot$ ml^{-1} penicillin and 50 μg $\cdot$ ml^{-1} streptomycin. Cells were cultured in 5% CO_2. Cytosine arabinoside (10 μM) was added within 24 h of plating.

2.2.2. Medium changes and drug additions

At 7 days in vitro, cultures were switched to fresh medium (DMEM supplemented with 10% fetal bovine serum, 10 mM glucose, 2 mM glutamine and penicillin-streptomycin). Control cultures were supplemented with 25 mM KCl. Inhibitors (genistein, herbimycin A or cycloheximide) were added a few minutes before LiCl or as indicated in the legends. Equal solvent concentrations (dimethylsulfoxide (DMSO) 0.1% or methanol 0.5%) were added to controls to check for solvent effects. Cells were incubated in fresh medium for 24 h before assessment of cell viability.

2.2.3. Cell viability assay

Cell respiration, an indicator of cell viability, was assayed with MTT (Mosmann, 1985). Briefly, MTT stock solution (1.5 mg $\cdot$ ml^{-1}) was added to yield a final concentration of 0.2 mg $\cdot$ ml^{-1} and plates were kept for another 30 min in the incubator. After removal of the medium, cells were lysed in 0.75 ml DMSO and absorbance was read at 570 nm on a Milton Roy spectrophotometer against DMSO as a blank. Linearity of the response with respect to cell concentration was confirmed in preliminary experiments.

2.2.4. Data expression and analysis

Absorbance values are normalized as percentage of controls (30 mM K^+). Data are presented as means ± S.E.M. of n determinations, each in duplicate. The Li^+ effect in the presence of an inhibitor (e.g., genistein) was calculated as: absorbance of (Li^+ + inhibitor) cultures minus absorbance of (5 mM K^+ + inhibitor) cultures and expressed as percentage of controls (30 mM K^+). Multiple statistical comparisons used the Kruskal-Wallis analysis, followed by post-hoc Mann-Whitney U-test when overall comparison was statistically significant.

3. Results

3.1. Li^+ prevents low K^+ induced neuronal loss

Using the MTT assay as an indicator of cell viability, we confirmed the findings of D'Mello et al. (1994): cultures switched to low K^+ at 7 days in vitro were severely affected, with some 30% cell loss after 24 h; LiCl, when added at the time of medium change, protected neurons in a dose-dependent fashion, with a significant effect ($P < 0.01$) at 1.5 mM Li^+ and above (Fig. 1).

3.2. Tyrosine kinase inhibitors prevent Li^+ protection of cerebellar granule cells

Genistein, a widely used tyrosine kinase inhibitor, dose dependently abolished the positive effect of 5 mM Li^+ on neuronal survival (Fig. 2). Half-maximal inhibition was in the 10–30 μM range, with essentially complete inhibition at 100 μM. Herbimycin A, a structurally different tyrosine kinase inhibitor, also inhibited the Li^+ effect at all concentrations tested. The lower concentration used here (1 μM) significantly prevented the Li^+ effect, while inhibition was complete at 10 μM. Conversely, genistein or herbimycin A did not compromise the viability of low K^+ cultures at any concentration up to 100 μM or 10 μM, respectively (data not shown).

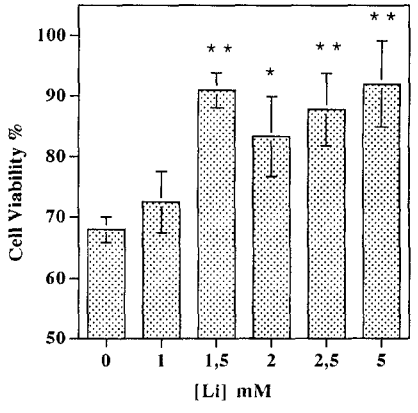

Fig. 1. Protective effect of LiCl on cerebellar granule neurons switched to non-depolarizing conditions. Cerebellar granule neurons were grown in 30 mM K^+ and switched to 5 mM K^+ medium at 7 days in vitro with or without added LiCl. Cell viability was assessed 24 h later with the MTT assay and expressed as percentage of control (30 mM K^+) cultures. Means ± S.E.M. of 4–8 determinations. Overall significance (Kruskal-Wallis): $P < 0.01$; * different from 5 mM K^+ at $P < 0.05$; ** different from 5 mM K^+ at $P < 0.01$.

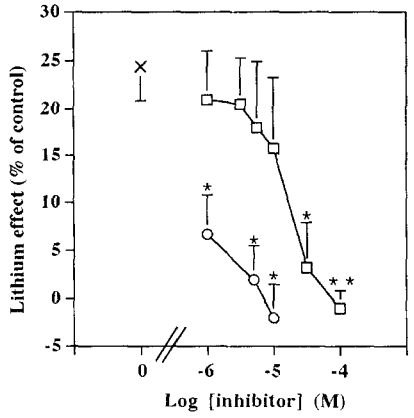

Fig. 2. Inhibition of the Li^+ effect by tyrosine kinase inhibitors. Cells were switched to non-depolarizing medium as in Fig. 1. The protective effect of 5 mM LiCl was assessed in the absence (x) or in the presence of genistein (□) or herbimycin A (○). Results are means ± S.E.M. of 3–9 different determinations. Kruskal-Wallis: $P < 0.01$; different from Li^+ alone at * $P < 0.05$ or * * $P < 0.01$.

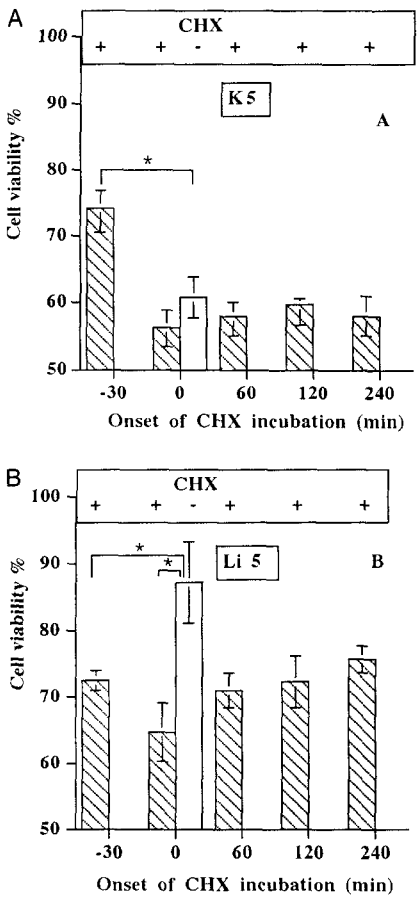

Fig. 3. Effect of cycloheximide. Cultures were incubated with (hatched bars) or without (open bars) cycloheximide (1 μM) at indicated times before (−30 min) or after (0–240 min) medium switching. Results are means ± S.E.M. of 3–9 determinations. Data were analyzed together but are presented separately for convenience. Kruskal-Wallis: $P < 0.001$. (A) 5 mM K^+ cultures; * different from control cultures; $P < 0.05$. (B) 5 mM Li^+-treated cultures; * different from 5 mM Li^+ alone; $P < 0.05$.

3.3. Cycloheximide differentially affects the survival of low K^+- and Li^+-treated cells

Tyrosine kinases are part of the signalling cascades which often result in transcriptional activation and de novo protein synthesis. Since the Li^+ effect was antagonized by tyrosine kinase inhibitors, we assessed the effect of the translation inhibitor cycloheximide on Li^+-induced cell survival, a somewhat intricate issue since cycloheximide and anisomycine are known to exert specific effects in this model (see discussion). Results are summarized in Fig. 3. In low K^+ medium without LiCl addition, cycloheximide (1 μM) *increased* cell viability, in line with its well-known effect of preventing programmed cell death (Fig. 3A). In sharp contrast, in Li^+-treated cells, cycloheximide significantly *decreased* cell viability; this effect was still apparent, but tended to be less prominent, when cycloheximide was added up to 4 h after Li^+ addition (Fig. 3B).

4. Discussion

The present report confirms the previous finding of D'Mello et al. (1994) that Li^+ protects cerebellar granule cells switched to non-depolarizing medium. These authors also showed that optimal Li^+ concentrations were different if cultures were maintained in serum-supplemented medium (Li^+ effect in the 2.5–5 mM range) or in serum-free medium (Li^+ effect in the 5–10 mM range). Indeed, in our hands, Li^+ increased granule neuron survival in the presence of fetal bovine serum at concentrations as low as 1.5 mM (Fig. 1).

This effect was dose dependently inhibited by genistein and herbimycin A, which are specific inhibitors of tyrosine kinases at the concentrations used here. Genistein inhibited lithium chloride's action with a half-maximal effect in the 10–30 μM range, which compares favorably with previously published results on inhibition of epidermal growth factor receptor or the non-receptor tyrosine kinase $pp60^{v\text{-}src}$ (22 and 26 μM, respectively) (Akiyama et al., 1987). This finding and the fact that genistein and herbimycin A inhibited the Li^+ effect without compromising low K^+ cell viability at the concentrations used here suggest that tyrosine kinase(s) are specifically involved in the Li^+ protection of cerebellar granule neurons.

Cycloheximide substantially prevented the protection by LiCl of granule neurons under our conditions, which suggests that this effect requires de novo protein synthesis (see later). Conversely, in low K^+ cultures, cycloheximide exerted a partial protection against cell death, a finding consistent with previous reports for the same model, and an acknowledged feature of some forms of programmed cell death (Dragunow and Preston, 1995). The protection imparted by cycloheximide was however incomplete and required preincubation: the latter methodological point was mentioned by Dessi et al. (Dessi et al., 1992, 1993),

whereas the former point somewhat contradicts previously published evidence of complete protection by cycloheximide in this model (Kharlamov et al., 1995). While minor experimental differences (time of medium switching, higher K^+ or cycloheximide concentrations) may contribute to this partial discrepancy, it must be stressed that the complete protective effect of cycloheximide was documented with methods specifically sensitive to apoptosis such as ApopTag (Kharlamov et al., 1995) or Hoechst 33258 (Copani et al., 1995) staining. Conversely, we have assessed overall cell viability, which may decrease as a result of necrosis, a cycloheximide resistant process (Dessi et al., 1993), as well as apoptosis.

Genistein has been shown to modulate such events as neurotransmitter release (Bare et al., 1995) synaptic plasticity (O'Dell et al., 1991) or NMDA receptor sensitivity (Wang and Salter, 1994). One definite possibility is therefore that Li^+ exerts its influence through post-translational modulation of some undefined event in signal transduction.

This is not likely to be the sole mechanism, since the cycloheximide inhibition of the Li^+ effect strongly suggests involvement of de novo protein synthesis. It is therefore tempting to speculate that Li^+ acts through enhancement of some neurotrophic factor. Indeed, as mentioned above, Li^+ provides full protection at notably different concentrations in serum-free or serum-complemented cultures, which suggests that Li^+ and serum act synergistically to enhance granule neuron survival. Of special interest, Calissano et al. (1993) showed that the serum enhancement of cerebellar neuron survival was partly inhibited by anti-insulin-like growth factor-I (IGF-I) monoclonal antibodies, thereby suggesting that part of the serum effect is achieved through IGF-I or IGF-I-like activity. Li^+ might therefore enhance the positive trophic effect of endogenous IGF, which is known to involve tyrosine protein phosphorylation. Investigation of the relevant pathways will require additional experimental work.

In summary, we have shown that the Li^+ enhancement of cerebellar granule cell survival is abolished by genistein and herbimycin A at concentrations consistent with the involvement of a tyrosine kinase. Moreover, the cycloheximide inhibition of the Li^+ effect suggests that neuron protection involves transcriptional activation. Further investigation of this effect might unravel a new mechanism to be added to the numerous cellular effects of the lithium ion.

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