PART II: Lithium action on ERK pathway in neural cells
Introduction

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1.1 Introduction to pharmacological targets of lithium.

Lithium has the simplest structure of any therapeutic agent, and its bioactive properties have been known for over a century. Lithium salts have proven to be a valuable tool for the treatment of bipolar disorder, formerly known as manic depressive illness. As the name implies, people who suffer from bipolar disorder experience drastic mood swings, from extreme happiness to extreme depression. Between 0.4 and 1.6% of the population suffer from this psychiatric condition. The use of lithium as a stabiliser of mood dates from 1949, when Australian physician John Cade observed the calming effect that lithium had on animals and on himself (that is the way how he tested the safety of lithium) [133].

In the central nervous system, lithium has been reported to confer protection to neural cells against a wide variety of insults, and to induce synaptic remodelling in neurons, which may account for its positive clinical effects in the treatment of mood disorders [134]. Apart from its mood-stabilising properties, profound developmental, metabolic, and hematopoietic effects are reported to be caused by millimolar concentrations of lithium. Lithium is found to profoundly affect the development of diverse lower organisms such as *Dyctiostelium* and *Xenopus* [135, 136], but surprisingly few teratogenic effects are reported in humans. Perhaps the most striking metabolic effect attributed to lithium is the stimulation of glycogen synthesis, thus mimicking insulin action [137]. In humans, it is reported to increase the number of circulating granulocytes and pluripotent hematopoietic stem cells [138, 139].

Unfortunately, the molecular mechanisms underlying all these effects is still a matter of debate. In 1971, some researchers reported reduced brain inositol levels in lithium-treated rats [140]. Nowadays, lithium ions are still one of the few pharmacological tools available to investigate the metabolic pathways involved in the phosphoinositide cycle, and the ability of lithium to inhibit myo-inositol monophosphatase (IMPs) at therapeutically relevant concentrations (0.5-1.5 mM) is now well established [141]. Glycogen synthase kinase–3 (GSK-3) is another protein whose in vitro activity has been reported to be inhibited by lithium ions, and this action seems to account for the profound developmental alterations lithium causes in *Dyctiostelium* or *Xenopus* [142]. IMPs and GSK-3 require from metal ions for catalysis and are inhibitited by lithium in an uncompetitive manner, most likely by displacing metal ions from the catalytic core. In central nervous system and in peripheral
tissues the actions of lithium can be mediated through inhibition of IMPs or GSK-3, but probably the picture is far more complex than that. Now these putative molecular targets of lithium are presented.

1.1.1 Lithium modulation of phosphoinositide cycle: inhibition of inositol monophosphate phosphatases (IMPs).

Phosphoinositide-based signaling brings to the cell an incredibly rich modulation potentiality, keeping in mind the presence of six hydroxyl groups on the inositol ring which can be found in a phosphorylated state. The first round of phosphoinositide-derived second messengers arrived with inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) and DAG. They derive from PLC hydrolysis of PtdIns(4,5)P$_2$, a minor lipid constituent of cell membranes. The former releases Ca$^{2+}$ from intracellular stores, while the later activates certain PKC isoforms. Ins(1,4,5)P$_3$ can be further phosphorylated to inositol (1,3,4,5)tetrakisphosphate, a molecule that may also have second messenger functions. It can in turn be a substrate for phosphatases and kinases and yield inositol(3,4,5,6)tetrakisphosphate, whose functions in the inhibition of the ionic conductance through Ca$^{2+}$-activated chloride channels is well reported [143]. It seems likely that a family of phosphoinositols downstream of Ins(1,4,5)P$_3$ could be used by cells to modulate specific functions [144].

But not only water soluble phosphates can be used by the cell to exert metabolic control, lipids can also do the job, this time as tethers for proteins at cellular membranes. Pleckstrin homology (PH) domains present in many different proteins (including PLC and PLD) bind to PtdIns(4,5)P$_2$ or PtdIns(3,4,5)P$_2$, and permit or facilitate their anchorage to membranes [145]. PtdIns(3)P specifically binds to a module called FYVE domain, also present in many proteins [146]. Alternatively, those lipid groups might be important for catalysis, and it seems to be the case for enzymes like PLD [14].

So, both inositolts and inositol lipids provide a complex level of regulation to the cell. Therefore, it looks feasible that interference in the phosphoinositide cycle could easily lead to deregulation of cellular functions. The nervous system is a site of intense inositol turnover, since the transduction pathways of numerous neurotransmitters, neuropeptides and hormones involve receptor-mediated hydrolysis of PtdIns(4,5)P$_2$ by PLC isoforms. The resultant production of Ins(1,4,5)P$_3$ is rapidly metabolized and returned to free inositol as a
‘shut down’ mechanism. The myo-inositol can then be incorporated to phosphoinositides by means of phosphatidylinositol synthase, thereby closing the cycle (see Fig. 7: phosphoinositide cycle). Lithium has been shown to inhibit effectively IMPs, thus breaking up the cycle and resulting in the accumulation of inositol monophosphates [147]. A lithium inhibition of purified inositol polyphosphate 1-phosphatase, which metabolizes Ins(1,4)P$_2$, Ins(1,3)P$_2$ and Ins(1,3,4)P$_3$, has also been reported [148, 149]. Many studies also clearly demonstrate an increase in inositol 1-phosphate (Ins1P) and inositol 4-phosphate (Ins4P) in brain following systemic injections of lithium to rats [150, 151]. Four hours after lithium administration a 15-30 fold increase in inositol monophosphates can be observed when compared to saline treated animals, and a concomitant decrease of inositol is also found. Based of these findings, the inositol depletion hypothesis was suggested. This hypothesis considers that in tissues with relative deficiency of inositol, persistent activation of PLC in the presence of lithium would lower cellular inositol concentration, leading eventually to the depletion of PtdIns(4,5)P$_2$ and the impairment of DAG/calcium signaling [152]. Lithium inhibits IMPs in vitro with a $K_i$ (0.8mM), which is within the therapeutic range (0.5-1.5mM) for lithium treatment of patients with bipolar disorder [153]. Additional support for the inositol depletion hypothesis comes from the marked effects of lithium on developing organisms. Exposure to millimolar concentrations of lithium during an early stage of development causes expansion and duplication of dorsal and anterior structures in Xenopus embryos. This lithium concentrations can inhibit IMPs in vivo and cause 30% drop of inositol levels [154]. Interestingly, those teratogenic effects of lithium in Xenopus could be overcome simply by coinjection of inositol [154]. Similarly, effects of lithium (in addition to valproic acid and carbamazepine, two other drugs commonly used in the treatment of bipolar disorder) on neuronal plasticity, in particular the observed increase in growth cone area of sensory neurons, can be counteracted by inositol addition, thus implicating inositol depletion in their actions [155]. Further support for the inositol depletion hypothesis comes from the observation that neurotransmitter-stimulation of PLC in presence of lithium leads to a dramatic accumulation of CDP-DAG [156]. PtdIns synthase reaction therefore mirrors inositol depletion (see fig 7).

Although inositol depletion is an attractive model, some inconsistencies make it possible that inhibition of IMPs is necessary but not sufficient to account for the effects of
lithium in some settings. The most direct is that selective inhibitors of IMPs (Bisphosphonates) have no effect on the development of *Xenopus* embryos, despite complete inhibition of IMPs [157]. A novel hypothesis to explain lithium actions on embryonic development and other cellular functions is based on the observation that lithium is a direct inhibitor of GSK-3. This is now discussed in next section.

**Figure 7.** The phosphoinositide cycle. Ligand binding to a surface receptor activates PLC, which hydrolyzes the phospholipid PtdIns(4,5)P$_2$ to yield diacylglycerol (DAG) and inositol-1,4,5 trisphosphate (IP$_3$). IP$_3$ is rapidly dephosphorylated by means of phosphatases to yield free inositol, which is then incorporated back to PtdIns by the action of PtdIns synthase. As shown in this simplified model, Li$^+$ ions inhibit inositol monophosphate phosphatase phosphatase (IMPs) and inositol polyphosphate 1-phosphatase. Inositol depletion hypothesis considers that the stimulation of PLC-coupled receptors in presence of lithium ions would result in the accumulation of inositol monophosphates and in the reduction of the pool of free inositol. The net effect of lithium would be the blockade of ligand-dependent signaling through PKC and IP$_3$/Ca$^{2+}$. 1, PtdIns 4-kinase; 2, PtdIns 5-kinase; 3, DAG kinase; 4, CTP-phosphatidate citidyltransferase; 5, PtdIns synthase.
1.1.2 Lithium inhibition of glycogen synthase kinase-3 beta (GSK-3β).

The developmental phenotype that is induced by lithium in *Xenopus* is similar to that caused by alteration in the expression of Wnt proteins [158, 159]. GSK-3 is a serine/threonine kinase that controls glucose utilisation, cell survival and cell fate through involvement in multiple signaling pathways [160]. It was named for its ability to phosphorylate, and thereby inactivate, glycogen synthase (GS), a key enzyme for the synthesis of glycogen [161-163]. GSK-3 is phylogenetically close to cyclin-dependent protein kinases (Cdk5s) and MAPKs. Two isoforms sharing 97% sequence similarity have been cloned, GSK-3α and GSK-3β. We now know that insulin treatment results in the phosphorylation of GSK-3 at a N-terminal serine residue (Ser21 in GSK3α and Ser⁹ in GSK3β) by protein kinase B (PKB, also called Akt). Thus, in response to insulin, PtdIns 3-kinase dependent pathway is activated, PKB is turned on promoting GSK-3 phosphorylation (that renders it inactive) resulting in the dephosphorylation of GS and thus in the stimulation of glycogen synthesis. Insulin, by inhibiting GSK-3, also stimulates the dephosphorylation and activation of eukaryotic synthesis initiation factor 2B (eIF2B), contributing to an increased rate of protein synthesis [163]. MAPK-activated protein kinase-1 (MAPKAP-K1, also called RSK) provides a route for the inhibition of GSK-3 by growth factors and other signals that activate MAPK pathway [164]. Another kinase that phosphorylates GSK-3 at Ser21/ Ser⁹ *in vitro* is p70 ribosomal S6 kinase-1 (S6K1). S6K1 is regulated by the mammalian target of rapamycin (mTOR), a protein that senses the presence of amino acids in the medium. Therefore, S6K1 phosphorylation of GSK-3 may underlie the observed inhibition of GSK-3 induced by aminoacids [165]. Figure 8 summarises some of the proposed signaling pathways that inhibit GSK-3. Downstream targets of GSK-3 are also included.

Being clarified its metabolic role on glycogen synthesis, GSK-3 reemerged this time as an essential kinase in embryonic development and patterning in studies conducted in *Xenopus* and *Drosophila*, and also involved in cell cycle control in mammals [160]. GSK-3 is now regarded as an essential component of the Wnt signaling pathway. Figure 9 depicts a simplified model of Wnt pathway in mammalian cells. In the absence of Wnt signal,
active GSK-3 is present in a multiprotein complex that targets β-catenin for ubiquitin-mediated degradation. This complex includes the scaffolding protein axin, the product of the adenomatous polyposis coli (APC) gene and β-catenin itself. In vitro, GSK-3 phosphorylates β-catenin on residues 33, 37 and 41, and this is proposed to permit β-catenin ubiquitination. GSK-3 requires a priming phosphorylation in position +4. Casein kinase I in vitro phosphorylates Ser45. Thus, the combined phosphorylation of β-catenin by casein kinase I first and then by GSK-3, seems to be required for β-catenin ubiquitin-mediated degradation [166]. The binding of Wnt to the seven-transmembrane-spanning receptor frizzled causes inactivation of GSK-3 leading to increased levels of cytoplasmatic
β-catenin and its translocation to the nucleus, where it associates with TCF/LEF transcription factors and enhances transcription of target genes. Thus, GSK-3 must be inactivated for the Wnt signalling to proceed. Disturbance in the Wnt signalling of *Xenopus* or *Dyctiostelium*, either by mutation or drug inhibition of any of the molecular components, causes severe developmental abnormalities [160]. In mammals, GSK-3 has been implicated in cell cycle regulation, supported by the finding that a surprisingly high rate of mutations of elements of the Wnt pathway have been found in different tumours [167, 168]. Therefore, a tight control on β-catenin levels by GSK-3 seems important for a normal cell cycle to proceed. Cancer causing mutations often enable β-catenin to accumulate, resulting in the transcription of oncogenic genes under TCF/LEF control (see fig. 9). Identified mutational hotspots are proteins of the complex that targets β-catenin to the proteasome, like APC. As an example, some 80% of colon cancers contain a mutation in APC gene [168].

The elucidation of GSK-3 three dimensional structure shed some light on its inhibition mechanism. Inhibition of GSK-3 by insulin and growth factors is mediated by phosphorylation at Ser⁹, turning the N-terminus into a pseudosubstrate inhibitor that competes for binding with the ‘priming phosphate’ of substrates. In contrast, Wnt proteins are thought to inhibit GSK-3 in a completely different way, since no phosphorylation on Ser⁹ has been reported in response to Wnt glycoproteins [169].

Lithium, which inhibits uncompetitively GSK-3 in *vitro*, has been a valuable tool in identifying potential roles of GSK-3. The Kᵢ is reported to be within the effective range for lithium actions (1-2 mM) [142, 157]. In *vivo*, millimolar concentrations of lithium have also been reported to inhibit GSK-3 [170]. Lithium, by inhibiting GSK-3, behaves as a mimic of Wnt signaling during embryogenesis, while also capable of mimicking the actions of insulin on activation of GS. In *Xenopus*, lithium causes duplication of the dorsal mesoderm while in *Dyctiostelium* exposure to lithium during early development blocks spore cell fate and promotes formation of stalk cells [142]. Since GSK-3 has been involved in the embryonic patterning, those teratogenic effects can be explained by lithium inhibition of GSK-3. In mammalian cells, lithium leads to nuclear accumulation of β-catenin, affecting cell proliferation either positively [171] or negatively [172, 173] depending on the
cell type. In BAEC cells, lithium arrested cell cycle and this effect was attributed to lithium-induced accumulation of β-catenin and to stabilisation of p53 [173].

**Figure 9 The Wnt signaling pathway in mammalian cells.** Wnts are a family of secreted glycoproteins involved in embryonic patterning, axonal remodelling and regulation of cellular proliferation. In the absence of Wnt, GSK-3β phosphorylates β-catenin resulting in its rapid degradation via the proteasome. Binding of Wnt to the frizzled receptor leads to the inhibition of GSK-3β and allows stabilisation of β-catenin and its accumulation in the nucleus, where binds to TCF/LEF transcription factors and turns on the expression of target genes, i.e. C-myc oncogene and cyclin D (CycD). Lithium ions can inhibit GSK-3 activity *in vitro* and *in vivo*. SB216763 and SB415286 are specific inhibitors of GSK-3 developed by SmithKline Beecham Pharmaceuticals.

In summary, the evidence in support of GSK-3 as a relevant target of lithium action includes: a) *in vitro* inhibition, b) *in vivo* inhibition, c) lithium mimicking the embryonic phenotype of GSK-3β loss of function mutants, d) downstream inhibition of Wnt signalling blocking the effects of lithium, e) alternative inhibitors of GSK-3 (SB216763, SB45286) mimicking lithium actions (reviewed in [142]).

**1.2. The Ras/Raf/MEK/ERK pathway.**

**1.2.1 General view of MAP kinase signaling pathways.**

Activation of mitogen-activated protein kinases (MAPKs) transmits information from surface receptors to nuclear transcription factors, and has been associated with stimulation of cell proliferation, differentiation, migration and cell death [173]. The core of the MAPK cascade consists of an evolutionally conserved module of three sequentially activated protein kinases. The activation of the MAPK requires phosphorylation on conserved
tyrosine and threonine residues localised to the so called activation loop, and is catalysed by a dual-specificity MAPK kinase (MAPKK). MAPKK, in turn, is under control of a MAPKK Kinase (MAPKKK), which, in turn, is regulated by G proteins and in some cases, by a MAPKKK kinase (MAPKKKK). Three of these modules are present in mammalian cells: extracellular signal regulated kinases (ERKs), N-terminal c-Jun kinases (JNKs), and p38 (see fig. 10A).

ERKs, the first to be cloned, were initially characterised as kinases that phosphorylated microtubule associated proteins (i.e. MAP2). Their actions have been best examined in the context of growth factor signaling via receptor tyrosine kinases (RTKs). The archaetypal ERK cascade involves the activation of the small GTPase Ras upon agonist stimulation of RTK receptors. This stimulation leads to autophosphorylation of the RTK in tyrosine residues and recruitment of adaptor proteins bearing SH2 and SH3 motifs (Shc and Grb2) that bind to an exchange factor for Ras, SOS. SOS allows nucleotide exchange-dependent activation of the MAPKKK Raf-1. Raf-1 is a kinase with serine/threonine specificity that catalyses the activating phosphorylation of MEK1/2, which ultimately phosphorylates ERK1/2 on its activation loop. Amplification via this signaling cascade is such that it is estimated that activation of solely 5% of Ras molecules is sufficient to induce a full activation of ERKs [174]. In resting cells, ERKs are anchored in the cytoplasm via association with MEK1/2, but following activation they dissociate from the cytoplasmatic anchoring complex and enter the nucleus, the site for signal termination [175]. Figure 10B depicts in a simplified model the activation of ERKs in response to growth factors.

The second subgroup of MAPKs, JNK proteins, were first described as kinases that phosphorylate serine residues on N-terminus of c-Jun transcription factor following UV exposure. The third subgroup, p38, were found to be activated in response to different forms of stress. Both JNK and p38 are given the more clarifying name SAPKs (stress activated protein kinases). Rho family members (Rho, Rac, Cdc42) are thought to be involved in SAPK activation in response to cytokines or cellular stress [173].
Figure 10. A) Schematic view of MAPK signalling pathways in mammalian cells. B) Archaetypical activation of ERK pathway by growth factors (GFs). TKR tyrosine kinase receptors.
1.2.2 MAPKs in the regulation of cell cycle.

The cell cycle is controlled by a class of nuclear enzymes called cyclin-dependent kinases (cdks), expressed constitutively but present in inactive form unless combined with their cyclin partners. The stimulatory effects of cyclins are counteracted by the inhibitory effects of cdk inhibitory proteins (CKIs), of which two families are well described: Ink4 and Cip/kip (p21) [176]. Progression through G1 phase is especially affected by extracellular signals, and is regulated by cdk4/CycD, cdk6/CycD and cdk2/CycE complexes. Most studies indicate that integrins and growth factors, by activating MAPK pathways, typically control expression of Cyclin D1 and/or downregulate CKIs. In this regard, the activation of the MEK/ERK pathway has been linked to the induction of cyclin D1 transcription, since expression of dominant negative mutants of MEK and ERK prevented growth factor-dependent transcription of the cyclin D1 gene [177]. The formation of active cyclinD/Cdk4 complex is considered rate-limiting for cell growth [178, 179]. Recent studies indicate that the critical determinant in the induction of cyclin D1 is the duration of the ERK signal. Activation of ERK can be transient or sustained, depending on the stimulus. For example, serotonin induces a transient (10 min) stimulation of ERK in CCL39 cells, while thrombin induces a far more sustained activation that peaks 3-4 hours after addition to cells [180]. Some studies have observed a correlation between the strength of the mitogenic signaling and the duration of the ERK stimulation. While non-mitogenic factors induce a transient activation of ERKs (less than 15 min) that does not lead to cell cycle entry, mitogens induce cell proliferation and sustained ERK activation (up to 6 hours) [181].

From these studies, an important link between MEK/ERK pathway and cell cycle machinery was established, and furthermore, that sustained ERK activation was a common characteristic elicited by mitogens that would permit entry and progression through G1 phase of the cell cycle (see fig. 11).
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Figure 11. ERKs on cell cycle progression. ERKs phosphorylate AP1 and ETS transcription factors turning on the expression of target genes including cyclin D (CycD). Association of CycD with its cyclin dependent kinase partners (cdk), cdk4 and cdk6, results in the phosphorylation of retinoblastoma protein (pRB). Its phosphorylation is required for progression through G1 phase of the cell cycle. Other Cyc/cdk complexes (CycE/Cdk2, CycA/Cdk2) are required for progression through late G1 and S phases.
OBJECTIVES
In the first part of this thesis we concluded that PLD activation is not a general requirement for astrocyte proliferation, although it can take place concomitantly following mitogen exposure. We wanted to keep on exploring molecular mechanisms mediating astrocyte growth, in particular those involving signaling lipids. Since phosphoinositide cycle can be impaired by lithium ions, we proposed initially to study the effects of lithium on the proliferation of cultured astrocytes.
RESULTS
Lithium inhibits the MEK-ERK pathway in astrocytes by a mechanism independent of GSK-3 and inositol depletion

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SUMMARY

Exposure of cerebellar granule neurons to milimolar concentrations of lithium enhanced MEK and ERK phosphorylation in a concentration-dependent manner. In asynchronous, proliferating cortical astrocytes, lithium inhibited $[^3]$H]thymidine incorporation into DNA, induced a G2/M cell cycle arrest, and inhibited phosphorylation of MEK and ERK, but did not affect phosphorylation of Akt. In quiescent astrocytes, lithium inhibited cell cycle re-entry as stimulated by endothelin-1, and also prevented stimulation of MEK and ERK phosphorylation, but did not affect endothelin-1 or EGF activation of Ras. Lithium inhibition of the astrocyte MEK-ERK pathway was not due to inositol depletion, as it was not counteracted by exogenous addition of inositol. Treatment of astrocytes with compound SB-216763 resulted in the inhibition of Tau phosphorylation at Ser$^{396}$, and the stabilization of cytosolic β-catenin, consistent with the inhibition of glycogen synthase kinase-3β, but failed to reproduce lithium effects on MEK and ERK phosphorylation and cell cycle arrest. These opposing effects of lithium in neurons and astrocytes make lithium treatment a promising strategy to favor neural repair and reduce reactive gliosis after traumatic injury.

Running title: Lithium inhibits astrocyte ERK

Keywords: Lithium, ERK, MEK, astrocytes, GSK-3, inositol

INTRODUCTION

Lithium has been used in the treatment of bipolar mood disorder for decades, but despite its therapeutic efficacy, the molecular mechanisms underlying its actions remain unclear (Jope, 1999; Phiel and Klein, 2001). In this regard, based on the observation that lithium inhibits inositol monophosphatase and inositol polyphosphate 1-phosphatase, thereby blocking inositol 1,4,5-trisphosphate recycling to inositol, the inositol depletion hypothesis considered that persistent activation of phosphoinositide phospholipase C in the presence of lithium would lower the cellular inositol concentration, leading eventually to the depletion of phosphatidylinositol 4,5-bisphosphate and the impairment of calcium signaling (Berridge et al., 1989; Atack, 1996). This hypothesis has received some support recently,
after the finding that lithium, carbamazepine, and valproic acid, all three drugs used in the
treatment of bipolar disorder, increase growth cone area in sensory neurons, in a manner
that was counteracted by inositol addition, and mediated probably by inhibition of prolyl
oligopeptidase by an as yet unknown mechanism (Williams et al., 2002).
In the past few years, however, lithium has emerged as a remarkable neuroprotective agent:
it was first shown to protect cerebellar granule neurons from undergoing apoptotic cell
death when cultured under non-depolarizing potassium conditions (D’Mello et al., 1994),
and soon thereafter similar observations were extended to many other apoptotic insults like
ischemia (Nonaka and Chuang, 1998), anticonvulsants (Nonaka et al., 1998) C2-ceramide
(Centeno et al., 1998), or β-amyloid (Alvarez et al., 1999), among others, where lithium
effects were independent of inositol depletion (Nonaka et al., 1998; Centeno et al., 1998).
Glycogen synthase kinase-3β (GSK-3β) is inhibited by lithium (Klein and Melton, 1996;
Stambolic et al., 1996), and this discovery has greatly fueled the interest on the
biochemistry of this classic therapeutic agent. In many instances, the activation of GSK-3β
taking place after disruption of the phosphatidylinositol 3-kinase/Akt pathway appears
related to apoptotic cell death, and it is generally acknowledged today that this kinase
constitutes the primary site of action underlying the cytoprotective actions of lithium
(Grimes and Jope, 2001). Lately, more selective GSK-3β inhibitors have been developed
with potent cytoprotective effects, lending further support to the involvement of GSK-3β in
the regulation of cell survival and apoptosis (Cross et al., 2001). Interestingly, inhibition of
GSK-3β may not be the sole mechanism underlying lithium neuroprotection, as the ion also
potentiates Akt phosphorylation in cerebellar neurons (Chalecka-Franaszek and Chuang,
1999; Mora et al., 2001). The relevance of GSK-3β inhibition in bipolar therapy, however,
is still a matter of controversy, although it is supported by the fact that valproate, another
drug used in the treatment of bipolar disorder, has been reported to inhibit GSK-3β in vitro
(Chen et al., 1999a) and also in vivo (Hall et al., 2002; De Sarno et al., 2002). The
inhibitory action of lithium and valproate on GSK-3 is not restricted to neuroprotection
against apoptotic insults: lithium has been shown to mimic the action of WNT-7a,
promoting axonal remodeling and synaptic differentiation (Hall et al., 2000). This
neuroplastic action of anti-bipolar drugs may be therapeutically relevant, as there is
evidence supporting the occurrence of neuronal loss and/or atrophy in patients with mood
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disorder (Drevets et al., 1997; Ongur et al., 1998). In fact, valproic acid has been shown to promote neurite growth in neuroblastoma cells by a mechanism that implies activation of the MEK-ERK cascade of mitogen-activated protein kinases (Yuan et al., 2001), a signaling pathway whose relevance in the regulation of neuronal function, synaptic plasticity, and survival is receiving growing attention (Grewal et al., 1999).

The present study was undertaken to explore whether lithium, like valproic acid, does stimulate ERK in neural cells. Experimental studies on the plasticity and regeneration of the nervous system often disregard the role of glial cells as trophic support for neurons or as source of inflammatory cytokines, so we compared the effects of lithium in neurons and astrocytes. We found that lithium strongly potentiates ERK phosphorylation in cerebelar granule neurons, but to our surprise, it exerts opposite effects in astroglial cultures, inhibiting MEK and ERK phosphorylation, DNA synthesis, and inducing cell cycle arrest.

MATERIALS AND METHODS

Materials
Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, penicillin, streptomycin, and glutamine were obtained from Gibco. Endothelin-1 (ET-1) was from Alexis. MEK inhibitors U0126 and PD98059, tyrphostin AG1478 and antibody against phosphoserine 396-Tau were from Calbiochem. Antibodies against total and phosphorylated ERK, MEK, and Akt were from Cell Signaling Technology. Anti Ras antibody was from Oncogene, antibody against β-catenin was from BD Transduction Laboratories, and antibody against β-tubulin was from BD PharMingen. Secondary peroxidase-conjugated antibodies were from Transduction Laboratories. [Methyl-3H]thymidine (87.0 Ci/mmol) was from Amersham Pharmacia. GSK-3 inhibitor SB216763 was kindly donated by GlaxoSmithKline, Stevenage, Herts (UK).

Cell culture
Primary cultures of cerebral cortical astrocytes were prepared from newborn (<24 h) Sprague-Dawley rats as previously described (Servitja et al., 2000). Cells were plated into 176 cm² dishes (12.5 x 10⁶ viable cells/dish) in DMEM supplemented with 10% FCS, 33
mM glucose, 2 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. After 24 h, the medium was changed to remove non-adhered cells. Before confluence, cells were replated into polylysine-coated 6-well plates (10^6 cells/well). At this time, immunocytochemical staining for glial fibrillary acidic protein revealed that cultures consisted of 90-95% astrocytes. Cells were maintained in serum-containing DMEM with or without LiCl, and then processed for Western blot analysis. In some experiments, cells were serum-starved for 2 days before being stimulated with ET-1. Each independent experiment was performed using a separate culture. Primary cultures of rat cerebellar granule cells were prepared as previously described (Masgrau et al., 2000). Briefly, cells were seeded at 2 x 10^6 cells/well in 6-well plates in DMEM medium containing 10% FCS, 25mM KCl, 5mM glucose, penicillin (50 units/ml), streptomycin (50 µg/ml), sodium pyruvate (0.1 mg/ml), 7mM p-aminobenzoic acid, and 2mM glutamine. 24 hours after plating, 10 µM cytosine arabinofuranoside was added to prevent growth of glial cells, and neurons were maintained for 7 days.

**Western blot analysis**

Astrocytes were washed twice with ice-cold phosphate-buffered saline prior to scraping and lysis. For ERK, MEK and Akt immunoblots, lysis buffer consisted of 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100, 2mM EDTA, 2mM EGTA, 50mM NaF, 10mM β-glycerophosphate, 5mM sodium pyrophosphate, 1mM sodium orthovanadate, 0.1% (v/v) β-mercaptoethanol, 250 µM PMSF, 10 µg/ml aprotinin and leupeptin. For immunoblots of cytoplasmic β-catenin, hypotonic lysis buffer was 50 mM Tris-HCl, pH 7.5, with 2mM EDTA, 2mM EGTA, 0.5mM NaF, 10mM β-glycerophosphate, 5mM sodium pyrophosphate, 1mM sodium orthovanadate and the same protease inhibitors as above. Cell lysates were centrifuged at 15000xg for 15 min at 4°C. Equal amounts of protein (10-20 µg) were subjected to sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore). Blots were blocked in 5% non fat dry milk /TBST buffer (20 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween-20) and immunoblotted for: total ERK1/2, phosphothreonine 202/phosphotyrosine 204-ERK 1/2, total MEK 1/2, phosphoserine 217/221 MEK 1/2, total Akt, phosphoserine
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473 Akt, Ras, β-tubulin, or phosphoserine 396-Tau. Blots were then labeled with anti-mouse IgG:HRP or anti-rabbit IgG:HRP, incubated for 1 min with ECL reagent (ECL, Amersham-Pharmacia) according to the supplier’s instructions, exposed to Hyperfilm ECL and developed. Quantification was performed by densitometric scanning of the film using a GS-700 Imaging Densitometer with Multi-Analyst 1.1 software (BioRad).

[^3]H Thymidine incorporation into DNA
As an indication of mitogenic induction in astrocytes, we measured the incorporation of [methyl-[^3]H]thymidine into DNA. Astrocytes were incubated 7 days in serum-containing medium with or without lithium and pulse labeled with 1 µCi/ml of [^3]H thymidine during the last 24h. At the end of the incubation, cells were washed with ice-cold Krebs-Henseleit buffer and treated with ice-cold HClO₄ (1 M) for 30 min. The acid-precipitated material was washed with ethanol and solubilized with 0.5 M NaOH. An aliquot was collected for liquid scintillation counting.

Cell cycle analysis
The cell cycle distribution was determined by flow cytometric analysis of propidium iodide-labeled cells. Briefly, astrocytes were plated in six-well plates 28h prior to treatment with either NaCl or LiCl for the indicated doses and times. After trypsinization, cells were collected, washed twice in ice cold PBS, and fixed in ice-cold 70% ethanol. Cells were then washed twice with ice-cold PBS, incubated at 37ºC for 30 min with propidium iodide (20 mg/ml) PBS solution containing 0.1 % (v/v) Triton X-100, 100 units/ml RNase A, and analyzed by flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The results were analyzed with CellQuest and Modfit LT Software.

Ras activation
Astrocytes were serum-starved for 2 days, and upon stimulation cells were lysed at 4 ºC in a buffer containing 20 mM HEPES, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycerophosphate, 20 mM MgCl₂, 1 mM Na₃VO₄, 1 mM dithiothreitol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF. Lysates were incubated for 30 min with 10 µg of a GST fusion protein including the Ras-binding domain of Raf-1 (RBD) previously
bound to glutathione-Sepharose beads (Upstate Biotechnology cat#14-278), followed by three washes with lysis buffer. Total Ras levels in the cell lysates and GTP-bound forms of Ras associated with GST-Raf-1-RasBD were quantified by Western blot analysis using an anti-Ras antibody.

RESULTS

Lithium stimulates ERK phosphorylation in neurons.

Treatment of cerebellar granule neurons with lithium (1-10 mM) for 3 hours resulted in a concentration-dependent increase of the phosphorylation status of ERK and MEK (Fig. 1), in agreement with the reported ERK-stimulating effects of valproic acid (Yuan et al., 2001). In contrast, lithium did not affect Akt phosphorylation. Lithium stimulatory effects on the neuronal MEK-ERK pathway were not mimicked by the GSK-3β inhibitor SB216763 (Fig. 1).

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Figure 1. Lithium induces MEK and ERK phosphorylation in cerebelar granule neurons.

A) Primary cultures of cerebellar granule neurons were treated for 3h with the indicated LiCl concentrations, or with 3 μM SB216763, and then cells were lysed and processed for Western blot analysis with antiphosphothreonine 202/tyrosine 204-ERK 1/2, antiphosphoserine 217/221 MEK 1/2, antiphosphoserine 473 Akt, or with the corresponding anti-ERK 1/2, anti MEK 1/2 or anti Akt antibodies. 20μg of protein were loaded on each lane. B) Densitometric analysis of phospho-ERK isoforms and phospho-MEK, relative to each total protein. Open bars represent the effect of SB216763. The results shown are from an experiment that was repeated twice with similar results.
Lithium inhibits DNA synthesis and induces a G2/M cell cycle arrest in asynchronous astrocytes.

Stimulation of ERK in dividing cells is expected to constitute a mitogenic stimulus, and in fact pituitary astrocytes have been shown to proliferate in response to lithium (Levine et al., 2000). Thus, we decided to study the effect of lithium on astrocyte DNA synthesis by measuring [\(^3\)H]thymidine incorporation. In our initial experiments, we found no stimulatory effects of lithium in serum-deprived astrocytes (not shown), and looked for effects in subconfluent, asynchronous astrocytes that had been exposed to various LiCl concentrations for 3 days. Compared to controls, the amount of [\(^3\)H]thymidine incorporation into DNA in 5 mM lithium-treated astrocytes was reduced by more than 60%, while with 10 mM the reduction was about 90%, as shown in Fig.2A (left). Since lithium is known to interfere inositol 1,4,5-trisphosphate recycling to myo-inositol, we checked whether lithium inhibitory effects on DNA synthesis in astrocytes could be due to intracellular inositol depletion. As shown in Fig. 2A (right), supplementation of the culture medium with 3 mM myo-inositol did not affect lithium inhibition of [\(^3\)H]thymidine incorporation, but reverted the potentiation due to 5 mM LiCl towards the accumulation of [\(^3\)H]CDP-diacylglycerol in [\(^3\)H]cytidine-prelabeled, carbachol-stimulated astrocytes (not shown), an effect that mirrors inositol depletion (Godfrey, 1989; Claro et al., 1993).

To further characterize the inhibitory effect of lithium on astrocyte proliferation, we monitored the cell cycle profiles of subconfluent, asynchronous astrocytes that had been treated for 3 days with different LiCl concentrations. As shown in Fig 2B, the percentage of tetraploid cells increased with lithium in a dose-dependent manner. The greater effect was observed with 10 mM LiCl, with 17.2% occurrence of cells in the G2/M phase compared to the 5.8% observed under control conditions. The cell cycle phase distribution was also monitored after a 7-day lithium treatment and showed similar results (14.3% of cells in G2/M in 5mM LiCl treated cells, compared to 4.9% in control cells, not shown). No signs of sub-diploid (apoptotic cells) were observed in any of the conditions assayed. Also, we found no impairment of cell adhesion in lithium-treated cells, indicating that lithium-induced cell cycle arrest occurred in the absence of cytotoxicity.
Results: lithium actions on ERK pathway in neural cells

Figure 2. Lithium inhibits astrocyte DNA synthesis and induces a G2/M cell cycle arrest.

A) Determination of [3H]thymidine incorporation in asynchronous secondary astrocytes. Subconfluent astrocytes were treated with LiCl at the indicated doses for 3 days in presence/absence of 3 mM added myo-inositol. The cells were pulse-labeled with 1 µCi/ml of [3H]thymidine during the last 24h of treatment. [3H]Thymidine incorporated into DNA was determined as shown in Methods. The results are means± S.D. of three independent experiments. B) Cell cycle analysis. Asynchronous astrocytes were treated with the indicated concentrations of LiCl for 3 days. After trypsinization, the cells were ethanol fixed and labeled with propidium iodide for quantification of DNA content by flow cytometry. The percentage of cells in each phase of the cell cycle was determined using Modfit LT software. The flow cytometric profiles shown are representative of two independent experiments.

Chronic lithium reduces ERK and MEK phosphorylation in astrocytes.

Given the essential role of the MEK-ERK signaling cascade in the stimulation of DNA synthesis and cell cycle progression (Chang and Karin, 2001), we hypothesized that lithium could exert opposite effects in this pathway in neurons and astrocytes. As shown in Fig. 3, a
chronic (7 days) treatment of proliferating, asynchronous astrocytes with lithium resulted in an effective inhibition of ERK and MEK phosphorylation. This was already apparent at 1 mM lithium, while a 10 mM lithium treatment reduced these phosphorylated kinases to barely detectable levels. In contrast, lithium did not significantly affect the phosphorylation status of Akt (Fig. 3), indicating that, rather than being the outcome of a general collapse of the ATP balance, the effect of lithium is somehow selective on the MEK-ERK pathway.

![Figure 3. Chronic lithium treatment blocks ERK 1/2 and MEK phosphorylation in asynchronous astrocytes.](image)

Secondary astrocytes were incubated for 1 week with the indicated concentrations of LiCl in DMEM containing 10% FCS. Cell extracts were processed as indicated in Methods, and subjected to Western blot analysis for determination of phospho-ERK 1/2, phospho-MEK 1/2, phospho-Akt, and the corresponding total proteins. The blots shown are representative of 5 independent experiments.

*Chronic lithium prevents ET-1 stimulated ERK phosphorylation.*

We next investigated whether lithium may preclude mitogen activation of the MEK-ERK pathway. ET-1 is a well-characterized mitogen to astrocytes, whose actions are especially relevant on reactive gliosis taking place upon central nervous system damage (Stanimirovic et al., 1995), and therefore ET-1 was chosen as the stimulus. Astrocytes were arrested in G0 by switching to serum-free DMEM for 24h, and were further maintained in this medium for 7 days in the presence of different LiCl concentrations, then challenged with ET-1 for 3h. As shown in Fig. 4, chronic lithium at all concentrations tested prevented ET-1-stimulated ERK phosphorylation. 1mM LiCl reduced phospho-ERK levels by 20% while 10mM dropped its levels to those of quiescent astrocytes.
Lithium does not affect Ras activation

Lithium effects on ET-1 stimulated ERK phosphorylation were rapid, as they could be reproduced in acute treatment with a high concentration (10 mM). As shown in Fig. 5A, phospho-ERK and phospho-MEK levels were reduced even when ET-1 and lithium were added simultaneously, while a 24-hour lithium treatment totally abolished ET-1 stimulated MEK and ERK phosphorylation. To gain some insight into the mechanism whereby lithium interferes with MEK-ERK signaling, we checked Ras activation under conditions (10 mM LiCl, 24h preincubation) that precluded ET-1 mediated MEK and ERK phosphorylation. As shown in Fig. 5B, a 3-min stimulation of quiescent astrocytes with ET-1 induced a clear increase in GTP-bound Ras, which was not affected by lithium pretreatment. Lithium also failed to prevent the robust activation of Ras in EGF-stimulated cells, in contrast to the total effectiveness of the EGF receptor kinase inhibitor tyrphostin AG1478. Taken together, these results indicate that lithium exerts its inhibitory effects on the astrocyte MEK-ERK pathway at a level downstream of Ras.

Figure 4. Chronic lithium treatment prevents ET-1-stimulated phosphorylation of ERK 1/2. Secondary astrocytes were serum-starved for 24h in DMEM, and then incubated in the same medium for 1 week in the presence or absence of the indicated LiCl concentrations. Upon stimulation with 25 nM ET-1 for 3 h, the cells were lysed and processed for immunoblot analysis. 10 µg of protein were loaded on each lane of a SDS-PAGE and immunoblotted for phospho-ERK and total ERK. The lower panel shows a densitometric analysis of phospho-ERK.
GSK-3 inhibition does not account for lithium effects on MEK-ERK and cell cycle.

To gain insight into the pharmacological target underlying lithium effects, we tested whether GSK-3 inhibition in astrocytes could uncouple the MEK-ERK pathway. For this purpose, we took advantage of the recently developed compound SB216763, which has been shown to inhibit GSK-3 and protect neurons from apoptosis induced by inhibition of phosphatidylinositol 3-kinase (Cross et al., 2001). As shown in Fig. 6A, pretreatment of proliferating, asynchronous astrocytes with 10 mM lithium for 3 hours resulted in a reduction in phospho-ERK and phospho-MEK levels, while a 3-hour exposure to 3µM SB216763 not only did not reduce ERK and MEK phosphorylation, but actually increased it slightly. Further, SB216763 also failed to reproduce the delay induced by lithium on the cell cycle re-entry stimulated by ET-1 (Table 1). Nevertheless, treatment of astrocytes with SB216763 induced cytosolic β-catenin stabilization and completely prevented Tau
phosphorylation in Ser$^{396}$ (Fig. 6B), which correlates with the effective inhibition of GSK-3β (Hart et al., 1998; Ikeda et al., 1998; Torreilles et al., 2000).

**Figure 6. Lithium effects on ERK signaling are not mediated by GSK-3 inhibition.**

A) Asynchronous astrocytes were left untreated (CNL), or treated for 3 hours with 10 mM LiCl, 3 µM SB216763, or with the MEK inhibitors U0126 (20µM) or PD98059 (50µM). 10µg of protein were loaded on each lane of a SDS-PAGE and immunoblotted for phosphoERK, phosphoMEK and the corresponding total proteins. The immunoblots shown are representative of three independent experiments.

B) Asynchronous astrocytes were left untreated or treated for 3 hours with 3µM SB216763. Cells were hipotonically lysed and the levels of cytosolic β-catenin were determined, or alternatively, cells were lysed in a 0.1% Triton X-100 containing lysis buffer to determine phosphorylation of Tau on Ser396 using a phospho-specific antibody. An antibody against β-tubulin was used in the same Western blot as a loading control. This experiment was performed three times with identical results.

**DISCUSSION**

The present study shows that lithium exerts different effects on the MEK-ERK pathway in cerebellar granule neurons and in cortical astrocytes. This is the first report showing lithium stimulation of MEK and ERK phosphorylation in neurons, although a similar effect has been found in SH-SY5Y neuroblastoma cells treated with valproate (Yuan et al., 2001). In that case, stimulatory effects of valproate were shown to take place well upstream of the pathway, as the drug was ineffective in dominant negative Raf and Ras mutants. We are currently investigating whether lithium stimulatory effects in the pathway are present at the Ras and/or Raf levels. It is well known that both lithium and valproate stimulate AP-1 binding activities (Ozaki and Chuang, 1997; Asghari et al., 1998; Chen et al., 1999b). Thus,
as ERK is involved in the activation of AP-1 (Frost et al., 1994), stimulation of neuronal ERK could contribute substantially to the modulation of gene expression and plasticity by antidepressant drugs. Regarding the pharmacological target of lithium, we show that SB216763, a selective inhibitor of GSK-3 (Cross et al., 2001), fails to stimulate neuronal MEK or ERK phosphorylation. Further, we have observed that inositol supplementation of the culture medium does not revert lithium effects in neuronal ERK. Although inositol supplementation may seem a rather indirect way to test for lithium actions mediated by inositol phosphatase inhibition, it is fully effective to revert lithium-dependent, agonist-stimulated accumulation of CDP-diacylglycerol (Godfrey, 1989; Claro et al., 1993; Centeno et al., 1998), and its use is justified by the absence of selective inositol monophosphatase inhibitors other than the poorly permeable L-690,330 (Atack et al., 1993). It is important to note, however, that inositol depletion may not be the only effect of inositol phosphatase inhibition: inositol polyphosphate 4-phosphatase has been shown to be 120 to 900 fold more effective to hydrolyze phosphatidylinositol 3,4-bisphosphate (PtdIns3,4P2) than inositol polyphosphates (Norris and Majerus, 1994), and therefore it is conceivable that lithium inhibition of this enzyme could induce a buildup of PtdIns3,4P2, promoting the activation of Akt (Nystuen et al., 2001). This would in turn act synergistically with lithium inhibition of GSK-3 to mimic WNT signaling, and could contribute to the stimulatory effects of lithium on axon and growth cone remodeling (Hall et al., 2000). Such action on inositol polyphosphate 4-phosphatase could explain in part the reported increases in Akt phosphorylation in neurons treated with lithium (Chalecka-Franaszek and Chuang, 1999; Mora et al., 2001). In our hands, however, we see that lithium potentiation of MEK and ERK phosphorylation in neurons is not accompanied by an increase in phospho-Akt, in agreement with De Sarno et al. (2002). Taken together, our observations suggest that lithium stimulation of neuronal MEK-ERK is mediated by targets other than GSK-3β and inositol monophosphatases.

Lithium effects on the astrocyte MEK-ERK pathway were opposite to those in neurons, leading ultimately to the inhibition of cell cycle progression. Interestingly, lithium was effective to rapidly downregulate this mitogenic cascade in asynchronous, proliferating astrocytes, and also to block its activation by a mitogenic stimulus. Similar to what we
found in neurons, lithium effects were neither reproduced by a selective inhibition of GSK-3 with SB216763, nor they were abrogated by exogenous addition of inositol. Again, taking into account that lithium did not increase Akt phosphorylation, our results argue against the involvement of GSK-3 or inositol monophosphatases on the inhibitory effects of lithium in the astrocyte MEK-ERK pathway. In contrast with the situation in SH-SY5Y neuroblastoma cells, where valproate stimulatory effects were already patent at the Ras level (Yuan et al., 2001), lithium inhibitory effects in astrocytes were clear regarding MEK phosphorylation, but Ras activation by either EGF or endothelin-1 remained unaffected. This points at Raf as the putative primarily affected component of the astrocyte pathway, and suggests that these lithium effects in astrocytes take place by a mechanism that is not simply the opposite to that occurring in neurons.

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**TABLE 1: Lithium, but not the GSK-3β inhibitor SB216763, delays cell cycle re-entry.**
Astrocytes deprived of serum for 3 days were treated with 10 mM LiCl or 3 μM SB216763 for 24 h. Then, cell cycle re-entry in synchronized astrocytes was initiated by addition of 10% FCS or 25 nM endothelin-1, and phase distribution was analyzed at different times after stimulation. Results are means of 2 independent experiments, and represent percent occurrence of cells in G0/G1 (diploid), G2/M (tetraploid), or S.
Although lithium inhibition of the MEK-ERK pathway has not been reported before, lithium has been shown to induce cell cycle arrest in bovine aortic endothelial cells by a mechanism involving stabilization of p53 (42). This antitumor transcription factor is stabilized by different mechanisms, including the Akt-GSK-3-β-catenin cascade. However, this mechanism does not seem to account for our results, as the GSK-3 inhibitor SB216763 failed to arrest the astrocyte cell cycle, while effectively stabilized β-catenin and inhibited Tau phosphorylation. However, p53 may be involved in lithium-induced astrocyte cell cycle arrest: it has been reported that ERK inhibition contributes with the activation of p38 MAP kinase in the stabilization of p53 induced by nitric oxide (Kim et al., 2002), and in fact lithium may stimulate p38 (Nemeth et al., 2001). We have preliminary evidence that lithium-induced inhibition of DNA synthesis in astrocytes is partly reversed by the p38 inhibitor SB-203580, suggesting that both ERK inhibition and p38 activation could be contributing to cell cycle arrest.

It would be worth testing whether lithium, a drug able to favor neuronal survival and plasticity and, at the same time, inhibit astrocyte proliferation, would be effective in the treatment of CNS traumatic injuries: these result in a rapid proliferative response from resident astrocytes, a process known as reactive astrogliosis or glial scarring, that has been suggested to be an attempt made by the CNS to restore homeostasis through isolation of the damaged region (Fitch and Silver, 1997). Upon CNS damage involving destruction of the brain-blood barrier, endothelium-derived ET-1 and plasma components intrude into parenchyma and initiate astrogliosis. The subsequent formation of a glial scar may interfere with any subsequent neural repair (McGraw et al., 2001). Current experimental approaches to attenuate gliotic reaction, however, do not constitute viable therapeutic interventions. In this context, lithium therapy could contribute to axonal regeneration after its own neuroplastic action and the inhibition of reactive astrogliosis.
ACKNOWLEDGMENTS
R.P. and B.R. are recipients of pre-doctoral fellowships from Universitat Autònoma de Barcelona and the Spanish Ministry of Science and Technology (SMST), respectively. This work has been supported by Grant BFI 2001-2017 from the SMST. We appreciate the generous gift of compound SB216763 by GlaxoSmithKline, Stevenage, Herts (U.K.), the help of Dr. Joan-Marc Servitja with setting up the assay of GTP-bound Ras, and the excellent technical assistance of Cristina Gutíérrez with cell culture and Manuela Costa with the FACS facility.

REFERENCES
Results: lithium actions on ERK pathway in neural cells


Results: lithium actions on ERK pathway in neural cells


Part I. Role of PLD in cellular proliferation and membrane ruffling.

Is PLD involved in signal transduction?

PtdCho is the main lipid constituent of eukaryotic membranes, accounting for 40-70% of total membrane phospholipids. Its turnover is mostly controlled by PLD, which catalyses its cleavage leading to the production of choline and PtdOH, a lipid that plays a central role in the synthesis of the major membrane phospholipids. PLD activation takes place within seconds in response to an incredibly rich variety of extracellular signals in different cell types (see table 2 on page 20). This observation suggested that PLD activation, well apart from its housekeeping functions, may be part of the complex signal transduction machinery that allow the cell to adapt and to respond to a changing environment. The requirement for PLD in several cellular events, such as vesicular trafficking, exocytosis or cell proliferation, has been proposed and studied in the last ten years. The methodologies used in studies on PLD cellular functions include the use of alcohols, that act by diverting the production PtdOH, antisense oligonucleotides or RNAi approaches to reduce its endogenous expression, or overexpression of wild-type and catalytically inactive forms. Unfortunately, the use of alcohols remains as a frequently used approach to unmask the putative role of PLD in a particular cellular event, and it is forced by the lack of more specific inhibitors. This strategy is rather contentious, since alcohols, besides being PLD substrates, are pleiotropic compounds with marked actions on different metabolic pathways. The appropriate concentrations should be the optimal for the transphosphatidylation reaction, an that appears not always to be the case for some studies on PLD actions.

A) Role of PLD in cellular proliferation

Many reports have been published providing evidences for a role of PLD in cellular proliferation, and for some of those papers the postulated implication of PLD is via production of lysophosphatidic acid, a lipid messenger with well known proliferating actions on different cell types [94]. Raf-1 can also provide a link between PLD
activation and activation of the MAPK pathway, since PtdOH greatly enhances Raf-1 kinase activity in vitro [9, 99]. The specific interaction site for PtdOH was mapped to a domain rich in basic and hydrophobic amino acids (residues 389-423) and is suggested to facilitate and stabilise the translocation of Raf-1 to the membrane [9, 98]. Recently mTOR has been added to the group of proteins that are controlled by PtdOH that could provide a new link connecting PLD activation to mitogenic signal transduction. mTOR senses amino acid presence in the medium and mediates nutrient-dependent cell growth by controlling mRNA translation initiation [100].

We adressed the study of PLD in transduction of mitogenic signaling in cultured astrocytes. As early mentioned, astrocyte proliferation is a naturally occurring phenomenon in response to certain pathological situations such as traumatic injury to the central nervous system. Cultured astrocytes display a wide variety of receptors coupled to PLD activation and exhibit a robust proliferative response following mitogen exposure. We took advantage of this and determined the ability of a set of different stimuli (including growth factors, other mitogens and non-mitogenic agonists) to induce DNA synthesis and to stimulate PLD activity. The aim of this strategy was to determine if correlation between PLD activation and mitogenesis did occur. We found a lack of correlation between both responses, supporting the idea of mitogenesis and PLD activation being independent phenomena. This lack of correlation has been observed by others [118]. We also made use of alcohols to divert the PtdOH formation elicited by mitogens. The protocol was designed taking special care on the side-effects associated to the use of alcohols. We therefore allowed just short alcohol incubations, since for most of the cases short time mitogen stimulation was sufficient to elicit a full response. This strategy proved to be devoid of cytotoxicity when leakage of cytosolic markers or viable dye staining were analysed. Regarding thymidine incorporation into DNA, primary alcohol treatment along with mitogens gave no significant differences when compared to non-PLD substrate alcohols, supporting again the non requirement of PtdOH formation in mitogenic signaling.

We assume that these findings are not conclusive by themselves, and that additonal data are needed to clearly establish the absence of any link in the cell system studied (reduction of endogenous expression of PLD isoforms, overexpression of catalytically
inactive forms of PLDs, overexpression of mutant ARFs defective in PLD activation). In particular, we have addressed without success an antisense approach to reduce the expression of PLD in a cell line. The results presented are restricted to the astrocyte model, and should not be extended to other cell types. Is is possible that for particular cell types with strong PtdOH deacylation activity to yield lysoPtdOH, a positive link between PLD activation and proliferation could occur. The mechanism of deactivation of PtdOH could be cell-type specific. Therefore, the existence of a relationship between proliferation and PLD activation could have more to do with the specific pathways employed by each cell type to metabolise PLD-derived PtdOH than simply to the activation of PLD. We should also bear in mind that alcohol inhibition of PtdOH formation is never complete. Taking into consideration that amplification mechanisms between PLD activation and DNA synthesis may exist, the remaining PtdOH produced might be enough for the transduction of mitogenic signals. A role of PLD in sustained PKC activation was regarded as a possible mechanism underlying the potential mitogenic actions of PLD. Recently this hypothesis lost support, as PLD-derived DAG is rich in alkyl linkages, which are unable to activate classical or new PKC isoforms [9].

**B) Role of PLD in membrane ruffling.**

We addressed the study of other cellular functions where PLD could be involved, in particular the membrane ruffling, which, for some cell types (e.g. mast cells and related cell lines), accompanies the fusion of secretory vesicles with the plasma membrane. The results presented were developed in collaboration with a foreign lab providing extensive facilities and background knowledge on mast cell physiology. Mast cells and their related cell lines are good models to study secretory processes and show extensive ruffling upon Fc receptor crosslinking [182]. When analysed, not only ruffling, but also PtdIns(4,5)P$_2$ synthesis were profoundly affected following butan-1-ol exposure, while they remained unaffected when butan-2-ol was used. The observations that **A)** time-courses of PLD activation and ruffling coincided in time following antigen stimulation and **B)** that ruffling resumed after butan-1-ol removal, led us to the idea that continuous PLD activation was required for membrane ruffling to take place. Studies on the
subcellular localisation of tagged-PLD isoforms identified PLD2 as the potential isozyme involved. We demonstrated that both isoforms, PLD1 and PLD2, can be regulated by antigen stimulation, but while PLD2 localised mainly to the plasma membrane, PLD1 was found in an intracellular vesicular compartment and did not change dramatically after antigen addition. This pattern of PLD1 and PLD2 localisation has recently been described by others [42]. We do not exclude a role of PLD1 in the process, since some works provide evidence of PLD1b colocalisation with markers of secretory granules and plasma membrane recruitment following stimulation [44, 183], something that could not be reproduced in our hands.

We report that ARF6 is able to reconstitute PtdIns(4,5)P$_2$ synthesis from cytosol-depleted cells challenged with antigen. PLD-generated PtdOH production seems to be required, as butan-1-ol, but not butan-2-ol, blocked PtdIns(4,5)P$_2$ synthesis stimulated with antigen plus ARF6. Since from all the ARF isoforms, ARF6 is the only one reported to localise to the plasma membrane upon stimulation [66, 82], we propose that both ARF6 and PLD2 are required for the synthesis of PtdIns(4,5)P$_2$ at the plasma membrane. When assayed in streptolysin-O-permeabilised cells, ARF1 but not ARF6 was found to leak out of the cell, presumably because of its association with intracellular structures. Endogenous ARF6 has been found at sites of intense cortical actin rearrangement and has been involved in cell motility events [184, 185]. Furthermore, ARF GTPase cycle is reported to control an endosome/plasma membrane recycling pathway [109]. This plasma membrane recycling pathway is active during ruffling. Interestingly, we have observed PLD2-containing endocytic structures in PLD2-transfected cells undergoing ruffling.

These results prompted us to propose a model for membrane ruffling. As already mentioned in the introduction, membrane ruffling requires from two independent events: reorganisation of the cortical actin plus an active membrane recycling pathway. Both GTP-ARF6 and GTP-Rac1 are plasma membrane localised proteins that have been involved in actin rearrangements [109] and both can mediate antigen stimulation of PLD in RBL cells [183]. We consider that the plasma membrane is a site for recruitment of proteins required for actin dynamics, since the tips of growing cortical actin filaments are just a few nm away from the inner surface of the plasma membrane. We propose that
GTP-ARF6 (and possibly GTP-Rac1) would guarantee PLD2 activation at the plasma membrane. The formed PtdOH would contribute to ARF6-mediated PIP 5-kinase activation, and the subsequent PtdIns(4,5)P2 formation would contribute to a second step of PLD2 activation, as a cooperative loop. PtdIns(4,5)P2 would also facilitate recruitment of actin-binding PH domain-containing proteins (gelsolin, profilin, ARNO, ASAP members of ARF-GAPs), therefore contributing to the cortical actin remodelling. In addition, an ARF6 cycle along with PIP5-kinase and PLD2 might account for the membrane recycling processes. To conclude, the concomitant operation of a Rac cycle, an ARF cycle, and activation enzymes that modify plasma membrane composition (PLD and PIP 5-kinase) might be required for membrane ruffling to take place and inhibition of any of them would halt ruffling. This conclusions support the observation that PLD2-overexpressing cells, while displaying high basal activity and a four-fold increase in PtdOH levels, are still dependent on antigen stimulation to undergo ruffling.

Interestingly, we show that ruffling and exocytosis, while a prominent feature elicited by engagement of IgE receptors on RBL-2H3 cells, are not always coupled phenomena, since phorbol ester stimulation could induce ruffling and lamellipodial extensions but not secretion.

As long as I am aware, this is the first time a study includes measures of PtdOH and PtdBut formation in cells transfected with PLD isoforms. As expected, we found that transfected cells upregulate mechanisms to compensate the enhanced PLD activity by increasing the rate of PtdOH removal. This explains why no further increase in PtdOH levels is observed in transfected cells after antigen stimulation, while a clear increase in PtdBut formation is still observed.

C) Oleic acid activation of PLD2 but not PLD1.

Another finding from our studies is the confirmation that oleic acid is a selective activator of PLD2. Oleic acid, when used at millimolar concentrations, greatly enhanced PLD activity from PLD2- but not PLD1-overexpressing cells and these results were reproduced in membrane preparations from PLD1 and PLD2 overexpressing cells.
Furthermore, the oleic acid stimulation of PLD in membranes from various cell types showed correlation with PLD2 immunoreactivity levels when examined. We took advantage from this property to study whether the overexpressed PLD2 colocalised with the endogenous enzyme. Membrane fractionation of homogenates from PLD2-overexpressing cells demonstrated that overexpressed PLD2 fractionated in an identical location to the endogenous PLD2, which was found to overlap with plasma membrane markers. These results support that overexpression does not lead to mislocalisation of the enzyme. Taken together, we can conclude that oleic acid is a useful tool to localise PLD2 and to identify whether a particular cell type has PLD2. This is specially relevant in a situation where the availability of antibodies is restricted and detection of endogenous forms is difficult because of low levels of expression. We observed that the concentration of oleic acid required for optimal PLD2 activation is critical and clearly depends on protein concentration. This fact could explain the remaining controversy regarding the identity of the oleate-activated PLD, since some researchers reported inhibition of PLD2 [13] while others have reported stimulation [186]. From our studies, it is clear that oleic acid should be carefully titrated, taking into consideration the protein concentration used in the assay. We assume that the oleate sensitive PLD is none other than PLD2. Unfortunately, the use of oleic acid on living cells is restricted due to cytotoxicity. We found that oleic acid could stimulate short-lived ruffling on its own, but clear cytotoxic effects could be observed soon after addition. The optimal concentration of oleic acid for stimulation of PLD2 in living cells is in the millimolar range when added exogenously. On a membrane preparation, the optimal concentration is reduced to 10-50 µM, depending on protein concentration. We consider that oleic acid (or other unsaturated fatty acids such as arachidonic acid), if released intracellularly by a PLA\textsubscript{2} activity, could be physiologically relevant in the modulation of PLD2.

**Part II. Modulation of the ERK pathway in neural cells by lithium.**

Having observed a lack of correlation between cell mitogenesis and PLD activation, and a positive link regarding membrane ruffling, we continued the study of the
involvement of signaling lipids on the molecular mechanisms regulating astrocyte proliferation. Since lithium impairs the phosphoinositide cycle, we used this agent with the initial aim of exploring a possible role of PLC-elicited signals. We found that lithium effectively prevented thymidine incorporation into DNA in asynchronous astrocytes. A 3-day incubation with 10 mM lithium completely abolished DNA synthesis. Results obtained from flow cytometric analysis were consistent with a G2/M cell cycle arrest, since a build up in tetraploid cells was observed. Furthermore, we observed that lithium delays mitogen-elicited cell cycle re-entry in serum deprived astrocytes. Astrocytes can become quiescent (G0 phase) by removing the serum from the culture medium for 3 days, and can then be forced to re-enter cell cycle by adding back serum. If cycle phase distribution is analysed in those synchronised cells 24 hours after serum addition, a typical 20% of the cells reach the S phase and a 22% reach the G2/M phase. Lithium-pretreatment of the cells results in a delay in their progress through cell cycle, since virtually no cells can be observed in the G2/M phase. Those effects of lithium on cell cycle showed dose-dependency and lacked cytotoxicity when measured by viable-dye staining. Also, flow cytometry revealed no signs of apoptosis associated to lithium incubations (no significant population of subdiploid cells, evidencing DNA degradation, could be observed).

We explored the molecular mechanisms underlying lithium effects on astrocyte cell cycle. To date, IMPs are widely accepted pharmacological targets of lithium ions that may mediate its clinical and cytoprotective properties [134, 142]. However, inhibition of IMPases could not account for lithium effects on the cell cycle, since they were not reverted after inositol supplementation of the culture medium. GSK-3 is also regarded as a molecular target mediating lithium actions with implications on cell cycle control [170, 187]. One of the jobs of GSK-3 is to target the β-catenin protein to ubiquitin-mediated degradation. Cancer causing mutations enable β-catenin to accumulate, which leads to increased transcription of TCF/LEF genes and cell cycle deregulation [167, 168]. This could be a possible mechanism underlying the effects of lithium on astrocyte proliferation. However, from flow cytometric analysis of cell cycle phase distribution, the selective inhibition GSK-3 by SB216763 could account for lithium-induced cell
cycle arrest. These results were validated by showing that SB216763 affected downstream targets of GSK-3. In particular, SB216763 completely prevented the phosphorylation of Tau protein and effectively stabilised β-catenin.

The search for the mechanisms underlying the observed cell-cycle arrest ended up with the finding that lithium prevented signalling through the canonical ERK pathway. Reduced phosphorylation on ERK and MEK proteins was observed in lithium-treated quiescent astrocytes challenged with mitogens (endothelin-1 or serum). The shut down of the pathway upon challenge with those mitogens was already seen after short incubation times (3h) with high lithium concentrations (10mM) or alternatively after long incubations (1 week) with therapeutic lithium concentrations (1mM).

Being clarified that lithium effects were not mediated by its most commonly recognised targets, we analysed further in detail lithium actions on upstream components of the ERK pathway, in particular its effects on Ras activation. We showed that lithium could not preclude activation of Ras in conditions that completely abolished ERK/MEK phosphorylation of quiescent astrocytes challenged with ET-1, indicating that lithium effects might take place downstream of Ras activation, namely at the Raf level. We are now currently investigating whether the kinase activity of Raf isoforms is sensitive to lithium and its expression levels in lithium-pretreated cells. Depending on the results of these experiments, the search can continue with modulators of Raf, as Rap1.

Our study has been completed showing the dual effects of lithium on ERK pathway in two different neural cell types, cerebellar granule neurons and astrocytes. In the former, ERKs activation is reported to confer neuroprotection against different kinds of insults (ischemia [188], C2-ceramide [189], anticonvulsants [190], β-amyloid peptides [191], non-depolarising conditions [192], glutamate [193]) and are also involved in synaptic plasticity events [194, 195]. In the latter, ERKs are mainly involved in the transduction of mitogenic signals following growth factor receptor stimulation. Glial proliferation is a physiologically relevant situation observed in vivo upon central nervous system damage. Traumatic injuries to the central nervous system typically result in a robust proliferative response from resident astrocytes, a process known as reactive astrogliosis or glial scarring, which can interfere with neural repair and axonal
regeneration [196]. The expression of extracellular matrix molecules or soluble mediators by reactive astroglia might account for the lack of neuronal regeneration [197]. Current experimental approaches to attenuate the gliotic reaction constitute no viable therapeutic interventions. In that sense, lithium could prove to be a valuable drug since it would contribute to axonal regeneration by preventing neuronal cell death and at the same time, it would attenuate astrocyte proliferation. We plan to test this hypothesis in vivo, in animals with traumatic spinal cord or central nervous induced lesions. In one group of animals lithium will be included in the regular diet. The extension of the damaged lesion and morphological features of the glial scar will be compared to control animals. Hopefully, we will be able to corroborate whether what is seen in the petri dish does take place in vivo.
GENERAL CONCLUSIONS
1. **PLD activation is not a general requirement for agonist-elicited cell proliferation** of astrocytes. This conclusion is supported by two findings. First, a lack of correlation between PLD activation and enhancement of DNA synthesis is observed for variety of stimuli including growth factors, G protein-coupled receptor agonists and the tumour promoter PMA. Second, short chain primary alcohol exposure under non cytotoxic conditions, while diverting up to 60% PLD-catalysed formation of PtdOH, did not reduce mitogen-stimulated thymidine incorporation into DNA.

2. **Long term alcohol exposure affects viability of astrocytic cultures.** Therefore, these methodologies should not be used when studying the putative role of PLD in a particular cellular function.

3. **Continual production of PtdOH by PLD is required for antigen-stimulated membrane ruffling in RBL mast cells.** Butan-1-ol but not butan-2-ol reversibly inhibited antigen-mediated membrane ruffling and it resumed soon after butan-1-ol removal. Time-course patterns of antigen mediated PLD activation and membrane ruffling are tightly coupled.

4. Antigen can stimulate both isoforms of PLD, but only PLD2 is found in membrane ruffles of RBL mast cells upon antigen stimulation. Overexpressed GFP-PLD1 localises to an intracellular vesicular compartment and does not translocate following stimulation. **We therefore assume that PLD2 is the isoform involved in membrane ruffling.**

5. **PLD-catalysed PtdOH formation is not sufficient to drive the dynamic actin cytoskeletal rearrangements and membrane recycling events characteristic of ruffling.** PLD2 transfected cells, despite displaying four-time higher basal levels of PtdOH when compared to mock transfected cells, were still dependent on antigen stimulation to undergo ruffling. Antigen-mediated activation of other pathways, namely Rac1 GTPase, must then be required.
6. **PLD-derived PtdOH is required for PtdIns(4,5)P₂ formation in antigen-stimulated RBL mast cells.** In cytosol-depleted RBL cells, which had lost the capacity to synthesise PtdIns(4,5)P₂ after stimulation with antigen, the addition of ARF1 or ARF6 recombinant proteins could restore the ability to synthesise PtdIns(4,5)P₂, but it did not occur in presence of butan-1-ol.

7. **Oleic acid is a selective activator of PLD2 but not PLD1.** Optimal concentrations are dependent on protein concentration in the *in vitro* assay and lie within the micromolar range. *In vivo*, optimal concentrations lie in the millimolar range, probably because oleic acid has to gain access to the inner leaflet of the plasma membrane, where PLD2 is located. This property can be used to detect the presence of PLD2 in subcellular preparations but its use is limited *in vivo* because of cytotoxicity.

8. **Lithium inhibits DNA synthesis and induces a G2/M cell cycle arrest in asynchronous astrocytes.** In quiscent astrocytes, lithium prevented ET-1-elicited cell cycle re-entry.

9. **Chronic or acute lithium treatments prevented ERK and MEK phosphorylations in both asynchronous and quiscent astrocytes challenged with ET-1.** The blockade occurs downstream of Ras, since lithium did not affect ET-1 or EGF-stimulated Ras activation. This effect was cell-type dependent, as demonstrated by the fact that lithium enhanced signaling through the ERK pathway in cerebelar granule cells.


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(AGRADECIMIENTOS)
AGRADECIMIENTOS

En la cuestión de agradecer me pasaré al idioma materno, ya le he hecho bastantes concesiones al inglés en esta tesis. Toda tesis es como la gestación de un bebé pero que en lugar de durar nueve meses, se prolonga durante cuatro años o más, con el agravante que los dolores del parto se extienden unos tres meses, el tiempo que se tarda en darle contenido a la susodicha. Durante todo ese tiempo muchas personas pasan por tu vida, hay muchas cosas que agradecer, así que intentaré no dejarme a nadie, aunque probablemente lo haga.

Empezaré por Fernando, una persona que me permitió dar a luz a este mamotreto. No lo fecundó, porque la semillita que te impulsa a lanzarte al mundo de la ciencia es anterior, y en mi caso al menos no sé de donde procede. En cualquier caso Pica me introdujo en el mundillo científico y aprendí muchas cosas estando a su lado. Surgieron diferencias, pero espero que no guarde un mal recuerdo de mi paso por su laboratorio. Este agradecimiento es del todo sincero y no forzado por la situación, que quede bien claro.

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Publications:


- Pardo R., Ramos B., Picatoste F., Claro E. Lithium inhibits the MEK/ERK pathway in astrocytes by a mechanism independent of GSK-3 and inositol depletion. Submitted for publication.

- Pardo R., Servitja J.M., Masgrau P, Sarri E., Picatoste F. Phospholipase D activation is not a general requirement for agonist-elicited proliferation in cultured astrocytes. Submitted for publication.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis gene product</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine trisphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>DAG</td>
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<tr>
<td>EGF</td>
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<td>T-cell factor</td>
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<td>TLC</td>
<td>Thin layer chromatography</td>
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Front cover.

**Top left:** Mid-plane confocal section of endogenous ERKs in astrocytes in culture. A 60x Leica immersion oil objective was used.

**Top right:** Single RBL-2H3 cell transfected with GFP-PLD2 undergoing membrane ruffling upon PMA stimulation.

**Bottom:** Bright field image of RBL-2H3 cells by using Nomarsky optics. The cells were viewed in an Olympus microscope with a 100x immersion oil objective.