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ORIGINAL PAPER

# Lithium and Valproate Protect Hippocampal Slices Against ATP-induced Cell Death

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**Abstract** Lithium and valproate (VPA) are the most commonly prescribed mood-stabilizing drugs. Recently, several studies have reported their neuroprotective properties in several models of neural toxicity and, in some pathological conditions, large amounts of intracellular ATP can be released from damaged cells. In the present study, we investigate the potential neuroprotective effect of lithium and VPA against ATP-induced cell death in hippocampal slices of adult rats. Acute (*in vitro*) and chronic (*in vivo*) treatment at therapeutic doses with lithium or VPA significantly prevent the ATP-induced cell death. Lithium and VPA also exerted a synergic effect in the prevention of ATP-induced cell death. Moreover, hippocampal slices prepared from rats chronically treated with lithium or VPA presented a significant reduction in cell death in the presence of cytotoxic extracellular ATP. Although further investigations are necessary, our results show the neuroprotective effect of lithium and VPA against neuronal death induced by extracellular ATP, probably through a different pathway, and suggest novel uses of these drugs in neurogenerative diseases.

**Keywords** Lithium · Valproate · ATP · Cell death · Neuroprotection

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## Introduction

Lithium ion and valproate (VPA), a short-chain fatty acid, are commonly used mood-stabilizing drugs for bipolar disorder [1–3]. In addition, several studies have reported neuroprotective properties of mood stabilizers in several models of neural toxicity [4]; however, the biochemical basis of their therapeutic effect is poorly understood. A large number of studies, encompassing *in vitro*, *in vivo*, and clinical analyses have concluded that lithium is neuroprotective. Given the vastly different methodologies and parameters employed by these studies, the basic finding that lithium is neuroprotective is strongly supported [5]. In vitro studies have shown that lithium has neuroprotective effects in multiple cell lines, including cerebellar granule cells, cerebral cortical cells, hippocampal neurons [6], PC 12 cells [7], and neuroblastoma cells [8] from both humans and rodents. Furthermore, *in vitro* lithium treatment has successfully provided neuroprotection against a wide variety of insults, such as oubain [4, 9], anticonvulsants [6], potassium deprivation [10, 11], α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate [12], β-amyloid peptide [13, 14], β-bungarotoxin [15], and ischemia [16].

Sodium VPA has also been reported to have neuroprotective properties, including cerebral ischemia [2, 17, 18]. VPA may have a role in the treatment of excitotoxic states in the hippocampus. Chronic treatment with VPA led to a dose-dependent increase in hippocampal glutamate uptake capacity [19]. In addition, a close similarity exists between the molecular effects of lithium compared with VPA, both showing benefits in manic-depressive illness [19].

Adenine nucleotides and nucleosides represent an important class of extracellular molecules involved in the modulation of signaling pathways that are crucial for normal functioning of the nervous system [20]. Besides the

well established effects of ATP in the cell metabolism, there is evidence to show that extracellular ATP can stimulate mitogenesis and cellular proliferation or cause cytotoxic effects, depending on the cell type and the ATP concentration [21]. In some pathological conditions, large amounts of intracellular ATP can be released from damaged cells [22, 23], forming part of an important response mechanism to cell lysis, as is the case for astrocyte responses to injury in CNS. High concentrations of extracellular ATP can induce cell death in different cell types, including thymocytes, hepatocytes, microglial, and myeloid cells [24, 25].

Recently, Neary and Kang [26] reported that ATP, released upon traumatic injury, hypoxia and cell death contributes to the gliotic response by binding to specific cell surface astrocytic P2 nucleotide receptors. These authors showed that the ability of ATP to kill cells involves both necrosis and apoptosis by activation of $P2X_1$ and $P2X_7$ receptors. There is some evidence to indicate that the $P2X_7$ receptor can mediate cell death by lytic pore formation in cells (independently of $Ca^{+2}$) or by stimulating excessive $Ca^{+2}$ influx after prolonged activation of the receptor [22].

The events induced by extracellular adenine nucleotides are controlled by the action of ecto-enzymes, which play a central role in modulating the extracellular levels of these important signaling molecules [27]. We have recently shown that chronic treatment with lithium promoted an enhancement of ATP and AMP hydrolysis in synaptossomes from the hippocampus of rats, suggesting that the purinergic system may be involved in the potential neuroprotective lithium action in hippocampal lesions [28].

Since ATP is known to be released in the extracellular milieu of the CNS, both in physiological and pathological conditions and lithium and VPA have also been reported to have neuroprotective properties, the objective of present study was to investigate the potential neuroprotective effect of treatment lithium and VPA in a model of ATP-induced cell death in hippocampal slices of adult rats.

## Experimental procedures

### Preparation and incubation of slices

Male Wistar rats (2 months; weighing 220–260 g) were maintained under standard conditions (12 h light/dark, 22 ± 2°C). The rats had free access to food (standard lab rat chow) and water. Procedures for the care and use of animals were adopted according to the guide for the care and use of laboratory animals (National Research Council). Rats were killed by decapitation and the brain was isolated in a modified Krebs–Henseleit solution containing (in mM): 120.0 NaCl, 2.0 KCl, 3.0 $CaCl_2$, 25.0 Hepes, 1.2 $MgSO_4$, 1.18 $KH_2PO_4$, 11.0 glucose (pH 7.4). The hippocampi were quickly dissected out and transverse sections (400 μm) were prepared using a McIlwain tissue chopper. Hippocampal slices were placed into separate 24-well plates and maintained for 30 min in the same modified Krebs–Henseleit solution (incubation medium). In all experiments, the slices were incubated in a humidified atmosphere at 37°C.

### Acute *in vitro* treatment of slices with lithium or VPA

After 30 min of pre-incubation, as described above, the medium was replaced by incubation medium containing 1.0 mM lithium or 0.5 mM VPA (these concentrations represent therapeutic blood levels in patients treated with these drugs [29]) and then maintained for 30 min at 37°C. The control group was maintained in the same conditions (without lithium or VPA). After 30 min, the medium was replaced by an incubation medium containing 5.0 mM ATP (final concentration) and the slices were maintained at 37°C for 18 h. After this time, an aliquot of the incubation medium was withdrawn and the activity of lactate dehydrogenase (LDH) was determined.

### Chronic treatment of rats with lithium or VPA

#### *Lithium*

The rats were divided into two groups: one group received standard rat chow and the other group had lithium chloride (2.5 mg/g of chow) and NaCl (17 mg/g of chow) added to the food. This previously described treatment lasted for 4 weeks [30], the animals remained healthy and the serum lithium levels at the end of the period were determined by atomic absorption spectrometry. The serum lithium concentration was 1.18 ± 0.05 mEq/l (mean ± SEM, $n = 18$), which is in the range observed in lithium treated patients.

#### *Valproate*

A dose of 300 mg/kg of VPA [31] or saline, as a vehicle control, was injected intraperitoneally (i.p.) daily, or every 12 h for 14 days.

After chronic treatments with lithium or VPA the animals were killed by decapitation and hippocampal slices were prepared and incubated with ATP as described above.

### Cell viability

The degree of cellular damage was evaluated by measuring the activity of LDH (EC 1.1.1.27) [32]. Briefly, after different times of incubation with ATP, the incubation medium was collected and LDH activity was evaluated by a colorimetric method, according to the procedure of

Whitaker [33]. The results were expressed as the ratio of released activity to total activity obtained after freeze-thawing the slices (100% of cell death).

### Statistical analysis

All experiments were carried out at least 6—9 times in triplicate and data are expressed as mean ± SEM Data were analyzed by unpaired ANOVA followed by post hoc comparisons (Student–Newman–Keuls test) using an Instat software package (GraphPad Software, San Diego, CA, USA).

## Results

### Cytolytic effect of extracellular ATP

To establish the experimental conditions of ATP-induced cell death, the slices were treated with different concentrations of ATP (0.1, 0.5, 3.0, 5.0 mM) for 24 h. The presence of 0.1 or 0.5 mM of ATP did not cause a significant increase in the LDH released when compared with control slices, while a significant increase in LDH release was observed with 3.0 or 5.0 mM of ATP (data not shown). Since extensive cell death was also observed in control slices after this long period of incubation, the subsequent experiments were performed with 5.0 mM of ATP for 18 h of treatment.

Since the ATP added to the incubation medium may be degraded by slices to its metabolites [34], we evaluated the effect of 3.0 mM of ADP, AMP or Adenosine (Ado) on cell lysis by evaluating LDH release. Figure 1 shows that ADP, AMP, and Ado did not cause a significant increase in the LDH release when compared with control slices, confirming that the cell death observed in our experiments is the consequence of ATP and not its metabolites.

### Lithium and VPA prevent ATP-induced cell death

Initially, the abilities of lithium or VPA (acute in vitro treatment) to protect hippocampal slices against ATP-induced cell death were evaluated. As shown in Fig. 2a, slices pre-treated with 1.0 mM of lithium before the exposure to ATP demonstrated a significant reduction in LDH release (11 ± 5%), compared with slices treated only with 5.0 mM of ATP (57 ± 12%). Similarly, pre-treatment with 0.5 mM of VPA significantly reduced cell death to 36 ± 7% when compared with the respective control (67 ± 7%) (Fig. 2b).

Since the *in vitro* treatment with lithium or VPA significantly prevented ATP-induced cell death, we evaluated

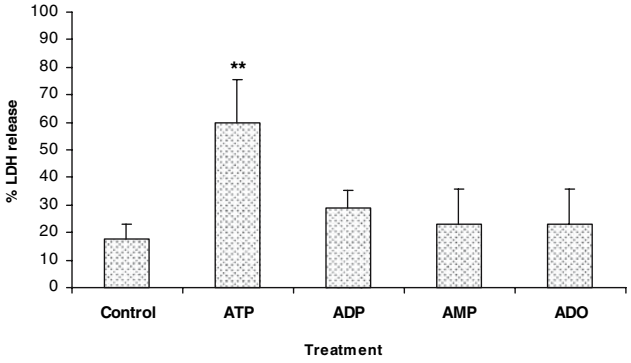


**Fig. 1** Effect of ATP metabolites on cell death. The slices were prepared as described in Materials and methods and treated with 5.0 mM of ATP, 3.0 mM of ADP, 3.0 mM of AMP or 3.0 mM of Adenosine (*ADO*). After 18 h, the incubation medium was collected and LDH activity was measured. Leakage of LDH is represented as a percentage of the total releasable enzyme. The data are the mean ± SEM of at least six experiments run in triplicate. **Significantly different from the control group for $P < 0.01$ (Student–Newman–Keuls test)

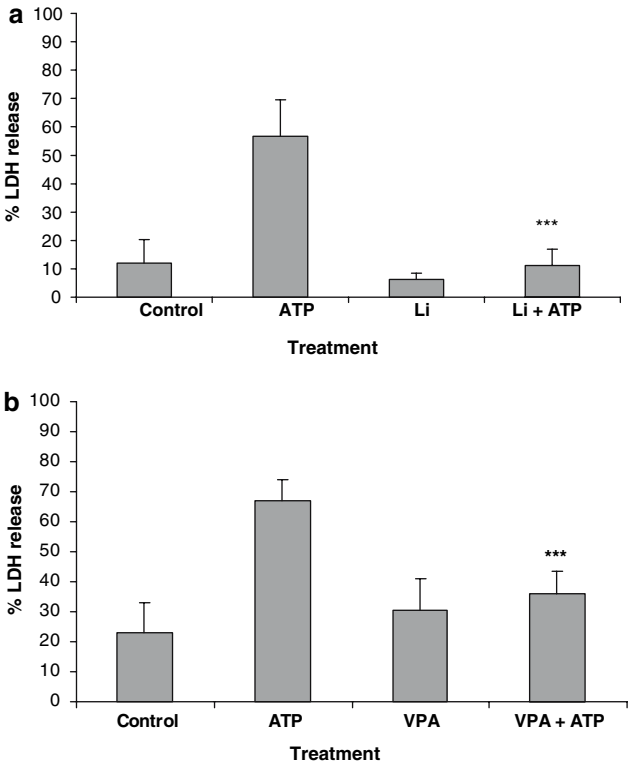


**Fig. 2** Effect of pre-treatment of hippocampal slices with lithium or valproate on the ATP-induced cell death. The slices were prepared as described in Materials and methods and pre-treated: (**a**) with 1.0 mM of lithium (*Li*) or (**b**) 0.5 mM of valproate (*VPA*) for 30 min before the treatment with 5.0 mM of ATP. After 18 h, the incubation medium was collected and LDH activity was measured. Leakage of LDH is represented as a percentage of the total releasable enzyme. The data are the mean ± SEM of at least six experiments run in triplicate. ***Significantly different from the respective ATP group for $P < 0.001$ (Student–Newman–Keuls test)

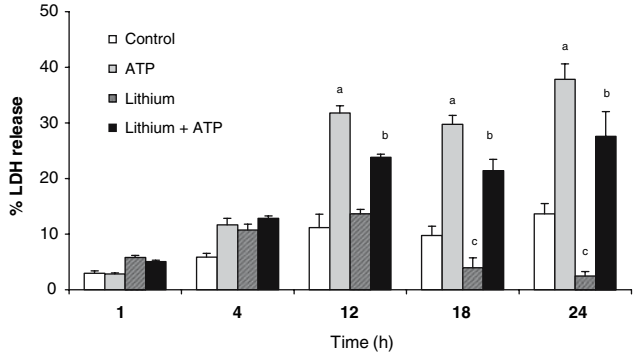


**Fig. 3** Effect of chronic treatment with lithium on ATP-induced cell death. Rats received standard rat chow (control group) or lithium chloride (2.5 mg/g of chow) plus NaCl (17 mg/g of chow) added to the food for 4 weeks (lithium group). Slices prepared as described in Materials and methods and, following 1, 4, 12, 18 or 24 h of treatment with 5.0 mM of ATP, the incubation medium was collected and the LDH activity was measured. Leakage of LDH is represented as a percentage of the total releasable enzyme. The data are the mean ± SEM of at least six experiments run in triplicate. The effect was statistically determined by Student–Newman–Keuls test. [a]Significantly different from the control group ($P < 0.001$); [b]Significantly different from the ATP group ($P < 0.05$); [c]Significantly different from the control group ($P < 0.05$)

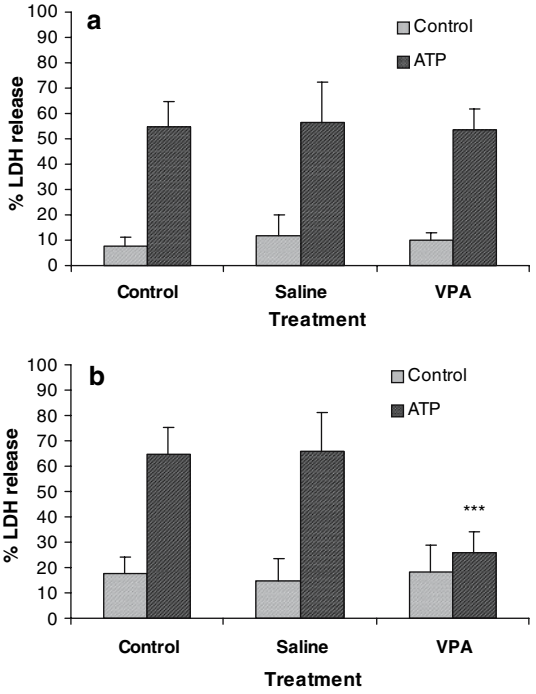


**Fig. 4** Effect of chronic treatment with valproate on ATP-induced cell death. Rats were treated with 300 mg/kg of valproate (*VPA*) or saline (vehicle control) every 24 h (**a**) or 12 h (**b**) for 14 days. The slices were prepared as described in Materials and methods and treated with 5.0 mM of ATP. After 18 h, the incubation medium was collected and the LDH activity was measured. Leakage of LDH is represented as a percentage of the total releasable enzyme. The data are the mean ± SEM of at least six experiments run in triplicate. [***]Significantly different from the slices treated with ATP in the control group and saline group, for $P < 0.001$ (Student–Newman–Keuls test)

whether chronic treatment of hippocampal slices of rats with lithium or VPA (*in vivo* treatment) demonstrated the same protection against the cell death induced by extracellular ATP. Figure 3 shows that hippocampal slices prepared from rats chronically treated with lithium presented a significant reduction in cell death in the presence of cytotoxic extracellular ATP. This protective effect was similar after 18 and 24 h of treatment with ATP. It is important to note that slices from rats chronically treated with lithium, but not exposed to ATP, demonstrated a significant reduction in LDH release when compared with the respective controls, suggesting that lithium is also able to prevent the basal cell death that occurs during slice preparation (Fig. 3).

To investigate the effects of chronic treatment with VPA on ATP-induced cell death, rats received a dose of 300 mg/kg of VPA or saline (vehicle control) i.p. daily [31] (Fig. 4a) or every 12 h (Fig. 4b) for 14 days. Results show that chronic treatment with 300 mg/kg of VPA every 12 h was able to prevent, significantly, ATP-induced cell death (Fig. 4b), reducing LDH release from 65 ± 10 to 26 ± 7%. Conversely, this effect was not observed when rats were injected with just one dose of VPA *per* day (Fig. 4a) for the same period of treatment (14 days).

## *In vitro* lithium and VPA treatment prevents BzATP-induced cell death

In order to investigate the involvement of the $P2X_7$ receptor in ATP-induced cell death, hippocampal slices were treated with BzATP, a specific agonist of $P2X_7$ for 1, 4, 12, 18 or 24 h (Fig. 5a). The treatment of slices with 0.1 mM BzATP caused a significant increase in the percentage of the LDH released after 4, 12, 18, and 24 h, when compared to the respective controls. This effect is significantly reduced by the simultaneous presence of 500 nM Brilliant Blue G, a specific P2X7 antagonist [35] (Fig 5b). Subsequently, the possible neuroprotective effect of lithium and VPA on BzATP-induced cell death was investigated. As observed in Fig. 5c, slices pre-treated with 1.0 mM of lithium or 0.5 mM of VPA for 30 min showed a significant reduction in LDH release (22 ± 13 and 26 ± 14%, respectively) when compared with slices treated with 0.1 mM BzATP (60 ± 11%).

## Synergic effect of lithium and VPA

Since the exact mechanism of lithium and VPA action remains unknown, we evaluated the effect of simultaneous *in vitro* pre-treatment with lithium and VPA on ATP-induced cell death. As shown in Fig. 6, when the slices were

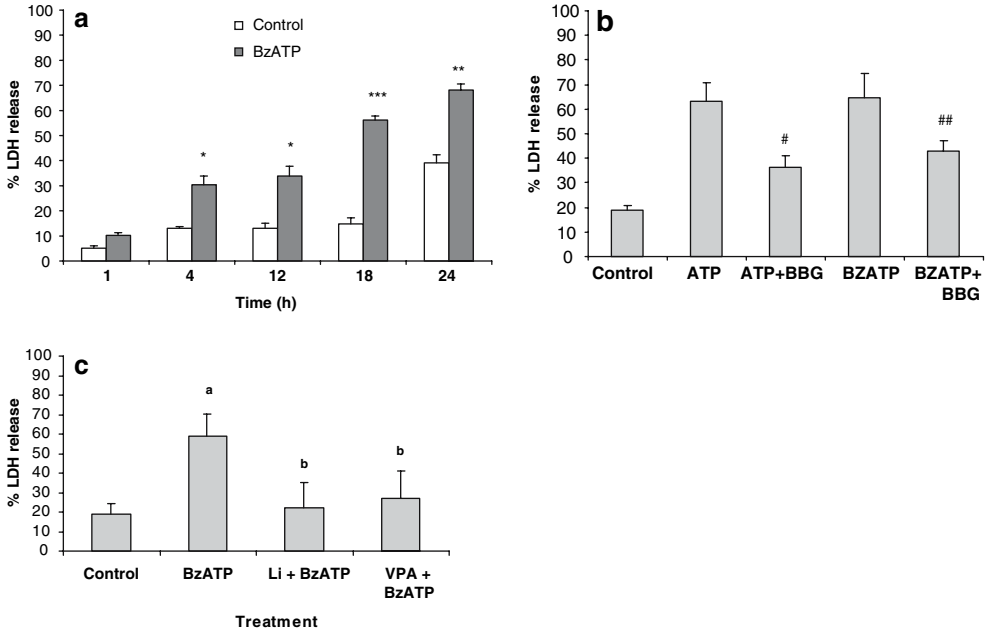


**Fig. 5** Effect of pre-treatment with lithium or valproate on BzATP-induced cell death. (**a**) The slices were prepared as described in Materials and methods and treated with 0.1 mM of BzATP for different times (1, 4, 12, 18 or 24 h) before determining LDH activity. (**b**) The slices were pre-treated with 500 nM of Brilliant Blue G (*BBG*) for 30 min before the treatment with 5.0 mM of ATP or 0.1 mM of BzATP. (**c**) The slices were pre-treated with 1.0 mM of lithium (*Li*) or 0.5 mM of valproate (*VPA*) for 30 min before the treatment with 0.1 mM of BzATP. In (**b**) and (**c**), after 18 h, the incubation medium was collected and the LDH activity was measured. Leakage of LDH is represented as a percentage of the total releasable enzyme. The data are the mean ± SEM of at least nine experiments run in triplicate. The effect was statistically determined by Student–Newman–Keuls test. $^{*}$Significantly different from the respective control group ($P < 0.05$); $^{**}$Significantly different from the respective control group ($P < 0.01$); $^{***}$Significantly different from the respective control group ($P < 0.001$); $^{\#}$Significantly different from the ATP group ($P < 0.001$). $^{\#\#}$Significantly different from the BzATP group ($P < 0.01$). $^{a}$Significantly different from the control group ($P < 0.001$); $^{b}$Significantly different from the BzATP group ($P < 0.01$)

pre-treated with lithium and VPA simultaneously, a significant reduction in LDH release (20 ± 6%) was observed when compared with slices pre-treated with lithium (48 ± 10%) or VPA (45 ± 8%), separately.

## Discussion

Initially, we established a model to study ATP-induced cell death in hippocampal slices by measuring the release of the cytosolic enzyme LDH, a well established marker of cell injury and death. Our results confirmed the cytolytic activity of extracellular ATP in hippocampal slices, a preparation that maintains the interactions among neural cells and preserves the integrity of neural tissue. Hydrolysis of extracellular ATP cannot explain the results reported here since ADP, AMP, and Ado did not have cytolytic effects (Fig. 1). Since cell death potentially elevates the extracellular concentration of ATP to millimolar levels [24], the data obtained here, allow us to use this *in vitro* model to study neuronal injury in which an increase of extracellular ATP is involved [36] and to investigate the neuroprotective effect of lithium and VPA.

The treatment of acute disorders such as ischemia/hypoxia and brain trauma requires a rapid clinical intervention. To test the effects of acute treatment, we treated the slices with lithium or VPA at therapeutic concentrations, for 30 min (*in vitro* treatment) before submitting them to cytotoxic ATP concentration. Significant reductions in LDH release were observed when compared with respective controls (Fig. 2), indicating that it is possible to prevent the cytolytic effect of extracellular ATP acutely; however, the relevance of this observation in a therapeutic approach requires further study.

Additionally, we treated adult rats with lithium and VPA to evaluate the effects of chronic treatment (*in vivo* treatment) on the neural protection against cell death induced by extracellular ATP. As shown in Figs. 3 and 4, a significant reduction in LDH release was also observed in slices prepared from rats chronically treated with therapeutic doses of lithium or VPA. These results may be compared with another study from our group, in which the neuroprotective effect of chronic treatment with lithium was shown in a model of neuronal death using organotypic slice cultures exposed to oxygen and glucose deprivation [16].

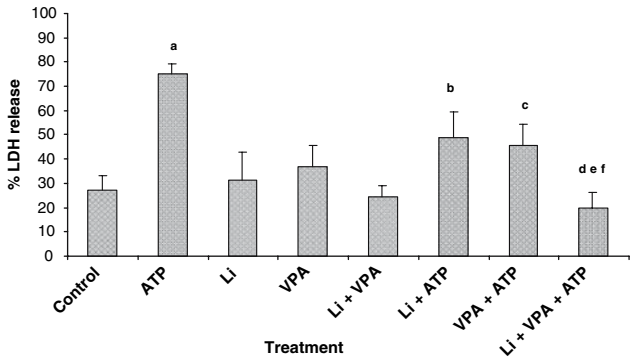


**Fig. 6** Synergic effect of pre-treatment with lithium and valproate on ATP-induced cell death. The slices were prepared as described in Materials and methods and pre-treated with: 1.0 mM lithium (*Li*), 0.5 mM valproate (*VPA*), 1.0 mM lithium and 0.5 mM valproate simultaneously (*Li + VPA*) for 30 min before the treatment with 5.0 mM ATP. After 18 h, the incubation medium was collected and the LDH activity was measured. Leakage of LDH is represented as a percentage of the total releasable enzyme. The data are the mean ± SEM of at least six experiments run in triplicate. The effect was statistically determined by Student–Newman–Keuls test. [a]Significantly different from the control group ($P < 0.001$); [b]Significantly different from the ATP group ($P < 0.01$); [c]Significantly different from the ATP group ($P < 0.001$); [d]Significantly different from the ATP group ($P < 0.001$); [e]Significantly different from the Li + ATP group ($P < 0.05$); [f]Significantly different from the VPA + ATP group ($P < 0.05$)

Since the cytotoxicity of ATP may be induced by the activation of $P2X_7$ receptors [24, 37], we submitted hippocampal slices to BzATP, a specific agonist of $P2X_7$, to evaluate the participation of this receptor in the effect of lithium and VPA. As observed in Fig. 5c, the pre-treatment of slices with 1.0 mM of lithium or 0.5 mM of VPA for 30 min caused a significant reduction in the LDH release after incubation with BzATP, suggesting the involvement of this receptor in the cell death induced by ATP. The application of BBG, known specific P2X7 receptor antagonist, caused a significant but partial blockage on cell death induced by ATP and BzATP. Although the inhibition of BBG on $P2X_7$ receptors is very slowly reversible [35] it is possible that the partial blockage observed could be due to the long period of incubation (18 h). However, the potential involvement of other P2 receptors than $P2X_7$ in the cell death evoked by ATP, as it was proposed by Amadio et al. [38], cannot be excluded.

According to Rowe and Chuang [5], lithium produces its neuroprotective effects not only via multiple pathways, but possibly though a direct influence at several levels of the same pathway. The effects of lithium on neuronal transduction systems have been extensively studied in animal models and many hypotheses have been proposed to explain its therapeutic action. Studies indicate that lithium has both direct and indirect actions that contribute to inhibit GSK3$\beta$. Since GSK3$\beta$ has recently been identified as an important proapoptotic enzyme [8, 39, 40], it has been suggested that the inhibition of GSK3$\beta$ by lithium and VPA may account, in part, for the neuroprotective actions of these agents, as well as contributing to their therapeutic effects in bipolar disorder [8, 41, 42].

Lithium and VPA are the two most prevalently used therapeutic agents in the treatment of bipolar mood disorder. Therefore, many investigators have an interest in determining whether these two agents share common sites of action [43]. Here, we showed that when slices were pre-treated with lithium and VPA simultaneously, a significant reduction in LDH release was observed when compared with slices pre-treated with lithium or VPA separately (Fig. 6). These results demonstrate that therapeutic doses of lithium and VPA, *in vitro*, exert a synergic effect in the prevention of ATP-induced cell death, suggesting the involvement of different signaling pathways; these findings are in agreement with data from the literature indicating different mechanisms for the neuroprotective actions of lithium and VPA [9, 44–47]. Many reports have shown the neuroprotective effects of these drugs in chronic treatment [4, 17, 18]. An important finding of the present study was the demonstration of the protective effects of lithium and VPA during acute treatment. Although more studies are necessary, these findings could have clinical relevance.

The mechanisms involved in neuronal death are complex and some hypotheses may be raised to explain the neuroprotective effects observed in the present study. As reviewed by Le Feuvre et al. [36], ATP and $P2X_7$ receptors can potentially regulate interleukin-1-mediated neuropathologies and, thus, may act as mediators of cell death following pathological insults, such as inflammation, trauma, and stress. We suggest that the effects observed, at least for the chronic treatment with lithium, may be explained by the fact that lithium treatment increases the physiological antagonist of the interleukin-1 receptors [48], therefore antagonizing the stimulatory effect of the cytotoxic ATP inflammatory process.

Another possible explanation for the neuroprotection induced by lithium against ATP signalization is the potential stimulation exerted by ATP and ADP via P2 receptors coupled to PLC, which produces diacylglycerol, in turn activating PKC [49] and resulting in the closure of $K^+$ channels. A classical explanation for the neuroprotective effects of lithium involves the hypothesis of the damping of the signal transduction via PIP2 with a consequent decrease in PKC activity. Finally, although the role of PKC in regulating the P2X7 receptor remains elusive, it is possible that the neuroprotective effect of lithium and/or VPA could be the consequence of a decrease in PKC activity that, in turn, decreases the P2X7-mediated $Ca^{2+}$ signaling [50].

Our *in vitro* results may also be an important tool for the understanding of the mechanisms involved in the cellular death induced by ATP, considering that the pathways involved with P2X7 receptors functions may be modulated by lithium and VPA.

Our knowledge of the molecular mechanisms involved in the neuroprotective action of lithium and VPA are incomplete, but our results support the hypothesis that these drugs could be used in the prophylaxis and/or treatment of neurodegenerative diseases that involve increases in ATP levels.

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