Polyphosphoinositide Metabolism and Golgi Complex Morphology in Hippocampal Neurons in Primary Culture is Altered by Chronic Ethanol Exposure

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Abstract — Aims: Ethanol affects not only the cytoskeletal organization and activity, but also intracellular trafficking in neurons in the primary culture. Polyphosphoinositide (PPIn) are essential regulators of many important cell functions, including those mentioned, cytoskeleton integrity and intracellular vesicle trafficking. Since information about the effect of chronic ethanol exposure on PPIn metabolism in neurons is scarce, this study analysed the effect of this treatment on three of these phospholipids. Methods: Phosphatidylinositol (PtdIns) levels as well as the activity and/or levels of enzymes involved in their metabolism were analysed in neurons chronically exposed to ethanol. The levels of phospholipases C and D, and phosphatidylylethanol formation were also assessed. The consequence of the possible alterations in the levels of PtdIns on the Golgi complex (GC) was also analysed. Results: We show that phosphatidylinositol (4,5)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate levels, both involved in the control of intracellular trafficking and cytoskeleton organization, decrease in ethanol-exposed hippocampal neurons. In contrast, several kinases that participate in the metabolism of these phospholipids, and the level and/or activity of phospholipases C and D, increase in cells after ethanol exposure. Ethanol also promotes phosphatidylylethanol formation in neurons, which can result in the suppression of phosphatidic acid synthesis and, therefore, in PPIn biosynthesis. This treatment also lowers the phosphatidylinositol 4-phosphate levels, the main PPIn in the GC, with alterations in their morphology and in the levels of some of the proteins involved in structure maintenance. Conclusions: The deregulation of the metabolism of PtdIns may underlie the ethanol-induced alterations on different neuronal processes, including intracellular trafficking and cytoskeletal integrity.

INTRODUCTION

Inositol phospholipids, or polyphosphoinositide (PPIn), are low-abundance membrane phospholipids, which play essential roles as regulators in a wide range of cellular functions, including signalling, cell survival, motility, cytoskeleton organization and membrane trafficking (Takenawa and Itoh, 2001; Fisher et al., 2002; Downe et al., 2005; Di Paolo and De Camilli, 2006; Vicinanza et al., 2008; Skwarek and Boulianne, 2009). PPIn are formed by the phosphorylation or dephosphorylation of phosphatidylinositol (PtdIns) at the D-3, D-4 or D-5 positions of the inositol ring by different phosphoinositide kinases or phosphatases, respectively, generating seven distinct phosphoinositide species: PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2 and PtdIns(3,4,5)P3. These enzymes are also responsible for regulating PPIn metabolism. In addition, and as Fig. 1 and 2 depict, PtdIns(4,5)P2 is transformed into inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG) by the action of phospholipase C (PLC) (Crockhardt, 2006; Berridge, 2009). Moreover, DAG can be used to resynthesize PtdIns via phosphatidic acid (PA).

PPIn perform their functions by interacting with specific protein domains, many of which are located in specific organelles, resulting in a predominant intracellular localization of all these molecules, (Le Roy and Wrana, 2005; Di Paolo and De Camilli, 2006; Vicinanza et al., 2008). Thus, for example, whereas PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are primarily enriched in the plasma membrane, where they control reactions such as the generation of intracellular second messengers, exocytosis, endocytosis and actin cytoskeleton reorganization, PtdIns(4)P is enriched in the Golgi complex (GC), where PtdIns(4,5)P2 also plays a central role in its structure and functional maintenance (Mayinger, 2009). Moreover, PPIn play a central role in the biology of neurons, where PPIn metabolism modulates membrane traffic, microtubules organization and the actin cytoskeleton and synapse function (Osborne et al., 2006; Wen et al., 2011). Finally, dysfunctions in the control of the PPIn level have been reported to possibly result in several pathologies, including diabetes, myopathies and neuropathies (Pendarties et al., 2003; Nicot and Laporte 2008; Vicinanza et al., 2008; Ooms et al., 2009).

Alcohol (ethanol) consumption during pregnancy produces abnormal central nervous system development, and affects different cellular processes, such as neurogenesis and astrogligenesis, neuronal migration and synaptogenesis, leading to a variety of phenotypes termed foetal alcohol spectrum disorders (FASDs) (Goodlett and Horn, 2001; Guerri et al., 2009; Goodlett, 2010). Thus, ethanol is considered as one of the commonest substances that has an impact on the developing brain, and prenatal alcohol exposure is a leading preventable cause of birth defects, mental retardation and neurodevelopmental disorders (American Academy of Pediatrics, 2000; May et al., 2009). Although no single mechanism has proved sufficient to account for these effects (Goodlett et al., 2005; Guizzetti and Costa, 2007; Martínez and Egea, 2007; González and Salido, 2009; Guerri et al., 2009), one such mechanism suggests that ethanol perturbs intracellular vesicular trafficking, including anomalies in the structure and function of the GC (Tomás et al., 2005; Marín et al., 2008; Ballestín et al., 2011; Esteban-Pretel et al., 2013).
In astrocytes and neurons in the primary culture, these effects on intracellular traffic have been related to the primary alcohol-induced alteration of the actin cytoskeleton and microtubules organization and function, among other mechanisms (Guasch et al., 2003; Tomás et al., 2003, 2005; Martínez et al., 2007; Marín et al., 2010; Romero et al., 2010; Loureiro et al., 2011). Although the mechanisms underlying these effects remain to be clarified, it has been demonstrated that cytoskeleton actin disorganization in astrocytes induced by chronic exposure to ethanol is accompanied by alterations in the levels and/or activity of several small GTPases, and in the levels of PtdIns(4,5)P$_2$ (Martínez et al., 2007). This result is interesting given the demonstration of functional interplay between small GTPases and PPIn (Di Paolo and De Camilli, 2006). Ethanol also affects the phospholipase D (PLD)-mediated biosynthesis of PA in different cell types, including astrocytes, through phosphatidylcholine (PC), the major membrane phospholipid. This is due to the fact that, in the presence of ethanol, PLD converts PC into an abnormal phospholipid, phosphatidylethanol (PEth), thereby disrupting the normal PLD-mediated signalling cascade (Fig. 2) (Alling et al., 1983; Gustavsson, 1995; Guizzetti et al., 2004; Klein, 2005; Newton and Messing, 2006; Sambuy, 2009). PEth increases membrane fluidity, although the presence of PEth in the membrane lipid bilayers may also confer some tolerance to ethanol-induced membrane disruption, inhibits ethanol activation of Na$^+$-K$^+$ ATPase, stimulates phosphoinositide hydrolysis and activates protein kinase C and phospholipase A$_2$, thus suggesting a role for PEth in ethanol modulation of signal transduction (Asaoka et al., 1988; Omodeo-Sale et al., 1991; Lundqvist et al., 1993; Aroor and Baker, 1996; Chang et al., 2000). Thus, it has been proposed that this phospholipid is a candidate cytotoxic substance that might be responsible for some of the adverse effects exerted on several cell types by alcohol (Alling et al., 1983; Gustavsson, 1995; Aradottir et al., 2002; Sambuy, 2009). However, it is unknown whether this mechanism exists in neurons and whether ethanol is able to alter the PPIn metabolism in these cells.

Given the importance of PPIn in the organization and function of both the actin cytoskeleton and protein trafficking, we propose that the previously described effects of ethanol on both processes in neurons (Marín et al., 2010; Romero et al., 2010) could be related to the ethanol-induced alterations of these phospholipids. Accordingly, this study analysed the effect of chronic ethanol exposure during the first 14 days of in vitro cultures on the levels of PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$, as well as the activity and/or level of enzymes phosphoinositide3-kinase (PI3K), phosphoinositide4-kinase (PI4K), phosphoinositide5-kinase (PIP5K1) and PLC in these cells (Fig. 1). In addition, the levels of PLD and PEth formation were also assessed (Fig. 2). Finally, after considering the relevance of PtdIns(4)P and PtdIns(4,5)P$_2$ in the function and structure of the GC, and since this organelle plays a central role in vesicular traffic, we also analysed the effect of alcohol on not only qualitative and quantitative morphology, but also the levels of the several proteins involved in the maintenance of their structure (Ramírez and Lowe, 2009; Burman et al., 2010; Nakamura, 2010; Xiang and Wang, 2011).
MATERIALS AND METHODS

Reagents

Primary antibodies

(a) Neuronal and astroglial markers: an anti-microtubule-associated protein 2 (MAP2) mouse monoclonal antibody and an anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody were obtained from Sigma-Aldrich (Spain). Anti-synaptosome-associated protein of 25 kDa (SNAP-25) and anti-postsynaptic density (PSD)-95 mouse monoclonal antibodies were purchased from Abcam (Cambridge, UK).

(b) PIPIn-related antibodies: anti-PtdIns(4)P, anti-PtdIns(4,5)P₂ and anti-PtdIns(3,4,5)P₃, mouse monoclonal antibodies were acquired from Echelon Biosciences, Inc. (Salt Lake City, USA). The anti-PI3K rabbit polyclonal antibody was purchased from Abcam, the anti-PI4K rabbit polyclonal antibody was acquired from Cell Signaling (Danvers, MA, USA) and anti-PIP5K1 was obtained from BD Transduction Laboratories (Erembodegem, Belgium). The anti-PLD2 mouse monoclonal antibody was acquired from Abnova GmbH (Germany), while the anti-PEth human monoclonal antibody was obtained from Echelon Biosciences Inc.

(c) GC proteins: monoclonal antibodies against GM130 and p115 were obtained from BD Transduction Laboratories. The anti-Scyl1 mouse monoclonal antibody and the anti-SAC1 goat polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Finally, the anti-GRASP65 and anti-giantin monoclonal antibodies were acquired from Abcam.

Secondary antibodies

Alkaline phosphatase-conjugated anti-rabbit, anti-mouse and anti-goat IgG were acquired from Sigma-Aldrich. Alexa Fluor® 488 chicken anti-mouse IgG and Alexa Fluor® 594 chicken anti-mouse IgG were obtained from Molecular Probes (Invitrogen SA, Spain). Finally, fluorescein anti-human IgG came from Vector Laboratories, Inc. (Burlingame, USA).

Other reagents

For the quantitative detection of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, two ELISA Kits from Echelon Bioscience were used. The Mass Strip Kit for the determination of PtdIns(4)P...
was obtained from the same supplier. To measure PLC activity, PtdIns(4,5)P₂ and [³H]PtdIns(4,5)P₂ were obtained from Sigma-Aldrich and PerkinElmer, respectively.

The culture media reagents (Neurobasal medium, B-27 supplement, Glutamax and antibiotics) were obtained from Gibco (Invitrogen Life Technologies, Spain). The chemicals used for electron microscopy were acquired from Electron Microscopy Science (EMS, PA, USA). All the remaining chemicals were ordered from Sigma-Aldrich.

Primary culture of hippocampal neurons and alcohol treatment

The primary cultures of hippocampal neurons were prepared from the foetuses of female rats on Day 16 of gestation, as described elsewhere (Braza-Boils et al., 2006; Marín et al., 2010; Romero et al., 2010). Cells were cultured in 5-cm diameter Petri plates in a humidified atmosphere (5% CO₂ and 95% air) at 37°C in the Neurobasal medium supplemented with B27 and Glutamax. In this medium, the astroglial cell growth at 5 days reduced to less than 1.0%, resulting in a nearly pure neuronal population (Marín et al., 2010). The medium was changed every 2 days and cells were maintained until Day 14 (14 days in vitro or DIV). To analyse the possible toxic effects of chronic alcohol exposure on PPIn metabolism during neuronal development, some cells were grown in the presence of ethanol, which was added to the culture medium when cells were plated. The ethanol concentration in the medium was carefully checked daily using a kit from Sigma-Aldrich (NAD-ADH Reagent Multiple Test Vial N7160) and was adjusted to a final concentration of 30 mM (138 mg/dl; ethanol evaporation after 24 h was 10–20%), similar to the blood levels reported in pregnant chronic drinkers. This is considered moderate alcohol consumption (Eckardt et al., 1998). Moreover, this ethanol concentration was in the range of the concentrations used in many in vitro and in vivo studies on FSADs and alcohol effects (Guerr et al., 1999; Zhou et al., 2003; Camarillo and Miranda, 2008; D’Addario et al., 2008; Kim et al., 2010; Valenzuela et al., 2011). Neuron culture purity was assessed by immunofluorescence using the anti-GFAP and anti-MAP2 antibodies (Marín et al., 2010). The anti-SNAP-25 and anti-PSD-25 antibodies were used as pre- and post-synaptic markers, respectively (see Marín et al., 2010). Our results indicate that cell cultures contained 97–99% of neurons, and that both synaptic markers were present in most of the cells examined (data not shown). Cell viability was determined by the trypan blue exclusion test. In some cultures, the possible cytotoxic effect of alcohol exposure on neurons was also assessed with a cytotoxicity assay kit (Sigma-Aldrich, Cat. Num. TOX7). These tests indicate that an excellent long-term survival was achieved in the control and alcohol-exposed cells after 2 weeks in the culture with more than 95% viability. No changes in the cell number were observed when cells were treated with ethanol when compared with control plates.

All the animal experiments were performed in accordance with the guidelines established by the European Communities Council Directive (86/609/EEC) and by Spanish Royal Decree 1201/2005. All the experimental procedures were approved by the Ethical Committee of Animal Experimentation of the Foundation La Fe Research Center (authorization number SAF2005-00615).

Fluorescence microscopy

The analysis of neuronal/astroglial and GC markers, and the presence of PtdIns(4)P, PtdIns(4,5)P₂, PtdIns(3,4,5)P₃ and Peth in both control and alcohol-exposed neurons was analysed by fluorescence microscopy, as previously described (Romero et al., 2010). The neurons growing on coverslips were quickly fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature (RT), and were washed in PBS (3 × 5 min each) and in PBS containing 50 mM NH₄Cl (10 min) to block any free aldehyde group. Subsequently, cells were permeabilized for 15 min with PBS containing 0.1% Triton X-100, 1% BSA and 10% FBS. To visualize the different proteins studied, cells were first incubated with the corresponding primary antibody and then with a secondary antibody conjugated with Alexa 488 nm or FITC.

Finally, coverslips were rinsed several times in PBS and mounted using Mowiol. Microscopy and imaging were performed with a Leica SP5 confocal microscope. To analyse those samples processed for the demonstration of Peth, correlative brightfield or differential interference contrast microscopy (DIC or Nomarski microscopy) and fluorescence microscopy were used. To assess the GC morphology, a total of 106 control cells and 110 alcohol-exposed neurons stained with anti-giantin, a widely used marker for the GC (Linstedt and Hauri, 1993; Valderrama et al., 2001; Schaub et al., 2006), were examined by conventional fluorescence microscopy at ×1000.

PPIn extraction

For PPIn extraction, the plates containing neurons were washed with cold PBS and incubated with 0.5 M ice-cold trichloroacetic acid (TCA). After 5 min incubation on ice, samples were scrapped and centrifuged at 200 g for 5 min, and pellets were washed in 5% TCA + 1 mM ethylenediaminetetraacetic acid (EDTA) twice. Neutral lipids were extracted by vortexing three times for 10 min at RT in MeOH:CHCl₃ (2:1), followed by centrifugation. Acidic lipids were also extracted in the same manner by vortexing four times for 15 min at RT in MeOH:CHCl₃:HCl (80:40:1), followed by centrifugation. A phase split was performed on the supernatant by adding CHCl₃ + 0.1 M HCl, vortexing and spinning down at 200 g for 5 min. The lower organic phase was collected and dried in a vacuum dryer. Dried lipids were stored at –20°C until processed.

Analysis of PPIn levels

PPIn samples were reconstituted, vortexed and sonicated in the appropriate solution immediately before use according to the manufacturer’s recommendation. The amount of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ in the purified reconstituted samples was measured by ELISA using the PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ Mass ELISA Kits (Echelon Biosciences) according to the supplier’s protocol. For the PtdIns(4)P quantifications, lipid samples were reconstituted in CHCl₃:MeOH:H₂O (1:2:0.8) and spotted onto membranes from the PtdIns(4)P Mass Strip Kit (Echelon Biosciences).
PLC activity

PLC activity was measured as described elsewhere (Huang et al., 2011), with minor modifications. Briefly, cells were detached from the plastic with a rubber policeman in lysis buffer (50 mM HEPES, pH 7.2, 0.1% Triton X-100, 0.5% Cholate and 5 μl/ml of mammalian protease inhibitor cocktail), vortexed and incubated with agitation at 4°C. After 30 min, samples were centrifuged for 5 min at 10,000 g and supernatants were frozen at −20°C until use. To carry out the assay, the phospholipid vesicles containing a mixture of PtdIns(4,5)P₂ and [³²P]PtdIns(4,5)P₂ were prepared by sonication in 0.5% cholate. Samples were diluted in assay buffer (assay final concentration 133 μg/ml of fatty-acid free BSA, 50 mM HEPES, pH 7.2, 70 mM KCl, 3 mM CaCl₂, 3 mM EGTA and 2 mM DTT). To begin reactions, the lipid stock was mixed with samples (1:1) to obtain the final assay conditions (300 μM of PtdIns(4,5)P₂ and ~20,000 cpm of [³²P] PtdIns(4,5)P₂) in 60 μl. Reactions were performed at 37°C for 5 and 10 min, and stopped by addition of 100 μl of 10 mg/ml BSA and 200 μl of 10% (v/v) TCA to precipitate uncleaved lipids and protein. Centrifugation of the reaction mixture isolated soluble [³²P]Ins(1,4,5)P₃, which was quantified using liquid scintillation counting.

Western blotting

Both control and alcohol-exposed neurons were washed with PBS and homogenized in extraction buffer (6 mM Tris-buffer, 10 mM EDTA and 2% sodium dodecyl sulphate, pH 7.0) with 5 μl/ml of mammalian protease inhibitor cocktail. Then, the proteins in cell lysates were quantified (Lowry et al., 1951) and processed for western blotting, as previously described (Tomáš et al., 2005; Marín et al., 2010). We analysed the levels of the different proteins which comprised the following: (a) two proteins involved in the GC architecture, GRASP65 and p115; (b) the Golgi matrix protein GM130; (c) proteins involved in the maintenance of the GC structure, Scy1 and SAC1 and (4) kinases participating in the PPIn metabolism (PI3K, PI4K and PIP5K1) (Fig. 1). To increase the accuracy of the specific protein measurements by western blot, each experimental situation was reproduced by quadruplicate in different culture plates and equal amounts of protein from each plate were loaded in the corresponding gel lanes. Consequently, each specific protein has been quantified four times.

Gel quantification was conducted using the ImageJ 1.43u programme (National Institute of Health, USA). Linearity ranges were established by blotting different amounts of each sample. The results are shown as the mean values of at least four different experiments per group.

Electron microscopy and stereology

For the ultrastructural and stereological studies, six plastic culture dishes per treatment (0 and 30 mM ethanol) containing neurons were randomly selected and washed in PBS. Cells were fixed in situ as monolayers with 1.5% glutaraldehyde + 1.0% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, 310 mOsm, for 60 min at 4°C. After washing in this buffer, cells were post-fixed in 2% osmium tetroxide containing 0.8% potassium ferrocyanide in cacodylate buffer for 60 min at 4°C. Neurons were then treated with 0.1% tannic acid in buffer for 1 min at RT, washed in buffer and stained in block with 2.0% aqueous uranyl acetate for 120 min at RT, washed, dehydrated in ethanol and embedded in Epon 812 (Marín et al., 2010). Then, the resin dishes containing cells were detached from the plastic and cut into fragments. After microscopic examination, fragments were glued onto Epon blocks or clamped into a holder for ultramicrotomy (Deitch and Banker, 1993; Bozzola, 1999; Romero et al., 2010). Ultrathin sections (60 nm) were double counterstained with uranyl acetate and lead citrate and examined at 80 kV in a Philips CM 100 Electron Microscope.

To quantitatively assess the effects of alcohol on GC morph-ology, stereological methods were used (Renau-Piqueras et al., 1987; Babia et al., 1999; Durán et al., 2003; Tomás et al., 2005). The micrographs of cells (×16,000, magnification in the microscope screen) containing the GC profiles were obtained by a successive selection of cultures, blocks and sections using systematic uniform random sampling (revised in Lucocq, 2008; Mayhew, 2011). The total GC (tg) was defined as a group of cisternae organized into stacks containing tubular and vesicular structures, with an arbitrary border in the cytoplasm (Renau-Piqueras et al., 1987; Babia et al., 1999; Orí et al., 2000; Durán et al., 2003). The intermediate elements continuous with the rough endoplasmic reticulum were excluded. These micrographs were obtained from the ultrathin sections taken along the apical-basal cell axis (i.e. cells were cut perpendicularly to the bottom of the culture dish). The following stereological parameters were determined using standard point-counting procedures (Williams, 1977; Weibel, 1979; Renau-Piqueras et al., 1987): the volume density (Vv) of the tg in relation to the cytoplasm (Vv tg,cyt); the Vv of the cister-nae in relation to the cytoplasm (Vv cist,cyt) and the surface density (Sv) of the cisternae in relation to the cytoplasm (Sv cist,cyt). The numerical density of the cisternae was estimated by two methods. With the first, the number of cisternae per μm² of the cytoplasm (Na) of the GC was calculated (Kang et al., 2009; Tang et al., 2010). In the second method, the number of cisternae per μm³ of the cytoplasm (Nv) of the GC was determined according to Weibel and Gomez (1962) (see also Weibel, 1979). However in the case of the GC cisternae, this method must be considered an approximation because it was developed for cylinders or ellipsoids of revolution, whereas GC cisternae are discoid or disc-shaped structures (Derganc et al., 2006). To determine any possible changes in the morphology of the cisternae profiles, the coefficient form (CF) of these structures was calculated using the CF = 4π area/perimeter² expression developed for circular or elliptical profiles (Renau-Piqueras et al., 1985) (the CF for a circle is 1.0 and CF = 0.8 for an ellipse of a = 1 and b = 0.5). The minimum sample size was determined by the progressive mean technique (confidence limit ≤5%) (Williams, 1977). The number of micrographs determined by this procedure was 20 for control cells and 22 for alcohol-exposed neurons. The results are expressed as means ± SD.

Statistical analysis

The results are expressed as mean ± SD. For statistical compu-tation and estimation of significance, we used the online GraphPad software (GraphPad Software, www.graphpad.com). Statistical significance was accepted when a P-value of <0.05 was obtained using the Student’s t-test.
RESULTS

Fluorescence microscopy of PPIn and PEth

Conventional fluorescence microscopy and confocal fluorescence microscopy were used to demonstrate the presence of PtdIns(4)P, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and PEth in control and alcohol-exposed neurons. As Fig. 3 reveals, all these PPIn were present in the cell body, axons and dendrites of both the neuron populations and no alcohol-induced qualitative differences were found in the morphological distribution pattern of labelling. The labelling distribution of PtdIns(4)P and PtdIns(4,5)P_2 appeared as punctata. Conversely, in those neurons incubated with anti-PtdIns(3,4,5)P_3, immunolabelling appeared much more diffuse throughout the cell. Moreover, in the neurons incubated with anti-PtdIns(4)P antibody, in addition to the distribution of antibody-binding sites in the cell body, axons and dendrites, the accumulation of fluorescence in some perikaria areas was noted. This pattern was not evident in the cells processed to demonstrate other PPIn. Finally, it is interesting to note that PtdIns(4,5)P_2 was the only PPIn also located in the nucleus.

PLD-mediated PEth production in both the control and treated neurons was analysed using an anti-PEth antibody. Confocal microscopy and DIC (Nomarski) microscopy showed that, whereas no signal was detected in the control neurons, staining markedly increased in both the soma and processes in ethanol-treated cells (Fig. 4B).

Alcohol affects PPIn levels

To analyse the possible effect of alcohol on PPIn metabolism, we measured the levels of PtdIns(4)P, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, which provide a general overview of the complete metabolism of these compounds. As Fig. 5 illustrates, alcohol exposure considerably lowered the levels in the three analysed PPIn, which dropped by 38.2, 31.4 and 28.3%, respectively, if compared with the control cell values.

Effects of ethanol on phosphoinositide kinases (PIK), PLC and PLD

In addition, the level and/or activity of several of the enzymes involved in the PPIn metabolism were measured by western blotting (PI4K, PIP5K1, PI3K and PLD2) and/or isotopic methods (PLC) (Fig. 6). All the measured enzymes showed a significant alcohol-induced increase if compared with controls (28.1, 36.9, 27.0, 21.0 and 51.5%, respectively).

Fig. 3. Confocal fluorescence microscopy of the control (A, C and E) and alcohol-exposed (B, D and F) cultured neurons processed for the demonstration of PtdIns(4)P, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. As shown, all these PPIn are present in control and alcohol-exposed neurons, and no remarkable alcohol-induced qualitative differences were found in the morphological distribution pattern of labelling. Bar represents 10 µm.

Fig. 4. Differential interferential contrast (DIC) and fluorescence microscopy of the neurons processed to demonstrate PEth formation. Presence of PEth was analysed using an anti-PEth antibody and immunofluorescence microscopy. Panels A and C show the features of the control (A) and alcohol-exposed (C) neurons seen by DIC. The same neurons processed for immunofluorescence are depicted in B and D. As shown, no signal was detected (B) in the absence of ethanol, whereas most cells showed intense fluorescence in those neurons cultured in the presence of ethanol (D). Bar represents 10 µm.
Fig. 5. A histogram showing the effect of chronic alcohol exposure on the levels of PtdIns(4)P, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. As illustrated, alcohol induces a significant decrease in all the PPIs studied. The data used in the statistical analyses are the mean ± SD of three independent experiments. Asterisks indicate significant differences (P < 0.05). Student’s t-test.

Fig. 6. Graph summarizing the effect of alcohol on the levels and/or activity of the several enzymes involved in the PPIn metabolism. (A) The levels of PI4K, PIP5K1 and PI3K were determined by western blotting. Note the presence of two bands in the gel corresponding to PIP5K1, which agrees with the data provided by the manufacturer. Histogram showing the quantification of both bands. (B) PLC activity was analysed by isotopic methods using [3H]PtdIns(4,5)P_2 as a substrate, whereas the PLD levels were measured by immunoblotting (C). In all cases, alcohol exposure induced a significant increase. The data used in the statistical analyses are the mean ± SD of four independent experiments for western blotting, and of three independent experiments for isotopic determinations. Asterisks indicate significant differences (P < 0.05). Student’s t-test.
Ethanol alters the GC morphology

Since alcohol exposure decreases the levels of PtdIns(4)P₂, the main PPIn in the GC, we analysed the effect of ethanol on the morphology of this cell component (Siddhanta et al., 2003). For this purpose, cells were immunostained for GC marker giantin. Our results, as shown in Fig. 7, indicate that alcohol induced a fragmentation of the GC in a large number of neurons. Thus, in the cultures exposed to ethanol, 68.2% of neurons showed a fragmented GC, whereas in the control cultures, this alteration was found only in 5.6% of the cells examined.

Moreover, ethanol induced important changes in the GC ultrastructure of most treated neurons, which appeared disorganized if compared with control cells. Thus, ethanol treatment led to the appearance of small cisternae and to the swelling of part of the GC cisternae (Fig. 8). The morphometric and stereological analyses of GC electron microscopy micrographs confirmed these qualitative observations (Fig. 9), indicating that chronic ethanol exposure caused a significant increase in the volume density of both the GC in relation to the cytoplasm (Vvtg,cyt) and of the cisternae in relation to the cytoplasm (Vcist,cyt) (Fig. 9A). In contrast, the surface density of the cisternae membranes diminished in relation to the cytoplasm (Svcist,cyt) after alcohol treatment was observed (Fig. 9B). Furthermore, when analysing the number of cisternae and minicisternae per cytoplasm unit of area (Na) or volume (Nv) (Fig. 9C), this parameter in the alcohol-exposed neurons significantly increased. Finally, the CF values calculated for the cisternae from control and alcohol-exposed cells were 0.56 ± 0.05 and 0.62 ± 0.1, respectively, while the λ values in both populations were estimated to be 3.4 ± 0.4 and 3.0 ± 0.4, respectively (Weibel, 1979). In both cases, values were significantly different (P < 0.05).

Analysis of GC proteins

We also analysed by western blotting whether the changes induced by ethanol in the GC morphology actually related to the variations in the amount of the several proteins responsible for the architecture and maintenance of the integrity of this morphology. As Fig. 10A shows, ethanol exposure significantly increased the levels of stacking protein GRASP65 and the p115 a tethering factor by 13.2 and 15.8%, respectively. Moreover, the GM130 matrix protein levels were not affected by ethanol (Fig. 10A), and no changes were noted in the amount of Scy11 and SAC1, which participate in maintaining the GC structure (Fig. 10B).

DISCUSSION

The present study shows that chronic exposure to ethanol alters PPIn metabolism in dissociated foetal rat hippocampal neurons in culture during their development by not only reducing the amount of several PPIn species, but by also increasing the level of those kinases involved in their interconversion. Moreover, the level and/or activity of phospholipases C and D, which likewise participate in the PPIn metabolism, also are altered after ethanol exposure. Finally, we also demonstrated that chronic alcohol exposure induces PEth formation in neurons which, as discussed below, could constitute an important mechanism involved in alcohol neurotoxicity.

As previously mentioned, of the three PPIn species analysed, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ concentrate in the plasma membrane and have been linked to most of the processes occurring in this structure, which also depend on the integrity of the actin cytoskeleton (Di Paolo and De Camilli, 2006; Mandal and Yan, 2009). In line with this, several studies reveal that exposure to PtdIns(4,5)P₂ phosphatase or the microinjection of PtdIns(4,5)P₂ antibodies results in actin cytoskeleton disruption (Gilmore and Burridge, 1996; Raucher et al., 2000) and, in addition, in a significant reduction of NMDAR-mediated currents when neurons were investigated (Mandal and Yan, 2009). Moreover, the depletion of PtdIns(4,5)P₂ in several cell types by adopting different strategies has been reported to result in a rapid disassembly of the coated pits in the plasma membrane, and to lead to the formation of new endocytic clathrin-coated pits and to important defects in the internalization and recycling of transferrin, a marker of clathrin-mediated endocytosis (Boucrot et al., 2006; Kim et al., 2006; Zoncu et al., 2007; Abe et al., 2008). Furthermore, this effect reverts after the addition of exogenous PtdIns(4,5)P₂ (Boucrot et al., 2006). One such strategy is based on exposing cells to either 1-butanol or other small primary alcohols which, as discussed later, could be capable of inducing an inferior PtdIns(4,5)P₂ formation (Siddhanta et al., 2003; Boucrot et al., 2006). All these and other studies demonstrate the key role played by both PtdIns in the actin cytoskeleton organization.
and activity and in intracellular traffic, including diverse endocytic routes (Di Paolo and De Camilli, 2006).

Along these lines, the present work demonstrates that chronic alcohol exposure significantly reduced the levels of PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$, which can be related to previous results obtained by our group, indicating that chronic alcohol exposure results in the alteration of not only clathrin- and caveolin-mediated endocytosis, but also in the assembly and organization of actin and tubulin cytoskeletons in cultured neurons (Marín et al., 2010; Romero et al., 2010). Thus, we have shown that exposure to ethanol affects not only the levels of these PPIns in neurons, but also several proteins, such as small Rab GTPases and small Rho GTPases (Marín et al., 2010; Romero et al., 2010), which cooperate with PtdIns(4,5)P$_2$ in either endocytosis or the dynamics of actin filaments (Weernink et al., 2000; Di Paolo and De Camilli, 2006; Oude Weernink et al., 2007; Compagnon et al., 2009).

The third PPIn studied was PtdIns(4)P, which is the most abundant phospholipid in the GC (Mayinger, 2009). PtdIns4P is a key regulator of the secretory pathway and plays an essential role in the trafficking from the GA. The central elements of this regulation are specific PI4Ks, which generate PtdIns4P and Sac1, which dephosphorylates at the 4-OH positions of the inositol ring (Piao and Mayinger, 2012). SAC1 is a transmembrane polyphosphoinositide phosphatase (Mayinger, 2009) that is predominantly located at the cisternal Golgi membranes (Cheong et al., 2010). So together, SAC1 and PI4K are required in yeast, and probably in mammals, for the metabolic reciprocal control of PtdIns(4)P in GA (Mayinger, 2009). Thus, PtdIns(4)P vastly contributes to plasma membrane composition (Di Paolo and De Camilli, 2006; D’Angelo et al., 2008; Farhan and Rabouille, 2011). Therefore, changes in the levels of this phospholipid and/or in the levels of PtdIns(4,5)P$_2$ result in alterations to the structural and functional characteristics of the GC and the trans-Golgi network (Siddhanta et al., 2003; Di Paolo and De Camilli, 2006; Daboussi et al., 2012). The current study shows that chronic alcohol exposure induces a drop in the levels of both phospholipids in neurons, which is accompanied by important changes in the GC morphology, mainly swelling and fractionation of cisternae, suggesting a causal relationship between the two effects, similarly to that described after treating GH3 rat pituitary tumour cells with primary alcohols (Siddhanta et al., 2003). Indeed, our stereological data confirm the immunofluorescence results using both anti-giantin and qualitative ultrastructural observations, indicating that alcohol treatment induces the swelling of some cisternae and the appearance of mini-cisternae, probably due to a fragmentation process. In addition, the ethanol-induced transformation of some cisternae from one elliptical profile to another more spherical one (swelling) was confirmed by the CF and the mean axial ratio ($\lambda$) values. Similar morphological alterations have been reported in other cell types exposed to ethanol, and have been related to the inhibition of vesicular traffic (Renau-Piqueras et al., 1987; Tomás et al., 2005; Esteban-Pretel et al., 2011; Tomás et al., 2012). However, the link between these effects of

Fig. 8. Electron microscopy micrographs showing the alterations induced by ethanol exposure on the GC morphology. As shown in the control neurons (A and B), the GC was composed of a number of flattened cisternae and vesicles. Moreover, some multivesicular bodies were also present. In contrast, the GC of alcohol-exposed cells (C and D) showed abundant swollen cisternae (arrows) and some minicisternae. This deleterious effect was observed in most treated cells. Bar represents 500 nm.
alcohol exposure and the drop in PtdIns(4)P after this treatment remains to be explored. In addition, we found variations in the levels of several proteins involved in the GC architecture and function, including p115 and GRASP65, which agrees with previous results in PC12 cells, from our group (Tomás et al., 2012). The p115 protein is essential for both exocytic transport and maintenance of the stacked structure of GC (Shorter and Warren, 2002). It acts in the endoplasmic reticulum to GC and intra-GC transport, and directly interacts with two GC membrane proteins: GM130 and giantin (Nakamura et al., 1997; Linstedt et al., 2000). GM130 is anchored by its C-terminal domain to the cytoplasmic face of the membrane through an interaction with protein GRASP65 (Barr et al., 1997). The increase in GRASP65 and p115 levels could be related to an internal cell mechanism to compensate for the altered architecture following ethanol exposure. This together with an alcohol-induced decrease in the amount of F-actin (Romero et al., 2010) could also contribute to the effects of alcohol on the GC. In fact, actin plays a key role in maintaining GC architecture and function, as demonstrated by different ways, including treatment with F-actin disrupters such as cytochalasin D, Latrunculin B or jasplakinolide, which caused GC cisternae fragmentation and swelling (Valderrama et al., 1998; Egea et al., 2006; Lázaro-Diéguez et al., 2006; Egea and Ríos, 2008). In this context, it is also important to note that while ethanol exposure increases the PI4K levels, such exposure does not alter the amount of SAC1.

One important question to be discussed is that of the mechanisms by which alcohol exposure affects balance in the PPP metabolism. Given the complexity of this metabolism and its regulation, different possibilities could be considered. Thus, for example, PtdIns(4,5)P2 levels are determined by a balance between synthesis and hydrolysis, and they can diminish in many ways (Mao and Yin, 2007; see also Cell Signalling Pathways in www.cellsignallingbiology.org). One mechanism that we consider to be more likely involved in the alcohol-induced alterations shown herein has been previously proposed, as discussed below, to explain various effects of ethanol on astrocytes (Klein, 2005). This mechanism is based on the fact that PLD generates phosphatidylalcohols...

Fig. 9. Quantitative morphological analysis of the GC in both control and alcohol-treated neurons, indicating significant changes in several stereological and morphometric parameters. Thus, these analyses indicate that the volume density (Vv) of the GC cisternae increased in the treated neurons (A), while the amount of surface membrane (Sv) lowered in these cells (B). Finally, both the numerical density per volume unit (Nv) and the numerical density per area unit (Na) reveal that the number of cisternae increased in ethanol-exposed cells (C). The data used in the statistical analyses are the mean ± SD. Asterisks indicate significant differences (P<0.05). Student’s t-test.

Fig. 10. The effects of alcohol exposure on the amount of some of the representative proteins involved in the architecture and maintenance of the GC structure were analysed by immunoblotting. (A) Ethanol exposure significantly increased the levels of stacking protein GRASP65 and tethering factor p115, but did not affect the levels of matrix protein GM130. (B) The levels of Scyl1 and SAC1, which participate in the GC structure maintenance, were not significantly affected by ethanol. The data used in the statistical analyses are the mean ± SD of four independent experiments. Asterisks indicate significant differences (P<0.05). Student’s t-test.
in the presence of primary alcohols instead of PA (Boucrot et al., 2006). In this sense, PLD has more than a 1000-fold preference for ethanol over water (Kötter and Klein, 1999; Klein, 2005; Boucrot et al., 2006; Newton and Messing, 2006) and, in the presence of ethanol, it produces PEth, which is readily incorporated into cell membranes where it accumulates, affecting the biophysical properties of the membrane (Omodeo-Salé et al., 1991; Gustavsson, 1995; Frohman et al., 1999; Newton and Messing, 2006).

Utilization of ethanol by PLD may also affect cellular functioning by diminishing PA formation, the precursor of PtdIns biosynthesis or by PEth-induced hydrolysis of PtdIns (Omodeo-Salé et al., 1991; Boucrot et al., 2006). Besides, an alcohol-induced decrease in this precursor could induce a compensatory mechanism that results in increased activity/levels of those enzymes involved in PtdIns synthesis, which also occurs with the P44K, PIP5K1, PI3K levels in the present study. In fact, there is considerable evidence of adaptive/compensatory responses in the cell metabolism after chronic or acute alcohol exposure (Valenzuela, 1997; Maturu et al., 2002; Parkhomenko et al., 2011; Yakovleva et al., 2011; Maturu and Varadacharyulu, 2012). For PLC, although the mechanism involved in the alteration of this enzyme is not clear, there are several studies reporting alcohol-induced changes in its activity/levels (Pandey, 1998; Kelm et al., 2010). On the other hand, due to the importance of PA in GC maintenance, vesicle trafficking, endocytosis and vesicular transport from the endoplasmic reticulum (Jenkins and Frohman, 2005; Yang et al., 2008), lower ethanol-induced PA production could also affect these processes. This mechanism has been proposed mainly in astrocytes (Kötter and Klein, 1999; Martínez et al., 2007; Guizzetti et al., 2010). The present work and previous studies (Klein, 2005) demonstrate the presence of PLD in neurons and, for the first time, we also demonstrate herein that chronic exposure to ethanol induces PEth formation in neurons. Although the plasma membrane composition of neurons probably differs from that of astrocytes, one possible conclusion is that the mechanism described in this latter cell type involving PEth formation in the presence of ethanol also exists in neurons, which affects its levels and its functions in protein trafficking and cytoskeleton organization, just as the PtdIns cycle does. Conversely, PLD activity is regulated by PtdIns(4,5)P$_2$ and other factors, including small RhoGTPases (Klein, 2005; Oude Weernink et al., 2007). For example, it has been reported that the reduction in PtdIns(4,5)P$_2$ levels by several mechanisms results in inhibited PLD activity (Exton, 2002; Jenkins and Frohman, 2005; Di Paolo and De Camilli, 2006; Oude Weernink et al., 2007). In addition to the alcohol-induced decrease in PtdIns(4,5)P$_2$ levels described herein, previous studies into astrocytes and neurons in the primary culture have shown that chronic exposure to ethanol significantly lowers the levels and activity of those GTPases (Martínez et al., 2007; Romero et al., 2010), which could, therefore, result in the altered PLD activity and in PtdIns biosynthesis.

As a general conclusion, these results suggest that the PPIn metabolism, the PPIn signal-transduction pathways and, therefore, the different processes depending on these pathways, including intracellular traffic and cytoskeleton organization and function, are affected by chronic ethanol exposure in neurons in the primary culture.

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