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ETHANOL POTENTIATES SEROTONIN STIMULATED INOSITOL LIPID METABOLISM IN PRIMARY ASTROGLIAL CELL CULTURES

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Abstract—Serotonin-stimulated activation of phospholipase C in primary astroglial cell cultures was studied as a mean of evaluating the effect of acute ethanol exposition on this signal transduction system. The addition of 50–150 mM ethanol prior to stimulation with 10^{-5} M serotonin led to a potentiation of the serotonin-induced [^{3}H]-inositol phosphate formation and an increased incorporation of [^{3}H]-inositol into the three phosphoinositides studied. This potentiating effect of ethanol was observed only when ethanol was added together with serotonin. No stimulatory effect of ethanol *per se* was found. Furthermore, ethanol had no effect on arginin-vasopressin, bradykinin or phenylephrine stimulated inositol lipid metabolism.

The relationship between alcoholism and the serotonergic system has been discussed with increasing intensity during the last decade (for review, see [1]). Clinical studies on alcoholics have revealed reduced concentrations of serotonin in blood [2], 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid [3, 4] and reduced activity of brain monoamine oxidase [5, 6]. Chronic administration of ethanol is also known to inhibit serotonergic metabolism in rat brain [7]. Furthermore, serotonin uptake inhibitors reduce voluntary alcohol intake in heavy drinkers [8].

Little is known about the influence of ethanol on serotonin receptors. Using radioligand binding, Hirsch [9] found that high concentrations of ethanol (>200 mM) inhibited the binding of [^{3}H]-LSD, a ligand with high affinity for the serotonin$_2$ receptor. In rats exposed for longer periods of time to ethanol, no clear-cut conclusions could be drawn concerning the binding of [^{3}H]-serotonin to brain membranes [10, 11]. We have however found a down-regulation of the serotonin$_2$ receptor on platelets from alcoholics [12].

In several tissues, including CNS, the serotonin$_2$ receptor is coupled to a transduction system which works via activation of phospholipase C [for review, see 13, 14]. This enzyme hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to the formation of two intracellular messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG) [15]. Although several reports have been published regarding the effects of ethanol on this transduction system, no general mode of action has emerged [16–27]. In hepatocytes, exposed acutely to ethanol in the absence of receptor agonists, Hoek and coworkers found a transient increase in the formation of IP_3 [22, 23]. In an astroglial cell culture model similar to the one used in this study, Ritchie *et al.* [27] found a potentiation of the noradrenaline stimulated IP_1 formation after long-term exposure but no effect on acutely exposed cells. On the other hand, several reports have indicated that neurotransmitter stimulated IP formation in brain preparations rather seems to be inhibited after acute ethanol exposure [16–21]. In addition, conflicting results were obtained when different regions of mice brain were examined [20]. The choice of agonist also proved to be of importance for the final results [19, 20]. These findings indicate that so far no unifying mechanism of ethanol on this transduction system can be postulated.

In order to elucidate the effect of ethanol on signal transduction in an isolated cell type from CNS, we have studied serotonin stimulated inositol lipid metabolism in cultured astrocytes from the cortex of neonatal rats. The astrocyte has emerged as a pharmacologically active cell with functional receptor complexes for a variety of neurotransmitters [28–33]. We have also recently shown that these cells possess serotonin$_2$ receptors coupled to phospholipase C [34].

MATERIALS AND METHODS

Cultivation of astrocytes. The primary astroglial cultures were made from newborn rat cerebral cortex (Sprague–Dawley strain, A-lab, Sweden). They were cultivated in Petri dishes (35 mm diameter, Nunc AS, Denmark) for 14 days in a humidified atmosphere [34, 35]. The cells were grown in Eagle's minimum essential medium (MEM, Flow Lab, Scotland) supplied with extra substances to make up the following final composition: double concentrations of amino acids, 2 mM glutamate, 7 mM glucose, quadruple concentrations of vitamins, 250,000 IU penicillin/l, 0.5% streptomycin and 20% (v/v) fetal calf serum (Gibco Bio Cult. Lab, U.K.). The pH was set at 7.3 and the medium was changed three times a week.

Stimulation of astrocytes. Cells were preincubated for 24 hr in medium containing 5 μCi/ml myo-[^{3}H]-inositol (New England Nuclear, Boston, MA, specific activity 15–17 Ci/mmol). This period of time has been shown to result in a steady-state labelling of astrocytes

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Table 1. Serotonin (10^{-5} M) stimulates the hydrolysis of [^{3}H]-labelled inositol lipids and the formation of [^{3}H]-IPs. Astroglial cell cultures were stimulated for 5 min in medium containing 10 mM LiCl and 10^{-5} M nialamide. Inositol lipids and IPs were analysed as described in Methods. Results are expressed as mean ± SE of 4 experiments, each done in triplicate

	Unstimulated cells (cpm/μg protein)	Stimulated cells (cpm/μg protein)	Percentage change after stimulation
IP_3	1.08 (0.24)	2.24 (0.38)	+107
IP_2	0.72 (0.26)	1.56 (0.20)	+117
IP_1	3.74 (0.53)	5.76 (0.23)	+54
PIP_2	2.12 (0.41)	1.56 (0.26)	−26
PIP	1.79 (0.19)	1.51 (0.25)	−19
PI	44.2 (5.5)	39.8 (1.8)	−10

[29]. On the day of stimulation, new medium without fetal calf serum, containing 10^{-2} M LiCl and 10^{-4} M nialamide (Sigma Chemical Co., St. Louis, MO) was added. When used, ethanol was administered at the same time as the medium. After 15 min of incubation, cells were challenged with 10^{-5} M serotonin (Sigma) as this concentration has been shown to lead to maximal receptor activation. In preliminary studies, the greatest formation of inositol phosphates was found after 5 min of stimulation and this time was therefore selected for this set of experiments. Stimulation was performed on a hot-plate at 37°. The reaction was stopped by aspiring the medium and rinsing the culture dishes three times with ice-cold glucose-saline buffer (pH 7.4) while keeping them on ice. Cells were scraped in 0.5 ml buffer and pipetted into 2.0 ml chloroform:methanol:conc. HCl (100:200:2,v:v:v). After extraction with 0.5 ml chloroform and 0.5 ml water, the two phases formed were taken into separate tubes for further analysis.

Analysis of [^{3}H]*-inositol phosphates.* Inositol phosphates were separated essentially according to Berridge *et al.* [36]. The water-soluble phase was diluted by 2.5 ml water and placed on 0.5 g Dowex sitol standards (Sigma) were added to facilitate identification of lipids after thin layer chromatography. Two aliquots were taken for the determination of total incorporation of radioactivity into the lipids. After evaporation, the lipids were dissolved in 30 μl chloroform:methanol (2:1, v:v) and spotted on oxalate-treated Silica Gel 60 HPTLC plates. The solvent system used consisted of chloroform:methanol:conc. NH_3:H_2O (65:47:2.5:15, v:v:v:v). Lipids were visualised by exposure to iodine vapour and the radioactivity determined by liquid scintillation counting.

RESULTS

The addition of 10^{-5} M serotonin to astrocytes labelled with [^{3}H]-inositol resulted in an increase in all inositol phosphates studied with a parallel hydrolysis of inositol lipids (Table 1). To study the influence of acute ethanol exposition, ethanol was added to the cell medium 15 min prior to serotonin stimulation. This pretreatment led to a 35–70% potentiation of the serotonin stimulated formation of inositol phosphates (Fig. 1). This increased accumulation was significant 1–8x (100–200 mesh, in the formate form, Karl Roth KG, Karlsruhe, B.R.D.). Elution of [^{3}H]-inositol phosphates was performed as described earlier [12] with the exception that the volume of each eluate was increased to 12 ml. Radioactivity was determined by scintillation counting. To compensate for the slight variation in cell content in different dishes, all values were correlated to the protein content as determined according to Lowry *et al.* [37] with bovine serum albumin as standard (Sigma). By eluting in fractions, it was possible to obtain an eluation profile with four peaks. The recovery of [^{3}H]-inositol 1,4,5-trisphosphate (NEN) added to cells was approximately 80%. Only minute amounts of [^{3}H]-inositol 1,4,5-trisphosphate were found in the eluates corresponding to the other inositol phosphates.

Analysis of [^{3}H]*-inositol lipids.* The lower lipid-phase obtained after extraction was dried under N_2 and dissolved in 2 ml chloroform:methanol (2:1, v:v). Phosphoinositide and phosphatidylino-

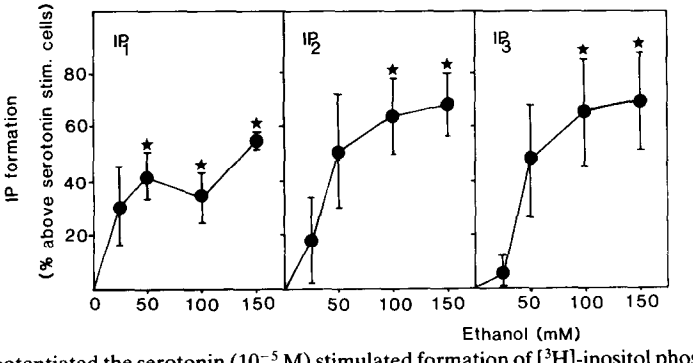

Fig. 1. Ethanol potentiated the serotonin (10^{-5} M) stimulated formation of [^{3}H]-inositol phosphates (IP). Data is expressed as increase above values found in unexposed cells that had been stimulated by serotonin for 5 min. Ethanol was added 15 min before stimulation. Results are expressed as mean ± SE of 3–4 separate experiments, each done in triplicate. (* = $P < 0.05$, Student's *t*-test). Basal values in serotonin stimulated but not ethanol exposed cells were 5057, 1670 and 2400 cpm/mg protein for [^{3}H]-IP_1, [^{3}H]-IP_2 and [^{3}H]-IP_3, respectively.

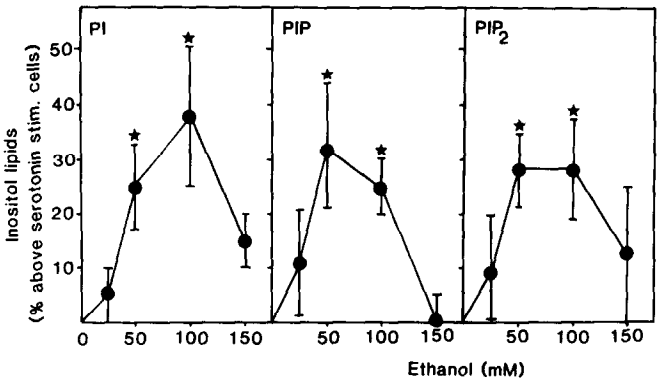

Fig. 2. Ethanol increased the incorporation of [^{3}H]-inositol into inositol lipids in astrocytes stimulated with serotonin (10^{-5} M). Data is expressed as increase above values found in unexposed cells that had been stimulated by serotonin for 5 min. Ethanol was added 15 min before stimulation. Results are expressed as mean ± SE of 3–4 separate experiments, each done in triplicate. (* = $P < 0.05$, Student's t-test). Basal values in serotonin stimulated but not ethanol exposed cells were 40609, 2585 and 2990 cpm/mg protein for [^{3}H]-PI, [^{3}H]-PIP and [^{3}H]-PIP_2, respectively.

Table 2. Ethanol added in the absence of agonist did not influence the radiolabelling of IPs or inositol lipids. Ethanol was added together with 10 mM LiCl to [^{3}H]-labelled astroglial cell cultures. After 20 min, the cells were harvested and analysed as is described in Methods. Results are expressed as percent change compared to values from ethanol-free dishes and are mean ± SE of two experiments, each done in triplicate

	Ethanol (mM)		
	50	100	150
IP_3	−4.0 (4.0)	−3.5 (7.1)	+3.5 (28)
IP_2	−6.5 (8.5)	−10.5 (8.5)	−15.5 (17)
IP_1	+1.5 (3.5)	+1.9 (11)	−10.2 (5.0)
PIP_2	+16 (8.5)	−7.0 (6.6)	+3.1 (6.9)
PIP	+8.9 (12)	−14 (3.9)	+7.4 (16)
PI	+2.5 (13)	+7.3 (4.2)	+12 (10)

already at an ethanol concentration of 50 mM. Under these conditions, an increased [^{3}H]-inositol labelling of PI, PIP and PIP_2 was observed (Fig. 2). However, the increased radiolabelling was consistently found to be biphasic with a maximal effect at 50–100 mM ethanol while, at 150 mM, levels tended to return to values seen in stimulated cells not exposed to ethanol. On the other hand, no effect was seen on cell cultures exposed only to ethanol for 20 min and not stimulated by serotonin (Table 2).

To elucidate if the potentiating effect of ethanol was restricted to the serotonin$_2$ receptor complex, three other receptors were studied. Arginin-vasopressin (AVP, 10^{-6} M), bradykinin (BK, 10^{-6} M) and phenylephrine (phe, 10^{-5} M), an α_1 agonist, all stimulated the hydrolysis of phosphoinositides and the formation of inositol phosphates (Figs 3 and 4). However, no effect of 100 mM ethanol was seen on IP formation induced by these agonists while serotonin, studied in the same sets of experiments, increased IP accumulation (Fig. 3). Furthermore, ethanol increased the labelling of inositol lipids only in cells challenged with serotonin (Fig. 4).

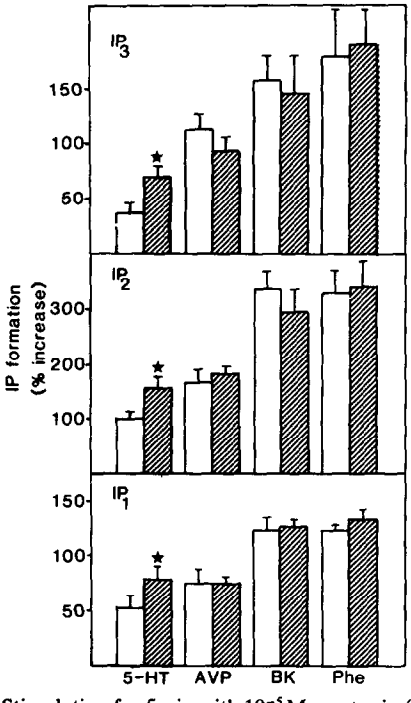

Fig. 3. Stimulation for 5 min with 10^{-5} M serotonin (5-HT), 10^{-6} M arginin-vasopressin (AVP), 10^{-6} M bradykinin (BK) and 10^{-5} M phenylephrine (phe) resulted in an increased formation of [^{3}H]-IPs (open bars). Ethanol (100 mM) added 15 min before stimulation significantly increased serotonin stimulated IP accumulation but had no effect on the other receptor responses (hatched bars). Results are expressed as mean ± SE of 3 separate experiments, each done in triplicate. * = $P < 0.05$ (Student's t-test).

DISCUSSION

The formation of inositol phosphates and reduction of PIP_2 further strengthens the evidence that serotonin receptors on primary astrocytes use a signal transduction system which is coupled to phospholipase C. However, the IP formation after

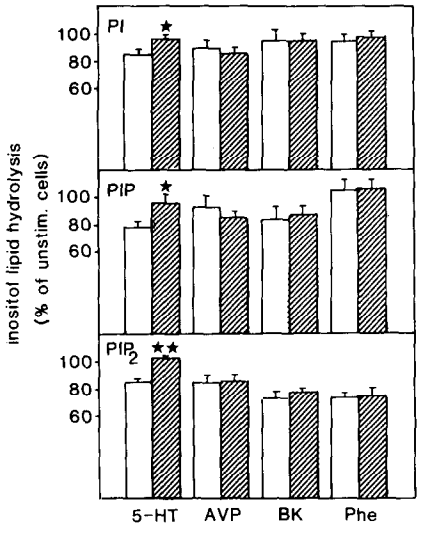

Fig. 4. Stimulation for 5 min with 10^{-5} M serotonin (5-HT), 10^{-6} M arginin-vasopressin (AVP), 10^{-6} M bradykinin (BK) and 10^{-5} M phenylephrine (phe) resulted in a hydrolysis of [^{3}H]-labelled inositol lipids (open bars). Ethanol (100 mM) added 15 min before stimulation significantly increased the radiolabelling of inositol lipids in serotonin treated cells but had no effect on the other receptor responses (hatched bars). Results are expressed as mean ± SE of 3 separate experiments, each done in triplicate. * = $P < 0.05$, ** = $P < 0.01$ (Student's t-test).

serotonin stimulation was not as great as after stimulation with AVP, BK and phe (Fig. 3).

Ethanol potentiated the serotonin stimulated accumulation of all three inositol phosphates studied. Interestingly, this effect could be observed already at ethanol concentrations similar to those found in man after ethanol intake, making it likely that this effect is of clinical relevance. One explanation of this potentiation would be a facilitation of the signal transduction through the serotonin$_2$ receptor complex resulting in a potentiated activation of phospholipase C. Similar explanations have been put forward to explain the stimulating influence of ethanol on receptor-coupled adenylate cyclase [38, 39]. However, a simple potentiation of the activity of phospholipase C would lead to an increase in the hydrolysis of the inositol phosphate precursor PIP_2. As can be seen in Figs 2 and 4, quite the opposite result was found with an increased radioactivity in the inositol lipids. This speaks against a single, isolated ethanol effect on the signal transduction but rather indicates a complex mode of action not only on phospholipase C but also on the phosphokinases responsible for phosphoinositide resynthesis. Recently, Rubin and Hoek [40] have presented similar results from studies on hepatocytes. In these cells, ethanol stimulated not only the activation of phospholipase C, but also induced an increase in [^{32}P]-PIP and, at ethanol concentrations of 400 mM, in [^{32}P]-PIP_2. Several possible explanations exist but none has so far been investigated in detail. However, it is known that agents that elevate cAMP in human platelets increase the phosphorylation of phosphoinositides [41, 42]. Since adenylate cyclase activity is elevated by ethanol [38], one plausible explanation of our results is that ethanol increases the intracellular concentration of cAMP leading to a stimulation of the phosphoinositide kinases. This would provide more substrate for phospholipase C and thus increase the formation of IP_3. However, this mechanism would lead to more substrate for all transduction systems using phospholipase C and thus result in a potentiation not only of the serotonin response but also of AVP, BK and phe signalling. The specificity of the ethanol potentiation on the serotonin$_2$ system also speaks against other explanations. For example, it is unlikely that the increased IP levels could be due to an inhibition of the phosphatases responsible for the elimination of IPs as this would lead to an increase of all agonist-induced responses studied. Furthermore, the potentiation found in the present study does not seem to be a direct alcohol influence on transduction systems coupled to phospholipase C as ethanol on its own had no detectable effect on inositol phosphate (Table 2).

Taking these considerations into account, it therefore seems likely that the stimulation of inositol lipid turnover observed at low ethanol concentrations is a specific interaction between ethanol and the activated serotonin$_2$ transduction system. From this study it is not possible to state the target of ethanol on the serotonin receptor but Rubin and Hoek recently reported a direct and transient ethanol-induced activation of the GTP-binding protein in human platelets [43]. A selective facilitation of the serotonin signal transduction through the membrane, possibly through a subclass of GTP-binding proteins associated with the serotonin receptor would lead to results similar to those presented above.

As we repeatedly saw a potentiation of the inositol lipid metabolism after serotonin stimulation of astrocytes and as neither Ritchie *et al.* [27] nor we found any effect on other receptors, it is tempting to speculate that the serotonin$_2$ system has a selective sensitivity to ethanol. This could explain the down-regulated serotonin$_2$ receptor response found in platelets from alcoholics [12] and would further point towards the importance of the serotonergic system for the understanding of alcoholism.

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