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Adenosine A_1 receptor agonist treatment up-regulates rat brain metabotropic glutamate receptors

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Abstract

Chronic R-N^6-phenylisopropiladenosine (R-PIA) subcutaneous injection for 6 days significantly increased total glutamate receptor number (180% of control) in rat brain synaptic plasma membranes (SPM), without affecting receptor affinity. A higher increase in metabotropic glutamate (mGlu) receptor number (258% of control) was also detected, indicating that mGlu is the main type of glutamate receptor affected by this treatment. On the other hand, the observed increase in basal and calcium- and Gpp(NH)p-stimulated phospholipase C (PLC) activity after treatment was associated with a significant increase in PLC β_1 isoform, detected in SPM by immunoblotting assays. Moreover, an increase in PLC activity stimulation with *trans*-ACPD, in the absence and in the presence of Gpp(NH)p, was detected after R-PIA treatment. These results show that mGlu receptors and its effector system, PLC activity, are up-regulated by chronic exposure to an adenosine A_1 receptor agonist and suggest the existence of a cross-talk mechanism between both signal transduction pathways in rat brain.

Keywords: Adenosine A_1 receptor; Metabotropic glutamate receptor; Phospholipase C; Up-regulation; Rat brain

1. Introduction

Glutamate is the main excitatory neurotransmitter in the central nervous system which has been implicated in different physiological and pathological processes [1,2]. The different actions of glutamate are mediated through glutamate receptors, which have been classified into ionotropic and metabotropic. Metabotropic glutamate (mGlu) receptors are coupled, through G-proteins, to different effector systems, including phospholipase C (PLC) activation and adenylyl cyclase (AC) inhibition [3,4]. In the central nervous system, several modulators regulate glutamate release, and activation of adenosine A_1 receptors play an important role [5,6]. Four receptor types mediate adenosine actions, named A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 receptors are coupled through a Gi/o protein to AC inhibition, while A_{2A} and A_{2B} receptors are coupled to AC stimulation through a Gs protein [7,8].

Receptor-mediated activation of a given signal transduction pathway by an agonist, not only modulates its own signal transduction pathway but may also modulate cellular responses through other different signalling pathways [9,10]. Previous studies have shown cross-talk processes between transduction pathways mediated through different G-protein coupled receptors, mainly involving β-adrenergic receptors [11–15]. Changes elicited by these cross-talk processes can affect all the transduction system components (e.g. receptors, G-proteins and enzymatic activities). Effects at the post-receptor level have been well documented. Thus, sensitization of the AC system after stimulation of PLC through transfected m5 muscarinic receptors in murine L-cells has been reported [16]. On the other hand, Gi-protein down-regulation has been described as a mechanism of heterologous regulation in adipocytes [17]. Finally, although less studied, changes in receptors have also been detected. Thus, activation of β_2-adrenergic receptors leads to down-regulation of m1 mAChR in CHO cells [13] and an increased level of α-adrenergic receptor mRNA in DDT_1 MF-2 cells [11].

We have previously described in vitro the existence of cross-talk between β-adrenergic/AC and mGlu/PLC path-

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ways in C6 glioma cells [18] and other authors have described an increase in β-adrenergic receptors after persistent activation of adenosine A_1 receptors in DDT_1 MF-2 cells [12]. Although several reports have shown the existence of cross-interaction between mGlu and adenosine receptors in vitro or ex vivo systems [19–23], including formation of functionally interacting complexes [24], however, little is known about cross-talk between these receptors after chronic in vivo activation of one of these signal transduction pathways.

The aim of the present work was to determine the effect of chronic R-PIA administration to rats on mGluR/PLC transduction pathway. We report that adenosine A_1 receptor activation with R-PIA causes an up-regulation of both mGlu receptors and PLC activity in rat brain, suggesting that a cross-talk mechanism is operating between glutamate- and adenosine-mediated transduction pathways.

2. Materials and methods

2.1. Animal treatment

Three-month-old male rats (180–200 g of body weight), kept on a 12 h light/12 h dark cycle and with free access to food and drinking water were treated by subcutaneous injection with R-PIA (one dose of 187 μg/day) during 6 days. Control rats were injected in parallel with vehicle. The 7th day rats were sacrificed by cervical dislocation and synaptic plasma membranes (SPM) from theirs brains were isolated. All experiments followed the European Community regulations about animal experimentation.

2.2. Materials

Phosphatidyl [2-^{3}H] inositol 4,5-bisphosphate (1 Ci/mmol) was purchased from Amersham. L-[^{3}H]glutamic acid (54.7 Ci/mmol) was from List Biological Laboratories (New England Nuclear). Non-hydrolyzable guanine nucleotides were from Roche. *Trans*-ACPD was from Tocris (Essex, UK). Nitrocellulose membrane and electrophoresis reagents were from Biorad Laboratories. Monoclonal antibody to PLC β_1 isoform was from UBI (Lake Placid, NY, USA). R-PIA was from Sigma. ECL Western blotting detection system was from Amersham. All other reagents were analytical grade.

2.3. Synaptic plasma membranes isolation

Brains were homogenized (1:10 w/v) in TDE buffer (5 mM Tris–HCl buffer pH 7.4, containing 1 mM dithiothreitol, 1 mM EGTA, 100 μM PMSF, 7.3 mU/ml bacitracin) and 10% sucrose (w/v) with a motor-driven Teflon glass tissue grinder. SPM were isolated as described earlier [25]. The homogenate was centrifuged at $800 \times g$ for 10 min. Pellet was dissolved in TDE buffer and centrifuged at $9000 \times g$ for 20 min. The washed pellet was resuspended in TDE buffer pH 8 and incubated at 4 °C for 30 min. This membrane preparation was adjusted to 34% (w/v) sucrose in TDE buffer and placed at the bottom of nitrocellulose tubes. A discontinuous sucrose gradient was formed and centrifuged at $60{,}000 \times g$ for 110 min at 4 °C in a SW40 rotor (Beckman). The material layered at the interface between the 34% and 28.8% sucrose was collected. This fraction was diluted 3-fold with TDE buffer and centrifuged at $150{,}000 \times g$ for 60 min. The pellet was resuspended in TDE and frozen to −70 °C until assays were performed.

2.4. L-[^{3}H]glutamate binding assays to synaptic plasma membranes

L-[^{3}H]Glutamate binding assays to rat membranes were performed as described previously [26]. Briefly, membranes were treated with 0.04% Triton X-100 to facilitate the removal of endogenous glutamate [27]. To determine mGluR binding, 60–100 μg of protein were incubated for 60 min at 25 °C in the presence of 100 μM α-amino-3-hydroxy-5-methyl-isoxazole-4 propionic acid (AMPA), 100 μM kainate, and 100 μM *N*-methyl-D-aspartic acid (NMDA), in order to block ionotropic glutamate binding, and different L-[^{3}H]glutamate concentrations (40–1500 nM) with 10 mM potassium phosphate pH 7.4, in the presence or in the absence of unlabeled L-glutamate to obtain non-specific binding. All assays were performed in the presence of 1 mM DL-threo-β-hydroxyaspartic acid (THBA), a L-glutamate uptake inhibitor [28].

2.5. Phospholipase C activity determination

PLC activity in SPMs was assayed as described previously [29], using [^{3}H]PtdInsP_2 as exogenous substrate. Assays were carried out for 10 min at 37 °C incubating 7.5 pmol [^{3}H]PtdInsP_2 with 10–15 μg of protein in a final volume of 100 μl of Tris–HCl pH 6.8 buffer, containing 1 mM sodium deoxycholate, 100 mM NaCl, 40 mM LiCl, 1 mM EGTA and the indicated concentrations of free calcium. Free calcium concentrations were set using a Ca^{2+}-EGTA buffer system as described by Herrero et al. [30].

2.6. Immunodetection of PLC β_1 isoform

Fifty micrograms of protein were subjected to 7.5% polyacrilamide gel electrophoresis in the presence of SDS. Western blotting was performed as described earlier [18]. Nitrocellulose membranes were incubated with isoenzyme-specific monoclonal anti-PLC antibody to PLC β_1 (1:400). After washing, blots were incubated with horseradish peroxidase-coupled goat anti-mouse antibodies (1:3000). Antigen was visualized using the ECL chemiluminescent detection kit from Amersham and specific bands were quantified by scanning densitometry.

2.7. Protein determination

Protein concentration was measured by the method of Lowry, using bovine serum albumin as standard.

2.8. Statistical and data analysis

Data statistical analysis was performed using the Student's *t*-test. Differences between mean values were considered statistically significant at $P<0.05$. The binding data were analyzed with the GraphPad Prism 3.02 program (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

Sprague–Dawley rats were daily treated with R-PIA, an adenosine A_1 receptor agonist. After 6 days of treatment, animals were sacrificed, brain SPMs were isolated and the status of different components of the mGlu receptor/PLC pathway was studied.

First, binding assays were performed using L-[^{3}H]glutamate as radioligand in order to determine specific binding to both total (ionotropic and metabotropic) and mGlu receptors in rat brain SPM. When assays were performed with a single concentration of radioligand (40 nM), an increase in total and mGlu receptor binding was detected after R-PIA treatment. This increase was of the same order (64–77%) with all mGlu receptor agonists assayed, as it can be observed in Table 1. To further study the effect of chronic adenosine A_1 receptor activation on mGlu receptors, saturation curves were performed with increasing L-[^{3}H]glutamate concentrations in control and treated SPM and in conditions in which we detected either total or mGlu receptors. R-PIA treatment caused a significant increase in B_{max} value of total glutamate receptors without a change in K_D value, suggesting no variation in receptor affinity (Fig. 1A and Table 2). Increase in B_{max} value was also observed when mGlu receptors saturation curves were determined, as it is shown in Fig. 1B and Table 2. However, K_D value increased significantly after treatment suggesting a decrease in mGlu receptors affinity. Therefore, higher specific binding of L-[^{3}H]glutamate shown in Table 1 can be explained by an increase in total glutamate receptor number, which can be attributed to the increase in metabotropic binding, this glutamate receptor type being mainly affected by R-PIA treatment. These results reveal that chronic R-PIA administration causes a mGlu receptor up-regulation. Accordingly, persistent adeno-

Table 1
R-PIA treatment effect on glutamate specific binding to SPM from rat brain

	Specific binding (pmol/mg protein)	
	Control	R-PIA treated
Total glutamate binding	1.13 ± 0.17	1.95 ± 0.20*** (172%)
Metabotropic glutamate binding		
L-Glutamate	0.63 ± 0.11	1.12 ± 0.06*** (177%)
Quisqualate	0.35 ± 0.03	0.59 ± 0.04*** (164%)
Trans-ACPD	0.38 ± 0.08	0.67 ± 0.03*** (172%)
Ibotenic acid	0.60 ± 0.08	1.01 ± 0.11*** (167%)

SPMs from control and R-PIA-treated rat brains were incubated with 40 nM L-[^{3}H]glutamate in binding conditions to all glutamate receptors subtypes and specifically to mGlu receptors, as described in Materials and methods, in the absence and in the presence of different mGlu receptor agonists at 100 μM to determine non-specific binding. Data are the mean ± S.E. of at least eight independent experiments performed in duplicate. ***$P<0.001$ significantly different from control. Percentage respect to each control is also indicated.

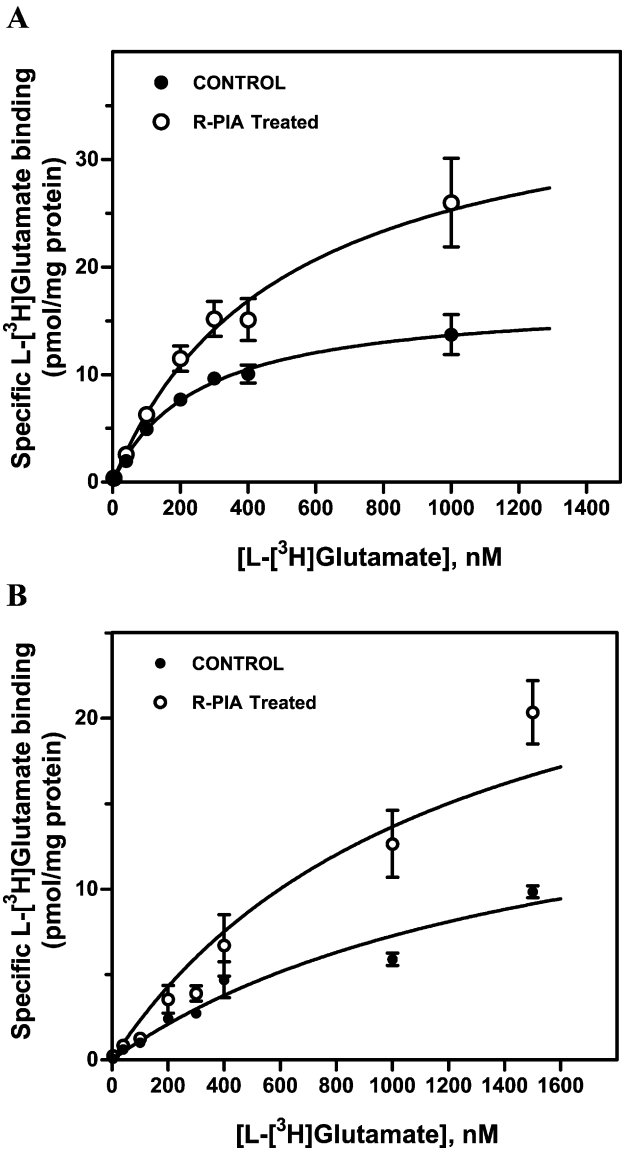

Fig. 1. R-PIA treatment effect on the saturation binding curve for both total and metabotropic glutamate receptors. SPM (60–100 μg protein) from control and R-PIA-treated rat brain were incubated with L-[^{3}H]glutamate in a concentration range from 4 to 1000 nM (panel A, total glutamate receptor binding), and from 4 to 1500 nM (panel B, metabotropic glutamate receptor binding) as described in Materials and methods. Data points are the mean ± S.E. of at least four independent experiments performed in duplicate, each using different membrane preparations. K_D and B_{max} values from Scatchard analysis of saturation curves are shown in Table 2.

Table 2
R-PIA treatment effect on kinetic parameters from saturation curves for total and metabotropic glutamate receptors

	Control	R-PIA treated
Total glutamate binding		
K_D (nM)	315.5 ± 31.4	434.2 ± 112.6
B_{max} (pmol/mg protein)	18.6 ± 1.4	33.6 ± 6.2**
Metabotropic glutamate binding		
K_D (nM)	663.3 ± 61.7	1074.7 ± 113.2***
B_{max} (pmol/mg protein)	9.5 ± 0.2	24.6 ± 3.1***

K_D and B_{max} values were determined by Scatchard analysis of data from saturation curves in Fig. 1. Data points are the mean ± S.E. of at least four independent experiments performed in duplicate, each using different membrane preparations. $^{**}P<0.005$, $^{***}P<0.001$ significantly different from control values.

sine A_1 receptor activation with R-PIA increased β_2-adrenergic receptors number in DDT_1 MF-2 cells in a time- and dose-dependent manner and decreased adenosine A_1 receptor number [12]. Similarly, we previously reported that chronic in vivo R-PIA treatment, at the same doses and administration method as that used in this work, causes a decrease (ca. 50% of control) in total adenosine A_1 receptor number and functionality in rat brain SPM. This desensitization was associated with a significant increase in cAMP level determined in SPM after treatment in basal, GTP- and GTP plus forskolin-stimulated conditions (137.5%, 120% and 132.2% of control, respectively) [31]. Basal as well as forskolin- and isoproterenol-stimulated cAMP levels were also significantly increased in adipocytes from R-PIA infused rats [32], but significantly decreased in DDT_1 MF-2 cells incubated with 3-isobutyl 1-methylxanthine (IBMX), an adenosine receptor antagonist [33]. Moreover, a mGlu receptor up-regulation following long-term β-AR activation with isoproterenol has also been reported in rat C6 glioma cells, where a B_{max} increase of 67% was detected after 24 h of isoproterenol treatment [18], when cAMP levels were also higher in treated than in control cells although β-ARs were strongly down-regulated [34]. Therefore, cross-talk between adenosine A_1 receptor and mGlu receptors detected in rat brain after R-PIA treatment could be related to increased cAMP levels.

One of the most widely described effector systems coupled to mGlu receptors is the PLC β_1 activity. Group I mGlu receptors activation causes an increase in PLC activity and inositol phosphate production through G-protein interaction, mainly $G_{q/11}$ type [26,35]. In order to study whether R-PIA treatment also causes any modulation in the PLC system, we determined this enzymatic activity in SPM from control and treated rats, using [^{3}H]PtdInsP$_2$ as exogenous substrate. First, we performed PLC assays using different free calcium concentrations. As Fig. 2A shows, PLC activity was calcium-modulated in a similar manner to that described in plasma membranes from several tissues [30,36] and intracellular organelles such as coated vesicles [37]. At low concentrations of calcium, we detected a concentration-dependent increase in PLC activity with maximum values observed at the micromolar range, while at higher calcium levels, we detected a lower PLC stimulation. This biphasic profile of concentration-dependent free calcium modulation of PLC activity was very similar in R-PIA-treated membranes. However, PLC activity was higher than those detected in control conditions at all calcium concen-

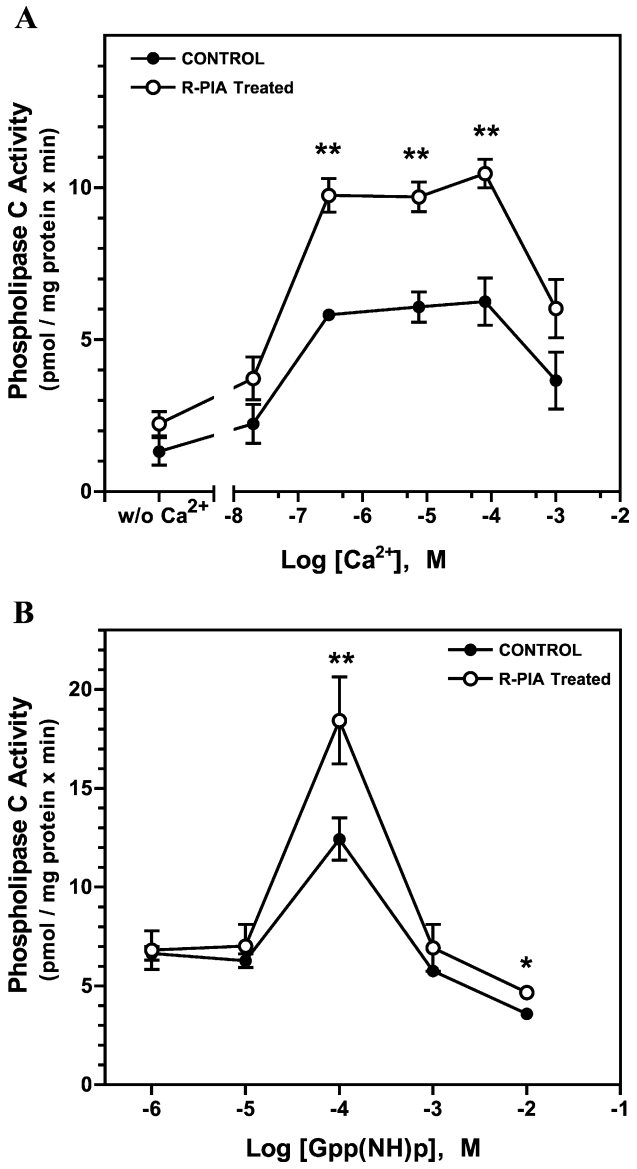

Fig. 2. R-PIA treatment effect on calcium and Gpp(NH)p modulation of PLC activity from rat brain SPMs. Ten to fifteen micrograms of SPM from control and treated rat brains were incubated with [^{3}H]PtdInsP$_2$ as exogenous substrate in the absence (w/o Ca^{2+}) and in the presence of increasing concentrations of calcium (panel A), and in the absence and in the presence of different concentrations of Gpp(NH)p at 0.3 μM free calcium (panel B). [^{3}H]PtdInsP$_2$ hydrolysis was determined as described in Materials and methods. Basal PLC activities in the absence of Gpp(NH)p were 5.29 ± 0.15 and 7.77 ± 1.30 pmol/mg protein × min in control and treated membranes, respectively. Data are the mean ± S.E. of at least four experiments performed in duplicate. $^{*}P<0.05$, $^{**}P<0.005$ significantly different from control.

trations assayed, and this difference was significant in the micromolar range (Fig. 2A), suggesting a possible modulation of PLC activity due to R-PIA treatment.

In order to determine the R-PIA treatment effect on G-protein/PLC coupling, we measured PLC activity in the presence of different concentrations of Gpp(NH)p, non-hydrolyzable analogue of GTP, and in the presence of 0.3 μM free calcium. As it can be observed in Fig. 2B, in both control and treated membranes, PLC activity is also modulated by guanine nucleotides in a biphasic manner in the concentration range assayed, being significant and maximum at 100 μM Gpp(NH)p. These results agree with previous reports in rat cerebral cortex [38], membranes prepared from rat salivary glands, GH3 cells, neutrophils, hepatocytes and cerebral cortical tissue [39] and membranes or coated vesicles from bovine brain [29]. However, an increase in PLC activity was observed after R-PIA treatment, again suggesting a positive modulation by adenosine A_1 agonist treatment. Basal and Gpp(NH)p-stimulated PLC activity were increased similarly in R-PIA-treated versus control membranes. These results suggest that this enhanced activity could be due to an increase in the steady-state level of the enzyme and, therefore, to a sensitization of the enzymatic system. In order to verify this point, we performed immunoblotting assays using specific PLC β_1 isoform antibody. As Fig. 3 shows, a significant increase in the

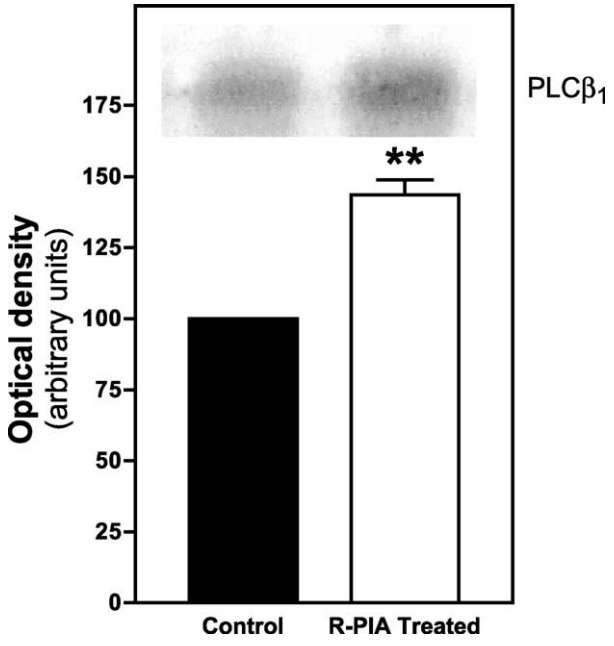

Fig. 3. Immunodetection of PLC β_1 isoform in SPMs from rat brain. Identical quantities (50 μg) of SPM from control and R-PIA-treated rat brain membranes were subjected to 7.5% SDS-PAGE, electrophoretically transferred to nitrocellulose and probed with the isoform-specific monoclonal antibody against PLC β_1 isoform, as described in Materials and methods. The figure shows densitometric quantification of four independent experiments performed with different membrane preparations. $^{**}P<0.005$ significantly different from control. Insert shows bands of a representative experiment.

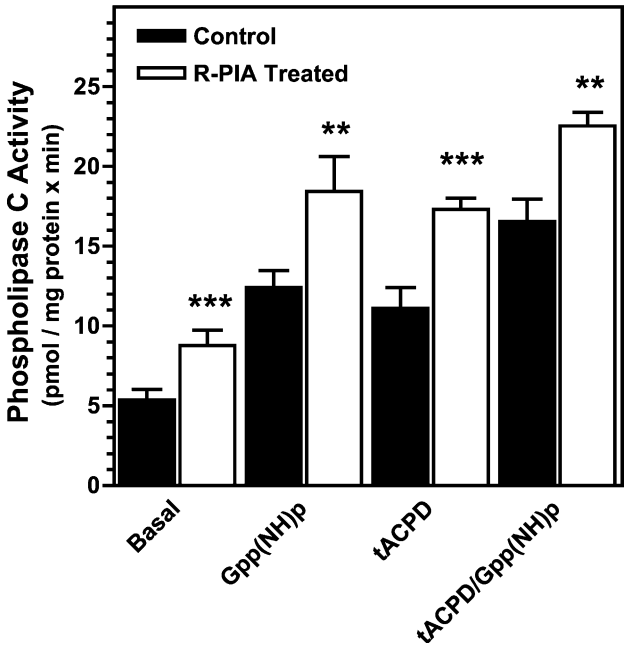

Fig. 4. R-PIA treatment effect on mGlu receptors/PLC activity coupling. Ten to fifteen micrograms of SPM membranes from control and treated rats were incubated with [^{3}H]PtdInsP$_2$ at 0.3 μM free calcium, in the presence of 100 μM Gpp(NH)p, 100 μM *trans*-ACPD or both, as described in Materials and methods. Data are the mean ± S.E. of at least four experiments performed in duplicate. $^{**}P<0.005$, $^{***}P<0.001$ significantly different from control. All data were significantly different ($P<0.001$) from its respective basal value in control and treated membranes.

quantity of β_1 isoform was obtained after treatment, approximately of the same order as that detected in PLC basal activity determined using [^{3}H]PtdInsP$_2$ as substrate, confirming that observed variation in PLC activity was due, at least partially, to an increase in the steady-state level of β_1 isoform in membranes and suggesting a cross-talk mechanism between cAMP and InsP$_3$ second messenger pathways. Similar results have been described in C6 glioma cells in which not only a sensitization of PLC activity but also a significant increase in the PLC β_1 isoform was detected after prolonged stimulation of AC pathway through β-adrenergic receptors [18]. Moreover, the increase in the PLC β_1 isoform in C6 cells was associated with an increase in cAMP levels [34], which, as commented above, were also increased in rat brain after in vivo R-PIA treatment [31]. Accordingly, continuous activation of AC system in CATH.a cells results in the desensitization of the cyclic AMP pathway and sensitization of the inositol phosphate signal, which was also obtained after 16 h incubation of cells with 1 mM Br-cAMP [40]. All these results suggest that cAMP increase could mediate PLC sensitization detected in rat brain after chronic in vivo adenosine A_1 receptor activation.

Finally, in order to study the effect of R-PIA treatment on mGlu receptor functionality, we determined the stimulatory effect exhibited by *trans*-ACPD, selective mGlu receptor agonist, on PLC activity. As it can be seen in Fig. 4, *trans*-

ACPD stimulated PLC activity (207% of basal activity) in control membranes and this effect was higher in the presence of Gpp(NH)p (309% of basal activity), therefore confirming the mGluR/PLC coupling through a G-protein. In the same figure, it can be observed that R-PIA treatment caused a significant increase in PLC activity detected in the presence of *trans*-ACPD, Gpp(NH)p or both Gpp(NH)p and *trans*-ACPD, the percentage of increment after R-PIA treatment being similar in all cases assayed. This means that mGluR/PLC pathway responsiveness to mGlu receptor agonists is maintained after treatment, reaching higher values of activity due to an increased level of PLC β_1 protein and mGlu receptor number. This increase in PLC system responsiveness has been reported in several cell cultures and related to increased intracellular cAMP levels [9]. Thus, chronic β-AR/AC stimulation with isoproterenol enhanced both basal and mGluR mediated PLC activity in C6 glioma cells [18], prolonged PACAP receptor/AC stimulation in CATH.a cells caused an increase in both basal and PACAP receptor mediated inositol phosphate production [40], and, finally, long-term incubation with forskolin or dibutyryl cAMP resulted in an increase in phosphoinositide hydrolysis stimulated by bradykinin in parallel with an increase in bradykinin receptors number in MC3T3-E1 osteoblast-like cell line [41].

Taken together, results presented herein suggest that chronic in vivo R-PIA treatment positively modulates mGluR/PLC pathway, causing an up-regulation in both its own receptor and effector system. The highlight of this work is that an in vivo cross-talk mechanism between adenosine A_1 receptor and mGlu receptor operates in rat brain after prolonged adenosine A_1 receptor activation, which could be mediated, at least partially, through increased cAMP levels. However, other effects of adenosine A1 receptors (e.g. control of calcium influx and glutamate release from nerve terminals) could also be implicated. Because both adenosine A_1 and mGlu receptors are implicated in neurodegenerative disorders, the existence of cross-modulation processes between these receptor-mediated transduction pathways in vivo could be important to be considered in future pharmacological treatments of those disorders.

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