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ARTÍCULO

Cross-talk between glutamate and nucleotide receptors in cerebellar granule neurons in culture

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ABSTRACT

ATP elicits Ca^{2+} transients in cultured cerebellar granule neurons acting through specific ionotropic (P2X) and metabotropic (P2Y) purinergic receptors. In these neurons, application of L-Glutamate (L-Glu) immediately before ATP induced a prolonged reduction of ATP-mediated responses that remains at least 5 minutes after L-Glu wash out. alpha-amino-3-hydro-5-methyl-4-isoxazolpropionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) and 3,5-dihydroxyphenyl-glycine (DHPG), selective agonists of ionotropic non-NMDA, NMDA and Group I metabotropic glutamate receptors respectively, mimicked Glu-induced attenuating effects. The activity of calcium-calmodulin dependent protein kinase II (CaMKII) seems to be involved, at least at long term, because inhibitors of CaMKII, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4phenylpiperazine

(KN-62) and N-[2-[[[3-(4'-chlorophenyl)-2-propenyl]methylamino] methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxybenzenesulfonamide (KN-93), abolished the inhibitory effect of L-Glu on ATP-mediated responses. However, it is likely that other protein kinases could be involved in the cross-talk process between both groups of receptors at short term. Therefore, these results demonstrate that the activation of glutamate receptors is able to modulate nucleotide responses in cerebellar granule neurons.

Keywords: Interaction; P2 receptors; Glutamate Receptors; Calcium-calmodulin-kinase II.

RESUMEN

Interacción entre receptores de glutamato y receptores de nucleótidos en neuronas granulares de cerebelo en cultivo

El ATP induce un incremento de Ca^{2+} en neuronas granulares de cerebelo en cultivo actuando a través de receptores purinérgicos específicos ionótropicos (P2X) y metabotrópicos (P2Y). En estas neuronas, la aplicación de L-Glutamato (L-Glu) inmediatamente antes del ATP induce una prolongada disminución de las respuestas mediadas por ATP que se mantiene al menos durante cinco minutos tras el lavado del L-Glu. Los agonistas selectivos de los receptores ionotrópicos de glutamato no-NMDA, NMDA y del Grupo I, el ácido alfa-amino-3-hidro-5-metil-4-isoxazolpropiónico (AMPA), el *N*-metil-D-aspartato (NMDA) y el 3,5-dihidroxifenil-glicina (DHPG), respectivamente, mimetizan los efectos atenuantes inducidos por el glutamato. La actividad de la proteína calcio-calmodulina quinasa II (CaMKII) parece estar implicada en este proceso, al menos a largo plazo, puesto que los inhibidores de la CaMKII, 1-[N,O-bis(5-isoquinolinesulfonil)-N-metil-L-tirosil]-4fenilpiperazine (KN-62) y N-[2-[[[3-(4'-clorofenil)-2-propenil]metilamino]metil]fenil]-N-(2-hidroxietil)-4'-metoxibenzenosulfonamida (KN-93), revierten el efecto inhibitorio del L-Glu sobre las respuestas mediadas por ATP. Sin embargo, es probable que puedan estar implicadas otras proteín quinasas en los procesos de interacción entre ambos grupos de receptores a corto plazo. Por lo tanto, estos resultados demuestran que la activación de los receptores de

glutamato son capaces de modular las respuestas a nucleótidos en neuronas granulares de cerebelo.

Palabras clave: Interacción; Receptores P2; Receptores de Glutamato; Calcio-calmodulina-quinasa II.

1. INTRODUCTION

Cerebellar granule neurons have a critical role in cerebellar function coordinating afferent input to and motor output from the cerebellum. Thus, granule cells receive excitatory glutamatergic contacts from mossy fibres and they, in turn, contact synaptically with the dendrites of Purkinje neurons via parallel fibres. Excitatory synaptic transmission between mossy fibres and granule neurons is mediated by L-Glu (1). The actions of L-Glu are mediated by a group of receptors named glutamate receptors which have been subclassified into ionotropic glutamate receptors (NMDA and non NMDA) and metabotropic glutamate receptors that includes Group I, II and III. Cerebellar granule cells express both Ca^{2+} permeable NMDA and non-NMDA glutamate receptors (2). Furthermore, group I metabotropic glutamate receptor involved in the glutamate activation of phospholipase C (PLC) and Ca^{2+} mobilization have also been described in granule cells growth *in vitro* (3). Recently, we have shown the presence and expression of functional nucleotide receptors in highly purified cultures of cerebellar granule neurons (4-8). The family of nucleotide receptor includes ionotropic receptors, P2X, which form ligand gated cation channels and mediate fast excitatory responses; and metabotropic nucleotide receptors, P2Y, which belong to the G-protein-coupled receptor superfamily (9, 10).

As our understanding of cellular signalling pathways has advanced, it has became increasingly apparent that, far from regulating linear transduction pathways, receptor activation can result in much more complex patterns of signalling within cells. Thus, the activation of neurotransmitter receptors by their own transmitter has been shown to be modulated by distinct receptors when they are also activated by their own transmitter. This mechanism, generally termed cross talk, provides a fast and efficient

way to adapt transmitter signalling to change functional needs. Previous works have shown a close interaction between nucleotidic receptors and acetylcholine (ACh) nicotinic receptors or GABA receptors (11-14), which was paralleled by a co-release of ATP and ACh or GABA from central terminals and spinal cord (15, 16). Co-release of ATP with L-Glu from nerve terminals (17), raised the possibility of interaction between glutamate and nucleotide receptors. This was further supported by double immunofluorescence experiments with confocal laser microscopy which revealed co-localization at the membrane level between the $P2Y_4$ and NMDA receptors, in both homologous (cerebellar granule neurons) and heterologous (HEK-293) cellular systems (18).

In the present work we have analyzed the possible cross-talk mechanism between glutamate and nucleotide receptors. Results obtained suggest that L-Glu evoked a prolonged depression of subsequent ATP-evoked calcium rises in granule neurons in a process in which calcium-calmodulin dependent protein kinase II seems to be involved.

2. MATERIALS AND METHODS

2.1. Cerebellar granule neurons in culture

All experiments carried out at the Universidad Complutense of Madrid followed the guidelines of the International Council for Laboratory Animal Science (ICLAS).

Cerebellar cultures from three Wistar rat pups (7 days old) were prepared following procedures described previously (19).

2.2. Calcium imaging

9 days *in vitro* (div) cerebellar granule neurons attached to glass coverslips were washed, under normal K^+ conditions, with Locke's solution (in mM: NaCl, 140; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; glucose, 5.5; HEPES, 10; pH 7.4) and loaded with 7.5 μM Fura-2 AM (Molecular Probes; Eugene, OR, USA) for 45 min

at 37º C. The coverslip was placed in a small superfusion chamber in the stage of a NIKON TE-200 microscope and were superfused with different nucleotide and glutamate receptors agonists, such as adenosine triphospate (ATP), L-Glu, alpha-amino-3-hydro-5-methyl-4-isoxazolpropionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) and 3,5-dihydroxyphenyl-glycine (DHPG), all of them obtained from Sigma (St. Louis, MI, USA). When the role of CaMKII on cross-talk was studied, granule neurons were preincubated with the CaMKII antagonists, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4phenylpiperazine (KN-62) and N-[2-[[[3-(4'-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxybenzenesulfonamide (KN-93) (both obtained from Tocris; Bristol, UK), for 20 minutes before to start the stimulations, in order to assay a full blockade of CaMKII.

A pulse of 30 mM KCl was applied at the end of each experiment to confirm the functionality and viability of the neurons under study.

The images were obtained using microscope 40 X lens and setting the incoming light at 340 and 380 nm. Images were captured using an ORCA-ER C 47 42-80 camera from Hamamatsu (Hamamatsu City, Japan) controlled by MetaFluor 6.2r6 PC software (Universal Imaging, Cambridge, UK). Time course data represent the average fluorescence intensity in circular regions located in each soma.

2.3. Immunocytochemical studies and fluorescence quantification

9 div cerebellar granule neurons were fixed for 15 min with 4% PFA (Sigma) in PBS (w/v). After several washes in PBS, cells were incubated for 1 h in PBS containing 5% donkey serum (v/v), 3% BSA (w/v) and 0.1% Triton X-100 (v/v). After that, neurons were incubated with the primary antibodies as follows: anti-vesicular glutamate transporter-1 (VGLUT1) at 1/1000 dilution and anti-vesicular glutamate transporter-2 (VGLUT2) at 1/500 dilution (both antibodies were obtained from Synaptic Systems; Goettingen, Germany). As secondary antibody we used TRITC-goat anti-rabbit IgG, at 1/500 dilution (Sigma). We used as a presynaptic marker an anti-synayptophysin primary antibody, 1/500 (Sigma), revealed with a FITC-goat anti-mouse IgG 1/500 as secondary antibody (Sigma).

For the immunocytochemical detection of P2X7 and $P2Y_1$, cells were incubated with primary antibodies that recognized the specified rat proteins: rabbit anti-P2X7 (1/100) and rabbit anti-$P2Y_1$ (1/100) (Chemicon International, Temecula, CA, USA); mouse anti-synaptophysin (1/500). As secondary antibodies were used: goat anti-mouse IgG fluorescein conjugated (1/500), goat anti-rabbit IgG rhodamine conjugated (1/500).

Immunocytochemical detection of phospho-CaMKII and total CaMKII in granule neurons stimulated with L-Glu (100 µM, 5 min) was carried out following procedures described previously (6). Primary antibodies used in this work recognized the specified rat proteins: rabbit anti-phospho-CaM-kinase II a/b (Thr286/287) (1/100) (Upstate Charlottestville, VA, USA) and mouse anti-b-CaM-kinase II (1/100) (Zymed Laboratories, San Francisco, CA, USA).

In all cases, incubations with primary and secondary antibodies were done at 37 ºC for 1 h. In every experiment incubation with the control antigen was made following the manufacturer´s instructions. Controls were performed following the same immunocytochemical procedure but replacing primary antibodies by the same volume of PBS-BSA solution.

Immunofluorescence images were captured digitally using a Kappa DX2 camera controlled by Kappa Image Base Control software. For each culture, acquisition parameters were adjusted in order to prevent brightest fluorescence from saturating the mean intensity of granule neuron. These conditions were maintained with the subsequent images. Three or four images under specific conditions were captured in each one of the cultures. Fluorescence quantification was performed as previously described (6).

2.4. Statistical analysis

Data are presented as mean ± SEM, and differences were tested by one-way ANOVA with Bonferroni´s post test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. General characteristics of cultured granule neurons

Cerebellar granule neurons were cultured following protocol described by Hervás and co-workers (4). Thus, in our culture conditions, no cells exhibited the glial marker protein GFAP (not shown), but all contained the neuronal vesicular marker synaptophysin and exhibited a glutamatergic nature since they were labelled with antibodies to the vesicular glutamate transporters, VGLUT1 and VGLUT2 (Figure 1). Furthermore, granule neurons exhibited a good response to K^+ ions (100%), and most cells to 100 μM L-Glu (90-95%). The prominent calcium entrance response induced by K^+ ions was an additional control to demonstrate the neuronal nature of the single cell under study.

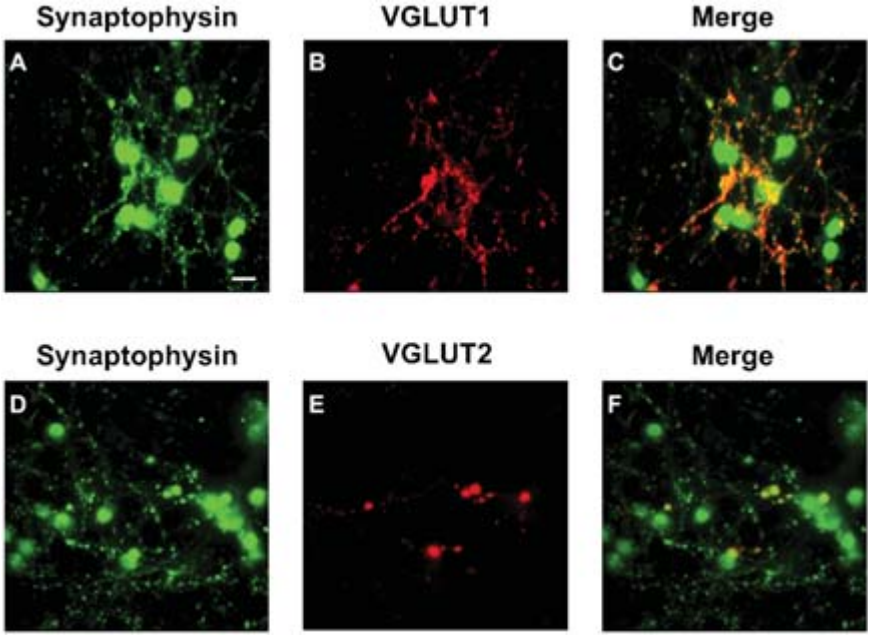

Figure 1. Immunodetection of VGLUT1 and VGLUT2 in 9 div cerebellar granule neurons. A, D) Immunostaining of granule neurons with the presynaptic marker synaptophysin (green). **B, E)** Detection of VGLUT1 and VGLUT2 (red), respectively, in granule neurons. **C)** Co-localization of synaptophysin and VGLUT1, showed in yellow. **D)** Idem for synaptophysin and VGLUT2. Scale bar: 10 μm.

Immunocytochemical studies also demonstrate the presence of ionotropic (such as P2X7, Figure 2A) and metabotropic (such as $P2Y_1$, Figure 2D) purinergic receptors in rat cultured cerebellar granule neurons. However, these cells showed different calcium responses to ATP. To determine the dose-response relations, neurons were challenged with several concentrations of ATP. Results, expressed as a percentage of cells responding per total number of cells tested for each concentration, have shown the following values: a) 1 μM ATP, 10%; b) 100 μM ATP, 30%; c) 200 μM ATP, 50% (not shown). It is necessary to consider that we are only measuring responses to added nucleotides, not to the hydrolysis products (mainly adenosine) that could appear due to ectonucleotidases action. The continuous superfusion of the cell chamber avoids concentration of any hydrolytic product in the extracellular medium.

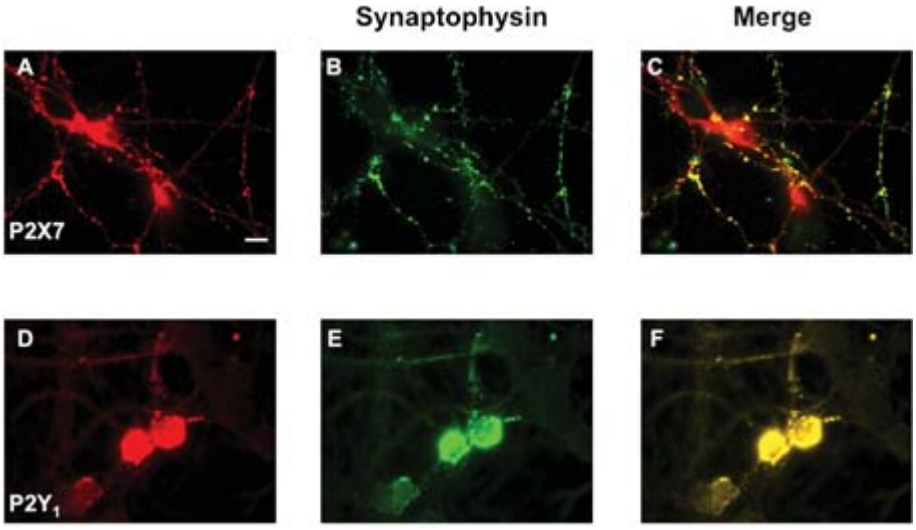

Figure 2. Immunocytochemical location of purinergic receptors in fibres and somas of 9 div cerebellar granule neurons. In red is shown the presence of ionotropic P2X7 **(A)** and metabotropic $P2Y_1$ **(D)** receptors. The immunolocation of the presynaptic marker synaptophysin is shown in green **(B, E)**. The co-localization of synaptophysin and P2X7 and $P2Y_1$ subunits is shown in yellow (**C, F**, respectively). Scale bar: 5 μm.

3.2. Activation of glutamate receptors decreases the ATP-induced intracellular calcium increase

Using doses of ATP to which 50% of the cells responded, we investigated by microfluorimetric experiments whether the calcium entrance mediated by glutamate receptor could modify the $[Ca^{2+}]_i$ increase induced by ATP. To perform this study granule neurons were stimulated first with 200 μM ATP, followed by a second double pulse of 100 μM L-Glu and 200 μM ATP, and afterwards by 200 μM ATP in a third pulse. Before each ATP pulse, the preparation was always washed with the Locke's solution for five minutes. As it is shown in Figure 3A, the mean calcium increase induced by the first application of 200 μM ATP was 209 ± 38 nM (n = 33 neurons), once Grynkiewicz's equation (20) had been applied. Similarly, when granule neurons were challenged with 100 μM L-Glu, the mean calcium increase observed was 210 ± 34 nM (n = 33 neurons). However, when L-Glu (100 μM) was applied immediately before ATP (200 μM), ATP-calcium transients were significantly reduced to a value that was 60 ± 3% of their initial value ($p < 0.001$, n = 33 neurons). In these neurons, ATP-mediated responses remained depressed 5 min after L-Glu (100 μM) application (by 73 ± 3%; $p < 0.001$, n = 33 neurons).

In order to investigate the possible inhibitory effect of L-Glu on metabotropic P2Y receptor activation, ATP (200 μM) was assayed in a virtually Ca^{2+}-free medium ($[Ca^{2+}] \sim 50$ nM) by using a Ca^{2+} chelator mixture of 5 mM EGTA/5.5 mM Tris (21). As Figure 3B shows, in the presence of EGTA, individual granule neurons responded to ATP which confirmed the presence of functional P2Y receptor, accordingly with previous results (4). When ATP (EGTA)-mediated responses were analysed after L-Glu stimulation, a significant reduction could be detected in both times assayed (0 min, 76 ± 6%, $p < 0.001$, n = 34 neurons; 5 min, 78 ± 4%, $p < 0.01$, n = 34).

The effect of L-Glu on ATP-mediated responses was concentration dependent. Thus, when granule neurons were challenged with L-Glu 10 μM, the second peak of calcium induced by ATP declined to a value that was 72 ± 5% of their initial peak amplitude ($p < 0.01$, n = 126 neurons). However, ATP-mediated response was fully recovered five minutes after granule neurons were stimulated with

10 μM L-Glu (105 ± 3%, $p > 0.05$, n = 126 neurons) (Figure 3C). As it can be observed in Figure 3D no calcium increases in response to 1 μM L-Glu were detected in neither of cells studied (n = 132 somas). Besides, ATP-mediated responses did not undergo any significant variations after exposition to 1 μM L-Glu. The pooled average data compared with the first ATP-induced current were 108 ± 4% ($p > 0.05$, n = 132 neurons) and 111 ± 4% ($p > 0.05$, n = 132 neurons).

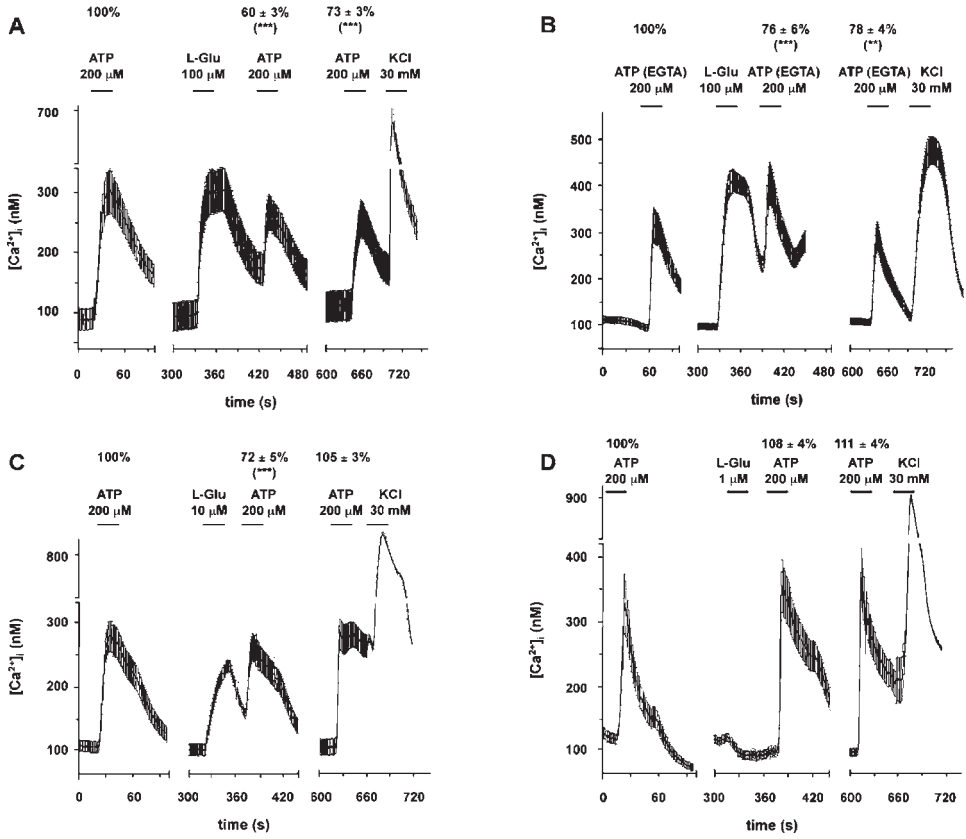

Figure 3. Cross-talk between ATP and L-Glu responses in somas of 9 div cultured granule neurons. Effects of several concentrations of L-Glu (100, 10 and 1 μM) on ATP-mediated responses measured in presence **(A, C, D)** or absence of Ca^{2+} **(B).** ATP-induced currents were studied 30 s and 5 min after L-Glu application and were compared with the first ATP-response for each neuron. Statistical significance was calculated by anova test: ***, $p < 0.001$; **, $p < 0.01$, significantly different from first ATP-mediated response.

3.3. Both NMDA and non-NMDA ionotropic glutamate receptors reduce the ATP-induced intracellular calcium increase

L-Glu acts as a mixed agonist for all classes of ionotropic glutamate receptor. To investigate the glutamate receptor involved in the L-Glu inhibitory effects on nucleotide receptors, we tested the actions of AMPA, NMDA and non-NMDA receptors agonists respectively, on ATP-induced responses. NMDA (100 μM) applied immediately before ATP (200 μM) increased $[Ca^{2+}]_i$ by itself (214 ± 20 nM; n = 70 neurons) and significantly suppressed the $[Ca^{2+}]_i$ increase induced by ATP (Figure 4A). The pooled average depression compared with the control ATP-induced response were 70 ± 11% ($p < 0.01$, n = 70 neurons) and 75 ± 2% ($p < 0.05$, n = 70 neurons) at 0 and 5 min, respectively. Similar results were observed when AMPA (100 μM) was assayed (Figure 4B). Thus, AMPA was also able to increase $[Ca^{2+}]_i$ by itself (254 ± 29 nM; n = 57 neurons) and to depress ATP-induced currents in a similar extent at both times studied (77 ± 2%, $p < 0.001$, n = 57 neurons at 0 min and 77 ± 4%, $p < 0.001$, n = 57 neurons at 5 min).

3.4. Group I mGluR activation decrease ATP-induced responses

Previous works have shown the presence of functional Group I mGluR in cerebellar granule neurons (3) where it is probably that they are involved in synaptic plasticity (22). The possible participation of Group I mGluR in the L-Glu inhibitory effects on nucleotide receptors was studied using 100 μM DHPG as specific agonist (23). As it is shown in Figure 4C, DHPG (100 μM) increased $[Ca^{2+}]_i$ (133 ± 58 nM) and also significantly decreased ATP-induced responses to a value that was 52 ± 5% of their initial value ($p < 0.001$, n = 50 neurons). This loss of responsiveness remained 5 min after DHPG (100 μM) application (60 ± 5%, $p < 0.001$, n = 50 neurons).

3.5. The inhibitory effect of glutamate on ATP-mediated responses is not associated to P2 receptor desensitization

Since different P2X and P2Y receptors can undergo a decline in current amplitude in response to repeated applications of ATP (24, 25), we investigated if this desensitization process could be involved

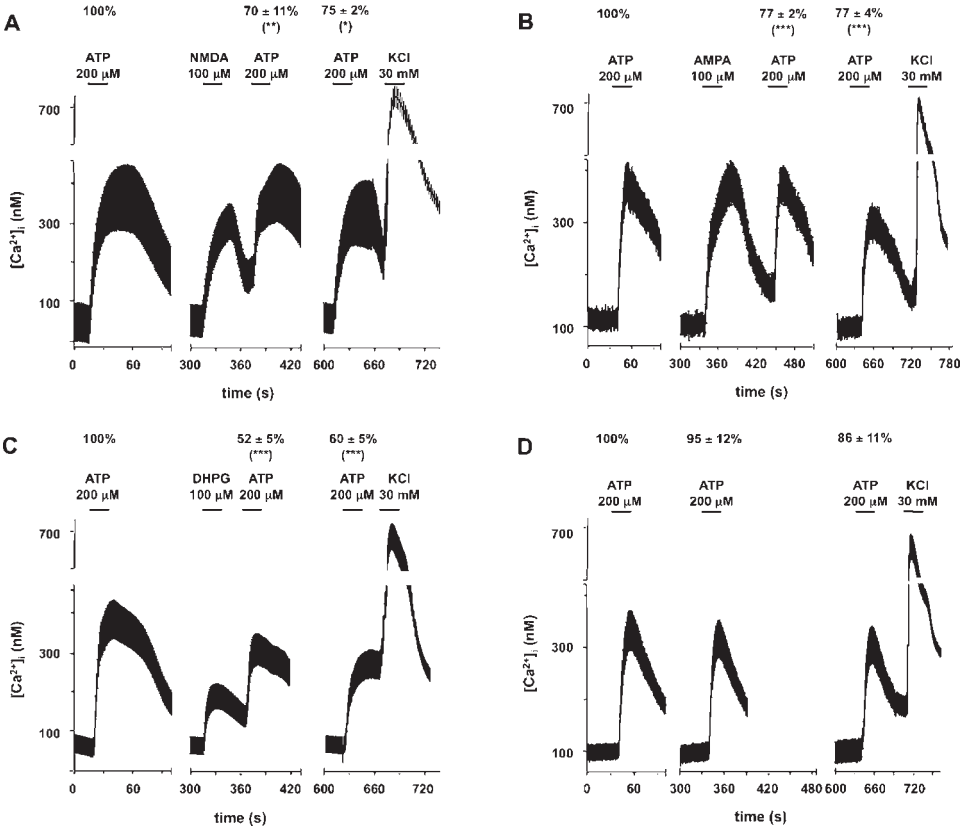

Figure 4. Effects of 100 μM NMDA (A), 100 μM AMPA (B) and 100 μM DHPG (C) on ATP-mediated responses in somas of 9 div cultured granule neurons. ATP-induced currents were studied 30 s and 5 min after glutamate receptor agonist application and were compared with the first ATP-response for each neuron. Changes in $[Ca^{2+}]_i$ in Fura-2-loaded granule neurons following repetitive stimulation with 200 μM ATP (D). ***, $p < 0.001$; **, $p < 0.01$: *, $p < 0.05$ significantly different from first ATP-mediated response.

in the decrease of ATP-mediated responses observed after L-Glu exposition. Thus, granule neurons were stimulated with 200 μM ATP three times at 5 min-intervals. Although both second and third peaks of calcium resulted slightly reduced compared to the first one (95 ± 12% and 86 ± 11%, respectively), statistical analyses did not reveal any significant differences between them (Figure 4D).

3.6. CaMKII is involved in the cross-talk between glutamate and nucleotide receptors

In order to find a possible mechanism in the cross-talk between glutamate and nucleotide receptors, we searched within the different receptors sequences for possible phosphorylation consensus sequences by protein kinases. Prediction of possible phosphorylation sites were carried out by using computer programs such as GPS 1.10 (26), Scansite 2.0 and ExPASy (27, 28). Thus, GPS (cut-off value 2.5) and Scansite (low stringency) analyses identified different residues in both P2X and P2Y receptors susceptible to be phosphorylated by CaMKII, whereas ExPASy checked that these residues faced the cytoplasm (Table 1).

Table 1. **Sequences for possible phosphorylation consensus sequences by protein kinases.** Prediction of possible phosphorylation sites were carried out by using computer programs such as GPS 1.10, Scansite 2.0 and ExPASy.

Receptor	Site	Sequence	GPS	Scansite	ExPASy
				Score	Cytoplasmatic
P2X2	Ser-431	PRPC**S**ISAL	4.35	0.56	348-472
P2X3	Thr-382	SDQA**T**VEKQ		0.54	339-397
	Thr-388	EKQS**T**DSGA	3.64	0.58	
P2X7	Ser-560	WRFV**S**QDMA	3.81	0.54	356-595
	Ser-23	TRIQ**S**VNYG	3.27	0.59	1-25
$P2Y_1$	Ser-258	LRRK**S**IYLV	3.08	0.55	239-265
$P2Y_2$	Ser-351	RKDL**S**ISSD	3.68	0.49	309-374
$P2Y_6$	Thr-59	RRTL**T**RSAV	3.38	0.60	49-62

Additionally, we investigated if calcium entry after glutamate receptor activation was able to induce the phosphorylation of CaMKII. Results showed that L-Glu (100 μM) was able to induce an important increase in CaMKII phosphorylation in granule neurons, at both experimental times, 1 and 5 min (219 ± 33% and 387 ± 19%, respectively) (Figure 5A and 5B). As a control, immunostaining studies —with the antibody that recognizes both non-phosphorylated and phosphorylated forms of CaMKII— showed no differences in the labelling intensity between control and L-Glu-stimulated cells, as was expected for total enzyme detection (Figure 5A).

Then, we used the selective CaMKII antagonists, KN-62 (10 μM) and KN-93 (10 μM). The effective inhibitory concentration values of both antagonists have been reported to be 1 μM (29, 30) with no significant effect observed at concentrations 100 μM on activities of other kinases, such as MLCK, PKC, or cAMP-dependent protein kinase. As it can be observed in the Figures 5C and 5D, when ATP-mediated responses were measured five minutes after L-Glu (100 μM) exposition in granule neurons pre-treated with KN-62 and KN-93, the inhibitory effects of L-Glu on ATP-induced currents were abolished. The pooled average data compared with the first ATP-induced current were: KN-62, 92 ± 3% ($p > 0.05$, n = 67 neurons); KN-93, 87 ± 5% ($p > 0.05$, n = 45 neurons). However, neither KN-62 nor KN-93 was able to abolish the inhibitory effect of L-Glu on ATP-mediated responses measured immediately after L-Glu exposition.

4. DISCUSSION

The present study confirms the existence of a negative cross-talk between nucleotide receptors activated by ATP and glutamate receptors on granule neurons growth in culture. The relevance of these results relies not only because it demonstrates a new type of interaction between receptors with different structure and function but also because it allows to speculate the presence of a similar interaction between both group of receptors in other brain regions, such as hippocampus and cortex, where ATP-containing synaptic vesicles can coexist with L-Glu-containing ones and, therefore, release of both neurotransmitters can occur simultaneously (31).

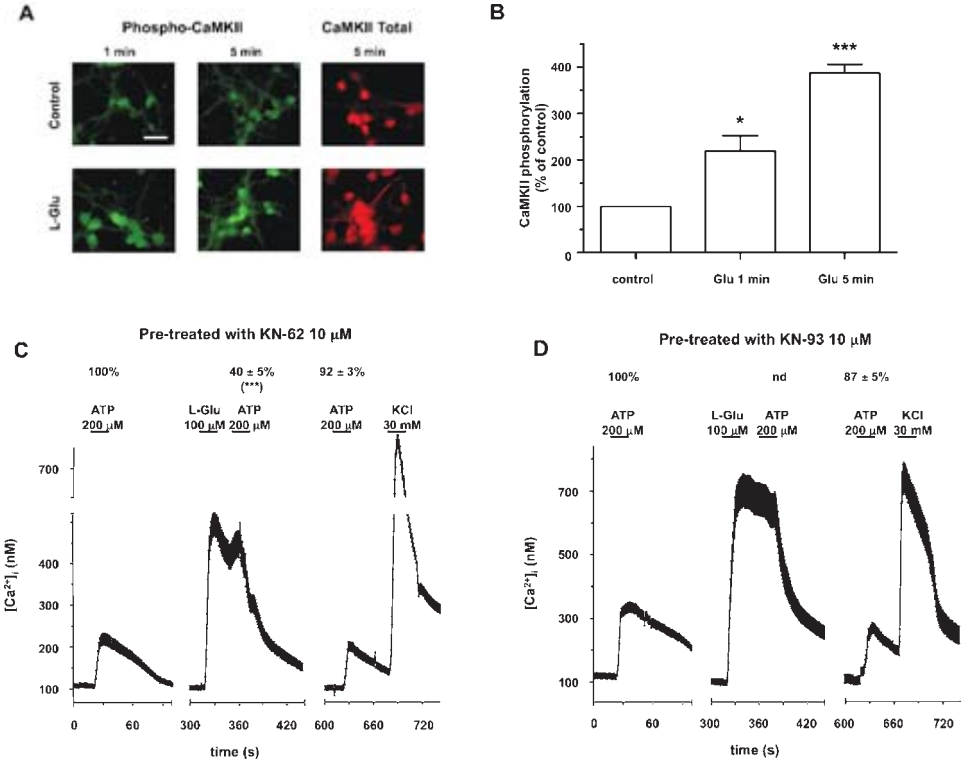

Figure 5. Effect of L-Glu on CaMKII phosphorylation at Thr286/287 9 div cerebellar granule cells. Neurons were stimulated with L-Glu 100 μM for 1 and 5 min, fixed with PFA and treated with antibodies that recognized either phospho-CaM-Kinase II a/b (Thr286/287) or the total CaMKII **(A)**. **(B)** Respectively, the quantification of fluorescence obtained in cellular somas. Bars indicate the mean ± SEM of three independent experiments. Statistical significance was calculated by anova test: ***, $p < 0.001$; *, $p < 0.05$. **(C, D)** Effect of L-Glu (100 μM) on ATP-mediated responses measured in somas after pre-treatment with KN-62 10 μM (C) and KN-93 10 μM (D). ATP-induced currents were studied 30 s and 5 min after L-Glu application and were compared with the first ATP-response for each neuron. Statistical significance was calculated by anova test: ***, $p < 0.001$; significantly different from first ATP-mediated response; nd, not significantly different from first ATP-mediated response. Scale bar: 30 μm.

We have used in this study cerebellar granule neurons which express both glutamate and nucleotide receptors (2-4). With these cells we measured the functional response by monitoring the agonist-induced Ca^{2+} release in microfluorimetric experiments. Accordingly with previous works, granule cells showed a heterogeneous pattern

of calcium responses to ATP (4, 8). Although we have not investigate the inhibitory effect of L-Glu on individual nucleotide receptors, results obtained have clearly shown that all ATP responses, including both ionotropic and metabotropic nucleotide receptors, undergo a significant decrease after L-Glu addition, suggesting, therefore, that inhibition of ATP-mediated responses evoked by L-Glu was a general phenomenous and not associated to a specific group of nucleotide receptor. The inhibitory effect of L-Glu on ATP-mediated responses was dose-dependent. Thus, 100 μM and 10 μM L-Glu caused a strong/medium effect on ATP-evoked Ca^{2+} rises whereas 1 μM L-Glu did not affect responses induced by ATP. These data suggest, therefore, that L-Glu concentrations play an important role in the interaction between both groups of receptors. The possibility that L-Glu inhibited ATP-mediated Ca^{2+} rises by a direct action on nucleotide receptors was excluded when different agonists of glutamate receptors were tested and data showed that they were also able to depress ATP currents.

In the present work glutamate receptor agonist not only acutely depresses ATP-elicited calcium rises, but also induces a depression in ATP responsiveness that remains even after 5 minutes the agonist gone. By using selective agonists of ionotropic glutamate receptor, NMDA and AMPA, and Group I-mGluR, DHPG, we could investigate the individual contribution of each receptor to the cross-talk process. Thus, both ionotropic agonists, NMDA and AMPA, caused a similar decrease on ATP-mediated responses whereas the inhibitory effect of DHPG was slightly higher. Another interesting point treated was if the loss of ATP responsiveness observed after L-Glu exposure could be due to desensitization of nucleotide receptor and not by a cross-talk process between both groups of receptors. In that sense, previous works have shown that different P2X and P2Y receptors can undergo a decline in current amplitude in response to repeated applications of ATP (24, 25). Results obtained have allowed excluding this possibility since the amplitude of the ATP-mediated response remains unchanged in granule neurons challenged three consecutives times with ATP. Therefore, these results demonstrate that L-Glu through binding ionotropic (both NMDA and non-NMDA receptors) and metabotropic glutamate receptor (Group I) caused a prolonged heterologous desensitization in nucleotide receptors.

Once demonstrated that glutamate modulates nucleotide receptors signalling in cerebellar granule neurons, the main question was to understand the biochemical components involved in their interaction. Several evidences suggested that CaMKII could participate in the cross-talk process. Thus, P2X2, P2X3, P2X4, P2X7 and $P2Y_1$, which distribution in somatic regions from granule neurons have been previously shown (4, 6), posses sequences susceptible to be phosphorylated by CaMKII. Furthermore, a large number of proteins have been described as being associated with P2X7 (32). One of these proteins is a-actinin, which also interacts with CaMKII and may be a way to locate CaMKII near its substrates (33). Concerning to metabotropic P2Y receptor, a previous report has shown that endocytosis of the $P2Y_1$ receptor is controlled by the activity of CaMKII (25). Finally, CaMKII is phosphorylated in response to glutamate receptor activation. Based on these data, it is probably that CaMKII was involved in the cross-talk mechanism between glutamate and nucleotide receptors. To verify the phosphorylation hypothesis, we analyzed the effect of CaMKII antagonists, KN-62 and KN-93. The reversion of the long-term L-Glu-inhibitory effects on nucleotide receptor response, by inhibiting the CaMKII, confirmed the role of this protein kinase on the reported effect. However, it is interesting to note that the immediate inhibitory effects induced by L-Glu were not abolished by CaMKII antagonist suggesting the involvement of other kinases. It should be noticed that although KN-62 has been described as antagonist of human P2X7 receptor (34) the differences in the amino acid sequence of the first 335 residues between human and rat P2X7 receptor have been described to change completely the sensitivity to KN-62. Thus, in HEK 293 cells stably transfected with rat P2X7 receptor KN-62 did not antagonize neither ATP activation of cation currents nor ethidium influx induced by ATP (35). In any case, we have additionally verified the lack of antagonism of KN-62 on P2X7 receptor in microfluorimetric experiments where the extracellular Ca^{2+} entrance induced by BzATP was not decreased in the presence of KN-62 (data not shown).

Multiple temporarily regulated mechanisms are used to modulate the efficiency of synaptic transmission; in the present work we have described a new one which involves glutamate and nucleotide

receptors and that result in a prolonged inhibitory effect of $[Ca^{2+}]_i$ increase. In the mechanism involved for this control is participating the CaMKII, although other kinases seem to be involved, at least at short term. Additional work will be necessary to investigate the possibility of glutamate/ATP receptors interaction in cortex and hippocampus where release of both neurotransmitters can occur simultaneously.

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