INVITED REVIEW

Esmerilda G. Delicado · M. Teresa Miras-Portugal ·
Luz María G. Carrasquero · David León ·
Raquel Pérez-Sen · Javier Gualix

Dinucleoside polyphosphates and their interaction with other nucleotide signaling pathways

Abstract Dinucleoside polyphosphates or Ap_nA are a family of dinucleotides formed by two adenosines joined by a variable number of phosphates. Ap_4A, Ap_5A, and Ap_6A are stored together with other neurotransmitters into secretory vesicles and are co-released to the extracellular medium upon stimulation. These compounds can interact extracellularly with some ATP receptors, both metabotropic (P2Y) and ionotropic (P2X). However, specific receptors for these substances, other than ATP receptors, have been described in presynaptic terminals form rat midbrain. These specific dinucleotide receptors are of ionotropic nature and their activation induces calcium entry into the terminals and the subsequent neurotransmitter release. Calcium signals that cannot be attributable to the interaction of Ap_nA with ATP receptors have also been described in cerebellar synaptosomes and granule cell neurons in culture, where Ap_5A induces CaMKII activation. In addition, cerebellar astrocytes express a specific Ap_5A receptor coupled to ERK activation. Ap_5A engaged to MAPK cascade by a mechanism that was insensitive to pertussis toxin and required the involvement of src and ras proteins. Diadenosine polyphosphates, acting on their specific receptors and/or ATP receptors, can also interact with other neurotransmitter systems. This broad range of actions and interactions open a promising perspective for some relevant physiological roles for the dinucleotides. However, the physiological significance of these compounds in the CNS is still to be determined.

Keywords ATP receptors · Calcium · CaMKII · Dinucleotides · Diadenosine pentaphosphate · ERK activation · Growth factors · Nucleotide receptors

E. G. Delicado · M. T. Miras-Portugal (✉) ·
L. M. G. Carrasquero · D. León · R. Pérez-Sen · J. Gualix
Departamento de Bioquímica, Facultad de Veterinaria,
Universidad Complutense,
28040 Madrid, Spain
e-mail: esmerild@vet.ucm.es
Tel.: +34-1-3943892
Fax: +34-1-3943909

Overview of dinucleotides

Dinucleotides or dinucleoside polyphosphates, commonly abbreviated as Np_nNs, comprise a group of compounds formed by two nucleoside moieties linked by their ribose 5′-ends to a variable number of phosphates, which can change from two to seven. The most abundant and widely studied of these dinucleotides contain either one or two adenine bases in their structure. They are naturally occurring substances ubiquitously present in the cytoplasm of prokaryotic and eukaryotic cells where they are synthesized by some aminoacyl-tRNA synthetases and other enzymes [60]. The cytosolic levels of the dinucleoside polyphosphates are modified under a variety of physiological and pathological conditions and these compounds have emerged as putative intracellular signaling implicated in the regulation of essential cellular process [40, 45]. Some of the intracellular functions of adenine dinucleotides may be related to environmental stress [3, 4, 70–72]. Apart from their well-known intracellular roles, diadenosine polyphosphates also exert important extracellular actions, which are of special relevance in the vascular system. These compounds are stored in the secretory dense granules of platelets and are released into the circulation when platelets become activated. Circulating diadenosine polyphosphates have deep effects on platelet aggregation and are involved in the control of vascular tone and myocardial function [21, 64, 69]. Our group has been largely working on the presence and functional significance of diadenosine polyphosphates in the nervous system. We have demonstrated that these compounds fulfil with the requirements to be considered as signaling molecules in the CNS [46]. Diadenosine polyphosphates are co-stored with ATP and classical neurotransmitters in the secretory granules of chromaffin cells, the cholinergic synaptic vesicles from *Torpedo marmorata* electric organ, and in rat brain synaptic terminals [50, 57, 62]. They are co-released to the extracellular media in a calcium-dependent manner and can interact extracellularly with membrane receptors. Finally, their effects are terminated by the action of ecto-Ap_nA-hydrolases, rendering adenosine

as final product. This nucleoside is re-uptaked into the cells by means of the nucleoside transporter [46].

Due to their close structural relationship, some of the extracellular actions of diadenosine polyphosphates can be attributed to the interaction of these compounds with ATP receptors. The ATP-sensitive receptors are divided into two families, ionotropic P2X (multimeric ligand-gated cationic channels) and metabotropic P2Y receptors (G-protein-coupled 7TM receptors) [61]. Both P2X and P2Y receptors are present in the nervous system and their expression change during development [9, 75]. Seven P2X subunits have been cloned to date (P2X$_1$, P2X$_2$, P2X$_3$, P2X$_4$, P2X$_5$, P2X$_6$, and P2X$_7$), which can assemble in different combinations to form homomeric or heteromeric P2X receptors. Similarly to the great variety on ionotropic receptors, eight metabotropic P2Y receptors have been currently characterized (P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$). Among the great diversity of nucleotidic receptors, P2X$_1$, P2X$_3$, and also P2Y$_1$ receptors have been described as possible targets for dinucleotides. This opens a broad spectrum of actions for dinucleotides in the CNS considering the wide distribution of multiple types of P2X and P2Y receptors at this level. The question regarding the role of these dinucleotides in the CNS that needs to be addressed is: are there specific receptors for diadenosine polyphosphates different from ATP receptors? This issue presented some difficulties to overcome, first due to the poor available tools, specific agonists and antagonists, which could allow to clearly distinguish the actions mediated through the different types of nucleotidic receptors.

Specific targets for dinucleotides at the presynaptic level in the nervous system

In spite of these initial problems, the presence of a specific receptor for dinucleotides of ionotropic characteristic was firstly described in the pioneering work of Pintor and Miras-Portugal (in 1995), who found specific responses for dinucleotides in midbrain synaptic terminals. The increase in the intrasynaptosomal calcium concentration mediated by diadenosine polyphosphates was unaffected after cross-desensitization with ATP, when Ap$_n$A and ATP were consecutively applied, though each agonist was able to desensitize itself. Additional proof for the presence of independent presynaptic receptors for ATP and Ap$_n$A was provided by the use of the classical P2 antagonists PPADS and suramin. These compounds did not affect the Ca^{2+}-increase evoked by diadenosine polyphosphates but blocked Ca^{2+} entry induced by ATP and its synthetic analogues [54]. The synthesis of a new series of dinucleotides denoted diinosine polyphosphates, which resulted by enzymatic deamination of the corresponding diadenosine polyphosphate, supposed a novel contribution in the characterization of dinucleotide receptors. These substances did not show any agonistic effect on the rat synaptosomal receptors but effectively antagonized the responses induced by Ap$_n$A. Diinosine pentaphosphate (Ip$_5$I) was the most potent among these substances, being able to block Ap$_n$A responses at concentrations in the nanomolar range (IC$_{50}$=4 nM). In contrast, micromolar concentrations of the antagonist were required to abolish ATP-elicited responses in the same preparation [52]. In addition, a series of experiments using structural analogs of the diadenosine polyphosphates demonstrated that the Ap$_n$A receptor is also sensitive to guanosine polyphosphates (Gp$_n$G) as agonists [51]. This is noteworthy, as the different P2 receptors described so far (either P2X or P2Y receptors) only show sensitivity to adenine or uridine nucleotides (ATP, ADP, UTP, and UDP) but none of them respond to guanine nucleotides. Taken together, these differences between the actions of Ap$_n$A compounds and ATP and the inability of methylxanthines to block dinucleotide actions (excluding the participation of adenosine receptors) strongly suggested that there are specific receptors for diadenosine polyphosphates in the rat midbrain synaptic terminals.

In the following years, there was some discrepancy over the nomenclature to be adopted for this new type of receptor and several terms have been used in the literature. According to its pharmacological properties and in the absence of any structural data, we propose it is not appropriate to define this receptor as a mere variant of the ATP receptors. We consequently prefer to use the term dinucleotide receptor over any other cited in the literature [55].

The presence of an independent dinucleotide receptor at the presynaptic level has important implications in neural transmission. In fact, the ionotropic character of the dinucleotide receptor implies Ca^{2+} entry through the receptor, which depolarizes the terminal and activates Na$^+$- and Ca^{2+}-voltage-dependent channels [16, 54, 58, 59]. It seems reasonable to propose that dinucleotides acting through this mechanism of action may modulate the release of the neurotransmitters present in the synaptic terminal. We explored the interaction between dinucleotide receptor activation and the exocytotic release of other neurotransmitters. Using microfluorimetric Ca^{2+} measurement techniques combined with immunocytochemical studies, we showed the presence of the dinucleotide receptor in aminergic terminals isolated from basal ganglia [23]. A specific receptor for diadenosine polyphosphates is also present in cholinergic terminals from rat midbrain as well as in GABAergic or glutamatergic terminals obtained from the same source. It is interesting that the stimulation of the midbrain synaptosomes with Ap$_5$A leads to the exocytotic release of the stored neurotransmitter: glutamate, GABA, or acetylcholine [17, 24, 27].

Several reviews on extracellular and neurotransmitter roles of dinucleotides acting through presynaptic ionotropic receptors, their specific dinucleotide receptor, and/or ionotropic nucleotide P2X receptors have been previously published [46, 55]. We refer the reader to these works to better understand some aspects, which are only briefly mentioned here. We will specifically address this review on the new interactions of the presynaptic ionotropic dinucleotide receptor with the nucleotide and other neurotransmitter signaling systems; we will also describe the new

findings on the actions of dinucleotides in the cerebellum. In addition, we will pay special attention on dinucleotide signaling in glial cells where a new specific metabotropic receptor for these substances has been described.

Modulation of the presynaptic dinucleotide receptor by other neurotransmitter systems

Previous work in rat midbrain synaptic terminals has demonstrated that the dinucleotide receptor was submitted to acute regulation at presynaptic level. In this sense, the dinucleotide receptor activity is modulated by effectors of protein kinases and phosphatases [53]. While activation of protein kinases A and C led to inhibition of Ap_5A-induced responses, protein kinase inhibitors as well as protein phosphatase modulators clearly potentiated these responses. To continue with these regulatory studies, modulation of the dinucleotide receptor by presynaptic purinergic receptors of adenosine and ATP was investigated. ATP, acting on a not fully characterized P2 receptor, induces a decrease in the maximal response elicited by Ap_5A in these terminals. Adenosine, on its hand, shows a dramatic effect on the affinity of the dinucleotide receptor for its ligand. This nucleoside, acting presumably on A_1 receptors, allows the dinucleotide receptor to attain a high-affinity state and, thus, to be stimulated by lower concentrations of diadenosine polyphosphates, even in the picomolar/low nanomolar range [15]. In the absence of A_1 receptor activation, the dinucleotide receptor in rat midbrain synaptosomes is only sensitive to Ap_nA concentrations in the micromolar range [51, 54]. Thus, the increased affinity induced by adenosine could confer the dinucleotide receptor the capacity to respond to more physiologically relevant Ap_nA concentrations. The ability of adenosine to induce a high-affinity state of the dinucleotide receptor has also been described in rat hippocampal nerve terminals [14].

Changes in dinucleotide receptor affinity have also been observed after the activation of presynaptic $GABA_B$ receptors [25]. The sigmoidal concentration response for Ap_5A ($EC_{50}=44$ μM) changes into a biphasic curve in the presence of the $GABA_B$ agonist baclofen. Thus, when $GABA_B$ receptors are activated, the curve shows a high-affinity component in the picomolar range ($EC_{50}=77$ pM) and a low-affinity component in the micromolar range ($EC_{50}=17$ μM). The effect of baclofen was blocked by the selective antagonist saclofen and by preincubation with pertussis toxin, clearly demonstrating the implication of $GABA_B$ receptors in these effects. Both A_1 and $GABA_B$ are G_i/G_o-coupled receptors that decrease the activity of adenylate cyclase and, as a consequence, reduce cAMP levels. It seems to be reasonable that a decreased cAMP concentration reduces PKA activity leading to a lower degree of phosphorylation of the dinucleotide receptor that could modify its affinity to Ap_5A. Experiments performed with modulators of the adenylate cyclase and PKA activities have demonstrated the involvement of this signaling pathway in the potentiatory effect of baclofen

[25]. Thus, it could be a general mechanism of regulation of the dinucleotide receptor activity (Fig. 1).

The functionality of the dinucleotide receptor cannot only be modulated by stimulation of metabotropic receptors present at the same terminal, but a fine-tuning interaction between the dinucleotide receptor and epibatidine-responding nicotinic receptors has also been described in rat midbrain cholinergic terminals [18]. Dinucleotide receptors and epibatidine-sensitive nicotinic receptors are coexpressed in 19% of the total of rat midbrain cholinergic terminals, as determined by a combination of immunological and microfluorimetric techniques. Activation of each independent receptor induces intrasynaptosomal $[Ca^{2+}]_i$ increases and acetylcholine release in a dose-dependent way. However, stimulation of the cholinergic terminals with both agonists induces a response that is significantly reduced with respect to the expected signal (the summatory of the individual responses for each agonist). The mutual negative control of these two ionotropic presynaptic receptors is perhaps the physiological way to turn off the synaptic excitability and prevent an excessive release of neurotransmitters (Fig. 1). This inhibitory cross-talk is reverted in the presence of specific inhibitors of calcium/calmodulin-dependent protein kinase II (CaMKII), such as KN-62 and KN-93. These results demonstrate the participation of this kinase in the mechanism of reciprocal regulation between the presynaptic dinucleotide and nicotinic receptors [18].
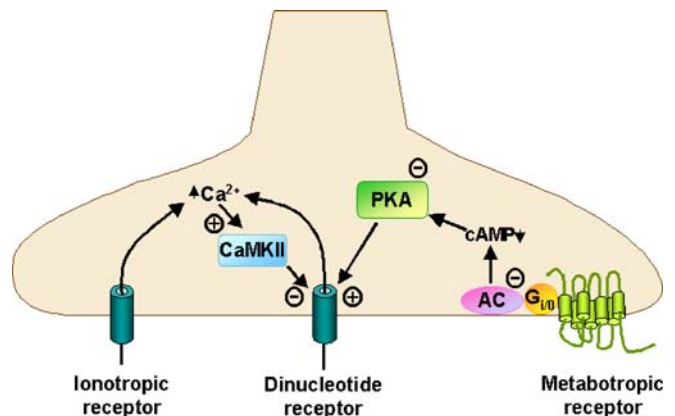

Fig. 1 Modulation of the presynaptic dinucleotide receptor. Activation of the dinucleotide receptor induces calcium entry into the terminals with the subsequent activation of CaMKII, finally leading to the inhibition of the calcium signaling through the dinucleotide receptor. A similar inhibition of the functionality of Ap_nA receptors can be achieved after activation of calcium-permeable ionotropic receptors that could be present at the same terminal, as is the case of the acetylcholine nicotinic receptors [18]. On the other hand, stimulation of second messenger systems that are negatively coupled to adenylate cyclase leads to a reduction of PKA activity and this allows the dinucleotide receptor to reach a higher-affinity state, being sensitive to lower diadenosine polyphosphate concentrations, even in the nanomolar range [15, 25]

566

Dinucleotide actions in cerebellum

Although, as mentioned before, the pharmacological and molecular characterization of native nucleotide receptors, both ionotropic P2X and metabotropic P2Y receptors, have been greatly delayed by the lack of specific agonists and antagonists for the different receptor subtypes, several binding studies carried out with the available tools, such as a nonhydrolyzable ATP analog, $[^3H]\alpha\beta$-meATP, and the dinucleotide $[^3H]Ap_4A$, revealed that the cerebellum, particularly the cerebellar cortex, was one of the structures exhibiting highest levels of specific binding in the brain [63]. Based on these findings, we decided to study the functional responses to nucleotides in this area.

We developed enriched cultures in the two major neuronal types of cerebellum, Purkinje neurons, at the beginning, and granule neurons, more recently. Astrocyte cultures have been carried out in parallel to separately study the nucleotide receptors present in neurons and glia, the two components of the brain multifunctional unit. We found that all of them expressed multiple functional nucleotide receptors. Purkinje and granule neurons co-expressed ionotropic P2X and metabotropic P2Y receptors, whereas astrocytes only expressed P2Y receptors [29, 34, 44]. This affirmation made for cerebellar astrocytes several years ago can now be ruled out. The presence of functional P2X$_7$ receptors has been recently detected, which are currently being investigated [11]. However, neurons and glial cells differed in their sensitivity to dinucleotides. In our experimental conditions, Purkinje neurons were completely insensitive to dinucleotide stimulations (Ap$_5$A and Ap$_4$A stimulations) and granule neurons displayed some intracellular responses to the dinucleotide Ap$_5$A; but, there can be no doubt that astrocytes have resulted to be one of the most active "sensors" of extracellular dinucleotide concentrations.

Cerebellar granule neurons

Beginning with granule neurons, among the great diversity of nucleotidic receptors functionally expressed in these cells, there are possible targets for dinucleotides, such as P2Y$_1$, P2X$_1$, and P2X$_3$ [29]. Among metabotropic receptors, the major population of nucleotidic responding cells presented a pharmacological profile typical of P2Y$_1$ receptors, being mainly found at the cell bodies. Concerning ionotropic receptors, although P2X$_2$ and P2X$_3$ were also present at the somas, the preferential distribution pattern of P2X subunits in granule cells were along the cell fibers. Significant responses at this level were found for $\alpha\beta$-meATP and BzATP, which could activate P2X$_{1/3}$ and P2X$_7$ receptors, respectively (unpublished observations). Taking into account that along the cell fibers of granule neurons in culture it is not possible to differentiate between dendritic and axonal compartments, the responses found for nucleotidic agonists could be both of presynaptic and/or postsynaptic nature. However, the characteristic punctuated immunostaining pattern observed for P2X$_3$ and P2X$_7$ receptors, which exhibited a high degree of colocalization with the synaptic marker synaptophysin, suggested a presynaptic function for these receptors in granule neurons. This was further confirmed in synaptosomal preparations of rat cerebellum. In fact, the only ionotropic nucleotidic receptors significantly expressed in glutamatergic cerebellar synaptic terminals were P2X$_3$ and P2X$_7$ receptors. The immunostaining pattern was corroborated by calcium recordings in single cerebellar synaptosomes, in which the responses found for the specific agonists, $\alpha\beta$-meATP and BzATP, fitted well with the distinctive pharmacology of P2X$_3$ and P2X$_7$ receptors, respectively [30].

Assuming the high percentage of P2X$_3$ receptor expression in cerebellar synaptosomes, experiments were carried out to challenge for dinucleotide responses. In this model, a significant population of synaptosomes was able to respond to the dinucleotide Ap$_5$A, either in the presence or absence of previous ATP responses. The fact that around 44% of these responses remained in cross-desensitization studies with ATP gives evidence in favor of the presence of a specific dinucleotide receptor at cerebellar synaptic terminals (unpublished results). Considering the high proportion of cerebellar synaptosomes exhibiting P2X$_3$ immunostaining and the lack of P2X$_1$ receptor expression with the antibodies commercially available, P2X$_3$ receptors are good candidates for assuming the remaining 56% Ap$_5$A responses [30].

Coming back to the granule neuron model, recent studies have shown that, beyond the great diversity and compartmentalization of nucleotidic and/or dinucleotidic receptors between cell bodies and fibers, the calcium signals primarily triggered for all these nucleotidic agonists become confluent in a specific calcium-derived signal, that is CaMKII. The phosphorylation and subsequent activation of CaMKII by different nucleotidic agonists spatially correlates with the distribution of the nucleotide receptors that they activate. Although the highest levels of phosphorylation were found for 2MeSADP and BzATP, P2Y$_1$ and P2X$_7$ agonists, respectively, a significant response was observed for the dinucleotide Ap$_5$A, and the P2X$_{1,3}$ agonist $\alpha\beta$-meATP, reaching values of about 70–80%, increased for both agonists (see Fig. 2) [42]. It is interesting to mention the similarity found for $\alpha\beta$-meATP and Ap$_5$A in the phosphorylation pattern of CaMKII at the granule cell fibers, which appeared in some but not all fibers and in a dotted appearance. This pattern fitted well with the immunostaining distribution of P2X$_3$ subunits along granule cell fibers [29]. In addition, the effect of both agonists on CaMKII was partially sensitive to Ip$_5$I inhibition at 100-nM concentration, with the inhibition being significantly higher, although not complete, in the case of Ap$_5$A. These results are consistent with $\alpha\beta$-meATP and Ap$_5$A acting through P2X$_3$ receptors to activate CaMKII and also imply that another receptor, possibly the previously cited specific dinucleotide receptor, could be accounted for the effect seen with Ap$_5$A, as demonstrated at the presynaptic level in synaptosomes.

The above data are consistent with the dinucleotide Ap$_5$A acting mainly through P2X$_3$ receptors and mediating
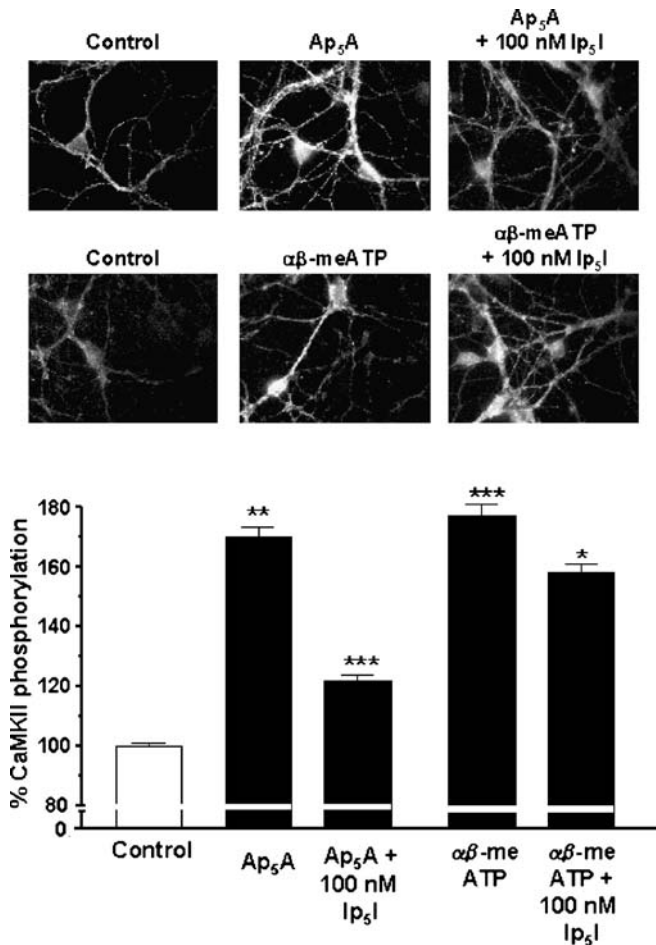
Fig. 2 CaMKII activation by Ap$_5$A and αβ-meATP in cerebellar granule neurons. CaMKII phosphorylation at Thr$^{286/287}$ was detected by immunocytochemistry and quantified as described in [42], after the treatment of granule cells with Ap$_5$A and αβ-meATP at 100 μM concentrations for 5 min. Although the phosphorylation pattern for both agonists was similarly distributed along the cell bodies and fibers of granule cells, Ip$_5$I, used at concentrations (100 nM) that inhibited more specifically the dinucleotide receptor over the P2X$_3$ receptors, exhibited a higher inhibitory effect on Ap$_5$A responses in comparison to that observed for αβ-meATP

a broad spectrum of actions in granule cell neurons. The putative modulatory functions will depend on the specific cellular compartment or microdomain in which Ap$_5$A could act. In addition, the possible actions of Ap$_5$A acting through the specific dinucleotide receptor described in cerebellar synaptic terminals cannot be excluded, which enriched even more the versatility of this dinucleotide as a wide modulator of cerebellar neurotransmission.

Considering the convergent localization of Ap$_5$A responses in cerebellar synaptic terminals of glutamatergic nature, it is tempting to speculate a possible interaction between glutamate and nucleotides, at the level of glutamate release. Taking into account that Ap$_5$A is coupled to the activation of CaMKII, it is well documented that this enzyme actively participates in synaptic vesicle mobilization for neurotransmitter release. In fact, between the targets of CaMKII at the presynaptic level are synaptic vesicle proteins, such as synapsin I, which, upon phos-

phorylation by CaMKII, stops interaction with actin filaments, releasing the synaptic vesicle and, thus, facilitating the vesicle movement towards the active synaptic sites. In this context, preliminary results have shown CaMKII-dependent phosphorylation of synapsin I by several nucleotides, 2MeSADP, αβ-meATP, and BzATP, in granule neurons. These nucleotidic agonists are also involved in the modulation of glutamate release in this neuronal model (León et al., manuscript in preparation). Whether Ap$_5$A acting through a specific dinucleotide receptor or through nucleotidic receptors, P2Y$_1$ and P2X$_3$, can be coupled to glutamate release modulation will be the aim of future studies.

In addition to these promising perspectives for Ap$_5$A, it has to be considered that its main action localizes at the postsynaptic level and partially acting through P2X$_3$ receptors. At this level, CaMKII is only the first step in the nucleotide-derived signaling following calcium influx. This enzyme is a link for different intracellular signaling pathways, such as MAPK kinases, NFκB, CREB, etc., and leads its actions even at the transcriptional level. Due to its effects on CaMKII activation, the nucleotides effectively become potential modulators of a great range of actions, as this enzyme has been involved as a critical mediator of synaptic plasticity, survival, and neuritogenesis [6, 20]. A recent study has demonstrated a role for CaMKII as a regulator of the increased expression of plasma membrane P2X$_3$ receptors, which leads to potentiation of ATP-mediated responses in dorsal root ganglion neurons [77]. This resembles the effect of CaMKII on the increase in synaptic AMPA receptor transmission, by facilitating the delivery of additional AMPA receptors to the synapse, an early event occurring in long-term potentiation [65]. This allows us to hypothesize that, similarly to the well-known actions of AMPA receptors as modulators of synapse formation and spine morphogenesis, nucleotide receptors could be playing a relevant role in these processes. In this sense, CaMKII is directly involved in promoting dendritic growth and maintenance on granule neurons, and this effect has been attributed to its regulatory effect on the transcription factor NeuroD involved in axonal and dendritic morphogenesis [22].

Cerebellar astrocytes

As mentioned previously, we select purified astrocyte cultures as a model of glial cells. Astrocyte cultures have been widely used for the characterization of receptors present in astroglia, such as glutamate, GABA, histamine, ATP, etc. [73]. Over the past decade, it has been concluded that astrocytes are the "new stars" in the nervous system. Neuron-to-astrocyte signaling in the brain represents a multifunctional unit, modulating synaptic activity and exerting a dynamic control of brain microcirculation [19]. Cerebellar astrocytes could perhaps be good candidates to study nucleotide signaling. In fact, in our first studies, we detected not only the presence of functional metabotropic ATP receptors in all tested cells but also the existence of an

interaction with dinucleotides in a subpopulation of astrocytes, which accounted for 65% of cells [32].

Dinucleotides potentiate the P2Y-mediated calcium responses in single astrocytes

Preincubation of astrocytes with 100 nM Ap_5A for 3 min and co-stimulation with previous ineffective ATP concentrations (0.1–1 μM, depending on the cell) induced remarkable $[Ca^{2+}]_i$ increases, which ranged from 40–60% of the maximal ATP response obtained at 100 μM ATP. The potentiation was independent of extracellular calcium and, once it occurred, persisted for a prolonged period (several hours), during which the cells continued to be sensitive to low ATP concentrations that were previously ineffective (Fig. 3, the upper panel shows an example of the described effect). The potentiation was exclusive for Ap_5A and was not reproduced by any other dinucleotide. These findings prompted us to characterize the receptors mediating ATP calcium responses and the "target" and mechanism triggered by Ap_5A. Using different approaches, we have found that all cerebellar astrocytes co-express several subtypes of metabotropic nucleotide receptors, ADP-sensitive receptors, the $P2Y_1$ and/or the $P2Y_{13}$-like receptors, which are heterogeneously distributed among astrocyte population, and the ATP/UTP-sensitive receptors, the $P2Y_2/P2Y_4$ receptors, which are homogeneous distributed in all the cells [8, 34].

Searching into the Ap_5A "target", at first, the fact that the potentiation was triggered by nanomolar concentrations of the dinucleotide induced us to think that Ap_5A could be interacting with A_1 adenosine receptors, which exhibited high affinity for the nucleoside. At that time, same potentiatory actions of adenosine had been reported. However, neither nanomolar adenosine concentrations nor specific A_1 receptor agonists or antagonists had any effect on ATP calcium responses. ATP calcium responses were surprisingly potentiated by simple co-stimulation with micromolar adenosine concentrations or other signals coupled to Gs proteins. Although, in contrast to that found for Ap_5A, this potentiation was totally reversible and was not exclusive for the ATP receptors, $P2Y_2/P2Y_4$ receptors, as other G-protein-coupled receptors mediating PLC stimulation were also potentiated, such as ADP and bradykinin receptors, which are also present in cerebellar astrocytes [13, 33].

Another candidate for Ap_5A target could be the ADP-sensitive $P2Y_1$ receptors, at which diadenosine polyphosphates can act as agonists or even as antagonists, as is the case of the $P2Y_1$ receptor present in rat brain endothelial cells [56, 74]. However, the possible Ap_5A interaction with this receptor could also be discarded. As mentioned before, all astrocytes were sensitive to ADP stimulations, whereas only around 65% were sensitive to Ap_5A, indicating that they are acting through different receptors. In addition, specific agonists of $P2Y_1$ receptor, such as ADP and
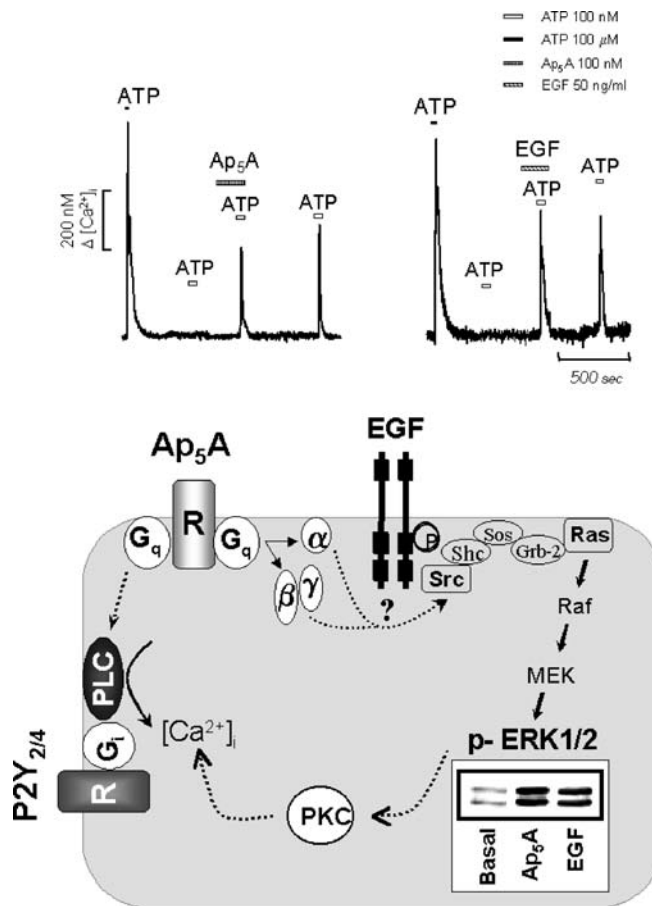

Fig. 3 Cross-talk among Ap_5A, EGF, and ATP nucleotide receptors in cerebellar astrocytes. The *upper panels* show the potentiation of ATP calcium responses by Ap_5A and EGF in individual cerebellar astrocytes. Cells were stimulated with different ATP concentrations: the maximal effective concentrations (100 μM, *solid bars*) and an ineffective concentration (100 nM, *open bars*) for 30 s. Where indicated, cells were preincubated with 100 nM Ap_5A or 50 ng/ml EGF for 3 min before co-stimulation with ineffective ATP challenges. The basal $[Ca^{2+}]_i$ levels were around 80 nM and the magnitude of the potentiated ATP responses amounted to 304±18 nM. These cells are representative of the astrocyte subpopulation in which the potentiation was observed. The *lower panel* shows a model representing the intracellular signaling triggered by Ap_5A in cerebellar astrocytes, adapted from [12]. The specific metabotropic diadenosine pentaphosphate receptor can adopt several active conformations, which can be differently stabilized in a ligand concentration manner to discriminate among various intracellular signaling pathways. At nanomolar concentration, Ap_5A engaged to ERK signaling via src and ras proteins, whereas at micromolar concentrations, it activated the typical intracellular calcium mobilization dependent on PLC activation. The *inset* shows the phosphorylated ERK1/2 forms detected by immunoblotting from a representative experiment, in which cells were stimulated with 100 nM Ap_5A or 50 ng/ml EGF for 3 min, lysed, and with p-ERK1/2 forms detected as described in [35]

2MeSATP, were unable to reproduce the dinucleotide potentiatory effect. These results suggested the existence of a specific metabotropic receptor for Ap_5A in this astrocyte subpopulation.

Metabotropic Ap$_5$A signaling cascades: analogies and divergences with EGF

Trying to find out the intracellular signaling triggered after the stimulation of the "putative" Ap$_5$A receptor in astrocytes, considering that the potentiation was maintained for hours and the numerous examples of cross-talk between GPCR and tyrosine receptor kinases resulting in ERK activation (as examples, see [43, 78]), we examined ERKs as possible mediators of this event. The stimulation of cells with 100 nM Ap$_5$A for 3 min induced ERK activation, in a similar extent to that produced by EGF under the same experimental conditions (Fig. 3, insert in lower panel). ERK activation induced by Ap$_5$A was concentration-dependent (EC$_{50}$ value of 9.8±2.4 nM) but exhibited a bell-shaped curve, reaching the maximal effect at 100 nM Ap$_5$A, just the concentration at which the potentiaton was displayed. At concentrations higher than 100 nM, ERK activation was considerably reduced. Additional studies using specific inhibitors for several steps of MAPK cascade activation showed that Ap$_5$A engaged to MAPK cascade by a mechanism that was insensitive to pertussis toxin, independent of EGFR transactivation, and required the involvement of src and ras proteins [12, 35]. This signaling is quite distinct from those reported for some members of the P2Y nucleotide receptor family [26, 41, 66, 67]. The "putative" Ap$_5$A receptor also exhibited another peculiar characteristic, when it was stimulated with micromolar concentrations of the dinucleotide also coupled to PLC, and evoked intracellular calcium mobilization (Fig. 3, lower panel). This switch was not observed for any other nucleotide receptor. Although P2Y$_1$ and P2Y$_2$/P2Y$_4$ receptor stimulation also induced ERK activation in cerebellar astrocytes, it ran parallel to PLC activation (unpublished results).

Ap$_5$A and EGF mediate similar potentiation on ATP calcium responses

Continuing with the potentiation phenomenon, whether Ap$_5$A induced ERK activation in a similar extent to those induced by EGF, it could be plausible that EGF or other growth factors reproduced the potentiation. As expected, it was exactly reproduced by EGF and NGF [12]. When cells were preincubated with 50 ng/ml EGF or NGF and co-stimulated with previous ineffective ATP concentrations, intracellular calcium responses ranging from 55–60% of the maximal ATP response (obtained at 100 μM ATP) were elicited. The potentiation was also permanent, as is the case of Ap$_5$A (Fig. 3, upper panel). In all the cases, an acute (3 min) stimulation of the "priming" receptor (dinucleotide or growth factor receptor) was required before co-stimulation with ATP. The potentiation of ATP responses by Ap$_5$A and EGF was completely abolished by the MAP kinase (MEK) inhibitor U-0126, proving that ERK activation is a required step for the potentiation. It was also sensitive to src-like kinases inhibitors, such as herbimycin, p21ras farnesyltransferase inhibitor peptide, and some PKC inhibitors. Taken together, these data revealed that Ap$_5$A triggered the potentiation of ATP calcium responses through an insensitive pertussis toxin G protein and required src protein-mediated ERK activation and the participation of atypical protein kinase C isoforms activated down-stream from ERK. The question that still remains open is: which are the substrate or the target of the activated PKC? We speculated that PKC could phosphorylate the RGS2 protein, a member of the regulators of G protein signaling (RGS). This phosphorylation decreased its capacity to reduce GTP-stimulated PLC activation, with the corresponding enhancement in inositol phosphate accumulation, as have been proposed by Cunningham et al. [10]. This could be feasible based on the fact that the potentiation required co-stimulation with the agonist. Independently of the intracellular mechanism that took place, these results clearly suggest that Ap$_5$A at nanomolar concentrations could act as a growth factor in these glial cells. In this sense, dinucleotides have been also proposed as growth-promoting extracellular mediators [31]. Besides, the interaction with growth factors represents a novel regulation of nucleotide signaling that could be relevant in both physiological and pathological conditions. Although several interactions between growth factors, cytokines, and nucleotide signaling have been previously reported [36, 37, 41], all of them described long-term effect, which required long incubation periods, and are very far from the acute effect reported here.

Conclusions and perspectives

The aim of this review has been to present an overview of the dinucleotide actions in the nervous system and their numerous cross-talks resulting in the majority of the cases in a considerable alteration of calcium signaling. Considering that dinucleotides are more resistant to hydrolysis than nucleotides, these interactions would contribute to maintain the functionality of nucleotide receptors, when the extracellular ATP levels fall because of hydrolysis and/or diffusion. The interactions revised here are isolated examples, which would represent only a small part among the great diversity of signals and mechanisms that must be integrated in vivo. However, this fact does not diminish the possible physiological implications, which unfortunately remain to be elucidated. Nucleotide responses and functional receptors have been described in intact preparations, but there was a controversy concerning the receptors implied [39, 48]. As noticed previously, the nucleotide receptor characterization is even more complex than those for any neurotransmitter, first by the lack of specific agonist and antagonist and, also, by other factors [1, 38, 47]. Nucleotide and dinucleotide actions depend on the local environment at which are released the receptors coexisting in this particular "microdomain", on the presence of ectoenzymes responsible for their hydrolysis and even also on the sources, as have been described for glutamatergic neurotransmission. Excellent works from Araque and colleagues have revealed that astrocyte Ca^{2+}

signal can be spatially localized and compartmentalized in restricted regions of the cells—the subcellular microdomains—that constitute the elementary units of the Ca^{2+} elevations. Furthermore, astrocytes can also discriminate between synaptically released glutamate from different afferent axons [2, 49]. In this sense, the nucleotide sources are even more diverse than those of glutamate. Nucleotides or dinucleotides can be released from healthy cells in a calcium-dependent or calcium-independent manner from neurons, glial and endothelial cells, and also from damaged or dead cells, in all the cases reaching sufficient extracellular concentrations to activate both ionotropic and metabotropic receptors and, after being metabolized in the extracellular media, also give products that continue being "active", such as ADP and, finally, adenosine, which can also act on specific receptors, modulating nucleotide responses at presynaptic level in neurons and also in glial cells [14, 33]. Thus, it is quite difficult to determine the setpoint of nucleotide signaling and to determine the "basal" activity of any cellular responses to nucleotides. This complexity of purinergic system itself together with the multiple interactions that could be produced in a local and temporal localization set us into thinking that any cellular response, the different types of neurotransmission, secretion, migration, etc., results from the integration of the multiple extracellular signals.

To finish this review, we would like to highlight another aspect of nucleotide signaling, the existence of scarce data concerning the subcellular distribution of the receptors, their intracellular trafficking, etc. In this way, the interactions revised here would bring out some light about this. The interactions described with GABA, acethylcholine, glutamate, and EGF receptors suggest that they must be colocalized within the same membrane domains facilitating the functional interactions. Considering that several of them have been localized in lipid rafts, one would speculate that the nucleotide receptor subtypes co-interacting must be also there. The first candidates would be the $P2Y_2/P2Y_4$ receptor, which is potentiated by EGF and Ap_5A, as well as the metabotropic Ap_5A receptor present in cerebellar astrocytes. EGF receptor and src family kinases were ones of proteins primarily identified in the lipid rafts [76]. These findings have opened new perspective in our research. Lipid rafts have also been identified in neurons, which appear to be crucial for some functions, including neurotrophic factors, adhesion, axon guidance, vesicular trafficking, and protein sorting. It has been described that lipid rafts are essential for the maintenance of $\alpha 7$ nicotinic acethylcholine receptor clusters in somatic spines in ciliary ganglion neurons and for the synaptic stability of the AMPA-type glutamate receptors in hippocampal neurons [7, 28]. In addition, the $GABA_B$ receptors have also been associated with lipid rafts in cerebellum [5]. The evidence of functional cross-talk between the presynaptic dinucleotide receptor and $GABA_B$ receptors described before could be based on the co-localization of both receptors in specific microdomains. This assumption is feasible as $P2X_3$ and $GABA_B$ receptors

have been found to be associated with lipid rafts in cerebellar granule neurons [68].

All these data suggest that the models under study, synaptosomal preparations, astrocytes, and granule neuron cultures from cerebellum, provide good models in which to explore the open new perspectives for different aspects of nucleotide and dinucleotide receptors.

References

1. Alvarado-Castillo CP, Harden TK, Boyer JL (2005) Regulation of $P2Y_1$ receptor-mediated by the ecto-nucleoside triphosphate diphosphohydrolase isozymes NTPDase1 and NTPDase 2. Mol Pharmacol 67:114–122
2. Araque A, Martin ED, Perea G, Arellano JI, Buño W (2002) Synaptically-released acetylcholine evokes Ca^{2+} elevations in astrocytes in hippocampal slices. J Neurosci 22:243–2450
3. Baker JC, Jacobson MK (1986) Alteration of adenyl dinucleotide metabolism by environmental stress. Proc Natl Acad Sci U S A 83:2350–2352
4. Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohta M, Huebner K (1996) Fhit, a putative tumor suppressor in humans, is a dinucleoside $5',5'''-P^1,P^3$-triphosphate hydrolase. Biochemistry 35:11529–11535
5. Becher A, White JH, McIlhinney JAJ (2001) The γ-aminobutyric acid receptor B, but not the metabotropic glutamate receptor type-1, associates with lipid rafts in the rat cerebellum. J Neurochem 79:787–795
6. Borodinsky LN, Coso OA, Fiszman ML (2002) Contribution of Ca^{2+}-calmodulin protein kinase II and mitogen-activated protein kinase kinase to neural activity-induced neurite outgrowth and survival of cerebellar granule cells. J Neurochem 80:1062–1070
7. Bruses JL, Chauvet N, Rutishauser U (2001) Membrane lipid rafts are necessary for the maintenance of the (alpha)7 nicotinic acetylcholine receptor in somatic spines of ciliary neurons. J Neurosci 21:504–512
8. Carrasquero LMG, Delicado EG, Jiménez AI, Pérez-Sen R, Miras-Portugal MT (2005) Cerebellar astrocytes co-express several ADP receptors. Presence of functional $P2Y_{13}$-like receptors. Purinergic Signalling 1:153–159
9. Cheung KK, Cahn WY, Burnstock G (2005) Expression of P2X purinoceptors during rat brain development and their inhibitory role of motor axon outgrowth in neural tube explant cultures. Neuroscience 133:937–945
10. Cunningham ML, Waldo GL, Hollinger S, Hepler JR, Harden TK (2001) Protein kinase C phosphorylates RGS2 and modulates its capacity for negative regulation of $G\alpha_{11}$ signaling. J Biol Chem 276:5438–5444
11. Delicado EG, Carrasquero LMG, Pérez-Sen R, Miras-Portugal MT (2005) Identification of functional P2X7 receptor in rat cerebellar astrocytes. International proceedings of VII European meeting on glial cell function in health and disease, Amsterdam, The Netherlands. Medimond, pp 71–78
12. Delicado EG, Jiménez AI, Carrasquero LMG, Castro E, Miras-Portugal MT (2005) Cross-talk among epidermal growth factor, Ap_5A, and nucleotide receptors causing enhanced ATP Ca^{2+} signaling involves extracellular kinase activaion in cerebellar astrocytes. J Neurosci Res 81:789–796
13. Delicado EG, Jiménez AI, Castro E, Miras-Portugal MT (2001) Cerebellar astrocytes coexpress different purinoceptors: crosstalk between several transduction mechanism. Drug Dev Res 52:114–121

14. Diaz-Hernandez M, Pereira MF, Pintor J, Cunha RA, Ribeiro JA, Miras-Portugal MT (2002) Modulation of the rat hippocampal dinucleotide receptor by adenosine receptor activation. J Pharmacol Exp Ther 301:441–450

15. Diaz-Hernandez M, Pintor J, Miras-Portugal MT (2000) Modulation of the dinucleotide receptor present in rat midbrain synaptosomes by adenosine and ATP. Br J Pharmacol 130:434–440

16. Diaz-Hernandez M, Pintor J, Castro E, Miras-Portugal MT (2001) Independent receptors for diadenosine pentaphosphate and ATP in rat midbrain single synaptic terminals. Eur J Neurosci 14:918–926

17. Diaz-Hernandez M, Pintor J, Castro E, Miras-Portugal MT (2002) Co-localisation of functional nicotinic and ionotropic nucleotide receptors in isolated cholinergic synaptic terminals. Neuropharmacology 42:20–33

18. Diaz-Hernandez M, Sanchez-Nogueiro J, Pintor J, Miras-Portugal MT (2004) Interaction between dinucleotide and nicotinic receptors in individual cholinergic terminals. J Pharmacol Exp Ther 311:954–967

19. Fellin T, Carmignoto G (2004) Neurone-to-astrocyte signaling in the brain represents a distinct multifunctional unit. J Physiol 559:3–14

20. Fink CC, Meyer T (2002) Molecular mechanisms of CaMKII activation in neuronal plasticity. Curr Opin Neurobiol 12:293–299

21. Flores NA, Stavrou BM, Sheridan DJ (1999) The effects of diadenosine polyphosphates on the cardiovascular system. Cardiovasc Res 42:15–26

22. Gaudilliére B, Konishi Y, de la Iglesia, N, Yao G-I, Bonni A (2004) A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. Neuron 41:229–241

23. Giraldez L, Diaz-Hernandez M, Gomez-Villafuertes R, Pintor J, Castro E, Miras-Portugal MT (2001) Adenosine triphosphate and diadenosine pentaphosphate induce $[Ca^{2+}]_i$ increase in rat basal ganglia aminergic terminals. J Neurosci Res 64:174–182

24. Gomez-Villafuertes R, Gualix J, Miras-Portugal MT (2001) Single GABAergic synaptic terminals from rat midbrain exhibit functional P2X and dinucleotide receptors, able to induce GABA secretion. J Neurochem 77:84–93

25. Gomez-Villafuertes R, Pintor J, Gualix J, Miras-Portugal MT (2004) GABA modulates presynaptic signaling mediated by dinucleotides on rat synaptic terminals. J Pharmacol Exp Ther 308:1148–1157

26. Grobben B, Claes P, van Kolen K, Roymans D, Fransen P, Sys SU, Slegers H (2001) Agonists of the $P2Y_{AC}$-receptor activate MAP kinase by a ras-independent pathway in rat C6 glioma. J Neurochem 78:1325–1338

27. Gualix J, Gomez-Villafuertes R, Diaz-Hernandez M, Miras-Portugal MT (2003) Presence of functional ATP and dinucleotide receptors in glutamatergic synaptic terminals from rat midbrain. J Neurochem 87:160–171

28. Hering H, Lin CC, Sheng M (2003) Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. J Neurosci 28:3262–3271

29. Hervas C, Pérez-Sen R, Miras-Portugal MT (2003) Co-expression of functional P2X and P2Y nucleotide receptors in single cerebellar granule cells. J Neurosci Res 73:384–399

30. Hervas C, Pérez-Sen R, Miras-Portugal MT (2005) Presence of diverse functional P2X receptors in rat cerebellar synaptic terminals. Biochem Pharmacol 70:770–785

31. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold H, Sachinidis A, Zidek W, Schlüter H (2001) Dinucleotides as growth-promoting extracellular mediators. Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granules. J Biol Chem 276:8904–8909

32. Jiménez AI, Castro E, Delicado EG, Miras-Portugal MT (1998) Potentiation of adenosine 5'-triphosphate calcium responses by diadenosine pentaphosphate in individual rat cerebellar astrocytes. Neurosci Lett 246:109–111

33. Jiménez AI, Castro E, Mirabet M, Franco R, Delicado EG, Miras-Portugal MT (1999) Potentiation of ATP calcium responses by A_{2B} receptors stimulation and other signals coupled to Gs proteins in type-1 cerebellar astrocytes. Glia 26:119–128

34. Jiménez AI, Castro E, Communi D, Boeynaems JM, Delicado EG, Miras-Portugal MT (2000) Coexpression of several types of metabotropic nucleotide receptors in single cerebellar astrocytes. J Neurochem 75:2071–2079

35. Jiménez AI, Castro E, Delicado EG, Miras-Portugal MT (2002) Specific diadenosine pentaphosphate receptor coupled to extracellular regulated kinases in cerebellar astrocytes. J Neurochem 83:299–308

36. John GR, Scemes E, Suadicani SO, Liu JSH, Charles PC, Lee SC, Spray DC, Brosnan CF (1999) IL-1β differentially regulates calcium wave propagation between primary human fetal astrocytes via pathways involving P2 receptors and gap junction channels. Proc Natl Acad Sci U S A 96:11613–11618

37. John GR, Simpson JE, Woodroofe MN, Lee SC, Brosnan CF (2001) Extracellular nucleotides differentially regulate interleukin-1β signaling in primary human astrocytes: implications for inflammatory gene expression. J Neurosci 21:4134–4142

38. Joseph SM, Buchakjian MR, Dubyak GR (2003) Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. J Biol Chem 278:23331–23342

39. Kawamura M, Gachet C, Inoue K, Kato F (2004) Direct excitation of inhibitory interneurons by exracellular ATP mediated by $P2Y_1$ receptors in the hippocampal slices. J Neurosci 24:10835–10845

40. Kisselev LL, Justesen J, Wolfson AD, Frolova LY (1998) Diadenosine oligophosphates (Ap_nA), a novel class of signaling molecules? FEBS Lett 427:157–163

41. Lenz G, Gottfrie C, Luo Z, Avruch J, Rodnight R, Nie WJ, Kang Y, Neary JT (2000) P2Y purinoceptor subtypes recruit different Mek activators in astrocytes. Br J Pharmacol 129:927–936

42. León D, Hervás C, Miras-Portugal MT (2006) Activation of $P2Y_1$ and $P2X_7$ receptors induce calcium/calmodulin-dependent protein kinase II phosphorylation in cerebellar granule neurons. Eur J Neurosci (In press)

43. Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor and Gβγ subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 271:19443–19450

44. Mateo J, García-Lecea M, Miras-Portugal MT, Castro E (1998) Ca^{2+} signals mediated by P2X-type purinoceptors in cultured cerebellar Purkinje cells. J Neurosci 18:1704–1712

45. McLennan AG (2000) Dinucleoside polyphosphates—friend or foe? Pharmacol Ther 87:73–89

46. Miras-Portugal MT, Gualix J, Pintor J (1998) The neurotransmitter role of diadenosine polyphosphates. FEBS Lett 430:78–82

47. Ostrom RS, Gregorian C, Insel PA (2000) Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. J Biol Chem 275:11735–11739

48. Pankatrov Y, Castro E, Miras-Portugal MT, Krishtal O (1998) A purinergic component of the excitatory postsynaptic current mediated by the P2X receptors in the CA1 neurons of the rat hippocampus. Eur J Neurosci 10:3898–3902

49. Perea G, Araque A (2005) Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. J Neurosci 25:2192–2203

50. Pintor J, Diaz-Rey MA, Torres M, Miras-Portugal MT (1992) Presence of diadenosine polyphosphates-Ap_4A and Ap_5A-in rat brain synaptic terminals. Ca^{2+} dependent release evoked by 4-aminopyridine and veratridine. Neurosci Lett 136:141–144

51. Pintor J, Gomez-Villafuertes R, Miras-Portugal MT (2001) Pharmacological profile of the dinucleotide receptor present in rat brain isolated synaptic terminals. Anal Pharmacol 2:85–92

52. Pintor J, Gualix J, Miras-Portugal MT (1997a) Diinosine polyphosphates, a group of dinucleotides with antagonistic effects on diadenosine polyphosphate receptor. Mol Pharmacol 51:277–284

53. Pintor J, Gualix J, Miras-Portugal MT (1997b) Dinucleotide receptor modulation by protein kinases (protein kinases A and C) and protein phosphatases in rat brain synaptic terminals. J Neurochem 68:2552–2557

54. Pintor J, Miras-Portugal MT (1995) A novel receptor for diadenosine polyphosphates coupled to calcium increase in rat midbrain synaptosomes. Br J Pharmacol 115:895–902

55. Pintor J, Miras-Portugal MT (2000) Receptors for diadenosine polyphosphates P2D, P2Y$_{ApnA}$, P4 and dinucleotide receptors: are there too many? Trends Pharmacol Sci 21:135

56. Pintor J, King BF, Miras-Portugal MT, Burnstock G (1996) Selectivity and activity of adenine dinucleotides at recombinant P2X2 and P2Y1 purinoceptors. Br J Pharmacol 119:1006–1022

57. Pintor J, Kowalewski HJ, Torres M, Miras-Portugal MT, Zimmermann H (1992b) Synaptic vesicle storage of diadenosine polyphosphates in the *Torpedo* electric organ. Neurosci Res Commun 10:9–15

58. Pintor J, Puche JA, Gualix J, Hoyle CH, Miras-Portugal MT (1997c) Diadenosine polyphosphates evoke Ca^{2+} transients in guinea-pig brain via receptors distinct from those for ATP. J Physiol 504(Pt 2):327–335

59. Pivorun EB, Nordone A (1996) Brain synaptosomes display a diadenosine tetraphosphate (Ap$_4$A)-mediated Ca^{2+} influx distinct from ATP-mediated influx. J Neurosci Res 44:478–489

60. Plateau P, Blanquet S (1992) Synthesis of Np$_n$N′ (n=3 or 4) in vitro and in vivo. In: McLennan AG (ed) Ap$_4$a and other dinucleotide polyphosphates. CRC, Boca Raton, pp 63–79

61. Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492

62. Rodriguez del Castillo A, Torres M, Delicado EG, Miras-Portugal MT (1988) Subcellular distribution studies of diadenosine polyphosphates-Ap$_4$A and Ap$_5$A-in bovine adrenal medulla: presence in chromaffin granules. J Neurochem 51:1696–1703

63. Rodríguez-Pascual F, Cortes R, Torres M, Palacios JM, Miras-Portugal MT (1997) Distribution of [^{3}H]diadenosine tetraphosphate binding sites in rat brain. Neuroscience 77:247–255

64. Schluter H, Offers E, Bruggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W (1994) Diadenosine phosphates and the physiological control of blood pressure. Nature 367:186–188

65. Sheng M, Lee SH (2001) AMPA receptor trafficking and the control of synaptic transmission. Cell 105:825–828

66. Short SM, Boyer JL, Juliano RL (2000) Integrins regulate the linkage between upstream and downstream events in G protein-coupled receptor signaling to mitogen-activated protein kinase. J Biol Chem 275:12970–12977

67. Soltoff SP, Avraham H, Avraham S, Cantley LC (1998) Activation of the P$_{2Y2}$ receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. J Biol Chem 273:2653–2660

68. Vacca F, Amadio S, Sancesario G, Bernardi G, Volonté C (2004) P2X$_3$ receptor localizes into lipid rafts in neuronal cells. J Neurosci Res 76:653–661

69. van der Giet M, Schmidt S, Tölle M, Jankowski J, Schlüter H, Zidek W, Tepel M (2002) Effect of dinucleoside polyphosphates on regulation of coronary vascular tone. Eur J Pharmacol 448:207–213

70. Varshavsky A (1983) Diadenosine 5′, 5″-P^1, P^4-tetraphosphate: a pleiotropically acting alarmone? Cell 34:711–712

71. Vartanian A, Alexandrov I, Prudowski I, McLennan A, Kisselev L (1999) Ap$_4$A induces apoptosis in human cultured cells. FEBS Lett 456:175–180

72. Vartanian AA, Suzuki H, Poletaev AI (2003) The involvement of diadenosine 5′,5‴-P^1,P^4-tetraphosphate in cell cycle arrest and regulation of apoptosis. Biochem Pharmacol 65:227–235

73. Verkhratsky A, Orkand RK, Kettenmann H (1998) Glial calcium: homeostasis and signaling function. Physiol Rev 78:99–141

74. Vigne P, Breittmayer JP, Frelin C (2000) Diadenosine polyphosphates as antagonists of the endogenous P2Y$_1$ receptor in rat brain capillary endothelial cells of the B7 and B10 clones. Br J Pharmacol 129:1506–1512

75. Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 43:647–661

76. Westover EJ, Covey DF, Brockman HL, Brown RE, Pike LJ (2003) Cholesterol depletion in site-specific increases in epidermal growth factor receptor phosphorylation due to membrane level effects. Studies with cholesterol enantiomers. J Biol Chem 278:51125–51133

77. Xu G-Y and Huang L-YM (2004) Ca^{2+}/calmodulin-dependent protein kinase II potentiates ATP responses by promoting trafficking of P2X receptors. PNAS 101:11868–11873

78. Zwick E, Hackel PO, Prenzel N, Ullrich A (1999) The EGF receptor as central transducer of heterologous signaling systems. Trends Pharmacol Sci 20:408–412