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Neuroscience Letters 389 (2005) 51–56
Neuroscience Letters
www.elsevier.com/locate/neulet

Phosphorylation of AMPA receptor subunits is differentially regulated by phospholipase A_2 inhibitors

Caroline Ménard [a,*], Christian Patenaude [a], Guy Massicotte [a,b]

[a] *Département de chimie-biologie, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Que., Canada G9A 5H7*
[b] *Département de pharmacologie, Faculté de médecine, Université de Montréal, Montréal, Que., Canada*

Received 8 April 2005; received in revised form 3 June 2005; accepted 8 July 2005

Abstract

Our laboratory recently discovered that the phosphorylation of subunits forming the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) subtype of glutamate receptors is regulated by constitutive phospholipase A_2 (PLA_2) activity in rat brain sections. In the present investigation, arachidonyl trifluoromethyl ketone (AACOCF3) and bromoenol lactone (BEL) were used to compare the influence of calcium-dependent ($cPLA_2$) and calcium-independent ($iPLA_2$) enzymes on phosphorylation of AMPA and *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors. Incubation of rat brain sections with 3 μM BEL enhanced phosphorylation on the serine (Ser) 831 residue of the AMPA receptor GluR1 subunit in synaptosomal P2 fractions, whereas AACOCF3 at the same concentration resulted in increased phosphorylation on residues Ser880/891 of GluR2/3 subunits. These effects were restricted to the AMPA receptor subtype as no changes in phosphorylation were elicited on the NMDA receptor NR1 subunit. The effects of BEL and AACOCF3 were not occluded during blockade of protein phosphatases since AMPA receptor phosphorylation was still apparent in the presence of okadaic acid, indicating that the PLA_2 inhibitor-induced increase in AMPA receptor phosphorylation does not rely on a decrease in dephosphorylation reactions. However, pretreatment of rat brain sections with a cell-permeable protein kinase C (PKC) inhibitor prevented BEL- and AACOCF3-induced phosphorylation on the Ser831 and Ser880/891 sites of GluR1 and GluR2/3 subunits, respectively. These results suggest that constitutive $cPLA_2$ and $iPLA_2$ systems may differentially influence AMPA receptor properties and function in the rat brain through mechanisms involving PKC activity.

Keywords: Phospholipases A_2; Glutamate receptor; Phosphorylation; Brain sections

Phospholipases A_2 (PLA_2s) are a large and diverse superfamily of enzymes which primarily catalyze the hydrolysis of membrane phospholipids at the sn-2 position to generate lysophospholipids and free fatty acids [13]. These enzymes are well known for their involvement in the regulation of inflammation, immune function and smooth muscle contraction through the production of arachidonic acid and its subsequent metabolism to eicosanoids (prostaglandins, leukotrienes, etc.) via cyclooxygenase and lipoxygenase enzymes [13]. More than 19 isoforms of PLA_2s have been identified so far and classified in three main groups, namely, the cytosolic calcium-dependent group ($cPLA_2$), the calcium-independent group ($iPLA_2$), and the secreted group. In the brain, $cPLA_2$ and $iPLA_2$ systems have attracted considerable attention because of their possible involvement in learning and memory [7], the formation of long-term potentiation [15], and the development of several neurological diseases [23].

Recent experimental results have provided evidence that constitutive $iPLA_2$ activity interacts with synaptic function by modulating phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) subtype

Abbreviations: AACOCF3, arachidonyl trifluoromethyl ketone; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; BEL, bromoenol lactone; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; PLA_2, phospholipase A_2; $cPLA_2$, calcium-dependent phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2; PP, protein phosphatases; Ser, serine

* Corresponding author. Tel.: +1 819 376 5053; fax: +1 819 376 5084.
E-mail address: Caroline_Menard@uqtr.ca (C. Ménard).

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doi:10.1016/j.neulet.2005.07.012

of glutamate receptors in pyramidal neurons of the hippocampus [16,22]. Although the molecular link between $iPLA_2$ and AMPA receptor phosphorylation remains to be elucidated, it has been found that endogenous $iPLA_2$ activity controls AMPA-mediated synaptic transmission by limiting phosphorylation on serine (Ser) sites of the GluR1 subunit. This observation is of particular interest because AMPA receptor modulation represents one of the basic mechanisms regulating glutamatergic responses and toxicity in many neuronal networks [10]. Whether such modulation occurs under the influence of calcium-dependent forms of the enzyme ($cPLA_2$) remains, however, largely unknown. In this respect, the aim of the present study was to further investigate the influence of PLA_2 systems on the properties of glutamate receptors. In particular, we compared the effects of $cPLA_2$ and $iPLA_2$ inhibitors on the phosphorylation of both AMPA and *N*-methyl-D-aspartate (NMDA) receptor subunits.

Male Sprague-Dawley rats were purchased from Charles River Canada (St-Constant, Quebec) at the age of 1 month (100–125 g) and were used 1 week after their arrival. They were kept in individual cages under a 12:12-h light-dark cycle in a facility that met laboratory standards (NIH Publication No. 86-23, revised 1985) and Canadian Council on Animal Care guidelines. They were anesthetised, and their brains were quickly removed and frozen in isopentane at −20 °C. Horizontal 30-μm thick brain sections were cut in a cryostat, thaw-mounted on chrome-alum gelatin-coated-slides, and stored at −70 °C. Only sections obtained at the level of the dorsal hippocampus were used. Generally, 12 sections were placed on four slides to obtain enough tissue for immunoprecipitation and Western blotting.

Adjacent rat brain sections were preincubated for 60 min at 35 °C in 45 mL of 100 mM Tris–acetate buffer (pH 7.4) containing 100 μM EGTA with or without PLA_2 inhibitors; the decision to use EGTA was based on previous reports indicating that AMPA receptor subunits are very sensitive to C-terminal degradation by calcium-dependent proteases [3]. Moreover, biochemical studies showed that, even at concentrations exceeding millimolar levels, EGTA only slightly interfered with $cPLA_2$ activity in the brain [8]. Tissue was collected, homogenized and centrifuged to obtain membrane fractions [9]. Homogenates were centrifuged at $1000 \times g$ for 10 min, and the supernatants were centrifuged at $11{,}500 \times g$ for 20 min. The resulting pellet, P2, was defined as the crude synaptosomal fraction, and protein levels were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario). For immunoprecipitation, 50–100 μg of synaptic membranes were suspended in lysis buffer (100 mM Tris–HCl, 100 μM EGTA, pH 7.4) containing 1% sodium dodecyl sulfate, heated at 100 °C for 5 min, and diluted in cold lysis buffer containing 2% Triton X-100. Protein A/G-agarose (Upstate Biotechnology, Lake Placid, NY) and antibodies recognizing the C-terminal domain for the AMPA receptor GluR1 (0.5 μg/mL, Upstate Biotechnology) or GluR2 subunit (0.5 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA), and for the NMDA receptor NR1 subunit (0.5 μg/mL, Santa Cruz Biotechnology) were added to the supernatant and incubated overnight with agitation at 4 °C. Immunoreactive complexes were recovered by centrifugation and washed with lysis buffer. Proteins were finally eluted in 4× sample buffer and heated at 100 °C for 5 min.

For Western Blotting, the aliquots were subjected to sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in phosphate-buffered saline containing 5% dry non-fat milk to block nonspecific sites. They were then incubated with primary antibodies: phospho-GluR1 Ser831 (0.5 μg/mL, Upstate Biotechnology), phospho-GluR2/3 Ser880/891 and phospho-NR1 Ser896/897 (0.5 μg/mL, Santa Cruz Biotechnology). Bands corresponding to the GluR and NR subunits were detected with a peroxidase-conjugated secondary antibody (Sigma–Aldrich, St. Louis, MO) and a chemiluminescent peroxide substrate (MJS Biolynx, Brockville, Ontario). The immunoblots were analyzed semi-quantitatively by densitometry with a microcomputer imaging device (Imaging Research, MCID, St. Catherines, Ontario) providing peak areas and apparent molecular weights.

The PLA_2 inhibitors AACOCF3 (IC_{50}, 2.5 μM) and bromoenol lactone (BEL, IC_{50}, 0.1–1 μM) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA) and dissolved in DMSO (0.2% final concentration). Their chemical structures appear in Fig. 1A. Okadaic acid, the inhibitor of protein phosphatases (PP)—PP1, IC_{50}: 42 nM; PP2A, IC_{50}: 0.51 nM; PP2B, IC_{50}: 5000 nM; PP2C, IC_{50}: 10,000 nM-and Ro 31-8220, the cell-permeable protein kinase C (PKC) inhibitor, IC_{50}: 10 nM, were also purchased from BIOMOL Research Laboratories. All other chemical reagents were obtained from Sigma–Aldrich.

Differences in subunit phosphorylation levels were compared by analysis of variance, followed by Bonferroni's post hoc analysis (GraphPad Prism 4) with conventional criteria of statistical significance: P-values <0.05.

The influence of PLA_2 enzymes on the phosphorylation of AMPA and NMDA receptors was compared by incubating frozen-thawed rat brain sections with the $iPLA_2$ inhibitor BEL or the $cPLA_2$ inhibitor AACOCF3 (Fig. 1A). As shown in Fig. 1B, in synaptosomal P2 fractions, phosphorylation was markedly enhanced (by about 75%) on residue Ser831 of AMPA receptor GluR1 subunit present in synaptic membranes when brain sections were incubated with 3 μM BEL. In the same sections, phosphorylation on residues Ser880/891 of GluR2/3 subunits was not affected by the $iPLA_2$ inhibitor BEL. Interestingly, treatment of rat brain sections with 3 μM AACOCF3 produced the opposite effect, as incubation with the $cPLA_2$ inhibitor resulted in a 51% increase of GluR2/3 Ser880/891 phosphorylation but did not modify GluR1 phosphorylation (Fig. 1B). We also investigated, in the same sections, whether NMDA receptor phosphorylation was regulated by PLA_2 inhibitors. In contrast to AMPA receptors, neither BEL nor AACOCF3 modified phospho-