FEBS Letters 576 (2004) 428–432 FEBS 28887

Dual signaling by mGluR5a results in bi-directional modulation of N-type Ca^{2+} channels

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Received 7 September 2004; accepted 9 September 2004

Available online 7 October 2004

Edited by Maurice Montal

Abstract We have studied how N-type Ca^{2+} channels are modulated by the metabotropic glutamate receptor 5a (mGluR5a) in *Xenopus oocytes*. Stimulation of the receptor with glutamate initiated two parallel responses, a rapid inhibition followed by an upregulation of the Ca^{2+} current. Although a subsequent stimulation did not upregulate the Ca^{2+} current, it did still produce a reduction in the amplitude of the current. The upregulation of Ca^{2+} channels was prevented by the protein kinases inhibitor staurosporine and it was mimicked by the activation of PKC with phorbol esters. In contrast, the inhibition of the Ca^{2+} current was insensitive to staurosporine. These results show that mGluR5a exerts a bi-directional influence on Ca^{2+} channels, which may explain how group I mGluRs facilitate and inhibit glutamate release at central synapses.

Keywords: mGluR5a; N-type Ca^{2+} channel; Desensitization; PI hydrolysis; PKC; *Xenopus oocytes*

1. Introduction

The signaling pathways activated by group I metabotropic glutamate receptors (mGluR1 and mGluR5) include those involving phospholipase A [1], adenylyl cyclase [1], as well as others that inhibit Ca^{2+} channels [2,3]. These receptors are also coupled to phospholipase C (PLC), which mediates the hydrolysis of phosphoinositide (PI) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) [1,4,5]. As the consequence of activating such signaling pathways, group I mGluRs can either reduce or increase excitatory synaptic transmission in the CA1 hippocampal area [6–9]. Indeed, we have shown that facilitation of transmitter release is associated with an increase in diacylglycerol and in PKC activity, leading to an enhanced influx of Ca^{2+} [10]. In contrast, the inhibition of transmitter release is independent of this signaling pathway and linked to a decrease in the influx of Ca^{2+} [10]. We have proposed that both these actions are mediated by the same mGluR that undergoes a switch in activity upon exposure to its agonist [9,10]. Therefore, we would expect that the selective fading of specific signaling pathways has striking effects on the physiological activity of these receptors. Interestingly, it is well known that the hydrolysis of PI triggered by group I mGluRs desensitizes when agonist stimulation is prolonged [10–13] and that this is largely due to the phosphorylation of the receptor at multiple sites by PKC [13].

Given the importance of Ca^{2+} channels in the control of glutamate release, we have investigated whether group I mGluRs can differentially modulate these channels by activating different signaling pathways. As a result, we found that PKC-dependent and independent signaling by mGluR5a exerts bi-directional control of Ca^{2+} channel activity, which underlies group I mGluR-induced facilitation and inhibition of glutamate release at synapses.

2. Materials and methods

2.1. Expression of mGluR5a and Ca^{2+} channels in Xenopus oocytes

Adult *Xenopus laevis* were obtained from the CNRS *Xenopus* facility (Montpellier, France) and we obtained the oocytes from females as described previously [14]. After defolliculation, oocytes were allowed to recover for 24 h at 17 °C in standard ND96 medium buffered to pH 7.4 (96 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1.8 $CaCl_2$ and 10 mM HEPES), and supplemented with 2.5 mM sodium pyruvate, 0.1 mg/ml streptomycin and 100 U/ml penicillin. The cRNAs were injected in 50 nl (6–15 ng/oocyte) and the oocytes were maintained in standard medium at 17 °C for 2–6 days prior to performing electrophysiological recordings.

2.2. RNA synthesis

Prior to RNA synthesis, the plasmids containing the cDNAs were linearized: the plasmid containing the mGluR5a cDNA with *Xba*I (pBluescript SK-); the plasmid with the cDNA encoding the α1B-b subunit (Ca_v 2.2) of N-type Ca^{2+} channels with *Vsp*I (pBSTA); and that with the β_3 subunit of Ca^{2+} channels with *Eco*RI (pcDNA3). The cRNAs were then transcribed in vitro from these linearized plasmids using the mMessage mMachine kit from Ambion (Austin, TX, USA).

2.3. Solutions and data analysis

Two-electrode voltage-clamp recordings were performed using a Geneclamp 500 amplifier (Axon Instruments, Union City, CA), and analyzed with pCLAMP and Axoscope software (Axon Instruments). Cells were voltage clamped at −60 mV with the aid of two microelectrodes filled with 3 M KCl with a resistance typically in the range of 0.5–1.5 MΩ. To isolate the Ca^{2+} currents, recordings were performed in a solution containing (in mM) NaCl 96, KCl 2, $MgCl_2$ 2, $BaCl_2$ 10, TEA 10, and HEPES 10, supplemented with niflumic acid (0.4 mM) to block Cl^- channels. On occasions, Ba^{2+} currents showed rundown, which was corrected off-line by subtracting the slope resulting from the linear fit to the time course plot. For measuring purposes, leak currents were subtracted from original recordings.

All drugs were obtained from Sigma (St. Louis, MO), except for staurosporine and collagenase D, which were supplied by Boehringer Mannheim (Germany). The *Vsp*I and *Eco*RI restriction enzymes were

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0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.09.053

obtained from MBI-Fermentas (Lithuania) and *Xba*I was from New England Biolabs (USA).

3. Results

3.1. The Ca^{2+}-dependent Cl^- current induced by activation of mGluR5a

The activation of receptors coupled to PLC and PI hydrolysis enhances the production of IP_3 and diacylglycerol. Whilst the latter activates protein kinase C, IP_3 induces the release of calcium from intracellular stores, which in oocytes triggers an endogenous Ca^{2+}-dependent Cl^- current. Therefore, the amplitude of this current can be taken as an estimate of the activity of G protein-coupled receptors linked to PI hydrolysis [15]. When we expressed mGluR5a in oocytes, a single pulse of glutamate (100 μM, 1 min) induced a transient inward current due to the opening of endogenous Ca^{2+}-dependent Cl^- channels (3–4 μA in amplitude; Fig. 1A). This glutamate pulse desensitized the receptors, since a second addition of glutamate shortly afterwards (e.g., 2 min) produced little or no effect (Fig. 1A). However, when glutamate was applied 30 min later, the mGluR5a-induced current was largely recovered. Indeed, the reduction in the response to a second pulse of glutamate delivered 2–5 min after the first was $72.3 \pm 6.5\%$ ($n = 24$) but when the second pulse was administered 20–30 min later, the response only decreased by $21.3 \pm 7.1\%$ ($n = 4$; Fig. 1B). As shown previously, the decrease of the Cl^- mediated response is due to receptor desensitization rather than to the inactivation of Cl^- channels or the depletion of intracellular Ca^{2+} stores [13]. These data indicate that the recovery from agonist-induced desensitization of recombinant mGluR5a receptors in oocytes is slow and is similar to that previously seen in brain preparations [10,12].

3.2. The modulation of N-type Ca^{2+} channels by mGluR5a is bi-directional

A large body of evidence indicates that transmitter release is facilitated by mGluR-mediated induction of PI hydrolysis and PKC activation, while the inhibition of release is mostly independent of this signaling pathway [9,10,12,16,17]. Since the extent of neurotransmitter release is established through Ca^{2+} channel activity, we determined whether mGluR5 could exert a bi-directional influence on these channels. Oocytes were injected with cRNAs encoding for both the N-type Ca^{2+} channel subunits and mGluR5a receptors. The Ca^{2+} channels were then opened by applying 60 ms voltage steps to +20 mV from a holding potential of −70 mV and the current carried by Ba^{2+} was measured. Using this protocol, we observed a rapid inhibition of the Ca^{2+} current upon application of glutamate (100 μM, 1 min; Fig. 2C) that subsequently faded and promoted a clear facilitation. On average, glutamate initially inhibited Ca^{2+} channels by $22.4 \pm 3.4\%$ ($n = 6$; measured at the peak of inhibition 20–30 seconds after glutamate addition), while the subsequent facilitation was $16.9 \pm 3.3\%$ ($n = 6$; measured 2 min after glutamate addition: Fig. 2D). However, a subsequent application of glutamate provoked a sustained inhibition of the Ba^{2+} current with no further facilitation. The inability of this second glutamate application to facilitate the Ba^{2+} current was not due to the Ca^{2+}channels having already reached a maximum, since receptor independent activation of PKC with phorbol esters further enhanced Ba^{2+} currents. Indeed, the application of the phorbol ester PDBu (1 μM, 1 min) induced a slow developing upregulation of the Ba^{2+} current that glutamate was still capable of reversibly inhibiting (Fig. 2C). Thus, it seems likely that in response to a pulse of glutamate, two partially overlapping processes are set in motion: a rapid inhibition that depends on the presence of the agonist; and a more slowly developing and long-lasting upregulation of Ca^{2+} channels.

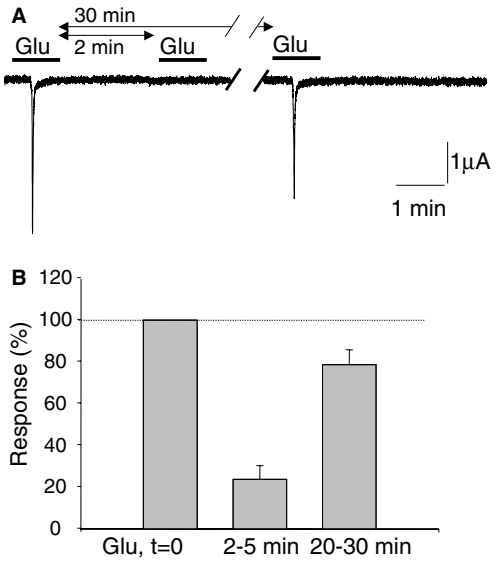

Fig. 1. Desensitization of mGluR5a-induced responses. (A) Electrophysiological responses to paired applications of 100 μM glutamate at the intervals indicated from a single oocyte injected with mGluR5 cRNA (horizontal bars). Note the complete desensitization of the response induced by the first exposure to glutamate and its slow recovery. (B) Bars represent means ± SEM of the Ca^{2+}-dependent Cl^- current amplitude induced by an initial ($n = 25$) and a subsequent application of glutamate delivered 2–5 min ($n = 24$) or 20–30 min ($n = 4$) after the first one.

The dual modulation of Ca^{2+} channel activity by mGluR5a was also observed after a longer application of glutamate (4 min; Fig 3A). Under these conditions, glutamate rapidly inhibited the Ba^{2+} current by $19.0 \pm 3.8\%$ ($n = 4$; Fig. 3B), however, a clear facilitation of the Ba^{2+} current developed afterwards ($17.0 \pm 2.4\%$, $n = 4$, measured 2 min after the application of glutamate commenced: Fig. 3B).

It appeared that the inhibition of Ca^{2+} channels was independent of the capacity of mGluR to activate Cl^- channels. In contrast, the facilitation of the Ca^{2+} current occurs when mGluR5a can activate Ca^{2+} dependent Cl^- channels. These data indicate that the facilitation and the inhibition of the N-type Ca^{2+} channels may be mediated by mGluR5a signaling through two distinct pathways: a PI hydrolysis-dependent and PI hydrolysis-independent pathway, respectively. To confirm this hypothesis, we first examined the influence that G-proteins might have on these events. The modulation of Ca^{2+} channels by mGluR5a was blocked by pertussis toxin (PTX, 500 ng/ml; overnight incubation; Fig. 3C). In the presence of PTX, the mean inhibition of the Ba^{2+} current was $3.1 \pm 1.1\%$ ($n = 5$), while the facilitation of the Ba^{2+} current was $0.5 \pm 0.5\%$ ($n = 5$; Fig. 3D). Thus, in contrast to the coupling of mGluR5a to Gq found in neuronal preparations [18], calcium channel modulation by mGluR5a in oocytes appeared to be largely mediated by the PTX-sensitive Gi or Go subtypes of G proteins [19].

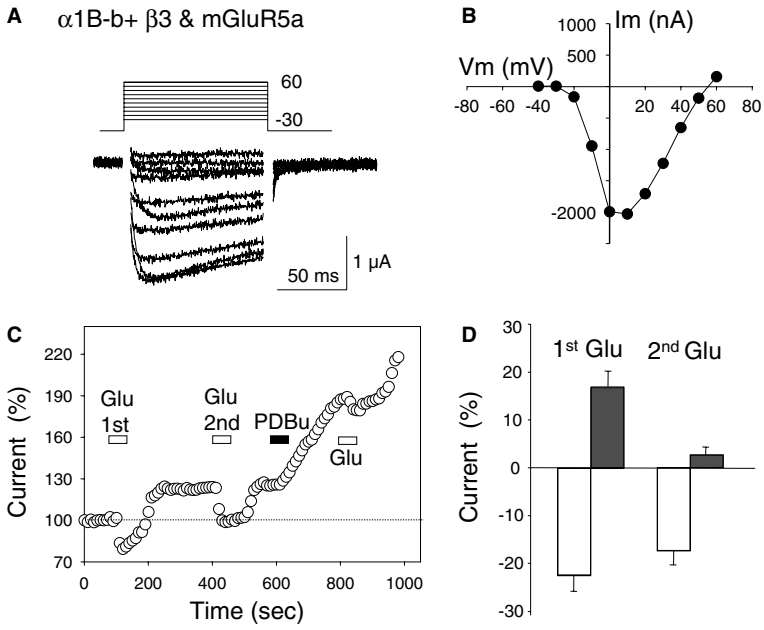

Fig. 2. mGluR5a induces the bi-directional modulation of N-type Ca^{2+} channels. (A) A family of Ba^{2+} currents in oocytes injected with cRNA for $\alpha_{1B\text{-}b}$ (Ca_v 2.2) and β_3, induced by 10 mV steps from a holding potential at −70 mV. (B) The current–voltage relationship for Ba^{2+}-currents. (C) Time-course of the changes in the amplitude of the Ba^{2+} currents (60 ms pulses to +20 mV) in mGluR5a-expressing oocytes following repetitive application of glutamate (100 μM, 1 min). When indicated, PDBu (1 μM, 1 min) was introduced into the recording chamber. (D) Fractional inhibition and facilitation after an initial and second pulse of glutamate. Bars are means ± SEM from six experiments. Measurements were taken 20 s (open bars) and 2 min (shaded bars) after glutamate addition in each case.

To further explore the signaling pathway used by mGluR5a in this bi-directional modulation of N-type Ca^{2+} channels, oocytes were treated with the broad-spectrum protein kinase inhibitor staurosporine (5 μM for 2 h). Interestingly, the addition of glutamate to staurosporine-treated oocytes caused a sustained inhibition of the Ba^{2+} current, which did not fade. Indeed, in these experiments the Ba^{2+} current was diminished by 23.33 ± 2.4% and 19.3 ± 2.7%, when measured 20 s and 2 min after glutamate application ($n = 3$; Fig. 3E and F). Furthermore, although the activation of PKC with PDBu (1 μM, 1 min) enhanced the Ca^{2+} current (46.8 ± 7.6%; $n = 5$; measured 5 min after the addition of glutamate), this effect was completely abolished by staurosporine (0.60 ± 0.70%; $n = 4$: data not shown). These results provide further evidence that the modulation of N-type Ca^{2+} channels by mGluR5a involves two distinct pathways: one that is PKC-dependent that upregulates Ca^{2+} channel activity; and the other that is PKC-independent that provokes the inhibition of Ca^{2+} channel activity. The upregulation of the Ca^{2+} current is only observed after the first application of the agonist, since it desensitizes and becomes tolerant to subsequent stimulation. On the other hand, the second pathway does not desensitize and could be readily activated by repeated applications of the agonist.

4. Discussion

The results presented here indicate that mGluR5a activates two independent pathways that modulate N-type Ca^{2+} channels. Specifically, mGluR5a activates a PKC-dependent facilitatory pathway and a PKC-independent inhibitory pathway. Moreover, Gi/o proteins are involved in both the facilitation and inhibition of N-type Ca^{2+} channels, since both activities are sensitive to PTX in oocytes. Facilitation of the Ca^{2+} current was associated with the ability of the receptor to promote hydrolysis of PI (i.e., PKC stimulation), whilst the inhibition of Ca^{2+} channels was observed even when the pathway leading to PI hydrolysis was desensitized.

In oocytes expressing mGluR5a, stimulation of PI hydrolysis induces the opening of endogenous Ca^{2+}-dependent Cl^- channels as a consequence of the IP_3-induced release of intracellular Ca^{2+}. The accumulation of IP_3 must be paralleled by the synthesis of diacylglycerol, which in turn would activate PKC. When we challenged the mGluR5a receptor with glutamate, Ca^{2+} channel activity was slowly facilitated. However, a second pulse of glutamate was ineffective in activating Ca^{2+}-dependent Cl^- channels, indicating that no PI hydrolysis or PKC activation occurred (see [13]). At the beginning of the first glutamate pulse, the Ca^{2+} current was initially inhibited and this inhibition was clearly unmasked during the second pulse. Although abolished by exposure to PTX, this inhibitory activity was resistant to the protein kinases inhibitor staurosporine. Hence, it seems likely that this inhibition results from a direct interaction between the G-protein βγ component and the pore-forming α_1 subunit of the channel [20–22], and that the slower facilitation probably arises from the PKC-mediated phosphorylation of Ca^{2+} channels [23]. Indeed, the facilitation of the Ca^{2+} currents by the initial application of glutamate was mimicked by exposure to phorbol esters and prevented by the general protein kinase inhibitor staurosporine. These results lead us to conclude that the bi-directional modulation of N-type Ca^{2+} channels is the consequence of dual signaling initiated by mGluR5a.

The desensitization of the mGluR5 provoked Ca^{2+}-dependent Cl^- current response is directly related to the phosphorylation of this receptor at PKC consensus sites in oocytes [13].

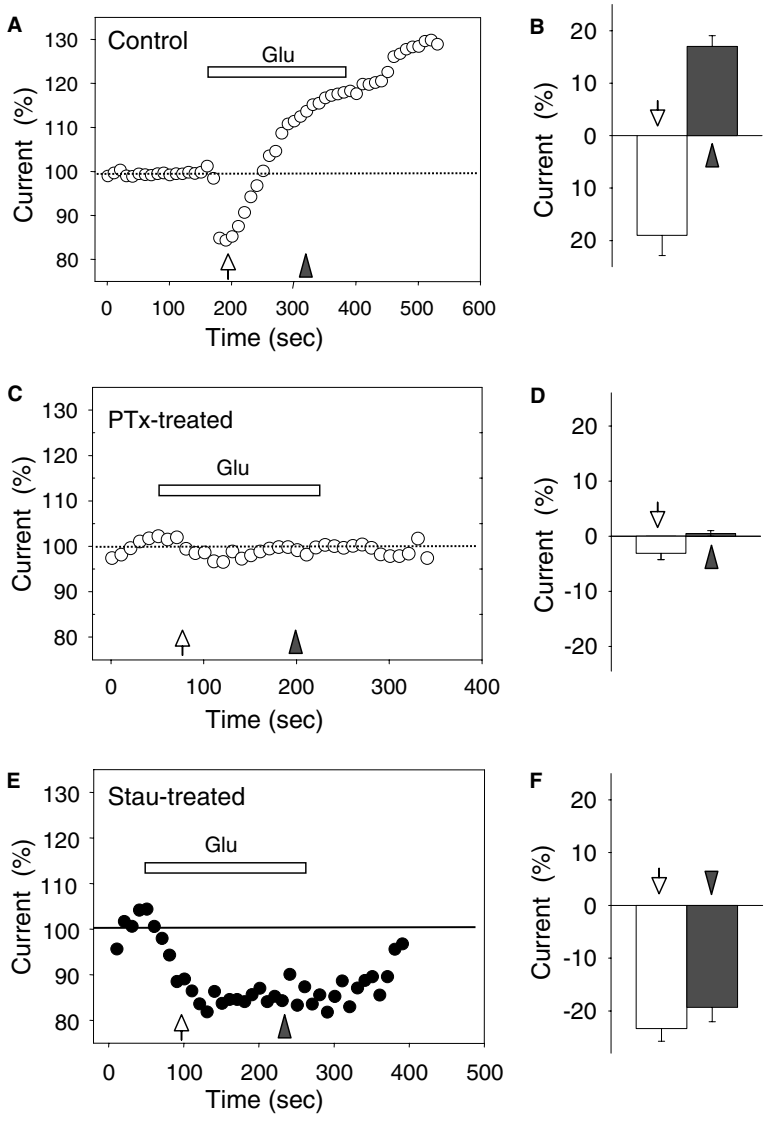

Fig. 3. Changes in Ba^{2+} currents (evoked by 60 ms pulses to +20 mV) induced by a long (4 min) application of glutamate in control oocytes (A, B), and in oocytes treated with pertussis toxin (1 μg/ml, 19 h) (C, D) or staurosporine (5 μM, 2 h) (E, F). Bars are means ± SEM of Ba^{2+} current amplitudes, measured 20 s (arrow, open bars) and 2 min (arrowhead, shaded bars) after glutamate addition, from three to five experiments.

Therefore, it seems likely that the phosphorylation of the receptor differentially affects the distinct signaling pathways. Indeed, a number of findings indicate that this dual signaling by mGluR5a could be switched on and off by selective desensitization of one of the pathways. For instance, in HEK 293 cells expressing mGluR1, the activation of αPKC selectively inhibits glutamate-induced stimulation of IP_3 production, but does not affect receptor signaling via cAMP [24]. A similar mechanism has also been proposed for β_2-adrenergic receptors, whose coupling to Gs or Gi proteins is switched by receptor phosphorylation by PKA, thereby modulating receptor signaling [25]. Similarly, in cultured astrocytes, mGluR5 enhances PI hydrolysis/Ca^{2+} signaling and the activation of the extracellular signal-regulated kinase 2 (ERK2) [26]. In this system, PKC plays an important role in the rapid desensitization of PI hydrolysis/Ca^{2+} signaling, but not in ERK2 phosphorylation.

The bi-directional modulation of N-type Ca^{2+} channels by mGluR5a accounts for the dual modulation of synaptic transmission in the CA1 hippocampal region. Here, perfusion with the group I mGluR agonist DHPG initially facilitates but then inhibits glutamate release [9]. However, the initial inhibition and subsequent potentiation of the N-type Ca^{2+} current observed in oocytes do not fit well with the sequential effect that group I mGluR agonists have in synaptic transmission in slices [9] and glutamate release in synaptosomes [10]. One possible explanation for this discrepancy could be that the exogenous activation of mGluRs in slices or synaptosomes is not attained as rapidly as in oocytes, precluding the temporal resolution of the initial inhibition. Alternatively, the more rapid onset of Ca^{2+} channel inhibition compared to the facilitation observed in oocytes could well reflect the failure of the receptor to couple optimally to the facilitatory pathway in this preparation.

Whatever the reason, the consequence of the dual signaling by mGluR5a accounts for the observed bi-directional modulation of synaptic transmission triggered by group I mGluR agonists. Group I mGluRs share signaling systems in that they

are coupled to similar G proteins. Therefore, the bi-directional modulation of N-type Ca^{2+} channels by these receptors may be a more generalized phenomenon.

Acknowledgements: We thank Dr. D. Lipscombe for the α_{1B} and β_3 cDNAs, and Dr. J.-P. Pin for the mGluR5a cDNA. This work was supported by grants from the Community of Madrid to J. S.-P. (08.5/0016/1998 and 08.5/0075.1/2000) and from the MCYT to JL (PM99-0106). AVP was a postdoctoral fellowship from the Community of Madrid.

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