Biochemical and Biophysical Research Communications 432 (2013) 275–280

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PIP_2 hydrolysis is responsible for voltage independent inhibition of $Ca_V2.2$ channels in sympathetic neurons

Oscar Vivas [a], Hector Castro [a], Isabel Arenas [a], David Elías-Viñas [b], David E. García [a,*]

[a] Department of Physiology, School of Medicine, Universidad Nacional Autónoma de México (UNAM), Apartado Postal 70250, C.P. 04510 Mexico, D.F., Mexico
[b] Section of Bioelectronics, Department of Electrical Engineering, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Apartado Postal 14740, México 07000, Mexico

ARTICLE INFO

Article history:
Received 28 January 2013
Available online 8 February 2013

Keywords:
Calcium channel regulation
$Ca_V2.2$ channel
SCG neuron
Voltage independent inhibition
PIP_2
diC8-PIP_2

ABSTRACT

GPCRs regulate $Ca_V2.2$ channels through both voltage dependent and independent inhibition pathways. The aim of the present work was to assess the phosphatidylinositol-4,5-bisphosphate (PIP_2) as the molecule underlying the voltage independent inhibition of $Ca_V2.2$ channels in SCG neurons. We used a double pulse protocol to study the voltage independent inhibition and changed the PIP_2 concentration by means of blocking the enzyme PLC, filling the cell with a PIP_2 analogue and preventing the PIP_2 resynthesis with wortmannin. We found that voltage independent inhibition requires the activation of PLC and can be hampered by internal dialysis of exogenous PIP_2. In addition, the recovery from voltage independent inhibition is blocked by inhibition of the enzymes involved in the resynthesis of PIP_2. These results support that the hydrolysis of PIP_2 is responsible for the voltage independent inhibition of $Ca_V2.2$ channels.

1. Introduction

Modulation exerted by calcium signaling is a major field in neurobiology. Conversely, calcium channel regulation leads to subsequent changes of related cellular responses such as short term plasticity [1–3]. Several neurotransmitters regulate voltage-gated calcium channels by activation of G-protein coupled receptors (GPCRs). Understanding the mechanisms by which calcium channels are regulated by G-proteins remains a major challenge since there are several molecular pathways upon GPCR activation, having pleiotropic effects [4,5]. The better understood pathway of calcium channel regulation, the so called voltage dependent inhibition, is mediated by $G_{\beta\gamma}$ subunits. It requires G-proteins of the $G\alpha_{i/o}$ family [6,7] and $G_{\beta\gamma}$ subunits [8,9] which subsequently bind to calcium channels in a membrane delimited mechanism [10]. Regulated calcium current by the voltage dependent pathway has particular features: the activation kinetic is slowed [11] and the voltage dependence of the channel is shifted to more positive values [12]. Also, binding between $G_{\beta\gamma}$ subunits and calcium channels is sensitive to strong and positive voltage changes [13,14].

In addition to this regulatory pathway, several authors have shown that there is a remaining fraction of inhibition after $G_{\beta\gamma}$ release, using a protocol in which a strong depolarizing pulse (voltages between +70 and +125 mV) was applied to release transiently $G_{\beta\gamma}$ subunits [13,15,16]. This remaining inhibition was named as voltage independent inhibition. Resistance to strong membrane depolarization suggests that this remaining inhibition involve another mechanism to modulate calcium channels different than $G_{\beta\gamma}$ subunits. Previous research has focused on muscarinic inhibition of calcium channels since muscarinic agonists inhibit calcium channels in a completely different pathway of that mediated by $G_{\beta\gamma}$ subunits. This regulation is sensitive to calcium chelators such as BAPTA [17], is insensitive to toxins blocking $G\alpha_{i/o}$ proteins such as the toxin from *Bordetella Pertussis* [6,18], is slow and uses a diffusible second messenger [10], in contrast to the membrane delimited mechanism mediated by $G_{\beta\gamma}$ subunits. This slow and PTX-insensitive pathway induced by muscarinic agonists seems to be similar to that described as voltage independent inhibition [6]. Whether these two pathways correspond to the same molecular cascade, it has not been firmly established.

In addition, muscarinic agonists inhibit both calcium and M current in sympathetic neurons [18,19]. M current inhibition induced by muscarinic agonists, through activation of muscarinic receptor type 1 (M_1R) [20,21], is also sensitive to calcium chelators [17] and PTX [22], is slow, uses a second messenger and is mediated by $G_{q/11}$ family proteins [23,24]. Now, it is well supported that KCNQ 2/3 channels, the molecular determinants for the M current [25], requires the phosphoinositide 4,5-bisphosphate (PIP_2) to open and that muscarinic inhibition of KCNQ 2/3 channel is mediated by PIP_2 hydrolysis [26–30]. Calcium channels also require PIP_2 [31] and their sensitivity to PIP_2 depends on the specific β subunit

* Corresponding author. Fax: +52 55 5623 2241.
E-mail address: erasmo@unam.mx (D.E. García).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.117

[32]. Nevertheless, whether PIP_2 is the underlying molecule of voltage independent inhibition of $Ca_V2.2$ calcium channels, it has not been established yet.

In this study we used a double pulse protocol as an experimental approach to test whether PIP_2 is the signaling molecule underlying the voltage independent inhibition of $Ca_V2.2$ channel in superior cervical ganglion (SCG) neurons. We manipulated the hydrolysis and resynthesis of PIP_2 and dialyzed diC8-PIP_2 to prevent hydrolysis of endogenous PIP_2 upon PLC activation induced by oxotremorine (oxo-M). In an additional experiment we assessed the effect of exogenous PIP_2 dialysis on muscarinic inhibition of KCNQ 2/3 current to compare it with the effect on calcium channels.

2. Materials and methods

2.1. Cell culture and plasmids

SCG neurons were isolated from 5-week-old male Wistar rats [18]. Rats were obtained from the animal breeding facility of the School of Medicine at the Universidad Nacional Autónoma de México (UNAM) and were handled according to the Mexican Official Norm for Use, Care and Reproduction of Laboratory Animals (NOM-062-ZOO-1999). Rats were anaesthetized with CO_2 and decapitated with a guillotine. After dissection, ganglia were desheathed and sliced into eight pieces. The tissue was transferred to a tube containing modified Hank's solution supplemented with 20 U/ml papain and after 20 min at 37 °C, it was transferred to a solution containing 1 mg/ml collagenase type I and 10 mg/ml dispase for another 20 min before mechanically disaggregating the tissue. The cell suspension was centrifuged at 180*g* for 3 min and washed twice in Leibovitz's L-15 medium and once in Dulbecco's modified Eagle's medium (DMEM), both supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. Cells were then plated on polystyrene culture dishes coated with poly-L-lysine and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were used up to 24 h after plating. Reagents were obtained as follow: L-15 and DMEM (Invitrogen Corp., Carlsbad, CA, USA), U73122 and U73343 (Merck KGaA, Darmstadt, Germany), diC8-PIP_2 (Echelon Biosciences Inc., Salt Lake City, UT, USA) and all other reagents were obtained from Sigma (St. Louis, MO, USA).

TsA-201 cells were grown in medium DMEM supplemented with 10% fetal bovine serum and 2% of penicillin/streptomycin. Cultures at 60–70% confluency were transfected with 1 μg DNA and Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA). KCNQ 2/3 currents were recorded 36–48 h after transfection. Mouse muscarinic receptor type 1 (M1R), human KCNQ 2, human KCNQ 3 and voltage sensitive phosphatase (VSP) coupled to GFP were provided by B. Hille (University of Washington, Seattle, WA).

2.2. Electrophysiology

$Ca_V2.2$ current was recorded in the whole-cell configuration of the patch-clamp technique [19] with a HEKA EPC 9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature. We used pipettes of borosilicate glass (kimble chase, Vineland, NJ, USA) with a resistance of 1.6–2.2 MΩ. Series resistance was 3–7 MΩ and compensated to > 60%. Currents were sampled at 100 kHz. Current recordings were filtered at 2.9 kHz. Cells were continuously bathed with control or test solutions at 2 ml/min rate. The control bath solution contained (in mM): 160 NaCl, 2.5 KCl_2, 10 HEPES, 8 glucose, 5 $CaCl_2$, 1 $MgCl_2$, 0.0001 TTX and 0.005 nifedipine, pH adjusted to 7.4 with NaOH. Oxo-M was diluted in bath solution to a final concentration of 10 μM. The pipette solution contained (in mM): 140 CsCl, 32 TEA-Cl, 10 HEPES, 0.1 BAPTA-4 Cs, 1 $MgCl_2$, 3 Na_2ATP, 3 Na_2GTP, and 0.1 leupeptin, pH adjusted to 7.4 with CsOH. $Ca_V2.2$ currents were defined as the component of the current sensitive to 100 μM $CdCl_2$ in the presence of 5 μM of nifedipine [10]. Calcium current was elicited with a double pulse protocol applied every 4 s. Steady-state current amplitude was calculated as the mean value of recorded points between 6 and 7 ms after the onset of the pulse. Percent of inhibition was calculated as the calcium current amplitude during oxo-M application subtracted from calcium current amplitude in control condition divided by calcium current amplitude in control condition. For KCNQ 2/3 recordings the pipette solution contained (in mM): 175 KCl, 5 $MgCl_2$, 5 HEPES, 0.1 BAPTA-4K, 3 Na_2ATP, 0.1 Na_2-GTP, and 0.1 leupeptin, pH adjusted to 7.4 with KOH. Results are presented as mean ± SEM.

3. Results

3.1. Oxo-M inhibition requires PLC activation

Muscarinic agonists reduce calcium current by two different components: one, fast and PTX-sensitive and the other slow, PTX-insensitive and calcium-dependent. The latter seems not to shift voltage dependence of the channels [5,10]. The current modulated under this muscarinic component has similar characteristics of the current regulated by the voltage independent pathway [6]. However, whether or not these two pathways correspond to the same molecular cascade has not been firmly established. Here, we were interested in the molecule responsible for muscarinic inhibition remaining after a depolarized pulse, which corresponds to the voltage independent inhibition. The protocol we used to iso-

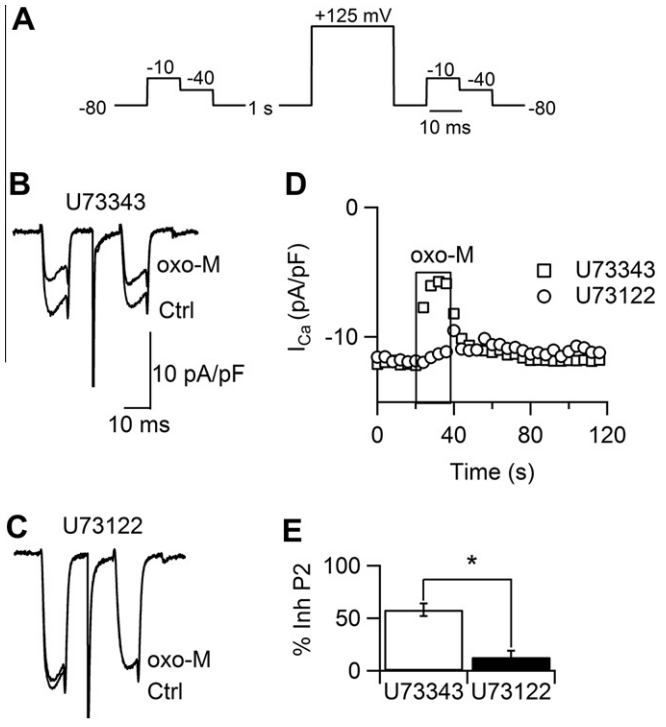

Fig. 1. PLC activation is required for muscarinic voltage independent inhibition of $Ca_V2.2$ calcium channel. (A) Double pulse protocol used to isolate voltage independent inhibition. (B and C) Representative superimposed currents under 10 μM oxo-M application and under control condition in neurons treated with U73343 (B) or U73122 (C). (D) Time course of calcium current amplitude in neurons treated with U73343 and U73122. (E) Summary of current inhibition in pulse 2 for each condition. Data plotted as mean ± SEM. * indicates $p < 0.05$.

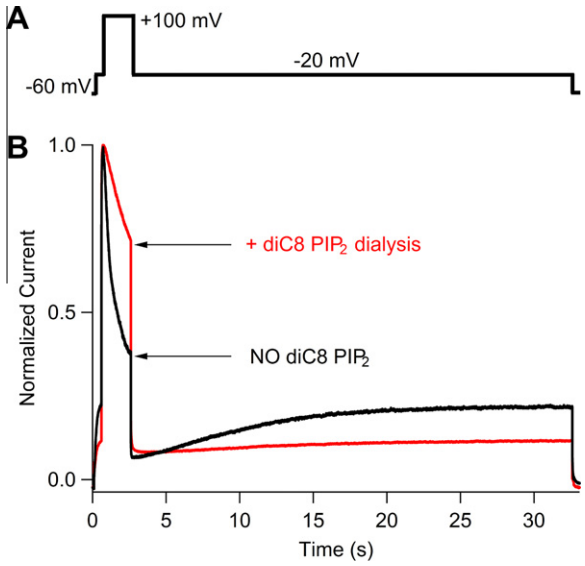

Fig. 2. Effect of diC8-PIP_2 dialysis on KCNQ 2/3 current. (A) Voltage protocol to activate KCNQ 2/3 channel and VSP. (B) Average superimposed currents in tsA-201 cells without (black, $n = 8$) and with (red, $n = 9$) 100 μM diC8-PIP_2 dialysis. Currents were normalized to the maximal current. diC8-PIP_2 was dialyzed up to 7 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

late the voltage independent inhibition consisted of two identical pulses from −80 to −10 mV for 10 ms, separated for 1 s each one, yet the second pulse was 10 ms anticipated by a 25 ms conditioning pulse to +125 mV (Fig. 1A). Since oxo-M inhibits calcium current in SCG neurons by activation of $G\alpha_{q/11}$ subunits [23,24] and since $G\alpha_{q/11}$ family activates phospholipase C (PLC), we tested the effect of an inhibitor of PLC, U73122, on voltage independent inhibition. Neurons were bathed with 5 μM of U73122, or with 5 μM of U73343, which is an inactive analogue, as negative control. Both compounds were diluted in DMSO. Final concentration (v/v) for DMSO was 0.2%. $Ca_V2.2$ current was not inhibited by oxo-M when cells where bathed with U73122 as can be seen in superimposed traces in Fig. 1C, whereas U73343 did not prevent current inhibition by oxo-M (Fig. 1B). Fig. 1D compares time courses for inhibition in pulse 2 between a cell in presence of U73343 and a cell in presence of U73122. In cells bathed with U73343 and exposed to oxo-M, calcium current was inhibited by 58 ± 6% ($n = 8$), however when cells were bathed with U73122 calcium current was inhibited by 13 ± 6% ($n = 6$, Fig. 1E). Therefore U73122 reduced calcium current inhibition by oxo-M, suggesting that PLC activation is required for voltage independent inhibition.

3.2. PIP_2 underlies voltage-independent inhibition

PLC activation leads to PIP_2 hydrolysis. Also, PIP_2 is a necessary cofactor for KCNQ 2/3 channels [26–30] and high voltage activated calcium channels [31]. We tested whether PIP_2 is the molecule responsible for the voltage independent inhibition of endogenous calcium channels in SCG neurons. To this end we filled the cell with 100 μM of diC8-PIP_2 through the recording pipette. This analogue of the endogenous PIP_2 is soluble in water at 1 mg/ml. The rationale of this experiment was that an extra source for the PLC substrate would hamper the hydrolysis of endogenous PIP_2 in the plasma membrane and therefore oxo-M could not inhibit calcium current. We first tested this experimental approach on the inhibition induced by oxo-M on KCNQ 2/3 channel. For this purpose KCNQ 2 and KCNQ 3 channels were transfected in TsA-201 cells. A voltage-sensitive phosphatase (VSP) was co-transfected to

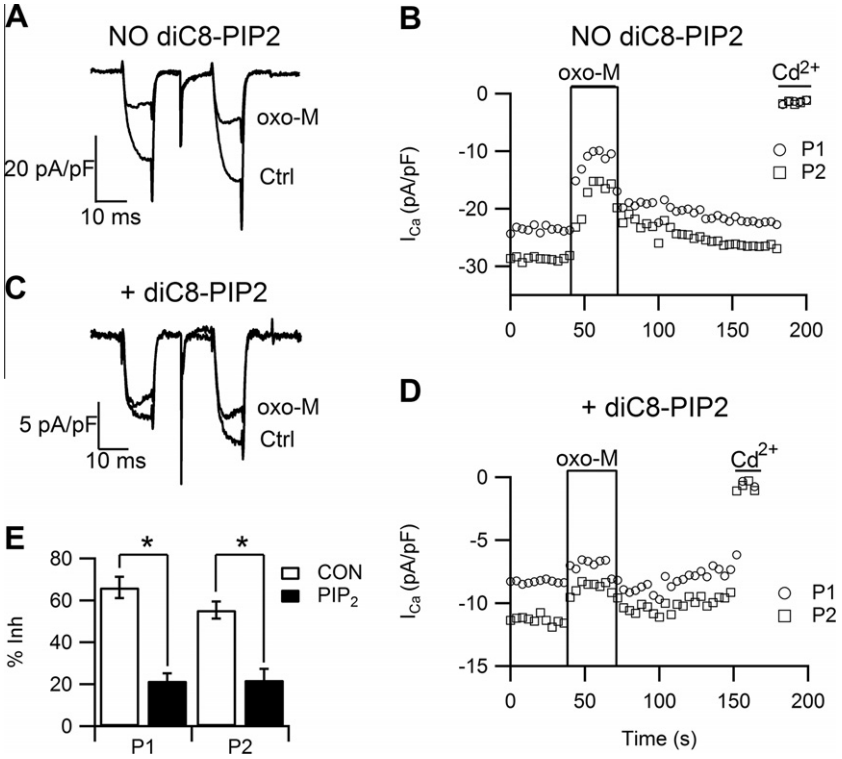

Fig. 3. Effect of diC8-PIP_2 dialysis on voltage independent inhibition of $Ca_V2.2$ channel. (A and C) Representative superimposed currents under oxo-M application and under control conditions in neurons without (A) or with (C) dialysis of 100 μM diC8-PIP_2. (B and D) Time courses of calcium current amplitude in neurons without (B) or with (D) dialysis of diC8-PIP_2. diC8-PIP2 was dialyzed up to 7 min. (E) Summary of current inhibition in pulse 1 (P1) and pulse 2 (P2) in neurons without (CON) or with (PIP_2) dialysis of diC8-PIP_2. Data plotted as mean ± SEM. * indicates $p < 0.05$.

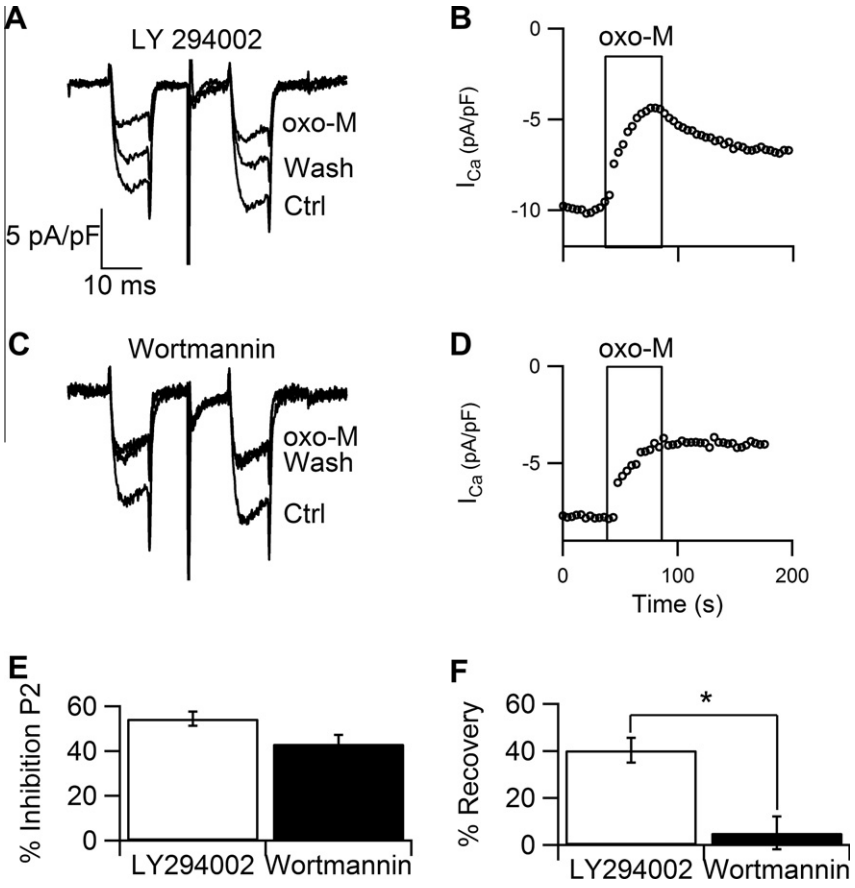

Fig. 4. Wortmannin inhibition of calcium current recovery. (A and C) Representative superimposed currents before (Ctrl), during (oxo-M) and after (Wash) 10 μM oxo-M application in neurons treated with LY294002 (A) or Wortmannin (C). (B and D) Time courses calcium current amplitude in neurons treated with LY294002 (B) or Wortmannin (D). (E) Summary of current inhibition in pulse 2 (P2) for each condition. (F) Summary of current recovery for each condition. Data plotted as mean ± SEM. * indicates $p < 0.05$.

diminish PIP_2 concentration at the plasma membrane. VSP was activated with a 2 s voltage pulse to +100 mV. KCNQ 2/3 current was recorded during a 30 s depolarizing pulse from −60 to −20 mV. After 500 ms at −20 mV, it was applied the pulse to activate the VSP. In this protocol we observed two signs of KCNQ 2/3 current inhibition; KCNQ 2/3 current decays during the pulse to +100 mV and it is much reduced upon return to −20 mV and recovered thereafter, as formerly reported [26]. In cells where diC8-PIP_2 was dialyzed ($n = 9$), KCNQ 2/3 current were less inhibited upon VSP activation (Fig. 2). This result suggests that filling the cell with diC8-PIP_2 through the recording pipette can be used as an experimental approach to test the role of PIP_2 in voltage independent inhibition of $Ca_V2.2$ calcium channels and that diC8-PIP_2 serves as a substrate source for enzymes binding endogenous PIP_2. Then, we used the same approach to test calcium current inhibition in SCG neurons. In PIP_2-dialyzed neurons, oxo-M application inhibited calcium current by 22 ± 5% ($n = 9$), whereas calcium current was inhibited by 55 ± 4% in non-dialyzed neurons ($n = 9$, Fig. 3). This reduction of muscarinic inhibition of $Ca_V2.2$ current suggests that PIP_2 hydrolysis is responsible of the voltage independent inhibition of $Ca_V2.2$ channels.

3.3. Recovery from voltage independent inhibition is blunted with wortmannin

Accordingly with a mechanism involving hydrolysis of PIP_2, recovery from voltage independent inhibition would need the activation of the phosphatidylinositol 4-kinase (PI4K) and the phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) to resynthesize PIP_2 from phosphatidylinositol. Wortmannin inhibits PI4K along with the phosphatidylinositol 3-kinase (PI3K), preventing the resynthesis of PIP_2 [33]. We bathed neurons with 10 μM of wortmannin and applied oxo-M to inhibit calcium current. Inhibition of calcium current was similar to control cells, however recovery was only 5 ± 7% ($n = 7$). On the other hand 50 μM LY294002, which inhibits specifically PI3K and should not affect PIP_2 resynthesis, did not affect recovery (40 ± 5%, $n = 10$) from the voltage independent inhibition induced by oxo-M (Fig. 4). Therefore, PIP_2 resynthesis is needed to recovery from voltage independent inhibition. All these results together suggest that the hydrolysis of PIP_2 is responsible for the voltage independent regulation of $Ca_V2.2$ channels after muscarinic signaling.

4. Discussion

In this paper we assayed PIP_2 hydrolysis as a possible mechanism underlying voltage independent inhibition on $Ca_V2.2$ channels induced by muscarinic activation. Voltage independent inhibition did not occur by preventing hydrolysis of phosphoinositides with a PLC inhibitor. As muscarinic PLC activation leads to phosphoinositide hydrolysis [34–36] and since we found that inhibition of PLC prevented oxo-M for inhibiting $Ca_V2.2$ channels, PIP_2 hydrolysis can explain voltage independent inhibition. Our result is in accordance with previous reports where PLC activation was required by oxo-M to inhibit M current [27], inhibition that is

mediated by PIP_2, and by bradykinin to inhibit neurotransmitter release in rat superior cervical ganglion neurons [37].

We also showed that diC8-PIP_2 into the recording pipette hampered inhibition induced by oxo-M on KCNQ 2/3 and $Ca_V2.2$ channels, presumably by means of binding with the enzyme, VSP or PLC, to avoid dephosphorylation or hydrolysis of endogenous PIP_2. DiC8-PIP_2 is a soluble phosphoinositide with two acyl chains composed with eight carbon atoms. The critical micellar concentration of PIP_2 in saline solutions is 30 μM [38]. Since we use 100 μM of diC8-PIP_2, we speculate that phosphoinositides are organized mainly in micelles. Once diC8-PIP_2 is into the cell it is able to incorporate with plasma membrane [39]. Upon activation of VSP or PLC neither potassium nor calcium current is inhibited when cells are filled with diC8-PIP_2, suggesting that this approach is blocking the effect of both enzymes acting on endogenous PIP_2, and therefore supporting that PIP_2 is the responsible molecule of voltage independent inhibition of $Ca_V2.2$ channels by oxo-M. In addition, diC8-PIP_2 did not affect tonic inhibition. Accordingly, PIP_2 seems to not affect voltage dependent inhibition although we did not test this idea further. Future experiments will be focused on the effect of neurotransmitters which induce voltage dependent and independent components in order to test PIP_2 as general molecule of voltage independent inhibition.

Releasing of $G_{\beta\gamma}$ also occurs in physiological conditions after the neuron is stimulated with a train of action potentials [1,40]. This suggests that some extent of calcium channel inhibition at synapses depends on circuit activity, but the remaining fraction, the voltage independent inhibition, remains despite neuron activity. Thus, after G-protein coupled receptors are activated and concomitant burst stimulation arrived into synapse, $Ca_V2.2$ channels might be still inhibited by voltage independent inhibition. Accordingly with our results, voltage independent inhibition is mediated by PIP_2 hydrolysis and the recover from inhibition need PIP_2 resynthesis. We propose that fine-tuning of PIP_2 concentration is a general mechanism to regulate synaptic transmission. In synapse, PIP_2 is located in microdomains and colocalized with syntaxin clusters [41]. Since neurotransmitter release occurs in functional microdomains, where synaptic molecules, such as syntaxin, and calcium channels are cluster together, and PIP_2 is also located at this microdomains, PIP_2 hydrolysis seems to be an important cue in regulating neurotransmitter release.

Acknowledgments

This work was supported by UNAM-DGAPA-PAPIIT IN200710 grant, and a grant from The Alexander von Humboldt Stiftung, Germany. We thank Manuel Hernandez, and Guillermo Luna for technical help. Ing. Gustavo Díaz for software support and Dr. Enrique Pinzon for excellent care of rats.

References

[1] D.L. Brody, D.T. Yue, Relief of G-protein inhibition of calcium channels and short-term synaptic facilitation in cultured hippocampal neurons, J. Neurosci. 20 (2000) 889–898.
[2] J. Han, M.D. Mark, X. Li, M. Xie, S. Waka, J. Rettig, S. Herlitze, RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating Gi/o-mediated inhibition of presynaptic Ca^{2+} channels, Neuron 51 (2006) 575–586.
[3] W.A. Catterall, A.P. Few, Calcium channel regulation and presynaptic plasticity, Neuron 59 (2008) 882–901.
[4] B. Hille, G protein-coupled mechanisms and nervous signaling, Neuron 9 (1992) 187–195.
[5] B. Hille, Modulation of ion-channel function by G-protein-coupled receptors, Trends Neurosci. 17 (1994) 531–536.
[6] D.J. Beech, L. Bernheim, B. Hille, Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons, Neuron 8 (1992) 97–106.
[7] G.G.t. Holz, S.G. Rane, K. Dunlap, GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels, Nature 319 (1986) 670–672.
[8] S. Herlitze, D.E. Garcia, K. Mackie, B. Hille, T. Scheuer, W.A. Catterall, Modulation of Ca^{2+} channels by G-protein beta gamma subunits, Nature 380 (1996) 258–262.
[9] S.R. Ikeda, Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits, Nature 380 (1996) 255–258.
[10] L. Bernheim, D.J. Beech, B. Hille, A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons, Neuron 6 (1991) 859–867.
[11] C. Marchetti, E. Carbone, H.D. Lux, Effects of dopamine and noradrenaline on Ca channels of cultured sensory and sympathetic neurons of chick, Pflugers Arch. 406 (1986) 104–111.
[12] B.P. Bean, Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence, Nature 340 (1989) 153–156.
[13] K.S. Elmslie, W. Zhou, S.W. Jones, LHRH and GTP-gamma-S modify calcium current activation in bullfrog sympathetic neurons, Neuron 5 (1990) 75–80.
[14] F. Grassi, H.D. Lux, Voltage-dependent GABA-induced modulation of calcium currents in chick sensory neurons, Neurosci. Lett. 105 (1989) 113–119.
[15] A. Formenti, E. Arrigoni, M. Mancia, Two distinct modulatory effects on calcium channels in adult rat sensory neurons, Biophys. J. 64 (1993) 1029–1037.
[16] J.I. Luebke, K. Dunlap, Sensory neuron N-type calcium currents are inhibited by both voltage-dependent and -independent mechanisms, Pflugers Arch. 428 (1994) 499–507.
[17] D.J. Beech, L. Bernheim, A. Mathie, B. Hille, Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons, Proc. Natl. Acad. Sci. USA 88 (1991) 652–656.
[18] E. Wanke, A. Ferroni, A. Malgaroli, A. Ambrosini, T. Pozzan, J. Meldolesi, Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons, Proc. Natl. Acad. Sci. USA 84 (1987) 4313–4317.
[19] A. Constanti, D.A. Brown, M-Currents in voltage-clamped mammalian sympathetic neurones, Neurosci. Lett. 24 (1981) 289–294.
[20] N.V. Marrion, T.G. Smart, S.J. Marsh, D.A. Brown, Muscarinic suppression of the M-current in the rat sympathetic ganglion is mediated by receptors of the M1-subtype, Br. J. Pharmacol. 98 (1989) 557–573.
[21] L. Bernheim, A. Mathie, B. Hille, Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons, Proc. Natl. Acad. Sci. USA 89 (1992) 9544–9548.
[22] D.A. Brown, N.V. Marrion, T.G. Smart, On the transduction mechanism for muscarine-induced inhibition of M-current in cultured rat sympathetic neurones, J. Physiol. 413 (1989) 469–488.
[23] M.P. Caulfield, S. Jones, Y. Vallis, N.J. Buckley, G.D. Kim, G. Milligan, D.A. Brown, Muscarinic M-current inhibition via G alpha q/11 and alpha-adrenoceptor inhibition of Ca^{2+} current via G alpha o in rat sympathetic neurones, J. Physiol. 477 (Pt 3) (1994) 415–422.
[24] J.E. Haley, P. Delmas, S. Offermanns, F.C. Abogadie, M.I. Simon, N.J. Buckley, D.A. Brown, Muscarinic inhibition of calcium current and M current in Galpha q-deficient mice, J. Neurosci. 20 (2000) 3973–3979.
[25] H.S. Wang, Z. Pan, W. Shi, B.S. Brown, R.S. Wymore, I.S. Cohen, J.E. Dixon, D. McKinnon, KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel, Science 282 (1998) 1890–1893.
[26] B.H. Falkenburger, J.B. Jensen, B. Hille, Kinetics of PIP2 metabolism and KCNQ2/3 channel regulation studied with a voltage-sensitive phosphatase in living cells, J. Gen. Physiol. 135 (2010) 99–114.
[27] B.C. Suh, B. Hille, Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis, Neuron 35 (2002) 507–520.
[28] B.C. Suh, T. Inoue, T. Meyer, B. Hille, Rapid chemically induced changes of PtdIns(4,5)P2 gate KCNQ ion channels, Science 314 (2006) 1454–1457.
[29] J.S. Winks, S. Hughes, A.K. Filippov, L. Tatulian, F.C. Abogadie, D.A. Brown, S.J. Marsh, Relationship between membrane phosphatidylinositol-4,5-bisphosphate and receptor-mediated inhibition of native neuronal M channels, J. Neurosci. 25 (2005) 3400–3413.
[30] H. Zhang, L.C. Craciun, T. Mirshahi, T. Rohacs, C.M. Lopes, T. Jin, D.E. Logothetis, PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents, Neuron 37 (2003) 963–975.
[31] B.C. Suh, K. Leal, B. Hille, Modulation of high-voltage activated Ca(2+) channels by membrane phosphatidylinositol 4,5-bisphosphate, Neuron 67 (2010) 224–238.
[32] B.C. Suh, D.I. Kim, B.H. Falkenburger, B. Hille, Membrane-localized beta-subunits alter the PIP2 regulation of high-voltage activated Ca^{2+} channels, Proc. Natl. Acad. Sci. USA 109 (2012) 3161–3166.
[33] G.B. Willars, S.R. Nahorski, R.A. Challiss, Differential regulation of muscarinic acetylcholine receptor-sensitive polyphosphoinositide pools and consequences for signaling in human neuroblastoma cells, J. Biol. Chem. 273 (1998) 5037–5046.
[34] J.G. Baird, D.G. Lambert, J. McBain, S.R. Nahorski, Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in a human neuroblastoma cell line SK-N-SH, Br. J. Pharmacol. 98 (1989) 1328–1334.
[35] J. Horwitz, S. Tsymbalov, R.L. Perlman, Muscarine increases tyrosine 3-monooxygenase activity and phospholipid metabolism in the superior cervical ganglion of the rat, J. Pharmacol. Exp. Ther. 229 (1984) 577–582.
[36] S.E. Moroi-Fetters, N.H. Neff, M. Hadjiconstantinou, Muscarinic receptor-mediated phosphoinositide hydrolysis in the rat retina, J. Pharmacol. Exp. Ther. 246 (1988) 553–557.

[37] S.G. Lechner, S. Hussl, K.W. Schicker, H. Drobny, S. Boehm, Presynaptic inhibition via a phospholipase C- and phosphatidylinositol bisphosphate-dependent regulation of neuronal Ca^{2+} channels, Mol. Pharmacol. 68 (2005) 1387–1396.
[38] P.D. Moens, L.A. Bagatolli, Profilin binding to sub-micellar concentrations of phosphatidylinositol (4,5) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate, Biochim. Biophys. Acta 1768 (2007) 439–449.
[39] U. Golebiewska, M. Nyako, W. Woturski, I. Zaitseva, S. McLaughlin, Diffusion coefficient of fluorescent phosphatidylinositol 4,5-bisphosphate in the plasma membrane of cells, Mol. Biol. Cell 19 (2008) 1663–1669.
[40] D.L. Brody, P.G. Patil, J.G. Mulle, T.P. Snutch, D.T. Yue, Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type Ca^{2+} channels in HEK 293 cells, J. Physiol. 499 (Pt 3) (1997) 637–644.
[41] K. Aoyagi, T. Sugaya, M. Umeda, S. Yamamoto, S. Terakawa, M. Takahashi, The activation of exocytotic sites by the formation of phosphatidylinositol 4,5-bisphosphate microdomains at syntaxin clusters, J. Biol. Chem. 280 (2005) 17346–17352.