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The antidepressant imipramine inhibits M current by activating a phosphatidylinositol 4,5-bisphosphate (PIP_2)-dependent pathway in rat sympathetic neurones

[1]Jania L. Quintero, [1]Maria Isabel Arenas & *,[1]David E. García

[1]Department of Physiology, Faculty of Medicine, UNAM, Apdo. Post. 70250, CP 04510 México, DF, México

1 Little is known about the intracellular actions of imipramine (IMI) in the regulation of ion channels. We tested the action of IMI on the intracellular cascade that regulates M current (I_M) in superior cervical ganglion neurones (SCGs).

2 Dialysis of the cells with GDPβS, a G protein signaling blocker, did not disrupt the inhibition of I_M.

3 When we incubated the cells with the phospholipase C (PLC) inhibitor U73122, it prevented the I_M inhibition by IMI. Also, when we dialyzed the cells with an intracellular Ca^{2+} chelator, it did not disrupt I_M inhibition by IMI, as occurs in the M_1 cascade.

4 When we incubated the cells with the generic kinase inhibitor wortmannin, it prevented the recovery of I_M from the inhibition by IMI. Also, when we applied phosphatidylinositol 4,5-bisphosphate (PIP_2) intracellularly, it diminished the inhibition of I_M by IMI.

5 Our findings suggest that PLC is the target for IMI, that recovery of I_M needs lipid phosphorylation for PIP_2 resynthesis, and that IMI inhibits I_M by activating a PLC-dependent pathway, likely by decreasing the concentration of PIP_2.
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Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane *N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA); DMEM, Dulbecco's modified Eagle's medium; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTP, guanosine 5′-triphosphate; I_M, M current; IMI, imipramine; Oxo-M, oxotremorine methiodide; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SCGs, superior cervical ganglion neurones

Introduction

It is well established that imipramine (IMI) and related tricyclic antidepressants potentiate the actions of biogenic amines by blocking their physiological inactivation, transport or reuptake, at nerve terminals (Horn *et al*., 1971; Barbaccia *et al*., 1983). Also, it has been shown that IMI can act on a variety of ion channels: L-type Ca^{2+} (Choi *et al*., 1992), hEAG and Ca^{2+}-sensitive potassium, SK/IK type (Gavrilova-Ruch *et al*., 2002); voltage-gated Na^+ (Ogata & Narahashi, 1989; Nicholson *et al*., 2002) and voltage-gated K^+ (Wooltorton & Mathie, 1993; Kuo, 1998; Teschemacher *et al*., 1999; Dreixler *et al*., 2000). Previously (Cuellar-Quintero *et al*., 2001), we reported that IMI inhibits the M-type K^+ current in superior cervical ganglion neurons (SCGs) and showed evidence that this effect can be attributed to intracellular actions. It has been long proposed that the K^+ ionic current, I_M (Brown & Adams, 1980) has a relevant role in modulating neuronal excitability (Brown & Higashida, 1988; Marrion, 1997). Nevertheless, until very recently the intracellular regulation of the M channel remained mostly obscure. Recent literature supports that phosphatidylinositol 4,5-bisphosphate (PIP_2) regulates the activity of a number of transporters and channels (Hilgemann & Ball, 1996; Ford *et al*., 2003; Loussouarn *et al*., 2003). The current hypothesis proposes that a reduction in PIP_2 concentration, as would occur after phospholipase C (PLC) activation (Willars *et al*., 1998), is responsible for the agonist-induced closure of the M channel and for the inhibition of I_M (Suh & Hille, 2002; Zhang *et al*., 2003; Suh *et al*., 2004). Despite the firmness of this hypothesis in model cells and expressed channels (KCNQ2/3), little has been shown in the native M channel, which is well documented in SCGs, and experimental evidence suggests that tricyclic antidepressants promote the activity of intracellular signaling pathways in several cellular types (Joshi *et al*., 1999). It has been reported that the antidepressant IMI activates PLC and the formation of inositol phosphate in cultured neurones, in slices of rat frontal cortex (Shimizu *et al*., 1993; Fukuda *et al*., 1994), and in fish olfactory cilia reconstituted into planar lipid bilayers (Cadiou & Molle, 2003). Therefore, the purpose of our study was to assess whether intracellular actions of IMI can affect the signaling cascade that regulates I_M in SCGs.

Methods

Cell culture

SCG neurones were taken from 4- to 5-week-old male rats (Wistar) and cultured for 1 day (Beech *et al*., 1991; Bernheim

*Author for correspondence; E-mail: erasmo@servidor.unam.mx

et al., 1991). Animals were anesthetized by CO_2 inhalation and decapitated as approved by the Institutional Animal Care and Use Committee. Ganglia were dissociated and the neurones suspended twice in DMEM supplemented with 10% heat-inactivated bovine serum. Cells were plated on glass coverslips coated with poly-L-lysine, incubated at 37°C (5% CO_2) overnight, and used within 24 h.

Electrophysiological recording

Patch-clamp recording of I_M was performed in the whole-cell configuration with a List EPC-7 or HEKA EPC-9 amplifier. Series resistance was 3–7 MΩ. Voltage pulse commands were generated and current records were sampled (5 kHz) and analyzed using BASIC-FASTLAB (Indec Systems, Capitola, CA, U.S.A.) and Sigma Plot (SPSS Inc.). I_M was activated by setting a holding voltage at −25 mV and deactivated by 500 ms command pulses from −25 to −60 mV every 4 s. I_M deactivation amplitude and its percentage inhibition were measured as described (Cruzblanca *et al*., 1998; Suh & Hille, 2002). All quantitative data are expressed as the mean ± s.e.m. Comparison between two groups was analyzed using Student's unpaired *t*-test, and differences were considered significant at a level of $P<0.05$.

To measure I_M we used solutions designed to minimize undesired currents. The pipette solution contained in (mM): 175 KCl, 5 $MgCl_2$, 5 HEPES, 0.1 BAPTA, 4 ATP, 0.3 GTP and 0.1 leupeptine, pH adjusted to 7.4 with KOH. The external solution contained (in mM): 160 NaCl, 2.5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 8 Glucose and 0.0002 TTX, pH adjusted to 7.4 with NaOH. IMI was applied by using a focal perfusion system (Automate Scientific, Inc., San Francisco, CA, U.S.A.), which locally exchanges the external solution within 1 s. IMI treatment was for 40–60 s allowing to attain its maximal effect. All experiments were performed at room temperature (23–25°C).

Reagents

IMI and Oxotremorine (RBI, Natick, MA, U.S.A.), GDPβS (SIGMA, St Louis, MO, U.S.A.), Bradykinin (Peninsula Laboratories, Belmont, CA, U.S.A.), BAPTA (Molecular Probes, Eugene, OR, U.S.A.), U73122, U73343, wortmannin and PIP_2 (α-phosphatidyl-D-*myo*-inositol-4,5-bisphosphate, triammonium salt from bovine brain) (Calbiochem, La Jolla, CA, U.S.A.). U73122 and U73343 were dissolved in chloroform to a final concentration of 5 mM and aliquoted into Eppendorf tubes. After evaporation of the solvent under N_2, tubes were stored at 4°C. On the day of experiment, one aliquot was dissolved in dimethyl sulfoxide to a concentration of 2.5 mM. PIP_2 was dissolved in chloroform, and aliquots were dissolved at −20°C under N_2. On the day of the experiment, the chloroform was evaporated with a stream of N_2 to leave a filmy residue of PIP_2. Recording solution was mixed with this residue for ~10 min before sonication on ice until the solution was clear (~30 min).

Results

IMI inhibits I_M without G protein activation

Several neurotransmitters affect the excitability of neurones through receptors coupled to G proteins and intracellular messengers (Wanke *et al*., 1987, Bernheim *et al*., 1991; Shapiro *et al*., 1994). It is known that the inhibition by the muscarinic agonist Oxo-M, an M_1 pathway activator (Caulfield *et al*., 1994) is mediated by a G protein non-PTX-sensitive Gαq (Haley *et al*., 2000). Furthermore, it has been long demonstrated that Oxo-M inhibits I_M (Hille, 1994). Thus, we used Oxo-M to evaluate G protein mediated responses. To assess whether IMI acts *via* a G-protein-linked mechanism, its effect was evaluated in neurones dialyzed for 10 min with the G protein signaling blocker GDPβS and the muscarinic inhibition was used as a positive control. Both Oxo-M and IMI readily evoked inhibition on I_M as expected for control conditions. This result is depicted in Figure 1a. It shows the effect of 10 μM Oxo-M and 10 μM IMI on the amplitude of I_M when the cells were internally dialyzed with the control solution containing GTP (0.3 mM). Figure 1b shows superimposed I_M traces representative of Figure 1a in the absence (1) or presence of Oxo-M (2) and IMI (3). On the other hand, GDPβS disrupted Oxo-M inhibition, whereas neither the extent nor the kinetics of I_M inhibition by IMI was changed, as shown in Figure 1c. It shows the effect of 10 μM Oxo-M and 10 μM IMI on the amplitude of I_M when cells were dialyzed

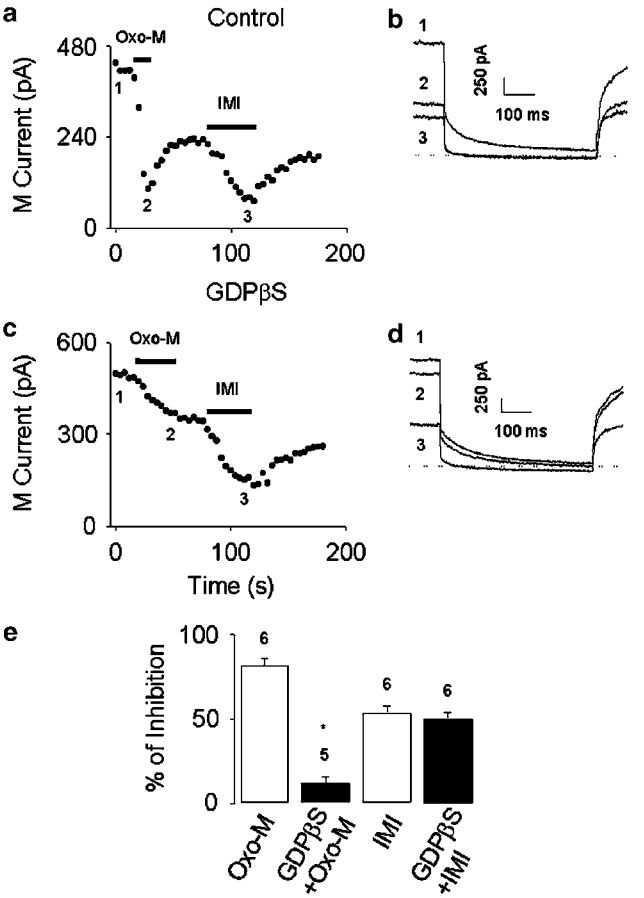

Figure 1 IMI response on I_M in the presence of GDPβS. Inhibition of I_M by 10 μM Oxo-M and 10 μM IMI in the absence (a) and the presence (c) of GDPβS (3 mM) in the patch pipette. (b, d) Current traces representative of (a, c) respectively, before (b1, d1) and during application of Oxo-M (b2, d2) or IMI (b3, d3). (e) Summary of experiments. Data plotted as mean ± s.e.m. The numbers above columns indicate the number of tested cells. *Indicates $P<0.05$; *t*-test vs control.

with an internal solution containing GDPβS (3 mM). Figure 1d shows superimposed I_M traces representative of Figure 1c in the absence (1) or presence of Oxo-M (2) and IMI (3). Aggregated data of the above experiments are shown in Figure 1e, which confirm the difference in G protein dependence between Oxo-M and IMI. Inhibition of I_M by Oxo-M and IMI with the control internal solution was 81.7 ± 4 ($n=6$) and 53.7 ± 4 ($n=6$), respectively. Consistently, the inhibition of I_M by Oxo-M and IMI in GDPβS conditions was 12.4 ± 4 ($n=5$) and 50.3 ± 3, respectively ($n=6$). Thus, these results indicate that G proteins do not underlie the effect of IMI on I_M and open the possibility that it affects PLC as it occurs in other preparations (Fukuda *et al*., 1994; Cadiou & Molle, 2003).Therefore, the intracellular target for IMI should be expected beyond the G protein activation step, possibly at PLC level.

IMI requires PLC activation to inhibit I_M

Current data show that IMI reversibly reduces I_M without a shift in voltage-dependence or kinetics in SCGs (Cuellar-Quintero *et al*., 2001) and stimulates PLC activity *in vitro* (Fukuda *et al*., 1994) with formation of IP_3 in rat frontal cortex (Shimizu *et al*., 1993). These effects are similar to those obtained by muscarinic agonists (Suh & Hille, 2002) and the neuropeptide BK (Jones *et al*., 1995; Haley *et al*., 2000). The above results indicate that IMI may affect a specific signaling pathway that involves depletion of PLC substrates. To test whether activation of PLC is required for suppression of I_M, we compared the effect of a PLC inhibitor U73122 with that of its inactive analog U73343. As a positive control we used BK, which is known to require the activation of PLC to reduce I_M (Jones *et al*., 1995; Cruzblanca *et al*., 1998; Haley *et al*., 2000). Figure 2 shows results obtained in SCG neurones incubated with U73343 (10 μM) (Figure 2a and b) or U73122 (10 μM) (Figure 2c) for 5 min. We found that the inhibition of I_M by 100 nM BK in cells treated with the inactive analog was of $38.1 \pm 7\%$ ($n=5$) (Figure 2a). This inhibition was reduced significantly ($P<0.05$) in cells treated with the PLC inhibitor ($2.7 \pm 2\%$, $n=10$) (Figure 2c). In the same way we found that inhibition of I_M by 10 μM IMI in control cells was $58.1 \pm 2\%$ ($n=9$) and in cells treated with the inactive analog was of $54.1 \pm 6\%$ ($n=10$; Figure 2b). This inhibition is significantly ($P<0.05$) smaller in cells treated with the PLC inhibitor ($14.2 \pm 4\%$, $n=8$; Figure 2c). Summary data of these experiments are illustrative (Figure 2d). Figure 2e–g shows superimposed I_M traces representative of Figure 2a–c, respectively. These results led us to propose that I_M inhibition by IMI can be attributed to PLC activation (see Figure 2d).

BK, a B_2 pathway activator (Jones *et al*., 1995), has been shown to inhibit I_M by a well-documented slow mechanism mediated by a Ca^{2+}-calmodulin system, depending on Ca^{2+} release from IP_3-sensitive stores (Cruzblanca *et al*., 1998). In order to test whether IMI inhibits I_M by a Ca^{2+}-sensitive pathway as BK does, we dialyzed neurones for 9 min with the intracellular Ca^{2+} chelator BAPTA, and used BK as a positive control. As expected, the inhibition of I_M by 50 nM BK was $43.1 \pm 2\%$ ($n=8$, Figure 3a and d) with 0.1 mM BAPTA and only $7.8 \pm 3\%$ ($n=11$) with 20 mM BAPTA (Figure 3c and d). Figure 3b shows that 50 μM IMI strongly suppresses I_M in a cell dialyzed with the standard internal solution containing 0.1 mM BAPTA. I_M inhibition using this standard internal

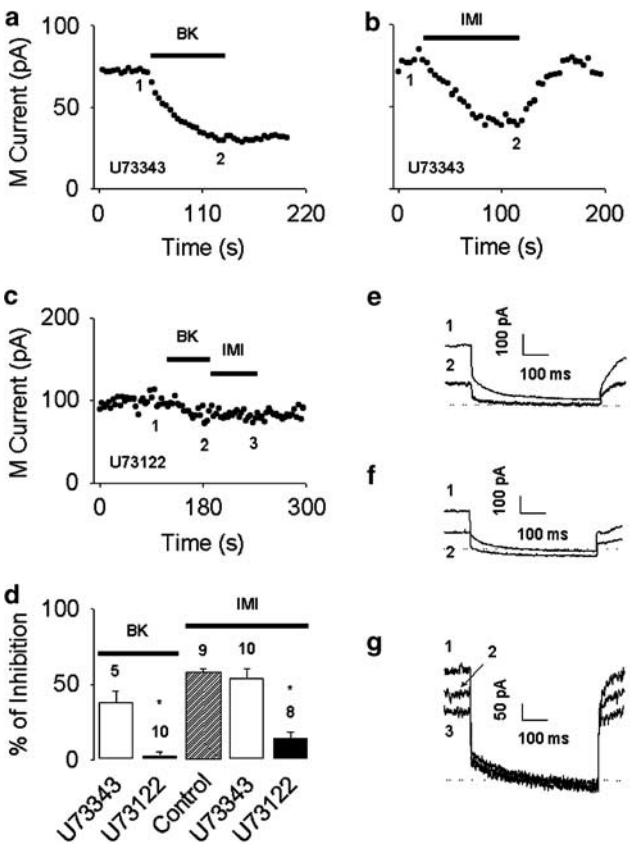

Figure 2 Effect of IMI on I_M in the presence of U73122. SCG neurones were incubated during 5 min with U73122 (10 μM) or its inactive analog U73343 (10 μM). (a, b) Effect of U73343 on the amplitude of I_M in the presence of 100 nM BK (a) or 10 μM IMI (b). Current traces representative of (a, b), respectively, before (e1, f1) and during application of BK (e2) or IMI (f2). (c) Effect of U73122 on the amplitude of I_M in the presence of 100 nM BK and 10 μM IMI. Current traces representative of (c) before (g1) and during application of BK (g2) or IMI (g3). (d) Summary of experiments. Data plotted as mean $\pm$ s.e.m. The numbers above columns indicate the number of tested cells. *Indicates $P<0.05$; *t*-test vs control.

solution was $78.1 \pm 2\%$ ($n=8$; Figure 1d). Similarly, with a pipette solution containing 20 mM BAPTA, the inhibition was $75.8 \pm 5\%$ ($n=7$) (Figure 1c and d). This results argues against a Ca^{2+}-dependence for IMI response on I_M. Nevertheless, although both Oxo-M and BK have been shown to inhibit I_M by activating a PLC, support has been provided to suggest different pathways being activated. M_1 stimulates PLCβ_1 (Berstein *et al*., 1992; Dippel *et al*., 1996) while B_2 stimulates PLCβ_4 (Haley *et al*., 2000). Furthermore, muscarinic agonists do not alter intracellular Ca^{2+} concentration at all in sympathetic neurones (Beech *et al*., 1991).Taking into account, the above results which show that IMI activates a PLC to inhibit I_M in a manner not sensitive to BAPTA lead us to compare the effect of Oxo-M and IMI in the presence of the aminosteroids U73343 and U73122. Figure 4 shows results obtained in SCG neurones incubated with U73343 (10 μM) (Figure 4a and b) or U73122 (10 μM) (Figure 4c) for 5 min. We found that the inhibition of I_M by 10 μM Oxo-M in cells treated with the inactive analog was of $82.9 \pm 4\%$ ($n=5$) (Figure 4a). This inhibition was reduced significantly ($P<0.05$) in cells treated with the PLC inhibitor ($6.7 \pm 3\%$, $n=5$) (Figure 4c). In the same way we found that inhibition of I_M by 10 μM IMI in

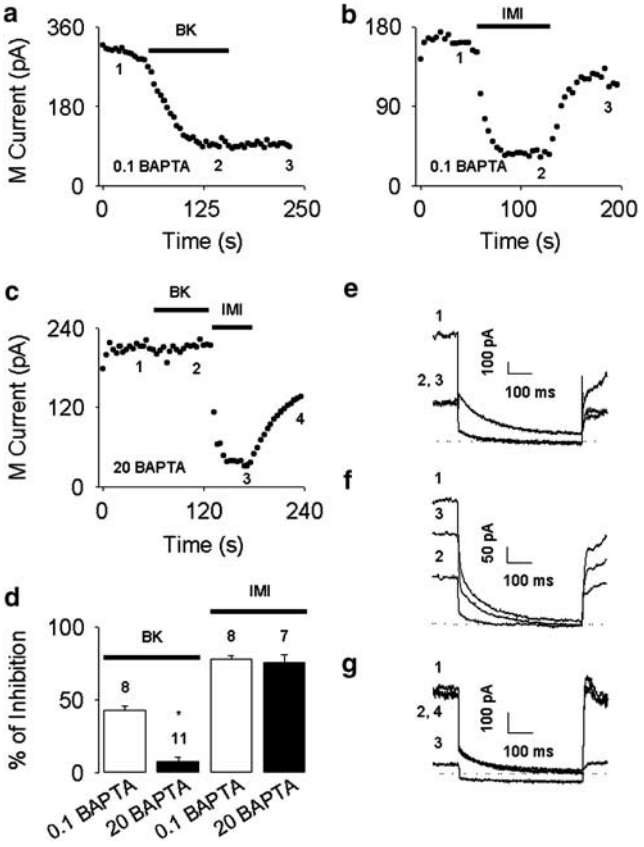

Figure 3 IMI response on I_M in the presence of high BAPTA. Inhibition of I_M by 50 nM BK or 10 μM IMI in the presence of 0.1 mM (a, b) or 20 mM (c) BAPTA in the patch pipette. (e–g) Current traces representative of (a–c), respectively, before (e1, f1, g1), during application of BK (e2, g2) or IMI (f2, g3) and during washout (e3, f3, g4). (d) Summary of experiments. Data plotted as mean $\pm$ s.e.m. The numbers above columns indicate the number of tested cells. *Indicates $P<0.05$; *t*-test vs control.

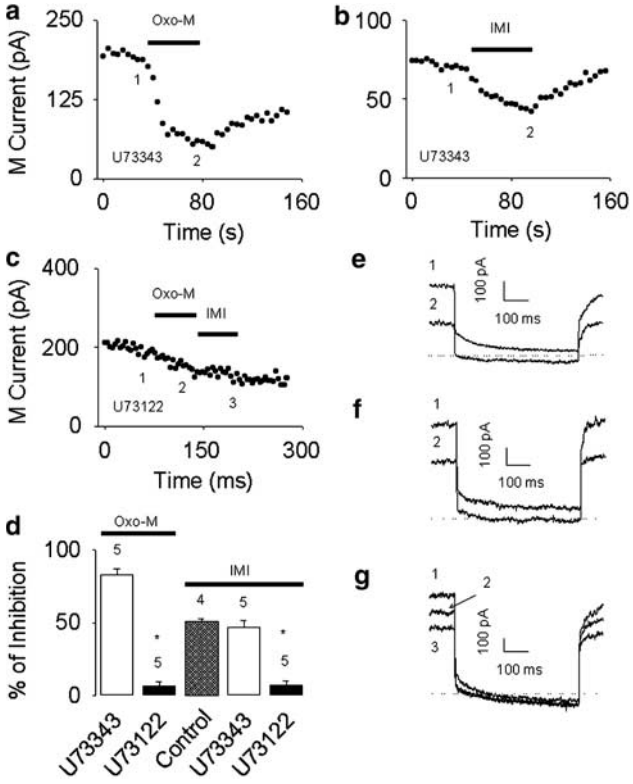

Figure 4 Effect of IMI response on I_M in the presence of U73122. SCG neurones were incubated during 5 min with U73122 (10 μM) or its inactive analog U73343 (10 μM). (a, b) Effect of U73343 on the amplitude of I_M in the presence of 10 μM Oxo-M (a) or 10 μM IMI (b). Current traces representative of (a, b), respectively, before (e1, f1) and during application of Oxo-M (e2) or IMI (f2). (c) Effect of U73122 on the amplitude of I_M in the presence of 10 μM Oxo-M or 10 μM IMI. Current traces representative of (c) before (g1) and during application of Oxo-M (g2) or IMI (g3). (d) Summary of experiments. Data plotted as mean $\pm$ s.e.m. The numbers above columns indicate the number of tested cells. *Indicates $P<0.05$; *t*-test vs control.

control cells was of 50.8 $\pm$ 7% ($n=4$) and in cells treated with the inactive analog was of 47 $\pm$ 4% ($n=5$; Figure 4b). This inhibition is significantly ($P<0.05$) smaller in cells treated with the PLC inhibitor (7.3 $\pm$ 3%, $n=5$; Figure 4c). Summary data of these experiments are illustrative (Figure 4d). Figure 4e–g shows superimposed I_M traces representative of Figure 4a–c, respectively. These results led us to propose that I_M inhibition by IMI can be attributed to PLC activation (see Figure 2d) likely using the M_1 pathway.

Intracellular application of PIP_2 prevents I_M inhibition by IMI

It has been proposed that a reduction in PIP_2 concentration, as would occur after PLC activation (Willars *et al*., 1998; Xie *et al*., 1999), could be responsible for agonist-induced closure of M channel (Suh & Hille, 2002; Zhang *et al*., 2003). In such a way, it has been shown that blockade of recycling agonist-sensitive pools of PIP_2 prevents the recovery of I_M from muscarinic inhibition (Suh & Hille, 2002). This antecedent along with the above results (see Figure 4) prompted us to ask if the inhibition of I_M by IMI was irreversible when blocking PIP_2 resynthesis by lipid kinase inhibitors. To address this question we applied in the bath, the kinase inhibitor wortmannin (Nakanishi *et al*., 1995) and used the muscarinic inhibition as a positive control. As expected, the recovery of I_M from inhibition by Oxo-M in control conditions was 82 $\pm$ 9% ($n=3$; Figure 5a), whereas only 32.5 $\pm$ 5% ($n=4$) was recovered 5 min after wortmannin (10 μM) treatment began and the recovery was slowed (Figure 5a). In order to discard effects due to desensitization in successive application of Oxo-M, this experiment was repeated in independent cells with the same result ($n=4$). Furthermore, this positive control with wortmannin has been extensively documented (Suh & Hille, 2002). Interestingly, the recovery of I_M from inhibition by IMI in control conditions was of 96 $\pm$ 2% ($n=5$; Figure 5b) whereas just 50 $\pm$ 0.3% ($n=5$) 5 min after wortmannin (10 μM, Figure 5b) treatment began. Cumulative data support this finding (Figure 5c). In Figure 5d and e, superimposed I_M traces representative of Figure 5a and b respectively are shown. These results support the hypothesis that recovery of I_M from inhibition by IMI requires recycling of agonist-sensitive pools of PIP_2.

Finally, in order to demonstrate the participation of PIP_2 on the response induced by IMI on I_M, we dialyzed SCG cells for 5 min with PIP_2 (182 μM) *via* the patch pipette and muscarinic inhibition was used as a positive control. Current evidence

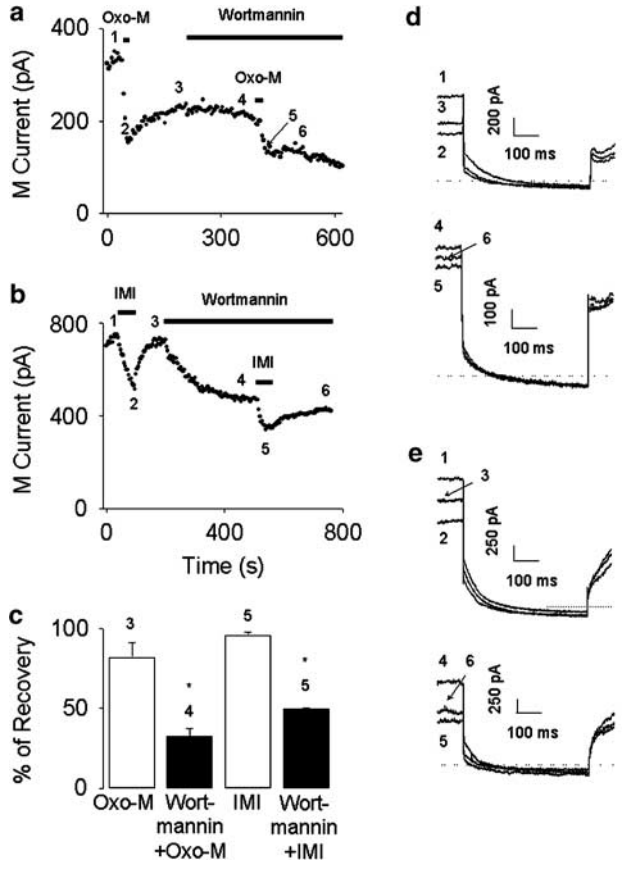

Figure 5 Recovery of I_M from inhibition by IMI in the presence of Wortmannin. (a) Inhibition and recovery of I_M by 10 μM Oxo-M in control conditions and under treatment with Wortmannin (10 μM). (d) Superimposed I_M traces of (a) before (d1, d2, d3) or under treatment with Wortmannin (d4, d5, d6); during control conditions (d1, d4), in the presence of Oxo-M (d2, d5) and after washout (d3, d6). (b) Inhibition and recovery of I_M by 10 μM IMI in control conditions and under treatment with Wortmannin (10 μM). (e) Superimposed I_M traces of (b), before (e1, e2, e3) or under treatment with Wortmannin (e4, e5, e6) during control conditions (e1, e4), in the presence of IMI (e2, e5) and after washout (e3, e6). (c) Summary of experiments. Data plotted as mean$\pm$s.e.m. The numbers above columns indicate the number of tested cells. *Indicates $P<0.05$; *t*-test vs control.

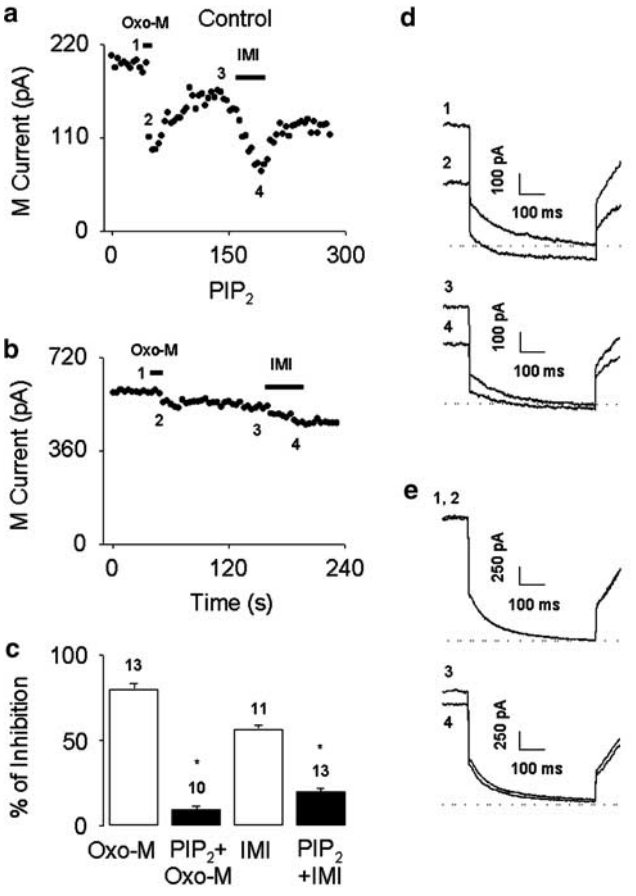

Figure 6 Effect of IMI on I_M under intracellular application of PIP_2. Inhibition of I_M by 10 μM Oxo-M and 10 μM IMI in the absence (a) and the presence (b) of PIP_2 (182 μM) in the patch pipette. (d, e) Superimposed I_M traces of (a, b) in the absence (d1, d3, e1, e3) or the presence of Oxo-M (d2, e2) and IMI (d4, e4). (c) Summary of experiments. Data plotted as mean$\pm$s.e.m. The numbers above columns indicate the number of tested cells. *Indicates $P<0.05$; *t*-test vs control.

demonstrating the effect of PIP_2 on K^+ channels has been obtained from expressed channels (Xie *et al*., 1999; Loussouarn *et al*., 2003). A direct interaction between an identified histidine residue in the C terminus proximal to the cytosolic end of the last transmembrane domain (S6) of the KCNQ channel and PIP_2 is proposed by Zhang *et al*., 2003. Mutations of this histidine residue significantly decrease the sensitivity of the channel to PIP_2 resulting in functionally silent channels. Thus, if PIP_2 hydrolysis underlies the effect induced by IMI on I_M, intracellular restoration of this phospholipid *via* the patch pipette would be expected to prevent or diminish the IMI effect. In cells dialyzed with the control internal solution, we found that the percent of inhibition of I_M by 10 μM Oxo-M (Figure 6a) was 80.1$\pm$3%; $n=13$. In the presence of PIP_2, this inhibition was reduced significantly ($P<0.05$; 9.6$\pm$2%; $n=10$) (Figure 6b). Notably, a similar result was found for IMI inhibition. In control cells, 10 μM IMI inhibited I_M by 56.6$\pm$2% ($n=11$; Figure 6a) whereas in the presence of PIP_2 only 20.1$\pm$2% ($n=13$; Figure 6b). Summary data of these experiments (Figure 6c) are illustrative and consistent with the above observations. In Figure 6d and e, some superimposed I_M traces representative of Figure 6a and b, respectively, are shown. This result supports our proposition that a decrease in PIP_2 concentration underlies the I_M inhibition by IMI.

Discussion

In a previous report (Cuellar-Quintero *et al*., 2001), we describe that IMI reversibly reduces I_M while voltage dependence or kinetics remains unchanged. Furthermore, we documented that IMI can access the intracellular side when applied either in the bath or in the pipette. In addition, we showed evidence that the neutral form of IMI applied extracellularly enhances its effectiveness in reducing I_M, likely by facilitating the passage of IMI across the membrane. These results prompted us to ask whether IMI might act on an internal substrate involved in the receptor-mediated inhibition of I_M. Here we show for the first time in a native system (SCGs) that, (1) a PLC inhibitor (U73122) prevents I_M from the inhibition by IMI, (2) a generic phosphatidylinositol kinases inhibitor (wortmannin) prevents the recovery of I_M

from inhibition by IMI, as some kinases are needed likely for PIP_2 resynthesis, and (3) internal dialysis of the cell with PIP_2 disrupts the I_M inhibition by IMI.

IMI targets PLC to inhibit I_M

To date it is widely accepted that the M-channel is linked to a G protein coupled receptor (Suh & Hille, 2002). Therefore, we asked whether IMI could inhibit I_M by G protein activation. Figure 1 shows that dialysis with the competitive inhibitor GDPβS does not alter the induced-inhibition of I_M by IMI. This indicates that IMI does not act on the G protein or the receptor, rather that its substrate is located downstream of the regulatory pathway. Accordingly, Fukuda *et al.* (1994) found that IMI directly activates PLCβ in cultured neurones of rat frontal cortex. This result prompted us to ask whether IMI is able to activate PLC also in SCGs. Figures 2c and 4c show that the PLC inhibitor U73122 prevents I_M inhibition by IMI. This finding is consistent with the hypothesis that IMI stimulates PLC activity to inhibit I_M. Thus, it is tempting to speculate whether this drug might exert its action through an already known G protein-coupled receptor pathway. It has long been proposed that the M channel is linked to membrane receptors through signaling cascades involving G proteins and second messengers (Hille, 1994). Presently, it is well known that muscarinic M_1 and Bradykinin B_2 receptors both couple to a heterotrimeric G protein, $G_{q/11}$ (Haley *et al.*, 2000) and exert modulatory influences on the M channel. Furthermore, it is documented that each cascade stimulates a different PLCβ, PLCβ_1 (Berstein *et al.*, 1992; Dippel *et al.*, 1996) or PLCβ_4 (Haley *et al.*, 2000). Thus, the initial claim by Fukuda *et al.* (1994) that IMI directly activates PLCβ_1 without receptor or G protein mediation in cultured neurones of rat frontal cortex, is supported by our results. There is some concern that IMI might promote intracellular actions by Ca^{2+} rise from internal stores, likely IP_3-activated. The B_2 pathway may be a possible target for IMI because it is part of the Ca^{2+}-dependent signaling cascade. However, unlike BK, no Ca^{2+}-dependence for IMI response on I_M was observed (Figure 3). Otherwise, muscarinic agonists do not alter intracellular Ca^{2+} concentration at all in sympathetic neurones (Beech *et al.*, 1991), and IP_3 produced by M_1 receptor-induced hydrolysis of PIP_2 has not been shown to have any consequence concerning I_M regulation. Therefore, the muscarinic M_1 cascade seems, with data presently available, the likely signaling pathway being altered by IMI in I_M regulation. Further experiments to confirm this proposition are beyond the scope of this work.

PIP_2 hydrolysis underlies the effect induced by IMI on I_M

To date, PIP_2 is the strongest candidate being investigated to modulate the M channel. This hypothesis implies that an agonist-induced reduction in PIP_2 concentration may be responsible for M-channel closure (Suh & Hille, 2002; Zhang *et al.*, 2003). Therefore, we wanted to test whether PIP_2 resynthesis inhibitors and analogs might alter the IMI response on I_M. Figure 5b shows that wortmannin prevents and slows I_M recovery from inhibition by IMI, indicating that a PI-kinase is required for I_M recovery. It is also known that wortmannin is an inhibitor of PI 3- and PI 4-kinases, as well as of the light chain kinase (Nakanishi *et al.*, 1995). In addition, there is supporting evidence that the PI4-kinase is the enzyme required for recovery of I_M from muscarinic inhibition, particularly for lipid phosphorylation and PIP_2 resynthesis (Suh & Hille, 2002; Zhang *et al.*, 2003). Our observation reinforces the proposition that IMI stimulates PLC, which in turn induces PIP_2 hydrolysis. It seems that continuous PIP_2 resynthesis is required for recovery of I_M from the inhibition by IMI, in a similar way as it has been proposed to occur for the M_1 muscarinic pathway. Therefore, our thinking was that an experimentally induced PIP_2 elevation might prevent changes elicited by IMI on the I_M. Dialyzing of the SCG cells with PIP_2 analogs made this experiment possible. Figure 6b confirms that internal loading of the cell with PIP_2 disrupts I_M inhibition by IMI. These results are consistent with the hypothesis that the amplitude of the KCNQ2/KCNQ3 current mirrors the PIP_2 level in the membrane (Suh & Hille, 2002; Zhang *et al.*, 2003). Therefore, PIP_2 acts as a membrane-diffusible second messenger to regulate directly the activity of KCNQ currents (Zhang *et al.*, 2003). Indeed, PIP_2 is thought to be a direct modulator of I_M activity and its hydrolysis cause current inhibition.

Finally, it is also interesting to contrast our results with what has been reported in other preparations. There is evidence (Wooltorton & Mathie, 1993; Kuo, 1998; Weinshenker *et al.*, 1999; Terstappen *et al.*, 2001) that IMI directly interacts with native and cloned K^+ channels. For example, it has been reported (Kuo, 1998) that IMI is an external open' A-type K^+ current (I_A) channel blocker in rat hippocampal neurones. Also, strong evidence has been shown recently (García-Ferreiro *et al.*, 2004) that IMI blocks the ether a go-go voltage-gated K^+ channel from the intracellular side of the membrane by binding in its charged form to a site located close to the inner end of the selectivity filter.

Taking together the present results, whatever the mechanism turns out to be, we show for the first time in a native system evidence that the tricyclic antidepressant IMI activates a route that involves PLC and PIP_2 in regulating I_M. Future design of drugs with specific action on PLC-β1 and the M-channel should be a powerful tool in modulating neuronal excitability.

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