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ARTICLE

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Phospholipid metabolism is required for M_1 muscarinic inhibition of N-type calcium current in sympathetic neurons

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Abstract The signal transduction cascade mediating muscarinic receptor modulation of N-type Ca^{2+} channel activity by the slow pathway has remained incompletely characterized despite focused investigation. Recently we confirmed a role for the G-protein G_q and identified phospholipase C (PLC), phospholipase A_2 (PLA_2), and arachidonic acid (AA) as additional molecules involved in N-current inhibition in superior cervical ganglion (SCG) neurons by the slow pathway. We have further characterized this signal transduction cascade by testing whether additional molecules downstream of phosphatidylinositol-4,5-bisphosphate (PIP_2) are required. The L-channel antagonist nimodipine was bath-applied to block L-current. Pretreating cells with pertussis toxin (PTX) minimized M_2/M_4 muscarinic receptor inhibition of N-current by the membrane-delimited pathway. Consistent with our previous studies, pharmacologically antagonizing M_1 muscarinic receptors (M_1Rs), $G_q\alpha$, PLC, PLA_2, and AA minimized N-current inhibition by the muscarinic agonist oxotremorine-M (Oxo-M). When cells were left untreated with PTX, leaving the membrane-delimited pathway intact and the same antagonists retested, Oxo-M decreased whole cell currents. Moreover, inhibited currents displayed slowed activation kinetics, indicating intact N-current inhibition by the membrane-delimited pathway. These findings indicate that the antagonists used to block the slow pathway acted selectively. PLA_2 cleaves AA from phospholipids, generating additional metabolites. We tested whether the metabolite lysophosphatidic acid (LPA) mimicked the inhibitory actions of Oxo-M. In contrast to AA, applying LPA did not inhibit whole cell currents. Taken together, these findings suggest that the slow pathway requires M_1Rs, $G_q\alpha$, PLC, PIP_2, PLA_2, and AA for N-current inhibition.

Keywords Arachidonic acid · Muscarinic receptors · N-current inhibition · Oxotremorine-M · Superior cervical ganglion neurons

Abbreviations *AA* arachidonic acid · *BAPTA* 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid · *BSA* bovine serum albumin · *DAG* diacylglycerol · *DEDA* 7,7-dimethyleicosadienoic acid · *ETYA* 5,8,11,14-eicosatetraynoic acid · *FPL* FPL 64176 · IP_3 inositol-1,4,5-trisphosphate · *L-channel* L-type calcium channel · *L-current* L-type calcium current · *LPA* lysophosphatidic acid · M_1R M_1 muscarinic receptor · *N-channel* N-type calcium channel · *N-current* N-type calcium current · *NMN* nimodipine · *OAG* 1-(*cis*-9-octadecenoyl)-2-acetyl-*sn*-glycerol · *OPC* oleoyloxyethyl phosphorylcholine · *Oxo-M* oxotremorine methiodide · PIP_2 phosphatidylinositol-4,5-bisphosphate · *PLC* phospholipase C · PLA_2 phospholipase A_2 · *PTX* pertussis toxin · *SCG* superior cervical ganglion

Introduction

The muscarinic receptors M_1R–M_4R are G-protein coupled receptors found in discrete areas of both the central and autonomic nervous systems (Caulfield and Birdshall 1998). One of their functions is to modulate the activity of both Ca^{2+} and K^+ channels, apparently by multiple signal transduction cascades, resulting in remarkably complex signaling (Hille 1994; Shapiro et al. 2001). Much of the work characterizing the biophysical properties of N-type Ca^{2+} channel (N-channel) activity and its modulation by muscarinic agonists has been performed on superior cervical ganglion (SCG) neurons. SCG neurons are ideal for studies of N-channel behavior because they are enriched in these channels and have few

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other Ca^{2+} channel types (Plummer et al. 1989; Delmas et al. 2000). Activation of a fast ($\tau < 1$ s), pertussis toxin (PTX)-sensitive, membrane-delimited pathway, mediated by M_2/M_4Rs and G_o/G_i-like GTP-binding proteins, selectively inhibits whole cell N-currents (Plummer et al. 1991; Beech et al. 1991; 1992; Bernheim et al. 1992; Shapiro et al. 1999; Liu and Rittenhouse 2003a). Inhibition occurs by the binding of at least $G\beta\gamma$ to the cytoplasmic linker between pseudo-subunits I and II of the N-channel's pore-forming subunit (see Furukawa et al. 1998a, 1998b; Jarvis and Zamponi 2001). Currents inhibited by this membrane-delimited pathway exhibit slowed activation kinetics and a shift in voltage sensitivity (Ikeda 1991; Beech et al. 1992).

An M_1R-signal transduction cascade both enhances and inhibits N-currents in SCG neurons (Shapiro et al. 2001; Liu and Rittenhouse 2003b). While little is known about how enhancement occurs, the changes in current kinetics and the signal transduction cascade for inhibition have been partially characterized. Current inhibition by the M_1R pathway, also called the slow pathway (Beech et al. 1992; Zhou et al. 1997), is mediated by the PTX-insensitive G-protein G_q (Shapiro et al. 1999; Haley et al. 2000) and phospholipase C (PLC) (Suh and Hille 2002; Wu et al. 2002; Liu and Rittenhouse 2003b; Zhang et al. 2003). This pathway modulates not only N-current but also L-type Ca^{2+} channel (L-channel) activity (L-current) and K^+ channel activity (M-current). Modulation of these currents appears to involve an unidentified diffusible second messenger because in cell-attached patches unitary L- and N-currents as well as the M-current decrease following bath application of a muscarinic agonist. Unlike the membrane-delimited pathway, the muscarinic agonist need not be in the pipette solution (Bernheim et al. 1991, 1992; Mathie et al. 1992; Liu and Ritttenhouse 2003a, 2003b). Agonist present in the bath solution binds to receptors outside of the patch pipette, activating a signal transduction cascade that gives rise to a diffusible signaling molecule. This diffusible second messenger is capable of migrating to the channels isolated under the patch pipette to act either directly on the channels or indirectly by stimulating additional downstream molecules that then modify the channel gating. The characteristics of inhibition also differ from the membrane-delimited pathway: the onset of this inhibition is slow ($\tau > 36$ s) and voltage-independent (Bernheim et al. 1991; Beech et al. 1992). Low levels of intracellular Ca^{2+} are required (tens of nanomolar), indicating the involvement of a Ca^{2+}-dependent molecule, e.g. calmodulin, a phosphatase, and/or a phospholipase (Beech et al. 1991; Bernheim et al. 1991). Because M_1R inhibition of L-, M- and N-currents shares the above properties, the same final pathway may modulate all of them. However, the identities of downstream molecules are uncertain, and despite more than a decade of research, the diffusible second messenger's identity remains controversial.

Attention has recently focused on the possibility that phosphatidylinositol-4,5-bisphosphate (PIP_2), the precursor of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), may itself be the diffusible second messenger that modulates the M-current (Zhang et al. 2003). PIP_2, normally bound to channels, is metabolized following M_1R activation of PLC; thus the loss of PIP_2 from channels, rather than the generation of a diffusible second messenger that modifies channels, may be the mechanism of action. Little is known about the role of PIP_2 in modulating the N-current. However, Wu et al. (2002) found that excising patches to the inside-out conformation decreased recombinant P/Q-type Ca^{2+} channel activity, which could be rescued with exposure to PIP_2. They then tested the hypothesis that loss of PIP_2 from bullfrog sympathetic neuronal membranes decreases whole cell Ca^{2+} currents. They found that lutenizing hormone releasing hormone (LHRH)-induced inhibition of whole cell current had a voltage-independent component that was sensitive to PLC blockers, but independent of IP_3 and protein kinase C (PKC). They concluded that inhibition by LHRH is most probably a direct consequence of the loss of PIP_2 molecules associating with channels rather than caused by downstream elements. In both the K^+ and Ca^{2+} channel studies, breakdown products of PIP_2, such as lysophospholipid, DAG, lysophosphatidic acid (LPA), or arachidonic acid (AA), were not conclusively excluded as participants in the slow pathway and therefore as its diffusible second messenger.

In our recent study of modulation of N-current by the slow pathway (Liu and Rittenhouse 2003b), we also identified PLC and thus its substrate PIP_2 as critical participants in this pathway and confirmed a role for M_1Rs and the G-protein G_q (Shapiro et al. 1999; Haley et al. 2000; Liu and Rittenhouse 2003b). However, we identified phopholipase A_2 (PLA_2) and AA as additional molecules critical for N-current inhibition by the slow pathway. This conclusion was based on the finding that pharmacologically blocking any of these molecules minimizes M_1R modulation of the N-current. Moreover, bath application of AA mimicked N-current modulation by Oxo-M. Because AA appears to participate in this pathway and can mimic the actions of Oxo-M, we hypothesize that PIP_2 breakdown may not be the final step in the pathway. Rather, additional steps leading to increases in free AA are required for N-current inhibition by the slow pathway. To probe this hypothesis, we have characterized the slow pathway in more detail while examining further whether AA could be the diffusible second messenger.

Materials and methods

Preparation of cells

Neonatal SCG neurons from 1- to 3-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were isolated as described in Liu and Rittenhouse (2003a). Cells were used within 12 h to avoid recording from neurons with processes. We have previously confirmed that muscarinic inhibition of peak current in

neonatal SCG neurons by the slow pathway (Liu and Rittenhouse 2003a) shows the same characteristics as observed in the adult (see Hille 1994).

Immunoblot analysis

SCG neurons were lysed in buffer containing the protease inhibitors calpain inhibitor II, phenylmethanesulfonyl fluoride, sodium orthovanadate, leupeptin, pepstatin A, aprotinin, and β-mercaptoethanol (Santa Cruz Biotechnology, Santa Cruz, Calif.). After determining the protein content (Bio-Rad RC/DC protein assay, Hercules, Calif.), lysates were loaded on 8% SDS-polyacrylamide gels and electrophoresed for 1 h. Separated proteins were transferred onto a PVDF membrane (Bio-Rad) using an electroblotting apparatus (Bio-Rad) according to the manufacturer's instructions. The membranes were blocked overnight in phosphate buffered saline (PBS) containing 5% (w/v) nonfat dry milk. Blots were probed with antibodies to M_1R (Santa Cruz Biotechnology) and $G_q\alpha$ (Calbiochem, La Jolla, Calif., or Santa Cruz Biotechnology). Primary antibodies were added at a dilution of 1:1000 in PBS with 0.1% (v/v) Tween-20 (PBST) and 5% (w/v) nonfat dry milk and incubated for 1 h. Following three 15-min washes with PBST, the membranes were treated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) diluted 1:15,000 in PBST. After three 15-min washes with PBST, membranes were developed using immuno-star HRP chemiluminescent kit (Bio-Rad) and exposed to film (Kodak).

Current recordings

Whole cell currents were recorded at room temperature (22–24 °C) as previously described (Liu and Rittenhouse 2003a). All current traces were stored and later analyzed on a Pentium computer using CED Patch (version 6.3) acquisition and analysis software (Cambridge Electronic Design, Cambridge, UK). Electrodes were made from borosilicate glass capillaries (Drummond Scientific Company, Broomall, Pa.) and fire-polished to a tip diameter of ~1 μm. The total pipette access resistance ranged between 2.0–2.5 MΩ for whole cell electrodes.

For some recordings, we isolated L-currents using a voltage protocol developed by Plummer et al. (1989) and modified by Liu et al. (2001). The membrane voltage was held at −90 mV, stepped to +10 mV for 20 ms, and then stepped back to an intermediate potential of −40 mV. Under these conditions and in the presence of a bath-applied L-channel agonist, FPL 64176 (FPL), we could elicit a long-lasting component of a tail current made up entirely of L-current (Liu et al. 2001), but FPL only minimally increased peak current (Liu et al. 2003). Command pulses were delivered at 4-s intervals. Before analysis, leak and capacitive transients were eliminated from all records by scaling up the current recorded at −110 mV and then subtracting it from individual sweeps. In some figures, residual transients that remained after leak-subtraction were removed. Current amplitudes were measured 15 ms after the start of the test pulse.

Data are expressed as the mean current amplitude or percent change in current ± standard error of the mean. Statistical significance was determined by either a Student's *t*-test for two means or a paired *t*-test; $P < 0.05$ was considered significant.

Drugs and solutions

The external solution contained 20 mM barium acetate (BaAc), 125 mM *N*-methyl-D-glucamine aspartate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.001 mM tetrodotoxin (293 mosmol). BaAc was used to minimize whole cell Ba^{2+} current contamination with Cl^- currents. The pipette solution comprised 123 mM cesium (Cs) aspartate, 10 mM HEPES, 0.1 mM BAPTA, 5 mM $MgCl_2$, 4 mM ATP (Sigma, St. Louis, Mo.) and 0.4 mM GTP (Aldrich, Milwaukee, Wis.) (264 mosmol). The pH of all solutions was adjusted to 7.5.

According to the requirements of each experiment, drugs were added to the external solution by gravity fed superfusion. FPL, NMN (RBI, Natick, Mass., or Sigma), U-73122, DEDA (Biomol, Plymouth Meeting, Pa.), and OPC (Calbiochem) were prepared from stock solutions made up in 100% ethanol and diluted with bath solution to a final ethanol concentration of less than 0.1%. The maximal final concentration of ethanol had no significant effect on the currents. Stocks of Oxo-M, ω-conotoxin-GVIA, tetrodotoxin (RBI or Sigma), GP antagonist-2A (Biomol), and PTX (List Biological Laboratories, Campbell, Calif.) were made in double-distilled water. Stocks were subsequently diluted to their final concentration by at least 1000-fold with external solution. BSA (essentially fatty acid free; Sigma) was added directly to either the bath or pipette solution.

Results

M_1Rs, G_q, and PLC mediate current inhibition by the slow pathway

Because the characteristics of AA-induced current inhibition were similar to those of Oxo-M-induced inhibition, we tested whether AA might be the unidentified diffusible second messenger mediating M_1R modulation of the N-current. To isolate modulation of the N-current via the slow pathway, cells were preincubated with 500 ng/mL PTX for at least 5 h to block the membrane-delimited pathway (Fig. 1A). The L-current was minimized by bath applying 1 μM NMN, a dihydropyridine antagonist of L-channel activity. Under these conditions, 10 μM Oxo-M reversibly inhibited the whole cell N-current (Fig. 1A). Decreased currents showed no slowing of activation kinetics, consistent with a successful block of the membrane-delimited pathway by PTX. When PTX-treated cells were exposed to the M_1R Mamba snake toxin MT-7 (100 nM), current inhibition was lost (Fig. 1B), confirming that, as with adult SCG neurons, M_1Rs mediate N-current inhibition in the neonate. When MT-7 was present in the bath and cells were not pretreated with PTX, Oxo-M inhibited currents with slowed activation kinetics (Fig. 1C), characteristic of current inhibition by the membrane-delimited pathway. Western blot analysis of homogenized SCG using an antibody directed against the carboxyl terminus of the M_1R identified a single band with the appropriate molecular weight for M_1R, indicating expression of this receptor in SCG neurons (Fig. 1D).

These findings confirm that M_1Rs mediate the slow pathway in neonatal SCG neurons. Moreover, the different results obtained with the two experimental protocols have allowed us to pharmacologically dissect the slow pathway. The pharmacological strategy is schematically shown in Fig. 2A. First, by pretreating cells with PTX and including the L-channel antagonist NMN in the bath, we examined the effects of inhibiting various signal transduction molecules on Oxo-M's ability to inhibit the N-current (Fig. 2B). Second, these same antagonists were checked for non-selectivity in cells not

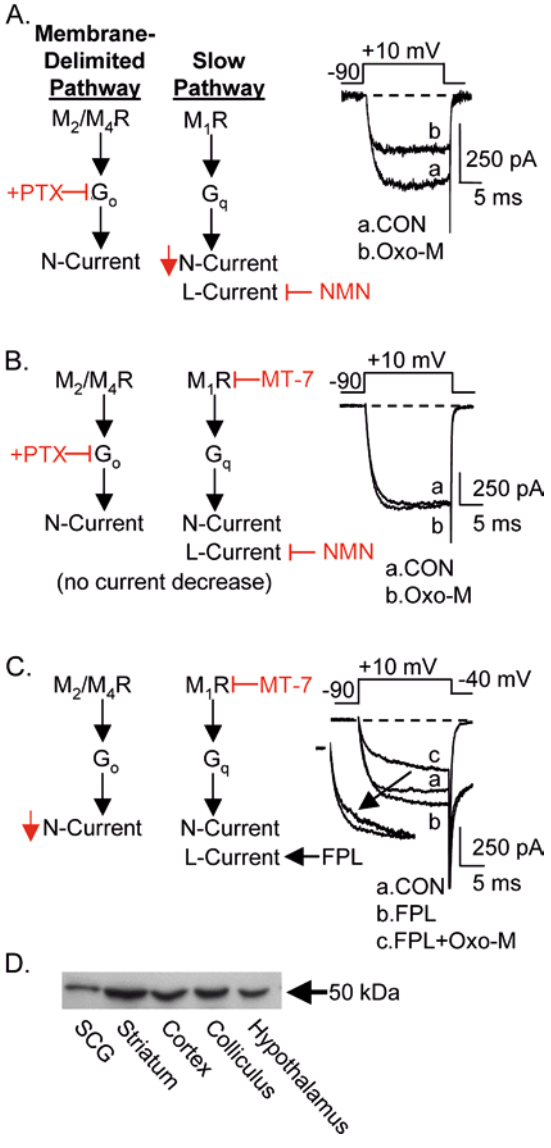

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Fig. 1A–D The muscarinic agonist Oxo-M inhibits whole cell Ba^{2+} currents in neonatal SCG neurons by two pathways, the membrane-delimited and slow pathways. See Materials and methods section for protocol details. **A–C** *Left and middle*: schematics for each experimental protocol illustrate inhibited or active pathways. **A** *Right*: pretreating cells with PTX for at least 5 h disables the membrane-delimited pathway (+PTX, *red*), leaving the slow pathway intact. Bath-applying 10 μM Oxo-M in the continued presence of 1 μM NMN inhibits currents (35%) via the slow pathway. Tail currents have been truncated in these and many of the subsequent traces. **B** *Right*: pretreating cells with PTX and including the M_1R antagonist MT-7 (100 nM) in the bath blocks both pathways, and no inhibition occurs in the presence of Oxo-M. **C** *Right*: blocking cells with MT-7 disables the slow pathway, leaving the membrane-delimited pathway intact. The presence of FPL (1 μM) in the bath allows the FPL-induced, long-lasting tail current, entirely composed of L-current, to be monitored for modulation by the slow pathway. Bath-applying 10 μM Oxo-M inhibits the current via the membrane-delimited pathway, as shown by the slowed activation kinetics. The *insert* illustrates the slowed activation kinetics, characteristic of the inhibition by the membrane-delimited pathway, when the inhibited current (*c*) is normalized to FPL levels (*b*). **D** Western blot analysis using antibodies to M_1R (1:1000) recognizes a single protein band whose molecular weight approximates that of M_1R. Lanes were loaded with 60 μg of adult SCG, striatum, cortex, colliculus, or hypothalamus homogenates

treated with PTX by demonstrating that inhibition by the membrane-delimited pathway remains intact (Fig. 2C). With these latter conditions, FPL was included in the bath to monitor the loss of L-current modulation by the slow pathway in the presence of various antagonists (data not shown). Using the pharmacological protocol shown in Fig. 2A, we identified PLC, PLA_2, and AA as additional components involved in the slow pathway and confirmed a role for the G-protein G_q (Haley et al. 2000; Liu and Rittenhouse 2003b). This conclusion is based on finding that selectively antagonizing any of these signaling molecules minimizes M_1R modulation of the N-current (Fig. 2A and B). In contrast, when cells were not treated with PTX, leaving the membrane-delimited pathway active, Oxo-M (10 μM) significantly inhibited whole cell currents when these antagonists were present (Fig. 2C). Furthermore, the inhibited currents in each case displayed slowed activation kinetics, consistent with inhibition occurring via the membrane-delimited pathway (data not shown). These results indicate that none of these antagonists acted non-selectively at the level of M_2/M_4 muscarinic receptors, G-proteins, or the N-channel. The details of these experiments are shown below.

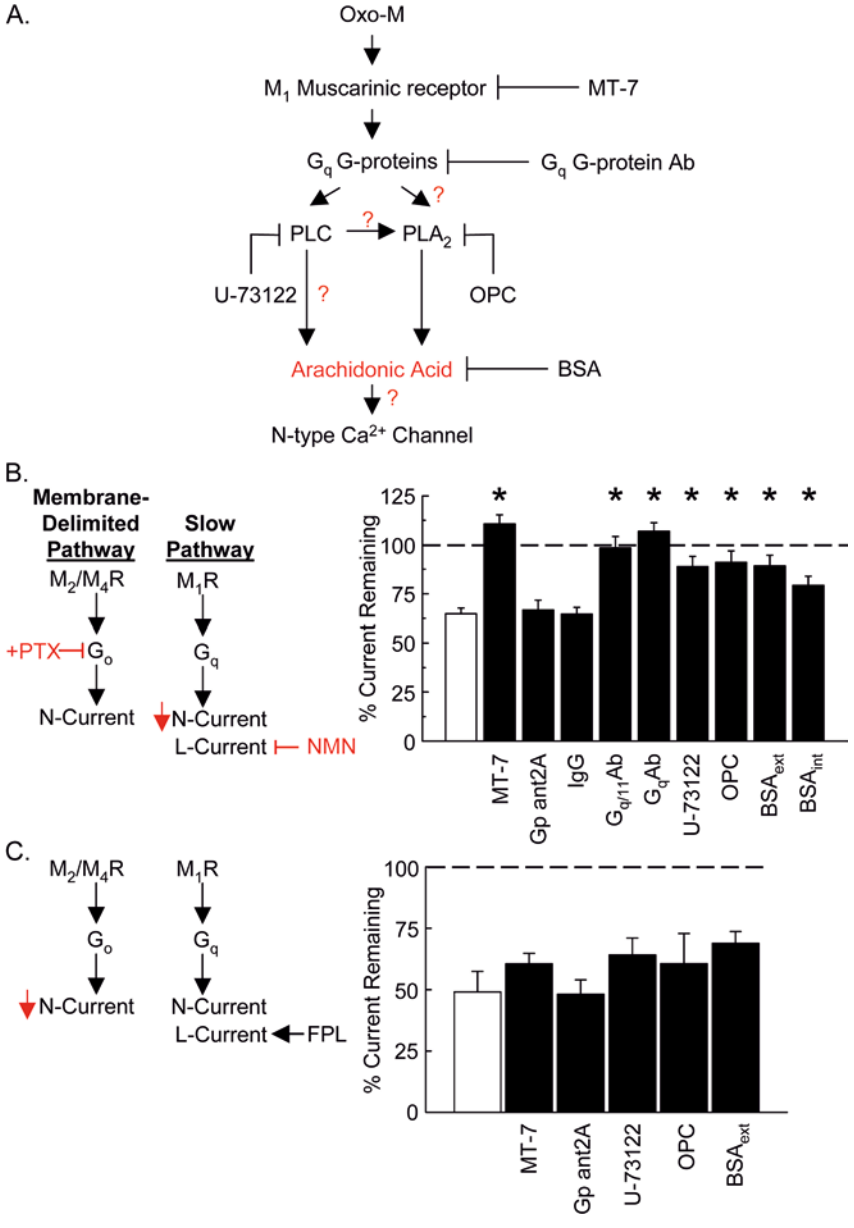
$G_q\alpha$ knockout animals, dialysis of $G_q\alpha$ antibodies into SCG neurons, and transfection of SCG neurons with G_q antisense mRNA all show that G_q is the

▶

Fig. 2A–C Summary of pharmacological blockade of N-current inhibition by the slow pathway. **A** Hypothesized slow pathway showing each antagonist's site of action: MT-7 (0.1 μM), selective for M_1Rs; Gp ant2A (10 μM), reportedly selective for $G_{q\beta\gamma}$; IgG (1:1000); $G_{q/11}$ antibody (Ab; 1:1000); $G_{q\alpha}$Ab (1:1000); U-73122 (2.5 μM), selective for PLC; OPC (10 μM), selective for PLA_2; BSA in the pipette solution (BSA_{int}; 1 mg/mL); BSA in the bath solution (BSA_{ext}; 1 mg/mL). *Question marks* indicate uncertainty about the relationship between a signaling molecule and a downstream effector. **B** Average percent of whole cell current remaining following bath application of Oxo-M (10 μM) in the absence (*open bar*) or presence (*solid bars*) of antagonist. Cells were pretreated with PTX (+PTX). NMN was present in the bath. Blocking M_1R, the G-protein $G_q\alpha$, PLC, PLA_2, and AA significantly decreased Oxo-M-induced inhibition of whole cell Ba^{2+} currents in SCG neurons ($^*P < 0.05$, compared to inhibition in the absence of antagonists; $n = 5$–12 recordings/group). **C** Summary of the current remaining when the membrane-delimited and slow pathways are active. Cells were not pretreated with PTX (−PTX), leaving the membrane-delimited and slow pathways intact. FPL (1 μM) was present in the bath solution. Bath applying each inhibitor in the continued presence of FPL did not significantly change the peak current amplitude from that seen with FPL alone ($P > 0.05$) (data not shown). In the continued presence of FPL and blocker, Oxo-M (10 μM) significantly decreased the peak current compared to unstimulated current levels; $^*P < 0.05$ (data not shown). These findings indicate that the inhibitors do not have non-specific effects on M_2/M_4R-mediated, membrane-delimited inhibition of the N-current. $n = 4$–11 recordings/group. *Open bar*, no antagonists present; *solid bars*, various antagonists used at the concentrations indicated in **A**

G-protein mediating muscarinic inhibition of the N-current by the slow pathway (Delmas et al. 1998; Haley et al. 2000; Liu and Rittenhouse 2003b). Upon stimulation, either the α or $\beta\gamma$ subunit of G_q can bind to and activate PLC. To determine whether $G_q\alpha$, $G_q\beta\gamma$, or both are required for M_1R stimulation of PLC, we dialyzed antibodies to $G_q\alpha$ or $G_q\beta\gamma$ into SCG neurons. Antibodies to $G_q\alpha$ minimized current inhibition by Oxo-M (Fig. 2A and B). Western blot analysis showed that this antibody produced a single band whose molecular weight approximates that of G_q (42 kD; Fig. 3A). In contrast, antibodies to $G_q\beta\gamma$ gave inconclusive results (data not shown). We therefore tested the effects of GP antagonist-2A (Gp ant2A), a peptide that can insert into the membrane and selectively antagonize $G_q\beta\gamma$ (Mukai et al. 1992; Tsunoda and Owyang 1995). When Gp ant2A (10 μM) was bath-applied to cells preincubated with PTX, Oxo-M inhibited the current (Fig. 3B–D). Under these conditions, moreover, no obvious change was seen in whole cell current kinetics (Fig. 3C), consistent with the inhibition occurring via the slow pathway. These findings indicate that activated $G_q\alpha$ is sufficient for transducing M_1R-induced inhibition of the N-current.

The N-current in SCG neurons, the Ca^{2+} current in frog sympathetic neurons, and the recombinant M-current require PLC to observe current modulation by the slow pathway (Suh and Hille 2002; Wu et al. 2002; Liu and Rittenhouse 2003b; Zhang et al. 2003). Figure 2B shows that inhibiting PLC with the selective antagonist U-73122 minimizes Oxo-M-induced current inhibition via the slow pathway. However, when the membrane-delimited pathway remains intact, Oxo-M inhibits current amplitude and slows the activation kinetics (Figs. 1C and 2C), demonstrating that U-73122 does not



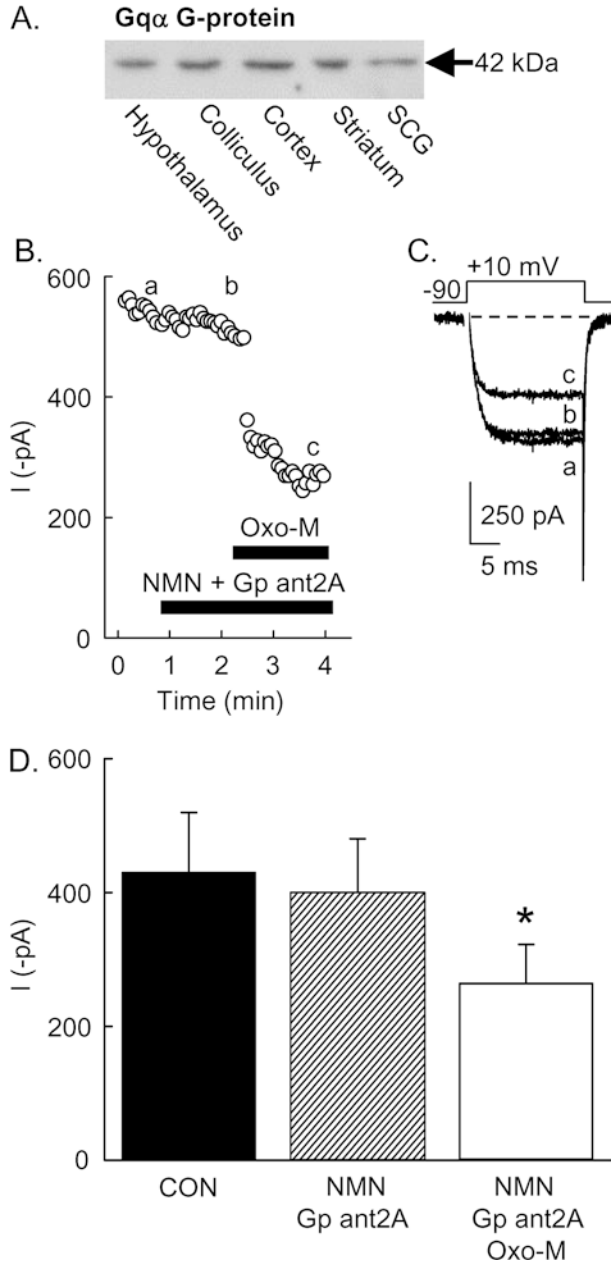


Fig. 3A–D The role of the α and *βγ* subunits of G_q in mediating N-current inhibition by the slow pathway. **A** Western blot analysis shows that the selective G_q antibody, which blocked current inhibition by the slow pathway, recognized a single protein band whose molecular weight corresponded to that of G_q. 60 μg of protein were loaded per well from adult brain regions and SCG. Antagonizing the function of *βγ* subunits of G_q with Gp ant2A did not block Oxo-M-induced current inhibition. Cells were preincubated with PTX and NMN (1 μM) was included in the bath solution. **B** Currents were elicited at +10 mV and plotted against time. The *solid bars* indicate bath application of Gp ant2A (10 μM) and Oxo-M (10 μM). **C** Individual sweeps taken from **B** where indicated. **D** Summary of the effects of Gp ant2A on current inhibition by Oxo-M. Bath-applying Oxo-M still inhibited currents by 33.3 ± 5.0% compared to Gp ant2A levels ($n = 8$, $^*P < 0.01$)

act non-specifically on this pathway. These findings indicate that a substrate for PLC such as PIP_2 participates in the slow pathway.

Antagonizing PLA_2 activity minimizes N-current inhibition by the slow pathway

In other systems, only a small percentage of phospholipid within the inner bilayer is PIP_2. Consequently, G_q-coupled receptor stimulation is thought to quickly deplete PIP_2. Additional phospholipases, such as PLA_2, which directly cleaves AA from phospholipids, are activated to maintain increased levels of free AA. M_1Rs in many cell types (leukocytes, cardiac myocytes, pancreatic acinar cells) and recombinant systems have been shown to stimulate phospholipase activity and AA release from cell membranes (Jelsema and Axelrod 1987; Kramer et al. 1987; Buck and Fraser 1990; Bernstein et al. 1992; Gurwitz et al. 1994; Tence et al. 1994; Biddlecome et al. 1996). Therefore, to investigate whether molecules downstream of PIP_2 were required for current modulation, we tested whether PLA_2 is also involved in the slow pathway.

We found that blocking PLA_2 activity with the selective antagonist oleoyloxyethyl phosphorylcholine (OPC) minimized Oxo-M-induced current inhibition (Fig. 2A and B). When OPC (10 μM) was bath-applied to cells not pretreated with PTX, Oxo-M significantly inhibited the whole cell current (Fig. 2C) with slowed activation kinetics (Fig. 4A), indicating that OPC did not disrupt the membrane-delimited pathway. We had previously found a loss of voltage-independent inhibition with OPC in the bath (Liu and Rittenhouse 2003a), inhibition that is resistant to a facilitating prepulse, which normally occurs with M_1R stimulation of the slow pathway. Lastly, the ability of bath-applied AA to inhibit current, as measured by the percent of current remaining following AA-induced inhibition (Liu and Rittenhouse 2000), remained in the presence of OPC (Fig. 4B and C). These latter findings are consistent with OPC antagonizing PLA_2 function, but having no effect on downstream events, such as the activity of AA.

Arachidonic acid may participate in N-current inhibition by the slow pathway

PLA_2 cleaves AA from the *sn*-2 position of phospholipids, giving rise to free AA and an additional phospholipid metabolite. Our finding that PLA_2 activity is required for muscarinic modulation of the N-current identifies AA as a potential participant in N-current inhibition. We therefore examined the consequences of decreasing the availability of AA in several ways. BSA, which is well known to rapidly bind free AA (see Spector 1975), was included either in the bath or pipette solution. BSA in the bath cannot cross the cell membrane. However, upon its liberation from phospholipids, AA diffuses or ''flips'' from the inner to the outer lipid layer of the cell membrane. Once there, BSA binds AA at the outer membrane/extracellular fluid interface, creating a concentration gradient that results in net movement of AA from the cell. BSA in the bath should thus limit the

availability of any AA released from cell membranes following M_1R stimulation. When BSA (1 mg/mL) was included in the bath solution, inhibition was minimized (Fig. 2B; see also Liu and Rittenhouse 2003b). In contrast, when cells were not pretreated with PTX, Oxo-M significantly inhibited the peak current with slowed activation kinetics, indicating that BSA in the bath did not block agonist interaction with muscarinic receptors (Figs. 2C and 5A).

In a third experiment, we back-filled electrodes with BSA (1 mg/mL) and dialyzed it directly into the cell. Under these conditions, BSA entering the cell should bind any free AA released from the inner membrane, thus lowering levels of free AA. Although significant inhibition occurred under these conditions ($P < 0.05$), dialyzing cells with BSA significantly ($P = 0.001$) reduced current inhibition by Oxo-M: $79.2 \pm 4.7\%$ current remained when BSA was included in the pipette solution compared to $64.7 \pm 2.9\%$ for control (see Figs. 2 and 5B–D). These findings are consistent with AA mediating the actions of Oxo-M.

When AA is cleaved from phospholipids by PLA_2, additional metabolites are created. It is therefore

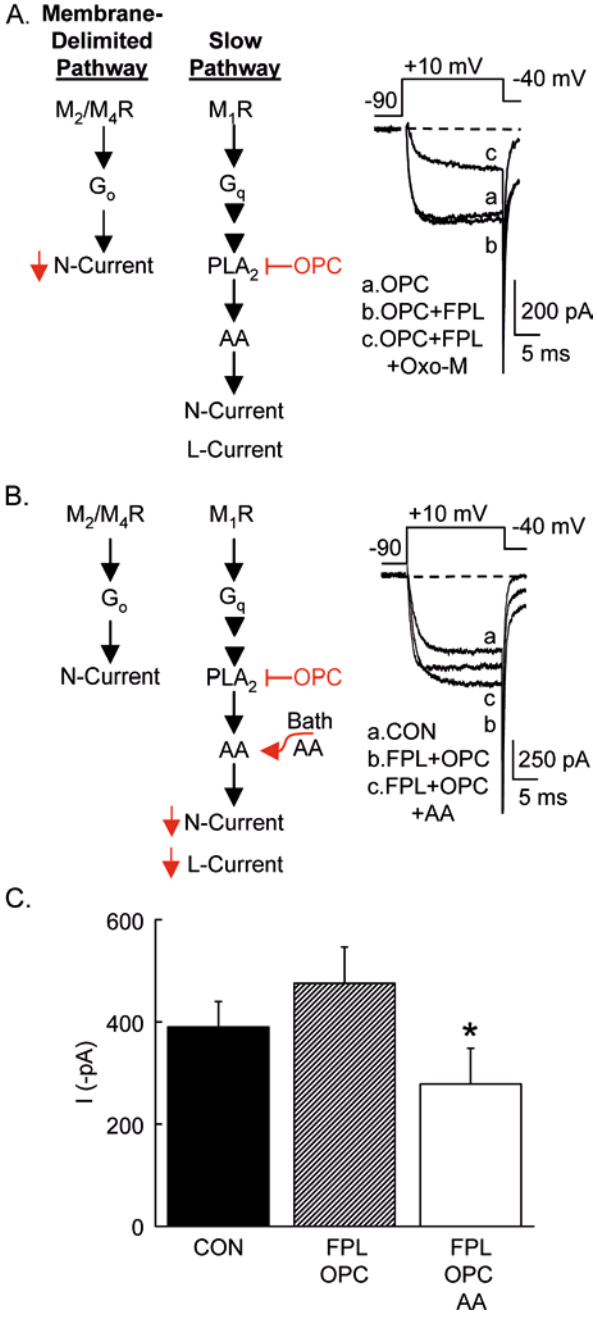


Fig. 4A–C OPC does not interrupt current inhibition induced by the membrane-delimited pathway or by AA. Cells were not pretreated with PTX and FPL was bath-applied. **A**, **B** *Left and middle*: schematics illustrate the experimental conditions and predicted outcome of L- and N-current modulation. **A** *Right*: under these conditions, Oxo-M (10 μM) decreased current amplitude; inhibited currents displayed slowed activation kinetics. **B** *Right*: superimposed sweeps illustrate that bath-applying 5 μM AA inhibits currents with OPC (10 μM) continuously in the bath. **C** Summary of AA's effects in the presence of OPC. AA still inhibited currents by $43.2 \pm 6.0\%$ compared to FPL + OPC conditions ($n = 6$, $^*P = 0.001$, two-way paired *t*-test)

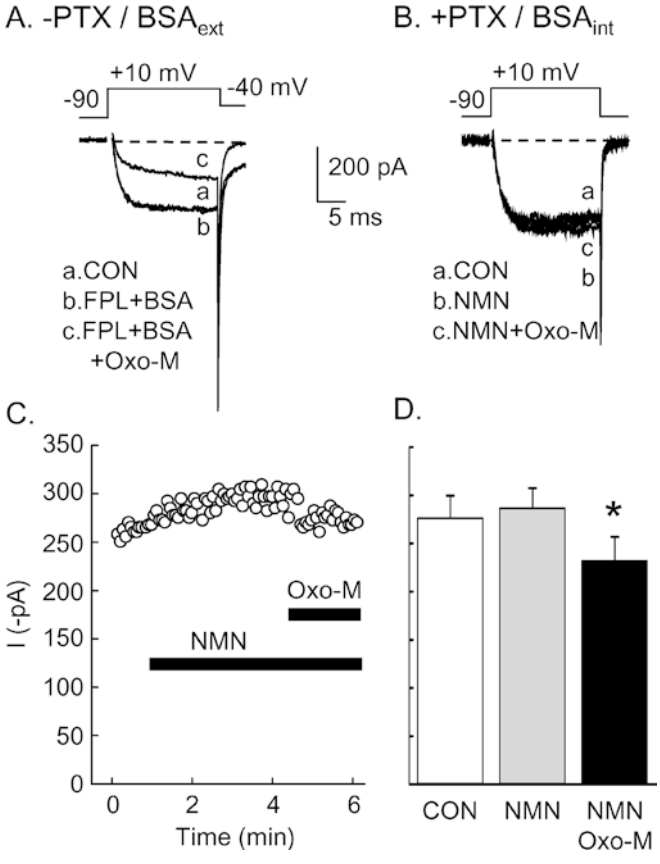


Fig. 5A–D BSA antagonizes N-current inhibition by the slow pathway, but not by the membrane-delimited pathway. **A** Oxo-M-inhibited currents displayed slowed activation kinetics when cells were not PTX-treated (−PTX) and both BSA (1 mg/mL; BSA_{ext}) and FPL (1 μM) were included in the bath. **B–D** In contrast, in cells preincubated with PTX (+PTX), Oxo-M-induced current inhibition was greatly reduced when BSA (1 mg/mL; BSA_{int}) was dialyzed into cells and NMN was present in the bath. **B** Individual sweeps taken from an example time course shown in **C**. **D** Summary of the effects of including BSA in the pipette solution. Current inhibition by Oxo-M remained significant compared to NMN levels ($n = 12$ recordings/group; $^*P < 0.05$)

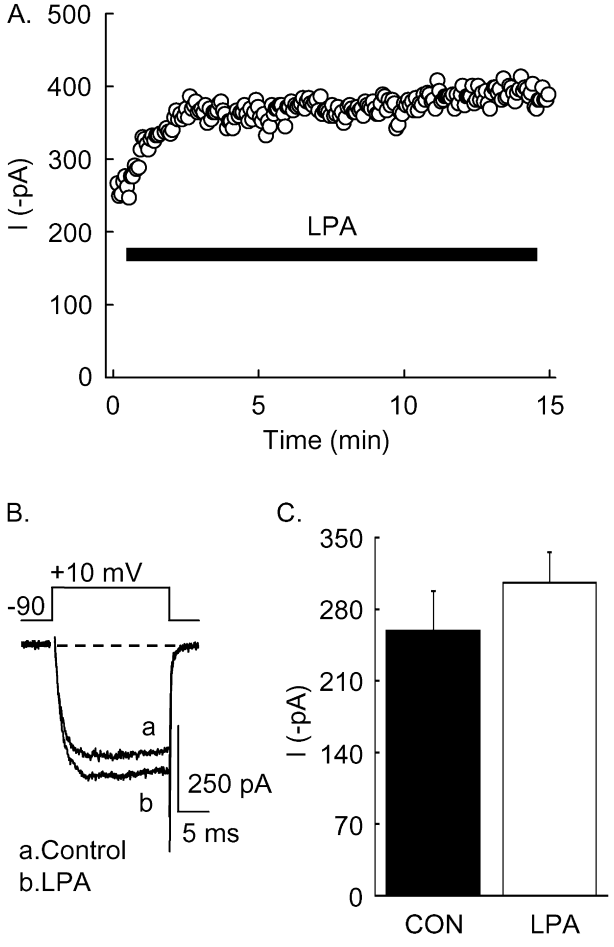


Fig. 6A–C LPA does not inhibit whole cell Ba^{2+} currents. This lack of current inhibition by LPA (10 μM) can be observed in the example plot of current vs. time (**A**), in the individual sweeps (**B**) taken from **A**, and in the bar graph (**C**) summarizing LPA's effects ($n = 6$)

possible that a metabolite other than AA is the critical molecule mediating the slow pathway. One of the most common by-products of PLA_2 activity is lysophosphatidic acid (LPA), which has biological activity of its own (Swarthout and Walling 2000). To test whether LPA can mimic the inhibitory actions of Oxo-M, we bath-applied 10 μM LPA and observed no current inhibition, but rather a slight, though insignificant, enhancement (Fig. 6). These results demonstrate that LPA does not mimic the actions of Oxo-M, whereas the same concentration of AA inhibits currents by 58% (Liu and Rittenhouse 2000).

Discussion

The steps leading from M_1R stimulation to modulation of N-channel activity have remained uncertain, despite the concerted efforts of many labs (see Shapiro et al. 2001). We have further characterized inhibition of the N-current by the slow pathway and tested whether molecules downstream of PIP_2 are required. Consistent with previous studies (Shapiro et al. 1999; Haley et al. 2000; Liu and Rittenhouse 2003b), we found that pharmacologically blocking M_1Rs, $G_q\alpha$ (but not $G_q\beta\gamma$), PLC, and PLA_2 minimized N-current inhibition by the slow pathway. These results were obtained under conditions where the N-current was isolated from the L-current by including NMN in the bath solution and where pretreating cells with PTX minimized muscarinic inhibition of the N-current by the membrane-delimited pathway. In contrast, when the membrane-delimited pathway was allowed to function, we found that Oxo-M decreased whole cell currents in the presence of the same antagonists. Moreover, inhibited currents displayed slowed activation kinetics, indicating that N-current inhibition by the membrane-delimited pathway remained intact. These latter findings demonstrate that the antagonists used to block putative components of the slow pathway appear to act selectively. Lastly, we found that LPA, a compound produced along with AA during phospholipid metabolism by PLA_2, did not inhibit whole cell currents, consistent with AA- rather than LPA-mediated, Oxo-M-induced current inhibition.

Our findings that M_1Rs, $G_q\alpha$, and PLC participate in the slow pathway in SCG neurons are consistent with previous studies that found PIP_2 is required for muscarinic modulation of recombinant M-current (Zhang et al. 2003) and Ca^{2+} current modulation by LHRH in frog sympathetic neurons (Wu et al. 2002). Both studies concluded that PIP_2 itself was most likely the unidentified diffusible second messenger since the products of PIP_2 metabolism, IP_3 and DAG or DAG analogs, did not mimic current modulation by PIP_2. Interest in the relationship between PIP_2 and ion channels grew after Hilgemann and Ball (1996) found that exogenous PIP_2 increased the K^+_{ATP} current, whereas its breakdown by PLC decreased the K^+_{ATP} current. These findings suggested that PIP_2 interaction with K^+ channels somehow favorably increases their probability of opening. Mutation of positive amino acid residues just interior to the inner membrane leaflet in both the amino and carboxy termini of K^+ channel subunits eliminated PIP_2 effects, indicating that both "ends" appear to bind PIP_2 (Lopes et al. 2002). These findings document a critical, stabilizing, structural role for PIP_2 interaction with K^+ channels, which may also be present in Ca^{2+} channels (Wu et al. 2002). ATP and PI 4-kinase, the kinase that synthesizes PIP_2, are required for M-current recovery from M_1R modulation (Suh and Hille 2002), suggesting that the loss of PIP_2 bound to channels may inhibit the M-current. Moreover, in a recombinant system where KCNQ2/3 subunits, which give rise to the M-current, and M_1Rs were co-expressed, current inhibition could be minimized by inhibitors of PLC or by exposing channels to exogenous PIP_2 but not DAG analogs (Zhang et al. 2003). Taken together, these findings indicate that PIP_2 participates in M_1R modulation of the M-current and raise the possibility that the loss of PIP_2 from the membrane may cause L- and N-current inhibition.

Our results with U-73122 indicate that PIP_2 metabolism by PLC participates in the slow pathway, consistent with previous studies (Suh and Hille 2002; Wu et al. 2002; Liu and Rittenhouse 2003b; Zhang et al. 2003). However, many of our findings also suggest a signal transduction cascade that may involve further processing of PIP_2. In contrast to the preparations used in the above-mentioned studies, bath application of the DAG analog OAG to SCG neurons causes a rapid reversible inhibition of both L- and N-currents (Plummer et al. 1991), similar to Oxo-M-induced inhibition of both currents by the slow pathway (Beech et al. 1991; Bernheim et al. 1991). We also found that AA and Oxo-M exhibit identical, unique changes in the N-channel current–voltage profile; with modulation, current enhancement is observed at negative test potentials and inhibition is observed at positive test potentials (Liu and Rittenhouse 2000; Barrett et al. 2001; Liu et al. 2001, 2003b). Thus, at least two components downstream of PIP_2 can mimic Oxo-M-induced inhibition. In contrast, we have found that other fatty acids (5–10 μM), e.g. oleic acid, myristic acid, the AA analog ETYA, and LPA, do not significantly inhibit the peak current in SCG neurons, indicating a selectivity for AA (Liu et al. 2001). Despite their mutual lack of PIP_2's phosphoinositol head group, which is hypothesized to interact with K^+ channels, it is possible that AA and OAG occupy a position around the channel, blocking interactions with PIP_2. On the other hand, AA's mechanism of action may be due to its binding to a distinct site on Ca^{2+} channels or activating additional downstream molecules such as a phosphatase (Petit-Jacques and Hartzell 1996).

The results from our BSA experiments suggest that PIP_2 may not be the final molecule mediating N-current inhibition by the slow pathway. We found that the presence of BSA either in the bath or pipette solution minimized inhibition of currents either by Oxo-M (Figs. 2 and 5) or AA (Barrett et al. 2001; Liu et al. 2001). Our interpretation of BSA's action is that it binds fatty acids effectively, in particular AA (Spector 1975), limiting their availability to bind to downstream signaling molecules or directly to channels. BSA does not appear to bind Oxo-M non-specifically since Oxo-M-induced current inhibition by the membrane-delimited pathway remains intact (Fig. 5A). The membrane-delimited pathway is a good marker for BSA-induced, non-specific disruption of Oxo-M interaction with muscarinic receptors since M_1R, M_2R, and M_4R have similar binding affinities for Oxo-M (Richards and van Giersbergen 1995). BSA is not known to bind to or remove PIP_2 from membranes. Nevertheless, if the loss of PIP_2 decreased channel activity, we would predict that BSA should *increase* the magnitude of current inhibition since BSA binds fatty acids and effectively removes them from solution, shifting the equilibrium and promoting further metabolism of PIP_2. Given these data, it is hard to reconcile the decrease in Oxo-M-induced current inhibition in the presence of BSA with a model where inhibition occurs upon PIP_2 dissociation from channels.

We have shown that a selective PLA_2 antagonist, OPC, antagonizes muscarinic modulation of the current in three experiments: (1) time-course studies of Oxo-M-induced inhibition in cells pretreated with PTX (Fig. 2B); (2) studies of inhibition observed in current–voltage plots (Liu and Rittenhouse 2003b); and (3) prepulse protocol experiments examining voltage-independent inhibition (Liu and Rittenhouse 2003a, 2003b). In contrast, time-course studies examining Oxo-M-induced inhibition under conditions where the membrane-delimited pathway was left intact showed normal inhibition by this fast pathway (Fig. 2C). Moreover, OPC had no effect on AA's ability to inhibit whole cell currents (Fig. 4B and C). Thus, OPC appears to have no effect up- or downstream from its putative site of action, or in any way that differs from other commonly used PLA_2 inhibitors, indicating that OPC is selectively antagonizing PLA_2 activity.

In summary, previous data and findings in this study indicate that (1) OAG and AA can mimic Oxo-M-induced N-current inhibition and (2) BSA, acting as an AA antagonist, and the PLA_2 antagonist OPC can minimize current inhibition by Oxo-M; therefore, the slow pathway appears to require at least phospholipid metabolism by PLA_2 and the production of AA. Proving a role for PLA_2 and AA in muscarinic inhibition of the N-current by the slow pathway in SCG neurons awaits future resolution. The participation of additional molecules upstream or downstream of AA remains to be determined.

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References

Barrett CF, Liu L, Rittenhouse AR (2001) Arachidonic acid reversibly enhances N-type calcium current at an extracellular site. Am J Physiol 280:C1306–C1318

Beech DJ, Bernheim L, Mathie A, Hille B (1991) Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons. Proc Natl Acad Sci USA 88:652–656

Beech DJ, Berheim L, Hille B (1992) Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. Neuron 8:97–106

Bernheim L, Beech DJ, Hille B (1991) A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. Neuron 6:859–867

Bernheim L, Mathie A, Hille B (1992) Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons. Proc Natl Acad Sci USA 89:9544–9548

Bernstein G, Blank JL, Smrcka AV, Higashijima T, Sternweis PC, Exton JH, Ross EM (1992) Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, Gq/11, and phospholipase C-beta 1. J Biol Chem 267:8081–8088

Biddlecome GH, Berstein G, Ross EM (1996) Regulation of phospholipase C-beta 1 by Gq and m1 muscarinic cholinergic receptor. Steady-state balance of receptor-mediated activation and GTPase-activating protein-promoted deactivation. J Biol Chem 271:7999–8007

Buck MA, Fraser CM (1990) Muscarinic acetylcholine receptor subtypes which selectively couple to phospholipase C: pharmacological and biochemical properties. Biochem Biophys Res Commun 173:666–672

Caulfield MP, Birdsall NJM (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290

Delmas P, Abogadie FC, Dayrell M, Haley JE, Milligan G, Caulfield MP, Brown DA, Buckley NJ (1998) G-proteins and G-protein subunits mediating cholinergic inhibition of N-type calcium currents in sympathetic neurons. Eur J Neurosci 10:1654–1666

Delmas P, Abogadie FC, Buckley NJ, Brown DA (2000) Calcium channel gating and modulation by transmitters depend on cellular compartmentalization. Nat Neurosci 3:670–678

Furukawa T, Nukada T, Mori Y, Wakamori M, Fujita Y, Ishida H, Fukuda K, Kato S, Yoshii M (1998a) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca^{2+} channels with G-protein alpha and beta gamma subunits. I. Molecular determination. J Biol Chem 273:17585–17594

Furukawa T, Miura R, Mori Y, Strobeck M, Suzuki K, Ogihara Y, Asano T, Morishita R, Hashii M, Higashida H, Yoshii M, Nukada T (1998b) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca^{2+} channels with G-protein alpha and beta gamma subunits. II. Evidence for direct binding. J Biol Chem 273:17595–17603

Gurwitz D, Haring R, Heldman E, Fraser CM, Manor D, Fisher A (1994) Discrete activation of transduction of pathways associated with acetylcholine M1 receptor by several muscarinic ligands. Eur J Pharmacol 267:21–31

Haley JE, Delmas P, Offermanns S, Abogadie FC, Simon MI, Buckley NJ, Brown DA (2000) Muscarinic inhibition of calcium current and M current in $G\alpha_q$-deficient mice. J Neurosci 20:3973–3979

Hilgemann DW, Ball R (1996) Regulation of cardiac Na^+, Ca^{2+} exchange and KATP potassium channels by PIP2. Science 273:956–959

Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci 17:531–536

Ikeda SR (1991) Double-pulse calcium channel current facilitation in adult rat sympathetic neurones. J Physiol (London) 439:181–214

Jarvis SE, Zamponi GW (2001) Interactions between presynaptic Ca^{2+} channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. Trends Pharmacol Sci 22:519–525

Jelsema CL, Axelrod J (1987) Stimulation of phospholipase A_2 activity in bovine rod outer segments by the beta gamma subunits of transducin and its inhibition by the alpha subunit. Proc Natl Acad Sci USA 84:3623–3627

Kramer RM, Checani GF, Deykin D (1987) Stimulation of Ca^{2+}-activated human platelet phopholipase A_2 by diacylglycerol. Biochem J 248:779–783

Liu L, Rittenhouse AR (2000) Effects of arachidonic acid on unitary calcium currents in rat sympathetic neurons. J Physiol (London) 525:391–404

Liu L, Rittenhouse AR (2003a) Pharmacological discrimination between muscarinic receptor signal transduction cascades with bethanechol chloride. Br J Pharmacol 138:1259–1270

Liu L, Rittenhouse AR (2003b) Arachidonic acid mediates muscarinic inhibition and enhancement of N-type Ca^{2+} current in sympathetic neurons. Proc Natl Acad Sci USA 100:295–300

Liu L, Barret CF, Rittenhouse AR (2001) Arachidonic acid both inhibits and enhances whole cell calcium currents in rat sympathetic neurons. Am J Physiol 280:C1293–C1305

Liu L, Gonzalez PK, Barret CF, Rittenhouse AR (2003) The calcium channel ligand FPL 64176 enhances L-type but inhibits N-type neuronal calcium currents. Neuropharmacology 45:281–292

Lopes CMB, Zhang H, Rohacs T, Jin T, Yang J, Logothetis DE (2002) Alterations in conserved Kir channel-PIP_2 interactions underlie channelopathies. Neuron 34:933–944

Mathie A, Bernheim L, Hille B (1992) Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. Neuron 8:907–914

Mukai H, Munekata E, Higashijima T (1992) G protein antagonists. A novel hydrophobic peptide competes with receptor for G protein binding. J Biol Chem 267:16237–16243

Petit-Jacques J, Hartzell HC (1996) Effect of arachidonic acid on the L-type calcium current in frog cardiac myocytes. J Physiol (London) 493:67–81

Plummer MR, Logothetis DE, Hess P (1989) Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. Neuron 2:1453–1463

Plummer MR, Rittenhouse AR, Kanevsky M, Hess P (1991) Neurotransmitter modulation of calcium channels in rat sympathetic neurons. J Neurosci 11:2339–2348

Richards MH, van Giersbergen PLM (1995) Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists. Br J Pharmacol 114:1241–1249

Shapiro MS, Loose MD, Hamilton SE, Nathanson NM, Gomeza J, Wess J, Hille B (1999) Assignment of muscarinic receptor subtypes mediating G-protein modulation of Ca^{2+} channels by using knockout mice. Proc Natl Acad Sci USA 96:10899–10904

Shapiro MS, Gomeza J, Hamilton SE, Hille B, Loose MD, Nathanson NM, Roche JP, Wess J (2001) Identification of subtypes of muscarinic receptors that regulate Ca^{2+} and K^+ channel activity in sympathetic neurons. Life Sci 68:2481–2487

Spector AA (1975) Fatty acid binding to plasma albumin. J Lipid Res 16:165–179

Suh BC, Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. Neuron 35:507–520

Swarthout JT, Walling HW (2000) Lysophosphatidic acid: receptors, signaling and survival. Cell Mol Life Sci 57:1978–1985

Tence M, Cordier J, Premont J, Glowinski J (1994) Muscarinic cholinergic agonists stimulate arachidonic acid release from mouse striatal neurons in primary culture. J Pharmacol Exp Ther 269:646–653

Tsunoda Y, Owyang C (1995) The regulatory site of functional GTP binding protein coupled to the high affinity cholecystokinin receptor and phospholipase A2 pathway is on the G beta subunit of Gq protein in pancreatic acini. Biochem Biophys Res Commun 211:648–655

Wu L, Bauer CS, Zhen XG, Xie C, Yang J (2002) Dual regulation of voltage-gated calcium channels by PtdIns(4,5)P2. Nature 419:947–952

Zhang H, Craciun LC, Mirshahi T, Rohacs T, Lopes CM, Jin T, Logothetis DE (2003) PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. Neuron 37:963–975

Zhou J, Shapiro MS, Hille B (1997) Speed of Ca^{2+} channel modulation by neurotransmitters in rat sympathetic neurons. J Neurophysiol 77:2040–2048