Dynamics of Phosphoinositide-Dependent Signaling in Sympathetic Neurons

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In neurons, loss of plasma membrane phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] leads to a decrease in exocytosis and changes in electrical excitability. Restoration of PI(4,5)P₂ levels after phospholipase C activation is therefore essential for a return to basal neuronal activity. However, the dynamics of phosphoinositide metabolism have not been analyzed in neurons. We measured dynamic changes of PI(4,5)P₂, phosphatidylinositol 4-phosphate, diacylglycerol, inositol 1,4,5-trisphosphate, and Ca²⁺ upon muscarinic stimulation in sympathetic neurons from adult male Sprague-Dawley rats with electrophysiological and optical approaches. We used this kinetic information to develop a quantitative description of neuronal phosphoinositide metabolism. The measurements and analysis show and explain faster synthesis of PI(4,5)P₂ in sympathetic neurons than in electrically nonexcitable tsA201 cells. They can be used to understand dynamic effects of receptor-mediated phospholipase C activation on excitability and other PI(4,5)P₂-dependent processes in neurons.

Key words: excitability; M-current; phosphoinositide metabolism; PI(4,5)P₂; superior cervical ganglion neurons

Significance Statement

Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] is a minor phospholipid in the cytoplasmic leaflet of the plasma membrane. Depletion of PI(4,5)P₂ via phospholipase C-mediated hydrolysis leads to a decrease in exocytosis and alters electrical excitability in neurons. Restoration of PI(4,5)P₂ is essential for a return to basal neuronal activity. However, the dynamics of phosphoinositide metabolism have not been analyzed in neurons. We studied the dynamics of phosphoinositide metabolism in sympathetic neurons upon muscarinic stimulation and used the kinetic information to develop a quantitative description of neuronal phosphoinositide metabolism. The measurements and analysis show a several-fold faster synthesis of PI(4,5)P₂ in sympathetic neurons than in an electrically nonexcitable cell line, and provide a framework for future studies of PI(4,5)P₂-dependent processes in neurons.

Introduction

We report the dynamics of phosphoinositide metabolism and signaling in single living neurons. We measure receptor-induced

changes of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], phosphatidylinositol 4-phosphate [PI(4)P], inositol 1,4,5-trisphosphate (IP₃), diacylglycerol (DAG), and Ca²⁺ and develop a kinetic description. Phosphoinositides are fundamental signaling components of all cellular membranes. PI(4,5)P₂ located at the plasma membrane is required by many membrane proteins to fulfill their functions. In neurons, binding of SNARE proteins and synaptotagmin to PI(4,5)P₂ allows vesicle docking and secretion (Di Paolo and De Camilli, 2006; Martin, 2015). Similarly, endocytosis (Cremona and De Camilli, 2001; Posor et al., 2015), actin filament assembly (Saarikangas et al., 2010), and ion channel activity (Hille et al., 2015) are regulated by PI(4,5)P₂.

PI(4,5)P₂ levels can be reduced through activation of receptors. Phospholipase C (PLC) activated by G-protein-coupled receptors and receptor tyrosine kinases cleave PI(4,5)P₂ into IP₃ and DAG, activating several signaling pathways (Michell, 1975; Berridge and Irvine, 1984). At least 80 types of PLC-coupled receptors are known (Pawson et al., 2014). Receptor tyrosine kinases acting through PI 3-kinases also use PI(4,5)P₂ as a pre-

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cursor to synthesize PI(3,4,5)P₃. Once the stimulus activating these receptors is gone, PI(4,5)P₂ levels are restored (Willars et al., 1998; Cantley, 2002). PI(4,5)P₂ is synthesized in two steps involving sequential phosphorylation of phosphatidylinositol (PI) and PI(4)P by the action of lipid 4- and 5-kinases, respectively. The kinase steps are opposed by lipid phosphatases that convert PI(4,5)P₂ back to PI(4)P (5-phosphatase) and PI(4)P to PI (4-phosphatase). Biochemical experiments show that the PI pool is by far the largest, comprising 90–96% of the total phosphoinositide, whereas PI(4)P and PI(4,5)P₂ make up much of the remainder (Willars et al., 1998; Xu et al., 2003), suggesting that primarily PI(4)P levels limit PI(4,5)P₂ synthesis.

In a scenario where neuromodulators are released from extra-synaptic or synaptic sites to bathe nearby neurons, the activation of PLC and subsequent hydrolysis of PI(4,5)P₂ will arrest PI(4,5)P₂-dependent processes. For example, in superior cervical ganglion (SCG) neurons a voltage-gated potassium channel can be turned off by PLC-mediated hydrolysis of PI(4,5)P₂ (Brown and Adams, 1980; Suh and Hille, 2002; Zhang et al., 2003; Winks et al., 2005). This greatly increases the excitability of SCG neurons, facilitating firing of spikes while PI(4,5)P₂ is low, a process that in the CNS would be akin to arousal. Despite the importance of PI(4,5)P₂ resynthesis for the duration of such neuronal arousal, relatively little is known about the dynamics of PI(4,5)P₂ replenishment in neurons.

The dynamics of PI(4,5)P₂ synthesis have been measured carefully in several cell lines (Willars et al., 1998; Xu et al., 2003; Jensen et al., 2009; Falkenburger et al., 2010a,b). After depletion by PLC, 63% of PI(4,5)P₂ is resynthesized within 120 s in these cells. Similarly in neurons, PI(4,5)P₂ is depleted and resynthesized after PLC activation (Micheva et al., 2001; Winks et al., 2005). Although the kinetics of PI(4,5)P₂ depletion have been characterized and modeled for sympathetic neurons (Winks et al., 2005), we still lack a quantitative description of the underlying dynamics of PI(4,5)P₂ resynthesis.

We ask the following questions: how long does it take for a neuron to replenish the pool of PI(4,5)P₂ at the plasma membrane? How do these dynamics compare with other cells, and how are they regulated? We decided to study these questions in superior cervical ganglion neurons as the expression of PI(4,5)P₂-dependent ion channels and PLC-activating G-protein-coupled receptors are well described in these cells, thus making them an ideal model system to characterize the phosphoinositide metabolism of a primary neuron. Our results show a several-fold faster phosphoinositide metabolism in SCG neurons compared with nonexcitable cell lines, leading us to the hypothesis that PI(4,5)P₂ dynamics are cell-type-specific and that neurons use faster kinetics of PI(4,5)P₂ synthesis to fine-tune electrical excitability and other PI(4,5)P₂-dependent processes. We develop a quantitative framework for the dynamics of physiological processes in neurons that depend on PI(4,5)P₂.

Materials and Methods

Cells. Animals were handled according to guidelines of the University of Washington Institutional Animal Care and Use Committee. Neurons were isolated from SCG of adult (7- to 12-week-old) male Sprague-Dawley rats by enzymatic digestion as described previously (Beech et al., 1991; Vivas et al., 2013). Isolated neurons were plated on poly-L-lysine (Sigma-Aldrich)-coated glass chips and incubated in 5% CO₂ at 37°C in medium supplemented with 10% fetal bovine serum.

SCG neurons were transfected by nuclear injection 24 h after isolation using an Eppendorf 5242 pressure microinjector and 5171 micromanipulator system (Eppendorf). cDNA was dissolved in 1 mg/ml dextran-fluorescein solution (10,000 kDa; Molecular Probes) to yield a final concentration of 50–150 ng/μl of DNA and microinjected into the nu-

cleus for 0.3 s at pressures of 80–120 hPa. TsA201 cells were cultured and transfected as described previously (Jensen et al., 2009). The following plasmids were used: TubbyR332H-YFP (provided by A. Tinker, University College of London, London, United Kingdom); human eCFP-PH (PLCδ1), eYFP-PH (PLCδ1), and CAAX-CFP (from K-Ras, provided by K. Jalink, The Netherlands Cancer Institute, Amsterdam, Netherlands); C1-YFP (from the C1 domain of PKCγ, provided by T. Meyer, Stanford University, Stanford, CA); the zebrafish voltage-sensitive phosphatase (Dr-VSP; provided by Y. Okamura, Osaka University, Osaka, Japan); and the IP₃ LIBRA version III (LIBRAvIII; provided by A. Tanimura, Health Sciences University of Hokkaido, Tobetsu, Japan).

Electrophysiology. The potassium current I_{KCNQ2/3} was recorded in whole-cell configuration as previously described for SCG neurons (Vivas et al., 2014) and tsA201 cells (Falkenburger et al., 2010b).

Lipid analysis by mass spectrometry. Lipid analysis was a modification of previously described methods (Clark et al., 2011; Laketa et al., 2014). Each lipid determination required two sympathetic ganglia or the cultured cells from one 35 mm ~70% confluent dish. Briefly, pairs of ganglia were mechanically minced, and neurons and tsA201 cells were pelleted by centrifugation for 3 min at 1000 × g and 4°C. Cell pellets were resuspended in water. Lipids were first extracted twice with *N*-butanol, followed by three extractions with chloroform. All extraction steps were performed on ice, and separation of aqueous and organic phases was achieved by centrifugation for 3 min at 20,000 × g and 4°C. Extracts were combined and solvents were evaporated under nitrogen. Lipids were methylated with (trimethylsilyl) diazomethane for 1 h at room temperature with suitable precautions. Lipids were separated by ultrahigh-pressure liquid chromatography (Acquity UPLC Protein BEH C4 column, 300 Å, 1.7 μm, 1 × 100 mm, Waters) and detected by mass spectrometry (Xevo TQ-S, Waters) with sodium formate infusion into the ionization chamber. To detect changes in phosphoinositide levels after activation of muscarinic receptors, whole SCG were cut into small pieces and treated for 1 min with 10 μM oxotremorine methiodide (Oxo-M) before the reaction was stopped by addition of ice-cold methanol/1 N HCl. TsA201 cells transiently transfected with muscarinic receptors (M₂R_s) were equally treated with Oxo-M for 1 min, followed by stoppage of the reaction ice-cold methanol/1 N HCl. Extraction of lipids was performed as described above. To normalize for cell numbers, 10% of each sample was removed before lipid extraction and genomic DNA was extracted with GeneJet Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. Genomic DNA amounts were quantified by spectrophotometry and used for normalization of lipid signal intensities.

FRET and calcium photometry. Optical measurements of calcium and Förster resonance energy transfer (FRET) were performed on single neurons by whole-cell photometry (not with images) as previously described (Falkenburger et al., 2010a,b, 2013; Dickson et al., 2013). Cells were illuminated by a grating-controlled monochromatic light source (Polychrome IV; TILL Photonics). For measurements of cytoplasmic-free Ca²⁺, neurons were loaded with 2 μM fura-2-AM (Invitrogen) dissolved in Ringer's solution containing 0.02% pluronic acid F-68. Fura-2 signals were reported as the fluorescence ratio with two wavelengths of excitation, F340/F380. FRET was measured as the ratio of corrected fluorescence from YFP and CFP after excitation of CFP molecules and is reported as F_{YFP}/F_{CFP} (FRET_r).

Western blot analysis. Cytoplasmic proteins from SCG neurons and tsA201 cells were isolated and purified with the Mem-PER Plus Membrane Protein Extraction Kit (Pierce Protein Biology Products, Thermo Scientific) according to the manufacturer's instructions. Membrane proteins were extracted as follows: cells were spun down at 300 × g and resuspended in 1 ml H₂O. Cell suspensions were frozen in liquid nitrogen and thawed at 37°C in a water bath. This step was repeated twice before samples were centrifuged for 3 min at 300 × g at 4°C. The supernatant was removed and spun again at 20,000 × g for 20 min at 4°C. The pellet was resuspended in HEPES-lysis buffer (150 mM NaCl, 10 mM HEPES, 0.5% Triton X-100, pH 7.4). Protein concentration was determined by BCA protein assay (Pierce Protein Biology Products, Thermo Scientific). Twenty micrograms of protein was separated by SDS-PAGE using standard techniques. The primary antibody against rat and human PLCβ1

Table 1. Rate constants and parameters for SCG neuron and tsA201 cell models

Parameter	Value (SCG)	Value (tsA201)	Rationale for parameter in SCG model
R (M_1 density)	$15.87 \mu\text{m}^{-2}$	$500 \mu\text{m}^{-2}$	From dose–response curve
G (G-protein density)	$40 \mu\text{m}^{-2}$	$40 \mu\text{m}^{-2}$	As previously by Falkenburger et al. (2013)
P (PLC density)	$3.12 \mu\text{m}^{-2}$	$10 \mu\text{m}^{-2}$	From Western blot
(free) PI(4,5)P ₂ (density)	$3232 \mu\text{m}^{-2}$	$5000 \mu\text{m}^{-2}$	Mass spectrometry and Xu et al. (2003)
Bound PI(4,5)P ₂ (density)	$6464 \mu\text{m}^{-2}$	$10,000 \mu\text{m}^{-2}$	$(\text{fold\_PIP}_2 - 1) \times 3232 \mu\text{m}^{-2}$
PI(4)P (density)	$4540 \mu\text{m}^{-2}$	$4000 \mu\text{m}^{-2}$	Mass spectrometry and Xu et al. (2003)
PI (density)	$226,975 \mu\text{m}^{-2}$	$140,000 \mu\text{m}^{-2}$	Mass spectrometry and Xu et al. (2003)
IP ₃ (basal, cytosol)	$0.02 \mu\text{M}$	$0.02 \mu\text{M}$	Steady state of basal PLC and IP ₃ ase
DAG (basal, plasma membrane)	$13 \mu\text{M}^{-2}$	$13 \mu\text{M}^{-2}$	Steady state of basal PLC and DAGase
Ca ²⁺ (basal, cytosol)	$0.101 \mu\text{M}$	$0.13 \mu\text{M}$	Wanaverbecq et al. (2003)
Ca ²⁺ (basal, ER)	$362 \mu\text{M}$	$400 \mu\text{M}$	Falkenburger et al. (2013) and fura-2 responses
LIBRAVlll	$6 \mu\text{M}$	$6 \mu\text{M}$	As previously by Falkenburger et al. (2013)
fura-2	$1 \mu\text{M}$	$1 \mu\text{M}$ fura-4F	As previously by Falkenburger et al. (2013)
C1 domain	$0.5 \mu\text{M}$	$0.5 \mu\text{M}$	As previously by Falkenburger et al. (2013)
Surface (area plasma membrane)	$4100 \mu\text{m}^2$	$1500 \mu\text{m}^2$	From neuron cell capacitance
size_cytosol (volume)	$6644 \mu\text{m}^3$	$2500 \mu\text{m}^3$	From surface–volume ratio
size_ER (volume)	$1196 \mu\text{m}^3$	$462 \mu\text{m}^3$	18% cytosol, Falkenburger et al. (2013)
k _{4K}	0.0008 s^{-1}	0.00026 s^{-1}	I _{KCNQ2/3} recovery after Oxo-M
k _{4P}	0.12 s^{-1}	0.03 s^{-1}	Steady state of basal PI(4,5)P ₂
k _{5K}	0.02 s^{-1}	0.02 s^{-1}	I _{KCNQ2/3} recovery after VSP
k _{5P}	0.028 s^{-1}	0.014 s^{-1}	Steady-state of basal PI(4,5)P ₂
k _{PLC}	$0.3 \mu\text{M}^2 \text{ s}^{-1}$	$0.2 \mu\text{M}^2 \text{ s}^{-1}$	Based on I _{KCNQ2/3} inhibition
k _{IP3ase}	0.13 s^{-1}	0.08 s^{-1}	Based on LIBRAVlll and fura-2 responses
k _{DAGase}	0.20 s^{-1}	0.05 s^{-1}	Based on C1-decay
Ca ²⁺ _buffer_high_affinity	$10 \mu\text{M}$	Absent	Based on fura-2 responses
Ca ²⁺ _buffer_low_affinity	$20 \mu\text{M}$	Absent	Based on fura-2 responses
K _D _Ca ²⁺ _high_affinity	$0.1 \mu\text{M}$	Absent	Wanaverbecq et al. (2003)
K _D _Ca ²⁺ _low_affinity	$1 \mu\text{M}$	Absent	Wanaverbecq et al. (2003)
K _D _IRBIT (IP ₃ R)	$0.5 \mu\text{M}$	Absent	Ando et al. (2003)
K _D _IP ₃ (IP ₃ R)	$0.1 \mu\text{M}$	$0.1 \mu\text{M}$	As previously by Falkenburger et al. (2013)
K _D _Ca ²⁺ (IP ₃ R activation site)	$0.2 \mu\text{M}$	$0.2 \mu\text{M}$	Bezprozvanny et al. (1991)
K _P (SERCA) (Ca ²⁺ pump site)	$1.3 \mu\text{M}$	$1.3 \mu\text{M}$	Duman et al. (2008)
v _P (SERCA)	$1 \mu\text{M} \mu\text{M} \text{ s}^{-1}$	$0.3 \mu\text{M} \mu\text{M} \text{ s}^{-1}$	Based on fura-2 signal
K _D _LIBRAVlll (IP ₃ site)	$0.5 \mu\text{M}$	$0.5 \mu\text{M}$	Tanimura et al. (2009)
K _D _C1 (DAG site)	$0.2 \mu\text{M}$	$0.2 \mu\text{M}$	Based on C1/CAAX FRET response
K _D _NCS-1 (Ca ²⁺ binding site)	$0.44 \mu\text{M}$	Absent	Aravind et al. (2008)
K _D _fura-2 (Ca ²⁺ site)	$0.145 \mu\text{M}$	$0.77 \mu\text{M}$ (fura-4F)	Life Technologies
K _D _TubbyR332H (PI(4,5)P ₂ site)	$1.5 \mu\text{M}$	Absent	Based on TubbyR332H/CAAX FRET signal
J _{max} 2 (IP ₃ R)	$80 \mu\text{M} \mu\text{M} \text{ s}^{-1}$	$200 \mu\text{M} \mu\text{M} \text{ s}^{-1}$	Based on Western blot and fura-2 responses

was used at a concentration of 1 $\mu\text{g}/\text{ml}$ (Abcam, ab140746); anti-IP₃R1 and anti-IP₃R2 were used at a dilution of 1:500 (Alomone Labs, ACC-019 and ACC-116), anti-IRBIT (Abcam, ab178693) was used at a dilution of 1:10,000, and all were detected using a horseradish peroxidase-coupled secondary antibody (1:5000, Santa Cruz Biotechnology) and chemiluminescence (Pierce ECL Western Blotting Substrate, Pierce Protein Biology Products, Thermo Scientific). Recombinant protein of human phospholipase C β 1 (PLC β 1) was used for Western blot analysis (OriGene Technologies).

Mathematical modeling. The Virtual Cell software environment (University of Connecticut Health Center, <http://www.nrcam.uchc.edu>) was used to develop a kinetic description of phosphoinositide metabolism in neurons. As a starting point and for comparison, we used a compartmental model (Dickson et al., 2013; Falkenburger et al., 2013) that had been developed to describe observations on the tsA201 cell line. Conditions that were changed to resemble the results obtained in SCG neurons are shown in Figures 3A,B, 5E, 8C,G, and Table 1. Model rate constants were chosen manually to fit experimental data. To compare modeled calculations to data obtained from FRET and electrophysiological recordings, we segmented the traces into onset and recovery of the effect and normalized to basal levels in each case to correct for incomplete recovery. The modified Virtual Cell Model “hillelab: KruseVivasTraynorKaplanHille_PI-metabolism_SCG_2015” is publicly available at <http://www.vcell.org/>.

Data analysis. We used IGOR Pro (IGOR Software, RRID:nlx_156887, WaveMetrics) and Excel (Microsoft) to analyze data. Statistics are given

as mean $\pm$ SEM. Student's *t* test was used to test for statistical significance. *p* values <0.05 were considered significant.

Results

We start by explaining the logic of the experiments. We first make empirical measurements of lipid metabolism and then bring in kinetic reasoning to develop a clearer understanding of several differences in the underlying steps in neurons. The lipids are followed by electrophysiological and optical indicators and by chemical analysis. Figure 1A shows the steps to be analyzed. They include the lipid 4- and 5-kinases (enzymes are shown in red) that phosphorylate PI to PI(4)P and PI(4,5)P₂, the lipid 4- and 5-phosphatases that reverse these reactions, and the PLC that hydrolyzes PI(4,5)P₂ into DAG and IP₃. We perturb lipid levels by activating enzymes. Thus, when PLC is activated by receptors, the pools of PI(4)P and PI(4,5)P₂ both become depleted (see the next section) and recovery of PI(4,5)P₂ is a two-step reaction that requires both the 4- and the 5-kinase. When we want to analyze the kinetics of the 5-kinase alone, we use a special voltage-sensing lipid phosphatase (VSP; Murata and Okamura, 2007) that dephosphorylates PI(4,5)P₂ to PI(4)P (Fig. 1A). Subsequent recovery of PI(4,5)P₂ then requires only the 5-kinase.

Fast recovery of $I_{KCNQ2/3}$ and $PI(4,5)P_2$ in SCG neurons

In the first experiments, we monitored the levels of $PI(4,5)P_2$ in the plasma membrane of SCG neurons using the endogenous $PI(4,5)P_2$ -dependent voltage-gated potassium channel $KCNQ2/3$ ($K_v7.2/7.3$) as a reporter (Suh and Hille, 2002; Zhang et al., 2003; Winks et al., 2005). These channels underlie the M-current in SCG neurons (Brown and Passmore, 2009). We depleted $PI(4,5)P_2$ by activation of M_1 Rs through a 20 s application of a muscarinic agonist, 10 μ M Oxo-M (Fig. 1B, left). During agonist application, $I_{KCNQ2/3}$ declined rapidly and almost completely, and upon removal of agonist, current recovered with a time constant of 42 ± 1 s at room temperature (Fig. 1C). Closure of these channels makes the neurons much more excitable (Brown et al., 2007), and their reopening terminates the period of raised excitability. The experiment was repeated for comparison in tsA201 cells transfected with $KCNQ2$, $KCNQ3$, and M_1 R. Now the current recovered with a much longer time constant of 123 ± 20 s upon removal of the agonist (Fig. 1C,D; Jensen et al., 2009). Thus, $PI(4,5)P_2$ pools are replenished more rapidly in the neurons than in tsA201 cells.

The experiments shown in Figure 1, C and D, were conducted with neurons cultured for 48 h after isolation. To test whether the culture conditions alter the time constant of M-current recovery, we repeated the experiments at different culture times, ranging from 1 to 72 h after isolation. In addition, we used only 1 μ M Oxo-M, which had previously been shown to cause half-maximal inhibition of M-current (Winks et al., 2005) and allowed us to observe potential alterations in M_1 receptor signaling better. In freshly dissociated neurons, cultured only for 1 h, the application of 1 μ M Oxo-M inhibited $\sim 50\%$ of M-current and the time con-

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Figure 1. Faster kinetics of $I_{KCNQ2/3}$ and $PI(4,5)P_2$ recovery in SCG neurons versus tsA201 cells. **A**, Overview of the analyzed reactions of PI metabolism. Red, Enzymes. **B**, Schematic of closure of $KCNQ2/3$ channels due to $PI(4,5)P_2$ hydrolysis. Left, Activation of phospholipase C after binding of the ligand Oxo-M to M_1 R. Right, Activation of a VSP by membrane depolarization. **C**, Normalized $KCNQ2/3$ tail current amplitude upon activation of M_1 receptors by 10 μ M Oxo-M measured with whole-cell recordings from SCG neurons and tsA201 cells. **D**, Time constants of tail current amplitude recovery after removal of Oxo-M. Numbers in brackets indicate number of experiments. **E**, Normalized $KCNQ2/3$ tail current amplitude upon activation of M_1 receptors by 1 μ M Oxo-M measured with whole-cell recordings from SCG neurons 1 h after isolation. **F**, Time constants of tail current amplitude recovery after activation of M_1 Rs with 1 μ M Oxo-M at different time points after isolation of SCG neurons (black) or percentage inhibition of M-current (red). **G**, Same as in **C** but with activation of VSP. **H**, Time constants of tail current amplitude recovery after VSP activation. **I**, SCG neurons were transfected by intranuclear injection with plasmids encoding TubbyR332H-YFP and CAAX-CFP to measure changes in plasma membrane $PI(4,5)P_2$ levels with ratiometric FRET recordings. Top, Schematic of FRET measurements before and after depletion of $PI(4,5)P_2$. Bottom, Negative contrast confocal image of fluorescence intensity distribution of TubbyR332H before (left) and after (right) application of Oxo-M. **J**, Line scans of TubbyR332H distribution before (black) and after (orange) application of Oxo-M along the lines indicated in **I**. **K**, Normalized FRET between TubbyR332H-YFP and CAAX-CFP upon application of Oxo-M to SCG neurons and tsA201 cells. **L**, Negative contrast confocal images of PH-PLC $\delta 1$ -YFP fluorescence in a SCG neuron before and 20 s after stimulation with 10 μ M Oxo-M. **M**, Line scans showing fluorescence intensity distribution of PH-PLC $\delta 1$ -YFP along lines indicated in **L** before (black) and after (orange) application of Oxo-M. **N**, Mean normalized FRET from SCG neurons transfected with PH-PLC $\delta 1$ -CFP and -YFP upon stimulation of cells with Oxo-M.

Figure 2. Recovery of $PI(4)P$ is faster in SCG neurons than in tsA201 cells. **A**, Negative contrast confocal images of the $PI(4)P$ indicator P4M-GFP in a SCG neuron before (left) and after (right) application of 10 μ M Oxo-M. **B**, Line scan showing P4M distribution along lines indicated in **A** before (black) and after (orange) Oxo-M application. **C**, Normalized FRET measured from SCG neurons (black) and tsA201 cells (blue) transfected with P4M-YFP and CAAX-CFP upon application of Oxo-M for 20 s.

stant of current recovery was 44 ± 9 s ($n = 9$; Fig. 1E). Culturing neurons for up to 72 h did not change the fraction of current inhibition or the time constant of M-current recovery (Fig. 1F), showing that the chosen culture conditions do not alter M_1 R signaling or the time constant of M-current recovery. Similar to recordings obtained from neurons cultured for 48 h and treated with 10 μ M Oxo-M, the time constant of current recovery was significantly faster than in tsA201 cells, suggesting that fast replenishment of $PI(4,5)P_2$ pools is an intrinsic property of sympathetic neurons.

Are both lipid kinases in Figure 1A faster in neurons? To isolate the contribution of endogenous lipid 5-kinases, we expressed the VSP in SCG neurons. During a depolarizing voltage step to +100 mV, VSP dephosphorylates $PI(4,5)P_2$ to $PI(4)P$, transiently depleting $PI(4,5)P_2$, and $KCNQ2/3$ channel activity decreases sharply (Fig. 1B, right). After the brief activation of VSP, $I_{KCNQ2/3}$ was strongly suppressed and then recovered with a time constant of 24 ± 5 s in SCG neurons and 10 ± 1 s in tsA201 cells (Fig. 1G,H; Falkenburger et al., 2010b). Apparently, the endogenous 5-kinase activity is lower in the neurons than in the tsA201 cells. Hence, we must hypothesize that the relative activity of the 4-kinase is markedly boosted in the neurons because the overall recovery of $I_{KCNQ2/3}$ after PLC-activation is several-fold faster than in tsA201 cells. We test this notion in the next section.

To confirm the kinetic measurements of $PI(4,5)P_2$, we repeated the experiments with two other indicators of $PI(4,5)P_2$. The first was a YFP-tagged $PI(4,5)P_2$ binding domain from Tubby (TubbyR332H-YFP; Hughes et al., 2007). To monitor plasma-membrane Tubby by a FRET assay (Fig. 1I), we coexpressed TubbyR332H-YFP (FRET acceptor) with a small plasma membrane localized CFP-tagged protein (CAAX-CFP, FRET donor). Under basal conditions, fluorescence of TubbyR332H-YFP was localized to the plasma membrane of SCG neurons (Fig. 1I,J) and the FRET ratio ($FRET_{YFP}/F_{CFP}$) was high. Then activation of M_1 R led to a translocation of the domain to the cytoplasm and a drop of FRET (Fig. 1K), indicating $PI(4,5)P_2$ hydrolysis. FRET recovery during the washout had a time constant of 53 ± 15 s in SCG neurons and a time constant of 127 ± 25 s in tsA201 cells, corresponding well with our findings for $I_{KCNQ2/3}$ recovery after receptor activation that overall $PI(4,5)P_2$ synthesis occurs more slowly in tsA201 cells.

As a second FRET-based assay for $PI(4,5)P_2$, we repeated the experiments with CFP- and YFP-tagged versions of pleckstrin

Figure 3. Estimation of PLC and M_1R expression levels in SCG neurons. **A**, Western blot of cytoplasmic lysates of tsA201 cells and SCG neurons with an anti-PLC $\beta 1$ antibody. Protein amounts of the two lysates were normalized for equal amounts; percentages indicate dilution steps. “PLC $\beta 1$ ” indicates detection of a recombinant PLC $\beta 1$ protein used as a positive control for the primary antibody. **B**, Percentage of PLC $\beta 1$ in SCG neurons compared with tsA201 cells as measured by analysis in **A**. Number in brackets indicates number of experiments. **C**, Left, Time course of KCNQ2/3 tail current amplitude recorded from a SCG neuron upon application of indicated concentrations of Oxo-M. Right, Current traces corresponding to time points indicated by letters on the left. Arrow indicates tail currents. **D**, $I_{KCNQ2/3}$ inhibition evoked by 20 s applications of different concentrations of Oxo-M in SCG neurons (black) or tsA201 cells (blue). **E**, Modeled $I_{KCNQ2/3}$ inhibition evoked by 20 s applications of different concentrations of Oxo-M for indicated densities of M_1R . Dotted line indicates M_1R density of 16 molecules μm^{-2} . **F**, Comparison of experimental (black) and modeled (red) dose-response curves for $I_{KCNQ2/3}$ inhibition by Oxo-M in SCG neurons. **G**, IC_{50} values for $I_{KCNQ2/3}$ inhibition by Oxo-M in SCG neurons as measured experimentally ($n = 5-25$) and from our model.

homology (PH) domains from PLC $\delta 1$. These experiments were done only in neurons. Similar to TubbyR332H, the PH-PLC $\delta 1$ domain probes were localized at the plasma membrane under basal conditions and FRET r was high, and the probes moved to the cytoplasm upon application of Oxo-M (Fig. 1*L,M*). The time constant of PH domain FRET r recovery was 47 ± 8 s (Fig. 1*N*), similar to the 53 s recovery of TubbyR332H FRET r and the 42 s recovery of $I_{KCNQ2/3}$. Hence, the FRET r recordings with TubbyR332H and PH-PLC $\delta 1$ confirm that $I_{KCNQ2/3}$ recovery

tracks PI(4,5) P_2 recovery in SCG neurons and that overall PI(4,5) P_2 resynthesis is appreciably faster in SCG neurons than in tsA201 cells.

Accelerated synthesis of PI(4)P in SCG neurons

Our analysis of $I_{KCNQ2/3}$ recovery after VSP stimulation implied that there is a faster lipid 4-kinase activity in SCG neurons. We used a recently developed fluorescent probe for PI(4)P (P4M; Hammond et al., 2014) to measure the kinetics of PI(4)P changes. In SCG neurons, a GFP-labeled version of P4M showed a significant component at the plasma membrane under basal conditions, which, as expected, became mainly cytoplasmic after application of Oxo-M to SCG neurons (Fig. 2*A,B*). In basal conditions, there also was intracellular labeling, likely at the Golgi. To measure the time course at the plasma membrane, we cotransfected SCG neurons with a YFP-tagged P4M-probe and the plasma-membrane localized CAAX-CFP. Under basal conditions there was FRET r between the two fluorophores as PI(4)P was present at the plasma membrane. Upon muscarinic stimulation, the FRET r decreased; P4M-probes were leaving the plasma membrane. Upon washout of the agonist, there was partial recovery with a time constant of 73 ± 15 s, reflecting resynthesis of PI(4)P (Fig. 2*C*). Similar experiments in tsA201 cells gave similar results except that the recovery of FRET r was threefold slower (Fig. 2*C*). With this direct measure we confirm that neurons synthesize PI(4)P faster than tsA201 cells.

Determination of PLC and M_1R expression levels in SCG neurons

We now develop a more quantitative description of the underlying events. To deconvolve the rates of enzymatic steps, we adapted our earlier kinetic model of phosphoinositide metabolism in tsA201 cells (Dickson et al., 2013; Falkenburger et al., 2013) to our observations in SCG neurons. An important initial step was to determine the relative amounts of PLC and M_1R proteins in SCG compared with tsA201 cells. We isolated cytoplasmic proteins from both cell types and detected PLC $\beta 1$ in these lysates by Western blot

analysis with an anti-PLC $\beta 1$ antibody. Comparison of PLC $\beta 1$ -signal intensities showed lower expression in superior cervical ganglia than in tsA201 cells when referred to a total protein basis (Fig. 3*A,B*). Accordingly we set the level of PLC $\beta 1$ in the model for SCG neurons to $\sim 31\%$ of the level used in the tsA201 cell model (Fig. 3*B*; Table 1).

The surface density of receptors was very high in our M_1R transiently transfected tsA201 cell model, 500 receptors μm^{-2} (Falkenburger et al., 2010a). As M_1R is endogenously expressed

Figure 4. Phosphoinositide levels determined by mass spectrometry. **A**, Ratio of total PI to total PIP as measured from SCG and tsA201 cells. **B**, Levels of PIP and PIP₂ relative to controls from SCG and tsA201 cells after 1 min application of 10 μ M Oxo-M. **C**, Relative amounts of total PI, PIP, and PIP₂ as percentages of total phosphoinositides for SCG and tsA201 cells. Number in brackets indicates number of experiments.

in SCG neurons, we anticipated that the surface density might be significantly lower there. Our method of estimation combined electrophysiological recordings and mathematical modeling, following the concept that the functional surface density of M₁R defines the concentration-response curve of $I_{KCNQ2/3}$ to graded concentrations of Oxo-M. We measured $I_{KCNQ2/3}$ inhibition with 20 s applications of different concentrations of Oxo-M (Fig. 3C), finding an IC_{50} for $I_{KCNQ2/3}$ inhibition of 1.2 ± 0.2 μ M Oxo-M (Fig. 3D), one order of magnitude higher than that needed in receptor-transfected tsA201 cells under similar conditions (0.1 μ M; Fig. 3D, data from Jensen et al., 2009). Our IC_{50} value for I_M in SCG neurons is approximately twofold higher than a previously measured value of 0.7 ± 0.1 μ M (Winks et al., 2005), a difference that is explained by the duration of Oxo-M application. We used only 20 s to permit for comparison to our previously measured IC_{50} in tsA201 cells, but not long enough for channel inhibition to reach a steady state at lower concentrations of Oxo-M. Winks et al. (2005) applied Oxo-M for >1 min, thereby measuring stronger $I_{KCNQ2/3}$ inhibition at the lowest concentrations of Oxo-M.

We next used modeling to estimate the surface density of M₁ receptors. We simulated the Oxo-M concentration-response curves for varying M₁R surface densities (Fig. 3E) and estimated a mean M₁R surface density of 16 ± 2 receptors μ m⁻² as the best fit to the experiments (Fig. 3F). This density predicts an IC_{50} for $I_{KCNQ2/3}$ of 0.9 μ M Oxo-M compared with the measured value of 1.2 ± 0.2 μ M (Fig. 3G). It was used for all following simulations.

Determination of phosphoinositide levels by mass spectrometry

We had found a faster synthesis of PI(4)P from PI in SCG neurons. Such a speed up relative to tsA201 cells might be explained in two ways: (1) by an increased amount of PI relative to PI(4)P in neurons, or (2) by an increased specific activity of the lipid 4-kinase (Fig. 1A). We evaluated the first possibility by measuring the phosphoinositides directly using mass spectrometry.

Extraction of lipids, followed by derivatization, allowed successful detection of phosphoinositides. Note that mass spectrometry does not distinguish the positions of phosphorylation so here we refer to PIP and PIP₂ without being able to distinguish regioisomers. We found a ratio of PI/PIP of 60 ± 12 ($n = 4$) for SCG neurons, and of 58 ± 8 ($n = 7$) for tsA201 cells (Fig. 4A). We confirmed that these lipids were accessible to hydrolysis by phospholipase C through activation of muscarinic receptors. Applica-

tion of 10 μ M Oxo-M reduced PIP to $27 \pm 8\%$ and PIP₂ to $16 \pm 3\%$ of control levels ($n = 3$; Fig. 4B). A similar reduction was observed in tsA201 cells transiently transfected with M₁ receptors. PIP was reduced to $39 \pm 8\%$ and PIP₂ to $41 \pm 5\%$ ($n = 3$; Fig. 4B). It should be noted that we extracted total cellular lipids from all cell types within the SCG. Histologic analyses by several different groups have shown that neurons occupy the majority of the volume of the SCG (Jacobowitz and Woodward, 1968; Ichikawa et al., 2009; Ke et al., 2015), suggesting that the reduction of PIP and PIP₂ detected by our measurements is mainly caused by activation of muscarinic receptors of neurons. In addition, we tested whether the relative abundance of different types of phosphoinositides of SCG neurons and tsA201 cells showed any significant differences. The three phosphoinositide classes PI, PIP, and PIP₂ made up the following percentages of the total: $96.2 \pm 1.2\%$, $1.8 \pm 0.6\%$, and $2.1 \pm 0.6\%$ in neurons and $96.9 \pm 0.9\%$, $1.5 \pm 0.3\%$, and $1.7 \pm 0.7\%$ in tsA201 cells, respectively (Fig. 4C). These values are similar to previously reported ratios of phosphoinositides in other cell types (Willars et al., 1998; Xu et al., 2003) and show no significant differences between neurons and tsA201 cells. We conclude that neurons have the same overall relative abundance of PI, PIP, and PIP₂ as other cells and the faster synthesis of PI(4)P observed in neurons is not caused by a superabundance of PI.

Modeling of phosphoinositide metabolism requires high activity of the lipid 4-kinase in SCG neurons

Having determined levels of PLC β 1 and M₁R for the model, and recognizing that the relative levels of PI, PIP, and PIP₂ are not significantly different between neurons and tsA201 cells, we turned our attention to the rate constants of the lipid kinases and phosphatases, abbreviated 4K, 4P, 5K, and 5P in Figure 1A. These rate constants were adjusted to approximate the measured time courses for $I_{KCNQ2/3}$ and the two FRET probes after M₁R or VSP activation in neurons. The simulations then agreed reasonably with the experimental kinetics of inhibition and recovery of $I_{KCNQ2/3}$ (Fig. 5A, B, red lines) as well as with the loss of FRET_r of Tubby-R332H and PH-PLC δ 1 and recovery of FRET_r (Fig. 5C, D, red line).

The major adjustments in the model were to increase the rate of phosphorylation from PI to PI(4)P by a factor of 4 and to decrease the rate of phosphorylation from PI(4)P to PI(4,5)P₂ by a factor of 2 for neurons (Fig. 5E; Table 1). As in the experiments, the neuron model shows a strikingly faster synthesis of PI(4)P

Figure 5. Modeling PI metabolism of SCG neurons requires faster lipid 4-kinase. **A**, Time course of normalized $I_{KCNQ2/3}$ amplitude upon stimulation of SCG neurons with Oxo-M. Comparison of experimental data from whole-cell recordings (black) and normalized $I_{KCNQ2/3}$ amplitude predicted by simulation (red). Traces were segmented into onset and recovery phases and normalized to basal levels to correct for partial recovery. **B**, Same as in **A**, but for activation of VSP. **C**, Time course of normalized FRET between TubbyR332H-YFP and CAAX-CFP upon stimulation of SCG neurons with Oxo-M as measured experimentally (black) and as predicted by simulation (red). Traces were segmented into onset and recovery phases and normalized to basal levels to correct for partial recovery. **D**, Same as in **C**, but for PH-PLC δ 1 domains as FRET reporters. The prediction plotted in red assumes no binding of PH-PLC δ 1 domains to IP $_3$. Note that the modeled traces approximate changes in FRET as a cooperative square law of the membrane-bound fraction of PH-PLC δ 1 domains as recently described (Itsuki et al., 2014). **E**, Comparison of rate constant of synthesis of PI(4)P and PI(4,5)P $_2$ between SCG neurons and tsA201 cells, expressed as fold-changes, SCG/tsA201. **F**, Simulated time courses of normalized levels of PI(4)P in SCG neurons (red, “neuron model”) and tsA201 cells (blue, “tsA201 model”) upon stimulation with Oxo-M.

(Fig. 5F), which leads to an overall faster recovery of PI(4,5)P $_2$ after transient depletion by PLC.

DAG turnover is faster in SCG neurons, but IP $_3$ turnover is not

Next, we turned to the two second messengers generated by hydrolysis of PI(4,5)P $_2$, DAG, and IP $_3$. We measured their production and degradation in neurons transfected with fluorescent FRET-probes for IP $_3$ (LIBRAVIII) and DAG (C1-YFP donor and membrane-bound CAAX-CFP acceptor; Fig. 6A). These probes indicated significant and rapid generation of IP $_3$ and DAG during muscarinic stimulation (Fig. 6B,E) and a relatively fast decay with time constants of 73 ± 9 s for IP $_3$ -bound LIBRAVIII and 15 ± 5 s for DAG-bound C1-domains. We used these measured kinetics together with the affinities of the probes (Table 1), to adapt our model for SCG neurons. The chosen PLC, M $_1$ R, and PI(4,5)P $_2$ levels (Table 1) turned out to be appropriate. We adjusted the rate constants of processes metabolizing DAG and IP $_3$ to reproduce the observed kinetics (Fig. 6C,D,F,G; Table 1). The

modeled kinetics of IP $_3$ production and degradation are almost identical for SCG neurons and tsA201 cells (Dickson et al., 2013), suggesting a similarity of the underlying machinery (see Fig. 7A). On the other hand, although DAG production follows a similar time course in these two cell types, its degradation is faster in SCG neurons (Fig. 7B). Hydrolysis of PI(4,5)P $_2$ and closure of PI(4,5)P $_2$ -dependent KCNQ2/3 channels also are kinetically indistinguishable between the two types of cells, suggesting that their endogenous PLCs have similar activity. Nevertheless, in the model, resynthesis of PI(4,5)P $_2$ happens significantly faster in SCG neurons than in tsA201 cells (Fig. 7C) as was observed, so simulated KCNQ2/3 channel activity recovers much more quickly (Fig. 7D).

Modeling suggests that regulation of IP $_3$ R-availability and cytoplasmic Ca $^{2+}$ -buffering limit Ca $^{2+}$ elevations from intracellular stores

As Oxo-M initiates fast and sizeable IP $_3$ production in SCG neurons with calculated peak concentrations of $\sim 3 \mu\text{M}$ one might expect a significant release of Ca $^{2+}$ from intracellular stores via IP $_3$ receptors (IP $_3$ R). In tsA201 cells, $\sim 2 \mu\text{M}$ IP $_3$ is sufficient to evoke almost maximal release of Ca $^{2+}$ from stores, and peak IP $_3$ concentrations of $\sim 13 \mu\text{M}$ do not lead to significantly larger Ca $^{2+}$ release. Accordingly, we loaded SCG neurons with fura-2-AM to measure changes in cytoplasmic Ca $^{2+}$ levels ratiometrically. In unclamped cells, we wanted to avoid action potential firing that would open voltage-gated calcium channels. Therefore, we bathed the cells with 100 nM tetrodotoxin (TTX). As others have reported (Cruzblanca et al., 1998; del Río et al., 1999; Zaika et al., 2007),

upon stimulation of these neurons with $10 \mu\text{M}$ Oxo-M there was only a small Ca $^{2+}$ rise, suggesting very little release of Ca $^{2+}$ from intracellular stores (Fig. 8A). To check whether the Ca $^{2+}$ -increase is due to opening of low-threshold voltage-gated calcium channels, which could still occur with TTX, we repeated the experiment replacing TTX with $100 \mu\text{M}$ Cd $^{2+}$, a blocker of voltage-gated calcium channels. We still observed a small cytoplasmic fura-2 signal under these conditions (Fig. 8B). Hence, it must be mediated by Ca $^{2+}$ release from intracellular stores, and we had to consider why it is so small compared with the large increase seen with the same Oxo-M stimulus in tsA201 cells (Dickson et al., 2013).

We tested alternative hypotheses with the model. First, we tested whether low IP $_3$ R levels can explain small Ca $^{2+}$ signals. The assumed protein level of IP $_3$ Rs had to be lowered to only 0.005% of the previous value to match the observed Ca $^{2+}$ signals (data not shown). However, Western blot analysis of neurons did not confirm such a reduction in IP $_3$ R protein levels (Fig. 8C). Instead, the protein amounts of IP $_3$ R1 and IP $_3$ R2 were reduced by

only 60% in SCG neurons. Therefore, we considered other explanations.

It has been proposed that M_1 receptors in SCG neurons are too far away from the ER membrane to activate IP_3 Rs (Delmas et al., 2002; Zaika et al., 2011; Zhang et al., 2013). This hypothesis requires a fast local degradation of released IP_3 . We developed a two-dimensional spatial model to ask how far IP_3 might diffuse before it eventually is degraded. We started with the published diffusion coefficient for IP_3 ($283 \mu m^2 s^{-1}$; Allbritton et al., 1992), and the experimentally determined degradation rate of IP_3 ($0.13 s^{-1}$, equivalent to a 7.7 s lifetime) in SCG neurons (Fig. 6B). Assuming that the IP_3 degrading enzymes are uniformly distributed, this calculation showed that IP_3 would diffuse beyond 50 μm before half of it is degraded (Fig. 8D). This distance is larger than the diameter of the soma of the neuron and several orders of magnitude larger than the expected distance between points of IP_3 -generation at the plasma membrane and the intracellular Ca^{2+} stores. Using a realistic geometry in a three-dimensional spatial model from an actual SCG neuron, the predicted profile of IP_3 showed an almost uniform rise throughout the cytoplasm (Fig. 8E,F). A second three-dimensional spatial model in which we simulated muscarinic receptors on only one side of the cell did reveal an initial gradient of IP_3 in the cytoplasm. However, within a couple of seconds IP_3 again became uniform throughout the cytoplasm (Fig. 9A–C). We conclude from these simulations that under the observed rate of IP_3 degradation, and no barriers impeding IP_3 diffusion, IP_3 should be able to bind to IP_3 Rs anywhere in the soma.

A third possibility is that IP_3 Rs of SCG neurons, though present, are inhibited. IRBIT (IP_3 R-binding protein released with inositol 1,4,5-trisphosphate), an inhibitor that interacts with IP_3 Rs and is displaced competitively when IP_3 binds, is expressed in the brain (Ando et al., 2003, 2006). We detected expression of IRBIT in superior cervical ganglia by Western blot (Fig. 8G), in agreement with previous reports showing expression of IRBIT by immunofluorescence (Zaika et al., 2011). When bound to an IP_3 R, IRBIT renders the IP_3 binding domain unavailable for IP_3 . Therefore, higher IP_3 concentrations are required to displace IRBIT and activate receptors. In addition, it has been shown that the intracellular Ca^{2+} -buffering of SCG neurons is due to two pools of Ca^{2+} -buffers with equivalent K_D values of 100 nM and 1 μM (Wanaverbecq et al., 2003). We tested by simulation whether competition by IRBIT or a similar molecule together with strong buffering of released Ca^{2+} could account for the small amplitude of the fura-2 signal observed upon muscarinic stimulation. Addition of a species with the properties of IRBIT and of two Ca^{2+} -buffering pools into our model (Fig. 8H; Table 1) sufficed to reproduce the experimental data (Fig. 8I,J). They predict an increase in free cytoplasmic Ca^{2+} of only 20 nM upon activation of M_1 R by a supramaximal concentration of ligand.

Figure 6. Kinetics of IP_3 and DAG signals after M_1 R activation. **A**, Schematic for detection of DAG and IP_3 by FRET recordings. **B**, Mean normalized FRET of LIBRAVIII in response to 10 μM Oxo-M in SCG neurons (inverted scale, F_{CFP}/F_{YFP}). **C**, **D**, Overlay of modeled LIBRAVIII response (red) on the time course of normalized inverted LIBRAVIII-FRET evoked by stimulation of SCG neurons with Oxo-M (black). Traces were segmented into onset (**C**) and recovery (**D**) phases and normalized to basal levels to correct for partial recovery. **E**, Same as in **B**, but SCG neurons were transfected with C1-YFP and CAAX-CFP to measure changes in DAG levels (F_{YFP}/F_{CFP}). Note different time scale. **F**, **G**, Same as in **C**, **D**, but for FRET data and modeled C1-YFP/CAAX-CFP signal.

It has been shown that the Ca^{2+} -binding protein neuronal calcium sensor-1 (NCS-1), which is highly expressed in SCG neurons, interacts with and stimulates lipid 4-kinases in its Ca^{2+} -bound form (Zhao et al., 2001; Rajebhosale et al., 2003; Strahl et al., 2003; Gamper et al., 2004; Winks et al., 2005). We used our model to address the question whether the small rise in cytoplasmic Ca^{2+} upon muscarinic stimulation would be sufficient to convert NCS-1 into its Ca^{2+} -bound form and stimulate lipid 4-kinases. We included a species with NCS-1's properties into our model and used the published K_D of NCS-1 for free Ca^{2+} (Aravind et al., 2008). Our simulations show that stimulation with 10 μM Oxo-M for 20 s increases the amount of Ca^{2+} -bound NCS-1 by only 2% (Fig. 9D,E). Interestingly, omitting the restriction of IP_3 -binding to IP_3 R by removing the species with IRBIT's properties from the model leads to a strong increase of Ca^{2+} -bound NCS-1 by 75% (Fig. 9D,E), illustrating the important role of a protein with IRBIT's properties for Ca^{2+} -dependent processes in SCG neurons. In conclusion, our simulations argue against a strong activation of NCS-1 by Ca^{2+} release from intracellular stores upon muscarinic stimulation, although they have not ruled out some role of NCS-1 for the fast phosphoinositide metabolism in SCG neurons.

Figure 7. Summary comparison of modeled phosphoinositide metabolism and signaling in SCG neurons and tsA201 cells. **A**, Modeled time courses of IP_3 in SCG neurons and tsA201 cells in response to a 20 s long $10 \mu M$ Oxo-M application. **B–D**, As in **A**, but for DAG (**B**), $PI(4,5)P_2$ (**C**), and $I_{KCNQ2/3}$ (**D**).

Discussion

PI(4)P replenishment is fast in SCG neurons

We found that PI(4)P, the $PI(4,5)P_2$ precursor, is restored faster in SCG neurons than in tsA201 cells. Because the PI pool is not larger, the most obvious explanation is that the enzymatic activity of the lipid 4-kinase is higher. The situation is subtle, because there are at least two sources of PI(4)P for $PI(4,5)P_2$ synthesis, the PM and the Golgi. In tsA201 cells, 70% of $PI(4,5)P_2$ is synthesized from the PI(4)P pool at the PM, and the remaining 30% of $PI(4,5)P_2$ is synthesized from the pool at the Golgi (Dickson et al., 2014). Our model, which neglects the presence of two cellular compartments, predicts a fourfold faster PI(4)P synthesis than in tsA201 cells, in close agreement with the threefold increase in the rate of recovery measured experimentally. Our experimental setup allows us to measure PI(4)P recovery at the PM but does not allow us to discriminate between synthesis of PI(4)P from PI at the PM or delivery of PI(4)P from an intracellular compartment. Refined experimental designs will be necessary to analyze these processes in greater detail as studies have described mechanisms for an acceleration of lipid 4-kinases, as well as an important role for PI transfer proteins in supporting restoration of PI(4)P levels as discussed below.

Several mechanisms might increase the activity of lipid 4-kinases in SCG neurons. General mechanisms include altered expression levels or posttranslational modifications. Other mechanisms depend on protein interaction for directed localization. One is palmitoylation acting on lipid 4-kinase type II (Barylko et al., 2009; Zhou et al., 2014). Others include interaction of the lipid 4-kinase type III with PKC (Xu et al., 2014), TMEM150 (Chung et al., 2015), or with neuronal calcium sensor 1 (Paterlini

et al., 2000; Zhao et al., 2001). We do not yet know whether any of these might apply here.

Faster PI(4)P synthesis at the PM requires a sufficient supply of the precursor, PI. Recent studies demonstrate an important role for PI transfer proteins, such as Nir2 (or PITPNM1), Nir3, and PITPNC1, in supplying PI to the PM from the ER and, under certain conditions, Golgi (Hardie et al., 2001; Garner et al., 2012; Chang et al., 2013; Kim et al., 2013, 2015; Chang and Liou, 2015). These proteins facilitate the transfer of PI to the PM at ER-PM junctions, and monitor $PI(4,5)P_2$ depletion by binding to phosphatidic acid, which stimulates their recruitment to ER-PM junctions. These papers demonstrate an essential role of PI transfer proteins in $PI(4,5)P_2$ recovery after its depletion, whereas they and other studies found only minor contributions of Golgi PI(4)P and vesicular transport for $PI(4,5)P_2$ replenishment (Szentpetery et al., 2010). Our concept of faster synthesis of PI(4)P at the PM might have to be extended to include faster transfer of phosphoinositides to the PM.

Implications of fast $PI(4,5)P_2$ replenishment in neurons

We used $KCNQ2/3$ channels as a tool to measure $PI(4,5)P_2$. Focusing on the role of these channels we can see the relevance of fast synthesis of $PI(4,5)P_2$ in excitable cells. $KCNQ2/3$ channels are the molecular determinant of M-current (Wang et al., 1998), which controls excitability in sympathetic and many central neurons (Brown and Adams, 1980; Marrion, 1997; Jentsch, 2000). Sympathetic neurons of the SCG receive cholinergic input from preganglionic neurons. Acetylcholine produces a fast and a slow response (Libet, 1964; Weight and Votava, 1970), with the slow response being mediated by activation of G-protein-coupled receptors that deplete $PI(4,5)P_2$ (Suh and Hille, 2002; Zhang et al., 2003). Thus, whereas $PI(4,5)P_2$ is depleted, M-current is inhibited and neurons are more excitable. Our analysis demonstrates that it takes ~ 40 s for SCG neurons to resynthesize enough $PI(4,5)P_2$ to reactivate most $KCNQ2/3$ channels, thereby restoring the resting membrane potential, ending the elevated excitability, and ending action potential firing. If instead, $PI(4,5)P_2$ synthesis rates were as measured for nonexcitable tsA201 cells, elevated electrical activity of SCG neurons would last threefold to fourfold longer.

We have seen that phosphoinositide metabolism differs between SCG neurons and tsA201 cells. Are all neurons the same? Given the special needs of the nervous system, it is tempting to imagine that many neurons have faster phosphoinositide dynamics than nonexcitable cells. In sensory neurons and photoreceptors, for example, TRP and other channels require phosphoinositides (Rohacs, 2014; Hille et al., 2015), and therefore the speed of phosphoinositide restoration in these neurons controls the dynamic range for sensory input (Yadav et al., 2015). In the brain, modulatory areas control other brain areas by releasing neurotransmitters and hormones, such as acetylcholine, norepinephrine, or serotonin, acting on G-protein-coupled receptors

Figure 8. Ca²⁺ signals after M₁R activation in SCG neurons. **A**, Cytoplasmic Ca²⁺ in SCG neurons loaded with fura-2-AM and stimulated with 10 μM Oxo-M. Fura-2 signals, measured in the presence of 100 nM TTX. **B**, Same as in **A**, but with 100 μM CdCl₂ as indicated instead of TTX. **C**, Western blot of membrane preparations of tsA201 cells and SCG neurons with anti-IP₃R1 (top left) and anti-IP₃R2 (bottom left) antibodies. Protein amounts of the samples were normalized for equal amounts. Right, Percentage of IP₃R in SCG neurons compared with tsA201 cells. Number in brackets indicates number of experiments. **D**, Steady-state IP₃ profile in a two-dimensional model of the cytoplasm of a neuron. IP₃-production is assumed to occur at the origin. Concentrations at different distances depend on the diffusion coefficient of IP₃ as well as the modeled activity of an IP₃-5-phosphatase. **E**, Calculated cytoplasmic IP₃ concentrations in one plane (*Figure legend continues.*)

Figure 9. Gradients of IP₃ during local activation of M₁R in a simulated SCG neuron. **A**, The image shows one plane of a three-dimensional spatial model using the geometry of a SCG neuron. Outlined are the plasma membrane as well as the nuclear membrane. The red field is the area in which the activating ligand 10 μM Oxo-M is present throughout the simulation. **B**, Map of cytoplasmic IP₃ concentrations for several time points during the simulation. The dark area inside the cytoplasm is the cell nucleus. **C**, Time course of IP₃ concentrations for two regions-of-interest (ROI). Inset, Localization of ROIs. **D**, Simulated relative levels of Ca²⁺-bound and Ca²⁺-unbound NCS-1 isoforms in response to a simulated application of 10 μM Oxo-M. Simulations were performed for presence (black) or absence (red) of IRBIT. **E**, Simulated increase in Ca²⁺-bound NCS-1 upon muscarinic stimulation in the presence or absence of IRBIT.

that activate PLC and hydrolyze PI(4,5)P₂. PI(4,5)P₂-dependent KCNQ2/3, Ca_v2.2, and Ca_v2.1 channels are widely expressed in the brain, emphasizing the importance for rapid adjustment of phosphoinositide levels for coordinated ion channel activity in the brain.

PI(4,5)P₂ depletion in neuronal and cardiac tissues

Studies on isolated neurons provide strong evidence for net PI(4,5)P₂ depletion upon activation of muscarinic receptors,

leading to closure of PI(4,5)P₂-dependent ion channels such as KCNQ2/3 or Ca_v channels in these cells (Suh and Hille, 2002; Gamper et al., 2004; Brown et al., 2007; Hughes et al., 2007; Zaika et al., 2007; Hille et al., 2015). However, it is not known to what extent natural stimuli cause net PI(4,5)P₂ depletion in intact neuronal tissues. Our simulations show that even a density of <20 M₁R/μm² is sufficient to cause net PI(4,5)P₂ depletion at low agonist concentrations and to modulate KCNQ2/3 channel activity. Thus our simulations support the concept that even short exposure to low concentrations of agonists can cause net PI(4,5)P₂ depletion in intact neuronal tissues.

In contrast to studies on neurons, studies on cardiac cells have failed to reveal evidence for net PI(4,5)P₂ depletion, although the cells express PI(4,5)P₂-dependent ion channels and PLC-activating G-protein-coupled receptors (Gertjederdes et al., 1979; Cho et al., 2002; Nasuhoglu et al., 2002; Hilgemann, 2007). It has been shown that cardiac cells respond to activation of certain Gα_q-coupled receptors with a slight increase in PI(4,5)P₂ levels, which mechanistically has been explained by enhanced activities of lipid 4- and 5-kinases upon activation of protein kinase C downstream of PLC activation (Xu et al., 2014). These temporally

(Figure legend continued.) of a three-dimensional model of a SCG neuron upon uniform activation of M₁Rs for the indicated time on the cell surface with a saturating concentration of Oxo-M. **F**, Calculated IP₃ concentrations at two selected regions-of-interest (ROI) in the 3-D diffusion model in **E**. **G**, Western blot of lysate of SCG neurons with anti-IRBIT antibody. **H**, Scheme of the components of the model involved in Ca²⁺-release from intracellular stores. Gray, Components included in tsA201 cell model; Red, additional components added in SCG model. **I**, Overlay of modeled fura-2 response (red) on the time course of normalized fura-2 signal evoked by stimulation of SCG neurons with Oxo-M (black) in the presence of 100 nM TTX. **J**, Same as in **I**, but for simulation of fura-2 signal measured in the presence of 100 μM CdCl₂ instead of TTX.

increased lipid kinase activities ensure the uninterrupted activity of PI(4,5)P₂-dependent ion channels. This stimulated PI(4,5)P₂ synthesis becomes understandable if one takes into account that the two main repolarizing potassium currents in the human heart, I_{Kr} and I_{Ks}, are conducted by the PI(4,5)P₂-dependent Herg and KCNQ1/KCNE1 channel complexes (Charpentier et al., 2010; Vandenberg et al., 2012). Any reduction in the activity of these ion channels could cause cardiac arrhythmias or even sudden death, which makes it necessary to remain sufficiently high PI(4,5)P₂ levels in cardiac cells. This situation is different for neuronal cells, which can use net PI(4,5)P₂ depletion as a mechanism to regulate ion channels and hereby neuronal activity. Perhaps in heart, the synthesis of phosphoinositides is significantly faster than in neurons or the maximum activity of PLC is significantly less.

Uncoupling of Ca²⁺ release and PI(4,5)P₂ hydrolysis

Most cell types respond to elevated levels of IP₃ by increases in cytoplasmic Ca²⁺ concentrations in the micromolar range via release of Ca²⁺ from intracellular stores (Berridge, 2009). It has been shown that activation of purinergic and bradykinin receptors in SCG neurons causes significant release of Ca²⁺ from intracellular stores, but does not cause net PI(4,5)P₂ depletion (Zaika et al., 2007, 2011; Zhang et al., 2013). Activation of M₁ receptors on the other hand causes net PI(4,5)P₂ depletion, but circumvents Ca²⁺ release from intracellular stores despite substantial IP₃ generation as shown in this and previous studies (Zaika et al., 2007, 2011). Although all of these receptors are coupled to the small G-protein Gα_q and activate PLC upon binding of their ligand, the subsequently activated signaling pathways are different. It is an interesting hypothesis that a protein like IRBIT could be a differentiating factor for the type of signals evoked by activation of these different receptors. It has been shown that bradykinin receptors physically interact with IP₃ receptors while M₁ receptors lack this interaction (Delmas et al., 2002; Zhang et al., 2013). One could speculate that the physical interaction between bradykinin and IP₃ receptors prevents the interaction of IRBIT with IP₃Rs, which would create a pool of IRBIT-free IP₃ receptors upon activation of bradykinin receptors. This receptor pool would then be available for IP₃ to evoke a larger cytoplasmic Ca²⁺ increase compared with IRBIT-restricted muscarinic stimulation as reported by several studies (Gamper and Shapiro, 2003; Zaika et al., 2011). A similar mechanism might be present for purinergic receptors for which a similarly large increase in cytoplasmic Ca²⁺ has been reported as for stimulation with bradykinin (Zaika et al., 2011). The differentiated response to PLC-activating stimuli could be a mechanism for SCG neurons to react combinatorially to incoming signals. In comparison, tsA201 cells lack this property as activation of PLC by any Gα_q-coupled receptor evokes release of Ca²⁺ from intracellular stores (Dickson et al., 2013). Apparently, neurons have a more nuanced response.

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