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N-PALMITOYL GLYCINE, A NOVEL ENDOGENOUS LIPID THAT ACTS AS A MODULATOR OF CALCIUM INFLUX AND NITRIC OXIDE PRODUCTION IN SENSORY NEURONS

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Running title: *N*-palmitoyl glycine an endogenous modulator of sensory neurons

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N-palmitoyl glycine (PalGly); *N*-stearoyl glycine (StrGly); *N*-oleoyl glycine (OlGly); *N*-linoleoyl glycine (LinGly); *N*-arachidonoyl glycine (NAGly); *N*-docosahexaenoyl glycine (DocGly); *N*-arachidonoyl dopamine (NADA); *N*-palmitoyl ethanolamine (PEA); 2-arachidonoyl glycerol (2-AG).

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Abstract

N-arachidonoyl glycine is an endogenous arachidonoyl amide that activates the orphan G-protein coupled receptor (GPCR) GPR18 in a pertussis toxin (PTX) -sensitive manner, and produces anti-nociceptive and anti-inflammatory effects. It is produced by direct conjugation of arachidonic acid to glycine and by oxidative metabolism of the endocannabinoid anandamide. Based on the presence of enzymes that conjugate fatty acids with glycine and the high abundance of palmitic acid in the brain, we hypothesized the endogenous formation of the saturated *N*-acyl amide *N*-palmitoyl glycine (PalGly). PalGly was partially purified from rat lipid extracts and identified using nano- high performance liquid chromatography/hybrid quadrupole time-of-flight (HPLC/QqTOF) mass spectrometry. Here we show that PalGly is produced following cellular stimulation and occurs in high levels in rat skin and spinal cord. PalGly was upregulated in fatty acid amide hydrlase (FAAH) Knockout (KO) mice suggesting a pathway for enzymatic regulation. PalGly potently inhibited heat-evoked firing of nociceptive neurons in rat dorsal horn. In addition, PalGly induced transient calcium influx in native adult DRG cells and a DRG-like cell line (F-11). The effect of PalGly on the latter was characterized by strict structural requirements, PTX-sensitivity and dependence on the presence of extracellular calcium. PalGly-induced calcium influx was blocked by the non-selective calcium channel blockers ruthenium red, SKF96365 and La^{3+}. Furthermore, PalGly contributed to the production of nitric oxide (NO) through calcium-sensitive nitric oxide synthase (NOS) enzymes present in F-11 cells, and was inhibited by the nitric oxide synthase inhibitor 7-NI.

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Introduction

Collectively, the acyl amides constitute a family of ubiquitous endogenous lipids present in varying levels throughout the body (for review, Di Marzo et al., 2007). These lipids are now recognized as potent modulators of pain and inflammation (Bradshaw and Walker, 2005; Hohmann et al., 2005). The subfamily of arachidonoyl amides is comprised of amide-conjugates of ethanolamine, glycine, dopamine, alanine, GABA, serine and taurine with arachidonic acid (Devane et al., 1992; Huang et al., 2001; Huang et al., 2002; Milman et al., 2006; Saghateailn et al., 2006). The earliest and best characterized among these is *N*-arachidonoyl ethanolamine (AEA; anandamide), an endogenous mammalian homolog to the analgesic phytocannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC; Devane et al., 1992). AEA activates the G-protein coupled receptors (GPCR) cannabinoid receptor 1 and 2 (CB_1, CB_2), GPR55, and the transient receptor potential vanilloid type-1 receptor (TRPV1; Devane et al., 1992; Ross et al., 2003; Ryberg et al., 2007). An oxygenated analog of AEA, *N*-arachidonoyl glycine (NAGly), was synthesized as part of a structure-activity relationship (SAR) study of AEA (Sheskin et al., 1997; Burstein et al., 1997) and shown to suppress pain induced by thermal and chemical stimuli in rodents (Burstein et al., 1997; Huang et al., 2001). NAGly was subsequently identified as an endogenous lipid in the mammalian nervous system (Huang et al., 2001) and found to be a high affinity ligand for the orphan GPCR GPR18 (Kohno et al., 2006).

While the subfamily of arachidonoyl amides has received considerable attention, much less is known about the presence and activity of their saturated counterparts

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(Bradshaw and Walker, 2005). The most studied member of the saturated acyl amides is *N*-palmitoyl ethanolamine (PEA), an endogenous lipid with anti-inflammatory properties recently identified as an activator of the peroxisome proliferator-activated receptor α and the orphan receptor GPR55. (Lo Verm et al., 2005; Ryberg et al., 2007). These findings highlight the growing number of bioactive *N*-acyl amides, a novel family of putative endogenous signaling lipids with multiple effects on pain, inflammation and other biological systems.

Several pathways for the formation of *N*-acyl glycines were described including: 1) production via the enzyme glycine *N*-acylase found in the mitochondria of bovine liver, and shown active with a variety of acyl-CoA donors including aliphatic short and medium chains (2-10 carbons) and aromatic acyl thioesters (Schachter and Taggart, 1954). 2) The enzyme bile acid CoA: amino acid *N*-acyl transferase (BACAT) found in microsomes and peroxisomes and shown to conjugate bile acids mainly to glycine and taurine (O'Byrne et al., 2003). BACAT was also shown to conjugate saturated 16-20 carbon fatty acids to glycine *in-vitro* but with only 20% of the activity reported for bile acids. 3) Cytochrome c, acting with hydrogen peroxide as a cofactor, produced *N*-oleoyl glycine (OlGly) and NAGly *in-vitro* when incubated with glycine and the respective fatty acid CoA (Mueller and Driscoll, 2007; McCue et al., 2008). 4) Oxygenation of *N*-acyl ethanolamines via the sequential enzymatic reaction of an alcohol dehydrogenase and aldehyde dehydrogenase (Burstein et al., 2000; Bradshaw et al., 2006). Based on the presence of the potent anti-inflammatory lipid signaling molecules PEA and NAGly, and

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the presence of several glycine-conjugating enzymes, we predicated the endogenous production of *N*-palmitoyl glycine (PalGly) as a putative signaling molecule.

Using partially purified lipid extracts from rat brain, we identified PalGly by nano-HPLC QqTOF mass spectrometry. By means of HPLC/MS/MS we determined that the levels of PalGly were 3 fold greater in brain and 100 fold greater in skin compared to those reported for the endocannabinoid AEA (Felder et al., 1996; Bradshaw et al., 2006). To determine if PalGly was metabolically regulated by fatty acid amide hydrolase (FAAH) we measured its levels in the brain of FAAH knockout (KO) mice and found them to be significantly higher in KO when compared to wild type (WT) animals. We hypothesized that PalGly may act as a signaling molecule in dorsal root ganglion (DRG) cells based upon the high levels in skin and spinal cord and its structural similarities to endocannabinoids and PEA, which produce anti-nociceptive and anti-inflammatory effects. Electrophysiological studies in intact animals showed that a sub microgram dose of PalGly administered subdermally in the paw of a rat potently inhibited heat-evoked firing of nociceptive dorsal horn wide dynamic range (WDR) neurons. Using a DRG-like cell line, F11, we revealed that PalGly induces transient influx of calcium followed by nitric oxide production via calcium-sensitive nitric oxide synthase enzymes. PalGly likewise produced calcium influx in native adult DRG cells.

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Materials and Methods

Chemicals- High performance liquid chromatography (HPLC) grade water, methanol, and acetonitrile used for mass spectrometric studies were purchased from VWR International (Plainview, NY). Mass spectrometry/HPLC grade acetic acid, formic acid, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). NAGly, [$^2H_8$]-NAGly and *N*-linoleoyl glycine (LinGly) were purchased from Cayman Chemical (Ann Arbor, MI). Fatty acids were purchased from Nu-Chek Prep (Elysian, MN). URB597, SK&F96365, ω-conotoxin MVIIC and ω-conotoxin GVIA were purchased from BIOMOL International (Plymouth Meeting, PA). Glycine, thapsigargin, ruthenium red, 4-phenyl-3-butenoic acid (PBA), and PTX were purchased from Sigma Aldrich. SR144528 and SR141716A were a gift from Sanofi Richerche (Montpellier, France). Fura-2 AM was purchased from Molecular Probes (Eugene, OR). 5'-iodoresiniferatoxin (I-RTX) and MK801 maleate were purchased from Tocris Cookson (Ellisville, MO) and LC Laboratories (Woburn, MA). The synthesis of *N*-acyl amino acid standards is detailed in the Supplemental data.

Antibodies: Antibodies of mouse nNOS were purchased from BD Transduction Laboratories (San Diego, CA). Antibodies of rabbit eNOS and iNOS were purchased from Abcam (Cambridge, MA). Antibodies, Rabbit anti-p38 MAPK antibody # 9212 and

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mouse anti-phospho-p38 MAPK # 9216 were both purchased from Cell Signaling Technologies (Danvers, MA).

Animals- Adult male and female Sprague-Dawley (Harlan, Indianapolis, IN) rats were used. All protocols were approved by the Indiana University Institutional Animal Care and Use Committee. Six male (300-450 g) and 6 female (250-300 g) Sprague-Dawley rats were used to conduct these studies. The FAAH WT and KO mice used in this study were littermates from the thirteenth generation offspring from intercrosses of 129SvJ-C57BL/6 FAAH KO and WT mice (Cravatt et al., 2001). Briefly, the generation of FAAH KO mice was achieved by isolating the FAAH gene from a 129SvJ genomic library first, and a 2.5-kb region encompassing the first exon was mapped and sequenced. The first FAAH exon (encoding amino acids 1-65) and ≈2 kb of an up-stream sequence were then replaced by inserting a PGK-Neo cassette between *Eco*RI and *Eco*RV sites located 2.3 kb apart. Homologous recombinant 129SvJ embryonic stem cell clones were identified by Southern analysis, and two clones were used to generate chimeric mice on a C57BL/6 background. Chimeras from both clones gave germline transmission of the mutated gene.

Partial Purification of PalGly from Mammalian Tissues: Six fresh male rat brains were homogenized in the methanol fraction of 20 volumes to weight of 2:1 chloroform: methanol and centrifuged for 15 min at 31,000 × g at 24 °C. Chloroform and NaCl (0.2 volume, 0.73%) were added to the supernatant and the solution was centrifuged at

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1,000 × g for 15 min. The upper phase was discarded and the interphase was washed twice. The lower phase was applied to diethylaminopropyl silica-based solid-phase extraction columns (DEA; Varian, Harbor City, CA) without prior column conditioning. The columns were sequentially washed with chloroform, methanol and 0.1% ammonium acetate in methanol and eluted with 0.5% ammonium acetate in methanol. HPLC-grade water (2.3 volumes) was added to the eluate, and the solution was loaded onto preconditioned C-18 Bond-Elut 500 mg solid phase extraction columns (SPE; Varian). The cartridges were washed with water and 60% methanol and eluted with 80% methanol. Samples were evaporated in a SpeedVac (Savant Instruments, Halbrook, NY), reconstituted in 1 ml chloroform, and applied to a 100 mg silica column (Bond-Elut SI; Varian, Harbor City, CA) with no preconditioning. The column was washed with chloroform and 10% (v/v) methanol in chloroform, and eluted with 25% (v/v) methanol in chloroform. The eluate was evaporated in a SpeedVac, reconstituted in acetonitrile and diluted to 2:1 HPLC-grade water:acetonitrile prior to analysis.

Nano-HPLC Quadrupole-TOF Analysis of PalGly: Exact mass measurements and structural characterization of PalGly from rat brain extract were performed with a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer (QSTAR Pulsar, Applied Biosystems-MDS Sciex; Foster City, CA). Extracts were chromatographed on a nano-HPLC C18 column (100 mm x 75 µm) coupled to a gradient nano-HPLC pump (Micro-Tech Scientific Inc.; Vista CA) operating at a flow-rate of 300 nl/min. Chromatographic gradients began with 30% mobile phase B (98% acetonitrile, 0.1% formic acid): 70% mobile phase A (2% acetonitrile, 0.1% formic acid) held for 10 minutes during sample

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loading, followed by a linear gradient from 30% B to 100% B over 30 minutes then held at 100% B for 30 min. The ESI voltage was +3000V generating positively charged $[M+H]^+$ molecular and fragment ions.

Quantification of tissue levels of PalGly- PalGly was extracted and quantified using methods similar to those developed and reported previously by this laboratory for extraction of NAGly (Huang et al., 2001; Bradshaw et al., 2006). In brief, each tissue sample was subjected to a methanol extraction and partially purified using C18 solid phase extraction columns (Varian, Harbor City, CA) with a final elution of 100% methanol. Rapid separation of analytes was obtained using 10 µl injections of analyte (Agilent 1100 series autosampler, Wilmington, DE) onto a Zorbax eclipse XDB 2.1 x 50 mm reversed phase column. Gradient elution (200 µl/min) was formed under pressure on a pair of Shimadzu (Columbia, Maryland) 10AdVP pumps. Mass spectrometric analysis was performed with an Applied Biosystems/MDS Sciex (Foster City, CA) API 3000 triple quadrupole mass spectrometer equipped with a heat-assisted electrospray ionization source. The MRM analyses used for quantification of tissue levels were conducted in negative ion mode with parent and fragment ions as follows: PalGly 312.2→74.2; [$^2H_8$]-NAGly 368.2→76.2. [$^2H_8$]-NAGly was used as an internal standard to calculate recovery. The percentage of analyte recovered based upon the internal standard was 73.4 ± 0.16 (mean % ± standard error).

Analysis of brain extracts from FAAH KO and WT mice- FAAH KO and WT mice were sacrificed when they were 6 weeks old. The brains were dissected and stored at -

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80 ºC until used. Lipid extraction, partial purification, and quantitation were performed as described above.

Effects of inhibition of FAAH and PAM on brain levels of *N*-palmitoyl glycine- Rats were injected with the FAAH inhibitor URB 597 (3 mg/kg in 1% dimethylsulfoxide (DMSO) in saline i.p.), the peptidylglycine α-amidating monooxygenase (PAM) inhibitor PBA (250 mg/kg in saline adjusted to pH 7.4 with sodium hydroxide i.p.), or the appropriate vehicle. Two hours later, the rats were decapitated, and brains were dissected and flash-frozen in liquid nitrogen, extracted, purified and analyzed as described above.

Electrophysiological recordings from spinal dorsal horn- Procedures for determination of the recording site in the dorsal horn, general electrophysiological methods, determination of the receptive field, assessment of heat-evoked firing rates, and data analysis were performed as reported by Huang and Walker (2006). In brief, neurons were classified as WDR nociceptive neurons if they exhibited increasing firing rates to application of increasing intensity of stimulation (brush, pressure, pinch, and heat) to the hindpaw skin. Following identification of a WDR neuron, heat-evoked responses were elicited using a computerized apparatus that applied increasing intensities of radiant heat to the receptive field on the plantar surface of the paw over a 30 sec interval. After the establishment of stable baseline responses (3 trials at 10 min intervals), PalGly (0.43 µg, n=4) or vehicle (1:1:18, ethanol: emulphor: saline, n=5) was injected into the receptive field of the neuron (hind paw) in a volume of 50 µl and heat-evoked responses were measured again. Post-drug heat-evoked responses were

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assessed at 10 min intervals for 30 min. Data from the three trials at 10, 20, and 30 min, were analyzed as postinjection responses and the three trials conducted prior to injection as baseline responses.

Cell culture- The F-11 cell line (rat dorsal root ganglion neuron X mouse neuroblastoma) was provided by Dr. Mark C. Fishman, Massachusetts General Hospital (Boston, MA). F-11 cells were cultured in Ham's F-12 (1X) with L-glutamine (Mediatech, Inc. Herndon, VA) containing 1% penicillin-streptomycin (Gibco, Carlsbad, CA), 2% HAT supplement (50X, Gibco, Carlsbad, CA) and 17% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA). Cells were sub-cultured every other day using non-enzymatic cell dissociation solution (Sigma Aldrich). Cells were grown under 5% CO_2 at 37˚C. Native adult DRG cells were harvested from 175-200 g male rats as described in detail by Burkey and colleagues (2004). Cells were grown in Adult Growth Medium containing: Ham's F12 medium with NGF (30 ng/ml), normocin O^{TM} (100 µg/ml; Invitrogen), 2 mM L-glutamine (Invitrogen), 1% penicillin-streptomycin (50 U/mL, 50 µg/mL; Gibco), 10 % horse serum, and the mitotic inhibitors 5-flouro-2'-deoxyuridine (50 µM; Sigma Aldrich), and uridine (150 µM; Sigma Aldrich). The HEK 293 cells stably transfected with human TRPV1 were a kind gift from Merck Research Laboratories (Whitehouse Station, NJ). The HEK 293 TRPV1 cell line was cultured in minimal essential medium, Eagle, modified with non essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 1.5 g/l sodium bicarbonate (ATCC, Manassas, VA), containing 1% penicillin-streptomycin, and 10% FBS. The mouse TRPV3-YFP, rat TRPV4 and mock-transfected pcDNA3 HEK293 cell lines were a kind gift from Dr.

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Michael J Caterina of Johns Hopkins University. These cells were cultured in DMEM 1X with L-glutamine (Mediatech, Inc. Herndon, VA), containing 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and 10% FBS. All HEK293 cells were passaged three times a week using 0.25% trypsin- EDTA 1X (Invitrogen) and grown under 5% CO_2 at 37°C.

Effects of neuron depolarization on PalGly levels in F-11 cells- F-11 cells were plated in 10 cm plates (650,000 cells/ plate) 24 h prior to stimulation. Cells were washed 3 X with Hank's balanced salt solution (HBSS; Invitrogen) and incubated for 10 min at room temperature with 5 ml Ham's F-12 (without FBS or antibiotics) containing 65 mM KCl or vehicle. The stimulated cells were collected, methanol extracts were partially purified on 500 mg C8 solid phase extraction columns, and PalGly levels were quantified using MRM on a triple quadrupole mass spectrometer as described above. The experiment was repeated three times. In one experiment, the media was changed 10 min after treating cells with KCl as described above, and the cells were examined the following day, which revealed that the cells recovered from treatment with KCl.

Measurement of Ca^{2+} influx in F-11 cells and transfected HEK293 cells- The calcium-sensitive fluorophore Fura-2 was used to measure intracellular calcium in F-11 cells using both single cell imaging and high-throughput 96-well plate assays. For single cell imaging, F-11 cells were plated on poly D-lysine- or collagen-coated glass cover slips 24 h before imaging. Immediately prior to imaging, cells were loaded with 3 μM Fura-2 AM in Ham's F12 (1X) with L-glutamine for 60 min at room temperature. Cells were then washed three times with Ham's F12 and mounted onto an inverted microscope (Nikon TS-100, Tokyo, Japan). Test compounds were added to the cells

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following 60 sec baseline recording. During recording, cells were alternately excited at 340 and 380 nm by switching optical filters mounted in a computer controlled Sutter wheel (Sutter Instrument Company, Novato, CA) using a xenon arc illuminator. Excitation light was reflected from a dichroic mirror through a 20 X plan fluor objective. Light emitted at an emission wavelength of 510 nm was projected onto a Cohu 4920 cooled CCD camera (Cohu, San Diego, CA) and intracellular Ca^{2+} concentrations were analyzed with the InCyt Im2 image acquisition and analysis software (Intracellular Imaging Inc., Cincinnati, OH). The system was calibrated using a Fura-2 calibration kit (Molecular Probes, Eugene, OR). Data were quantified by integrating the area under calcium concentration (nM) x time (s) curves. Stably transfected HEK293 cells expressing TRPV3, TRPV4 and hTRPV1 were imaged under the same conditions except that all procedures were carried out in HEPES-Tyrode buffer. The reagents 2-aminoethoxydiphenyl borate (2-APB; Calbiochem, EMD Biosciences, San Diego, CA), 4α-phorbol 12, 13-didecanoate (4αPDD; PKC Pharmaceuticals, Woburn, MA) and capsaicin (Sigma-Aldrich) were used as positive controls for TRPV3, TRPV4 and hTRPV1 expressing cells, respectively.

For high-throughput assays, F-11 cells were plated on CellBIND 96-well flat clear bottom black polystyrene microplates (Corning, Corning, NY) 24 h prior to imaging. Cells were then loaded for 60 min at room temperature with 3 μM Fura-2 AM in Ham's F12 (1X) with L-glutamine containing 0.05 % w/v Pluronic F-127 (Molecular Probes, Eugene, OR) and washed twice with Ham's F12 (1 X) with L-glutamine. The 340/380 fluorescence ratio was recorded for each well using a Flexstation II (Molecular Devices,

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Union City, CA). Data were quantified by integrating the area under 380/340 fluorescence ratio x time (s) curves.

The following conditions were used for drug administration: ruthenium red, glycine, MK801, SK&F96365 and ω-conotoxins were dissolved in H_2O; all other compounds were dissolved in DMSO. The final concentration of DMSO was less than 2% in all experiments (up to 2% DMSO did not affect calcium mobilization). In order to examine the effects of intracellular calcium store depletion, cells were pretreated and imaged with 500 nM of the Ca^{2+} -ATPase inhibitor thapsigargin. As expected, thapsigargin produced intracellular calcium release followed by a gradual return to baseline. After baseline levels were reestablished, cells were treated with PalGly. To test the effects of glutamate, cannabinoid receptor antagonists and ω-conotoxins, cells were treated with MK-801 (24 μM), SR144528 (150 nM), SR141716A (150 nM) or ω-conotoxins (2 μM) for 20 min prior to PalGly administration. The effects of SK&F 96365 (10 μM or 25 μM) and ruthenium red (10 μM) were examined by applying these compounds onto cells immediately before imaging. For assays testing dependency on extracellular-calcium, Fura-2-loaded cells were imaged in a HEPES-Tyrode solution with or without calcium ions (pH 7.4). To test the effects of pertussis toxin cells were incubated overnight with PTX (250 or 500 ng/ml) prior to the administration of PalGly.

Single cell measurement of Ca^{2+} influx in native DRG cells

Native adult DRG cells were plated on poly D-lysine (Sigma Aldrich), and laminin (Sigma Aldrich) -coated glass cover slips in Adult Growth Medium. Fura-2 was used to

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measure changes in intracellular calcium. Prior to imaging, cells were loaded with 3 µM Fura-2 AM in Ham's F12 (1X) with L-glutamine (no other supplements were added) for 60 min at room temperature. Cells were then washed three times with Ham's F12 with L-glutamine, and mounted onto the inverted microscope as previously described. In order to identify the neuronal cells for imaging, we identified and circled the cells under bright field conditions. We chose the cells with typical DRG rounded morphology, as shown by Burkey and colleagues (2004). Test compounds (16 µM PalGly or Vehicle) were manually added to the cells following 60 sec baseline recording. At 400 s, after a brief wash, capsaicin (50 nM) was applied to the DRG cells, and was used as a positive control in vehicle or PalGly treated cells.

P38 MAPK in cell western assay- F-11 cells were plated at 80 % confluence and treated with PalGly (10 nM-20 µM). Upon completion of the PalGly treatment, an in-cell Western assay was conducted by the following procedure: the media was removed and the cells immediately fixed with a solution of 3.7% formaldehyde in PBS for 20 min at 20°C. The cells were then washed with a solution of 0.1% Triton X-100 in PBS with moderate shaking for 5 min at 20°C, the wash solution was removed and washed 4 more times. Following the final wash, the cells were blocked with Odyssey blocking buffer (Cell Signaling Technologies) with moderate shaking for 90 min at 20°C. Primary antibodies anti-p38 and anti-phospho-38 were diluted in Odyssey blocking buffer 1:200. For all PalGly concentrations tested, the Odyssey blocking buffer was removed from the relevant wells in the plate and replaced with buffer containing primary antibodies, while again for each concentration tested corresponding control wells received no primary

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antibody. The plate was then incubated overnight with moderate shaking at 4°C. Following incubation with the primary, antibody the wells were then washed 5 times with PBS/0.1% Tween-20 for 5 min at 20°C. Fluorescently labeled antibodies (Odyssey 827-08367 goat anti-rabbit 680 nm antibody; 827-08364 goat anti-mouse 800 nm antibody) were diluted in Odyssey blocking buffer 1:800 containing 0.2% Tween-20 secondary antibody solution was added to all wells and incubated in the dark for 1 hour at 20°C. The wells were then washed 5 times with PBS/ 0.1% Tween-20 for 5 min at 20°C. The plate was then scanned using the Li-Cor Odyssesy Infrared Imaging System, using both 700 and 800 nm channels, a resolution of 169 μm, an intensity of 5, and focal offset of 4 mm. Changes in p38 MAPK were then determined by calculating the mean background fluorescence from all non- primary antibody containing control wells, for both 700 and 800 nm channels. Background fluorescence was then subtracted from the fluorescence measured in primary antibody containing PalGly wells, for both p38 MAPK and phospho-p38 MAPK. The changes in p38 MAPK phosphorylation were then obtained by constructing the ratio of p38:phospho-p38 MAPK fluorescence for all concentrations of PalGly tested.

Western blotting- Western blotting was carried out as described (Rimmerman et al., 2007) and optimized for the detection of phosphorylated proteins according to Prieto et al. (2007) by supplementing the lysis buffer with phosphatase inhibitor cocktails (type I and II, Sigma-Aldrich, St. Louis, MO) in concentrations suggested by the manufacturer. The blocking buffer consisted of TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20) containing 3% skim milk (Carnation, Nestle, Vevey, Switzerland) to

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detect non-phosphorylated proteins, or the blocking buffer consisted of TBST containing 1% BSA (Fisher) and 10 mM NaF, 2 mM Na_3VO_4, and 5 mM $Na_4P_2O_7$ to detect phosphorylated proteins. The membranes were incubated overnight at 4°C in the presence of primary antibody. The following antibodies were used for the Western blot analyses: anti-nNOS, (155 kDa, 1:2500 dilution), anti-phospho (Ser 847) nNOS (160 kDa, 1: 1000 dilution), anti-eNOS (133 kDa, 1: 1000 dilution), anti-iNOS (131 kDa, 1:500-1000 dilution).

Nitric oxide imaging- Nitric oxide production in F-11 cells was measured using the nitric oxide sensing fluorophore DAF-2 (Cayman Chemicals, Ann Arbor, MI). Briefly, F-11 cells were plated on 96-well plates (CellBIND) and grown overnight prior to experiments. Cells were incubated for one hour in serum free Ham's F-12 containing 5 µM DAF-2 DA and 0.002% Pluronic F-127 (Invitrogen). At the end of the incubation period, cells were washed once using Ham's F-12, which was also used as the recording medium. Fluorescence (excitation=485 nm, emission=530 nm) was recorded from each well for 240 sec using a Flexstation II (Molecular Devices, Sunnyvale, CA). Automated additions of drug occurred following 20 sec baseline recordings. All drugs were initially dissolved in DMSO and diluted in recording medium to a final DMSO concentration of 1%. To test the effects of the calcium entry blocker SK&F96365 (Biomol, Plymouth Meeting, PA), cells were loaded with DAF-2 as mentioned above and washed twice, followed by addition of the recording medium. SK&F96365 (28.6 µM) or the vehicle (water, 1.4%) was added to the wells followed immediately by data

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acquisition, during which drug additions lowered these concentrations to their final values of 20 µM SK&F96365 with a vehicle concentration of 1%. For experiments with the NOS inhibitor 7-nitroindazole (7-NI, Sigma-Aldrich, St. Louis, MO), serum-free F-12 preparations were made containing either 200 µM 7-NI or the vehicle DMSO (0.2%). Cells were first incubated for 3.25 h at 37º C in Ham's F-12 containing either 7-NI or DMSO, after which loading, washing and recording were conducted as described above but using the Ham's F-12 containing 7-NI or DMSO. Data from cell-based assays were normalized to the effect of the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) on DAF-2 fluorescence in cell-free assays. Fluorescence was measured in cell-free wells containing 5 µM DAF-2 (Cayman Chemicals) to which 2 mM SNAP or vehicle (DMSO, 1%) was added.

To ensure that pretreatment with 7-NI did not cause cell death, cell viability was measured using Trypan Blue (Sigma-Aldrich, St. Louis, MO). Cells were treated with 7-NI or DMSO exactly as for the fluorescence assays. However, after loading and washing, the cells were treated for 5 min with 0.2% Trypan Blue in F-12. The percentage of viable cells was calculated by examining random fields of view in the 7-NI and DMSO treatment conditions.

Data analysis- Results were analyzed using one-way ANOVA with Fisher's LSD, Bonferonni Dunnett post-hocs or student's t-test depending on the type of comparison. Data are presented as means ± standard error of the mean where $p < 0.05$ was considered statistically significant. Calcium data from single cell recording were analyzed using cubic spline interpolation (Matlab, 2004, Natick, MA). For each cell, data

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were normalized by estimating the baseline level of calcium and subtracting it from the post-treatment levels. Data from the DAF-2 fluorescence assays were analyzed using SoftMax Pro v4.8 (Molecular Devices, Sunnyvale, CA). Data points were expressed as a percentage of baseline fluorescence and area under the curve (AUC) was computed for each well. Drug effects were assessed as the AUC of drug treatment minus the AUC of vehicle.

Results

Isolation, identification and tissue distribution of PalGly

Methanol extracts of rodent brain were analyzed by mass spectrometry in a search for the 16-carbon saturated fatty acid conjugated to a glycine molecule through an amide bond, PalGly (Fig. 1A). By comparing the extracted analyte to the synthetic PalGly standard using LC/MS/MS, we found that both had matching mass spectra and retention times (Fig. 1 B-D). Exact mass measurements and product ion scans with nano-HPLC/qQTOF permitted further characterization of the extracted lipid (Fig. 1F-G). The mass of the molecular ion of each compound in the extract deviated from that of the theoretical exact mass of PalGly by < 5 ppm and fragment ions differed from the theoretically predicted ions by no more than 16 ppm (Fig. 1H). Based on the matching HPLC retention times, mass spectra, and exact masses of the tissue-derived analyte and synthetic standard we can conclude that PalGly is a naturally occurring constituent of mammalian tissues.

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PalGly is produced following neuronal depolarization and is metabolized by FAAH.

In light of observations indicating that signaling lipids such as the endocannabinoids AEA and 2-AG are made on demand in response to cellular stimulation (Di Marzo et al., 1999), we hypothesized that PalGly could be produced under similar conditions. We incubated the DRG-like cell line (F-11) with depolarizing media containing 65 mM KCl for 10 min. Quantification of the levels of PalGly revealed a significant increase (25.3%) in the KCl-stimulated cells over the unstimulated cells (unstimulated cells 335.0 ± 43.3 fmol/1 x 10^6 cells versus stimulated cells 419 ± 9.34 fmol/1 x 10^6 cells; $p<0.05$.)

Fatty acid amide hydrolase (FAAH) and peptidyl-glycine α-amidating monooxygenase (PAM) are enzymes known to metabolize other acyl-amides such as AEA, oleamide, and the *N*-acyl taurines (Ritenour-Rodgers, et al., 2000; McKinneyet al., 2005). Therefore we hypothesized that either FAAH or PAM would act on PalGly. Although the PAM inhibitor 4-phenyl-3-butenoic acid failed to alter brain levels of PalGly, 3 mg/kg of the FAAH inhibitor URB597 nearly doubled the levels of PalGly in brain compared to vehicle (pmol/g: DMSO: 47.51±6.56; URB597: 90.41±5.84; p≤0.05). Consistent with this observation, the levels of PalGly were markedly increased in the brains of FAAH KO mice compared to WT controls (pmol/g: WT 26.2±2.77; KO 41.1±3.63; p≤0.05).

PalGly levels are highest in skin, lung and spinal cord

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The level of PalGly was quantified in methanol extracts of 12 tissues and organs by HPLC/MS/MS. PalGly was found in all tissues tested, although wide variations of its levels in the different tissues were observed (Fig. 1E). For example, the concentration of PalGly in skin was approximately 1,600 pmol/g versus 50 pmol/g in brain. The levels of PalGly were approximately 3 fold greater in brain and 100 fold greater in skin compared to those of anandamide (Felder et al., 1996; Bradshaw et al., 2006) and NAGly (Huang et al., 2001; Bradshaw et al., 2006). However, comparable to the tissue distribution of NAGly (Huang et al., 2001), PalGly levels were highest in spinal cord, skin and intestine suggesting similarities in their biosynthetic pathways.

PalGly modulates heat-evoked responses of dorsal horn wide dynamic range neurons

Several acyl amides including AEA, NAGly and PEA were reported to act as endogenous signaling lipids mediating antinociception (Bradshaw and Walker, 2005). Hence, the relatively high levels of PalGly in skin and spinal cord suggested a potential role of PalGly in the modulation of nociceptive pathways. To test this hypothesis, PalGly was administered intradermally to anesthetized rats, and single wide dynamic range (WDR) neurons were selected for recording based on their increasing rate of firing in response to mechanical stimuli of increasing strength and their responses to noxious heat. Vehicle administration had no effect on neuronal firing compared to pre-vehicle responses. By contrast, intradermal administration of PalGly (0.43 µg in 50 µl vehicle) in the receptive fields of these WDR neurons suppressed the responses to a heat stimulus

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at noxious temperatures (45º - 52ºC, $p<0.01$, Fig. 2) when compared to pre-PalGly responses.

PalGly induces calcium influx in DRG-like (F-11) cells

To investigate the effect of PalGly on primary sensory neurons, calcium influx was recorded in the DRG-like F-11 cells. As shown in Fig 3A., PalGly (10 µM) induced a transient influx of calcium in F-11 cells. The effect was immediate, and the initial increase was followed by a gradual return to baseline after 5 min of PalGly addition with an EC_{50}= 5.5 µM (Hill slope= 4.7; R^2= 0.99; Fig. 3B). Single-cell calcium imaging further revealed a markedly lower level but still significant influx of calcium following application of *N*-stearoyl glycine (StrGly) and stearic acid (SA; Fig. 4). However, other related molecules including OlGly, *N*-linoleoyl glycine (LinGly), NAGly, *N*-docosahexaenoyl glycine (DocGly), and free glycine were inactive at concentrations up to 50 µM (Fig. 4). Palmitic acid (PA) and *N*-palmitoyl L-alanine also failed to alter calcium influx at concentrations up to 25 and 50 µM, respectively, demonstrating strict structural requirements for both the lipid and polar moieties of PalGly (Fig. 4). To test the possibility that the effects of PalGly were mediated by a GPCR linked to a pertussis toxin (PTX)-sensitive Gα subunit, F-11 cells were incubated overnight with PTX (250 or 500 ng/ml). The effect of PalGly was significantly reduced following PTX treatment but not abolished (Fig. 5). Treatment with the specific P/Q or N-type voltage-gated calcium channel blockers ω-conotoxin MVIIC or ω-conotoxin GVIA, did not block calcium influx (2 µM; Fig. 5). To determine the source of calcium influx, experiments were performed in the presence or absence of extracellular calcium and in cells treated with thapsigargin

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(500 nM) or cyclopiazonic acid (37 µM) to deplete intracellular stores. Calcium influx by PalGly was dependent on the presence of extracellular calcium ($t(146) = 9.4$, 2-tailed, $p<0.001$) and was not reduced following treatment with thapsigargin (500 nM) or cyclopiazonic acid (37 µM).

F-11 cells express several lipid-activated receptors such as CB_1 and CB_2 (Ross et al., 2001) as well as several transient receptor potential vanilloid (TRPV) subtypes (Jahnel et al., 2003), each of which regulates calcium levels. The CB_1 antagonist SR141716A (150 nM) and the CB_2 antagonist SR144528 (150 nM) failed to inhibit PalGly-induced calcium influx. The NMDA channel blocker MK801 (25 µM), and the TRPV1 specific blocker I-RTX (35 nM) also failed to block PalGly-induced calcium influx (Fig. 6D). By contrast, the nonspecific calcium channel blockers ruthenium red (10 µM) and La^{3+} (1 mM) blocked the influx of calcium produced by PalGly (Fig. 6A, B, D). In addition, the receptor or store operated calcium channel inhibitor SK&F96365 significantly attenuated the effects of PalGly in a dose-dependent manner (Fig. 6C, D). PalGly had no effect on p38 MAPK-phosphorylation in F-11 cells and did not induce calcium influx in HEK293 cells stably-expressing mouse-TRPV3-YFP, rat-TRPV4 or human-TRPV1 (Supplemental data).

PalGly dose-dependently increases nitric oxide production in-vitro

Nitric oxide (NO) inhibited neuronal firing in several sensory systems (Chaban et al., 2001; Clasadonte et al., 2007), and both calcium dependent nitric oxide synthase (NOS) isoforms (neuronal and endothelial) were detected in F-11 cells (Fig. 7A;

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Rimmerman et al., 2007). Therefore, we hypothesized that the reduction in firing rate of the WDR cells in response to PalGly seen *in vivo* may be mediated through the activation of calcium-sensitive NOS enzymes and production of NO. NO production was monitored using the fluorescence dye DAF-2. We observed that PalGly but not other *N*-acyl glycines induced NO production in a dose-dependent manner (Fig. 7B, C, D). NO production was blocked by the non-selective calcium channel blocker SK&F96365 (Fig. 7D; $t(10) = -2.3$, 2-tailed, $p<0.05$) and was attenuated by the NOS blocker 7-NI (Fig. 7E). 7-NI treatment did not affect cell viability, tested using Trypan blue as mentioned in the methods. The time course for calcium influx and NO production is shown in Fig. 7E by the overlay of a single cell calcium and a single well NO trace (Fig. 7B).

PalGly induces calcium influx in native DRG cells

PalGly induces calcium influx in native DRG cells

Finally we asked whether PalGly would cause similar influx of calcium in primary adult rat dorsal root ganglion (DRG) cells. We tested native adult DRG cells 24-48 hours after plating. We found that PalGly produced a transient calcium influx with a rapid return to baseline (Fig. 8A) in a manner that paralleled that observed with F11 cells. 50 nM capsaicin was used as a positive control. 15/34 (44%) of the DRG cells that were treated with PalGly (16 μM) responded with an average increase ≥ 50 nM above baseline (calculated across 60 sec post application). The average increase in calcium influx after PalGly application was significantly different than the average increase post vehicle treatment (Fig. 8B).

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Discussion

Based on the presence of glycine conjugating enzymes and saturated bioactive lipid conjugates such as PEA; we hypothesized the presence and bioactivity of PalGly in mammalian tissues. We found a significant rise (~25%) in the levels of PalGly following KCl-induced depolarization of F-11 cells. Additionally, PalGly was identified as an endogenous molecule in rat tissues under basal conditions and its levels were modulated by knockout of the gene for the lipid metabolizing enzyme FAAH *in vivo*. Huang and colleagues (2001) previously reported that NAGly, and less so PalGly and LinGly, inhibited the hydrolysis of anandamide by FAAH *in vitro*. These findings led us to investigate the potential role of PalGly as a biological mediator.

The high basal levels of PalGly found in rat skin and spinal cord were suggestive of its involvement in cutaneous signaling. Cutaneous signaling through peripheral receptors activated by lipid molecules (including TRP channels and GPCRs) modulates the firing rate of stimulated WDR neurons (For example, Elmes et al., 2004; Merrill et al., 2007; Huang et al., 2006). We used this *in-vivo* model to determine whether peripherally administered low doses of PalGly would produce upstream effects on heat-stimulated WDR neurons. Indeed, we discovered that a submicrogram dose of PalGly (0.43 µg in 50 µl) significantly suppressed heat-evoked responses of WDR neurons when the heat stimulus increased the temperature of the rat paw to above 43 C°.

WDR neurons receive innervations from peripheral C and A fibers that encode both noxious and innocuous stimuli and project to higher brain centers; therefore,

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inhibitory effects on these neurons can modulate upstream sensory processing (Willis, 2007; Elmes et al., 2004). In order to shed light on the molecular components leading to this inhibitory effect, we used the hybrid DRG X neuroblastoma cell line (F-11) as a model system for studying the effects of PalGly on cell signaling. PalGly caused an immediate and robust calcium influx with an EC_{50} of 5.5 μM. This level is similar to the endogenous concentration measured in skin under basal conditions ~ 1.6 μM. Given that the amount reported here in the skin was averaged across the entire dissected tissue it likely underestimates the local concentrations of this lipophilic molecule in specific cellular compartments. For example, we previously demonstrated that 2-AG clusters within lipid rafts (Rimmerman et al. 2007). Tissue levels of PalGly also do not account for the heterogeneity of cell types, down-regulation of PalGly by FAAH or other enzymes, or up-regulation following cellular stimulation.

In-vitro metabolism of PalGly was previously demonstrated using a bacterial cytochtrome P450 (CYPBM-3). PalGly bound the enzyme with higher affinity than any other tested compound. The products of the enzymatic oxidation were ω-1, ω-2, and ω-3 -monohydroxylated metabolites of PalGly (Haines et al., 2001). It is yet to be determined whether PalGly can bind mammalian cytochrome P450 enzymes in the same manner and whether these metabolites take part in the signaling mechanisms of this abundant lipid.

The structure-activity relationship study using 6 other *N*-acyl glycine molecules indicated strict structural requirements for PalGly signaling which were suggestive of receptor activation. In these experiments, the addition of a single methyl group to the

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polar moiety (alanine versus glycine) led to a dramatic drop in activity, as did changes to chain length and degree of saturation in the lipid moiety. The influx of calcium by PalGly was inhibited by PTX (250-500 ng/ml) suggesting the involvement of a $G\alpha_{i/o}$-coupled signaling pathway. PalGly's effect was also dependent on extracellular calcium. A similar pathway was previously described in the neuroblastoma X glioma hybrid cell line (NG108-15) following treatment with the endocannabinoid 2-arachidonoyl glycerol (2-AG). In contrast to 2-AG the effect of PalGly was not inhibited by CB_1 and CB_2 antagonists. Moreover, PalGly-induced calcium influx was blocked by ruthenium red, $La3^+$, and SK&F 96365, which are non-selective antagonists of TRP channels and receptor-operated calcium channels (Merritt et al., 1990; Caterina et al., 1997).

Three pathways leading to cation influx through TRP receptor modulation have been described: ligand binding (e.g. Δ^9-THC, *N*-arachidonoyl dopamine, anandamide, capsaicin, diacylglycerol), direct activation (e.g. temperature, pH, osmolarity, membrane stretch), and indirect activation through GPCR-mediated pathways (e.g. through PLC, DAG production, IP3 production; Ramsey et al., 2006). We ruled out simple ligand binding of PalGly to human-TRPV1, mouse-TRPV3 and rat-TRPV4 receptors by screening stably transfected HEK293 cells. The complex signaling properties of PalGly including strict SAR, partial inhibition by PTX, and dependence on extracellular calcium are suggestive of GPCR-mediated cation channel activation.

An analogous pathway involving the neuropeptide head activator (HA), which drives cells into mitosis by its actions at GPR37, has been previously characterized (Rezgaoui et al., 2005). GPR37 activates a PTX-sensitive pathway regulating a TRPV-

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like calcium channel (the growth-factor-regulated calcium-permeable cation channel) that could be inhibited by SK&F 96365 (Boels et al., 2001). The influx of calcium induces the activation of a calcium-dependent K+ channel, leading to cellular hyperpolarization. We are currently exploring whether a similar set of events is plausible for PalGly: activation of a PTX-sensitive GPCR followed by activation of a second messenger which in turn modulates a TRP-like channel, promoting activation of a downstream channel leading to suppression of spinal circuits. Additional studies will investigate the role of PalGly on PPAR activation of calcium and as a ligand for GPR55 given the potent effects of the structural analog PEA in these systems.

The stimulation of nitric oxide production by PalGly is an additional downstream mechanism that could explain the heat-stimulated WDR neuronal firing inhibition produced by PalGly *in vivo*. The transient influx of calcium evoked by PalGly appears to activate calcium-dependent NOS enzymes present in F-11 cells, a conclusion based upon the failure of PalGly to induce production of NO in cells treated with the calcium channel blocker SK&F96365 and the similarity between the SARs of PalGly induced calcium influx and NO production. The constitutive NOS isoforms generate relatively smaller NO fluxes with a shorter time course compared to inducible NOS (Wink and Mitchell, 1998). The effects of NO on neuronal firing differ among cell types, but several studies indicate that NO production leads to inhibition of neuronal firing and transmission (e.g., Chaban et al., 2001; Clasadonate et al., 2007). Hence, it is possible that the PalGly-induced NO production may contribute to the observed inhibition of heat-stimulated WDR neuronal firing *in vivo*. This may occur via direct activation of

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soluble guanylate cyclase to produce cyclic GMP leading to protein kinase G (PKG) activation and modulation of K^+ channels. This pathway was implicated in peripheral antinociceptive effects of several compounds including indomethacin and dipyrone (Lorenzetti and Ferreira, 1996; Ventura-Martınez et al., 2004). Additional molecular studies testing cyclic GMP upregulation and PKG activation as well as physiological experiments using NOS inhibitors will be needed to test this hypothesis and investigate potential anti-nociceptive effects of PalGly.

In summary, we have provided mass spectrometric evidence for the novel endogenous lipid *N*-palmitoyl glycine that is produced throughout the body with the highest levels in skin and spinal cord, is hydrolyzed by FAAH, inhibits heat-evoked firing of WDR neurons, activates calcium influx in DRG cells and stimulates NO production. Together these data provide a framework for further investigations into this putative signaling lipid and provide additional evidence into the investigations of a potentially larger family of acyl amide signaling lipids.

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Footnotes.

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b) NR and HBB contributed equally to this work

c) This work is dedicated to Prof. J. Michael Walker (1950-2008)

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Figure legends

Figure 1. ***Chromatographic and mass spectrometric analysis revealed endogenous occurrence of N-palmitoyl glycine.*** A) PalGly: a 16-carbon saturated fatty acid conjugated to a glycine molecule through an amide bond. B) 500 fmol standard of PalGly using the LC/MS/MS MRM method and chromatographic gradient outlined in Methods. C) 10 µl injection of lipid extract from spinal cord using the same PalGly LC/MS/MS MRM method C. D) overlay of chromatograms from B and C. E) Constitutive production of PalGly was measured in partially-purified lipid extracts using LC/MS/MS using MRM methods. Values are reported as pmol/g dry tissue (mean ± SEM), estimated from the ratio of lyophilized to wet weight of each type of tissue. *Sm. Inst= small intestine*. F) Product ion scan mass spectrum of the positively charged molecular ion of the PalGly synthetic standard in which the arrows are directed to the peaks of the proposed molecular and fragment ions and labeled with the calculated exact mass. G) Product ion scan mass spectrum of the brain lipid extract tuned to the positively-charged PalGly molecular ion in which the arrows are directed to the peaks of the proposed molecular and fragment ions and labeled with the calculated exact mass. H) Mass measurements of the molecular and fragment ions together with the proposed formulae of these ions for PalGly found in lipid extract from murine brain. The parts per million (ppm) differences from the theoretical exact masses of each molecular ion and fragment ion are represented in parentheses.

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Figure 2. Peripheral administration of PalGly suppressed heat-evoked firing in spinal nociceptive neurons. Firing rates of WDR neurons in response to exposure of their receptive fields to a heat ramp. Heat-evoked firing rates were measured prior to and 30 min after intradermal injection of PalGly (0.43 µg). Mean firing rates were calculated before (pre) and after (post) PalGly administration. * $p < 0.05$ difference from pre-PalGly firing rates. Error bars indicate standard error of the mean.

Figure 3. PalGly induced a dose-dependent calcium influx in F-11 cells. A. Single cell calcium traces (assessed by Fura-2 fluorescence) of F-11 cells treated with either 10 µM PalGly or DMSO. Dashed line indicates the time of drug administration. B. A dose-response curve of F-11 cells to PalGly. The following formula was used to calculate the average change in nM calcium for each cell: [(the average response 60 sec after PalGly application) – (the average response 60 sec before PalGly application)]. All cells, regardless of their response rate, were averaged. Each data point represents an average of single cells (n=31-75) from at least three independent plates. The EC_{50} for PalGly= 5.5 µM. Error bars indicate standard error of the mean.

Figure 4. PalGly induced calcium influx in F-11 cells with strict structural requirements. Comparison of PalGly-induced calcium influx with calcium influx by structurally related molecules. Calcium influx was calculated from the integral of the time x calcium-concentration curve as described in the text. Free glycine was tested at 16 µM. All other compounds were tested at 10 µM. One-Way ANOVA with Bonferroni post hoc. *, $p<0.05$ compared to vehicle; ***, $p<0.001$ compared to vehicle; †††, $p<0.001$ compared to PalGly.

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Figure 5. PTX attenuated PalGly-induced calcium influx. The effect of PalGly (15 μM) was significantly attenuated by pertussis toxin (250 or 500 ng/ml) but not by ω-conotoxin MVIIC (2 μM) or ω-conotoxin GVIA (2 μM). ω-Conotoxin MVIIC and ω-conotoxin GVIA were tested in the presence of fatty acid free bovine serum albumin. Calcium influx was calculated by integrating the area under 340/380 fluorescence ratio x time curves. n=8 wells per condition, *** $p < 0.001$ difference from vehicle + PalGly. One-Way ANOVA with LSD post hoc. Error bars indicate standard error of the mean.

Figure 6. PalGly-induced calcium influx was inhibited by pretreatment with ruthenium red (10 μM), SK&F96365 (25 μM) and La^{3+} (1 mM). A) Single cell calcium traces for F-11 cells treated with 16 μM PalGly following administration of vehicle or 10 μM ruthenium red. B) A representative F-11 well population calcium influx of cells pretreated with La^{3+} or vehicle. C) Concentration-response curves for PalGly and PalGly + SK&F96365 (10 μM or 25 μM) show profound inhibition of PalGly-induced calcium influx in F-11 cells following treatment with 25 μM SK&F96365. n=3-20 wells per condition. D) PalGly (16 μM) induced marked calcium influx in the presence of MK801 (25 μM) and I-RTX (35 nM), whereas it lacked efficacy in the presence of SK&F96365 (10 μM, 24 μM) or ruthenium red (10 μM). Calcium mobilization was calculated from the integral of the calcium-concentration x time curve as described in the text. *** $p < 0.001$ difference from vehicle + PalGly. One-Way ANOVA with LSD post hoc. Error bars indicate standard error of the mean.

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Figure 7. PalGly treatment induced NO production in F-11 cells in a calcium-dependent manner. A) F-11 cells express calcium-dependent NOS isoforms. Western blots showed that eNOS, nNOS and Ser847-phospho-nNOS were present in F-11 cells. iNOS was not detected using Western blotting. B) NO release by F-11 cells upon PalGly treatment is calcium channel dependent. Traces from individual wells containing DAF-2-loaded F-11 cells displayed an increase in NO upon treatment with PalGly but not DMSO. Treatment of F-11 cells with the non-specific calcium channel blocker SK&F96365 (20 μM) suppressed PalGly-evoked NO release when compared to vehicle (water) treated cells. Data points are presented as the increase in fluorescence relative to baseline using a 9-point moving point average. C) NO production closely parallels calcium influx in F-11 cells. A calcium trace from a single cell is overlaid with a NO trace from a single well. D) PalGly causes dose-dependent release of NO in F-11 cells. Data are presented as a percentage of the effect of 2 mM SNAP in 5 μM DAF-2. PalGly at and above 10 μM caused significant elevations in NO release when compared to DMSO (** $p<0.01$, n=6, One-Way ANOVA, Dunnett t-test post hoc). The EC_{50} for PalGly's effect was 8.7 μM. E) NO release by PalGly in F-11 cells had rigid structural requirements. PalGly and stearic acid (SA), but not other structurally related compounds, significantly increased NO production when compared to DMSO vehicle (***, $p<0.001$, $n\geq9$, One-Way ANOVA, Dunnett t-test post hoc). All compounds were tested at 10 μM. Data are presented as a percentage of the effect of 2 mM SNAP in 5 μM DAF-2. F) NOS catalyzes PalGly-induced NO production. PalGly-induced NO

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production in F-11 cells was inhibited by pretreatment of cells with 200 μM 7-NI, a NOS inhibitor (*, $p<0.05$, n=32, One-Way ANOVA, LSD post hoc). Data are presented as a percentage of the maximum mean effect of PalGly in each experiment, which occurred in each case under DMSO treatment conditions. Error bars represent standard error of the mean.

Figure 8. PalGly induced calcium influx in a large subset of native adult DRG cells.A) A Single cell trace of a primary adult DRG cell treated with PalGly (16 μM) after 60 sec of baseline recording. B) The average increase in calcium influx after PalGly application (for cells that responded with an average > 50 nM calcium increase over baseline), was significantly different than the average increase after vehicle treatment (total cells; Figure 8C; $t(54) = 12.4$; two tailed; ***, $p<0.001$).

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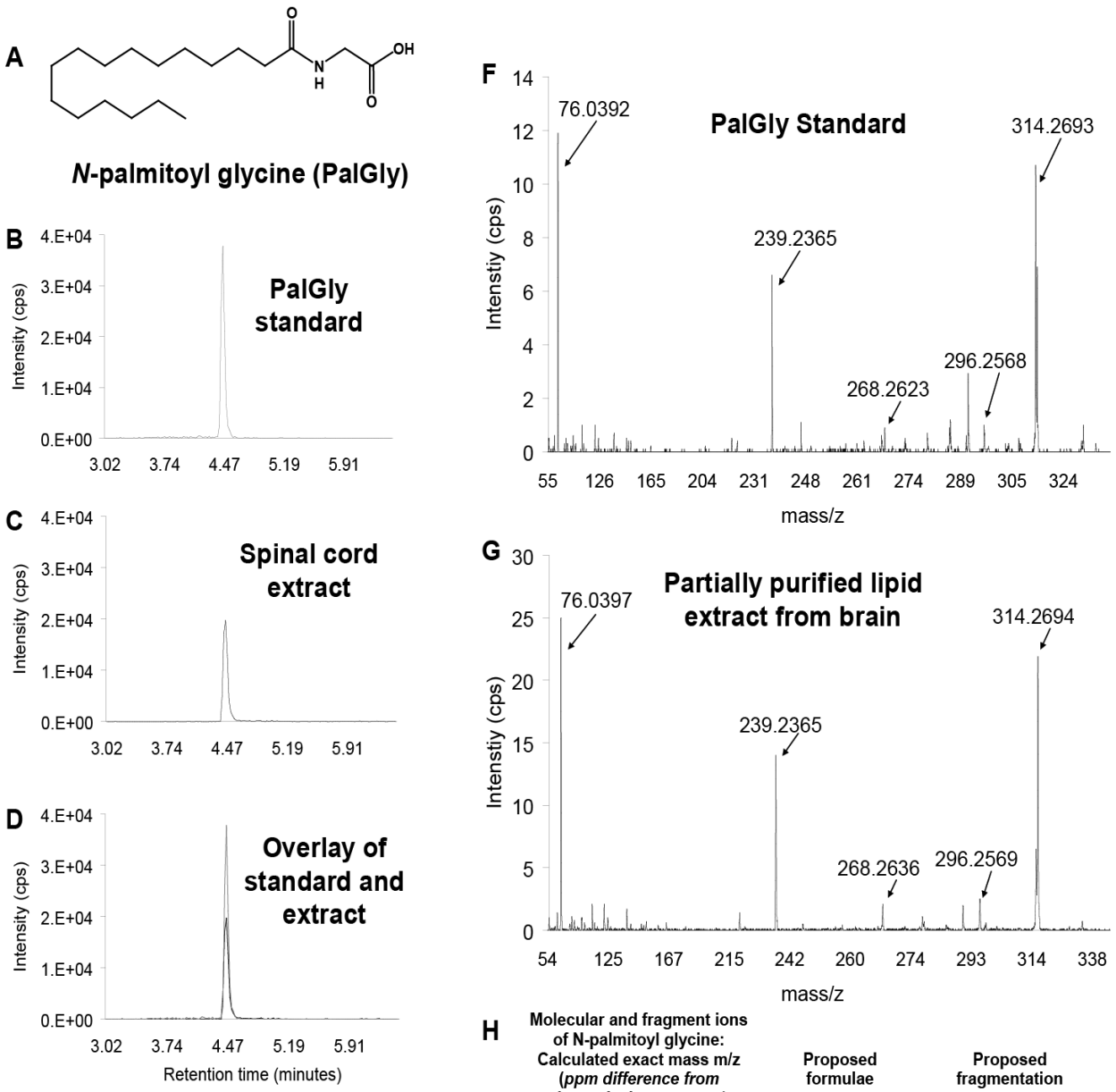
Figure 1



E

pmol PalGly per gram tissue

Skin	Lung	Spinal cord	Kidney	Liver	Ovaries
1612±174	895±181	684±120	424±37	388±20	261±11

Spleen	Sm Inst	Testes	Brain	Uterus	Heart
167±25	106±13	105±11	99±20	71±10	6±5

H

Molecular and fragment ions of N-palmitoyl glycine: Calculated exact mass m/z (*ppm difference from theoretical exact mass*)	Proposed formulae	Proposed fragmentation
314.2694 *(4.8659)*	$C_{18}H_{36}NO_3$	MH+
296.2569 *(-15.5476)*	$C_{18}H_{34}NO_2$	MH+ - H_2O
268.2636 *(1.8957)*	$C_{17}H_{34}NO$	MH+ - CH_2O_2
239.2365*(-3.9387)*	$C_{16}H_{31}O$	MH+ - C_2H_5N
76.0397 (3.8802)	$C_2H_6NO_2$	MH+ - $C_{16}H_{30}O$

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Figure 2

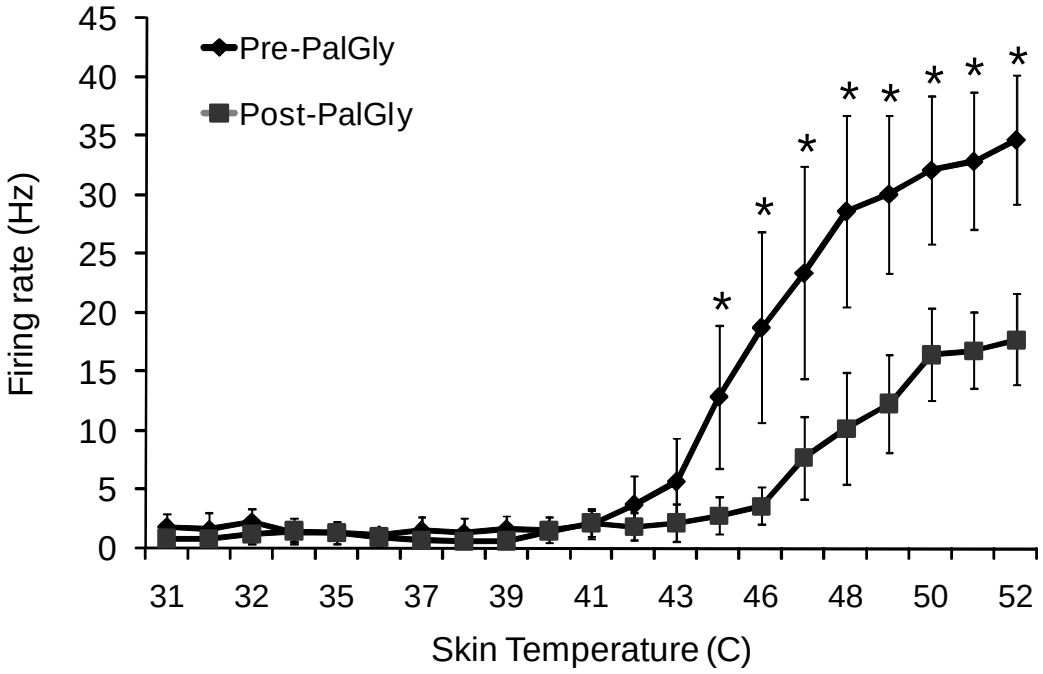

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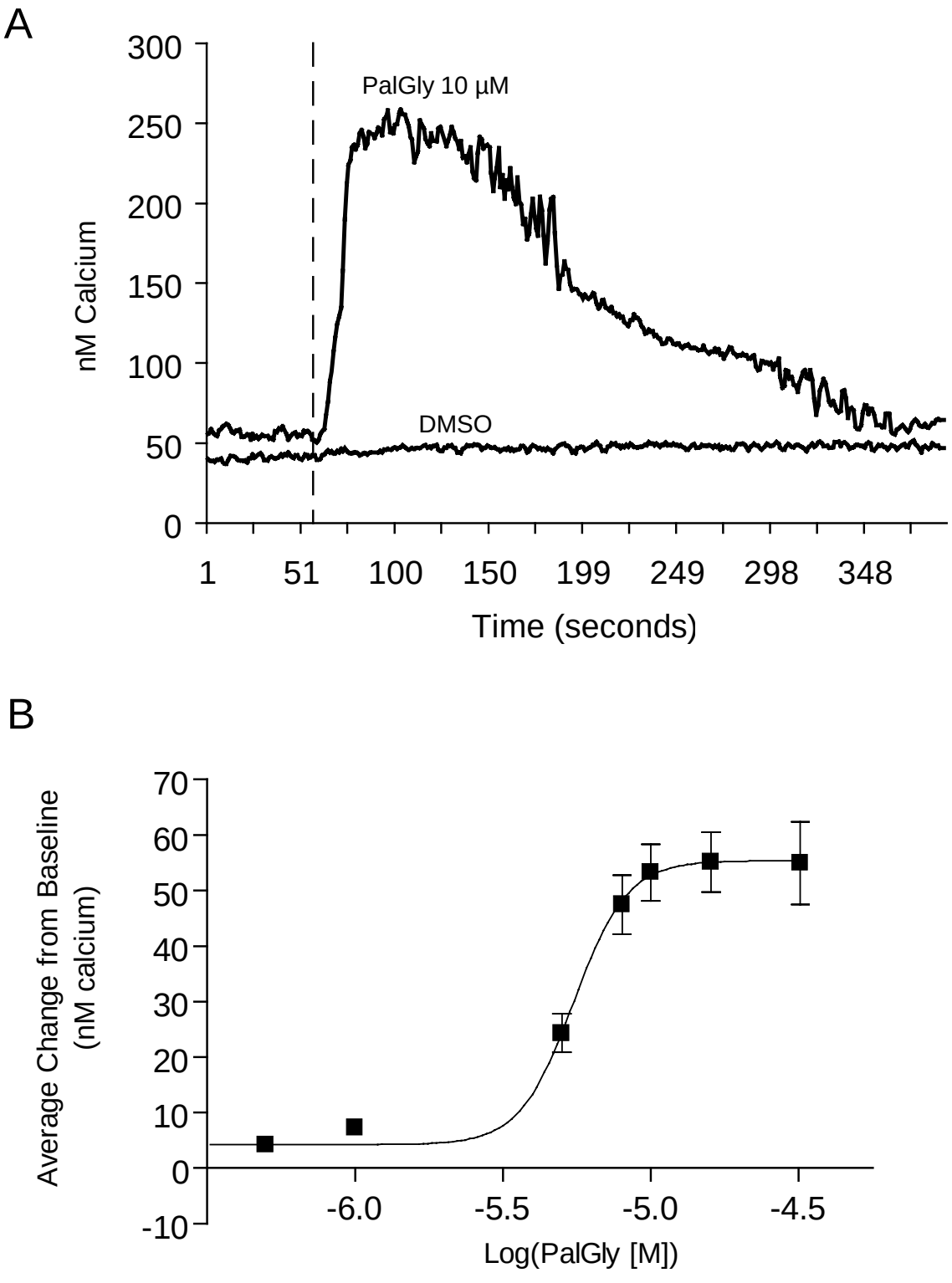
Figure 3



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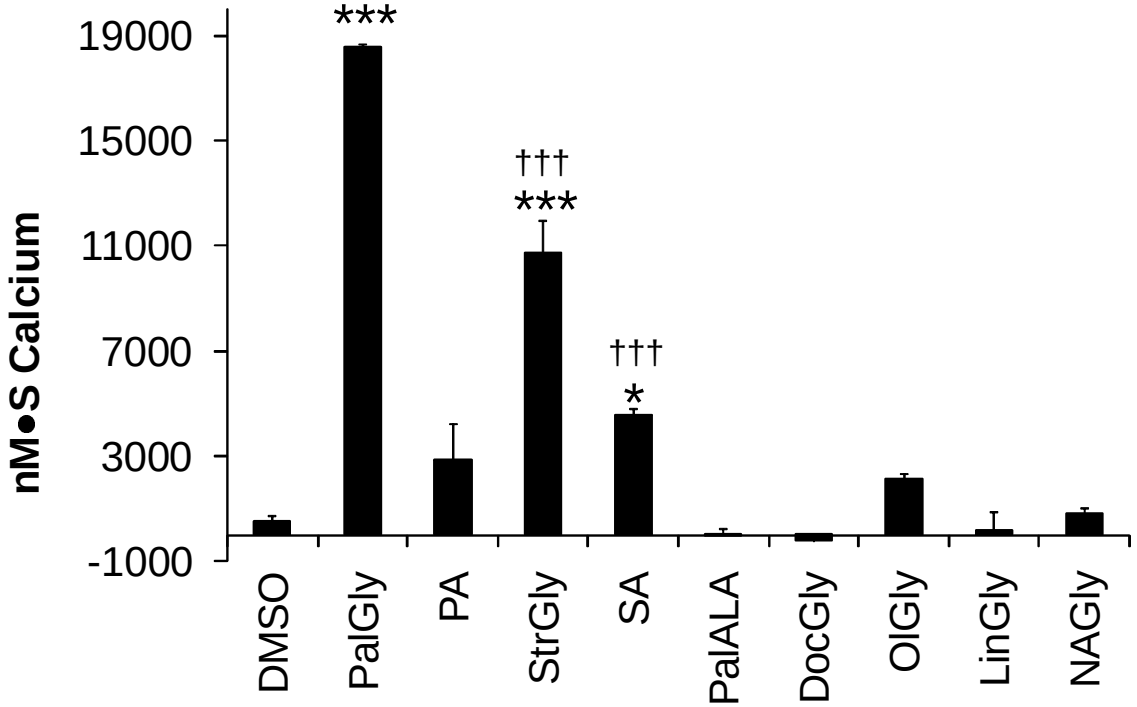

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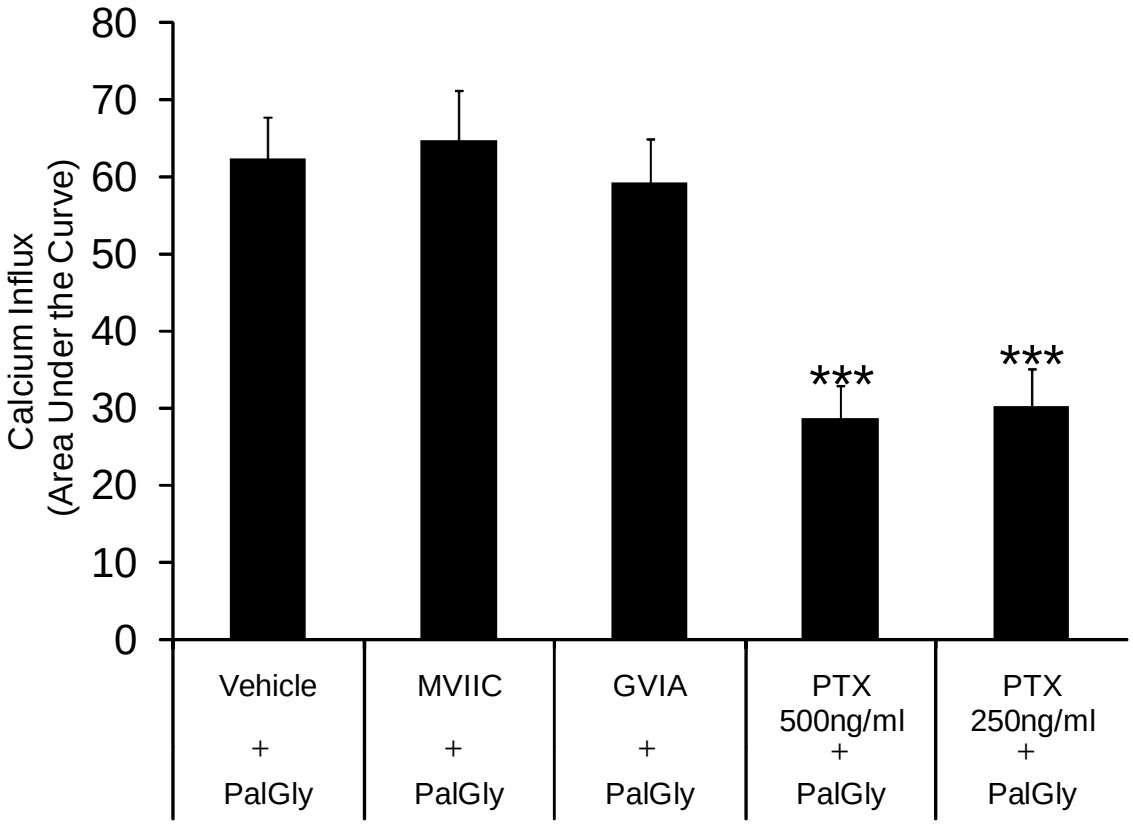

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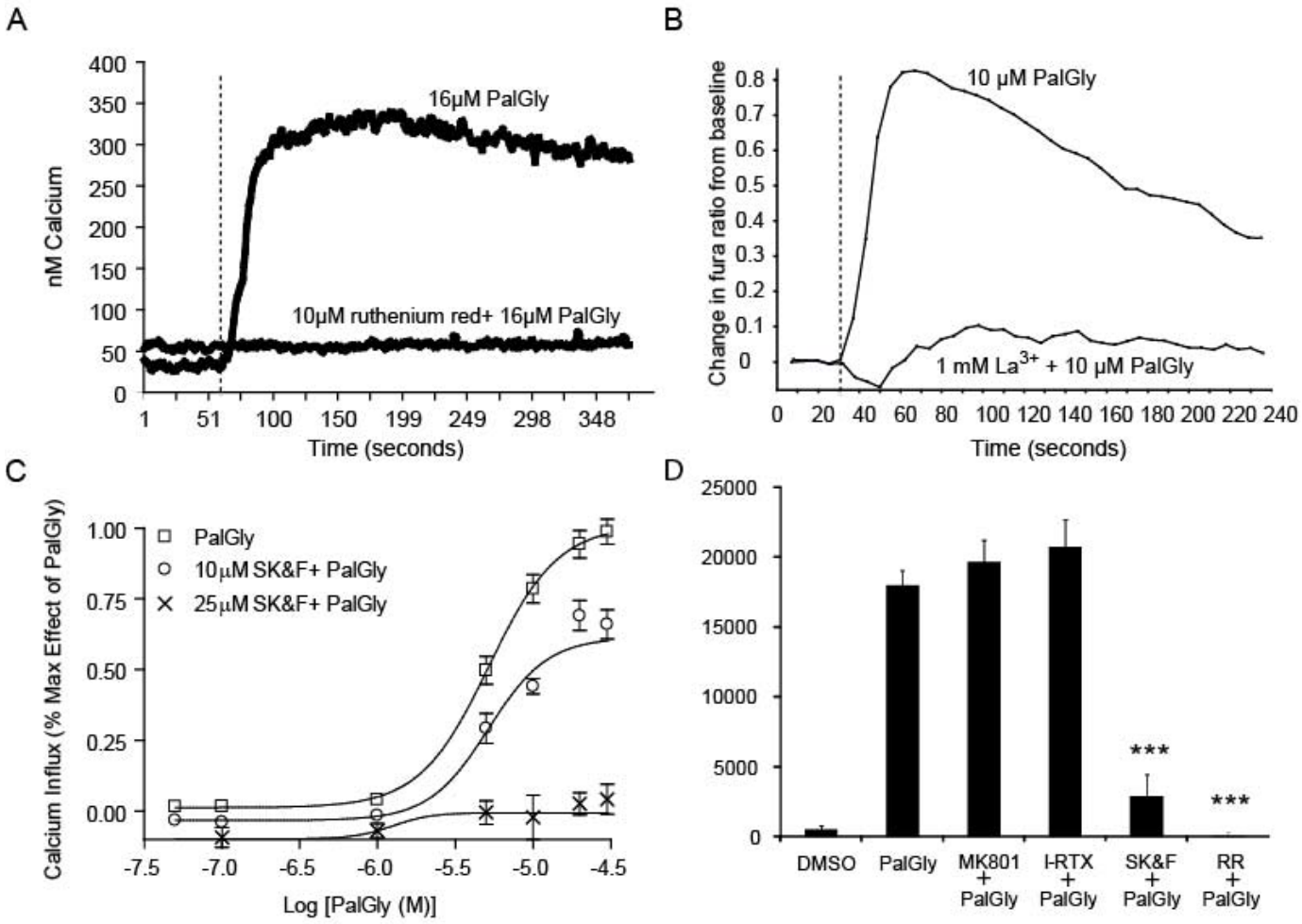

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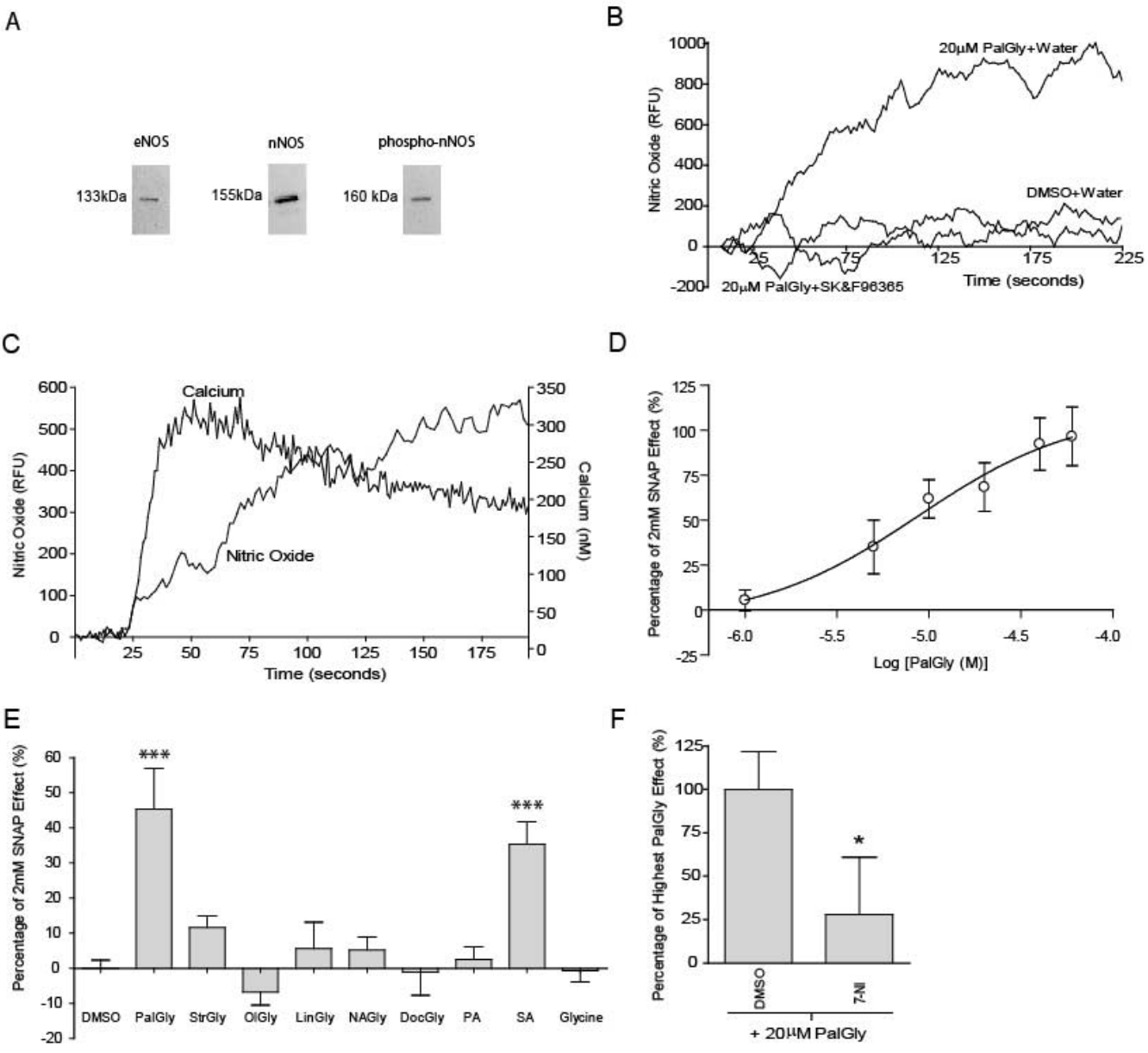

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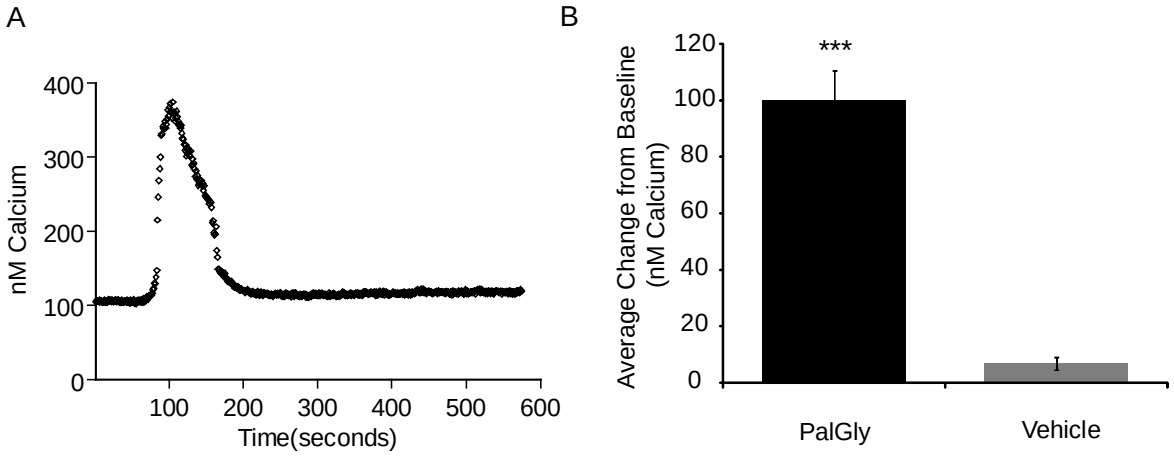

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