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**Phosducin and phosducin like-protein attenuate G-protein coupled receptor mediated inhibition of voltage-gated calcium channels in rat sympathetic neurons**

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Abbreviations: PDC, phosducin; PDCL, phosducin like-protein; GppNHp, guanylyl imidophosphate; SCG, superior cervical ganglion; mGlu<sub>2</sub>, metabotropic glutamate receptor 2; GPCR, G-protein coupled receptor; HEK, human embryonic kidney; GRK2, G-protein coupled receptor kinase 2; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein

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## Abstract

Phosducin (PDC) has been shown in structural and biochemical experiments to bind the G $\beta\gamma$  subunit of heterotrimeric G-proteins. A proposed function of PDC and phosducin like-protein (PDCL) is the sequestration of “free” G $\beta\gamma$  from the plasma membrane thereby terminating signaling by G $\beta\gamma$ . The functional impact of heterologously expressed PDC and PDCL on N-type calcium channel (Ca<sub>v</sub>2.2) modulation was examined in sympathetic neurons, isolated from rat superior cervical ganglia, using whole-cell voltage-clamp. Expression of PDC and PDCL attenuated voltage-dependent inhibition of N-type calcium channels, a G $\beta\gamma$ -dependent process, in a time-dependent fashion. Calcium current inhibition following acute exposure to norepinephrine was minimally altered by PDC or PDCL expression. However, in the continued presence of norepinephrine, PDC or PDCL relieved calcium channel inhibition when compared with control neurons. We observed similar results following activation of heterologously expressed metabotropic glutamate receptors with 100  $\mu$ M L-glutamate. Neurons expressing PDC or PDCL maintained suppression of inhibition following re-exposure to agonist. Unlike other G $\beta\gamma$  sequestering proteins that abolish the acute inhibition of Ca<sup>2+</sup> channels, PDC and PDCL require prolonged agonist exposure before effects on modulation are realized.

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## Introduction

G-protein coupled receptors (GPCRs) are expressed throughout the nervous system and influence systems important for homeostasis. The canonical G-protein signaling pathways consists of a ligand binding to the receptor, exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the heterotrimeric G-protein  $\alpha$ -subunit ( $G\alpha$ ), separation of the  $G\beta\gamma$  dimer ( $G\beta\gamma$ ) from the  $G\alpha$  subunit, and modulation of divergent downstream effector proteins by  $G\alpha$ -GTP and  $G\beta\gamma$ . For example, free  $G\beta\gamma$  can associate with N-type voltage-gated  $Ca^{2+}$  channels to reduce the probability of channel opening upon membrane depolarization. The strength and duration of this interaction will be influenced by competition with proteins possessing a high affinity for  $G\beta\gamma$  including  $G\alpha$ -GDP. Therefore, the duration of G-protein signaling is dependent upon the GTP hydrolysis rate since coalescence of the  $G\alpha\beta\gamma$  heterotrimer terminates signaling. Other proteins that bind  $G\beta\gamma$  with high affinity, such as the carboxyl-terminus of GRK2 (ctGRK2) or phospholipase C- $\beta$ 2, attenuate  $G\beta\gamma$  signaling (Ford et al., 1998). Here we examined how the expression of phosducins, a protein family known to interact with  $G\beta\gamma$ , influenced GPCR-mediated N-type  $Ca^{2+}$  channel modulation in sympathetic neurons. The goals of the study were to: 1) characterize the effects of PDC and PDCL expression within the context of protein-based optical sensor development, and 2) provide insight into possible physiological roles for PDC/PDCL modification of GPCR responses in neurons.

Phosducin (PDC) is a 28 kDa soluble protein expressed primarily in the retina and pineal gland (Schulz, 2001; Sokolov et al., 2004). Retinal PDC has been hypothesized to adjust the gain of luminosity perception in the vertebrate eye (Thulin et al., 2001) by sequestering transducin  $G\beta\gamma$  ( $G\beta_1\gamma_1$ ) from the rod outer segment plasma membrane following rhodopsin activation. PDC binds with high affinity to  $G\beta_1\gamma_1$  as well as several other  $G\beta\gamma$  combinations (Lee et al., 1987;

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Müller et al., 1996). The crystal structure of the PDC/G $\beta_1\gamma_1$  complex reveals that the N-terminus of PDC contains three  $\alpha$ -helices while the C-terminal portion is comprised of  $\beta$ -sheets with overall homology to bacterial thioredoxin (Gaudet et al., 1996, 1999; Loew et al., 1996). The N-terminal domain of PDC contacts regions of G $\beta$  common to the interface between G $\alpha$  and G $\beta\gamma$  in the heterotrimer. Thus, binding of PDC to G $\beta\gamma$  occludes re-association of G $\beta\gamma$  with the G $\alpha$  subunit (Xu et al., 1995). Structural analyses also indicate that PDC induces a conformational change in G $\beta\gamma$  that buries the isoprenyl group of G $\gamma$  within the seven-bladed  $\beta$ -propeller structure of G $\beta$  thereby decreasing the affinity of G $\beta\gamma$  for the lipid bilayer (Lukov et al., 2004).

A homolog of PDC, phosducin-like protein (PDCL), was discovered during a screen for ethanol responsive genes (Miles et al., 1993). Although less is known about its cellular functions, PDCL exhibits a more ubiquitous expression pattern (Schroder and Lohse, 2000), binds G $\beta\gamma$  with high affinity, and potentially regulates G-protein receptor function (Schroder et al., 1996; Shultz et al., 1998). The N-terminal domain of PDCL is alternatively spliced and larger than the N-terminal domain of PDC. At present, high-resolution structures of PDCL in complex with G $\beta\gamma$  are unavailable although biochemical studies indicate that heterologously expressed PDCL acts similarly to PDC by binding to and solubilizing G $\beta\gamma$  (Lukov et al., 2004; Thibault et al., 1997). Recent studies, however, indicate that natively expressed PDCL may act as a chaperone within the biosynthetic pathway of G $\beta$  and facilitate assembly with G $\gamma$  (Humrich et al., 2005; Knol et al., 2005; Lukov et al., 2005).

G-protein coupled receptors are popular targets for pharmacological agents. The above structural characteristics of PDC and PDCL provide a rational basis for their use as biosensors of G-protein activation and could facilitate the discovery of new GPCR targeted drugs via high throughput assays. Techniques such as FRET (Miyawaki, 2003) and protein complementation

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(Hu and Kerpolla, 2003) are amenable to high throughput detection and rely on the dynamic interaction of distinct sensor components. An assay based on these techniques could benefit from the bipartite structure of these G $\beta\gamma$  binding proteins. For example, knowledge of the crystal structure of the PDC-G $\beta\gamma$  complex makes conformational optimization via techniques such as circular permutation possible. Additionally, changes in binding affinity with phosphorylation (Gaudet et al., 1999; Thulin et al., 2001; Humrich et al., 2003), and promiscuity in G $\beta\gamma$  binding (Müller et al., 1996) may provide a means of producing a tunable and universal detector of free G $\beta\gamma$  following receptor stimulation.

In this report we show that heterologous expression of PDC or PDCL in rat superior cervical ganglia (SCG) neurons attenuates GPCR-mediated voltage-dependent inhibition of N-type Ca<sup>2+</sup> channels (Ca<sub>v</sub>2.2). Unlike other G $\beta\gamma$  scavengers, PDC and PDCL require prolonged application of agonist before significant effects on modulation were realized. Western blot analysis demonstrated the presence of PDCL, but not PDC, in SCG neurons suggesting a possible role for this protein in neurotransmitter signaling. We also discuss the possible use of these G $\beta\gamma$  binding proteins as biosensors for G-protein activation.

## Materials and Methods

**Vectors and DNA constructs.** The open reading frame (ORF) of human phosducin (PDC, accession number NM\_002597) and rat phosducin-like protein<sub>L</sub> (PDCL, accession number NM\_022247) were amplified by RT-PCR from retinal and dorsal root ganglion mRNA, respectively. The PDC and PDCL ORFs were subcloned into the mammalian expression vector pCI (Promega Corp, Madison, WI) at the *XbaI/SmaI* sites and *EcoRI/NotI* sites, respectively. Fusion protein constructs were made by ligating PDC and PDCL ORFs into the multiple cloning

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sites of various pEGFP vectors (Clontech/BD Biosciences, Palo Alto, CA). All clones were sequenced to verify the fidelity of the cloning process with a Beckman-Coulter DNA sequencer (Fullerton, CA).

**Isolation and injection of rat SCG neurons.** Adult male rats were used as a source for sympathetic neurons. Following dissection of the SCG, ganglia were de-sheathed, minced, and exposed to Earle's Balanced Salt solution containing 0.06 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN), 0.03 mg/ml trypsin (Worthington Biochemical Corp, Lakewood, NJ), and 0.005 mg/ml DNase I (Sigma-Aldrich, St. Louis, MO). The minced ganglia were then incubated in a shaking water bath for 1 hour at 37°C. Following incubation, the neurons were dissociated by mechanical disruption, isolated by centrifugation, re-suspended in Eagle's Minimal Essential Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin solution, and plated on 35 mm polystyrene tissue culture dishes coated with poly-L-lysine.. Following a static incubation of 3.5–6 hours at 37°C, plasmid DNA was introduced into single neurons via intranuclear injection (Ikeda, 2004) using an Eppendorf Femtojet injection system (Hamburg, Germany). Plasmid DNA, dissolved in TE (Tris 10 mM, EDTA 1 mM, pH 8.0) was injected at concentrations varying from 5–100 ng/μl. A computer driven stepper motor-based micromanipulator (Eppendorf 5171) was used to inject neuronal nuclei. Injection pressure was 125–200 hPa (1.8–3 psi) for 0.3 s. In order to visualize successfully injected neurons, 5 ng/μl of pEGFP-N1 was co-injected with the plasmid DNA under study. EGFP fluorescence was visualized 12–24 hours following injection with a filter cube consisting of an HQ480/40 excitation filter, Q505LP beam splitter, and HQ535/50m emission filter (Chroma Technology Corp, Rockingham, VT), and 100 W Hg arc lamp excitation source. Non-fluorescent neurons served as control cells.

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**Patch clamp electrophysiology.** Whole-cell currents were measured at room temperature (19–21°C) with an Axopatch 200A patch-clamp amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Recording pipettes were prepared from 7052 borosilicate glass (Garner Glass, Claremont, CA) with a Flaming-Brown puller (Sutter Instrument Co., Novato, CA) and had resistances of 1.5–3.0 M $\Omega$  when filled with pipette (internal) solution. Pipettes were coated with Sylgard<sup>®</sup> near the tip to reduce capacitance coupling with the bathing solution. The internal solution used to record voltage-gated Ca<sup>2+</sup> currents contained the following in mM: 120 N-methyl-D-glucamine, 20 TEA-Cl, 11 EGTA, 10 sucrose, 0.3 Na<sub>2</sub>GTP, 1 CaCl<sub>2</sub>, 4 MgATP, 14 creatine phosphate. The solution was adjusted to pH 7.2 with methanesulphonic acid, which served as the primary anion, and had an osmolality of ~305 mosm/kg. The extracellular (bathing) solution contained the following in mM: 140 TEA-OH, 10 CaCl<sub>2</sub>, 10 HEPES, and 15 glucose. The solution was adjusted to pH 7.4 with methanesulphonic acid and had an osmolality of 325–330 mosm/kg. The bathing solution was supplemented with 100 nM tetrodotoxin, to block endogenous voltage-gated Na<sup>+</sup> channels. Series resistance prior to electronic compensation (typically 80%) ranged from 2–6 M $\Omega$ . During recordings, neurons were continuously superfused with external solution. G-protein coupled receptors were activated by releasing external solution containing agonist over the cell surface using a gravity driven perfusion system positioned approximately 50  $\mu$ m from the soma. Drug delivery was controlled electronically via solenoid valves driven by software commands. Currents were low-pass filtered at 5 kHz, digitized at a frequency of 10 kHz, and stored on a Macintosh G4 computer using custom developed software. Data were analyzed using the Igor Pro software package (Wavemetrics, Lake Oswego, OR). L-glutamic acid,  $\pm$  norepinephrine HCl, and guanylyl imidophosphate (GppNHp) were purchased



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from Sigma-Aldrich. GppNHp was prepared daily at 100x final concentration. Stock solutions of receptor agonists were prepared at 1000x final concentration and stored at -20°C.

**Immunoblotting.** HEK293 cells were grown to 75–90% confluence and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Crude total protein from SCG neurons or transfected HEK293 cells was prepared by re-suspending cells in a lysis buffer containing 10 mM Tris, pH 6.8, supplemented with 0.1% Triton X-100, 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride, and a protease inhibitor cocktail (Sigma-Aldrich, #P8340). The cell suspension was sonicated six times and protein concentration determined with a bicinchoninic acid (BCA) reagent kit (Pierce Biotechnology Inc., Rockford, IL). Solubilized protein (20 µg/lane) was separated on SDS-polyacrylamide gels according to the method of Laemmli (1970). Separated proteins were electrophoretically transferred (90 mins at 25 V) to a polyvinylidene difluoride membrane (Hybond-P<sup>®</sup>, Amersham Pharmacia, Piscataway, NJ) in blotting buffer consisting of 25 mM Tris base, 191 mM glycine, and 20% (v/v) methanol using an Xcell II blot module (Invitrogen). The membrane was then blocked overnight at 4°C with a solution of 10% (w/v) nonfat dry milk in PBS (consisting of 136 mM NaCl, 10 mM KCl, 32 mM Na<sub>2</sub>HPO<sub>4</sub>, and 5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2) then washed three times (10 min/wash) with PBS containing 0.05% (v/v) Tween 20. Rabbit anti-PDC (7.5 µg/ml) or anti-(GST)-PDCL (10 µg/ml) polyclonal antibodies diluted in PBS containing 0.5% (v/v) goat serum, were used to probe the blot for 60 minutes at room temperature. The primary antibodies were generously provided by Dr. Barry Willardson (Brigham Young University, Provo, UT) and characterized by Thulin et al., (1999). After washing, the blot was incubated with a horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (0.5 µg/ml) diluted in PBS containing 0.5% (v/v) goat serum (Upstate, Lake

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Placid, NY). Antigen was visualized using TMB Stabilized Substrate for HRP (Promega Corp.).

**Microscopy.** Laser scanning two-photon imaging was performed using a Zeiss 510 META/NLO attached to an upright Zeiss Axioplan 2 microscope with a 20x, 0.5 NA water-immersion objective (Carl Zeiss AG, Jena, Germany). Fluorescent neurons were excited in two-photon mode with a Chameleon® Ti:Sapphire laser (Coherent Inc., Santa Clara, CA) mode-locked at 850 nm. Emitted photons were filtered with a 500–550 nm bandpass filter and detected on a micro-channel plate photomultiplier (Hamamatsu R3809U-52). The z-axis optical section depth was estimated to be 3.3  $\mu\text{m}$ .

## Results

**Phosducin expression did not alter maximal  $\text{Ca}^{2+}$  current inhibition in SCG neurons.** SCG neurons express  $\alpha_2$ -adrenergic receptors (Schofield, 1991) that couple to pertussis toxin-sensitive G-proteins and inhibit N-type voltage-gated  $\text{Ca}^{2+}$  channels. The inhibition is voltage-dependent and mediated by  $\text{G}\beta\gamma$  “released” from the activated G-protein heterotrimer. We utilized this well-characterized pathway to examine the impact of PDC or PDCL expression on neurotransmitter-mediated modulation of N-type  $\text{Ca}^{2+}$  channels.

Plasmid cDNAs encoding PDC and enhanced green fluorescent protein (EGFP) were injected into the nuclei of isolated rat SCG neurons. Uninjected neurons or neurons injected with EGFP cDNA alone served as control cells. It should be noted that some neurons considered “uninjected” may represent unsuccessful injections, i.e., neurons in which insufficient cDNA reached the nucleus to produce detectable fluorescence. Whole-cell voltage-clamp recordings were performed following overnight incubation at 37°C to allow for protein expression.  $\text{Ca}^{2+}$

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channel current (hereafter,  $I_{Ca}$ ) was evoked every 10 s with the voltage protocol illustrated in Figure 1A (top). The protocol, evoked from a holding potential of -80 mV, consisted of a 25 ms test pulse (denoted prepulse) to +10 mV, a 40 ms conditioning depolarizing step to +80 mV, a 10 ms return to -80 mV, and a second 25 ms test pulse (denoted postpulse) to +10 mV (Elmslie et al., 1990). Representative  $I_{Ca}$  traces recorded in the absence (con) or presence of norepinephrine are illustrated in Figure 1A. In control neurons, the mean amplitudes of the prepulse and postpulse current, measured isochronally 10 ms following the start of the test pulse, were  $-1.62 \pm 0.14$  and  $-2.07 \pm 0.17$  nA (n=41), respectively. The ratio of the postpulse to prepulse current amplitude in the absence of agonist (termed the basal facilitation ratio) averaged  $1.30 \pm 0.02$  and  $1.33 \pm 0.04$  in uninjected and EGFP-N1 (n=10) expressing neurons (summarized in Fig 1D). The enhancement of postpulse current amplitude (i.e., facilitation ratio > 1) in the absence of agonist has been shown to arise from tonic  $G\beta\gamma$  modulation of N-type  $Ca^{2+}$  channels in SCG neurons (Ikeda, 1991; Garcia et al., 1998).

Application of norepinephrine (10  $\mu$ M) to uninjected neurons decreased the prepulse  $I_{Ca}$  by  $63.4 \pm 1.6\%$  (n=41) and slowed the activation phase of the current (Fig. 1A, E). Conversely, the postpulse  $I_{Ca}$  was inhibited by only  $26.2 \pm 1.1\%$  and the activation phase remained rapid (Fig 1A). Similar results were obtained from neurons expressing EGFP alone (prepulse and postpulse  $I_{Ca}$  inhibitions of  $62.8 \pm 3.1$  and  $24.3 \pm 3.4\%$  (n=10), respectively). To determine the effects of expressing PDC on N-type  $Ca^{2+}$  channel modulation, a cDNA construct consisting of the PDC ORF was cloned into the mammalian expression vector pCI. PDC cDNA was injected into the nuclei of SCG neurons at concentrations ranging from 10–100 ng/ $\mu$ l. In 23 neurons co-injected with EGFP and PDC cDNA, application of 10  $\mu$ M norepinephrine decreased the pre- and postpulse  $I_{Ca}$  by  $56.8 \pm 3.3$  and  $24.1 \pm 2.1\%$ , respectively—values similar to control (Fig 1E). To

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ensure that PDC expression was occurring, the ORF of PDC was fused in-frame with EGFP and constructs injected as above. Fusion of EGFP to either termini of PDC results in small but significant decrease in channel modulation. Application of 10  $\mu$ M norepinephrine to EGFP-PDC or PDC-EGFP expressing neurons (10-100 ng/ $\mu$ L) resulted in  $54.2 \pm 3.5$  and  $54.3 \pm 2.8\%$  inhibition of prepulse  $I_{Ca}$ , respectively (Fig. 1C, E).

Expression of G $\beta\gamma$  binding proteins has been shown to reduce the N-type Ca<sup>2+</sup> current facilitation ratio in the absence of agonist. For example, expression of G $\alpha$  subunits (Ikeda, 1996; Jeong and Ikeda, 1999) or a myristoylated C-terminal constructs of G-protein coupled receptor kinase 2 (MAS-GRK2, Kammermeier and Ikeda, 1999) reduce the basal facilitation ratio. In neurons injected with 10-100 ng/ $\mu$ L of PDC cDNA, the mean basal facilitation ratio was  $1.19 \pm 0.03$ —a value significantly different from control neurons (Fig. 1D, one-way ANOVA, Dunnett's test). Similarly, expression of the EGFP fusion constructs reduced the basal facilitation ratio (summarized in Fig 1D).

In contrast, manipulations that increase free G $\beta\gamma$  result in a greater facilitation ratio. For example, activation of adrenergic receptors by agonists increases the ratio of post- to pre-conditioning pulse current amplitude. This maximal or agonist-induced facilitation ratio determined in the presence of 10  $\mu$ M norepinephrine was decreased by PDC expression (summarized in Fig 1F). The peak facilitation ratio increased to  $2.74 \pm 0.09$  and  $2.78 \pm 0.12$  in uninjected and EGFP-expressing cells, respectively, in the presence of agonist. These values averaged  $2.32 \pm 0.12$ ,  $2.22 \pm 0.16$ , and  $2.18 \pm 0.11$  in the PDC, EGFP-PDC and PDC-EGFP injection conditions, respectively. The relief of block following a strong depolarization (resulting in an increased facilitation ratio) and the slowed current activation time course are characteristic features of G $\beta\gamma$ -mediated voltage-dependent inhibition of Ca<sup>2+</sup> channels (reviewed by Jarvis and

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Zamponi, 2001).

**Phosducin expression produced a time-dependent decrease in voltage-dependent  $\text{Ca}^{2+}$  channel modulation.** Although PDC expression produced only modest effects on the maximal voltage-dependent inhibition of N-type  $\text{Ca}^{2+}$  channels by norepinephrine, more substantial effects were observed during prolonged agonist application. In control SCG neurons, a 5 minute continuous exposure to agonist (Fig. 2A) produced a sustained  $I_{Ca}$  inhibition that decreased gradually during the agonist application. The norepinephrine-mediated inhibition of the prepulse  $I_{Ca}$  decreased to  $41.9 \pm 2.1$  and  $39.9 \pm 4.5\%$  of initial values after 5 min of agonist exposure in uninjected (n=41) and EGFP-expressing (n=10) neurons, respectively (Fig. 2A). Expression of PDC, EGFP-PDC, or PDC-EGFP accelerated the rundown of prepulse  $I_{Ca}$  inhibition (example shown for PDC-EGFP in Figure 2B). At the fifth minute of drug application, the inhibition of  $I_{Ca}$  averaged  $28.4 \pm 3.4$ ,  $17.7 \pm 3.1$ , and  $23.2 \pm 2.5\%$ , in neurons injected with PDC (n=22), EGFP-PDC (n=20) and PDC-EGFP (n=25), respectively (summarized in Fig. 2C). In control neurons, the mean agonist facilitation ratio at the end of norepinephrine exposure was  $1.99 \pm 0.06$  and  $1.98 \pm 0.08$  in EGFP injected cells. The agonist facilitation ratio after 5-minute agonist exposure averaged  $1.52 \pm 0.07$ ,  $1.34 \pm 0.06$  and  $1.40 \pm 0.04$  in PDC (n=22), EGFP-PDC (n=20), and PDC-EGFP (n=25) expressing neurons (summarized in Fig. 2D). The decrease in agonist facilitation ratio during agonist application indicates a fading of the  $\text{G}\beta\gamma$ -mediated  $I_{Ca}$  inhibition process in PDC expressing neurons. Consistent with this, the prepulse current activated more rapidly in neurons expressing PDC at the end of the agonist application (data not shown).

**Expression of phosducin suppressed  $\text{Ca}^{2+}$  channel recovery from inhibition following agonist treatment.** It has been hypothesized that phosducin decreases the affinity of free  $\text{G}\beta\gamma$

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subunits with the plasma membrane thereby causing the heterodimer to dissociate from the plasma membrane (Lukov et al., 2004). If these events occur, then voltage-dependent inhibition of  $\text{Ca}^{2+}$  channels would be expected to diminish upon re-application of agonist. To probe this question, SCG neurons were exposed to norepinephrine for 5 minutes, washed with agonist free solution for 3 minutes, and then re-challenged with norepinephrine (Fig. 2E). During the second agonist application, the mean peak inhibition was  $55.6 \pm 1.9\%$  in control cells (n=21),  $58.8 \pm 4.0\%$  in EGFP injected cells (n=8),  $29.1 \pm 4.2\%$  in PDC injected cells (n=17),  $34.4 \pm 3.8\%$  in EGFP-PDC injected cells (n=13), and  $35.0 \pm 2.9\%$  in PDC-EGFP injected cells (n=18). In all PDC expression conditions, a statistically significant difference was observed in comparison to control measurements (ANOVA,  $p < 0.01$ , Dunnett's test). While control neurons nearly returned to peak inhibition conditions, neurons expressing PDC displayed partial recovery of inhibition after agonist washout (Fig. 2F).

**Phosducin-like protein affects N-type  $\text{Ca}^{2+}$  channel modulation mediated by  $\alpha_2$ -adrenoceptors.** PDCL is ~65% homologous with PDC and binds the  $\text{G}\beta\gamma$  heterodimer *in vitro* (Schroder et al., 1996). However, unlike PDC, PDCL has a more widespread expression pattern thus making it a candidate for modulating GPCR signaling in neurons. At present, it is unclear whether PDCL impacts GPCR mediated inhibition of  $I_{\text{Ca}}$ . Therefore, SCG neurons were injected with PDCL cDNA and N-type  $\text{Ca}^{2+}$  channel modulation was tested. Following expression of PDCL, peak  $I_{\text{Ca}}$  inhibition was unaffected. In neurons injected with 10–50 ng/ $\mu\text{l}$  pCI-PDCL cDNA, the mean prepulse inhibition was  $51.7 \pm 3.1\%$  (n=16, example in Fig. 3B) vs.  $55.1 \pm 2.5\%$  (n=32) for control neurons (Fig. 3A, D). The mean peak facilitation ratio during agonist application was also not altered by PDCL expression ( $2.28 \pm 0.10$  vs.  $2.00 \pm 0.13$  for control and PDCL-expressing neurons, respectively, ANOVA,  $p > 0.05$ ). In contrast, the mean basal

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facilitation ratio was decreased by PDCL expression (Fig. 3C). In control neurons, the mean basal facilitation ratio was  $1.21 \pm 0.02$  while in PDCL injected neurons this value decreased to  $1.12 \pm 0.02$  ( $p < 0.05$ , ANOVA). As with expression of PDC, PDCL caused a more rapid disinhibition of  $I_{Ca}$  in the continued presence of agonist (Fig. 3B). Following 5 minutes of exposure to  $10 \mu\text{M}$  norepinephrine, the peak prepulse inhibition averaged  $31.5 \pm 2.2\%$  ( $n=24$ ) in control cells while in those neurons expressing PDCL, peak inhibition averaged  $22.1 \pm 2.7\%$  ( $n=13$ , Fig. 3E). The corresponding mean facilitation ratio at the end of the drug application averaged  $1.56 \pm 0.04$  in control cells and  $1.25 \pm 0.04$  in PDCL-injected cells. As with PDC, expression of PDCL maintained suppression of inhibition upon re-challenging neurons with  $10 \mu\text{M}$  norepinephrine. During the re-challenging period, seventeen control neurons were inhibited by  $41.0 \pm 3.1\%$ , while in nine neurons expressing PDCL,  $I_{Ca}$  was inhibited by  $25.8 \pm 4.7\%$ . PDCL fusion proteins at the amino and carboxyl terminus with EGFP were also made and injected into SCG neurons. In six neurons injected with PDCL-EGFP, peak  $I_{Ca}$  inhibition by  $10 \mu\text{M}$  norepinephrine averaged  $62.5 \pm 4.8\%$ . In four neurons injected with the EGFP-PDCL, this value averaged  $59.8 \pm 6.1\%$ .

**Phosducin like-protein, but not phosducin, is endogenously expressed in SCG neurons.** To determine if PDC or PDCL was endogenously expressed in SCG neurons, Western blot analyses were performed. The affinity purified polyclonal antibodies against PDC (anti-PDC) and the N-terminus of PDCL (anti-PDCL) were a gift from Dr. Barry M. Willardson (Brigham Young University, Provo, UT) and have been previously characterized (Thulin et al., 1999). In HEK-293 cells transfected with PDC cDNA, the anti-PDC antibody recognized a protein with an apparent molecular weight of  $\sim 31$  kDa (Fig. 4A, lane 3), consistent with the expected size of the PDC protein (Thulin et al., 1999). An immunoreactive band corresponding to PDC was not

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detected in protein prepared from SCG neurons or HEK-293 cells mock transfected with an empty expression vector (Fig. 4A, lanes 1–2). As a test for antibody specificity, HEK-293 cells were transfected with a plasmid encoding PDCL. The anti-PDC antibody weakly detected a protein with an apparent molecular weight of ~42 kDa probably corresponding to overexpression of PDCL (Fig. 4A, lane 4). This weak cross-reactivity of anti-PDC for PDCL is in agreement with the previous characterization of the antibody (Thulin et al., 1999). Similar experiments performed on protein prepared from SCG neurons using an anti-PDCL antibody revealed two immunoreactive bands with apparent molecular weights of ~42 and ~40 kDa (Fig. 4B, lane 1). The positions of these two immunoreactive proteins are consistent with the expected sizes of the PDCL protein as seen by, and discussed in, Thulin et al. (1999). Immunoreactivity of comparable size was detected in HEK cells transfected with an empty expression vector or expression vectors containing PDC or PDCL (Fig 4B, lanes 2–4). In contrast to the anti-PDC experiment, and, despite weak immunoreactivity of the anti-PDCL for PDC during characterization of the antibody by Thulin et al. (1999), a 31 kDa PDC band was not observed in our system upon overexpression of PDC in HEK-293 cells. However, the specificity of the anti-PDCL is supported by the observation that in Figure 4B (lane 4) the HEK-293 cells overexpressing the PDCL protein clearly display a larger amount of antigen detected under identical conditions as HEK cell untransfected or transfected with PDC. If the antibodies were not specific for PDCL the same level of immunoreactive product would be present in all lanes loaded with protein from HEK-293 cells. Overall, these results suggest that PDCL is endogenously expressed in HEK-293 cells and SCG neurons.

The subcellular distribution of heterologously expressed PDC and PDCL were examined using confocal microscopy. Injection of either the amino- (EGFP-PDC) or the carboxyl terminus



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(PDC-EGFP) fusion constructs (10–100 ng/ml) resulted in green fluorescence throughout the cytoplasm and nucleus of SCG neurons (Figure 4C). Upon expression of PDCL-EGFP protein, green fluorescence was observed within the cytoplasm but restricted from the nucleus in both SCG neurons (Fig 4D) and HEK-293 cells (data not shown).

**Phosducin and phosducin like-protein expression disinhibited voltage-dependent  $I_{Ca}$  mediated by mGlu<sub>2</sub> activation.** To test the generality of PDC and PDCL effects on Ca<sup>2+</sup> channel modulation, we examined voltage-dependent inhibition mediated by metabotropic glutamate receptor type 2 (mGlu<sub>2</sub>), a type III GPCR. The mGlu<sub>2</sub> receptor couples to G $\alpha_{i/o}$  family of proteins and has been shown to inhibit N-type Ca<sup>2+</sup> channels in a voltage-dependent fashion that involves G $\beta\gamma$  in SCG neurons (Kammermeier et al., 2003). Heterologous expression of mGlu<sub>2</sub> in SCG neurons produces a robust inhibition upon glutamate application that is consistent and rapidly reversible.

The amplitude of  $I_{Ca}$  recorded from uninjected neurons were minimally affected by application of 100  $\mu$ M L-glutamate ( $2.9 \pm 1.1\%$  inhibition, n=11) supporting previous studies showing that SCG neurons do not express functional mGlu receptors. Prior to agonist application, the basal facilitation ratio averaged  $1.14 \pm 0.02$ ,  $1.03 \pm 0.02$  and  $1.12 \pm 0.03$  in mGlu<sub>2</sub> (n=14), mGlu<sub>2</sub>/PDC (n=10) and mGlu<sub>2</sub>/PDCL (n=13) conditions, respectively (mGlu<sub>2</sub>/PDC statistically different at  $p < 0.01$  from both mGlu<sub>2</sub> and mGlu<sub>2</sub>/PDCL, ANOVA with Dunnett's *post hoc* test). Following application of 100  $\mu$ M L-glutamate to neurons previously injected with pCI-mGlu<sub>2</sub> cDNA (50 ng/ $\mu$ l), prepulse  $I_{Ca}$  was inhibited by  $64.5 \pm 2.8\%$  (n=14; Fig. 5A). In the continued presence of L-glutamate for 5 minutes,  $I_{Ca}$  inhibition decreased to  $41.2 \pm 1.9\%$ ; a result similar to that seen with endogenous  $\alpha_2$ -adrenoceptor activation. PDC or PDCL cDNA (25–50 ng/ $\mu$ l) was co-injected with the mGlu<sub>2</sub> cDNA and EGFP. When mGlu<sub>2</sub> and PDC

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were co-expressed, the mean peak prepulse inhibition was  $61.1 \pm 2.5\%$  (n=10) and decreased to  $22.7 \pm 3.6\%$  in the continued presence of L-glutamate for 5 minutes (Fig 5D, E). When mGlu<sub>2</sub> and PDCL were co-expressed, peak  $I_{Ca}$  inhibition averaged  $51.4 \pm 3.1\%$  (n=13) and decreased to  $17.0 \pm 3.1\%$  by the fifth minute of agonist exposure (Fig. 5D, E). The decrease in the peak inhibition was significantly different when comparing mGlu<sub>2</sub> alone expressing neurons with mGlu<sub>2</sub>/PDCL expressing neurons (ANOVA, Dunnett's test  $p < 0.05$ , Fig 5D). The corresponding facilitation ratios at the end of the L-glutamate application averaged  $1.62 \pm 0.04$ ,  $1.20 \pm 0.05$  and  $1.24 \pm 0.04$  in the mGlu<sub>2</sub>, mGlu<sub>2</sub>/PDC and mGlu<sub>2</sub>/PDCL conditions, respectively (ANOVA, Dunnett's test,  $p < 0.001$  for both experimental conditions).

**Phosducin and phosducin like-protein delay voltage dependent inhibition mediated by direct G-protein activation.** The accelerated disinhibition kinetics shown above could result from PDC or PDCL acting directly on the receptor, e.g. desensitization, or coupling to the G-protein. To examine this possibility, GppNHp, a non-hydrolyzable analog of GTP, was included in the patch pipette solution thereby bypassing receptor activation as the initiator of modulation. Because GppNHp traps the G $\alpha$  subunit in an activated state, G $\beta\gamma$  subunits are free to interact with effectors such as Ca<sup>2+</sup> channels. The normal internal solution containing 300  $\mu$ M GTP did not alter the pre or post conditioned Ca<sup>2+</sup> current amplitude over 8–10 minutes of recording (data not shown). Conversely, inclusion of GppNHp (500  $\mu$ M) in the patch pipette resulted in a decrease in  $I_{Ca}$  amplitude, slowing of current activation, and increase in facilitation ratio within minutes of patch rupture (Fig. 6). Shown in Figure 6A are the time courses of facilitation ratio enhancement by GppNHp in control, PDC and PDCL expressing neurons. PDC and PDCL expression delays the onset of  $I_{Ca}$  inhibition. These data are consistent with PDC and PDCL acting in the absence of receptor activation.

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## Discussion

Here we provide evidence that: 1) PDC and PDCL attenuate  $G\beta\gamma$ -mediated N-type  $Ca^{2+}$  channel modulation during prolonged treatment with agonist while having minimal impact on short-term modulation; 2) both natively and heterologously expressed GPCR functions are modified by PDC and PDCL expression; 3) encumbering either the N- or C-terminus of PDC and PDCL with EGFP does not interfere with function; and 4) PDCL, but not PDC, is expressed in sympathetic ganglia. The motivations underlying the study were to provide : 1) information on the suitability of PDC or PDCL as the basis for a universal optical sensor of heterotrimeric G-protein activation by GPCRs, and 2) insight into possible physiological roles for PDC/PDCL modification of GPCR responses in neurons.

A protein-based optical sensor for G-protein activation would facilitate investigation into the temporal and spatial aspects of GPCR function in living cells and provide the basis for high-throughput drug screening. To be widely applicable across a broad spectrum of GPCRs, a suitable sensor should detect an event universal to G-protein activation rather than rely on interactions with downstream effectors specific to G-protein families. For example, changes in intracellular  $[Ca^{2+}]$  or  $[cAMP]$  are associated with the  $G_{q/11}$  and  $G_s/G_i$  families, respectively, and thus cannot be used as a universal monitor of GPCR activity without heterologously expressing chimeric  $G\alpha$  or providing  $G\alpha$ s such as  $G\alpha_{15/16}$  that promiscuously couple to receptors. We thus focused on the initial event in G-protein activation, dissociation of the  $G\alpha$  from the  $G\beta\gamma$  subunit. Although it is unclear whether the classical view of  $G\alpha$ -GTP and  $G\beta\gamma$  dissociating into “free” subunits is correct (Bünemann et al., 2003), there is general agreement that activation of heterotrimeric G-proteins involves both conformational changes resulting from GTP binding to  $G\alpha$  and exposure of regions of  $G\beta\gamma$  previously masked by tight association with  $G\alpha$ . Thus, a

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G $\beta\gamma$ -binding protein that bound to regions of G $\beta\gamma$  exposed upon receptor activation could detect activation of a wide variety of GPCRs.

Of the G $\beta\gamma$ -binding proteins for which a high resolution structures are available (PDC, G $\alpha$ , and GRK2), PDC was attractive for several reasons. First, PDC consists of two independent domains (Savage et al., 2000) that bind to distinct surfaces of G $\beta$  (Gaudet et al., 1996, 1999; Loew et al., 1996). The PDC N-terminus interacts with the top of the G $\beta$  propeller sharing many residues normally masked by G $\alpha$ -GDP in the heterotrimeric state. The C-terminus of PDC resembles a thioredoxin domain and interacts with residues on the side of the G $\beta$  propeller distinct from any of those utilized by G $\alpha$ -GDP. The two-domain structure would facilitate two common strategies used to develop protein-based optical sensors: fluorescence resonance energy transfer (FRET; see review by Miyawaki, 2003) and protein complementation (Hu and Kerpolla, 2003). Both strategies rely on bringing separate protein moieties (two different fluorescent proteins or two halves of the same fluorescent protein, respectively) into close proximity upon target binding. Knowledge of the PDC-G $\beta\gamma$  structure provides a rational basis for engineering a sensor using techniques such as circular permutation (Nagai et al., 2001) to optimize the proximity of fluorophores or protein domains upon PDC binding to G $\beta\gamma$ . Second, PDC, although interacting natively only with transducin G $\beta_1\gamma_1$ , binds to numerous different G $\beta\gamma$  combinations *in vitro* (Müller et al., 1996) suggesting the ability to modify activation by numerous G-protein families. Third, the affinity of PDC and PDCL for G $\beta\gamma$  is altered by phosphorylation of identified residues (Gaudet et al., 1999; Thulin et al., 2001; Humrich et al., 2003) thus providing a rational basis for fine-tuning the interaction.

To evaluate how heterologous expression of PDC and PDCL impacted GPCR function in neurons, N-type Ca<sup>2+</sup> channel function in sympathetic neurons was examined. In this system,

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voltage-dependent  $\text{Ca}^{2+}$  channel modulation via  $\text{G}\beta\gamma$  following GPCR activation has been well characterized (e.g., Ikeda, 1996) and the effects of  $\text{G}\alpha$ -GDP and the carboxyl-terminus of GRK2 (ctGRK2), two other  $\text{G}\beta\gamma$ -binding proteins, documented (Jeong and Ikeda, 1999; Kammermeier and Ikeda, 1999). The finding that PDC and PDCL had little effect on basal facilitation (an indicator of tonic  $\text{G}\beta\gamma$ -mediated  $\text{Ca}^{2+}$  channel inhibition) or maximal voltage-dependent inhibition of N-type  $\text{Ca}^{2+}$  channels (Fig. 1) was unexpected as heterologous expression of  $\text{G}\alpha$ s and ctGRK2 greatly attenuated peak inhibition. A possible explanation for this difference is that PDC and PDCL are cytosolic proteins whereas the latter molecules were targeted to the plasma membrane (the ctGRK used previously was targeted to the membrane via an N-terminal myristoylation sequence; Kammermeier and Ikeda, 1999). In support of this idea, Rishal et al. (2005) found that targeting PDC to the membrane (via a myristoylation sequence) increased the ability of PDC to modify basal and GPCR-activated GIRK-type  $\text{K}^+$  channels in *Xenopus* oocytes. Preliminary data from our laboratory using analogous constructs resulted in similar augmented effects on  $\text{Ca}^{2+}$  channels in sympathetic neurons (unpublished observation). Prolonged application of agonist indicate that PDC and PDCL function like other  $\text{G}\beta\gamma$  buffers but require more time before substantial effects are realized (Figs. 2, 3). Schulz et al. (1998) describe a mechanism whereby a PDC-EGFP fusion protein concentrates at the plasma membrane within 2–3 minutes of prostaglandin receptor stimulation in NG108 neuroblastoma cells—a result similar to the time course we observed in SCG neurons. The lack of an immediate effect on  $\text{Ca}^{2+}$  channel modulation provides evidence that heterologously expressed PDC and PDCL were not competing with  $\text{G}\alpha$ -GDP for  $\text{G}\beta\gamma$  and thereby uncoupling GPCRs from G-proteins prior to receptor stimulation. Such an effect would decrease the signal from an optical sensor as a significant fraction might be bound to  $\text{G}\beta\gamma$  prior to receptor stimulation.

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Fusing EGFP to either the N- or C-terminus of PDC/PDCL (Figs 1–3) had no discernable effect when compared with wild-type constructs. Thus, as predicted from high resolution structures, these regions do not seem to participate in the binding of PDC/PDCL to G $\beta\gamma$ . In addition, the steric bulk introduced by fusing EGFP did not seem to hinder accessibility to binding surfaces of G $\beta\gamma$  exposed following receptor stimulation. Since ECFP and EYFP variants represent the current choice for genetically encoded FRET-based sensors, the absence of effects following EGFP fusion suggest a compatibility with this approach. The subcellular fluorescence patterns observed with PDC or PDCL fusion proteins to EGFP (Fig. 4C, D) were consistent with PDCL, but not PDC, forming a complex with sufficient mass to be excluded from the nucleus. In regard to this observation, PDCL (but not PDC) was recently shown to complex with a chaperone, TCP1 $\alpha$ , which regulates the assembly of the G $\beta\gamma$  dimer (Lukov et al., 2005; Humrich et al., 2005). This finding makes PDCL a less desirable candidate for development as an optical sensor.

The generality of PDC and PDCL actions was tested by heterologously expressing a class III GPCR, the mGlu<sub>2</sub> receptor. In general, disinhibition of Ca<sup>2+</sup> channel modulation was similar to that observed with natively expressed class I  $\alpha_2$ -adrenoceptors (Fig. 5). This suggests that PDC and PDCL can generally influence GPCR mediated signaling—at least in regard to receptors that preferentially couple to G<sub>i/o</sub> proteins. It is unclear whether  $\alpha_2$ -adrenoceptors and mGlu<sub>2</sub> receptors couple to distinct G $\beta\gamma$  isoforms. However, these results are consistent with biochemical studies indicating that PDC can bind numerous G $\beta\gamma$  combinations (Müller et al., 1996). The results of the re-challenging experiments (Fig. 2E, F) demonstrate that the effects of PDC and PDCL did not readily reverse. This characteristic could decrease the temporal resolution of a sensor but provide some advantage for steady-state measurements. It has been

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suggested that PDC renders  $G\beta\gamma$  soluble by opening a binding pocket in the  $G\beta$  propeller that engulfs the prenyl group attached to  $G\gamma$  (Lukov et al., 2004). Thus the long term effects of PDC and PDCL may utilize such a mechanism to prevent the re-association of  $G\beta\gamma$  with  $G\alpha$  although our data do not specifically address this mechanism. The effects of PDC and PDCL on direct activation of G-proteins (Fig. 6) rule out receptor desensitization as the sole mechanism for these effects.

A second goal of these studies was to evaluate potential roles for PDCL in modulating G-protein mediated ion channel modulation. Western blot analysis indicated that PDCL, but not PDC, is expressed in sympathetic ganglia (Fig. 4) consistent with the ubiquitous expression pattern of the former and more restricted expression pattern (i.e., retina and pineal) of the latter. Although the effects of PDCL expression mimicked those of PDC, recent studies indicate the PDCL is involved in the assembly of  $G\beta$  with  $G\gamma$  (Humrich et al., 2005; Knol et al., 2005; Lukov et al., 2005). Thus, although our results are compatible with PDCL playing an analogous role in neurons to PDC in the retina, it seems likely that PDCL subserves a more restricted role. For example, siRNA directed against PDCL mRNA reduces  $G\beta\gamma$  protein expression (Lukov et al., 2005). Within this context,  $G\beta\gamma$  might be expected to be up regulated when PDCL is over-expressed in SCG neurons. However, the decrease in basal facilitation ratio observed following PDCL expression (Fig. 1) suggests this does not occur on the time scale of our experiments (~24 h).

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## Figure Legends

**Fig. 1. Effect of phosducin expression on N-type  $\text{Ca}^{2+}$  channel modulation in sympathetic neurons.** *A*, superimposed  $\text{Ca}^{2+}$  current ( $I_{Ca}$ ) traces from an uninjected control neuron recorded using the whole-cell patch-clamp technique.  $I_{Ca}$  was elicited from a holding potential of  $-80$  mV using the voltage protocol illustrated (*bottom*) in the absence (*con*) or presence of  $10 \mu\text{M}$  norepinephrine (*NE*).  $I_{Ca}$  elicited by the first test pulse to  $+10$  mV are denoted *prepulse* currents (*pre*). Following the depolarizing conditioning pulse to  $+80$  mV,  $I_{Ca}$  elicited by the subsequent test pulse are denoted *postpulse* currents (*post*). Facilitation ratio (*FR*), a measure of  $\text{G}\beta\gamma$ -mediated  $I_{Ca}$  modulation, was determined as the post:pre  $I_{Ca}$  amplitude ratio (see lower dashed line). Capacitance transient and peak tail currents have been truncated. The zero current level in this and subsequent figures is denoted by a dashed line. Vertical and horizontal calibration were  $0.5$  nA and  $20$  ms, respectively. *B*, superimposed sample currents from neurons expressing phosducin (*PDC*) in absence (*con*) or presence (*NE*) of  $10 \mu\text{M}$  norepinephrine. *C*, representative  $I_{Ca}$  traces from neurons expressing EGFP-PDC in the absence (*con*) or presence (*NE*) of  $10 \mu\text{M}$  norepinephrine. *D*, bar graph summary of the mean ( $\pm$ SEM) facilitation ratio in the absence of agonist (termed *basal FR*). *E*, summary bar graph of mean ( $\pm$ SEM) prepulse  $I_{Ca}$  inhibition evoked by norepinephrine. *F*, bar graph summary of the mean ( $\pm$ SEM) peak facilitation ratio determined in the presence of norepinephrine (termed *agonist FR*). The open and filled bars represent basal facilitation ratio from uninjected and EGFP-expressing neurons. The filled gray bars represent basal facilitation ratio from neurons expressing the indicated PDC constructs. Asterisks above bars in this and subsequent figures indicates significance of  $p < 0.05$ (\*) or  $p < 0.01$ (\*\*) compared with control responses as determined using one-way ANOVA and Dunnett's *post hoc* test. Numbers in parentheses denote the number of neurons tested.

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**Fig. 2. Effects of phosducin during prolong agonist application.** *A,B* time courses of  $I_{Ca}$  amplitude during a five minute agonist exposure. *Top*, the filled and open circles represent the prepulse and postpulse  $I_{Ca}$  amplitude, respectively. The solid bar indicates the duration of norepinephrine exposure. *Bottom*, the facilitation ratio (open squares) plotted as a function of time. Neurons were either uninjected (*A*) or injected with PDC-EGFP cDNA (*B*). Note the more rapid decay of  $I_{Ca}$  inhibition and facilitation ratio in *B*. *C*, summary bar graph of mean (+SEM)  $I_{Ca}$  inhibition following 5 minutes of continuous norepinephrine (10  $\mu$ M) application. *D*, bar graph summary of mean facilitation ratio (+SEM) following 5 minutes of norepinephrine exposure. Bar graph shading as in Fig. 1. *E,F* time courses of  $I_{Ca}$  amplitudes during a five minute and re-challenge agonist exposure. *Top*, prepulse (*filled circles*) and postpulse (*open circles*)  $I_{Ca}$  amplitudes from an uninjected neuron (*E*) and a neuron expressing EGFP-PDC (*F*). Solid bars represent the two periods of norepinephrine (10  $\mu$ M) application. *Bottom*, time course of facilitation ratio change.

**Fig. 3. Effects of PDCL expression on N-type  $Ca^{2+}$  channel modulation.** *A–B*, *Top*, superimposed  $I_{Ca}$  traces corresponding to time points denoted by lower case letters (*a–c*, *middle*). Vertical and horizontal calibration are 0.5 nA and 20 ms, respectively. *Middle*, time course of prepulse (*filled circles*) and postpulse (*open circles*)  $I_{Ca}$  amplitude during prolonged norepinephrine (10  $\mu$ M) application (*solid bar*) to a control (*A*) or PDCL-expressing (*B*) neuron. Lower case letters above plot correspond in time to  $I_{Ca}$  traces shown to the *top*. *Bottom*, the facilitation ratio (*open squares*) plotted as a function of time. Neurons were either uninjected (*A*) or injected with PDCL cDNA (*B*). *C–E*, bar graph summaries of mean (+SEM) basal facilitation



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ratio (C),  $I_{Ca}$  inhibition at the start of (D) or following 5 min exposure to norepinephrine (E). Statistical significant difference in (C,E) was determined using unpaired Student's t-test.

**Fig. 4. Native expression and subcellular localization of PDC and PDCL in sympathetic neurons.** A–B, Western blot of Triton X-100 soluble crude protein prepared from SCG neurons and HEK-293 cells probed with a rabbit anti-PDC (A) or rabbit anti-PDCL (B) antibody. An equivalent amount of protein (20  $\mu$ g) was analyzed from SCG neurons, mock transfected HEK-293 cells or HEK-293 cells transfected with pCI-PDC or pCI-PDCL cDNA as indicated (*left to right*). The mobility of molecular weight standard proteins (kDa) is indicated by *horizontal bars* to the left of each blot. C–D, images of SCG neurons expressing PDC-EGFP (C) or PDCL-EGFP (D). Scale bar corresponds to 10  $\mu$ m.

**Fig. 5. Phosducin and phosducin like-protein attenuate mGlu<sub>2</sub>-mediated voltage-dependent inhibition of Ca<sup>2+</sup> channels.** (A–C) *top*, superimposed  $I_{Ca}$  recordings from neurons co-injected with EGFP and (A) mGlu<sub>2</sub>, (B) mGlu<sub>2</sub>/PDC, and (C) mGlu<sub>2</sub>/PDCL, in the absence (*con*) or presence (*glu*) of L-glutamate (100  $\mu$ M). Letters adjacent to traces (*a–c*) denote time during which traces were sampled as annotated on the plots below. Horizontal and vertical calibration (A–C) are 0.5 nA and 20 ms, respectively. *Middle*, time courses of  $I_{Ca}$  amplitude during a five minute exposure to L-glutamate (*solid bar*). The filled and open circles represent the prepulse and postpulse  $I_{Ca}$  amplitude, respectively. Lower case letters correspond to  $I_{Ca}$  traces. *Bottom*, the facilitation ratio (*open squares*) plotted as a function of time. D,E, bar graph summaries of the mean (+SEM)  $I_{Ca}$  inhibition mediated by 100  $\mu$ M L-glutamate at the peak (D, *time “b”*) and after

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five minutes of exposure to agonist (*E*, time “*c*”) for mGlu<sub>2</sub> (*open*), mGlu<sub>2</sub>/PDC (*light gray*), or mGlu<sub>2</sub>/PDCL (*dark gray*) expressing neurons.

**Fig. 6. Phosducin and phosducin like-protein delay the onset of GppNHp-induced Ca<sup>2+</sup> current facilitation.** *A*, facilitation ratio time courses of five uninjected neurons (*open circles*) and five neurons injected with PDC or PDCL cDNA (*filled circles*) dialyzed with 500 μM GppNHp from the patch pipette. facilitation ratio was determined at 10 s intervals following rupture of the cell membrane. Lower case letters (*a–d*) correlate to *I*<sub>Ca</sub> traces obtained at the indicated times for a control neuron (*B*) and a neuron injected with PDC cDNA (*C*). Vertical and horizontal calibration are 1 nA and 20 ms, respectively.

Figure 1

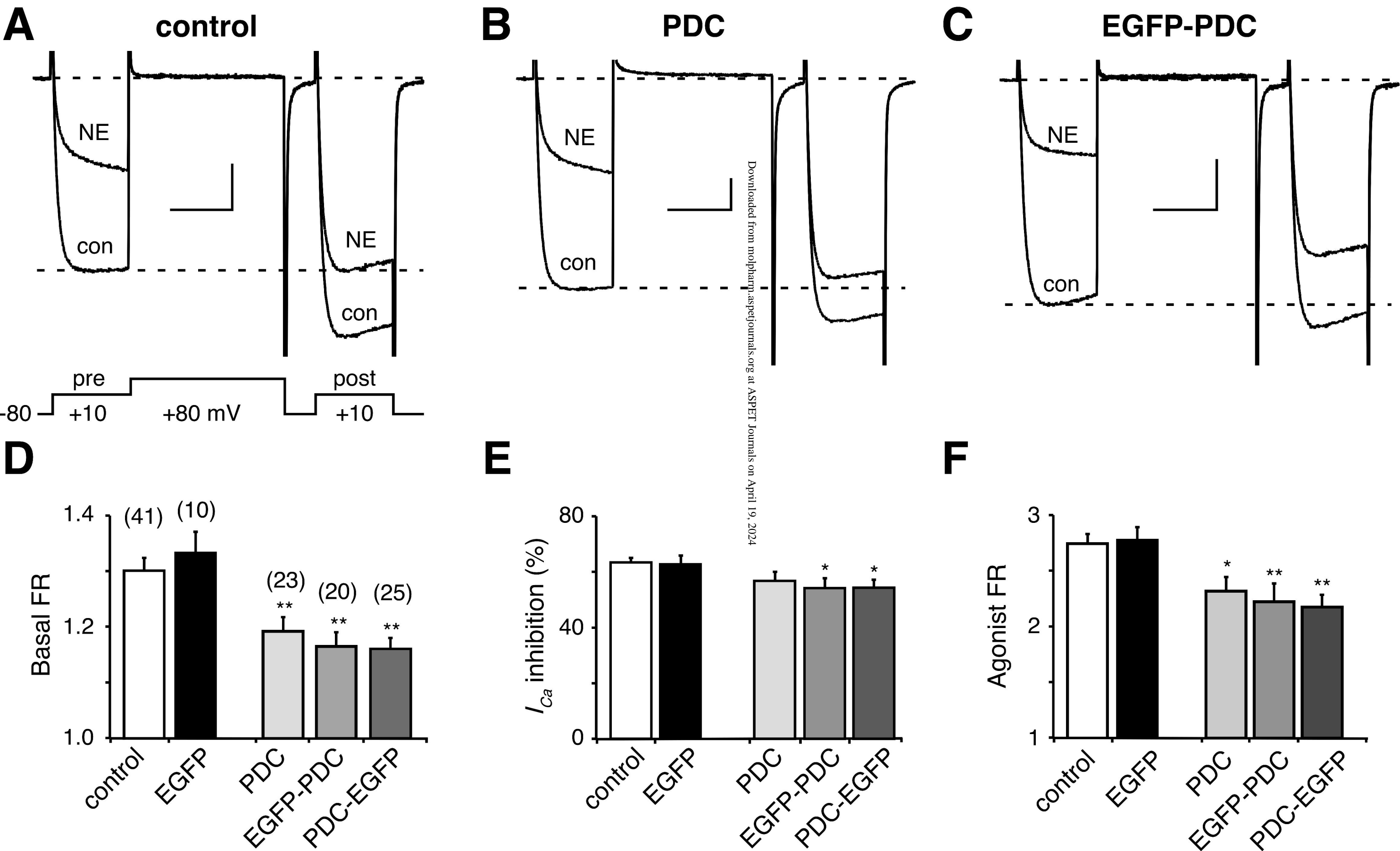
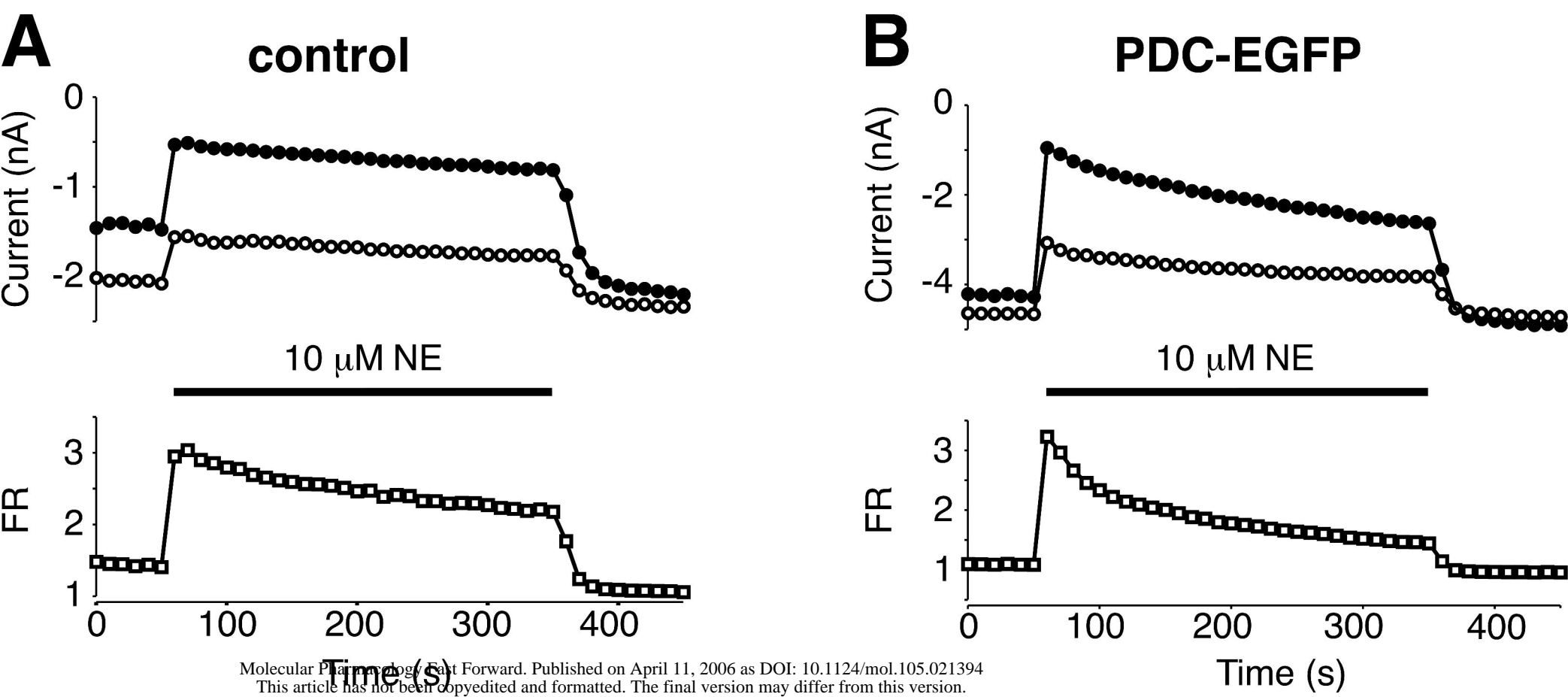
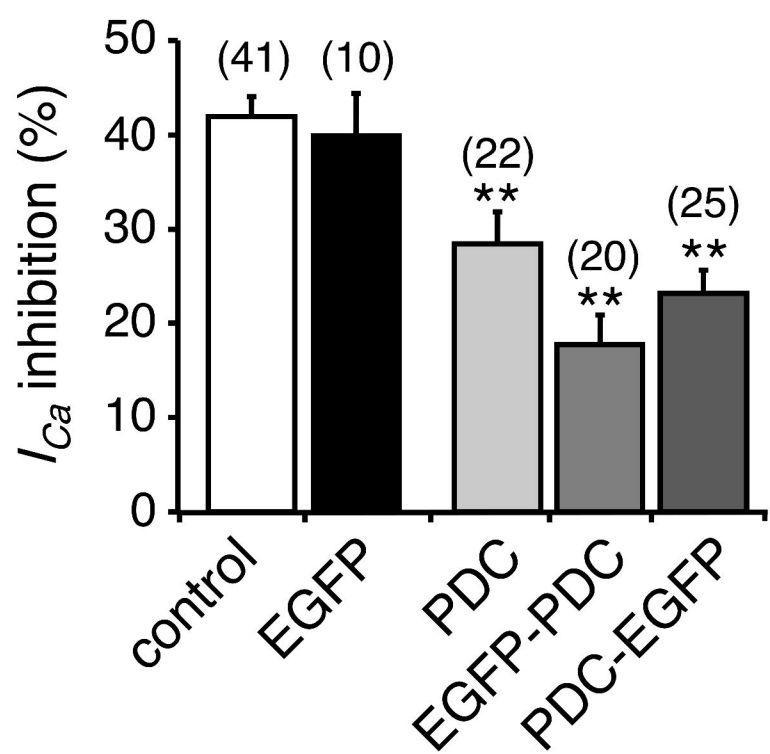


Figure 2



**C @ 5 min of agonist**



**D @ 5 min of agonist**

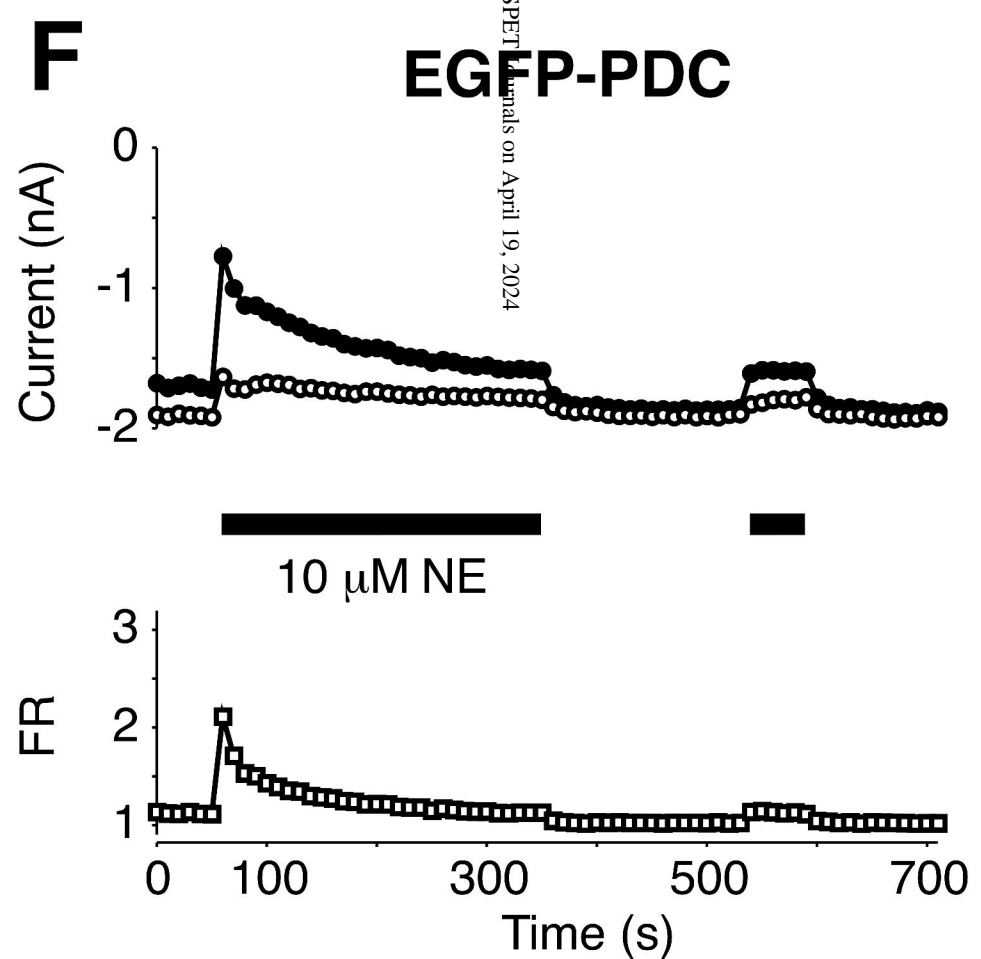
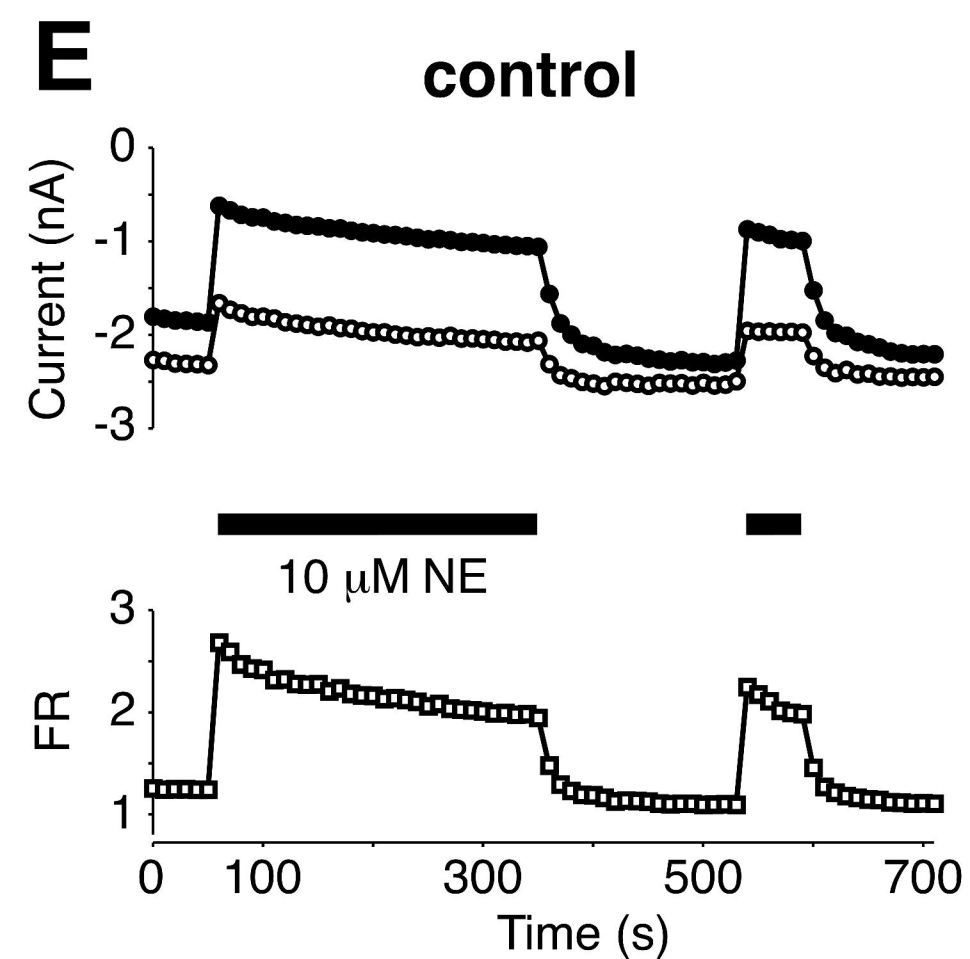
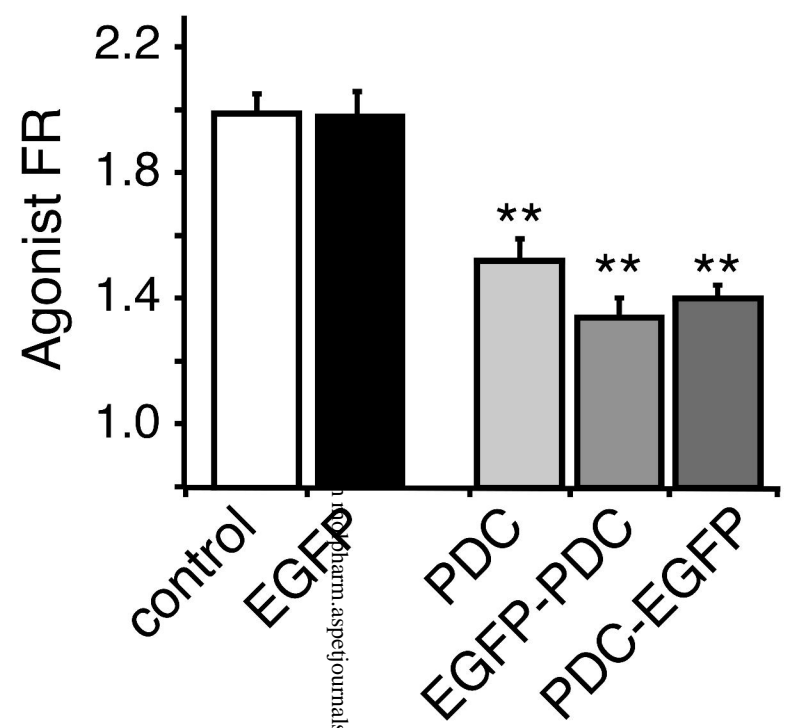
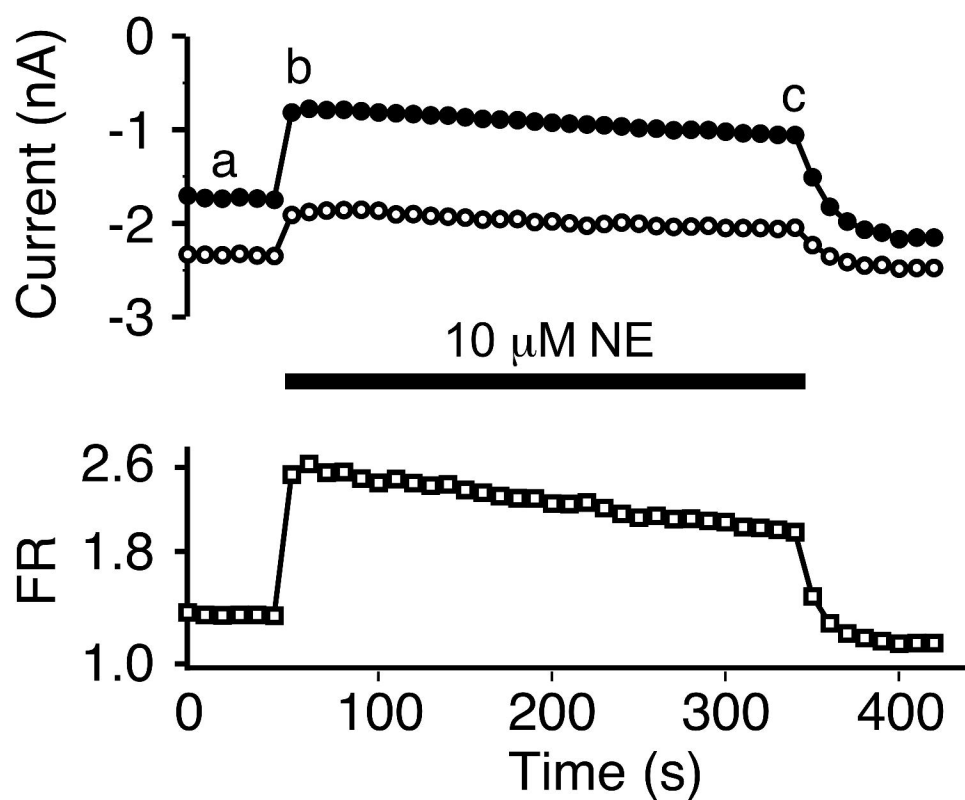
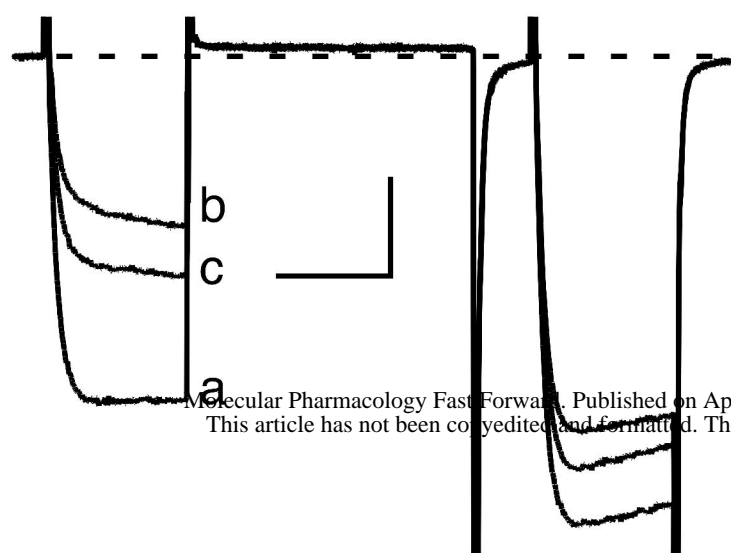


Figure 3

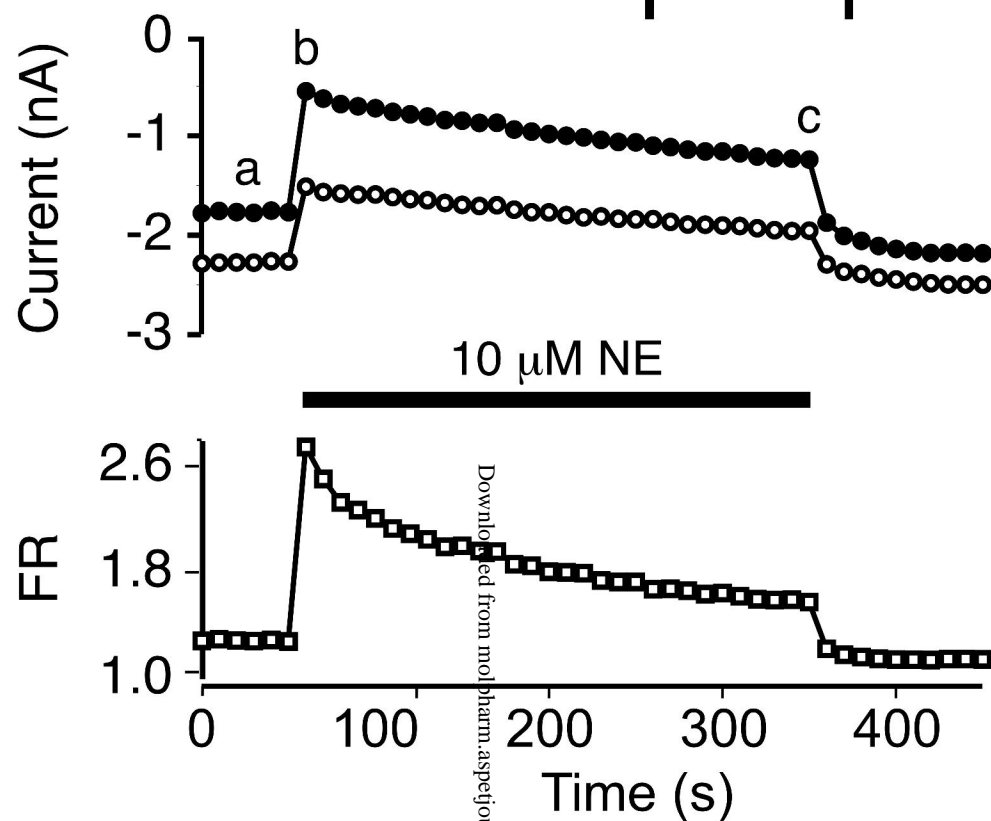
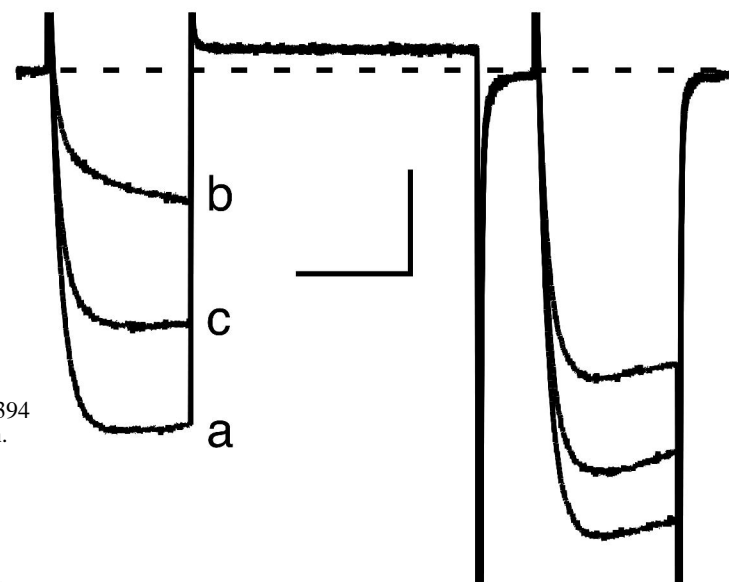
**A**

**control**

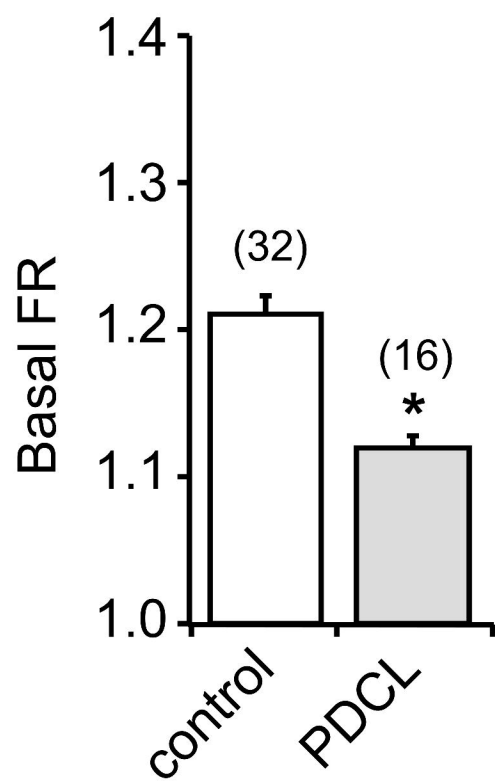


**B**

**PDCL**

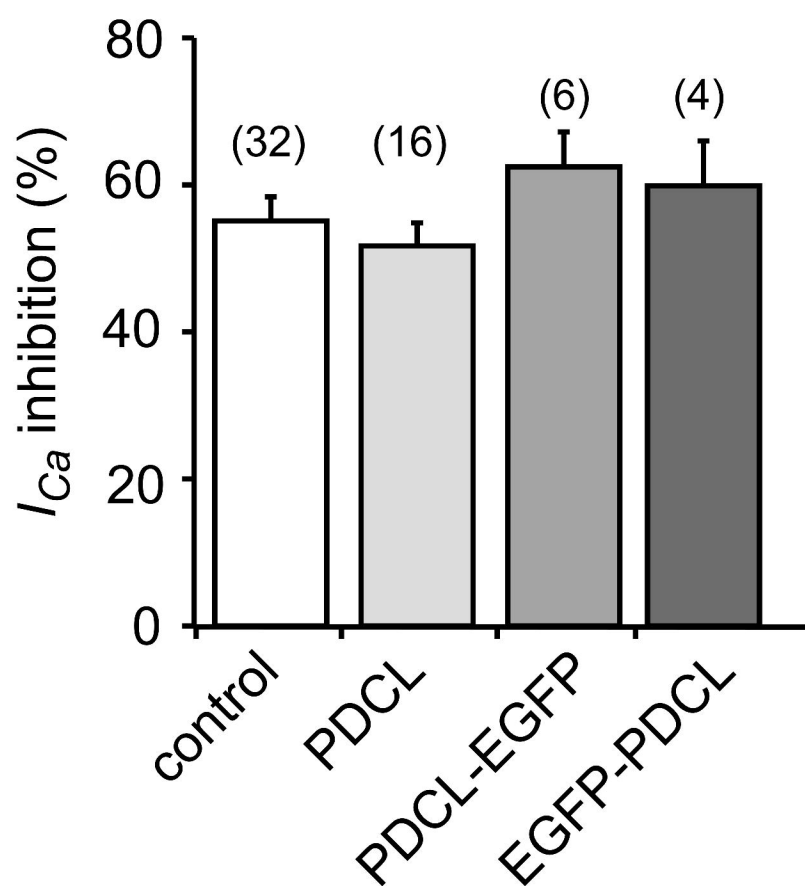


**C**



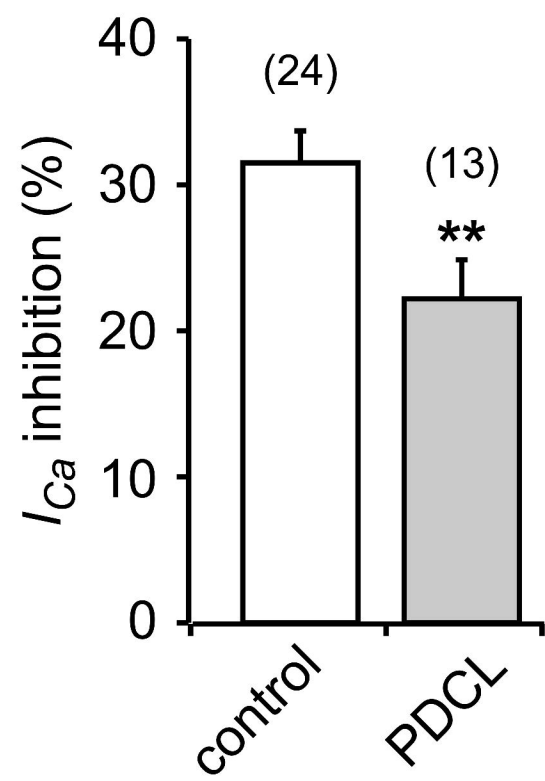
**D**

**peak**



**E**

**5 min**



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Figure 4

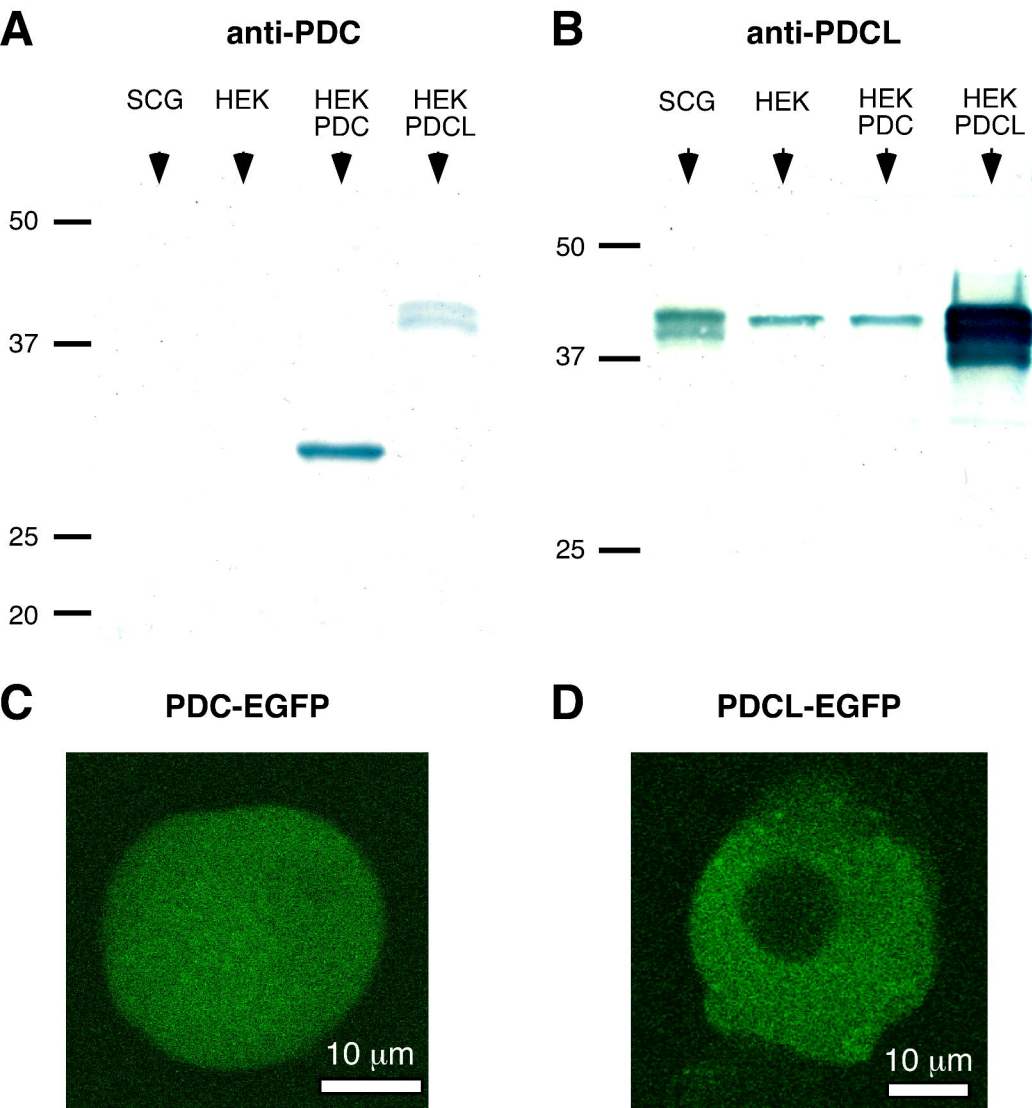


Figure 5

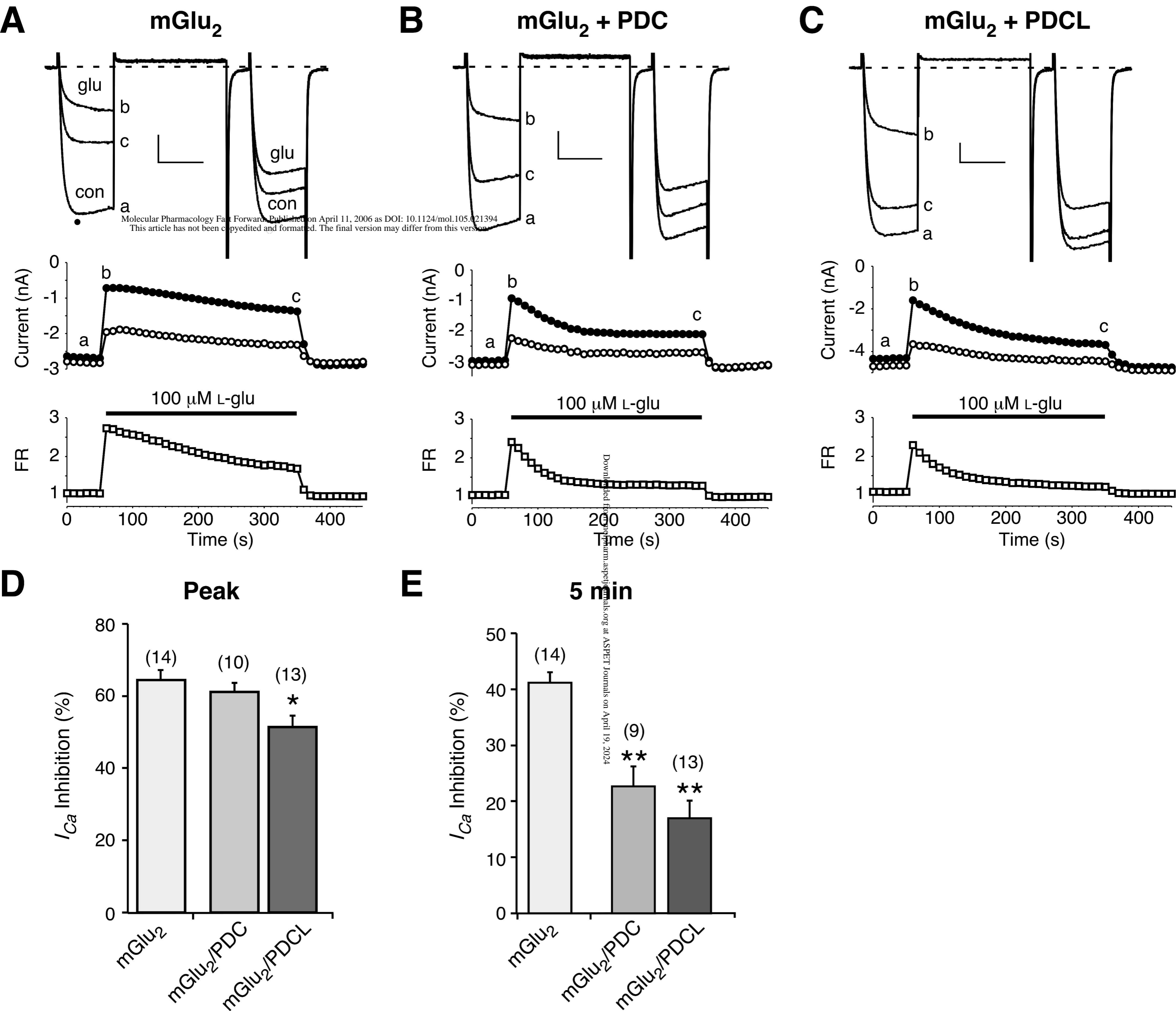


Figure 6

