

Somatostatin receptor subtype 4 modulates L-type calcium channels via $G\beta\gamma$ and PKC signaling in rat retinal ganglion cells

Spring R Farrell¹, Donald R Rankin¹, Nicholas C Brecha^{2,3}, and Steven Barnes^{1,2,3,4,*}

¹Department of Physiology & Biophysics; Dalhousie University; Halifax, NS, Canada; ²Department of Neurobiology; David Geffen School of Medicine; University of California; Los Angeles, CA USA; ³Veterans Administration; VAGLAHS; Los Angeles, CA USA; ⁴Department of Ophthalmology & Visual Sciences; Dalhousie University; Halifax NS, Canada

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Somatostatin subtype-4 receptors (sst₄) inhibit L-type calcium channel currents (I_{Ca}) in retinal ganglion cells (RGCs). Here we identify the signaling pathways involved in sst₄ stimulation leading to suppression of I_{Ca} in RGCs. Whole cell patch clamp recordings were made on isolated immunopanned RGCs using barium as a charge carrier to isolate I_{Ca}. Application of the selective sst₄ agonist, L-803 (10 nM), reduced I_{Ca} by 41.2%. Pretreatment of cells with pertussis toxin (Gi/o inhibitor) did not prevent the action of L-803, which reduced I_{Ca} by 34.7%. To determine the involvement of $G\beta\gamma$ subunits after sst₄ activation, depolarizing pre-pulse facilitation paradigms were used to remove voltage-dependent inhibition of calcium channels. Pre-pulse facilitation did not reverse the inhibitory effects of L-803 on I_{Ca} (8.4 vs. 8.8% reductions, ctrl vs. L-803); however, pharmacologic inhibition of $G\beta\gamma$ reduced I_{Ca} suppression by L-803 (23.0%, $P < 0.05$). Inhibition of PKC (GF109203X; GFX) showed a concentration-dependent effect in preventing the action of L-803 on I_{Ca} (1 μ M GFX, 34.3%; 5 μ M GFX, 14.6%, $P < 0.05$). When both PKC and $G\beta\gamma$ were inhibited, the effects of L-803 on I_{Ca} were blocked (1.8%, $P < 0.05$). These results suggest that sst₄ stimulation modulates RGC calcium channels via $G\beta\gamma$ and PKC activation. Since reducing intracellular Ca²⁺ is known to be neuroprotective in RGCs, modulating these sst₄ signaling pathways may provide insights to the discovery of unique therapeutic targets to reduce intracellular Ca²⁺ levels in RGCs.

Introduction

Somatostatin, also known as somatotropin release inhibiting factor (SRIF), is a neuropeptide transmitter mediating biological actions throughout the nervous system.¹ Many biological actions regulated by SRIF are in the endocrine system,² such as hypothalamic SRIF release inhibiting growth hormone secretion from the pituitary gland,³ or pancreatic SRIF inhibiting insulin and glucagon secretion.⁴ However, there are also significant effects of SRIF in the retina.⁵

In order to produce effects in a cell, SRIF must stimulate one of its 5 G-protein coupled receptor subtypes, sst₁₋₅, which are distributed throughout the nervous system.^{2,5,6} Previous studies have shown that selective stimulation of somatostatin receptor subtype 4 (sst₄) reduces calcium influx through voltage-gated calcium channels,^{7,8} and that in the retina, sst₄ is expressed exclusively in retinal ganglion cells (RGCs).⁷ Since RGC degeneration due to Ca²⁺ dysregulation can be a significant contributor to vision loss, understanding the mechanisms of sst₄ stimulation may aid in the development of cell-selective neuroprotective therapies.

L-803,087 (L-803), a selective agonist for sst₄, was shown to modulate Ca²⁺ influx via inhibition of L-type calcium channels, but not N-type calcium channels in RGCs.⁷ The intracellular signaling mechanisms through which sst₄ modulates these channels has yet to be determined. Most sst subtypes have been shown to couple to the inhibitory GTP-binding protein G_{i/o},⁹ suggesting that sst₄ stimulation could modulate calcium channels through inhibition of cAMP-dependent actions. G_{oi} inhibits adenylyl cyclase, reducing levels of cAMP and thus diminishes the activation of PKA, which may in turn lead to a reduction of I_{Ca}.¹⁰ Another mechanism through which some sst subtypes have been reported to act is via G α q.¹¹ When a G_q coupled receptor is activated, G α q stimulates phospholipase C (PLC) resulting in increased levels of DAG and PKC activity, which can also lead to reduction of I_{Ca}.^{6,12} GTP-binding proteins also contain $\beta\gamma$ subunits, which may directly inhibit calcium channels¹³ or activate other signaling molecules such as PLC.^{14,15} Voltage-dependent $G\beta\gamma$ inhibition of calcium channels can be identified by applying strong depolarizing voltage prepulses that briefly displace the $G\beta\gamma$ inhibition and show an increase, or facilitation, of I_{Ca}. There are also voltage-independent $G\beta\gamma$ interactions with

*Correspondence to: Steven Barnes; Email: sbarnes@dal.ca

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calcium channels, which can be blocked using pharmacological agents.^{16,17} Thus, $G_{i/o}$, G_q and $G\beta\gamma$ are potential signaling pathways that sst_4 may utilize to inhibit I_{Ca} in RGCs.

The purpose of this study is to identify the intracellular signaling pathways through which selective sst_4 stimulation reduces Ca^{2+} influx through calcium channels. We performed whole cell patch clamp experiments on isolated rat retinal ganglion cells to identify the cytoplasmic components of sst_4 signaling. This report demonstrates that calcium channel inhibition in RGCs results from sst_4 acting not through a cAMP-mediated process,

but rather through a combination of $G\alpha_q$ activation of the PKC pathway and a voltage-independent $G\beta\gamma$ interaction causing direct inhibition of the calcium channels.

Results

The aim of the present study was to determine the intracellular signaling molecules involved in the sst_4 -mediated inhibition of L-type voltage-gated calcium channels in isolated retinal ganglion cells (RGCs). **Figure 1A** shows examples of voltage-clamp recordings from an acutely isolated adult rat RGC in the absence (black trace) and presence (red trace) of the selective sst_4 agonist (L-803, 10 nM). The voltage ramp protocol (-60 mV to $+40$ mV; 1 Hz) used to depolarize the cell membrane is shown below. Similar to our previous findings in cultured neonatal rat RGCs,⁷ mean data show that L-803 (10 nM, 2 min.) significantly reduced I_{Ca} in acutely isolated adult rat RGCs (**Fig. 1B**; $*P < 0.05$, 2-way RM ANOVA vs. control). In isolated adult rat RGCs stimulation of sst_4 by L-803 caused an average reduction in I_{Ca} of approximately 40% (**Fig. 7**).

To determine whether the sst_4 -mediated reduction in I_{Ca} involved a pertussis toxin-sensitive G-protein ($G_{i/o}$), RGCs were incubated with pertussis toxin (PTX; 0.5 μ g/mL overnight; as per ref. 18) to inactivate toxin-sensitive G-proteins. **Figure 2A** shows an example of I_{Ca} recordings from an isolated RGC pretreated with PTX in the absence (black trace) and presence (red trace) of L-803 (10 nM). Pretreatment with PTX did not affect I_{Ca} amplitudes when compared to control (no PTX pretreatment) recordings (data not shown). Mean data show that L-803 (10 nM, 2 min) significantly reduced I_{Ca} in RGCs that had been pretreated with PTX (**Fig. 2B**; $*P < 0.05$, 2-way RM ANOVA vs. PTX pretreatment). When compared to RGCs that had not been pretreated with PTX, L-803 administration showed similar reductions in I_{Ca} (**Fig. 7**; $34.7 \pm 3.8\%$ vs. $41.2 \pm 2.7\%$, PTX pre-treated vs. L-803 alone). Thus, sst_4 -mediated reduction of I_{Ca} in RGCs does not require the activation of a pertussis-sensitive G-protein.

It is also possible $G\beta\gamma$ proteins may be involved in the inhibition of L-type calcium channels in rat RGCs. Previous studies have shown voltage-dependent and -independent $G\beta\gamma$ interactions with Ca_v2 calcium channels that result in reduced I_{Ca} .^{19,20} To determine whether voltage-dependent $G\beta\gamma$ interactions with L-type (Ca_v1) calcium channels were involved in the sst_4 -mediated reduction in I_{Ca} , a pre-pulse facilitation voltage paradigm was designed. It has been shown previously that a short, strong depolarizing voltage step causes a conformational change in the N-type calcium channel which causes dissociation of the $G\beta\gamma$ protein from the channel resulting in a temporary increase in I_{Ca} .²¹ **Figure 4A** shows an example paired pre-pulse recording from an isolated RGC in the presence of L-803 (10 nM, 2 min). Prior to the depolarizing step, I_{Ca} may be influenced by $G\beta\gamma$; however, after the strong depolarization, I_{Ca} is increased due to the dissociation of $G\beta\gamma$ from the channels. Pre-pulse facilitation of I_{Ca} was measured as the increase in I_{Ca} after the depolarizing step (120 mV, 50 ms) as a percentage of the current prior to the

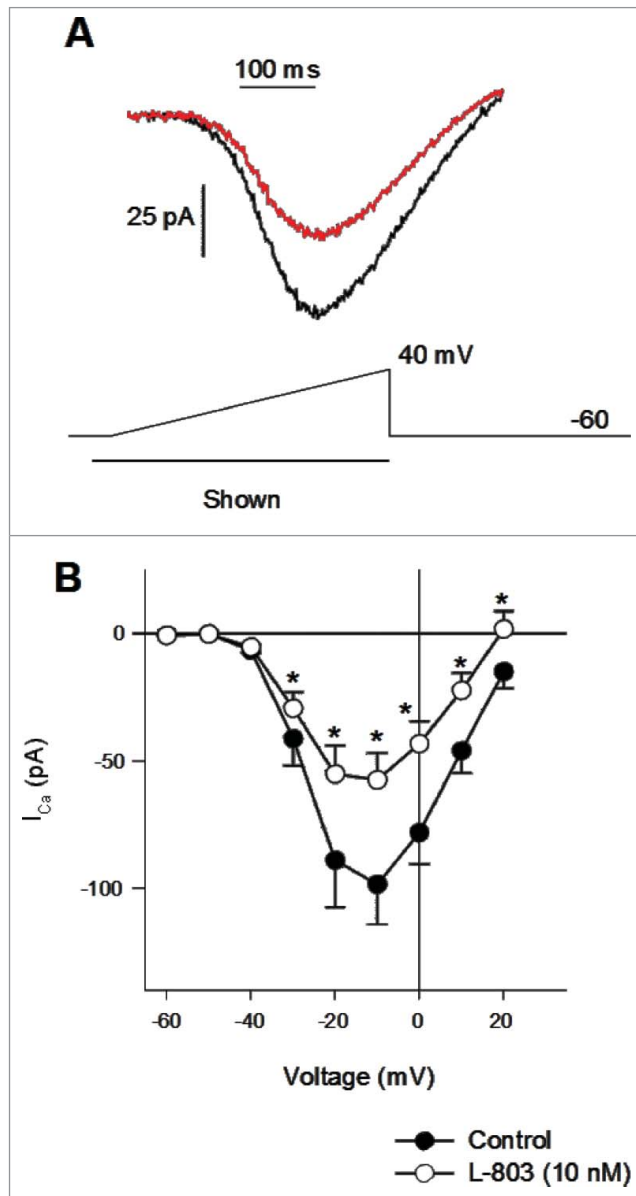


Figure 1. Calcium channel currents reduced by L-803,087 in isolated adult retinal ganglion cells (RGCs). **(A)** Calcium channel currents recorded during a voltage ramp from an isolated RGC in the absence (black) and presence of L-803,087 (gray, 10 nM). **(B)** The mean data show that L-803,087 produced a significant reduction of calcium channel current ($n = 27$; $*P < 0.05$, 2-way RM ANOVA vs. control).

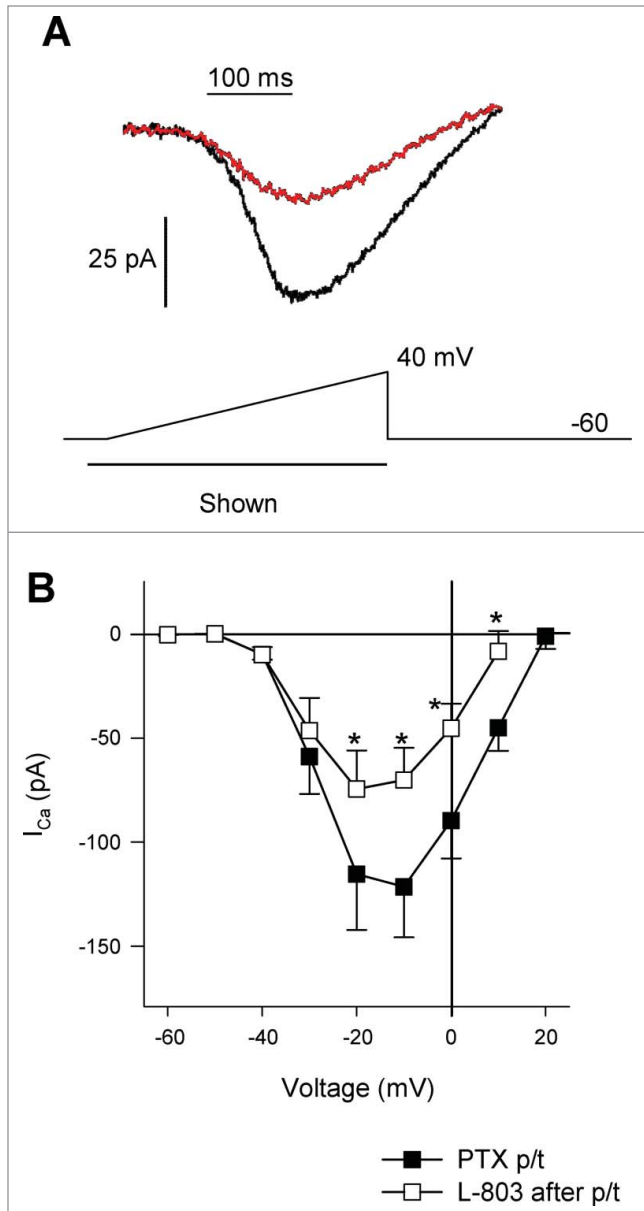


Figure 2. Pre-treatment of RGCs with pertussis toxin (PTX; Gi/o inhibitor) does not prevent the inhibitory actions of L-803,087 on calcium channel currents. **(A)** Calcium channel current in an isolated RGC pre-treated (p/t) with PTX. Administration of L-803,087 (gray, 10 nM) reduced calcium channel current compared to control (black trace). **(B)** The mean data show that L-803,087 significantly reduced calcium channel current ($n = 10$; $*P < 0.05$, 2-way RM ANOVA vs. PTX p/t).

pre-pulse. When comparing paired recordings in the absence and presence of L-803, the mean data show no significant change in pre-pulse facilitation (Fig. 4B). These results show that while pre-pulse facilitation of calcium channels occurs in RGCs, voltage-dependent G $\beta\gamma$ interactions with calcium channels are not involved in the L-803-mediated reduction in I_{Ca} in these cells.

Next, to determine whether G $\beta\gamma$ plays other roles in *sst*₄ signaling (e.g., via voltage-independent interactions between G $\beta\gamma$ and calcium channels or stimulation of other signaling

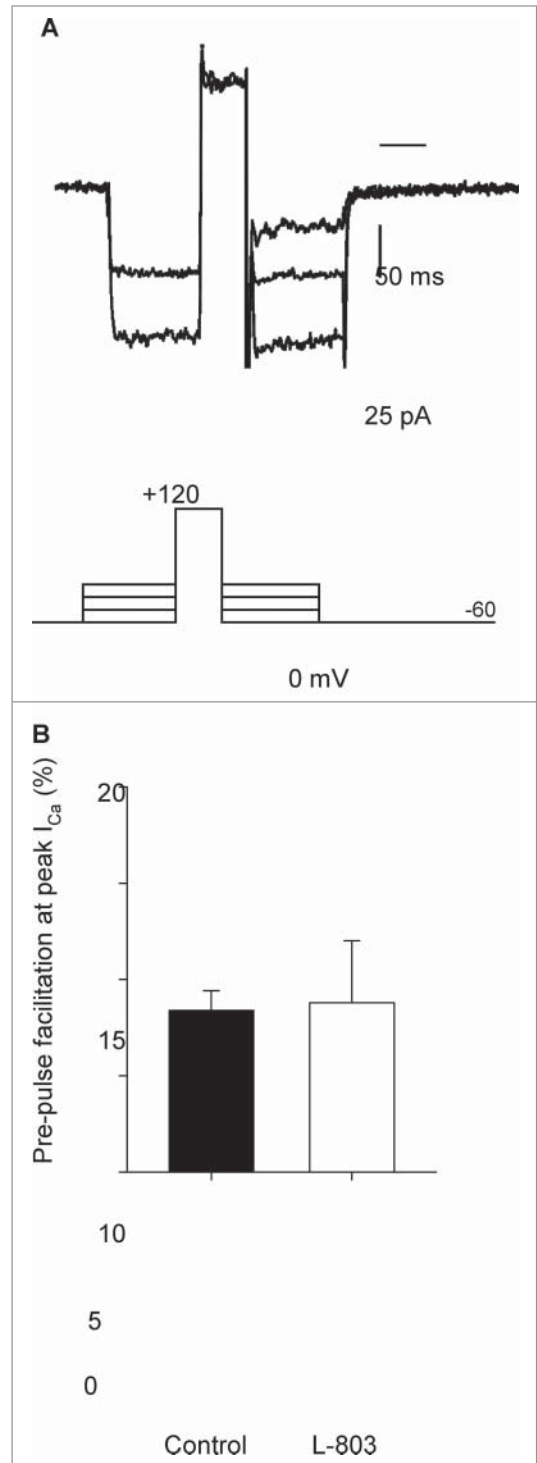


Figure 3. Depolarizing pre-pulse facilitates calcium channel current but has no influence on the suppression by L-803,087. **(A)** Calcium channel current recorded using a pre-pulse facilitation voltage command protocol (shown below current traces) in an isolated RGC in the presence of L-803. **(B)** Pre-pulse facilitation was measured as the percentage increase in calcium channel current after the depolarizing step (120 mV, 50 ms). The mean facilitation in the absence (control) and presence of L-803 was the same showing that pre-pulse facilitation did not reverse the suppression of calcium channel current caused by L-803 ($n = 5$; 8.4 ± 1.0 vs. $8.8 \pm 3.2\%$ ctrl vs. L-803, paired t-test).

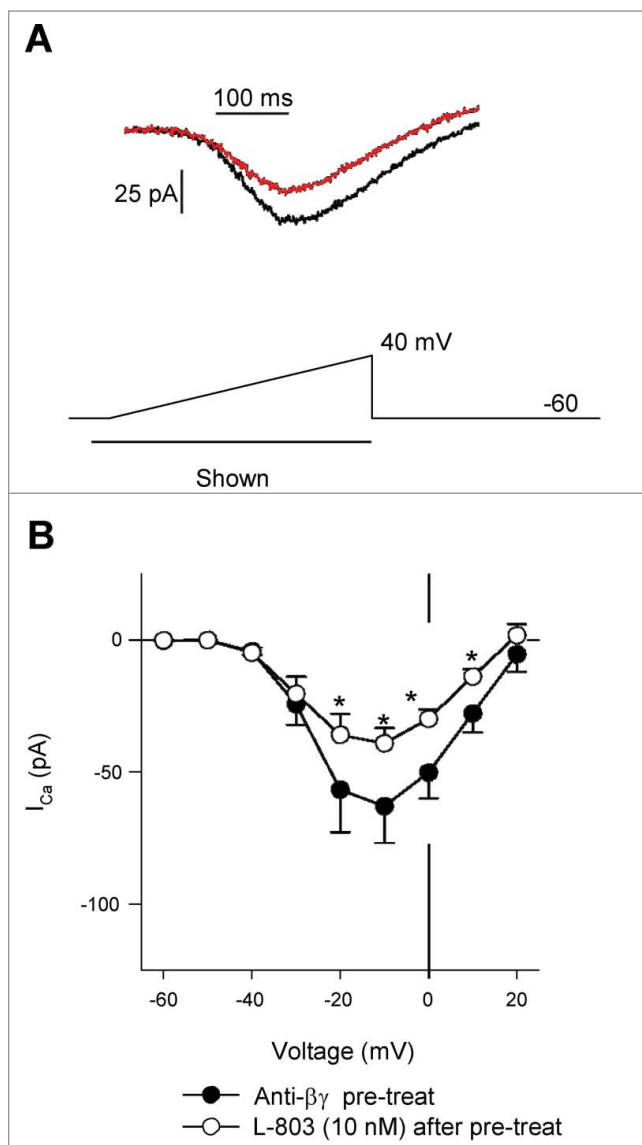


Figure 4. Pre-treatment of isolated RGCs with anti-G $\beta\gamma$ peptide reduced calcium channel suppression by L-803,087. **(A)** Calcium channel currents recorded from an isolated RGC pretreated with a combination of anti-G $\beta\gamma$ peptide (1 μ M) in the absence (black) and presence of L-803,087 (gray, 10 nM). **(B)** The mean data show that L-803,087 reduced calcium channel current in cells pre-treated with anti-G $\beta\gamma$ peptide (n = 10, * P < 0.05, 2 way RM ANOVA).

molecules), an inhibitor of G $\beta\gamma$ proteins was incubated with isolated RGCs prior to recording. Anti- $\beta\gamma$ peptide is a small membrane permeable peptide that interacts with G $\beta\gamma$, preventing its dissociation from the G-protein trimeric complex after receptor stimulation.^{16,17} **Figure 5A** shows a voltage-clamp recording from an isolated RGC after pre-treatment with anti- $\beta\gamma$ peptide (1 μ M, 15 min.) before (black trace) and after L-803 administration (red trace). Stimulation of *sst*₄ by L-803 (10 nM, 2 min.) significantly reduced I_{Ca} in RGCs pre-treated with anti- $\beta\gamma$ peptide (**Fig. 5B**, * P < 0.05, 2-way RM ANOVA vs. Anti- $\beta\gamma$ peptide pretreatment). However, the inhibitory effect of L-803 was

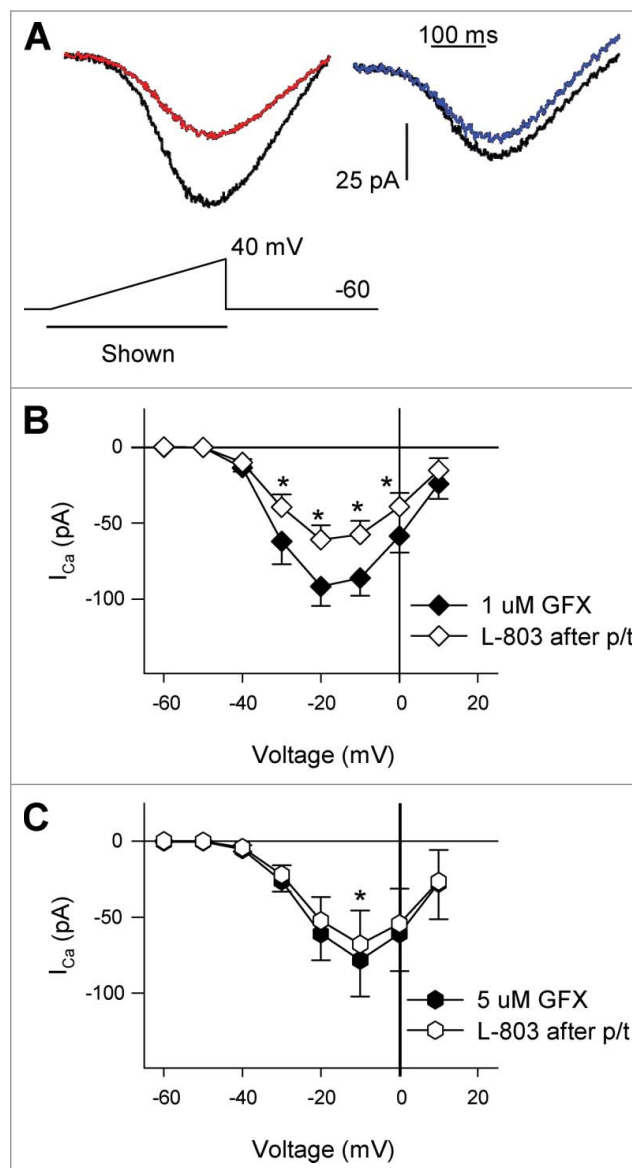


Figure 5. Pre-treatment of RGCs with PKC antagonist GF109203X reduced the actions of L-803, 087. **(A)** Calcium channel currents in isolated RGCs pretreated (p/t) with GFX (left: 1 μ M, right: 5 μ M). Administration of L-803,087 (left: gray; right: bold) reduced calcium channel currents compared to pre-treatment conditions (regular trace). **(B)** The mean data show that after cells were pre-treated with 1 μ M GFX, L-803,087 reduced calcium channel current (n = 11; * P < 0.05, 2-way RM ANOVA vs. 1 μ M GFX p/t). **(C)** Pre-treatment of cells with 5 μ M GFX reduced the effect of L-803,087 on calcium channel current (n = 8; * P < 0.05, 2-way RM ANOVA vs. 5 μ M GFX p/t).

less than that observed in cells that have not been pretreated (**Fig. 7**; $23.0 \pm 4.8\%$ vs. $41.2 \pm 2.7\%$, anti- $\beta\gamma$ peptide pretreatment vs. L-803 alone; * P < 0.05, ANOVA). Thus, these data suggest a role of G $\beta\gamma$ signaling in the *sst*₄-mediated inhibition of L-type calcium channels.

Several other intracellular signaling molecules could be responsible for the reduction in I_{Ca} observed after stimulation of the *sst*₄ receptor in RGCs. Previous studies have shown that *sst*₄

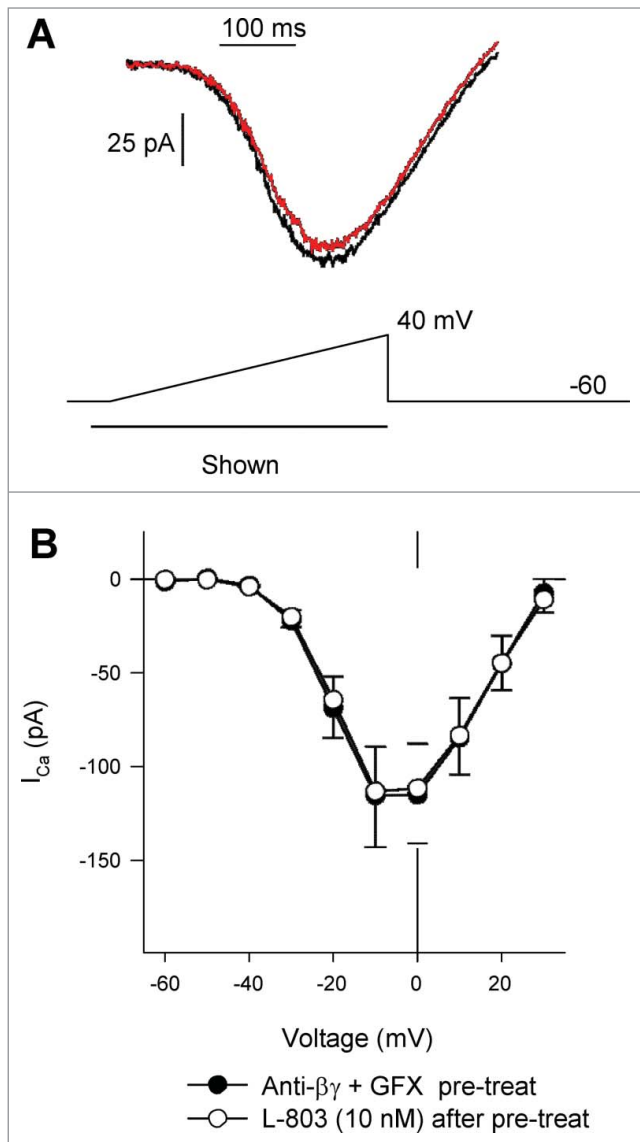


Figure 6. Pre-treatment of isolated RGCs with anti-G $\beta\gamma$ peptide and GFX blocked L-803,087 induced suppression of calcium channel current. **(A)** Calcium channel currents recorded from an isolated RGC pre-treated with a combination of anti-G $\beta\gamma$ peptide (1 μ M) and GFX (5 μ M) in the absence (black) and presence of L-803,087 (gray, 10 nM). **(B)** The mean data show that L-803,087 had no effect on calcium channel current in pre-treated cells (n = 6, NS 2 way RM ANOVA).

stimulation activates phospholipase C (PLC),⁶ which is an intermediate in the Gq signaling pathway resulting in the activation of protein kinase C (PKC). In addition, studies have shown that Gq and PKC are involved in the inhibition of L-type calcium channels in response to the stimulation of other G-protein coupled receptors.²²⁻²⁴ Thus, to determine whether PKC activation was required to reduce I_{Ca} during ss_4 stimulation, isolated RGCs were pretreated with the PKC antagonist GF109203X (GFX). First, isolated RGCs were incubated with a moderate concentration of GFX (1 μ M, 30 min.) prior to voltage-clamp recordings. Fig. 3A (left panel) shows an example of a recording

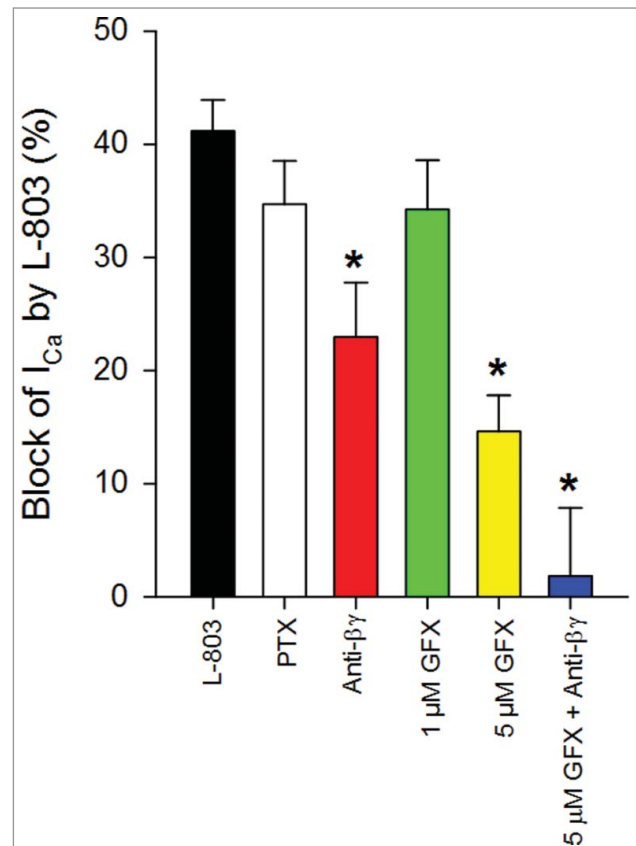


Figure 7. Summary of effects of signaling pathway modulators administered with L-803,087. L-803 reduced I_{Ca} in isolated RGCs (n = 27; 41.2 ± 2.7% vs. control). Pre-treatment with pertussis toxin (PTX) had no effect on the L-803-mediated calcium channel current reduction (n = 10; 34.7 ± 3.8%). The non-selective PKC antagonist, GF109203X had a concentration dependent effect on L-803-mediated current reduction (1 μ M: n = 11; 34.3 ± 4.4%; 5 μ M: n = 8; 14.6 ± 3.2%; * P < 0.05 vs. L-803 alone). Pre-treatment of cells with anti- β gamma (Anti-G $\beta\gamma$) peptide impaired the ability of L-803 to reduce calcium channel current (n = 10; 23.0 ± 4.8%; * P < 0.05 ANOVA vs. L-803 alone). Combination treatment with 5 μ M GFX and Anti-G $\beta\gamma$ peptide abolished the effects of L-803 (n = 6; 1.8 ± 6.0%; * P < 0.05 ANOVA vs. L-803 alone).

from an isolated RGC pretreated with 1 μ M GFX in the absence (black trace) and presence (red trace) of L-803 (10 nM). RGCs pretreated with 1 μ M GFX showed a significant reduction in I_{Ca} in the presence of L-803 (Fig. 3B; * P < 0.05, 2-way RM ANOVA vs. 1 μ M GFX pretreatment). The effect of L-803 in cells that had been treated with 1 μ M GFX was similar to that of non-pretreated cells (Fig. 7; 34.3 ± 4.4% vs. 41.2 ± 2.7%, 1 μ M GFX pre-treatment vs. L-803 alone).

Next, a higher concentration of GFX (5 μ M, 30 min.) was used to pretreat RGCs. Fig. 3A (right panel) shows I_{Ca} recorded from an isolated RGC which had been pretreated with 5 μ M GFX before (black trace) and after administration of L-803 (blue trace). The mean data show that L-803 caused a modest reduction in I_{Ca} in RGCs pretreated with the high concentration of PKC antagonist (5 μ M GFX; Fig. 3C). Pre-treatment of RGCs with 5 μ M GFX significantly reduced the effects of L-803

compared to cells that had not been pretreated (Fig. 7; $14.6 \pm 3.2\%$ vs. $41.2 \pm 2.7\%$, 5 μM GFX pre-treatment vs. L-803 alone; $*P < 0.05$, ANOVA). These data suggest that PKC is involved in the sst_4 -mediated reduction in I_{Ca} in RGCs.

Finally, to determine whether inhibition of both PKC and $\text{G}\beta\gamma$ could completely abolish the reduction of I_{Ca} by L-803, isolated RGCs were pretreated with both GFX (5 μM) and anti- $\beta\gamma$ peptide (1 μM). Figure 6A shows an example of a voltage-clamp recording from an isolated RGC pretreated with GFX and anti- $\beta\gamma$ peptide in the absence (black trace) and presence of L-803 (red trace; 10 nM, 2 min). The mean data show no change in I_{Ca} after L-803 administration in cells that had been pre-treated with the combination of GFX (5 μM) and anti- $\beta\gamma$ peptide (1 μM ; Fig. 6B). Blocking both PKC activation and $\text{G}\beta\gamma$ interactions with calcium channels abolished the sst_4 -mediated reduction in I_{Ca} (Fig. 7; $1.8 \pm 6.0\%$ vs. $41.2 \pm 2.7\%$, GFX + anti- $\beta\gamma$ peptide pre-treatment vs. L-803 alone; $*P < 0.05$, ANOVA). These results suggest additive roles for $\text{G}\beta\gamma$ and PKC in the sst_4 -mediated inhibition of L-type calcium channels.

Discussion

The present study characterized the intracellular signaling pathway of sst_4 -mediated inhibition of L-type calcium channels in RGCs. Activation of sst_4 in these cells caused activation of PKC via a pertussis-toxin insensitive G-protein (Gq) signaling cascade, which resulted in the inhibition of calcium channels. In addition, sst_4 -activated $\text{G}\beta\gamma$ proteins caused further inhibition of I_{Ca} via a voltage-independent mechanism. Together, these results demonstrate the roles of 2 potential intracellular targets for the selective modulation of L-type calcium channels in RGCs.

An earlier report⁷ showed that sst_4 stimulation selectively reduced I_{Ca} via L-type calcium channels in neonatal rat RGCs. In the present study we have extended our experimental model by using acutely isolated adult rat RGCs. In adult RGCs, we show selective sst_4 stimulation using a non-peptide agonist (L-803,087, 10 nM) caused a significant reduction in I_{Ca} , similar to findings in neonatal RGCs where L-803 (10 nM) caused an average reduction in I_{Ca} of 32.5%.⁷ In fact, when the effect of L-803 in these 2 groups was compared directly (percent block of I_{Ca} by L-803; adult vs. neonatal RGCs, ANOVA), no statistical difference was found between adult and neonatal cells. Thus, these findings indicate that, at least in RGCs, the sst_4 -mediated reduction of I_{Ca} does not change throughout development.

The present study aimed to characterize the G-protein coupled intracellular signaling pathway mediating the sst_4 -mediated inhibition of L-type calcium channels. As the net effect of sst_4 stimulation is a reduction in I_{Ca} , we began by investigating G-proteins known to cause inhibitory responses. Several studies report that all sst subtypes couple to Gi/o proteins, inhibiting cAMP formation.²⁵⁻²⁷ However, in the present study, isolated RGCs pretreated with pertussis toxin (0.5 $\mu\text{g}/\text{mL}$, 2.5–24 hr) to inhibit Gi/o proteins showed significant reductions in I_{Ca} after L-803 administration. It is possible that the concentration of PTX was insufficient to completely inhibit the G-proteins

involved in the sst_4 -mediated response. However, an identical PTX pre-treatment protocol (0.5 $\mu\text{g}/\text{mL}$, 18 hr) in isolated RGCs showed coupling of a glutamate receptor subtype to calcium channels via a PTX-sensitive G-protein.¹⁶ A significant inhibition of the glutamate-mediated response (a well characterized Gi-coupled receptor) in the presence of PTX was demonstrated; thus this pre-treatment protocol was deemed to be sufficient to inhibit Gi/o in the present study. The finding that L-803 reduced I_{Ca} in cells that had been pre-treated with PTX suggests that the sst_4 -mediated reduction in I_{Ca} involves a PTX-insensitive G-protein.

Inhibitory effects of $\text{G}\beta\gamma$ subunits on N-type calcium channels have been described previously.¹⁹⁻²¹ However, such an inhibitory role of $\text{G}\beta\gamma$ subunits in interactions with L-type calcium channels is not seen.^{20,28} The physical interaction between the $\text{G}\beta\gamma$ subunit and the N-type calcium channel stabilizes a closed state of the channel and smaller currents cross the membrane.²⁰ Studies have shown that strong depolarization of the cell can cause a conformational change in the N-type channel resulting in dissociation of the $\text{G}\beta\gamma$ protein, facilitating an increase in the net current through the channels.^{19,21,29,30} In the present study, we examined the possibility that this type of voltage-dependent $\text{G}\beta\gamma$ interaction may be involved in the L-803-mediated reduction in L-type I_{Ca} . Indeed, when large depolarizing voltage steps were applied, I_{Ca} increased, showing a transient pre-pulse facilitation (Fig. 3). However, the extent of facilitation was the same in cells before and after L-803 administration; thus, a direct voltage-dependent interaction between $\text{G}\beta\gamma$ proteins and the L-type channel was not responsible for the inhibition of I_{Ca} observed with sst_4 stimulation. Although this result suggested no voltage-dependent role of $\text{G}\beta\gamma$, previous reports describe a voltage-independent component of $\text{G}\beta\gamma$ inhibition of calcium channels^{19,29} while others demonstrate $\text{G}\beta\gamma$ activation of PLC,^{14,15} a known modulator of calcium channels.¹² This led us to further examine the potential involvement of $\text{G}\beta\gamma$ in the sst_4 signaling pathway. The anti- $\beta\gamma$ peptide used in the present study (Anti-BetaGamma MPS-phosducin-like protein C terminus peptide) has been shown previously to bind to and inactivate $\text{G}\beta\gamma$ signaling.^{16,17} Indeed, pretreatment of RGCs with anti- $\beta\gamma$ peptide reduced the effects of L-803 on I_{Ca} (Fig. 4), although not entirely. Thus, these results corroborate those of previous reports that there can be voltage-independent $\text{G}\beta\gamma$ signaling which influences calcium channels.^{12,14,15,19,29} In addition, these results demonstrate a role of $\text{G}\beta\gamma$ in the sst_4 -mediated inhibition of I_{Ca} in RGCs; however, further studies in native cell models are required to elucidate the precise $\text{G}\beta\gamma$ mechanism involved.

While pretreatment of RGCs with anti- $\beta\gamma$ peptide reduced the effects of sst_4 stimulation; there was still a significant reduction of I_{Ca} after L-803 administration, suggesting involvement of another intracellular signaling molecule. There is evidence that protein kinase C (PKC) phosphorylates calcium channels, which can result in either reduction or enhancement of current amplitude.^{31,32} Specifically, phosphorylation of L-type channels by PKC has been shown to reduce I_{Ca} ,³¹ thus, it seemed possible that PKC was involved in the sst_4 signaling pathway resulting in

inhibition of L-type calcium channels in the retina. Indeed, results of the present study showed a significant block of the effects of L-803 in the presence of a non-selective PKC antagonist. Since there are several isoforms of PKC, we chose an antagonist (GF109203X, GFX) with activity against all of the known PKC isoforms. In addition, GFX (1 μ M) has been shown to completely inhibit PKC activity involved in cytoskeleton dynamics in salamander retina.³³ In the present study, we observed that GFX had a concentration-dependent effect on the L-803 mediated I_{Ca} reduction (Fig. 5), with a significant block of L-803 activity at 5 μ M GFX. These results demonstrate that PKC is involved in the ss_4 -mediated reduction of I_{Ca} and suggest that PKC isoforms may be selectively involved in different cellular processes within the retina. In addition, the role of PKC is complex and can be significantly altered by changing a single amino acid in the target protein.³² Thus, further studies would be required to elucidate which PKC isoform is involved in the ss_4 signaling pathway in RGCs. This would be a significant challenge using a native cell model, such as the one used in the present study, as many antagonists are not specific enough to distinguish between PKC isoforms. However, results of the present study demonstrate a clear role for PKC in the modulation of retinal calcium channels via the ss_4 signaling pathway.

Finally, we treated RGCs with both anti- $\beta\gamma$ peptide and PKC antagonist (GFX, 5 μ M) to determine whether blockade of both aspects of the signaling pathway would completely prevent the effect of L-803 on I_{Ca} (Fig. 6). Studies have shown that there is significant cross-talk between PKC and $G\beta\gamma$ signaling pathways. A competitive interaction between PKC and $G\beta\gamma$ pathways has been shown where phosphorylation of N-type calcium channels by PKC prevented the interaction of $G\beta\gamma$ with the ion channel resulting in a net positive ionotropic effect of PKC.³² Results of the present study showed an additive effect of blocking 2 different signaling molecules ($G\beta\gamma$ and PKC) that contributes to the inhibition of the L-type calcium channel in isolated RGCs. Administration of anti- $\beta\gamma$ peptide and PKC antagonist together completely abolished the effects of L-803 compared to cells that had not been pretreated with antagonists (Fig. 7). While there have been multiple reports of the competitive signaling between PKC and $G\beta\gamma$,^{32,34,35} the present study is the first to report these signaling molecules acting in parallel to inhibit L-type calcium channels. These results suggest that cells may have signaling pathway redundancies to prevent over or under stimulation of cellular targets. Although these results add to our understanding of convergent signaling pathways, additional investigations will provide improved understanding of how these pathways integrate and function in this physiological network.

Results of the present study show that it is possible to selectively modulate L-type calcium channels via $G\beta\gamma$ and PKC by stimulating the ss_4 receptor. Since ss_4 is exclusively expressed in RGCs in the retina, selective modulation of this receptor may be a useful therapeutic target to regulate intracellular Ca^{2+} levels in RGCs. Further studies are warranted to determine whether ss_4 modulation or manipulation of the downstream signaling

pathways described here may provide neuroprotection in the setting of retinal injury or disease.

Methods

Purified retinal ganglion cell isolation

Experimental protocols were approved by the Dalhousie University Committee on Laboratory Animals and performed in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental.

Ganglion cells were isolated and purified using a modified panning procedure.³⁶ Briefly, young adult (6–8 weeks old) Long-Evans rats (Charles River, strain code 006) were exposed to an overdose of isoflurane prior to enucleation of the eyes. Retinas were dissected in Hibernate-A (Gibco, Life Technologies, catalog # A12475-01) and transferred to papain solution containing: Leibovitz (L-15) media (without glutamine; Lonza, catalog # 12-700F), 20 U/mL papain, 1 mM cysteine, 0.5 mM EDTA (Worthington, catalog # LK003178), 50 U/mL DNase (Worthington, catalog # LK003172; 37°C, pH \sim 7.4). Retinas were dissociated for 5–15 minutes (37°C); the papain solution was then removed and replaced with enzyme inhibitor solution containing: L-15 media, 0.5 mg/mL ovomucoid, 50 U/mL DNase (Worthington, catalog # LK003182; 37°C, pH \sim 7.4). The tissue was incubated in enzyme inhibitor solution for 5 minutes at room temperature (RT) prior to trituration in fresh enzyme inhibitor solution. The retinal cell suspension was transferred to a conical tube and centrifuged (2 \times 1000 rpm, RT, L-15 media), resuspending the pellet in supplemented media containing: L-15 media, 2% B-27 (Gibco, Life Technologies, catalog #0080085SA), 200 mM L-glutamine, 10,000 U/mL penicillin, 10 mg/mL streptomycin (SigmaAldrich, catalog #G1146), 50 U/mL DNase (37°C, pH \sim 7.4). The retinal cell suspension was then incubated (37°C, 1 hr) in cell culture dishes containing glass coverslips (Fisher Scientific, catalog # 12-545-82) coated with antibody against the RGC protein marker Thy-1 (goat anti-mouse IgM; Jackson ImmunoResearch, catalog #115-005-020; 4°C, overnight; mouse anti-Thy-1; produced from cell line T11D7e2, ATCC catalog #TIB-103; 37°C, 5% CO₂/O₂, 2 hrs). Coverslips were washed with L-15 media (37°C, pH \sim 7.4) to remove non-adherent cells and maintained at 37°C in supplemented L-15 media until electrophysiology recordings were made.

Electrophysiological recordings

Extracellular solution (containing: (mM) 137 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 22.2 glucose, and 5 HEPES, adjusted to pH 7.4 with NaOH) was perfused over isolated RGCs throughout all voltage-clamp experiments. Calcium channel currents (I_{Ca}) were isolated by switching the extracellular solution immediately surrounding the cell of interest using a rapid perfusion system (VC8-S, ALA Scientific) containing (in mM): 110 choline chloride, 5 KCl, 1 MgCl₂, 7 BaCl₂, 15 TEACl, 0.1 4-aminopyridine, 20 glucose, 10 HEPES and 1 μ M tetrodotoxin, adjusted to pH 7.4 with CsOH. Bath applied drugs were diluted in this barium

choline extracellular solution and administered to RGCs using the ALA perfusion system (21–25°C). Recordings were taken before (control recording), during and after bath applied drug administration; however, only recordings after steady-state had been reached (2–3 min. after drug application) were used in comparisons.

All electrophysiology recordings were made in whole-cell patch clamp mode, using borosilicate electrodes (5–10 M Ω ; Sutter Instruments; catalog #BF150-86-10). The intracellular pipette solution contained (mM): 128 CsCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 HEPES, 2 Mg²⁺-ATP, 0.4 Na⁺-GTP, and 10 phosphocreatine, adjusted to pH 7.2 with CsOH.

The bath reference electrode consisted of an agar bridge with an AgCl wire. An Axopatch-1D amplifier (Molecular Devices) was used to control membrane voltage and adjust whole-cell capacitance and series resistance. Voltage command protocols were initiated using pClamp 10 software (Molecular Devices). The current signal was filtered at 0.5 Hz (Ithaco 4302 Dual 24dB/octave filter, Ithaca, NY) and digitized at 1 kHz with Digi-data 1440 (Molecular Devices).

Drugs and chemicals

Routine chemicals and reagents were obtained from Sigma Aldrich Canada and Cedarlane Laboratories. L-803,087 (catalog #1979), GF109203X (GFX; catalog #0741) and pertussis toxin (PTX; catalog #3097) were purchased from R&D Systems. Tetrodotoxin citrate (TTX; catalog #T-550) was purchased from Alomone Labs. Anti-BetaGamma MPS-phosducin-like protein C terminus peptide (Anti- $\beta\gamma$ peptide, catalog #24178) was obtained from AnaSpec.

L-803,087 (0.001%) and GFX (0.01–0.05%) were prepared as DMSO stock solutions, frozen at -20°C and thawed immediately before experiments (numbers in parenthesis indicate percentage of DMSO in the final working solution). These levels of DMSO do not influence I_{Ca}. Pertussis toxin stock solution was stored at 4°C for no longer than 6 weeks. All other drugs and

reagents were prepared in double distilled water as stock solutions (stored at -20°C) and warmed to room temperature immediately before performing experiments.

Drug pretreatments were performed by administering drugs directly to the cell culture wells during incubation (37°C, supplemented L-15 media).

Data analysis

All data are reported as the means \pm SEM. Data analysis was performed using Clampfit 10 software (Molecular Devices). Graphing and statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc.). Specific statistical tests used are noted in the figure legend in the appropriate results sections. P values of less than 0.05 were considered statistically significant. All pair-wise multiple comparison procedures were performed in SigmaPlot, using the Holm-Sidak method.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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