Protein Kinase A Activity Controls the Regulation of T-type CaV3.2 Channels by Gβγ Dimers* S

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Low voltage-activated (LVA), T-type, calcium channels mediate diverse biological functions and are inhibited by Gβγ dimers, yet the molecular events required for channel inhibition remain unknown. Here, we identify protein kinase A (PKA) as a molecular switch that allows Gβγ dimers to effect voltage-independent inhibition of CaV3.2 channels. Inhibition requires phosphorylation of Ser1107, a critical serine residue on the II-III loop of the channel pore protein. S1107A prevents inhibition of unitary currents by recombinant Gβ2γ2 dimers but does not disrupt dimer binding nor change its specificity. Gβγ dimers released upon receptor activation also require PKA activity for their inhibitory actions. Hence, dopamine inhibition of CaV3.2 whole cell current is precluded by Gβγ-scavenger proteins or a peptide that blocks PKA catalytic activity. Fittingly, when used alone at receptor-selective concentrations, D1 or D2 agonists do not elicit channel inhibition yet together synergize to inhibit CaV3.2 channel currents. We propose that a dual-receptor regulatory mechanism is used by dopamine to control CaV3.2 channel activity. This mechanism, for example, would be important in aldosterone producing adrenal glomerulosa cells where channel dysregulation would lead to overproduction of aldosterone and consequent cardiac, renal, and brain target organ damage.

G-protein βγ dimers released upon receptor activation play a central role in regulating the activity of high voltage-gated calcium channels (HVA) 3 of the CaV2 family (N-type, P/Q-type, R-type) by directly interacting with intracellular domains of these channel pore proteins (1–6). Inhibition of CaV2 channels by Gβγ dimers depends on voltage and can be modulated by protein kinase C (7), and synaptic protein interactions (8) allowing for a range of CaV2 channel activity in neurons and neuroendocrine cells, and for the opportunity for presynaptic modulation. By contrast, LVA channels of CaV3 family have a less restricted distribution (9) and are expressed in peripheral tissues (10). They are prominently expressed in zona glomerulosa cells (ZG) of the adrenal gland (11) where CaV3.2 channel activity controls the production of aldosterone. LVA channel currents are inhibited consistently by a host of GPCR-linked hormones (dopamine, enkephalin, nociceptin, bradykinin, somatostatin, and corticotrophin) (12, 13). In each of these examples, channel inhibition depends on Gβγ activity, yet an understanding of the molecular events required for channel inhibition remains incomplete. For instance, dopamine inhibits nickel-sensitive LVA calcium channel currents in sympathetic and dorsal root neurons (14, 15), lactotrophs (16, 17), melanotrophs (18), retinal horizontal cells (19), and adrenal zona glomerulosa (ZG) cells (20, 21). Yet, as typified in ZG cells, Gβγ alone is not sufficient to mediate channel inhibition transduced by dopamine, and conflicting data posits the singular importance of activating D1- or D2-receptor subtypes that produce opposing changes in the level of the intracellular messenger cAMP (20, 21).

Using a heterologous expression system, we recently described a molecular mechanism for the inhibition of LVA channels that involves heterotrimERIC G-protein βγ dimers (22). Unlike inhibition of HVA channels, inhibition of LVA channel activity by Gβγ dimers is independent of voltage, specific for CaV3.2/α1H channels within the LVA channel family, and requires active Gβγ dimers that contain Gβ2. Yet, like Gβγ-induced HVA channel inhibition, LVA channel inhibition depends on a direct interaction of Gβγ dimers with the channel protein. Accordingly, recombinant Gβ2γ2 dimers inhibit CaV3.2 single channel activity when applied to membrane patches excised from HEK293 cells (23). Here, we test the possibility that the described molecular mechanism for the inhibition of CaV3.2 channels underlies dopamine-induced inhibition of CaV3.2 currents. Our results reconcile previous findings yet are unexpected. They demonstrate that: 1) protein kinase A (PKA) acts as a molecular switch to enable the inhibition of CaV3.2 currents by Gβ2γ2 dimers, and 2) they highlight a role for a novel cooperative action of D1 and D2 receptors in controlling CaV3.2 calcium channel activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—H295R cells (human adrenocortical carcinoma cells) were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 containing 10% cosmic calf serum, 1 μg/ml gentamicin. All culture vessels for H295R cells were coated with sterile 0.1% gelatin and washed with 1X (phosphate-buffered saline) before use. HEK293 cells (human

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 12, pp. 7465–7473, March 20, 2009

Received for publication, October 21, 2008, and in revised form, January 8, 2009 Published, JBC Papers in Press, January 8, 2009, DOI 10.1074/jbc.M808049200

1 Supported by a postdoctoral fellowship from the American Heart Association Grant AHA 053535ON (to W. E. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “in accord with 18 U.S.C. Section 1734 solely to indicate this fact.

2 To whom correspondence should be addressed: 1300 Jefferson Park Ave, Charlottesville, VA 22908. Fax: 434-982-3878; E-mail: pqb4b@virginia.edu.

3 The abbreviations used are: HVA, high voltage-gated calcium channel; CHAPS, 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid; PKA, cAMP-dependent kinase; LVA, low voltage-activated; ANOVA, analysis of variance; GST, glutathione S-transferase.

4 This work was supported, in whole or in part, by National Institutes of Health Grant HL36977 (to P. Q. B.). This work was also supported by American Heart Association Grant AHA 053535ON (to W. E. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

5 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

6 Supported by a postdoctoral fellowship from the American Heart Association Mid-Atlantic Affiliate.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 12, pp. 7465–7473, March 20, 2009

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embryonic kidney cells) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 1% pen/strep. Cells were transiently transfected with plasmids for wild-type or mutant Ca_{3.2}/Ca_{3.1} channels by using JetPEI reagent (Polyplus-transfection.com) and used for patching or immunoprecipitation 48 h after transfection.

Electrophysiology—Ca_{3.2} currents were recorded with an Axopatch 200A amplifier (Axon Instruments), and data collected with PCLAMP 9.2 software (Axon Instruments). All experiments were performed at room temperature. Data were analyzed by using Clampfit 9.2 (Axon Instruments) and OriginPro 7.5 (OriginLab) software. ANOVA was used for statistical examination with post hoc Dunnett’s test, where significance was taken as p < 0.05. Average data are given as mean ± S.E.

Whole Cell Recording—Steady-state channel activation was determined using tail currents in response to test depolarization in −90 mV increments (−60 mV to +10 mV) from a holding potential of −90 mV upon repolarization to −60 mV; steady-state channel inactivation was determined in response to +20 mV test depolarization (6 ms) from holding a potential incremented in 5 mV (−90 mV to −20 mV; 6 s) upon repolarization to −60 mV. To follow the time course of agonist-induced current inhibition, repetitive tail currents elicited upon repolarization to −75 mV from a test pulse of −15 mV were delivered every 6 s. The internal solution (in mM): 115 CsCl, 1 TBACl, 1 MgCl_{2}, 5 Mg-ATP, 1 Li-GTP, 0.9 CaCl_{2}, 20 HEPES, 11 BAPTA, pH 7.2 (adjusted with CsOH). The bath solution (in mM): 132 TEACl, 10 CaCl_{2}, 0.5 MgCl_{2}, 10 HEPES, 5 dextrose, 32 sucrose, pH 7.4 (adjusted with CsOH). Currents were filtered at 2 kHz and sampled at 12.5 kHz, and leak subtraction was performed on line by using scaled hyperpolarizing steps of one-fourth amplitude (P/N{4}) (22).

Single Channel Recording—Unitary currents from inside-out excised patches were elicited by a test pulse to −35 mV from −90 mV (200 ms, 6 s. interpulse). Currents were sampled at 100 kHz and filtered at 2 kHz. The pipette solution (in mM): 75 CsCl, 60 CaCl_{2}, and 10 HEPES, pH 7.4 (adjusted with CsOH). The bath solution (in mM): 140 K^{+}-aspartate, 5 MgCl_{2}, 10 EGTA, 20 HEPES, pH 7.4 (adjusted with KOH), 0.162 CHAPS (0.01%), and 0.04 dithiothreitol. CHAPS and dithiothreitol were added to the bath solution to maintain the stability and solubility of Gβγ recombinant proteins. Purified Gβγ subunits were added directly to the bath solution. Channel openings were detected with a 50% threshold crossing criterion. Open probability (N_{Po}) was calculated as the ratio of the sum of channel open time (per sweep) and the analyzed test pulse duration (23, 24).

Molecular Biology—Ca_{3.2}/Ca_{3.1} II-III loop channel chimera was made as reported (22). Mutations of putative PKA phosphorylation sites were introduced by using the QuikChange XL Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by sequencing.

Generation of Recombinant Gβγ—The recombinant Gβγ proteins were made and purified as reported (23). The activity of pure Gβγ dimer was verified by testing for PLC-β activation in synthetic lipid vesicles (25).

Immunoprecipitation and Binding—HEK293 cells transfected with FLAG-tagged Ca_{3.2} and Ca_{3.2} (S1107A) channels were stimulated with 10 μM 8-bromo-cAMP (10 min at 37 °C). Stimulation was terminated by snap-freezing dry-ice/methanol. Frozen cells were solubilized in lysis buffer (in mM: 150 NaCl, 25 Tris-HCl, 2 EDTA, pH 7.5, with protease and phosphatase inhibitors (0.025 leupeptin, 0.025 aprotinin, 20 β-glycerophosphate, 0.0005 microcystin, 10 pyrophosphate, 0.0001 vanadate) and 0.5% Triton-X (30 min, 4 °C). The lysate was centrifuged (5 min. at 10,000 × g), and the supernatant recovered. Four micrograms each of anti-Ca_{3.2} or goat IgG (Santa Cruz Biotechnology Inc) were incubated with 2 mg of supernatant (2 h, 4 °C), before incubation with protein G-Sepharose. The immunoprecipitates were washed (3×) with lysis buffer, and then (3×) with binding buffer (in mM: 140 K^{+}-aspartate, 5 MgCl_{2}, 10 EGTA, 20 HEPES, pH 7.4, 0.162 CHAPS (0.01%), and 0.04 dithiothreitol). Immunoprecipitates were incubated with 10 nM recombinant Gβ_{1,γ2} or Gβ_{2,γ2} (room temperature, 30 min.) in binding buffer (100 μL), and transferred to WIZARD minicolumns (Promega) for rapid washing (6×) with binding buffer. Immunoprecipitated channel and bound recombinant proteins were eluted with 2× SDS sample buffer at 80 °C. Samples were resolved by 4–15% SDS-PAGE, and analyzed by immunoblot using anti-FLAG (Sigma-Aldrich), anti-Gβ_{2,γ2} and anti-Gβ_{1,γ2} (Santa Cruz Biotechnology).

Phosphorylation Assay—Recombinant GST fusion proteins of Ca_{3.2} II-III loop and deletion mutants were purified as previously described (26). Each phosphorylation reaction (30 μL) contained (in mM): 50 Tris-HCl pH 7.4, 11 MgCl_{2}, 0.2 ATP, 0.2 μM recombinant protein, 0.5 μL PKA (Calbiochem, CA), 10 μCi [γ^{32}P]ATP. Reactions were incubated at 30 °C for 30 min and stopped by boiling in SDS loading buffer. Samples were separated by 10% SDS-PAGE and stained with Coomassie Blue. Phosphorylation levels were assessed by the Storm 820 Phosphorimager (Amersham Biosciences).

RESULTS

PKA Activity Enables the Inhibition of Ca_{3.2} Unitary Currents by Gβγ—In patches excised from HEK293 cells stably expressing Ca_{3.2} channels, recombinant Gβ_{2,γ2} subunits reduce the frequency of Ca_{3.2} unitary openings (23). Here, we confirm these findings in HEK293 cells that are transiently expressing recombinant Ca_{3.2} channels. Calcium current was elicited from a holding potential of −90 mV by a depolarizing test pulse to −35 mV. Following patch excision, control activity was measured for 5–6 min during bath perfusion with vehicle, before the direct application of Gβγ subunits. At −35 mV, active calcium channels displayed varied patterns of small openings. As illustrated in Fig. 1A from an exemplar recording of an excised patch that may have more than one active channel, channel openings were abundant early in the record producing a transient ensemble averaged current consistent with time-dependent inactivation. Gβ_{2,γ2} (1–2 nm) reduced (41%) the channel open probability (N_{Po}) per sweep, a value calculated from the ratio of the total open time and the test pulse duration, and increased (193%) the number of silent/null sweeps (denoted by the solid circles). Surprisingly, these findings were not replicated in human adrenocortical carcinoma cells (NCI-H295R) transiently expressing recombinant human Ca_{3.2} channels. Rather, we observed that Gβ_{2,γ2} failed to inhibit Ca_{3.2} single channel currents recorded from patches excised from unstimu-
lated NCI-H295R cells; neither the single channel probability (NPo) per sweep, nor the number of silent sweeps was changed by the application of \( G_{\beta\gamma} \) dimers (Fig. 1B). To reconcile these findings, we considered the possibility that activation of an additional second messenger system that remained quiescent in unstimulated H295R cells was required for current inhibition.

A role for PKA in the regulation of LVA current is supported by numerous electrophysiological studies; however, the resulting alteration in channel activity seems context dependent and complex. PKA increases recombinant currents expressed in \textit{Xenopus} oocytes (27), mammalian cells (HEK293 or CHO) (28), and bullfrog atrial myocytes (29) yet cAMP analogues do not increase native mammalian LVA currents (16, 20, 30–32). Nevertheless, pharmacologic inhibition of PKA activity can prevent the stimulation (33, 34) or even the inhibition (19, 20, 35) of native LVA currents induced by G-protein receptor activation. Accordingly, we considered the possibility that PKA mediated phosphorylation of the channel protein may be required for \( G_{\beta\gamma} \) dimer-mediated inhibition of Cav3.2 single channel currents. We treated H295R cells expressing Cav3.2 channels with \( 10^{-8} \) M 8-Br-cAMP for 10 min prior to patch excision, and tested for inhibition. \( G_{\beta\gamma} \) dimers (Fig. 1D) and concomitantly decreased NPo (diary plot, Fig. 1D) and increased the number of silent sweeps. The \( G_{\beta\gamma} \)-mediated decrease in sweep NPo averaged 35 ± 3% (\( n = 6 \)) and was accompanied by a 131 ± 44% increase in the number of null sweeps. These changes in channel open probability were not elicited by \( G_{\beta_1\gamma_2} \) dimers (Fig. 1E) and were not complemented by changes in: the first latency to open (50.7 ± 4.9 ms (cAMP), 50.4 ± 0.6 ms (cAMP + \( \beta_2\gamma_2 \))), the single channel conductance of (6.6 pS with 60 mM CaCl\(_2\)), or the open-state distributions of dwell times, which remained fitted to the sum of two exponentials with unchanged proportions (cAMP + \( \beta_2\gamma_2 \); \( \tau_1 = 0.60 ± 0.14 \) ms (62%), \( \tau_2 = 2.61 ± 0.26 \) ms).
Hence, modulation of Ca^v3.2 single channel currents by many native cells including the aldosterone producing ZG cell, human NCI-H295R adrenocortical carcinoma cells (38, 39). are expressed in the human and rodent adrenal cortex, and in we characterized inhibition of recombinant Cav3.2 channels protein G (Fig. 2 voltage-dependence of activation (Fig. 2 shown), and was not accompanied by either a change in the occurrence at all test potentials (4). Dopamineduced by endogenous dopamine receptors in H295R previously established in HEK293 cells (23).

FIGURE 2. Dopamine inhibition of Ca^v3.2 current. A, left panel, time course of Ca^v3.2 current inhibition by 50 μM dopamine (a–d). Note that application of 10 μM 8-Br-cAMP (a–c) or vehicle (a–b) alone was without effect. Right panel, representative current recordings from H295R cells. Currents were elicited by a test pulse to −15 mV from a holding potential at −90 mV following repolarization to −75 mV. B, dopamine evokes a dose-dependent inhibition of channel current. Numbers are cells recorded for each condition. *, p < 0.05 compares control with dopamine treatment by ANOVA. Both the steady-state activation (C) and inactivation (D) properties of Ca^v3.2 remain unaltered by 50 μM dopamine, p > 0.05, n = 7–9. Sample currents shown to the right (c) and left (d).

(38%) (supplemental Fig. S1). Nor did G^βγ alter the amplitude distribution of the test-pulse currents. Average midpoints for the primary conductance state (65% of openings) were not different from each other (−0.394 ± 0.004 pA, cAMP; −0.389 ± 0.005 pA, cAMP + G^βγ, at −35 mV). Hence, modulation of Ca^v3.2 single channel currents by G^βγ dimers in cAMP-stimulated model ZG cells displayed hallmark characteristics of native LVA currents (24, 29, 36) and G^βγ regulation of recombinant Ca^v3.2 unitary currents previously established in HEK293 cells (23).

Dopamine Induces a Voltage-independent Inhibition of Ca^v3.2 Current That Requires Active G^βγ Dimers and PKA— Dopamine mediates its actions via two distinct classes of receptors: D_1-like (D_1, D_3) that couple to the heterotrimeric G protein Gα_12, and D_2-like receptors (D_2, D_3, D_4) that couple to Gα_2/ Gα_13 (37). D_1-like receptors (D_1, and D_3) receptors are expressed in the human and rodent adrenal cortex, and in human NCI-H295R adrenocortical carcinoma cells (38, 39). Because dopamine inhibits nickel-sensitive LVA currents in many native cells including the aldosterone producing ZG cell, we characterized inhibition of recombinant Ca^v3.2 channels transduced by endogenous dopamine receptors in H295R cells that lacked native Ca^v3.2 channel expression. Dopamine produced current inhibition (Fig. 2, A and B) that was slow in onset (Fig. 2A), depended on dose (1–50 μM; Fig. 2B), occurred at all test potentials (−45 to +15 mV; data not shown), and was not accompanied by either a change in the voltage-dependence of activation (Fig. 2C) or inactivation (Fig. 2D). Therefore, this scaled reduction in Ca^v3.2 whole cell calcium current in H295R cells by dopamine mimics the voltage-independent inhibition of current induced by G^βγ dimers in HEK293 cells (22, 23) expressing human recombinant Ca^v3.2 channels.

Therefore, we perturbed dopamine signaling to determine whether cAMP and active G^βγ subunits are required for inhibition of Ca^v3.2 current by dopamine. We provided a “sink” for active G^βγ by transfecting H295R cells with either transducin, the sensory rhodopsin II Gα subunit, or βARKct, the C terminus of β-adrenergic receptor kinase-1 (40). The expression of either transducin or βARKct prevented the inhibition of Ca^v3.2 current induced by dopamine (Fig. 3A). Current levels remained indistinguishable from those of vehicle-treated cells transfected with empty vector (vehicle: 1.1 ± 1.5%, n = 8; βγ sinks combined: 6 ± 2.4%, n = 9). Because PKA plays a central role in dopamine signaling, and 8-Br-derivatives of cyclic nucleotides preferentially target nucleotide activated protein kinases (41), we evaluated PKA as the cAMP effector molecule. To prevent PKA activation by dopamine, we used PKI (5–24), a selective peptide inhibitor that binds to the catalytic subunit of protein kinase A. Inclusion of PKI in the patch pipette precluded channel inhibition induced by dopamine but failed to inhibit Ca^v3.2 channel current in vehicle-treated cells (vehicle: 2.7 ± 1.5%, n = 11; PKI: 0.4 ± 2.3%, n = 5; n.s.). Collectively, these data identify PKA as the cAMP effector and provide support for a cooperative role for active G^βγ subunits and PKA in the regulation of channel activity by dopamine.
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II-III Intracellular Loop Harbors a Critical PKA Phosphorylation Site for Channel Regulation—Inhibition of Ca₃.2 channels by heterologously expressed Gβ₂γ₂ dimers depends on the intracellular loop that connects transmembrane domains II and III. Replacing the II-III loop of Ca₃.2 channels with that of unregulated Ca₃.1 channels prevents current inhibition (22). To test the importance of the II-III loop in the regulation of LVA channels by dopamine, we evaluated the regulation of a chimeric Ca₃.2 channel (Ca₃.2 (Ca₃.1 II-III)) that contains the II-III loop from Ca₃.1 channels. Each of three channel constructs: wild-type Ca₃.1, wild-type Ca₃.2, or chimeric Ca₃.2, Ca₃.2(Ca₃.1 II-III) was transiently expressed in H295R cells and tested for dopamine-induced current inhibition (Fig. 4A). Dopamine reduced Ca₃.2 channel current by 21.3 ± 1.6% (n = 15), but failed to decrease current carried by Ca₃.1 channels (4.0 ± 3.0%, n = 9), Ca₃.2 chimeric channels also were refractory to inhibition by dopamine (4.5 ± 3.2%, n = 8) despite displaying gating properties similar to wild-type Ca₃.2 channels. These data are in agreement with channel subtype-specific regulation by Gβγ subunits, and the important role of the II-III loop in this mechanism of regulation.

The Ca₃.2 channel protein contains 25 phosphorylation sites that conform to the minimal consensus sequence for PKA phosphorylation (R/R/Y/K/S/T/T/Y/X- hydrophobic). We evaluated the highest stringency PKA site in the channel protein located on the C terminus (Arg-Arg-Arg-Thr²²¹⁸-Pro), and the two highest stringency sites located on the II-III loop (Arg-Arg-Ser¹¹⁴⁴-Trp; Arg-Arg-Gly-Ser¹¹⁰⁷-Ser), as potential sites for PKA regulation (Fig. 4A). These selected residues in the full-length channel protein were mutated alone or in combination, and currents carried by the expressed channel constructs were tested in H295R cells for agonist-induced inhibition. The combined mutation of residues: S1144A, S1107A, and T2214A, produced a triple mutant channel that had normal gating properties (data not shown). Notably, this mutant channel was refractory to modulation by dopamine. Following agonist exposure, current levels remained indistinguishable from those of vehicle-treated cells expressing wild-type Ca₃.2 channels (% inhibition: vehicle: 1.4 ± 1.5%, n = 17; triple mutant: 5.1 ± 1.5%, n = 7; n.s.). Channels harboring the Ser¹¹⁰⁷ mutation were similarly refractory to inhibition by dopamine (4.9 ± 3.9%, n = 8; n.s.). This inhibition of modulation was specific for the Ser¹¹⁰⁷ site, as calcium current carried by Ca₃.2 channels harboring either an adjacent mutation in the II-III loop (Ser¹¹⁴⁴) or one in the channel C terminus (Thr²²¹⁸, data not shown) were regulated by dopamine. To confirm that the Arg-Arg-Gly-Ser¹¹⁰⁷-Ser recognition motif on the II-III loop is indeed a site for PKA phosphorylation, we used GST fusion proteins that expressed either full-length or abbreviated II-III loop sequences that internally removed one or both of the PKA recognition motifs (~60 amino acids) as substrates in in vitro phosphorylation reactions. The catalytic subunit of PKA robustly phosphorylated the full-length loop (amino acids 1019–1300) and the abbreviated loop devoid of the S1144 recognition motif (d: 1142–1192) to nearly equal extent (Fig. 4B). By contrast, removal of the S1107 recognition motif alone (d:1059–1108) or in combination with that of S1144 (d:1097–1154) dramatically decreased the level of incorporated phosphate (Fig. 4B). Moreover, because deletion of the S1144 recognition motif reduced phosphate incorporation only when the S1107 motif was also removed, our data imply that Ser¹¹⁰⁷ is the preferred phosphorylation site in the full-length II-III loop. Collectively our mutagenesis studies highlight the critical role played by PKA-mediated phosphorylation and identify the phosphorylation state of residue Ser¹¹⁰⁷ as a molecular switch for the control of Ca₃.2 channel activity induced by dopamine.

Based on these findings, we hypothesized that the efficacy of Gβ₂γ₂ in the excised-patch also would be dependent on the phosphorylation state of Ser¹¹⁰⁷. Accordingly, Gβ₂γ₂ would not be expected to inhibit Ca₃.2 single channel currents carried by channels harboring the S1107A mutation. As predicted, S1107A mutant Ca₃.2 channels were refractory to modulation by Gβ₂γ₂ (Fig. 5, A–C) as they were refractory to modulation by dopamine. Importantly, this dependence on the phosphorylation state of Ser¹¹⁰⁷ for Gβ₂γ₂ inhibitory activity was not a specific requirement of H295R cells. S1107A mutant Ca₃.2 channels expressed in HEK293 cells were also refractory to modulation by Gβ₂γ₂ in the excised-patch (Fig. 5, A–C). Hence the high cAMP tone of HEK293 cells (43), likely promotes PKA activation and precludes the need for cellular pretreatment with cAMP, permitting the regulation of wild-type Ca₃.2 channels by Gβ₂γ₂ subunits. To provide additional experimental support for this rationale, we analyzed the state of phosphorylation of FLAG-tagged Ca₃.2 channels expressed in HEK293 cells using a phospho-(Ser/Thr) PKA substrate antibody. Wild-type, single (Ser¹¹⁰⁷), and triple (S1144A, S1107A, and T2214A) mutant channels expressed in HEK293 cells were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted with phospho-(Ser/Thr) PKA substrate antibody. As
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We questioned further whether phosphorylation of the channel by PKA was required for binding or inhibitory action of Gβ₂γ₂ subunits. We immunoprecipitated cAMP-phosphorylated Ca₃.2 channels from HEK293 cells and tested binding of recombinant Gβγ dimers to the channel protein in vitro. After extensive washing, bound Gβγ dimers were separated from FLAG-tagged channels by SDS-PAGE and detected in immunoblots using β-specific antibodies. Consistent with selective modulation of Ca₃.2 channels by Gβ₂-containing dimers, recombinant Gβ₂γ₂, but not Gβ₁γ₂, dimers, bound to the immunoprecipitated channel protein (Fig. 6A). The measured binding was specific to the channel immunoprecipitated as binding to an immunoglobulin immunoprecipitate was null. Importantly, binding of Gβ₂γ₂ dimers to S1107A mutant Ca₃.2 channels was equivalent to that of wild-type channels implying that phosphorylation of Ser₁¹⁰⁷, in addition to binding, is needed for Gβ₂γ₂ inhibitory activity (Fig. 6B). Collectively these data identify the mechanism underlying regulation of Ca₃.2 channels by Gβ₂γ₂ dimers and show that phosphorylation of Ser₁¹⁰⁷ acts as a molecular switch to enable Gβ₂γ₂ inhibitory activity.

Co-activation of D₁ and D₂ Receptors Is Required for Channel Inhibition—The relative contribution of D₁ versus D₂ receptors to current inhibition in ZG cells is ambiguous with data supporting roles for each receptor subtype (20, 21). Typically, D₁ and D₂ receptors transduce opposing alterations in cAMP signaling that underlies their antagonistic actions. However, Gαi-linked D₂ receptors can contribute to the activation of PKA (44) if cAMP is generated by specific adenylyl cyclase subtypes (AC2, AC4, AC7). These AC subtypes are resistant to inhibition by Goα subunits, and are activated by Gβγ dimers released from Gi/o-linked receptors (45). Notably, AC subtypes 2 and 3 are expressed robustly in the plasma membrane compartment of the human adrenal (39, 46), and in ZG cells activation of Gαo-linked D₂ receptors does not reduce cAMP formation illustrated in supplemental Fig. S2, wild-type Ca₃.2 channels demonstrated a high degree of phosphorylation, which in part, was attributable to PKA activity as the states of phosphorylation of the PKA-triple and the single S1107A mutant channel were proportionally reduced.

In vitro phosphorylation of full-length or abbreviated Ca₃.2 II-III loop GST fusion proteins by the catalytic unit of PKA. Numbers denote residue positions in the full-length channel. WT, full-length II-III loop, amino acids 1019–1121; S1107, amino acids 1059–1107 deleted; S1144, amino acids 1142–1192 deleted. For each condition, mean ± S.E. of percent inhibition of current density recorded from H295R cells 10 min after the application of 50 μM dopamine. Note, S1107 is permissive for dopamine-induced current inhibition. Numbers are cells patched for each condition. *, p < 0.05 compared to control with treatment group by ANOVA. Right panel, sample currents recorded at −15 mV from holding −90 mV following repolarization to −75 mV for each condition. B, in vitro phosphorylation of full-length or abbreviated Ca₃.2 II-III loop GST fusion proteins by the catalytic unit of PKA. Numbers denote residue positions in the full-length channel. WT, full-length II-III loop, amino acids 1019–1121; S1107, amino acids 1059–1107 deleted; S1144, amino acids 1142–1192 deleted. For each condition, mean ± S.E. of percent inhibition of current density recorded from H295R cells 10 min after the application of 50 μM dopamine. Note, S1107 is permissive for dopamine-induced current inhibition. Numbers are cells patched for each condition. *, p < 0.05 compared to control with treatment group by ANOVA. Right panel, sample currents recorded at −15 mV from holding −90 mV following repolarization to −75 mV for each condition.
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FIGURE 5. Inhibition of Ca_{3.2} channel activity in the excised patch by recombinant Gβγ dimers required PKA phosphorylation of Ser^{1107}. Shown are records and analysis of unitary currents of Ca_{3.2} channels elicited by repeated (6-s) test pulses to −35 mV from −90 mV. A, five consecutive sweeps and the ensemble average of 150 sweeps concatenated from three cells (bottom trace) recorded before (left) and after (right) reconstitution of 1 nmol Gβγ into the patch. Unitary currents recorded from mutant Ca_{3.2} channels (Ser^{1107} to Ala^{1107}) expressed in HEK293 cells (right panel) or H295R cells with 10 μM 8-Br-cAMP pretreatment (left panel). B, bar graph plots show the time course of NPo from the representative patch (H295R, left; HEK293, right) before and after adding Gβγ. Filled circles at 0.3 (NPo) show null/inactive sweeps. C, bar graph plots mean ± S.E. of percent reduction in NPo (left) or percent increase in nulls (right) elicited by Gβγ for wild-type versus mutant Ca_{3.2} channels recorded in either H295R or HEK293 patches. Numbers are patches recorded for each condition. *p < 0.05 compares wild-type versus mutant Ca_{3.2} channels by ANOVA.

FIGURE 6. Recombinant Gβγ dimers bound selectively to Ca_{3.2} channels in vitro. Immunoprecipitation of FLAG-tagged Ca_{3.2} channels from HEK293 cell lysates using a Ca_{3.2} channel specific antibody or a goat IgG. A, binding of 10 nmol of recombinant Gβγ dimers: Gβγ (left, green), or Gβγ (right, red) to the immunoprecipitate. Immunoblots show bound Gβ subunits detected with Gβ-specific antibodies after resolution on SDS-PAGE. B, binding of Gβγ dimers to wild-type and S1107A mutant channel proteins was compared. FLAG-tagged Ca_{3.2} channel protein in immunoprecipitates was detected with Anti-FLAG antibody. Shown is representative experiment (n = 4). Notably, S1107 was not required for Gβγ-specific binding.

μM) (37), but when added together synergized to inhibit Ca_{3.2} channel currents (10.2 ± 0.5%, n = 5). By contrast, at 2 μM each agonist alone failed to inhibit channel activity. Taken together, these data indicate that coactivation of D1 and D2 receptors is required for channel inhibition.

Based on the mechanism described above for channel inhibition that requires the dual activity of PKA and released Gβγ dimers, we examined further the contribution of Gαs-linked D2 receptors and Gβγ-linked D1 receptors to second messenger generation. We hypothesized that if active D2 receptors preferentially released Gβγ subunits, and D1 receptors preferentially generated cAMP (Fig. 7D), we would expect cAMP treatment to permit the inhibition of Ca_{3.2} channel activity by D2-receptor agonists but not D1-receptor agonists. 10 μM 8-Br-cAMP was ineffective alone (see Fig. 1A) and failed to facilitate current inhibition produced by the D1 receptor agonist SKF (1 μM), but enabled the inhibition of current produced by the D2 receptor agonist bromocriptine (1 μM, Fig. 7C). Moreover, this current inhibition induced by bromocriptine was prevented by preexposure to the D2 receptor antagonist, raclopride (20 μM) but not by the D1 receptor antagonist, SCH-23390 (10 μM). Thus, our data provide support for an underlying intracellular mechanism whereby each dopamine receptor subtype preferentially contributes one critical signal toward producing the inhibition of Ca_{3.2} channel activity.

DISCUSSION

Our results define a molecular mechanism by which PKA controls inhibition of Ca_{3.2} channels by Gβγ dimers and offer a cellular explanation for the regulation of LVA calcium channels by dopamine. These data describe how cAMP combines with active Gβγ dimers to inhibit Ca_{3.2} channels and unites previous observations that have suggested the importance of these signaling molecules in dopamine-induced current inhibition (20). We show that PKA is the effector protein for cAMP
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and that PKA activity acts as a molecular switch to permit the inhibition of Ca₃.2 channels by Gβ₂γ₂ subunits.

The phosphorylation state of a critical serine residue (Ser₁₁₀⁷) located on the II-III loop of the channel protein governs the regulation of channel activity by Gβγ subunits. Neither the phosphorylation state of an adjacent PKA site (Ser₁₁₄⁴) on the II-III loop with equivalent stringency nor the phosphorylation state of the highest stringency site on the channel protein (Thr₂₂₁⁴) is permissive for channel regulation by Gβγ subunits. Significantly, this requirement for channel phosphorylation provides new possibilities for precise tuning of calcium signaling via the control of cyclic nucleotide phosphodiesterase and/or protein phosphatase activities.

Gβ₂-containing Gβγ dimers are the only dimeric isoforms that bind to the channel II-III loop and reduce the open probability of Ca₃.2 channels (22, 23). Here we show that PKA activity does not change Gβ subtype selectivity but rather allows Gβ₂γ₂ dimers to silence channels in the membrane, decreasing their frequency of opening. This requirement for channel phosphorylation is not cell context dependent as our data show that Ca₃.2 channels expressed in HEK293 cells are basally phosphorylated and recombinant Gβ₂γ₂ dimers do not regulate unitary currents carried by S1107A mutant channels expressed in either H295R or HEK293 cells. Ser₁₁₀⁷ phosphorylation does not regulate Gβ₂γ₂ binding to the channel protein; hence we conclude that the phosphorylation of Ser₁₁₀⁷ in addition to Gβ₂γ₂ binding is required to inhibit Ca₃.2 channels. Interestingly, phosphorylation of Ser₁₁⁰⁸ in the II-III loop is critical for CaMKII regulation of Ca₃.2 channel activity (47) suggesting an important role for II-III loop phosphorylation in Ca₃.2 channel gating.

Although D₁/D₂ receptor co-activation typically results in antagonism at the cellular level (37, 48), our studies provide evidence for a novel cellular mechanism by which D₁/D₂ receptors coordinate to produce a cellular response that differs from other receptor coactivation paradigms previously reported that have underscored the production of either an amplified signal (44) or the generation of a novel second messenger that is not produced by activation of either receptor alone (32).

Here, we show that each receptor independently produces a signal (cAMP/active Gβ₂γ₂ subunits) that is required to mediate current inhibition. Notably, although our studies do not rule out the possibility that activation of Gβ₂γ₂ subunits by Gβ₂γ₂-linked D₂ receptors may contribute to cAMP formation by stimulating Gβγ-sensitive adenyl cyclase isoforms, our data indicate that cAMP-independent signaling also is required to elicit current inhibition, as the application of 8Br-cAMP alone failed to reduce either Ca₃.2 whole-cell or single channel current. Thus, the signaling paradigm outlined here differs from that described in the nucleus accumbens where coactivation of D₁ and D₂ receptors coordinate to increase spike firing by amplifying the production of a single second messenger, cAMP (44). Dual modulation of T-type calcium channels by PKA and Gγ₆ proteins also underlies amplified facilitation of LVA currents in frog atrial myocytes although the intracellular mechanism of this potentiation remains undefined (29).

In addition, our data highlight the previously underappreciated role of receptor co-activation in dopamine induced LVA current inhibition. Our proposed dual receptor mechanism is consistent with previously reported studies that either have focused solely on the effectiveness of D₁ or D₂ antagonism in preventing dopamine-induced current inhibition (19–21, 49); or have underscored the ineffectiveness of D₁-antagonism by circumventing D₁ receptor signaling with intracellular cAMP fixed at high levels (17). Nevertheless, in pituitary melanotrophs D₂ agonists used at receptor selective concentrations induce current inhibition without apparent D₁ receptor co-ac-
tivation (18). Because the most sensitive inhibitory current responses to dopamine have been reported in pituitary lactotrophs when cAMP is fixed at 200 μM (17), and because 10 μM 8-Br-cAMP potentiates current inhibition by dopamine in H295R cells, we speculate that differences in the expression of Gβγ-sensitive adenyl cyclase isoforms and hence PKA activity may obviate a requirement for D1 receptor activation in some cellular preparations.

Collectively our data show that protein kinase A acts as a molecular switch to enable the voltage-independent inhibition of Cav3.2 currents by Gβγ subunits and provides a mechanism for expanding the dynamic range of Cav3.2 channel activity. In the adrenal glomerulosa cell, where aldosterone production is driven by Cav3.2 channel activity, failure of this mechanism may contribute to aldosterone excess and the loss of inhibitory dopaminergic tone that is observed in some forms of human hypertension (50–54).

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*P. Q. Barrett, unpublished observations.*