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Title: **Protein kinase C-mediated inhibition of recombinant T-type $Ca_V3.2$ channels by neurokinin 1 receptors**

Authors: Azahel Rangel, Sergio Sánchez-Armass and Ulises Meza.

Affiliation: Departamento de Fisiología

Facultad de Medicina

Universidad Autónoma de San Luis Potosí

Av. Venustiano Carranza # 2405

San Luis Potosí, SLP, 78210, México.

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Running title: NK1R modulates $Ca_V3.2$

Corresponding author: Ulises Meza, Ph.D.
Departamento de Fisiología
Facultad de Medicina
Universidad Autónoma de San Luis Potosí
Av. Venustiano Carranza # 2405
San Luis Potosí, SLP, 78210, México
Phone number: +52-444-8262344
FAX number: +52-444-8176976
E-mail address: umeza@uaslp.mx

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Abbreviations: PKC, protein kinase C; NKA, neurokinin A; HEK, human embryonic kidney; EGFP, enhanced green fluorescent protein; GPCR, G-protein coupled receptor; $G\alpha_t$, rod transducin; RGS3T, regulator of G protein siganling protein 3T; RGS2, regulator of G protein siganling protein 2; Bis, bisindolylmaleimide; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; U73122, 1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; C_m, cell capacitance; R_s, series access resistance; SP, substance P; NK1R, neurokinin 1 receptor; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDD, phorbol -12, 13-didecanoate.

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Abstract

The voltage-activated T-type calcium channel ($Ca_V3.2$) and the G protein-coupled neurokinin 1 (NK1) receptor are expressed in peripheral tissues and in central neurons where they participate in diverse physiological processes including neurogenic inflammation and nociception. In the present report, we demonstrate that recombinant $Ca_V3.2$ channels are reversibly inhibited by NK1 receptors when both proteins are transiently coexpressed in human embryonic kidney (HEK293) cells. We found that the voltage-dependent macroscopic properties of $Ca_V3.2$ currents were unaffected during NK1 receptor-mediated inhibition. However, inhibition was attenuated in cells coexpressing either the dominant-negative $G\alpha_q$ Q209L/D277N or the regulator of G protein signaling proteins 2 (RGS2) and 3T (RGS3T) which are effective antagonists of $G\alpha_{q/11}$. By contrast, inhibition was unaffected in cells coexpressing human rod transducin ($G\alpha_t$), which buffers $G\beta\gamma$. Channel inhibition was blocked by U73122 and bisindolylmaleimide I, selective inhibitors of phospholipase $C\beta$ and protein kinase C (PKC), respectively. Inhibition was occluded by application of the PKC activator phorbol-12-myristate-13-acetate (PMA). Altogether, these data indicate that NK1 receptors inhibit $Ca_V3.2$ channels through a voltage-independent signaling pathway that involves $G\alpha_{q/11}$, phospholipase $C\beta$ and PKC. Our results provide novel evidence regarding the mechanisms underlying T-type calcium channel modulation by G protein-coupled receptors. Functional coupling between $Ca_V3.2$ channels and NK1 receptors may be relevant in neurogenic inflammation, neuronal rhythmogenesis, nociception and other physiological processes.

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The neurokinin 1 (NK1) receptor is a G-protein coupled receptor (GPCR) activated by the tachykinin peptides substance P (SP) and neurokinin A (NKA) (Pennefather et al., 2004). This receptor is mainly coupled to heterotrimeric G proteins of the $G_{q/11}$ family (Macdonald et al., 1996); hence stimulation of NK1 receptors typically activates signaling by phospholipase Cβ (PLCβ), inositol triphosphate (IP_3), diacylglycerol (DAG) and protein kinase C (PKC). NK1 receptors are widely expressed in diverse mammalian tissues, including peripheral and central nervous systems (Pennefather et al., 2004). NK1 receptors have been implicated in gastrointestinal motility and secretion, neurogenic inflammation, affective behaviors, and nociception (De Felipe et al., 1998; Shimizu et al., 2008).

Three distinct isoforms of T-type voltage-gated Ca^{2+} channels ($Ca_V3.1$-3.3) have been identified and cloned to date (Perez-Reyes, 2003). Like NK1 receptors, T-type channels are widely expressed (Talley et al., 1999; McKay et al., 2006) and play important roles in physiological processes including neuronal and cardiac pacemaker activity, vascular smooth muscle contraction, fertilization, and nociception (Perez-Reyes, 2003). Because T-type channels are activated by relatively small depolarizations from the cell membrane resting potential, abnormal T-type channel activity may give rise to abnormal cellular electrical excitability and may thereby contribute to disorders such as altered sensitivity to pain, absence epilepsy, and cardiac hypertrophy (Choi et al., 2007; Becker et al., 2008; Chiang et al., 2009).

It is now established that natively-expressed T-type channels can be modulated by G protein-dependent signaling pathways (Iftinca and Zamponi, 2009). However, there have

been conflicting reports related to this topic, possibly due to the presence of more than one type (or splice isoform) of T-channel, differences among receptors, G-proteins, downstream signaling proteins (e.g., kinases), or differences in recording conditions (Iftinca and Zamponi, 2009). Thus, activation of muscarinic receptors evoked either stimulation or inhibition of nickel-sensitive T-type currents in embryonic rat hippocampal neurons (Toselli and Lux, 1989) or human granulosa cells (Platano et al., 2005), respectively. Similarly, activation of SP receptors attenuated T-type currents in rat nucleus basalis neurons (Margeta-Mitrovic et al., 1997), whereas SP receptors increased T-type currents in rat spinal dorsal horn neurons (Ryu and Randic, 1990). Application of the PKC activator phorbol-12-myristate-13-acetate (PMA) stimulated T-type currents in neonatal rat ventricular myocytes (Furukawa et al., 1992) but inhibited T-type currents in both newborn rat dorsal root ganglion neurons (Schroeder et al., 1990) and MN9D dopaminergic cells (Kim et al., 2007). Other activators of PKC, such as 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and phorbol -12, 13-didecanoate (PDD) inhibited T-type channels in chick embryo dorsal root ganglion (DRG) neurons (Marchetti and Brown, 1988) and mouse thalamic neurons (Cheong et al., 2008), respectively. Thus, there is currently much uncertainty regarding the modulatory effects of a particular G protein-coupled receptor on an identified T-type channel.

Expression of recombinant channels with identified receptors provides an alternative approach that reduces the number of variables inherent to native systems. Heterologous coexpression of cDNAs encoding a single variety of voltage-gated Ca^{2+} channels with a single receptor type has yielded valuable insights into the modulation of both high voltage-activated Ca_V channels (Tedford and Zamponi, 2006), and low voltage-activated Ca_V3

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channels (Iftinca and Zamponi 2009). However, the signaling pathways that modulate Ca_V3 channels, and the effects that such modulation may have upon physiological processes, remain incompletely characterized.

Although it is generally accepted that both $Ca_V3.2$ channels and NK1 receptors are expressed in peripheral and central neurons and that both participate in neurogenic inflammation and nociceptive transmission, no functional interaction between these two proteins has been previously reported. Here, we demonstrate that stimulation of NK1 receptors reversibly inhibits recombinant $Ca_V3.2$ channels expressed in HEK293 cells. Inhibition is voltage-independent and requires signaling by $G\alpha_{q/11}$, phospholipase Cβ and PKC.

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Materials and Methods

Cell culture and transfection. Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in a humidified air atmosphere containing 5% CO_2. The culture medium contained 90% Dulbecco's modified Eagle's Medium (GIBCO-Invitrogen, Grand Island, NY), 10% fetal bovine serum (GIBCO-Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Once a week, cells of low passage number (< 20) were replated at low density (~ 20-30% coverage) on 35 mm culture dishes and transfected within 3-5 days using $Ca_3(PO_4)_2$ precipitation technique (CellPhect Kit, Amersham Biosciences, Buckinghamshire, UK). The transfection mixture included expression plasmids encoding $Ca_V\alpha 3.2$ calcium channel subunit (at 1.0 μg/dish), and separate plasmids that encoded the neurokinin 1(NK1) receptor (1.0 μg/dish) and enhanced green fluorescent protein (EGFP) (0.1 μg/dish). In selective experiments, transfection mixture was added with either rod transducin ($G\alpha_t$) (1.0 μg/dish) or the dominant-negative Gαq Q209L/D277N (1.0 μg/dish), or the aforementioned plasmids (excluding EGFP) plus the fusioned proteins EGFP-RGS2 or EGFP-RGS3T (1.0 μg/dish). One day later, transfected cells were briefly trypsinized and replated at low density onto 12 mm round glass coverslips. Electrophysiological recordings were performed 24-36 h later. Successfully transfected cells were visually identified by their green fluorescence under ultra-violet (UV) illumination. Exclusively isolated green cells were used for patch-clamp recording.

Expression Plasmids. Human $Ca_V\alpha 3.2$ (α_{1H}) (GenBank Accession # AF051946) was in pcDNA3.1 (Invitrogen). Human NK1 receptor (NM015727) was in pCI (Promega,

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Madison, WI). Jellyfish enhanced green fluorescent protein (U55763) was in pEGFP (Clontech, Cambridge, UK). Human RGS3T (U27655) was in pEGFP-C3 (Clontech). Human RGS2 (L13463) was in pEGFP-C2 (Clontech). Human rod transducin (X63749) was in pcDNA3.1. Human dominant-negative $G\alpha_q$ Q209L/D277N (U40038) was in pcDNA3.1$^+$. The last construction was obtained from the University of Missouri-Rolla cDNA resource center.

Voltage-Clamp Recordings. Large-bore patch pipettes were pulled from 100 µl borosilicate glass micropipettes (World Precision Instruments, Inc., Sarasota, FL) and filled with an intracellular solution containing (in mM) 155 CsCl, 10 Cs_2-EGTA, 4 Mg-ATP, 0.32 Li-GTP and 10 HEPES, with pH adjusted to 7.4 with CsOH. Aliquots of pipette solution were stored at -80°C, kept on ice after thawing, and filtered at 0.22 µm immediately prior to use. Filled pipettes had d.c. resistances of 1.2-1.5 MΩ. The bath solution contained (in mM) 185 NaCl, 10 $CaCl_2$, 2 KCl and 10 HEPES, with pH adjusted to 7.4 with NaOH. After forming a GΩ seal in the cell-attached configuration, residual pipette capacitance was compensated using the negative capacitance compensation circuit of the Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). No corrections were made for liquid junction potentials. Ca^{2+} currents were recorded in the whole-cell, ruptured-patch mode. The steady holding potential (HP) in all the experiments was -90 mV. Test depolarizations were delivered at 0.1-0.2 Hz. Macroscopic Ca^{2+} currents were filtered at 2-5 kHz using the built-in Bessel filter (4-pole low-pass) of the amplifier and sampled at 10 kHz using a Digidata 1200 analogue-to-digital board (Molecular Devices) installed in a personal computer. The pCLAMP software programs Clampex and Clampfit (Molecular

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Devices, v.9.2) were used for data acquisition and analysis, respectively. Figures, data fits, and statistical comparisons were performed using the software program Origin (Microcal, Northampton, MA; v.6.0 and v.7.5). Series resistance (R_S), linear cell capacitance (C_m), and time constant for decay of the whole-cell capacity transient (τ) values were determined directly from readings on the amplifier. R_S was minimized for each cell by using the series resistance compensation circuit of the amplifier. The average value of C_m was 19.1 ± 0.7 pF (mean ± SEM; n = 143). The average values of compensated τ and R_S were 61.5 ± 1.5 μs and 3.5 ± 0.1 MΩ, respectively. The Ca^{2+} currents were typically evoked by step depolarizations from -90 to -20 mV. The average maximal current, measured at the time of peak inward current for each cell, was 1.0 ± 0.6 nA, and the corresponding average maximal voltage error was 3.3 ± 0.1 mV. The *d.c.* resistance of the whole-cell configuration was typically >1 GΩ. The Ca^{2+} currents were corrected for linear capacitance and leakage currents using -P/4 subtraction. All experiments were performed at room temperature (20-23 °C).

Reagents. Neurokinin A (NKA), Bisindolylmaleimide (Bis) I, Bis V, and Phorbol-12-myristate-13-acetate (PMA) were purchase from Calbiochem (San Diego, CA). NKA was dissolved in 5% acetic acid to make a 1.0 mM stock solution, aliquoted, and stored at -80ºC. Bis I and Bis V were dissolved in dimethylsulfoxide (DMSO) to make 2.5 and 3.0 mM stock solutions, respectively, aliquoted, and stored at 4ºC. PMA was dissolved in ethanol to make 1.5 mM stock solution, aliquoted, and stored at -20ºC. The final concentration of all the vehicles was always < 0.001%, which did not significantly modify the basal properties of $Ca_V3.2$ channel or its modulation by NKA.

Data Analysis. Results are reported as mean ± SEM. Treatment residuals were tested for homogeneity of variances and normality using the Brown–Forsythe and Shapiro–Wilk procedures, respectively. Means were compared using one-tailed unpaired Student's *t*-test, single population test, or one-way ANOVA, as indicated. Statistical significance was set at $p < 0.05$. For multiple comparisons using successive *t*-test, α was maintained at the 0.05 level using the Bonferroni correction. The experimental design corresponded to generalized random blocks, where the blocking criterion was the cell transfection.

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Results

1. $Ca_V3.2$ currents are inhibited through activation of NK1 receptors.

Previously, it has been shown that T-type $Ca_V3.2$ channels are modulated by several different types of heterotrimeric G protein-coupled receptors (e.g., Welsby et al., 2003; Wolfe et al., 2003; Kim et al., 2006; Kim et al., 2007; Iftinca et al., 2007; Tao et al., 2008). However, little is currently known regarding modulation of T-type channels by tachykinin receptors. This is a significant issue since T-type channels and tachykinin receptors both play important roles in diverse physiological processes, including neurogenic inflammation and nociception. To address this gap in knowledge, we investigated the effects of activation of NK1 receptors on $Ca_V3.2$ channels. We used the natural agonist neurokinin A (NKA), because this peptide functions as a full agonist for NK1 receptors (Pennefather et al., 2004), yet its effects are easily reversible upon washout. As illustrated in Fig. 1A, 10 nM NKA inhibited $Ca_V3.2$ currents by 23.9 ± 1.3% (n = 59, $p < 0.05$). Inhibition was maintained throughout NKA applications and was fully relieved following washout (Fig. 1B). No evidence for receptor desensitization was observed during NKA applications lasting up to 3 min, in agreement with our previous report (Meza et al., 2007). As shown in Fig. 1C, NKA generated a dose-dependent inhibition of $Ca_V3.2$ channels. The averaged normalized dose-response data were fitted with a Hill equation, yielding an IC_{50}value of 0.62 nM, and a Hill coefficient value of 0.56. Importantly, NKA did not affect $Ca_V3.2$ currents in cells not transfected with the NK1 receptor (data not shown). These results demonstrate that $Ca_V3.2$ channels are reliably and reversibly inhibited through NKA-dependent activation of NK1 receptors.

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2. NK1 receptors produce voltage-independent inhibition of $Ca_V3.2$ currents.

Modulation of Ca_V channels by G protein-coupled receptors may involve voltage-dependent and/or voltage-independent mechanisms (Tedford and Zamponi, 2006; Iftinca and Zamponi, 2009). Therefore, we assessed the voltage-dependence of inhibition of $Ca_V3.2$ channels by NK1 receptors. Fig. 2A shows representative $Ca_V3.2$ currents generated by a series of test pulses, in the absence or presence of 10 nM NKA. A similar degree of inhibition was observed at all test potentials, suggesting that inhibition of $Ca_V3.2$ by NKA was predominantly voltage-independent. Accordingly, the normalized current-voltage and conductance-voltage relationships, obtained both before and after the application of NKA, were not different (Fig. 2B and 2D). The corresponding mean values of $V_{1/2}$ for maximal conductance were -31.3 ± 1.4 and -32.8 ± 1.8 mV, in the absence and presence of NKA, respectively (n = 9; $p > 0.05$). The values of the slop factor (s) were 6.6 ± 0.3 and 6.5 ± 0.4 mV before or after NKA, respectively ($p > 0.05$). Likewise, the normalized steady-state voltage-dependent inactivation relationship was not modified by NKA (Fig. 2C and 2D). The corresponding values of $V_{1/2}$ for maximal steady-state inactivation were -54.8 ± 0.8 and -55.6 ± 2.1 mV, before and after application of NKA, respectively (n = 5; $p > 0.05$). The values of s were 5.2 ± 0.5 and 6.0 ± 1.0 mV in the absence or presence of NKA, respectively ($p > 0.05$). Finally, we looked for effects of NKA on activation and inactivation kinetics of $Ca_V3.2$ currents. Neither activation nor inactivation rate were modified by NKA at any test potential (Fig. 2E and 2F). Altogether, these data indicate that inhibition of macroscopic $Ca_V3.2$ currents by NK1 receptors does not involve significant changes in channel voltage-dependence. Thus, NK1 receptor activation produces a voltage-independent inhibition of $Ca_V3.2$.

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3. $G\alpha_{q/11}$ subunits mediate inhibition of $Ca_V3.2$ by NK1 receptors.

It is well known that NK1 receptors mainly couple to heterotrimeric $G_{q/11}$ proteins (Macdonald et al., 1996). Accordingly, we recently reported (Meza et al., 2007) that both $G\alpha_{q/11}$ and $G\beta\gamma$ subunits are involved in mediating a complex modulation of $Ca_V2.3$ (R-type) calcium channels by NK1 receptors. To examine this question for $Ca_V3.2$, the dominant-negative $G\alpha_q$ Q209L/D277N (Kumar et al., 2008), or the scavenger proteins for either $G\alpha_{q/11}$ subunits (i.e., RGS2 or RGS3T) (Kammermeier and Ikeda, 1999; Meza et al., 2007) or $G\beta\gamma$ subunits (i.e., rod transducin) (Meza et al., 2007) were separately expressed with $Ca_V3.2$ channels and NK1 receptors in HEK293 cells. In cells expressing the dominant-negative $G\alpha_q$ Q209L/D277N inhibition of $Ca_V3.2$ currents by NKA was reduced to 7.3 ± 1.4% (n = 8), compared to 16.0 ± 3.1% (n = 9) in matching control cells ($p < 0.05$). In cells that expressed RGS3T, inhibition was reduced to 6.4 ± 2.1% (n = 6), compared to 30.3 ± 6.1% (n = 6) in matching control cells ($p < 0.05$) (Fig. 3A-C). Similarly, inhibition was significantly reduced in cells that expressed RGS2 (15.2 ± 2.1 %; n = 12 versus 24.7 ± 2.0%; n = 12 in matching controls; $p < 0.05$). In contrast, the magnitude of inhibition was not significantly reduced in cells expressing $G\alpha_t$ (19.8 ± 3.2%; n = 11 versus 24.9 ± 3.3 %; n = 11 in matched controls; $p > 0.05$) (Fig. 3D-F). These data suggest that inhibition of $Ca_V3.2$ channels by NK1 receptors requires signaling by $G\alpha_{q/11}$ but not $G\beta\gamma$.

4. Inhibition of $Ca_V3.2$ currents by NK1 receptors requires PKC.

Studies have reported that $Ca_V3.2$ channels are modulated by signaling through phospholipase C (PLC) and protein kinase C (PKC) (Park et al., 2003; Park et al., 2006;

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Chemin et al., 2007). To explore this subject in relation to $Ca_V3.2$ inhibition by NKA, cells that expressed both $Ca_V3.2$ channels and NK1 receptors were preincubated in culture medium containing 3 μM U73122, a specific inhibitor of PLCβ (Kumar et al., 2008). As illustrated in Fig. 4A-C, inhibition of $Ca_V3.2$ currents by NKA was significantly attenuated in these cells (7.8 ± 1.7%, n = 7) as compared with cells preincubated in vehicle (16.0 ± 0.8%, n = 7, $p < 0.001$). In addition, when 200 nM PMA, a well known activator of PKC (Kim et al., 2007), was directly applied in the bath, $Ca_V3.2$ currents were inhibited to an extent (25.9 ± 4.3%, n = 8) indistinguishable from that produced by application of 10 nM NKA (21.6 ± 2.5%, n = 8, $p > 0.05$) (Fig. 4D-F). Furthermore, channel inhibition by NKA was completely occluded by preapplication of PMA (Fig. 4D and 4E). Thus, the average percent of inhibition induced by simultaneous addition of PMA and NKA (22.0 ± 3.7) was not significantly different from that evoked by PMA alone (25.9 ± 4.3, n = 8; $p > 0.05$) (Fig. 4F), suggesting that both reagents were acting through a common signaling element (i.e., PKC). Finally, inhibition of $Ca_V3.2$ currents by NKA was significantly attenuated (6.1 ± 1.6%, n = 8, $p < 0.05$) in cells preincubated in 500 nM Bis I, a specific inhibitor of PKC (Sculptoreanu and de Groat, 2003) (Fig. 4G-I), whereas inhibition was not significantly reduced in cells preincubated in 500 nM Bis V (21.1 ± 3.2%, n = 8, $p > 0.05$), the inactive analogue of Bis I. Altogether, these results indicate that NK1 receptors inhibit $Ca_V3.2$ channels by inducing signaling by PLCβ and PKC.

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Discussion

It is known that T-type calcium channels in different cell types (expressed endogenously or heterologously) are modulated by diverse hormones and neurotransmitters acting through heterotrimeric G protein-coupled receptors, but the molecular mechanisms involved remain largely uncharacterized. In this study, we report that recombinant T-type $Ca_V3.2$ channels, transiently expressed in HEK293 cells, are reversible inhibited by $G_{q/11}$-coupled NK1 receptors, via a voltage-independent and PKC-mediated signaling pathway.

Previous studies have demonstrated that Ca_V channels are modulated (either enhanced or inhibited) by neurokinin receptors in various types of neurons, such as rat spinal dorsal horn neurons (Ryu and Randic, 1990), rat nucleus basalis neurons (Margeta-Mitrovic et al., 1997), rat dorsal root ganglions neurons (Sculptoreanu and de Groat, 2003), and rat nucleus tractus solitarius neurons (Endoh, 2006). Recently, we have reported that NK1 receptors induced a pertussis toxin- and cholera toxin-insensitive $G_{q/11}$-mediated modulation of high voltage-activated $Ca_V2.3$ channels coexpressed in HEK293 cells, through a complex mechanism that involved a fast Gβγ-mediated inhibition, and both slow inhibition and slow stimulation mediated by $G\alpha_{q/11}$ subunits (Meza et al., 2007). Our present results indicate that NK1 receptors evoked an inhibition of T-type $Ca_V3.2$ channels mediated by $G_{q/11}$ proteins via $G\alpha_{q/11}$ subunits that was independent from signaling by Gβγ. This conclusion is supported by the ability of the dominant-negative $G\alpha_q$ Q209L/D277N, RGS2 and RGS3T, but not $G\alpha_t$, to attenuate inhibition of $Ca_V3.2$ by NKA (Fig. 3). The absence of a Gβγ-mediated attenuation of $Ca_V3.2$ currents when NK1 receptors were activated differs from the previously reported inhibitory effect of D_1-dopamine receptors activation on

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$Ca_V3.2$ channels expressed in the human adrenocarcinomal cell line H295R, which was mediated selectively by $G\beta_2\gamma_2$ subunits (Wolfe et al., 2003). This difference could be attributed to the low endogenous level of $G\beta_2\gamma_2$ dimer expression in HEK293 cells (Wolfe et al., 2003). Our data also show that inhibition of $Ca_V3.2$ channels by NK1 receptors is mediated by PLCβ and PKC, since it was significantly diminished by their selective blockers U73122 and Bis I, respectively (Fig. 4). Furthermore, the inhibitory effect of NKA was totally occluded by preceding application of the PKC activator PMA (Fig. 4D-F). Overall, these results suggest that $Ca_V3.2$ channels were inhibited by NK1 receptors coupled to heterotrimeric $G_{q/11}$ proteins through the sequential activation of $G\alpha_{q/11}$, PLCβ, and PKC (Fig. 5). It is noteworthy that this signaling pathway differs from that formerly reported for slow inhibition of R-type $Ca_V2.3$ channels by NK1 receptors expressed in HEK293 cells, which was also mediated by $G\alpha_{q/11}$ subunits but it was independent from PKC activation (Meza et al., 2007).

The inhibition of $Ca_V3.2$ currents by NK1 receptors did not involve changes in their channel voltage-dependence (Fig. 2). This conclusion is consistent with previous reports for PKC-mediated modulation of Ca_V channels (e.g., Scultoreanu and de Groat, 2003; Kim et al., 2007). Several putative PKC consensus motifs localize to the II-III linker in Ca_V3 channels (Monteil et al., 2000) which could be involved in their regulation by this enzyme (Park et al., 2006). Interestingly, the II-III connecting loop is also a critical determinant in the PKA-, CaMKII-, and Rho-associated kinase-mediated modulation of Ca_V3 channels (Kim et al., 2006; Yao et al., 2006; Iftinca et al., 2007). The observed inhibition of $Ca_V3.2$ channels by NKA is probably not related to a change in the number of channels in the

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membrane, because the relatively rapid onset and reversal of the NKA modulation (Fig. 1B). Alternatively, it is possible that inhibition may indicate a decrease in the maximal channel opening probability and/or a reduction in the number of functional channels, as a consequence of phosphorylation events, either on the channel proteins themselves or on unidentified regulatory proteins (Churchill et al., 2009). It also remains to be explored whether NKA modulation influences the single channel conductance of $Ca_V3.2$.

The inhibition of $Ca_V3.2$ (or Ni^{2+}-sensitive) channels by GPCR signaling has been previously reported in rat nucleus basalis neurons by NK1 receptors (Margeta-Mitrovic et al., 1997), H295R cells by D_1-dopamine receptors (Wolfe et al., 2003), human granulosa cells by muscarinic receptors (Platano et al., 2005), rat thalamocortical neurons by metabotropic glutamate receptors type 1 (Cheong et al., 2008), and HEK293 cells by corticotrophin-releasing factor receptors 1 (Tao et al., 2008). Interestingly, in most cases, the inhibitory effect was never complete (i.e., < 50%). In the present experiments, we observed a similar partial inhibition (~ 25%) when a saturating concentration of NKA (1 μM) was applied (Fig. 1C). Further studies are necessary to elucidate whether the partial inhibitory effect of GPCR on $Ca_V3.2$ actually reflects an intrinsic quality of these channels.

The inhibition of $Ca_V3.2$ channels by PMA observed in our experiments (Fig. 4D-F) is consistent with other studies where PMA, and other activators of PKC (i.e., OAG, PDBu, and PDD), inhibited natively-expressed T-type currents in neuronal (Marchetti and Brown, 1988; Schroeder et al., 1990; Cheong et al., 2008) and non-neuronal cells (Kim et al., 2007). However, our data are also in contrast with studies in which $Ca_V3.2$ channels were

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either unaffected (Park et al., 2003) or significantly stimulated by PMA (Park et al., 2003; Park et al., 2006; Hildebrand et al., 2007). It is also interesting to mention the temperature-dependence of PMA effects. PMA had no effect on endogenous T-type channels in rat dorsal root ganglions neurons at room temperature, but inhibited these channels when temperature was raised to 29^{o}C (Schroeder et al., 1990). Similarly, PMA did not affect $Ca_V3.2$ channels expressed in At20 cells when applied at room temperature, but triggered significantly stimulation when cells were maintained for 10 min at 37^{o}C just prior to patch-clamp recording at room temperature (Chemin et al., 2007). These divergent results highlight the large variability among the experimental findings related to PKC-mediated modulation of T-type calcium channels. In future studies, it will be important to identify the specific isozymes of PKC or any other putative PKC-adaptors proteins (Churchill et al., 2009) activated by $G_{q/11}$-coupled receptors, or PMA, under different experimental conditions (e.g., distinct expressing cell lines, channel isoform, and temperature), to attempt to understand the origins of this variability (Iftinca and Zamponi, 2009).

Although the physiological relevance of the inhibitory action of NK1 receptors on $Ca_V3.2$ channels remains to be established, it is possible to postulate its participation in physiological processes where both native $Ca_V3.2$ channels and endogenous NK1 receptors are involved, i.e., neuronal rhythmogenesis and nociception. In this sense, it has been proposed that the attenuation of Ni^{2+}-sensitive T-type currents by NK1 receptors in nucleus basalis cholinergic neurons may decrease the activation of Ca^{2+}-dependent K^+ channels and thus maintain the neuronal excitability and prevent neuronal adaptation (Margeta-Mitrovic et al., 1997). In sensitive DRG neurons it is also expected that NK1 receptors modulate T-type $Ca_V3.2$ channels because both proteins are endogenously expressed in these cells

(Sculptoreanu and de Groat, 2003; Iftinca et al., 2007). Unexpectedly, T-type currents in DRG neurons of adult rat were not affected by SP (Sculptoreanu and de Groat, 2003). This quite surprising result is probably explained by the presence of unresponsive neurons to SP, given the significant heterogeneity of neuronal populations reported in DRG (Coste et al., 2006). In future studies, neuronal populations in DRG might be distinguished to better understand their electrophysiological characteristics and physiological function. Finally, the regulatory effect of NK1 receptors on T-type channels may also play a key role in neurotransmitter release (Bao et al., 1998), additional work is required to evaluate this issue.

In conclusion, we have shown that stimulation of NK1 receptors inhibited recombinant $Ca_V3.2$ channels through a voltage-independent mechanism that involved $G\alpha_{q/11}$, phospholipase Cβ and PKC signaling. Our data provide novel information that may help in understanding the physiological relationships between $Ca_V3.2$ channels and NK1 receptors and the molecular mechanisms underlying T-type calcium channel modulation by G protein-coupled receptors.

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Footnotes

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Address correspondence to: Dr. Ulises Meza, Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de San Luis Potosí, Av. Venustiano Carranza 2405, San Luis Potosí, SLP, 78210, México. E-mail: umeza@uaslp.mx

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Legends for figures

Fig 1. Inhibition of $Ca_V3.2$ channels by NK1 receptors. A, whole cell Ca^{2+} currents recorded in absence or presence of 10 nM NKA. Currents were evoked by voltage steps from -90 to -20 mV at 0.1 Hz. Cell capacitance (C_m) = 21.0 pF and series resistance (R_s) = 2.5 MΩ. B, time course of the inhibitory effect of NKA. The peak current amplitude was plotted as a function of time. Application of NKA is indicated by horizontal bar; same cell as in A. C, dose-response curve for inhibition. To eliminate the possibility of cumulative NKA effects, just one concentration on each recorded cell was added (n = 4 - 8). The solid line corresponds to the fit of the average data to the Hill equation: I_{Ca} (% inhibition) = D / [1+([NKA] / EC_{50})^h], where D represents maximal percent of inhibition, EC_{50} is the NKA concentration producing half-maximal inhibition, and *h* is the Hill coefficient.

Fig 2. The inhibitory effect of NKA on $Ca_V3.2$ channels is voltage-independent. A, current families recorded from the same cell before (upper) or after application of 10 nM NKA (lower). Currents were generated by depolarizations from -90 to -60 through +30 mV in 10 mV steps at 0.1 Hz. C_m = 17.8 pF and R_s = 3.3 MΩ. B, normalized I-V curve of $Ca_V3.2$ channels determined before (○) or during NKA application (●). Voltage protocol is as described in A. I-V curves were normalized to maximal control peak current amplitude for each cell. A scaled I-V curve during NKA application (□) is also shown for comparison. The maximal voltage error was 3.6 ± 0.4 mV (n = 8). C, currents evoked by a test pulse to -20 mV following a 2 s pre-pulse to indicated potentials were recorded from the same cell before (upper) or after application of NKA (lower). C_m = 11.3 pF and R_s = 4.5 MΩ. D, normalized activation and steady state inactivation curves in the absence (○) or presence of NKA (●). Voltage protocol is as described in A and C, respectively. The normalized

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conductance values were obtained for each cell by the chord conductance method; same cells as in B. The smooth lines of activation curves were calculated from the mean of the parameters determined by the fit of each individual data set (n = 9) to the Boltzmann equation: $G = 1 / (1+\exp [-(V-V_{1/2} / s])$. Where G is the normalized conductance, $V_{1/2}$ is the voltage for half maximal activation and s is the slope factor. Smooth lines of steady-state inactivation curves were calculated from the mean of the parameters determined by the Boltzmann equation: $I = 1/ (1+\exp [V-V_{1/2}]/s)$ to each individual data set (n = 5). Where $V_{1/2}$ is the voltage at which half of the channels are inactivated and s is a slope factor. E and F, time constant of activation (τ_{act}) or inactivation (τ_{inac}) is plotted as function of test potentials. Rates of activation (E) and inactivation (F) were measured from currents evoked by voltage steps from -90 to -40 through +30 mV, in 10 mV increments, before (○) or after application of NKA (●), by fitting a single exponential function to the currents; same cells as in B.

Fig 3. Inhibition of $Ca_V3.2$ channels by NK1 receptors involves $G\alpha_{q/11}$. A, currents from cells expressing $Ca_V3.2$ channels, NK1 receptors, and RGS3T proteins, before or during application of 10 nM NKA. Currents were evoked by a voltage step from -90 to -20 mV at 0.1 Hz. C_m = 30.8 pF and R_s = 2.4 MΩ. B, time course of the inhibitory effect of NKA; same cell as in A. C, pooled data for current inhibition in control (n = 6) or RGS3T expressing cells (n = 6, $*p < 0.05$). D, currents from cells expressing $Ca_V3.2$ channels, NK1 receptors, and $G\alpha_t$, before or after application of NKA. Same pulses protocol as in A. C_m = 22.8 pF and R_s = 2.9 MΩ. E, time course of inhibition by NKA; same cell as in D. F,

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pooled data for current inhibition in control (n = 11) or $G\alpha_t$ expressing cells (n =11, $p > 0.05$).

Fig 4. Inhibition of $Ca_V3.2$ by NKA requires PKC. A, inhibition is sensitive to U73122. $Ca_V3.2$ currents were recorded, before or after application of 10 nM NKA, from a cell which was preincubated in culture medium containing 3μM U73122 for 1 h at 37°C. Currents were evoked by a voltage step from -90 to -20 mV at 0.1 Hz. C_m = 21.4 pF and R_s = 2.9 MΩ. B, time course of the inhibitory effect of NKA on the same cell as in A. C, pooled data for $Ca_V3.2$ current inhibition by NKA in control cells (n = 7) or cells which were preincubated with U73122 (n = 7, ***$p < 0.001$). D, currents recorded under control conditions, during addition of 100 nM PMA, or after application of NKA in presence of PMA. Test pulses protocol as in A. C_m = 32.9 pF and R_s = 1.7 MΩ. E, time course of the inhibitory effect of NKA on the same cell as in D. Note the lack of effect of NKA when it was applied during the maximum steady-state inhibitory effect of PMA. F, pooled data for $Ca_V3.2$ current inhibition by 10 nM NKA (n = 8), 100 nM PMA (n = 8), or 100 nM PMA + 10 nM NKA (n = 8, $p > 0.05$). G, inhibition of $Ca_V3.2$ current by NKA is sensitive to Bis I. Currents were recorded, before or after application of 10 nM NKA, from a cell expressing $Ca_V3.2$ channels and NK1 receptors, before or after application of 10 nM NKA, from a cell which was preincubated in culture medium containing 500 nM Bis I for 1 h at 37°C. Same test pulses protocol as in A. C_m = 28.9 pF, and R_s = 2.2 MΩ. H, time course of the inhibitory effect of NKA on the same cell as in G. I, pooled data for $Ca_V3.2$ current inhibition by NKA in control cells (n = 8), or cells preincubated with 500 nM Bis I (n = 8) or 500 nM Bis V (n = 8, *$p < 0.05$, Bis I versus control and Bis V).

Fig 5. Schematic summary of results of $Ca_V3.2$ channels inhibition by NK1 receptors. Solid arrows indicate the well established signaling pathways associated to NK1 receptors activation. Dashed arrows indicate the possibilities of signaling pathways related to PKC. PKC could either act directly on $Ca_V3.2$ channel or via a regulatory protein (RP). The putative sites of action of dominant-negative G_q Q209L/D277L ($G\alpha_{qND}$), RGS2, RGS3T, U73122, PMA, and Bis I are also indicated.

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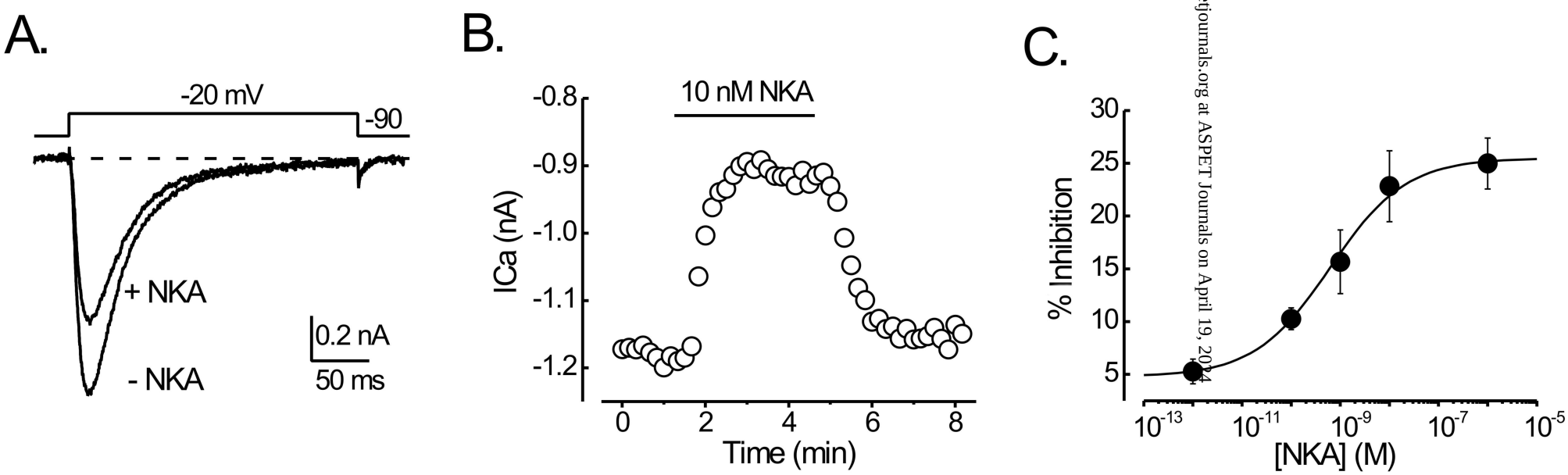

Fig 1.

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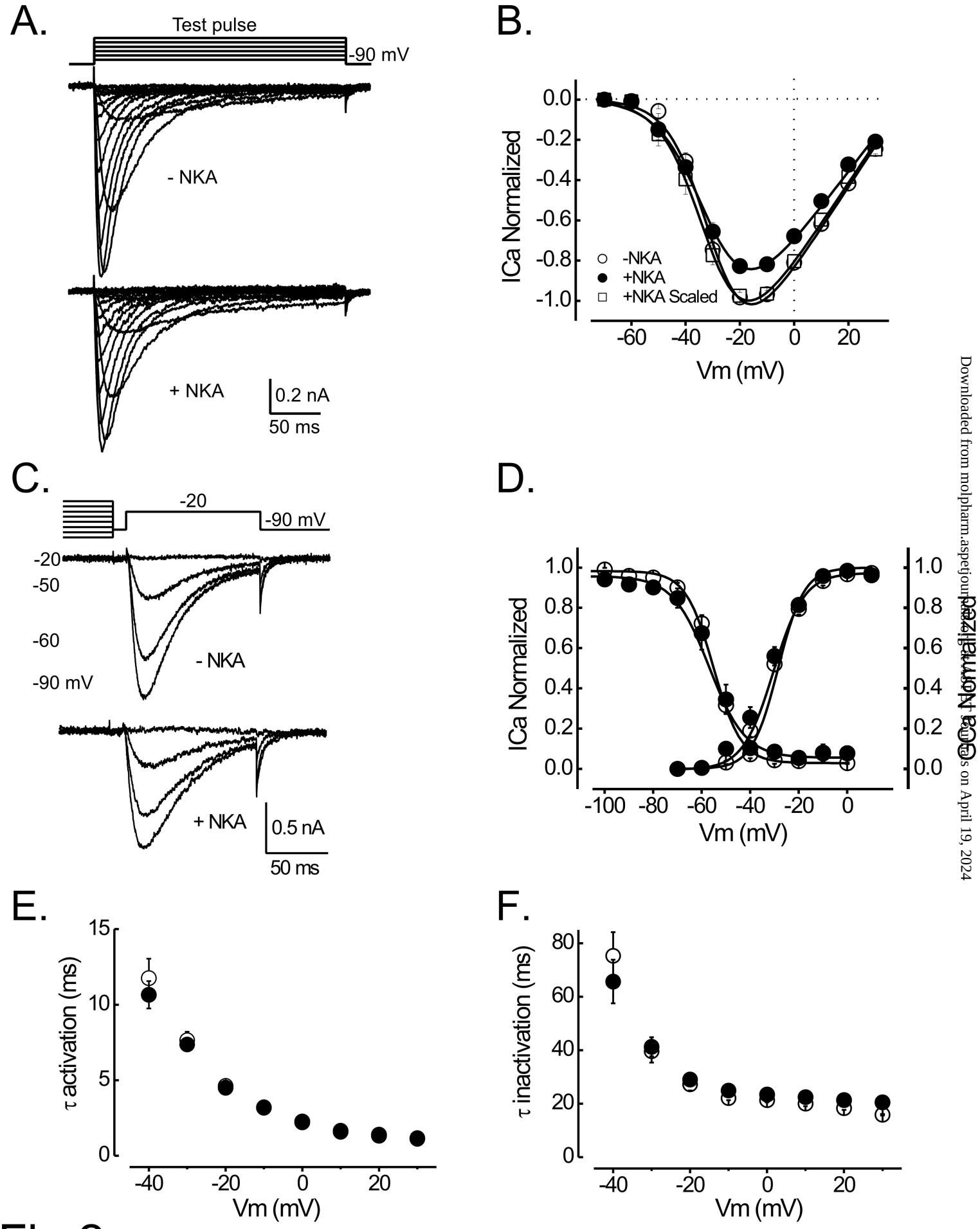

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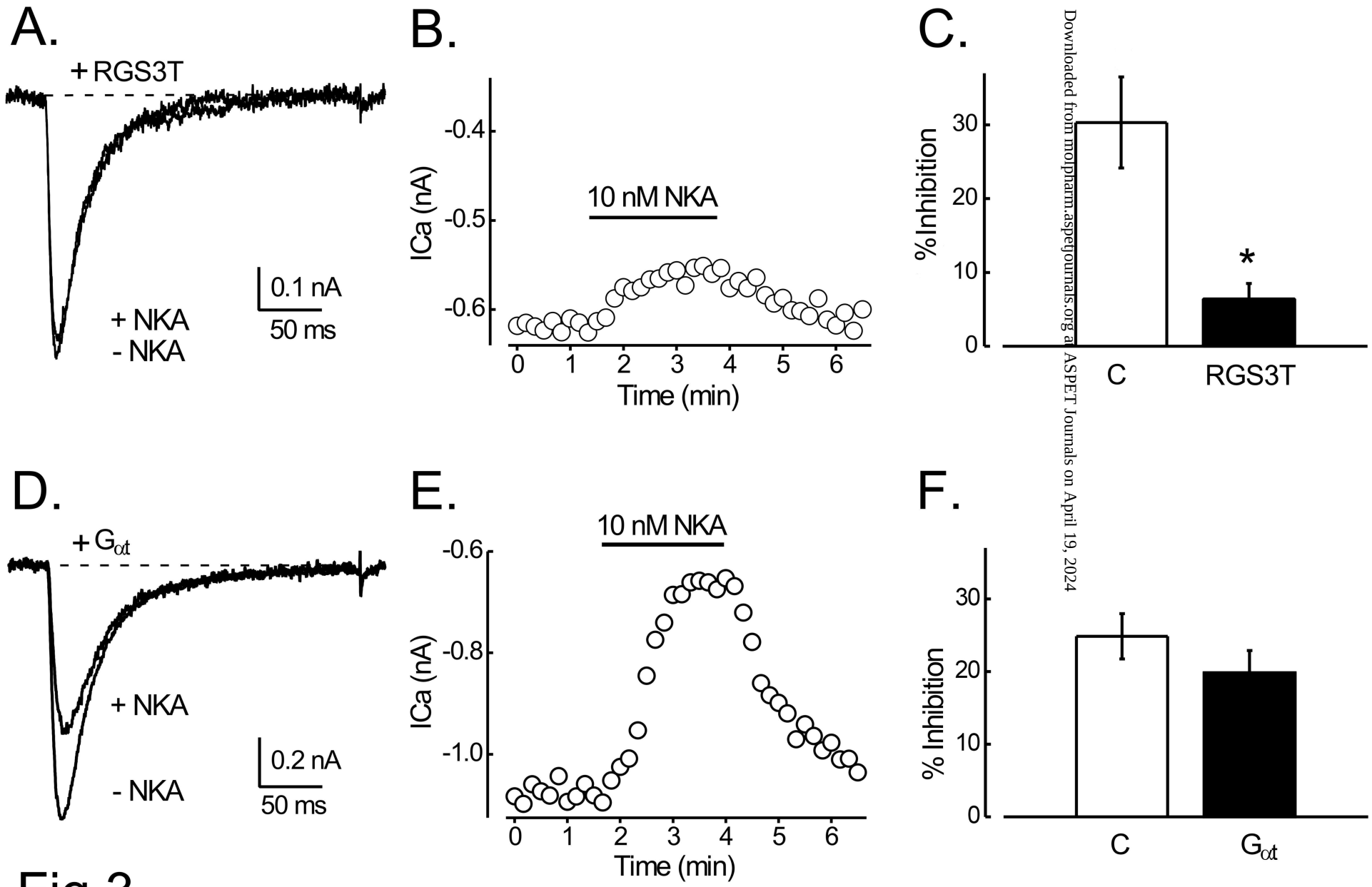

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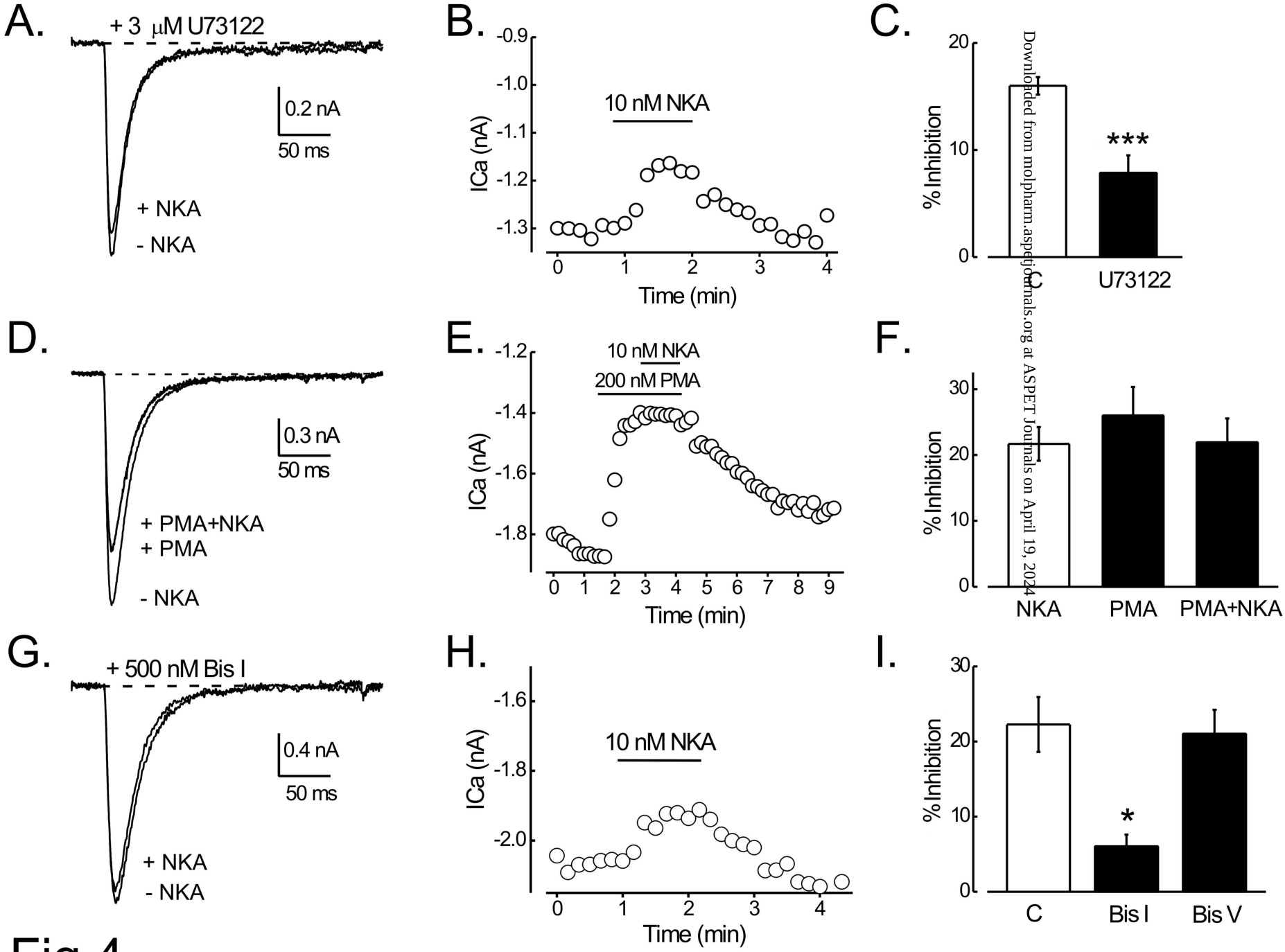

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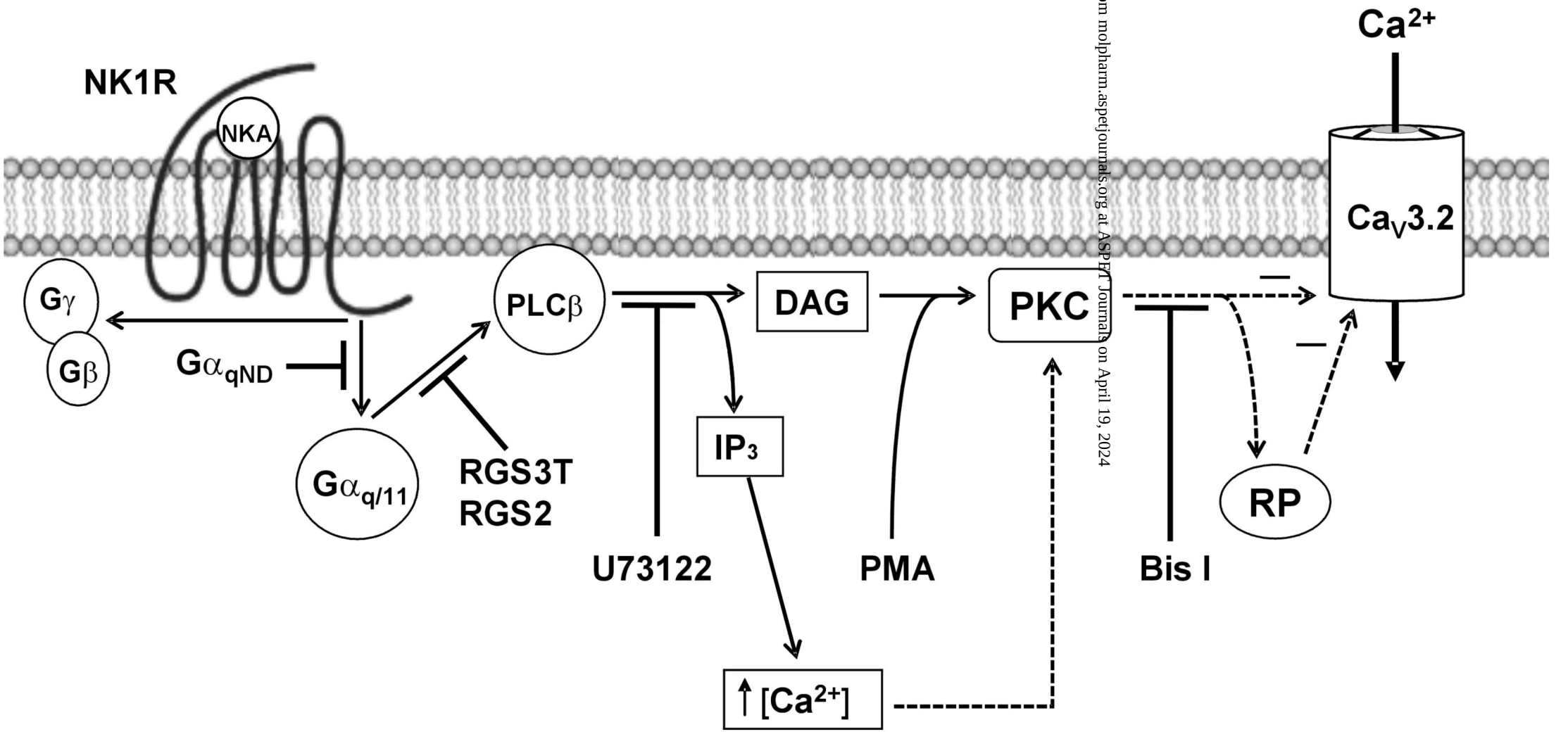

Fig. 5