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ROLE FOR PROTEIN KINASE C IN CONTROLLING *APLYSIA* BAG CELL NEURON EXCITABILITY

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Abstract—Targeting signalling molecules to ion channels can expedite regulation and assure the proper transition of changes to excitability. In the bag cell neurons of *Aplysia*, single-channel studies of excised patches have revealed that protein kinase C (PKC) gates a non-selective cation channel through a close, physical association. This channel drives a prolonged afterdischarge and concomitant neuropeptide secretion to provoke reproductive behaviour. However, it is not clear if PKC alters cation channel function and/or the membrane potential at the whole-cell level. Afterdischarge-like depolarizations can be evoked in cultured bag cell neurons by bath-application of *Conus textile* venom (CtVm), which triggers the cation channel through an apparent intracellular pathway. The present study shows that the CtVm-induced depolarization was reduced by nearly 50% compared to control following dialysis with the G-protein blocker, guanosine-5′-O-2-thiodiphosphate (GDP-β-S), or treatment with either the phospholipase C inhibitor, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), or the PKC inhibitor, sphinganine. Neurons exposed to the PKC activator, phorbol 12-myristate 13-acetate (PMA), displayed depolarization with accompanying spiking, and were found to be far more responsive to depolarizing current injection versus control. Immunocytochemical staining for the two typical *Aplysia* PKC isoforms, Apl I and Apl II, revealed that both kinases were present in unstimulated cultured bag cell neurons. However, in CtVm-treated neurons, the staining intensity for PKC Apl I increased, peaking at 10 min post-application. Conversely, the intensity of PKC Apl II staining decreased over the duration of CtVm exposure. Our results suggest that the CtVm-induced depolarization involves PKC activation, and is consistent with prior work showing PKC closely-associating with the cation channel to produce the depolarization necessary for the afterdischarge and species propagation.

Key words: *Conus* venom, membrane potential, bursting, immunocytochemistry, reproduction.

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Abbreviations: Ab, antibody; CtVm, *Conus textile* venom; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol bis (aminoethylether) tetraacetic acid; FITC, fluorescein isothiocyanate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GDP-β-S, guanosine-5′-O-2-thiodiphosphate; HEPES, N-2-hydroxyethlpiperazine-N′-2-ethanesulphonic acid; IgG, immunogammaglobulin; nASW, normal artificial salt water; PKA, protein kinase A; PKC, protein kinase C; PKC Apl I, *Aplysia californica* PKC isoform one; PKC Apl II, *Aplysia californica* PKC isoform two; PMA, phorbol 12-myristate 13-acetate; ROI, region of interest; tcASW, tissue culture artificial salt water; TFA, trifluoroacetic acid; U-73122, 1-[6-[[(17ß)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione.

A key means of controlling neuronal activity and excitability is the regulation of ion channel function through changes in cellular biochemistry (Hille, 2001; Levitan and Kaczmarek, 2002). The principal post-translational modification that influences channel gating and responsiveness is protein phosphorylation by kinases (Magoski and Kaczmarek, 2004). Protein kinase C (PKC), a lipid- and often Ca^{2+}-dependent enzyme (Takai et al., 1977, 1979; Sossin, 2007; Newton, 2010) was first shown to modulate ion channels by DeRiemer et al. (1985b). Subsequently, this kinase has been demonstrated to regulate essentially all types of ion channels, as well as plasticity, learning, and memory (Kaczmarek, 1986; Byrne and Kandel, 1996; Birnbaum et al., 2004; Derkach et al., 2007; Sossin and Abrams, 2009). Activation of typical PKC occurs when a G-protein coupled receptor provokes the membrane-bound enzyme, phospholipase C, to hydrolyze the membrane lipid, phosphatidylinositol-4,5-bisphosphate, into inositol triphosphate and diacylglycerol; subsequently, inositol triphosphate elevates intracellular Ca^{2+}, causing PKC to translocate to the membrane and bind diacylglycerol for activation (Levitan and Kaczmarek, 2002; Sossin, 2007; Newton, 2010). That stated, some PKC is always at the membrane (Oancea and Meyer, 1998); in part, this may constitute kinase targeted to signalling complexes (Reinhart and Levitan, 1995; Brandon et al., 1999; Higashida et al., 2005; Levitan, 2006).

PKC-dependent regulation of ion channels and neurotransmission has been extensively studied in neurons from the marine mollusc, *Aplysia californica*. This includes sensory neurons responsible for various defensive reactions (Byrne and Kandel, 1996; Sossin and Abrams, 2009) and the bag cell neurons, a class of neuroendocrine cells that initiate reproduction (DeRiemer et al., 1985b; Conn and Kaczmarek, 1989). Brief input to the bag cell neuron clusters triggers an ~30 min depolarization and period of action potential firing referred to as the afterdischarge (Kupfermann, 1967; Kupfermann and Kandel, 1970; Pinsker and Dudek, 1977). During this burst, egg-laying hormone is released into the circulation, where it acts on the ovotestis to cause egg deposition (Arch, 1972; Stuart et al., 1980; Rothman et al., 1983; Loechner et al., 1990; Michel and Wayne, 2002). PKC activation occurs in the bag cell neurons shortly after the start of the afterdischarge (Wayne et al., 1999), and these cells express the two typical *Aplysia* PKC isoforms, Ca^{2+}-dependent Apl I and Ca^{2+}-independent Apl II (Sossin et al., 1996; Nakhost et al., 1998).

Much of the ongoing drive for the afterdischarge comes from a non-selective cation channel permeable to Na^+, K^+, and Ca^{2+} (Wilson et al., 1996; Magoski et al., 2000;

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Geiger et al., 2009). This channel is gated by voltage, Ca^{2+}, and PKC-dependent phosphorylation (Wilson et al., 1996; Lupinsky and Magoski, 2006; Gardam and Magoski, 2009). Single-channel studies show that PKC enhances cation channel opening through a close-physical association which persists in excised, inside-out patch-clamp recordings (Wilson et al., 1998; Magoski et al., 2002; Gardam and Magoski, 2009). The cation channel-PKC association is more likely to occur in resting neurons versus cells that have recently undergone an afterdischarge, i.e., it is dynamic (Magoski and Kaczmarek, 2005). Intriguingly, an extract of the venom from the molluscivorous snail, *Conus textile*, evokes afterdischarges that are essentially indistinguishable from those produced by synaptic stimulation (Wilson et al., 1996). *Conus* venom is a well-established source of peptides that bind and affect both channels and receptors (Olivera and Cruz, 2001; Bogin, 2005). In cultured bag cell neurons, *Conus textile* venom (CtVm) liberates intracellular Ca^{2+} and elicits strong depolarization by opening the cation channel through an apparent intracellular pathway (Wilson et al., 1996; Magoski et al., 2000). Here, we test the hypothesis that the CtVm-induced depolarization of bag cell neurons involves PKC activation.

EXPERIMENTAL PROCEDURES

Animals and cell culture

Adult *Aplysia californica* weighing 150–500 g were obtained from Marinus Inc. (Long Beach, CA, USA), housed in an ~300 L aquarium containing continuously circulating, aerated sea water (Instant Ocean; Aquarium Systems, Mentor, OH, USA or Kent sea salt; Kent Marine, Acworth, GA, USA) at 15 °C on a 12/12 h light/dark cycle, and fed Romaine lettuce 5× a week. All experiments were approved by the Queen's University Animal Care Committee (protocol number Magoski-2005-050 or Magoski-2009-065) and conformed to the Canadian Council on Animal Care guidelines for the Care and Use of Experimental Animals. Every attempt was made to minimize the number of animals used and their suffering.

For primary culture of isolated bag cell neurons, animals were deeply anaesthetized by an injection of isotonic $MgCl_2$ (50% of body weight), the abdominal ganglion removed and treated with neutral protease (13.3 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN, USA) for 18 h at ~22 °C dissolved in tissue culture artificial sea water (tcASW) (composition in mM: 460 NaCl, 10.4 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 15 HEPES, 1 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, pH 7.8 with NaOH). The ganglion was then transferred to fresh tcASW for 1 h, after which time the bag cell neuron clusters were dissected from their surrounding connective tissue. Using a fire-polished glass Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto 35×10 mm polystyrene tissue culture dishes (430165; Corning, Corning, NY, USA). Cultures were maintained in tcASW in a 14 °C incubator and used for experimentation within 1–3 days. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH, USA), or Sigma-Aldrich (Oakville, ON, Canada or St. Louis, MO, USA).

Whole-cell current-clamp

Current-clamp recordings were made from bag cell neurons using an EPC-8 amplifier (HEKA Electronics; Mahone Bay, NS, Canada) and the tight-seal, whole-cell method. Microelectrodes were pulled from 1.5 mm external diameter/1.12 internal diameter, borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL, USA) and had a resistance of 1–3 MΩ when filled with intracellular saline (composition in mM: 500 K-aspartate, 70 KCl, 3.75 $CaCl_2$, 1.25 $MgCl_2$, 10 HEPES, 11 glucose, 10 glutathione, 5 EGTA, 5 adenosine triphosphate (ATP) (grade 2, disodium salt; Sigma-Aldrich), and 0.1 guanosine triphosphate (GTP) (type 3, disodium salt; Sigma-Aldrich); pH 7.3 with KOH; free Ca^{2+} concentration calculated as 300 nM using WebMaxC: http://www.stanford.edu/~cpatton/webmaxcS.htm). Pipette junction potentials were nulled immediately before seal formation. Voltage was filtered at 3 kHz by the EPC-8 built-in Bessel filter and sampled at 2 kHz using an IBM-compatible personal computer, a Digidata 1300 analogue-to-digital converter (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA) and the Clampex acquisition program of pCLAMP 8.1 or 8.2 (Axon Instruments). If necessary, the current injection function of the EPC-8 was used to maintain membrane potential at −60 mV prior to stimulation; however, Clampex was used to transiently inject current into the neurons (see Results for detail). Recordings were done in normal artificial sea water (nASW; composition as per tcASW, but with glucose and antibiotics omitted).

Immunocytochemistry

Cultured bag cell neurons were stained for PKC Apl I and PKC Apl II using a protocol adapted from White and Kaczmarek (1997) and Magoski and Kaczmarek (2005). Neurons were plated in the centre of small (20–40 μl) rings made from dental wax (92189; Heraeus Kulzer, South Bend, IN, USA) affixed to tissue culture dishes. For rapid solution changes, the dish was drained of all fluid except for the contents of the wax ring and new solution was delivered by Pasteur pipette directly onto the neurons. Neurons that were 1–2 days in culture were treated (see Results for detail) and then fixed for 25 min at room temperature with 4% (w/v) paraformaldehyde (04042; Fisher) in 400 mM sucrose/nASW (pH 7.5). They were then permeabilized for 5 min at room temperature with 0.3% (w/v) Triton X-100 (BP151; Fisher) in fix and washed twice with PBS (composition in mM: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.5 KH_2PO_4; pH 7.0 with NaOH). Neurons were blocked for 30–60 min at room temperature in a blocking solution of 5% (v/v) goat serum (G9023; Sigma-Aldrich) in PBS. Primary antibodies, rabbit anti-*Aplysia* PKC Apl I immunogammaglobulin (IgG) or rabbit anti-*Aplysia* PKC Apl II IgG (both kindly provided by Dr. WS Sossin, McGill University) were applied at a dilution of 1:50 for Apl I or 1:2500 for Apl II in blocking solution. Neurons were incubated in the dark at room temperature for 1 h and subsequently washed 4× with PBS. The secondary antibody (goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; #111-095-003, lot 66186; Jackson ImmunoResearch, West Grove, PA, USA)) was applied at a 1:100 dilution for Apl I or 1:200 for Apl II in blocking solution and incubated in the dark for 2 h at room temperature. Neurons were then washed 4× with PBS, the wax rings filled with VectaShield (H-1000; Vector Laboratories, Burlingame, CA, USA), and covered with a glass coverslip (#1; 48366045; VWR, West Chester, PA, USA).

Fluorescence microscopy

For standard fluorescence microscopy, stained neurons were imaged using a Leica DM IRB microscope (Leica Microsystems, Heidelberg, Germany) equipped with a NPLAN 20× (NA=0.4) objective. Neurons were excited with a 50 W Mercury lamp and a 490/15 nm band pass filter. Fluorescence was emitted to the eyepiece or camera through a 500 nm dichroic mirror and 525/20 nm emission filter. Images (1392×1040 pixels) were acquired at a focal plane that was as close as possible to the middle of the somatic vertical axis using a Retiga Exi camera (QImaging, Burnaby, BC, Canada) and OpenLab 4.0 (Improvision, Lexington, MA, USA). The exposure time to acquire images to disk for off-line

analysis was 300 ms and 400 ms for PKC Apl I and PKC Apl II stained neurons, respectively.

For confocal microscopy, stained neurons were imaged using a Leica TCS SP2 microscope (Leica Microsystems, Heidelberg, Germany) equipped with a HC PL FLUOTAR 10× (NA=0.4) objective. Neurons were excited with an Argon laser at 488 nm and fluorescence was emitted to the eyepiece or camera through a prism set to collect light in the 505–530 nm range. A Leica photomultiplier tube and Leica confocal software were used to acquire a stack of images (1024×1024 pixels) in 2 μm sections from the bottom to the top of the vertical axis of the soma. These images were saved to disk for off-line analysis.

Conus textile venom

Crude (CtVm) (Cruz et al., 1976) was bath applied to cultured bag cell neurons. CtVm lyophilate was generously provided by Ms. J Imperial and Dr. BM Olivera of the University of Utah. Adult specimens of the molluscivorous snail, *Conus textile*, were collected from the ocean around the Philippine island of Marinduque. Venom ducts were dissected out of an animal and placed on an ice cold metal spatula. The duct was then cut into 2 cm sections, the venom extruded by squeezing with forceps, lyophilized in a vacuum centrifuge, and stored at −80 °C. CtVm was extracted at 4 °C by adding 0.5% (v/v) trifluoroacetic acid (TFA) (T6508; Sigma-Aldrich) to the lyophilate for a final protein concentration of 5% (w/v). CtVm was vortexed for 2 min and sonicated for 2 min, in an alternating fashion, for a total of 18 min. The mixture was then centrifuged at 15,000 g for 12 min and the supernatant collected. A second aliquot of TFA was added to the pellet (final protein concentration 10% w/v) and the protocol repeated. The supernatants were pooled, aliquoted, and frozen at −80 °C. CtVm was introduced into the bath (culture dish) by pipetting a small volume (<10 μl) of the stock into the culture dish for a final protein concentration of ~100 μg/ml. Care was taken to pipette the stock near the side of the dish and as far away as possible from the neuron. In other studies, boiling CtVm eliminated the electrophysiological effects produced by this toxin (Wilson et al., 1996). Given this, and the paucity of the CtVm itself, the control for the present study was to simply expose neurons to <10 μl of water or water containing 0.5% TFA.

Reagents

All other drugs were made up as concentrated stock solutions and frozen at −30 °C. The G-protein blocker, guanosine-5′-O-2-thiodiphosphate (GDP-β-S; G7637; Sigma-Aldrich) was diluted down to 10 mM in intracellular saline and introduced into neurons via diffusion from the whole-cell pipette. Ethanol was used as the vehicle for the PKC inhibitor, sphinganine (D-6908; Sigma-Aldrich), while dimethyl sulfoxide (DMSO; BP231-1; Fisher) was used as the vehicle for the phospholipase C inhibitor, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122; U6756; Sigma-Aldrich), and the PKC activator, phorbol 12-myristate 13-acetate (PMA; P8139; Sigma-Aldrich). Neurons were exposed to 0.1% ethanol, 0.05–0.5% DMSO, 2.5 μM U-73122, 20 μM sphinganine, or 100 nM PMA by pipetting a small volume of stock solution into the bath as per CtVm. In this or other studies (Magoski et al., 2000, 2002; Gardam et al., 2008; Geiger and Magoski, 2008; Tam et al., 2009; Hickey et al., 2010) we observed no effects of TFA, ethanol, or DMSO on the electrical characteristics of cultured bag cell neurons.

Analysis

Membrane potential changes to stimulation by CtVm, PKC activation, or current injection were quantified with the Clampfit analysis program of pCLAMP. Two cursors were placed at either end of 30 s to 1 min of the baseline prior to stimulation, while an additional two cursors were similarly positioned over 30 s to 5 min of the peak response to stimulation (see Results for details). Clampfit then calculated the average voltage between the paired cursors. The maximal amplitude of the response was taken as the difference between these average values. In cases where the response to stimulation evoked action potentials, the Fetchan analysis program of pCLAMP was used to generate an all-points histogram from the region of firing. The Pstat analysis program of pCLAMP was then used to fit the largest peak of the histogram, with a Gaussian function by the least-squares method using a simplex search, to obtain the intra-action potential voltage. The maximal amplitude of the response was taken as the difference between the baseline and the peak depolarization. To measure neuronal input resistance, Ohm's law and the average steady-state voltage change produced by three, sequential −50 pA, 800 ms current steps was used.

The staining intensity of standard fluorescence images was quantified using ImageJ 1.32 (http://rsbweb.nih.gov/ij/). A region of interest (ROI) consisting of two circles was defined over the soma: a larger circle drawn around the outline of the edge of the membrane and a smaller circle drawn within the larger circle at two-thirds the diameter. Fluorescence at the membrane was maximized by taking the image from the middle of the vertical axis of the neuron. This ring-ROI served to capture signal, in arbitrary units, from the membrane at that focal plane as well as some signal from the membrane that curved above and below the focal plane. In addition, the cytoplasmic fluorescence was gathered with an ROI of a single circle drawn at two-thirds the soma diameter and placed centrally over the cell body. For confocal images, staining intensity was quantified using Image Pro 6.2 (Media Cybernetics; Silver Springs, MD, USA). Rather than an ROI, analysis was performed using a circle (either provided by the analysis feature of the program or drawn) which best fit the soma within 4 μm of the apparent edge of the membrane). Intensity, in arbitrary units, was determined for every pixel on the line of the circle. In all instances the middle slice of the soma, as determined by halving the total number of slices making up the vertical axis of the neuron, served as the image for analysis.

Data are presented as mean±standard error of the mean throughout. Statistical analysis was performed using Instat (version 3; GraphPad Software, San Diego, CA, USA). The Kolmogorov-Smirnov method was used to test data sets for normality. To test whether the mean differed between two groups, either Student's unpaired *t*-test (for normally distributed data) with the Welch correction as necessary (for unequal standard deviations) or the Mann–Whitney U-test (for not normally distributed data) was used. To test for differences between multiple means, a nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) was used, followed by Dunn's multiple comparisons test. Means were considered significantly different if the two-tailed *P*-value was <0.05.

RESULTS

G-protein blockade reduces the depolarization elicited by CtVm

The canonical signalling pathway for PKC activation is through heterotrimeric G-protein coupled receptors (Sossin and Abrams, 2009; Rosenbaum et al., 2009; Newton, 2010). If CtVm depolarizes bag cell neurons by metabotropically activating the cation channel, block of G-proteins is predicted to attenuate the response. Cultured bag cell neurons were whole-cell current-clamped at −60 mV and dialyzed for 30 min with either normal K^+-based intracellular saline or the same saline supplemented with 10 mM of the G-protein blocker, GDP-β-S (Eckstein et al., 1979). Similar concentrations of GDP-β-S have been used to

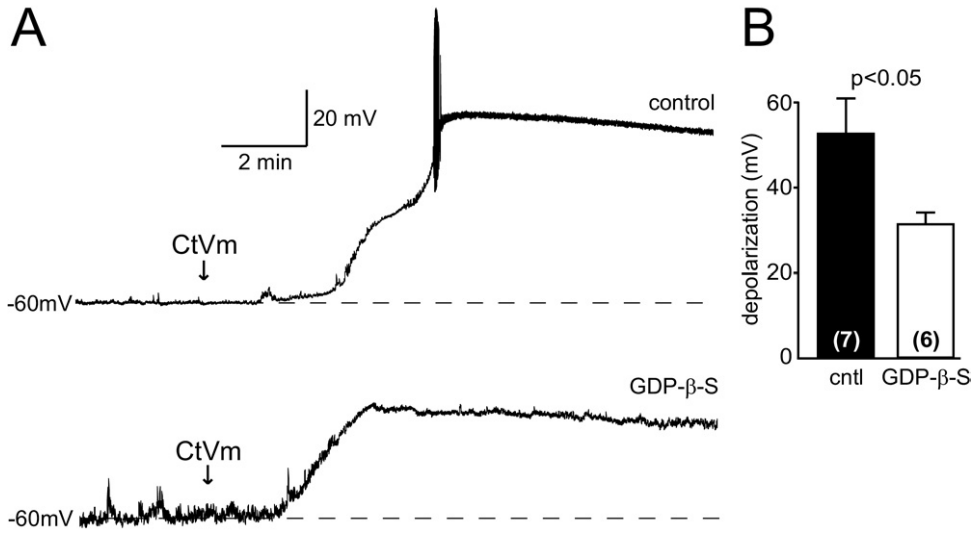

Fig. 1. The response to *Conus textile* venom involves G-proteins. (A) Sample voltage traces from cultured bag cell neurons under whole-cell current-clamp at −60 mV. Upper, application of 100 μg/ml (at arrow) of *Conus textile* venom (CtVm) evokes a marked depolarization and a brief burst of action potentials in a control neuron dialyzed for 30 min with regular intracellular saline. Lower, dialysis of a neuron for 30 min with intracellular saline containing 10 mM of the G-protein antagonist, GDP-β-S, lessens the response to CtVm. In two of seven control neurons, action potentials are evident during the CtVm response; similarly, one of six GDP-β-S neurons display spiking to CtVm. Scale bars apply to both traces. (B) Summary data of the mean depolarization following CtVm application. The presence of GDP-β-S inside the neurons leads to a significantly lower depolarization compared to control (unpaired *t*-test, Welch corrected). For this and subsequent bar graphs, the *n*-values are within a given bar.

inhibit metabotropically-gated currents in other *Aplysia* neurons (Kudo et al., 1991; Kehoe, 1994). Bath application of 100 μg/ml of CtVm (courtesy Ms. J Imperial and Dr. BM Olivera) produced a substantial depolarization in control neurons with an average of ~50 mV (n=7) (Fig. 1A). Our laboratory and others have found this dose of CtVm to reliably evoke cation channel-mediated depolarization in cultured bag cell neurons and afterdischarges from the bag cell neuron cluster in the intact nervous system (Wilson and Kaczmarek, 1993; Wilson et al., 1996; Geiger et al., 2009). The response in the present study developed within 2–5 min, and in the case of two out of seven controls, resulted in a <1 min burst of action potentials. The CtVm-induced depolarization recovered to a small extent over the course of the experiment, and generally left neurons well positive of pre-venom resting potential. In neurons dialyzed with GDP-β-S, the response to CtVm was diminished to ~30 mV (n=6), and exhibited action potential firing in only one out of six cases (Fig. 1A). Overall, the reduction in the CtVm-induced depolarization following block of G-proteins reached the level of significance compared to neurons that underwent control dialysis (Fig. 1B). However, the onset time of the response was similar between the two conditions. Moreover, the input resistance, an elementary measure of excitability, of control neurons (181±26 MΩ, n=6) was not different from neurons dialyzed with GDP-β-S (193±45 MΩ, n=6) (P>0.05, unpaired *t*-test), when measured prior to the application of CtVm.

Antagonizing phospholipase C or PKC reduces the CtVm-induced depolarization

As with G-proteins, if CtVm triggers PKC to turn on the cation channel, antagonizing either the pathway that leads to PKC activation or the kinase itself is similarly predicted to attenuate the depolarization. The PKC pathway was examined by treating cultured bag cell neurons for 20–30 min with either 2.5 μM of the phospholipase C inhibitor, U-73122 (Bleasdale et al., 1990), or 0.05% of the vehicle, DMSO (control). U-73122 prevents serotonin-induced enhancement of both *Aplysia* sensory-motor neuron synapses and motor neuron glutamate responses (Fulton et al., 2008). In bag cell neurons whole-cell current-clamped to −60 mV, the depolarization following 100 μg/ml CtVm was attenuated by U-73122 (n=5) when contrasted with control (n=5) (Fig. 2A). On average, the control depolarization was ~40 mV, whereas the U-73122-treated neurons was significantly less at ~15 mV (Fig. 2B). Action potentials were apparent following CtVm in two out of the five control neurons, while none of the five neurons exposed to U-73122 bursted. Nevertheless, before the introduction of CtVm, no difference in the input resistance was found between neurons in DMSO (258±38 MΩ, n=5) and those that in U-73122 (194±37 MΩ, n=5) (P>0.05, unpaired *t*-test).

To target PKC itself, cultured bag cell neurons were treated for 40–120 min with either 0.1% DMSO (the vehicle; control) or 20 μM sphinganine (a broad-spectrum PKC blocker) (Hannun et al., 1986) and whole-cell current-clamped at −60 mV. Previous work has shown that sphinganine lessens PKC-dependent responses in bag cell neurons (Conn et al., 1989; Zhang et al., 2002). We observed a reduction of the depolarization elicited by 100 μg/ml CtVm in neurons exposed to sphinganine (n=11) compared to control (n=9) (Fig. 3A). The mean depolarization in control neurons was just over 50 mV, while those exposed to sphinganine was ~30 mV—a difference that

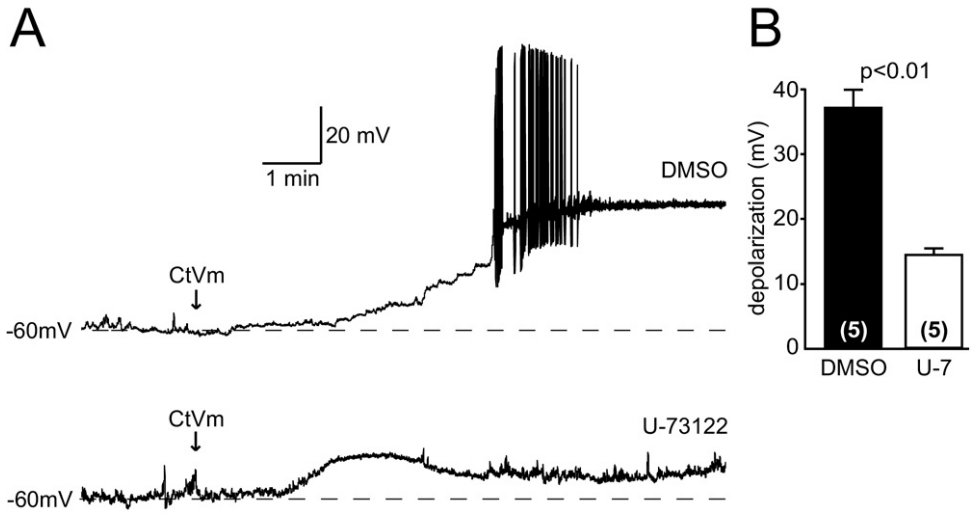

Fig. 2. A phosholipase C antagonist reduces the *Conus textile* venom depolarization. (A) Selected membrane potential recordings of cultured bag cell neurons whole-cell current-clamped to −60 mV. Upper, introduction of 100 μg/ml CtVm (at arrow) provokes depolarization and an action potential burst in a neuron treated with 0.05% DMSO for 20 min. Lower, the response to CtVm is far less in a neuron exposed for 20 min to 2.5 μM of the phospholipase C antagonist, U-73122. Ctvm results in action potentials in two of five neurons bathed with DMSO, but all five cells fail to spike following CtVm when previously exposed to U-73122. Scale bars apply to both traces. (B) Summary data of the mean CtVm-induced depolarization. The presence of U-73122 (U-7) leads to a smaller depolarization that is significantly different from control (unpaired *t*-test, Welch corrected).

reached the level of significance (Fig. 3B). CtVm evoked a burst of action potentials seven out of nine times in control neurons versus three of 11 occasions in neurons bathed in sphinganine. However, the input resistance under control conditions (317±55 MΩ, $n=9$) was not different from sphinganine (493±75 MΩ, $n=11$) ($P>0.05$, unpaired *t*-test) prior to delivering CtVm.

PKC activation depolarizes bag cell neurons

To reinforce the role of PKC in the depolarization, attempts were made to mimic the effects of CtVm by directly activating the kinase. Under whole-cell current-clamp at −60 mV, cultured bag cell neurons were exposed to either 100 nM of the PKC activator, PMA (Castagna et al., 1982) or 0.5% of the vehicle, DMSO. PMA is a potent and specific activator of *Aplysia* PKC in biochemical preparations derived from nervous system tissue or expression systems (DeRiemer et al., 1985a; Sossin and Schwartz, 1994; Manseau et al., 2001). Six neurons treated with PMA displayed a relatively slow depolarization accompanied by action potential firing, while the five parallel cells which saw DMSO did not show appreciable changes in resting poten-

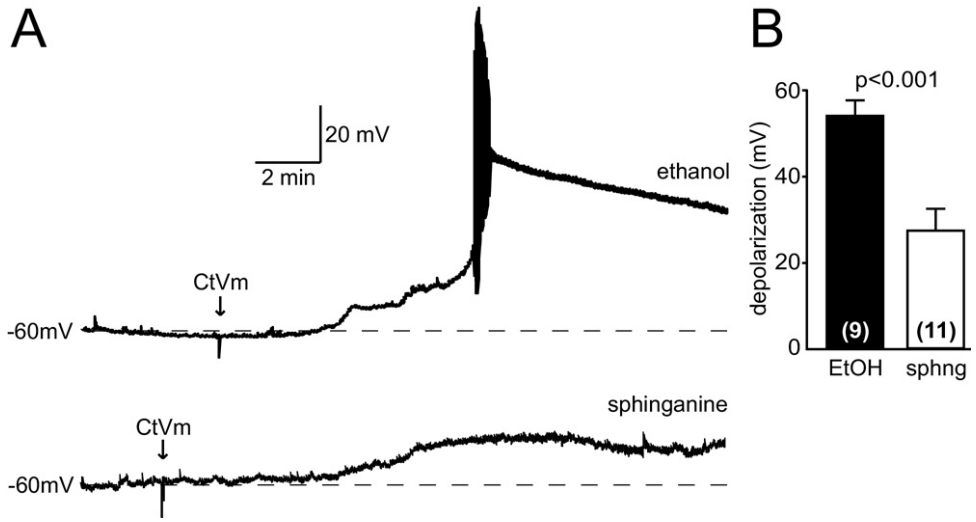

Fig. 3. Inhibition of PKC attenuates the *Conus textile* venom response. (A) Examples of voltage recordings from cultured bag cell neurons under whole-cell current-clamp at −60 mV. Upper, a control neuron exposed to 0.1% ethanol presents a robust depolarization and spiking following application of 100 μg/ml CtVm (at arrow). Lower, there is a decrease in the CtVm response when a neuron is treated for 120 min with 20 μM of the PKC blocker, sphinganine. Spiking is present during the CtVm response of seven of nine neurons subjected to ethanol, while CtVm produced action potential firing in only three of 11 neurons bathed with sphinganine. Scale bars apply to both traces. (B) Summary data of the averaged depolarization to CtVm application. The difference between the neurons that see ethanol versus sphinganine is significant (unpaired *t*-test).

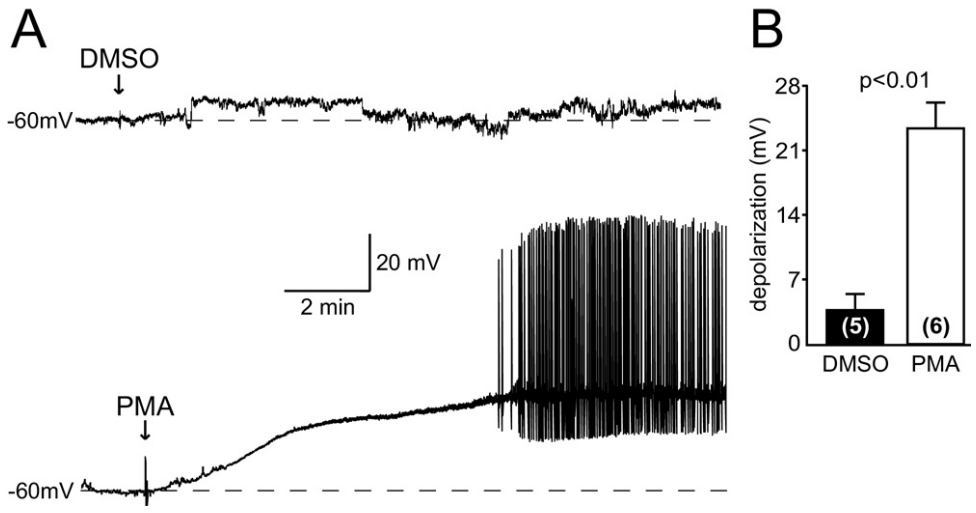

Fig. 4. PKC activation excites bag cell neurons. (A) Representative voltage traces of cultured bag cell neurons whole-cell current-clamped to −60 mV. Upper, delivery of 0.5% DMSO to a control neuron (at arrow) does not cause appreciable change in membrane potential. Lower, introduction of 100 nM of the PKC activator, PMA (at arrow), elicits a depolarization that eventually results in persistent action potential firing. Scale bars apply to both traces. (B) Summary data of the mean depolarization produced by the vehicle (DMSO) or PKC activation (PMA). There is a significant difference between control and neurons in which PKC is triggered (unpaired *t*-test).

tial (Fig. 4A). The average response to bath-applied PMA was ~25 mV, a figure significantly different from the ~3 mV change produced by DMSO (Fig. 4B).

Work by others on bag cell neurons does not report changes in membrane potential following PMA-induced PKC activation (DeRiemer et al., 1985b; Conn et al., 1989; Knox et al., 1992). Consistent with this, we observed an additional 11 neurons which showed no overt change in membrane potential following PMA application. However, when the response to depolarizing current injection of these neurons was compared to their parallel DMSO controls, the PMA-treated neurons displayed greater responsiveness. Specifically, cultured bag cell neurons were current-clamped to −60 mV and stimulated with a multi-phase current injection consisting of a 40 s ramp to 100 pA, a further 40 s of continuous 100 pA, followed by a 2.5 min ramp from 100 to 0 pA. None of the neurons in 0.5% DMSO (n=8) reached spiking threshold during the ramp protocol, while all of the neurons in 100 nM PMA (n=11) presented a maintained burst of action potentials (Fig. 5A). Furthermore, bag cell neurons treated with PMA had a mean peak depolarization of ~30 mV, which was significantly different from the <25 mV response observed in DMSO-treated neurons (Fig. 5B). Despite this enhanced responsiveness, there was no significant difference in the input resistance between control neurons (432±59 MΩ, n=8) and those cells that were given PMA (487±44 MΩ, n=6) (P>0.05, unpaired *t*-test). The ramp protocol represents an average version of the current responsible for induction of an activity-dependent prolonged depolarization we have previously characterized (Hung and Magoski, 2007; Tam et al., 2009). This stimulus was used because it is both more physiological and gradual enough to prevent the spike accommodation that sometimes occurs when depolarizing current is rapidly delivered (Magoski, unpublished observation).

Bag cell neurons express both isoforms of *Aplysia* PKC

The data thus far suggest that CtVm depolarizes bag cell neurons through metabotropic receptor activation of PKC. However, the electrophysiology does not provide information on which kinase isoforms may be involved. To address this, bag cell neurons were stained for the two typical *Aplysia* PKC isoforms, Apl I and Apl II, using specific antibodies (Kruger et al., 1991). These antisera (courtesy Dr. WS Sossin) have been employed to detect PKCs in Western blots of proteins derived from either *Aplysia* nervous tissue or cell lines expressing recombinant kinases (Sossin et al., 1993, 1996; Sossin and Schwartz, 1992, 1994; Manseau et al., 2001); moreover, they have been used to immunocytochemically localize *Aplysia* PKC in cell lines, sensory neurons, and bag cell neurons themselves (Zhao et al., 2006; Nakhost et al., 1998; Farah et al., 2009). In the current study, immunofluorescent staining of cultured bag cell neurons for either PKC Apl I or Apl II was dependent on the presence of both primary and secondary antibodies. Under standard fluorescent microscopy, essentially no signal was detected following incubation in the presence of secondary antibody (goat anti-rabbit IgG-FITC) alone or either of the primary antibodies (rabbit anti-*Aplysia* PKC Apl I IgG or rabbit anti-*Aplysia* PKC Apl II IgG) alone (Fig. 6A, B, E). Conversely, when either of the primary antibodies and the secondary antibody was applied in succession, the neurons fluoresced markedly brighter. Both PKC Apl I and Apl II were localized to the periphery of the soma (Fig. 6C, F), likely reflecting a combination of plasma membrane and cytoplasmic kinase, as well as reduced staining over the centrally-located nucleus. We focussed our attention on the soma, given that this is where the prior single-channel as well as whole-cell current- and voltage-clamp recording of the cation channel

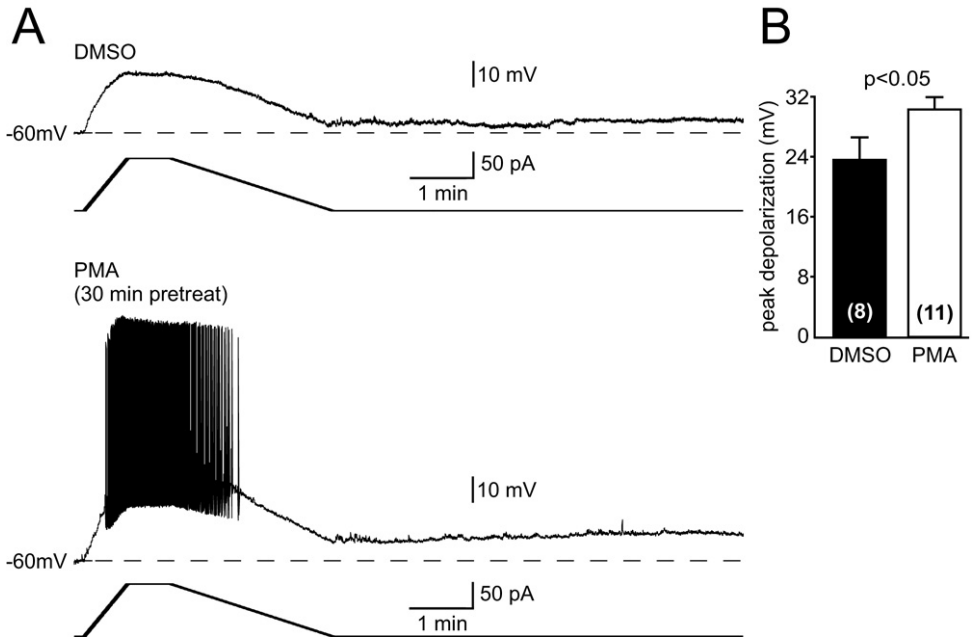

Fig. 5. PKC activation enhances the depolarization elicited by a current ramp. (A) Sample responses of cultured bag cell neuron membrane potential to an inverted idealized version of a prolonged depolarization current (see Results for detail). Upper, in the presence of 0.5% DMSO, the voltage faithfully follows the current ramp and depolarizes by ~20 mV, but fails to spike. Lower, prior application of 100 nM PMA results in a greater depolarization to the current ramp, as well as prominent spiking. These experiments represent neurons that did not show an immediate responses to PKC activation by PMA and their corresponding parallel controls. While none of the neurons exposed to DMSO show action potential firing during current injection, the ramp always causes firing in the presence of PMA. (B) Summary graph of peak depolarization elicited in DMSO and PMA treatment. The peak depolarization in PMA neurons exhibiting spiking activity is taken as the mean voltage over the plateau period during the current ramp protocol. The difference between the means is significant (unpaired *t*-test).

and CtVm-induced effects have been carried out (Wilson et al., 1996; Magoski et al., 2002; Magoski, 2004; Magoski and Kaczmarek, 2005; Geiger et al., 2009). Nevertheless, PKC Apl I staining could be seen in the growth cones and axons when the camera exposure time was lengthened greatly beyond the standard 300 ms duration (Fig. 6D).

CtVm increases PKC Apl I staining intensity

As mentioned in the Introduction, the cation channel that underlies the CtVm response is known to closely-associate with PKC (Wilson et al., 1998; Magoski et al., 2002; Gardam and Magoski, 2009). Therefore, a reasonable prediction would be that one or both of the PKC isoforms may localize to a greater extent at or around the plasma membrane following CtVm application. Cultured bag cell neurons were exposed to 0.5% TFA in water (control) or 100 μg/ml CtVm for 5, 10, and 30 min before being fixed and processed for PKC Apl I immunocytochemistry and standard fluorescent microscopy. Compared to control cells, the somatic staining for PKC Apl I following 5 min of CtVm was largely the same (Fig. 7A, B). However, neurons maintained in CtVm for 10 min presented an increase in PKC Apl I signal (Fig. 7C). This enhancement of PKC signal dissipated by 30 min, as the staining intensity fell in the continued presence of CtVm (Fig. 7D). PKC Apl I staining at the focal plane corresponding to the middle of vertical axis of the cell was quantified in a ring near the membrane (see Experimental procedures, Analysis for detail). Relative to control, the mean intensity of neurons treated with CtVm for 5 or 30 min did not differ significantly, but the average signal from the 10 min CtVm cells was larger and met the level of significance (Fig. 7E).

CtVm decreases PKC Apl II staining intensity

In part, PKC Apl I was initially examined because it is Ca^{2+}-dependent (Kruger et al., 1991), and both the afterdischarge as well as the CtVm response are associated with an increase in intracellular Ca^{2+} (Fisher et al., 1994; Magoski et al., 2000; Geiger and Magoski, 2008). This notwithstanding, we tested the possibility that the Ca^{2+}-independent PKC, Apl II (Kruger et al., 1991), is impacted by the introduction of CtVm. Cultured bag cell neurons were again exposed to either 0.5% TFA in water or 100 μg/ml CtVm for 5, 10, and 30 min prior to fixation and immunocytochemistry for PKC Apl II followed by standard fluorescent microscopy. As was the case for PKC Apl I, there was no obvious difference in the PKC Apl II staining pattern or intensity between control neurons and those experiencing CtVm for 5 min (Fig. 8A, B). In contrast, the cells bathed in CtVm for 10 or 30 min showed a decrease of the PKC Apl II signal (Fig. 8C, D). Analysis of the groups using a ring near the membrane (see Experimental procedures, Analysis for detail) revealed that the average signal of CtVm-treated neurons at the 5 min mark was not significantly different from control, while the mean intensity for the neurons in CtVm for 10 or 30 min diminished to the point of significance (Fig. 8E).

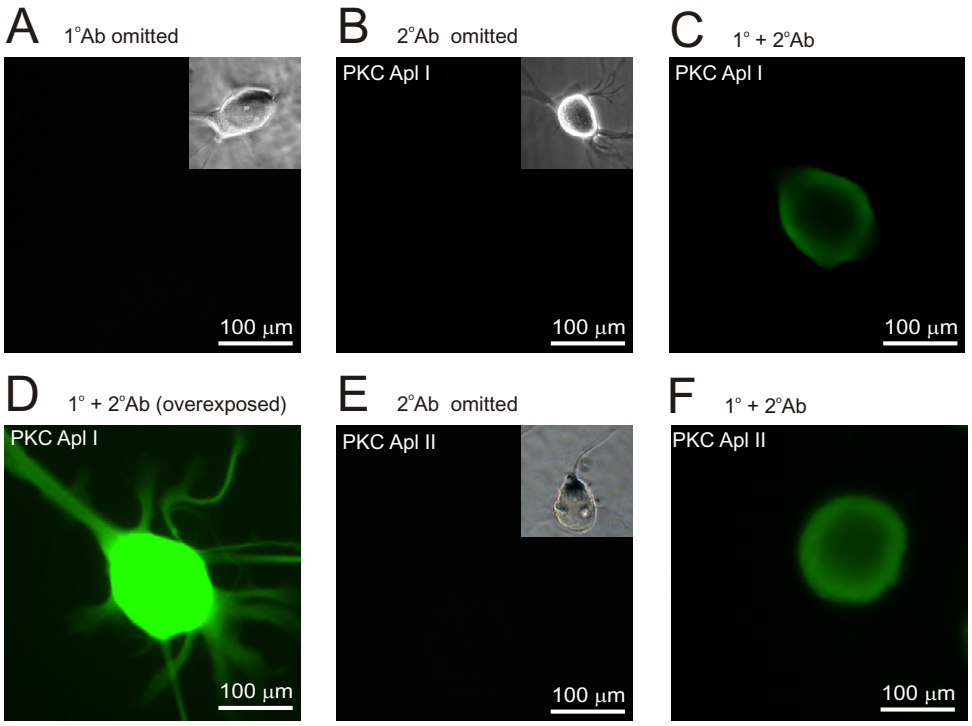

Fig. 6. Immunocytochemistry of cultured bag cell neurons shows expression of both PKC isoforms. Standard fluorescent microscopy of immunostained cultured bag cell neurons. (A) Lack of staining of a neuron when no primary antibody is used and only the secondary antibody (2°Ab; goat anti-rabbit IgG-FITC) is present at a 1:100 dilution (300 ms exposure). The inset in the upper right hand corner of this and panels (B, E) shows the phase image of the neuron in question. (B) Staining is also lacking when the secondary antibody is omitted and the primary antibody for PKC Apl I (1°Ab; rabbit anti-*Aplysia* PKC Apl I IgG) is present at 1:50 (300 ms exposure). (C) Treatment with the primary antibody for PKC Apl I at 1:50 followed by the secondary antibody at 1:100 reveals PKC immunostaining of the neuronal soma (300 ms exposure). This focal plane is essentially at the middle of the vertical axis of the cell body and shows staining concentrated at the periphery of the soma, with more a diffuse signal towards the centre. This relatively small amount of signal over the centre of the neuron is also due to a lack of staining in the nucleus. (D) When the same neuron from panel (C) is photographed using a very long exposure time (3 s), the signal from the soma saturates, but apparent staining is evident in the growth cones and axons. (E) There is again an absence of staining when the secondary antibody is omitted and only the primary antibody for PKC Apl II (1°Ab; rabbit anti-*Aplysia* PKC Apl II IgG) is present at 1:2500 (400 ms exposure). (F) Delivery of the primary antibody for PKC Apl II at 1:2500 followed by the secondary antibody at 1:200 also shows PKC immunostaining of the soma (400 ms exposure). As with PKC Apl I, the staining for PKC Apl II is more intense at the periphery of the soma, although there is an overall stronger signal even 20 μm in from the membrane. Again, the comparatively limited staining in the centre reflects the nucleus. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Confocal microscopy confirms changes to PKC Apl I and Apl II

The standard fluorescence microscopy images were taken by focussing at the middle of the vertical axis of the neuron (see Figs. 7 and 8), where light from above and below the focal plane would have contributed to the signal. As such, some neurons were re-examined using confocal microscopy, which provided a stack of 2 μm-thick images through the vertical axis of the soma. We choose to examine those CtVm-treated neurons, and their controls, that displayed a change under standard microscopy, i.e., the 10 min CtVm group stained for PKC Apl I and the 30 min CtVm group stained for PKC Apl II. Confocal images through the mid-section of neurons showed that the 10 min exposure to CtVm caused an increase in staining for PKC Apl I relative to control (Fig. 9A, B). Quantification of intensity along the line of a circle $\sim$4 μm from the membrane edge (see Experimental procedures, Analysis for detail) showed that the difference between control neurons and cells treated with CtVm for 10 min reached the level of significance (Fig. 9C). Similarly, confocal images of neurons bathed in CtVm for 30 min revealed a decrease in staining intensity for PKC Apl II compared to control (Fig. 9D, E). This drop in signal following 30 min of CtVm also attained the level of significance (Fig. 9F).

DISCUSSION

PKC is a widely-recognized intracellular regulator, known to moduate many types of ion channels (Numann et al., 1991; Yang and Tsien, 1993; Lu et al., 2000; Macica et al., 2003; Derkach et al., 2007). The initial description of PKC-dependent ion channel modulation was in fact made using bag cell neurons, i.e., DeRiemer et al. (1985b) found that turning on PKC with PMA enhances voltage-gated Ca^{2+} current. However, unlike the present study, they did not report any effects on membrane potential or neuronal responsiveness. Here, we suggest that CtVm evokes biophysical changes through recruitment of PKC.

Although often thought of as ion channel blockers, some *Conus* toxins are agonists or antagonists for adren-

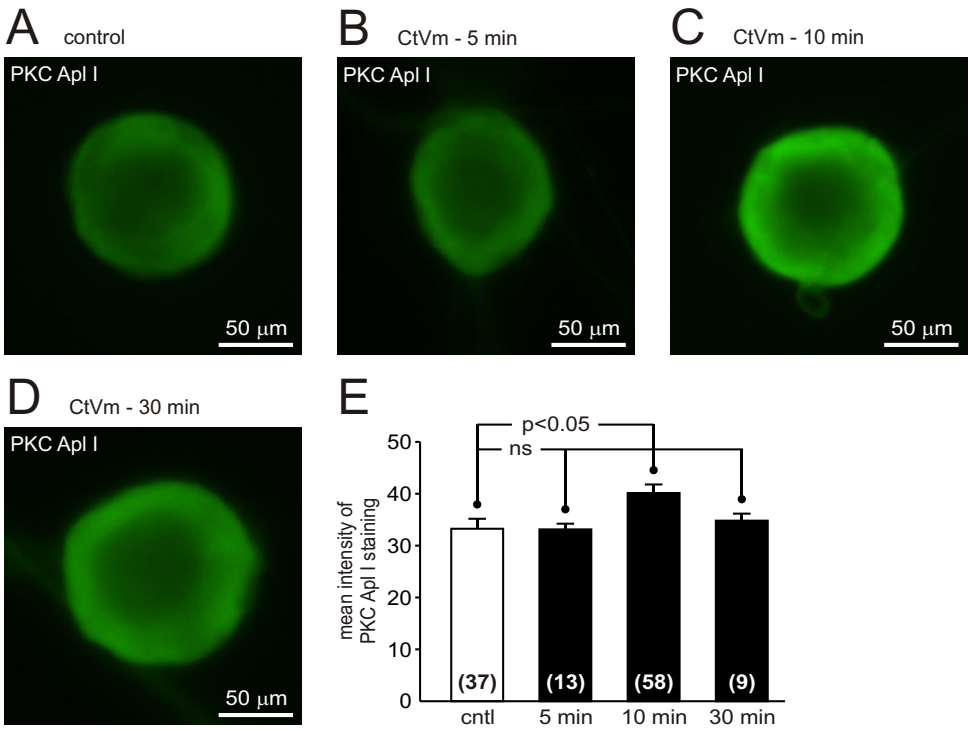

Fig. 7. PKC Apl I staining increases following exposure to CtVm. Standard fluorescent microscopy of cultured bag cell neurons immunostained for PKC Apl I as per Fig. 5C, that is, primary antibody against PKC Apl I at 1:50 followed by secondary antibody at 1:100. (A) In a control neuron exposed to water prior to fixation, the picture of staining for somatic PKC Apl I is similar to that observed in prior controls. (B) A separate neuron subjected to 100 μg/ml CtVm for 5 min; the staining pattern appears essentially the same as that of the control neuron. (C) Exposure of a different neuron to CtVm for 10 min results in an enhanced signal for PKC Apl I. (D) By 30 min of CtVm, another neuron presents a staining pattern that is not dissimilar to that of the control cells. (E) Summary data for the effect of CtVm on PKC Apl I staining demonstrates a significant rise in the signal intensity for the 10 min exposure, but not the 5 and 30 min time points, compared to control (nonparametric Kruskal-Wallis ANOVA, Dunn's multiple comparisons test). Measurements made using a ring-ROI over the soma (see Experimental procedures, Analysis for detail). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

ergic, oxytocin, vasopressin, and neurokinin receptors (Cruz et al., 1987; Craig et al., 1999; Sharpe et al., 2001). Previous work on bag cell neurons suggests that CtVm triggers the cation channel through a metabotropic receptor to cause depolarization. Cation channels excised using single-channel methods, then re-inserted or "crammed" back into the neuron, are activated by bath-applied CtVm (Wilson et al., 1996). Under these circumstances the extracellular face of the channel is contained within the patch-pipette and the only way CtVm can produce an effect is by initiating an intracellular biochemical response, presumably through a receptor. CtVm also brings about an increase in intracellular Ca^{2+} which persists in the absence of extracellular Ca^{2+} and is prevented by depletion of endoplasmic reticulum Ca^{2+} (Magoski et al., 2000). The fact that the pathway leading to PKC activation usually results in endoplasmic reticulum Ca^{2+} release (Levitan and Kaczmarek, 2002; Sossin, 2007; Newton, 2010), also supports a receptor-operated mechanism.

We now substantiate this reasoning by demonstrating that the CtVm response is diminished following block of G-protein signalling with GDP-β-S. Heterotrimeric G-proteins are activated when GTP exchanges for GDP on the α-subunit, allowing for separation from the $\beta\gamma$-subunits and interaction with effectors (Rodbell et al., 1971; Northup et al., 1980; Rosenbaum et al., 2009). Inhibition by GDP-β-S is a hallmark for G-protein involvement, as the nucleotide locks the complex in the inactive $\alpha\beta\gamma$ state by competing with GTP for binding (Eckstein et al., 1979). In molluscan neurons, GDP-β-S prevents coupling of *Aplysia* serotonin receptors to membrane-bound effector enzymes, and attenuates cholinergic, dopaminergic, FMRFamingeric, and serotonergic responses in *Aplysia*, *Lymnaea*, or *Planorbarius* (Lemos and Levitan, 1984; Brezina, 1988; Kudo et al., 1991; Bolshakov et al., 1993; Kehoe, 1994; Magoski et al., 1995).

Our experiments reveal that the ability of CtVm to depolarize bag cell neurons is lessened when the aminosteroid phospholipase C inhbitor, U-73122, or the sphingolipid PKC inhibitor, sphinganine, are employed to interfere with the PKC signalling pathway. Bleasdale et al. (1990) first showed that U-73122 inhibited phosphatidylinositol hydrolysis as well as inositol triphosphate and diacylglycerol accumulation in both platelets and neutrophils. Subsequently, U-73122 and PKC antagonists were found to block the serotonin-dependent intermediate facilitation of *Aplysia* sensory-motor neuron synapses and the motor neuron glutamate response (Fulton et al., 2008; Zhao et al., 2006; Villareal et al., 2009). Sphinganine blocks typical PKC isoforms (Hannun et al., 1986) and inhibits PKC-mediated changes to bag cell neuron voltage-gated Ca^{2+}

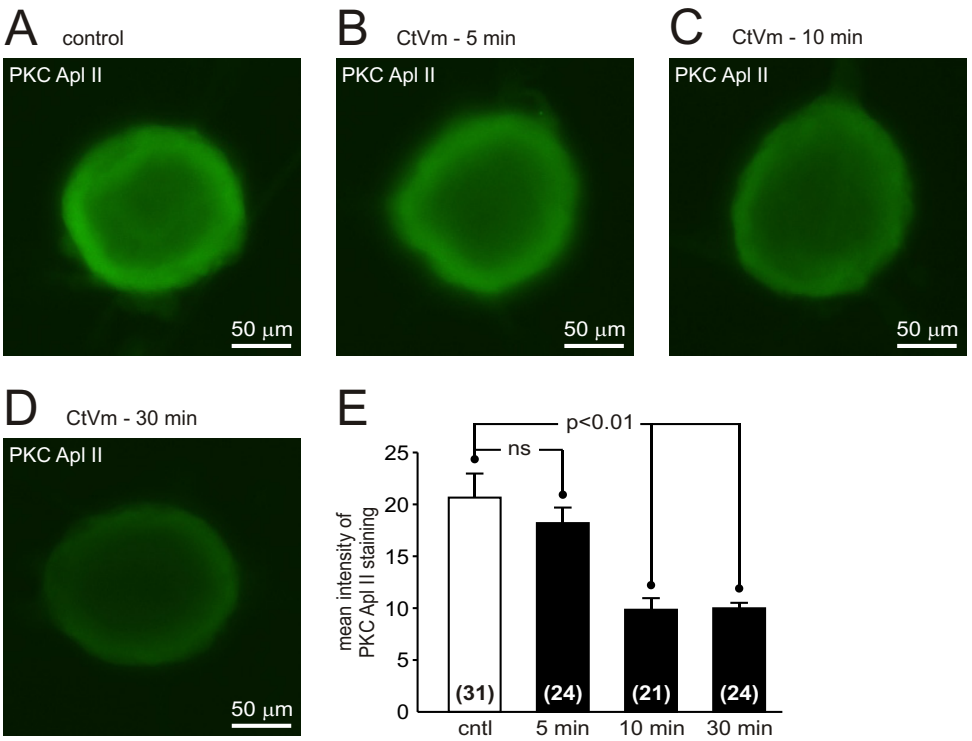

Fig. 8. PKC Apl II staining chronically decreases following exposure to CtVm. Standard fluorescent microscopy of cultured bag cell neurons immunostained for PKC Apl II as per Fig. 5F, i.e., primary against PKC Apl II at 1:2500 followed by secondary antibody at 1:200. (A) A neuron serving as control is exposed for 30 min to 0.5% TFA in water prior to fixation. The image of somatic PKC Apl II staining is like that of other controls. (B) Another neuron presented with 100 μg/ml CtVm for 5 min exhibits a staining pattern not all together different from that of the control neuron. (C, D) Two different neurons exposed to CtVm for 10 or 30 min. In both cases, the signal for PKC Apl II is lessened when compared to those cells under control conditions or in the presence of CtVm for only 5 min. (E) Average data for the result of CtVm on PKC Apl II staining reveals, compared to the control group, a significant drop in signal intensity for both the 10 and 30 min time points, but not at 5 min (nonparametric Kruskal-Wallis ANOVA, Dunn's multiple comparisons test). Measurements made using a ring-ROI of the soma (see Experimental procedures, Analysis for detail). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

channels and Ca^{2+}-activated K^+ channels (Conn et al., 1989; Zhang et al., 2002). While determining the channel-PKC protein-protein interactions, Magoski et al. (2002) showed that whole-cell dialysis of bag cell neurons with the pseudosubstrate PKC inhibitor peptide, PKC_{19-36}, also decreases the CtVm-induced depolarization. Thus, two structurally unrelated PKC antagonists, applied in distinct ways, attenuate the CtVm response. Importantly, blocking G-proteins, phospholipase C, or PKC fails to alter the input resistance of bag cell neurons. This indicates that disturbing the pathway does not indiscriminately lower excitability; rather, it prevents CtVm from accessing the cellular machinery necessary for initiating depolarization. In keeping, our laboratory finds that GDP-β-S does not change the ionotropic acetylcholine response in bag cell neurons (White and Magoski, 2009).

We examined the possibility that direct activation of PKC by PMA would mimic CtVm. PMA is a phorbol ester that frees the kinase domain by binding to the C1 regulatory domain (Castagna et al., 1982). This drug has been used to routinely activate *Aplysia* PKC, including biochemical assays of nervous system ganglia or bag cell neuron clusters (DeRiemer et al., 1985a; Sossin and Schwartz, 1992, 1994; Sossin et al., 1996). Furthermore, PMA turns on PKC in single bag cell and sensory neurons to modulate Ca^{2+} channels, Ca^{2+}-activated K^+ channels, and growth cone morphology (DeRiemer et al., 1985b; Critz and Byrne, 1992; Knox et al., 1992; Zhang et al., 2002). Consistent with a role for PKC in the CtVm response, we find that PMA initiates both a depolarization and sustained spiking in bag cell neurons. Perhaps because of persistent PKC activation, the PMA-induced firing is more intense than that produced by CtVm. As the immunocytochemistry suggests, CtVm only transiently activates PKC. Previous investigations did not report an effect of PMA on membrane potential or excitabilty; potentially contributing to this, is our using a higher concentration of PMA (100 nM) compared to some prior studies (Conn et al., 1989; Knox et al., 1992) (10–20 nM). Regarding other cation channel-mediated responses, metabotropic stimulation of nucleus tractus solitarius or prefrontal pyramidal neurons elicits depolarizations that are blocked by PKC inhibition or recapitulated by PKC activation (Yang et al, 2003; Yan et al., 2009).

A second consequence of PMA is a dramatic increase in the response of bag cell neurons to a multi-phase current ramp analogous to the activity-dependent current experienced after a short burst of action potentials (Whim and Kaczmarek, 1998; Hung and Magoski, 2007; Tam et al., 2009). For PMA-exposed neurons, this stimulus causes more prominent depolarization and leads to action

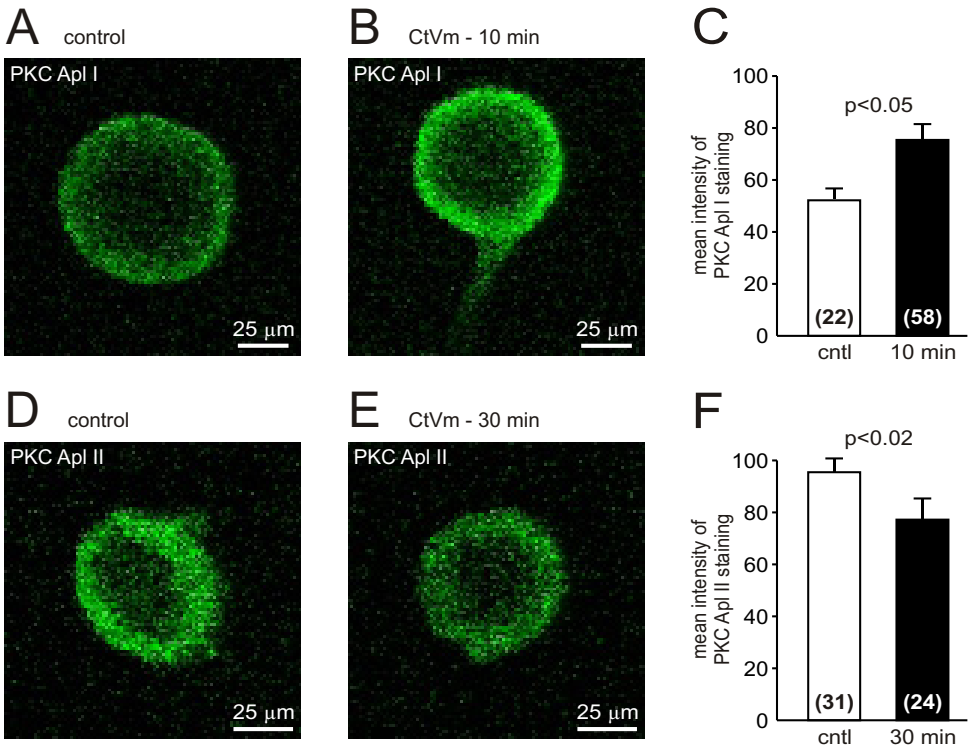

Fig. 9. Confocal fluorescent microscopy of immunostained neurons is consistent with an increase and decrease in PKC Apl I and Apl II, respectively. (A, B) Representative fluorescent confocal image slices acquired from the middle of the vertical axis of neurons under either control conditions or in the presence of 100 μg/ml CtVm for 10 min prior to fixation. Treatment with CtVm causes an increase in somatic staining for PKC Apl I. (C) Mean confocal data from the middle image slice of neurons stained for PKC Apl I demonstrates that the signal intensity is significantly greater for the 10 min CtVm group versus control (Mann–Whitney *U*-test). Quantification presented in this panel and panel (F) is taken from the line of a circle following the inner edge of the somatic membrane (see Experimental procedures, Analysis for detail). The neurons that make up this data set are the same as the control and 10 min CtVm cells depicted in Fig. 6; however, because of the accidental loss of some control dishes, the number of control neurons subjected to confocal imaging is less than the number seen in the original standard fluorescent microscopy data set. (D, E) Sample mid-vertical axis fluorescent confocal image slices obtained from control or neurons exposed to CtVm for 30 min prior to fixation. The CtVm induces a drop in staining for PKC Apl II. (F) Summary confocal data from the middle image slice of neurons stained for PKC Apl II. Unlike Apl I, the signal intensity for this kinase isoform at the membrane is significantly lower for the 30 min CtVm group compared to control (Mann-Whitney *U*-test). Measurements again taken from the line of a circle following the inner edge of the somatic membrane. This data set is comprised of the same neurons from the control and 30 min CtVm groups analyzed by standard fluorescent microscopy as depicted in Fig. 7. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

potential firing; however, PMA does not alter input resistance, suggesting that generic excitability is unchanged. The cation channel activated by CtVm is voltage-dependent, with a half-maximal voltage for activation of ~−30 mV (Gardam and Magoski, 2009). If PMA triggers the cation channel, additional inward current would arise when the ramp depolarizes the membrane potential into the activation range. Alternatively, the well-established PKC-mediated increase in voltage-gated Ca^{2+} current (DeRiemer et al., 1985b; Conn et al., 1989) could contribute to the PMA-induced enhancement of excitability we observe. As to why this sub-set of neurons did not respond to PMA at the outset may reflect a requirement of both PKC activation and elevated intracellular Ca^{2+}, which incidentally, is caused by CtVm delivery (Magoski et al., 2000). In addition, the PMA-induced enhancement of Ca^{2+}-activated K^+ channels documented by Zhang et al. (2002) could oppose cation channel opening in some neurons.

There are no pharmacological means to distinguish between PKC Apl I and Apl II (Manseau et al., 2001). Thus, we immunostained for both kinases to gain a better understanding of the isoforms potentially activated by CtVm. In agreement with prior reports employing Western blot or immunocytochemistry (Sossin et al., 1996; Nakhost et al., 1998), our staining also reveals both PKC isoforms in the soma and neurites of bag cell neurons. Similar to the PKC upregulation that occurs near the start of the afterdischarge (Wayne et al., 1999), there is a transient elevation of PKC Apl I following exposure to CtVm for 10 min. In contrast, PKC Apl II staining falls below control levels by 10 and 30 min. Confocal sections at the mid-soma vertical axis confirm the increase in PKC Apl I and decrease in PKC Apl II staining at 10 and 30 min of CtVm, respectively. Our quantification of the PKC signal focussed on a ring around the inside of the plasma membrane. Because binding of membrane lipids is necessary to turn on typical PKC, (Sossin, 2007; Newton, 2010), the changes could be interpreted as CtVm initiating PKC Apl I transition to the membrane so as to gate the cation channel through close association. When we measured the standard PKC Apl I

fluorescence staining over the cytoplasmic region (as a single circle of two-thirds the soma diameter, see Experimental procedures, Analysis for details), there was a small but significant decrease at 10 min of CtVm (20.8±1.0 U, $n=58$) compared to control (24.6±1.7 U, $n=37$) ($P<0.05$, unpaired t-test). However, it is quite possible that the difference reflects an increase in the synthesis of the kinase. As a case in point, within minutes to hours of serotonin, the synthesis of the neuropeptide, sensorin, is enhanced in *Aplysia* sensory neurons (Hu et al., 2006).

PKC Apl I is a classical PKC that requires diacylglycerol, acidic lipids, and Ca^{2+} for physiological activation, while PKC Apl II is considered novel because, despite needing lipids, it is Ca^{2+}-independent (Kruger et al., 1991; Sossin et al., 1993). A rationale for PKC Apl I involvement in the CtVm response is that intracellular Ca^{2+} is elevated by the toxin in cultured bag cell neurons and during the afterdischarge in intact clusters (Fisher et al., 1994; Magoski et al., 2000; Geiger and Magoski, 2008). Chelation of intracellular Ca^{2+} also attenuates the CtVm depolarization (Wilson et al., 1996). The cation channel is Ca^{2+}-permeable (Geiger et al., 2009) and may well attract PKC by acting as a Ca^{2+} hot spot. Differential PKC activation occurs in *Aplysia* pleural-pedal ganglia, where stimulating serotonin receptors causes only PKC Apl I to move to the plasma membrane (Sossin and Schwartz, 1992). Furthermore, in individual sensory neurons, serotonin alone up regulates PKC Apl II, whereas serotonin plus activity (Ca^{2+} influx) activates PKC Apl I (Zhao et al., 2006). Depending on the state of the synapse and type of training, the isoforms make distinct contributions to synaptic facilitation (Manseau et al., 2001; Zhao et al., 2006; Farah et al., 2009).

The role and cause of the CtVm-induced decrease in PKC Apl II staining is unclear. Nakhost et al. (1998) reported that phorbol ester causes a proportion of pedal ganglion PKC Apl II to translocate from the plasma membrane to actin microfilaments. In addition, insulin persistently activates then down-regulates PKC Apl II in bag cell neuron clusters (Sossin et al., 1996), but fails to depolarize cultured bag cell neurons (Jonas et al., 1997). If PKC Apl II is somehow inhibitory or competes with PKC Apl I for association with the cation channel, it might be necessary for CtVm to initiate PKC Apl II down-regulation or movement to the cytoskeleton. Our laboratory showed that protein kinase A (PKA) and PKC both associate with the cation channel, but in a mutually exclusive manner, suggesting only one kinase can be linked to the channel at any given time (Magoski, 2004; Magoski and Kaczmarek, 2005). The standard PKC Apl II fluorescence staining in the cytoplasmic region (measured as a circle of two-thirds the soma diameter) dropped by nearly one-third after 30 min of CtVm (15.8±0.9 U, $n=24$) versus control (23.7±2.4 U, $n=31$) ($P<0.01$, unpaired t-test, Welch corrected). Both this drop in kinase, as well as that nearer to the membrane, could be unrelated to translocation, and include enhanced degradation of PKC Apl II or some CtVm-induced alteration in the epitope that impacts antibody affinity.

To an extent, the CtVm-induced depolarization of cultured bag cell neurons is analogous to the afterdischarge in intact clusters: both involve lengthy depolarization, action potential firing, and ultimately result in the neurons becoming refractory to stimulation (Kupfermann and Kandel, 1970; Kaczmarek and Kauer, 1983; Wilson et al., 1996; Magoski et al., 2000). However, prolonged bursting to CtVm *in vitro* is rare, which in part may be a consequence of transient PKC Apl I activation. Similarly, the upregulation of PKC Apl I produced by serotonin and activity in sensory neurons is temporary; the enzyme reverts back to the cytosol once action potential firing stops (Zhao et al., 2006). *In vivo*, additional signals, such as PKA or calmodulin kinase (Kaczmarek and Strumwasser, 1981; DeRiemer et al., 1984), contribute to maintaining the afterdischarge and Ca^{2+}-influx, which in turn may promote long lasting PKC activation. Coordinated targeting of signalling molecules is essential for efficient transition from rest to periods of extended activity, especially for neurons which use long-term change to store information, process sensory input, and evoke motor or neuroendocrine commands (Morisset and Nagy, 1999; Egorov et al., 2002; Dembrow et al., 2004; Teruyama and Armstrong, 2005; Sidiropoulou et al., 2009). PKC activation in bag cell neurons has the potential to impact afterdischarge generation and, ultimately, reproduction.

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