Neuroscience 179 (2011) 41–55

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

A. K. H. TAM, K. E. GARDAM, S. LAMB, B. A. KACHOEI AND N. S. MAGOSKI*

Department of Physiology, Queen's University, Kingston, ON K7L 3N6, Canada

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.

*Corresponding author. Tel: +1-613-533-3173; fax: +1-613-533-6880.
E-mail address: magoski@queensu.ca (N. S. Magoski).

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;

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.01.037

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