Published in final edited form as:

Neuroscience. 2010 August 25; 169(2): 787–793. doi:10.1016/j.neuroscience.2010.05.018.

Atypical PKC expression in phrenic motor neurons of the rat

C.H. Guenther, S. Vinit, J.A. Windelborn, M. Behan, and G.S. Mitchell

Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI USA, 53706.

Abstract

Atypical protein kinase C (PKC) isoforms play important roles in many neural processes, including synaptic plasticity and neurodegenerative diseases. Although atypical PKCs are expressed throughout the brain, there are no reports concerning their expression in central neural regions associated with respiratory motor control. Therefore, we explored the neuroanatomical distribution of atypical PKCs in identified phrenic motor neurons, a motor pool that plays a key role in breathing. Diaphragm injections of cholera toxin B were used to retrogradely label and identify phrenic motor neurons; immunohistochemistry was used to localize atypical PKCs in and near labeled motor neurons (i.e. the phrenic motor nucleus). Atypical PKC expression in the phrenic motor nucleus appears specific to neurons; aPKC expression could not be detected in adjacent astrocytes or microglia. Strong atypical PKC labeling was observed within cholera toxin B labeled phrenic motor neurons. Documenting the expression of atypical PKCs in phrenic motor neurons provides a framework within which to assess their role in respiratory motor control, including novel forms of respiratory plasticity known to occur in this region.

Keywords

spinal cord; breathing; ventilatory control; astrocytes; microglia; cholera toxin

INTRODUCTION

Protein Kinase C (PKC) is a class of serine/threonine enzymes that regulate key cellular events via phosphorylation. At least 10 PKC isoforms have been identified and categorized into families based on structure and function (Nishizuka, 1995). The atypical PKCs are unique because they lack calcium and diacylglycerol binding sites, and are activated by a different regulatory mechanism than other PKCs. Although all PKCs contain a regulatory and catalytic domain, truncated PKC isoforms containing only the catalytic domain have been identified and are referred to as PKMs (Takai, *et al.*, 1977; Inoue, *et al.*, 1977). The atypical isoform, PKM ζ , is the single molecule identified to date that is essential for maintenance of long term memory and hippocampal long term potentiation (Ling, *et al.*, 2002; Pastalkova and Serrano, *et al.*, 2006).

Although atypical PKCs exist throughout the brain, there are no reports concerning their expression in central neural regions associated with respiratory motor control (Naik, *et al.*

Corresponding Address: G.S. Mitchell, Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, 2015 Linden Dr. West, Madison, WI 53706 USA, (608) 263-9826, mitchell@svm.vetmed.wisc.edu.

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2000). Given their role in synaptic plasticity, we questioned whether atypical PKCs exist in the phrenic motor nucleus, a key site for respiratory motor plasticity (Mitchell, *et. al.*, 2001; Fuller, *et. al.*, 2000; Feldman *et al.*, 2003; Mahamed and Mitchell, 2007). Documenting the expression of atypical PKCs in the phrenic motor nucleus is an important first step before we will be able to assess their potential role in respiratory motor control.

We analyzed the expression of atypical PKCs in the region of the phrenic motor nucleus. First, we identified the atypical PKC isoforms present in homogenates of ventral spinal segments C₃₋₅ by immunoblots. Injections into the diaphragm of the neuroanatomical tracer, cholera toxin B, were used to retrogradely label phrenic motor neurons in C₃₋₅, and then immunohistochemistry was used to localize atypical PKCs within the identified phrenic motor neurons. Astrocytes and microglia adjacent to identified phrenic motor neurons were revealed with cell-specific markers (Glial fibrillary acidic protein, GFAP, and OX-42, respectively) and possible expression of atypical PKC in these cells was explored. Here, we report that atypical PKC is expressed in identified phrenic motor neurons, but could not be detected in adjacent glia. These data were previously presented in abstract form (Guenther, *et. al.*, 2009).

METHODS

Animals

All procedures were approved by the University of Wisconsin Animal Care and Use Committee. A total of 10 adult male Lewis rats were used in this study (3–6 months of age, Harlan, Colony 202A, Indianapolis, IN, USA).

Immunoblot analyses of ventral spinal homogenates

Four rats were anesthetized with isoflurane and euthanized with an overdose of Beuthanasia-D (pentobarbital, at least 120mg/kg, i.c.). C₃₋₅ spinal segments were harvested and immediately placed on dry ice. The dorsal half of C₃₋₅ was removed, and the ventral half homogenized in RIPA buffer (1% nonylphenylpolyethylene glycol, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate in 10mM phosphate buffered saline, pH=7.4) with protein phosphatase (Pierce Biotechnology, Rockford, IL, USA) and protease (Roche Applied Science, Indianapolis, IN, USA) inhibitors. Samples were centrifuged, and supernatant aliquoted. Total protein concentration was determined by bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). Sample buffer was added to supernatant, and samples were boiled. Samples were loaded onto BioRad 4–20% gradient criterion gels. Protein was transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Blots were blocked in 6% milk buffer with Tween for 1 hour and probed with primary antibody (PKC ζ C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200 or PKC ι , 1:1000) overnight at 4°C. Immunoblots were rinsed in 1× TBST (20mM Tris, 500mM NaCl, 0.1% Tween) and probed with HRP-conjugated mouse anti-rabbit (for PKC ζ) or HRP-conjugated donkey anti-mouse (for PKC ι) secondary antibodies (Jackson Immuno, West Grove, PA, USA, 1:10,000) for one hour at room temperature. The immunoblot label was expressed using Pierce Super Signal West Dura extended duration Chemiluminescent kit (Pierce Biotechnology, Rockford, IL, USA). Chemiluminescence was detected and digitized using an AutoChemi Imaging system (UVP Bioimaging Systems, Upland, CA, USA) with LabWorks 4.6. Blots were stripped using Pierce Restore Western blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, USA) and reprobed with pan-actin (Chemicon, Billerica MA, 1:5000) to confirm equal loading.

Retrograde labeling of phrenic motor neurons

Four rats were used for retrograde labeling of phrenic motor neurons with cholera toxin B fragment (Kinkead, R., *et al.*, 1998). Rats were pretreated with 0.7–1.0ml of an analgesic solution containing an analgesic (buprenorphine, 50 µg/kg, s.c.), an anti-inflammatory drug (carprofen, 6.5mg/kg, s.c.), an antibiotic (enrofloxacin, 4 mg/kg, s.c.), and a sedative (dexmedetomidine, 100 µg/kg, s.c.) in lactated Ringer's solution. Anesthesia was induced in a closed chamber with isoflurane. Rats were intubated and anesthesia was maintained (1.5% isoflurane in 100% oxygen) while the rats were mechanically ventilated. A transversal laparotomy was performed to expose the diaphragm. A total of 4–5 intramuscular injections of 0.5% cholera toxin B fragment (List Biologicals, Campbell, CA, USA) in saline containing ~1% Evans blue were made bilaterally with a cannula (C-315 I, Plastics One Inc., Roanoke, VA, USA) connected to a 50µL Hamilton syringe (total volume injected: 20–25 µl on each side). Care was taken to prevent the cannula from piercing the diaphragm or adjacent blood vessels. A Q-tip was applied over the injection area in order to prevent leaking. Muscle and skin were sutured, and an active reversal of the dexmedetomidine sedative (atipamezole, 500 µg/kg, i.m.) was injected. Rats were treated for 2 days with 0.8ml of an analgesic solution containing buprenorphine (50 µg/kg, s.c.), carprofen (6.5 mg/kg, s.c.), and enrofloxacin (4 mg/kg, s.c.) in lactated Ringer's solution. Three days after surgery, the rats were deeply anesthetized with sodium pentobarbital (at least 120mg/kg, i.p.) and perfused with 150 ml of heparinized saline (4 IU/ml) followed by 4% paraformaldehyde solution in 0.1M phosphate buffer. Two additional, un-operated rats were perfused using the same procedure. Brains and spinal cords were removed and post-fixed in 4% paraformaldehyde solution overnight at 4°C. Tissues were then cryoprotected (30% sucrose in 0.1M PBS).

Immunofluorescence

Spinal segments C_{3–5} from retrogradely labeled and un-operated rats were sectioned coronally (40µm) using a sliding microtome (Leica SM2000R). Eight sections, primarily from the C₄ region and sampled rostral to caudal, from each retrogradely labeled rat (n=4) were double-labeled with anti-cholera toxin B fragment primary antibody and an anti-PKCζ primary antibody (1:100, Santa Cruz Biotechnology). Details of the primary antibodies are summarized in Table 1. To confirm atypical PKC expression, two sections from each rat were double-labeled with anti-cholera toxin B fragment and a second rabbit anti-PKCζ antibody (1:100, Abcam, Cambridge, MA, USA). Controls included pre-adsorption of primary antibody (PKCζ, Santa Cruz; Table 1) with its peptide immunogen (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the omission of the primary antibody (PKCζ, Santa Cruz and cholera toxin B; Table 1).

Six sections (primarily from the C₄ region) from each un-operated rat (n=2) were double-labeled with anti-PKCζ (1:100, Santa Cruz Biotechnology, CA), and one of the following anti-sera (markers of neurons and glia): GFAP, OX-42, synaptophysin, neurofilament. All secondary antibodies were conjugated to Alexa Fluor® fluorescent dyes (568 donkey anti-goat, 488 donkey anti-rabbit, 488 goat anti-mouse, 594 goat anti-rabbit; Invitrogen, Carlsbad, CA, USA).

Data analysis

All immunofluorescent images were viewed using a Nikon C1 laser scanning confocal microscope with lambda strobing in the EZ-C1 confocal imaging software; 1µm step increments were used for the z-series. Z-series images were constructed with the “Rocking 3D Project” plug-in in ImageJ (NIH, Bethesda, MD, USA) and intensity was adjusted using Photoshop CS3 (Adobe, San Jose, CA, USA).

Counts of cholera toxin B fragment labeled neurons were performed with the confocal microscope by two independent observers and were classified as phrenic motor neurons if they contained a visible nucleus. After cholera toxin B fragment labeled neurons were identified throughout the visible z-planes, the number of phrenic motor neurons expressing atypical PKC immunoreactivity were also counted.

RESULTS

Immunoblot identification of atypical PKC isoforms

At least three atypical PKC isoforms including PKC ζ , PKM ζ , and PKC ι , were detected in ventral spinal segments C₃₋₅ in each of four un-operated rats (Figure 1A). A control immunoblot, obtained by omission of primary antibody, confirmed that label in Figure 1A was not due to non-specific label from the secondary antibody (control data not shown). Using a different primary antibody (PKC ι ; Table 1), a separate immunoblot confirmed the presence of PKC ι (Figure 1B). Background staining from the secondary antibody was observed and distinguished from PKC ι label by comparing with control (Figure 1B). A dense band was also detected at 25kDa, which could represent another atypical PKC isoform. This 25kDa band limited the usefulness of this antibody for immunohistochemical studies describing the expression and localization of atypical PKC. Therefore, the PKC ζ antibody obtained from Santa Cruz (Table 1) was used for immunohistochemical studies.

Retrograde labeling of phrenic motor neurons

Retrogradely labeled neurons were found in a cluster in the ventrolateral grey matter of spinal cord segment C₄ in each of four rats (Figure 2A). An average of 62 ± 7.9 (mean $\pm$ S.D.) phrenic motor neurons in eight sections from each rat were labeled and analyzed.

Localization of atypical PKCs in neurons and glia

Atypical PKCs were expressed throughout the ventral horn of cervical segment C₄ (Figure 2A). Staining was less intense in the dorsal horn (data not shown). Within the ventral horn, atypical PKCs co-localized with all (100%) cholera toxin B fragment labeled phrenic motor neurons and were expressed in other presumptive phrenic motor neurons that were not labeled with cholera toxin B fragment. This co-localization included cell somas and primary dendrites (Figure 2B, D–F). Virtually identical findings were observed using a different primary antibody to PKC ζ (Abcam; data not shown). Atypical PKCs were also expressed in small unidentified neurons throughout the ventral horn, but did not colocalize with neurofilament (axonal marker) or synaptophysin (presynaptic marker) (Figure 3). Atypical PKCs could not be detected within glia. Specifically, atypical PKCs did not colocalize in GFAP-positive (astrocytes) or OX-42-positive (microglia) cells (Figure 4).

DISCUSSION

Here we demonstrate the presence of atypical PKC isoforms in identified phrenic motor neurons. This is one of the first demonstrations of atypical PKC expression in any type of vertebrate motor neuron. Previous studies examined the distribution of atypical PKCs in the brain, including mRNA analysis in mice (Oster, *et. al.*, 2004) and protein analysis in rats (Naik, *et. al.*, 2000). However, only a few studies have investigated spinal atypical PKC expression (Hu, *et. al.*, 2003; Wolf, *et. al.*, 2008; Narita, *et. al.*, 2004). Although these studies explored atypical PKCs in other spinal regions and in rat embryos, none specifically examined the anatomical distribution of atypical PKCs in regions of interest to respiratory motor control, such as the phrenic motor nucleus.

Phrenic motor neurons were identified by retrograde labeling. Diaphragm injections of cholera toxin B fragment label phrenic motor neurons, but not interneurons (Mantilla, *et al.*, 2009). Although atypical PKC and cholera toxin B fragment were colocalized (i.e. within phrenic motor neurons; Figure 2), atypical PKC staining was also observed in other neurons in the same region. These neurons may be unlabeled phrenic motor neurons, non-phrenic motor neurons or interneurons. The main differences between motor neurons and pre-phrenic interneurons are size and location, with motor neurons being larger (~30–40 μm) than interneurons (~20–30 μm ; Lane, *et al.*, 2008), and with pre-phrenic interneurons found around the central canal (Lane, *et al.*, 2008). Near the phrenic motor nucleus, unlabeled motor neurons and putative interneurons (based on size) express atypical PKC. However, atypical PKC was far less robust in neurons located near the central canal or in the dorsal horn, regions where most pre-phrenic interneurons are found (Lane, *et al.*, 2008). While labs have found some pre-phrenic interneurons in close proximity to phrenic motor neurons, Lane *et al.* excluded analysis of these interneurons since an “unequivocal distinction between putative interneurons and PhMNs could not be made.” Therefore, we conclude that atypical PKCs are expressed in identified phrenic motor neurons as well as in other, unidentified neurons near the phrenic motor nucleus; atypical PKC expression is less clear in neurons located in the dorsal horn or near the central canal.

The absence of atypical PKC in pre-synaptic terminals (synaptophysin), neurofilament-labeled processes or glia in the ventral spinal cord is of considerable interest, and suggests a post-synaptic, neuron-specific role for these PKC isoforms within respiratory motor nuclei. Several previous studies investigated atypical PKC expression in glial cultures. Our findings are in agreement with a study reporting no atypical PKC expression in astrocyte cultures from rat cerebral cortex (Asotra and Macklin, 1994), but contrast with reports that atypical PKCs are expressed in microglial (Slepko, *et al.*, 1999) and astrocyte (Slepko, *et al.*, 1999; Roisin and Deschepper, 1995; Chen, *et al.*, 1993, 1995; Ballestas and Benveniste, 1995) cultures from rat brain. Although many of these studies detected PKC ζ expression in astrocytes and microglia, they were performed with cultured cells from different CNS regions, including C₆ glioma cells (Chen, 1993). Ours would not be the first report of *in vitro* protein expression that is diminished or even nonexistent *in vivo* (e.g. Ridyard and Sanders, 2000). Cell culture conditions differ from the *in vivo* environment, potentially leading to altered protein expression. The intensity of labeling within phrenic motor neurons described here suggests that atypical PKC isoforms have the potential to be involved in important neuron-specific functions, such as the maintenance of long-term motor neuron plasticity (e.g. Feldman *et al.*, 2003; Mahamed and Mitchell, 2007).

Methodological Limitations

Determining which atypical PKC isoforms are expressed in phrenic motor neurons is of considerable interest since the isoforms have different functions. Murine PKC ι/λ knockouts are embryonic lethal (Soloff, *et al.* 2004), whereas the PKC ζ knockout is viable (Leitges, *et al.*, 2001). Thus, these atypical isoforms differ in function early in development. It is uncertain which of the three atypical PKC isoforms is/are expressed in phrenic motor neurons since available antibodies are not sufficiently specific to answer this question. The C-terminus is identical for both PKC ζ and PKM ζ and shares significant homology with PKC ι (Selbie, *et al.*, 1993). Therefore the Santa Cruz PKC ζ antibody used in this study, which targets the C-terminus of PKC ζ , can label all atypical PKC isoforms (Figure 1A). The PKC ι specific antibody detected an appropriate band for PKC ι , thus confirming its presence, but also detected another nonspecific 25 kDa band rendering immunohistochemical studies inconclusive (Figure 1B). At this time there are no PKC ζ /PKM ζ specific antibodies that do not also have the potential to cross react with PKC ι . Nonetheless, these studies are the first

demonstration that atypical PKCs in general are located within phrenic motor neurons, but not in adjacent glia.

There remains the possibility that unsampled phrenic motor neurons did not express atypical PKCs. The longitudinal distance of C₄ is approximately 1.5–2.0mm (unpublished); therefore, we sampled approximately 21% of the total distance (8 sections × 40μm/section, using 1.5mm total C₄ distance). One study found an average of 267 cells per phrenic nucleus at C₄, which is predominantly where the sections in this study were sampled (Goshgarian and Rafols, 1981). By extrapolating our cell counts to the total distance of C₄, we conclude that we have examined approximately 54% of the total number of C₄ phrenic motor neurons. Given the high percentage of co-localization in the 54% of motor neurons sampled, we are confident that our estimate is robust and that our conclusions extend to virtually all phrenic motor neurons within the phrenic nucleus.

Potential role of atypical PKCs in phrenic motor neuron function

Given the unique role of PKM ζ in the maintenance of hippocampal long term potentiation (Ling, *et al.*, 2002; Pastalkova and Serrano, *et al.*, 2006), the identification of atypical PKCs in phrenic motor neurons suggests the possibility that this protein may be involved in respiratory motor plasticity (Mitchell and Johnson, 2003; Feldman *et al.*, 2003; Mahamed and Mitchell, 2003). This work lays the foundation for further functional studies examining the role(s) of atypical PKCs in respiratory control.

Acknowledgments

Supported by National Institutes of Health (HL80209, HL69064 and HL07654). Dr. Stéphane Vinit is supported by a Craig H. Neilsen Foundation Fellowship.

Abbreviations

PKC	Protein Kinase C
GFAP	Glial fibrillary acidic protein

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Fig. 1. Immunoblot detection of atypical PKC isoforms in ventral spinal segments, C₃₋₅. (A) Representative immunoblot from four un-operated rats (Lanes 1–4). Note label for PKCζ/ι (~74kDa) and PKMζ (55kDa). Loading control, pan-actin, migrates at 42kDa. (B) Representative immunoblots from four un-operated rats (Lanes 1–4). Note label for PKCι (74kDa). Control immunoblot was obtained with the omission of primary antibody. A nonspecific band can be observed (~25kDa).

Fig. 2.

Atypical PKC expression and co-localization with cholera toxin B fragment in identified phrenic motor neurons. (A) Coronal section of spinal segment C₄. Atypical PKC immunolabel (green) is present throughout the gray matter of the ventral spinal cord. Phrenic motor neurons, retrogradely labeled with cholera toxin B fragment, are clustered in the ventrolateral region (yellow). (B) Higher magnification of phrenic motor neurons labeled in A showing co-localization (yellow) of cholera toxin B (red) and atypical PKC (green). (C) Single plane image of phrenic motor neurons labeled with cholera toxin B (red). In this section, the primary antibody for atypical PKC (green) was pre-adsorbed with blocking peptide. Images B and C were obtained from the same rat and were reacted at the same time. Images were captured at the same laser intensity level and gain settings. Images were post-processed with the same intensity adjustments. (D–F) z-stack images (39 confocal optical slices) of phrenic motor neurons labeled in A and B. (D) Cholera toxin B (red). (E) Atypical PKC (green). (F) Co-localization of cholera toxin B fragment and atypical PKC (yellow). Scale bar in A=100µm; B–F=40µm.

Fig. 3.

Localization of atypical PKC in neurons in the ventral spinal cord. (A) Coronal section of spinal segment C₄ in the region of the phrenic motor nucleus. Atypical PKC immuno-label (green) is localized in soma and dendrites. (B) Same section as in A stained for neurofilament (red). (C) Superimposed images of A and B. Note that neurofilament and atypical PKC do not colocalize. (D) Coronal section of spinal segment C₄ in the region of the phrenic motor nucleus. Atypical PKC immunolabel (green) is localized in soma and dendrites. (E) Same section as in D stained for synaptophysin (red). (F) Superimposed images of D and E. Note that synaptophysin and atypical PKC do not co-localize. Scale bar in A–F=20μm.

Fig. 4.

Localization of atypical PKC and glia in the ventral spinal cord. (A) Coronal section of spinal segment C₄ in the region of the phrenic motor nucleus. Atypical PKC immuno-label (green) is localized in soma and dendrites. (B) Same section as in A stained for GFAP (red). (C) Superimposed images of A and B. Note that GFAP and atypical PKC do not co-localize. (D) Coronal section of spinal segment C₄ in the region of the phrenic motor nucleus. Atypical PKC immunolabel (green) is localized in soma and dendrites. (E) Same section as in D stained for OX-42 (red). (F) Superimposed images of D and E. Note that OX-42 and atypical PKC do not co-localize. Scale bar in A–F=40μm.

Table 1

Characteristics of Antibodies

	Species/type	Antigen	Dilution	Source
Cholera toxin B	Goat polyclonal	Non-denatured Cholera Toxin B	1:10,000	Calbiochem
PKC ζ (C-20)	Rabbit polyclonal	C-terminus of PKC ζ	1:100	Santa Cruz
PKC ζ	Rabbit polyclonal	PKC ζ around pT560	1:100	Abcam
PKC ι	Mouse monoclonal	Human PKC ι aa 404–587	1:1000	BD Labs
GFAP	Mouse monoclonal	Purified GFAP	1:1000	Millipore
OX42	Mouse monoclonal	Cell peritoneal macrophages	1:200	Santa Cruz
Neurofilament	Mouse monoclonal	Purified neurofilament polypeptides	1:2000	Millipore
Synaptophysin	Mouse monoclonal	C-terminus	1:2000	Neuromics
Pan-actin	Mouse monoclonal	Actin aa 50–70	1:5000	Chemicon