Published in final edited form as:

Respir Physiol Neurobiol. 2018 October ; 256: 36–42. doi:10.1016/j.resp.2017.10.007.

Spinal activation of protein kinase C elicits phrenic motor facilitation

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Abstract

The protein kinase C family regulates many cellular functions, including multiple forms of neuroplasticity. The novel PKC θ and atypical PKC ζ isoforms have been implicated in distinct forms of spinal, respiratory motor plasticity, including phrenic motor facilitation (pMF) following acute intermittent hypoxia or inactivity, respectively. Although these PKC isoforms are critical in regulating spinal motor plasticity, other isoforms may be important for phrenic motor plasticity. We tested the impact of conventional/novel PKC activator, phorbol 12-myristate 13-acetate (PMA) on pMF. Rats given cervical intrathecal injections of PMA exhibited pMF, which was abolished by pretreatment of broad-spectrum PKC inhibitors bisindolymaleimide 1 (BIS) or NPC-15437 (NPC). Because PMA fails to activate atypical PKC isoforms, and NPC does not block PKC θ , this finding demonstrates that classical/novel PKC isoforms besides PKC θ are sufficient to elicit pMF. These results advance our understanding of mechanisms producing respiratory motor plasticity, and may inspire new treatments for disorders that compromise breathing, such as ALS, spinal injury and obstructive sleep apnea.

1. Introduction

Plasticity is a key feature of the neural system controlling breathing. Protein kinases play important roles in many forms of neuroplasticity, including hippocampal long-term potentiation (Bortolotto and Collingridge, 2000, Matthies and Reymann, 1993, Nayak et al., 1998, Sacktor et al., 1993), *Aplysia* sensorimotor long-term facilitation (Cai et al., 2011, Chain et al., 1999) and spinal sensitization of pain pathways (Coderre, 1992, Laferrière et al., 2011). In many neural systems, protein kinase C (PKC) activity plays a key role in plasticity (Sossin, 2007).

Contributions

MJD collected and analyzed all data. MJD and GSM contributed to ideas, data analysis and manuscript preparation. Both authors have approved the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

We recently demonstrated that the novel PKC isoform, protein kinase C theta (PKC θ), is necessary for a well-studied form of respiratory motor plasticity, phrenic long-term facilitation (pLTF) following moderate acute intermittent hypoxia (AIH; Devinney et al., 2015). pLTF is expressed as a prolonged increase in phrenic nerve burst amplitude lasting several hours post-AIH (Devinney et al., 2013, Mitchell et al., 2001). pLTF is serotonin-dependent (Mitchell et al., 2001, Feldman et al., 2003, Devinney et al., 2013), and requires spinal activation of metabotropic 5-HT₂ receptors (Baker-Herman and Mitchell, 2002, Fuller et al., 2001, Kinkead and Mitchell, 1999, MacFarlane et al., 2011). Although AIH-induced PKC θ activation could occur downstream from 5-HT₂ receptor activation and Gq protein-coupled phospholipase C activation (Farah and Sossin, 2011), recent unpublished evidence from our laboratory indicates that the relevant PKC θ is activated downstream from BDNF/TrkB signaling instead, most likely via the phospholipase C γ pathway (Agosto-Marlin and Mitchell, 2017, Leal et al., 2014, Reichardt, 2006, Santos et al., 2010). pLTF mechanisms downstream from PKC θ activation are not yet known, but may involve phosphorylation of synaptic targets such as NMDA or AMPA receptors, thereby enhancing synaptic strength between phrenic pre-motor and motor neurons (MacDonald et al., 2001, McGuire et al., 2005, McGuire et al., 2008, Neverova et al., 2007).

Although PKC θ activity is required for pLTF following moderate AIH (Devinney et al., 2015), this does not rule out contributions from other PKC isoforms. For example, distinct PKC isoforms contribute to spinal sensitization of pain pathways (Hua et al., 1999, Peng et al., 1997), and phrenic motor plasticity (Strey et al., 2012). Although atypical PKCs are not necessary for AIH-induced pLTF (Strey et al., 2012), prolonged inactivity elicits phrenic motor plasticity by a mechanism that requires activity of the atypical PKC isoform, PKC ζ (Strey et al., 2012).

Recently we have come to realize that multiple, distinct cellular cascades are capable of giving rise to phrenic motor facilitation (pMF; a more general term that includes AIH-induced pLTF; (Dale-Nagle et al., 2010, Devinney et al., 2013, Fields and Mitchell, 2015). For example, pMF can be induced pharmacologically via spinal injections of receptor agonists for Gq protein-coupled serotonin 2A and 2B receptors (Hoffman and Mitchell, 2011, MacFarlane et al., 2011, MacFarlane and Mitchell, 2009), or Gs protein-coupled serotonin 7 or adenosine 2A receptors (Golder et al., 2008, Hoffman and Mitchell, 2011). pMF can also be elicited via intrathecal injections of growth/trophic factors, such as brain-derived neurotrophic factor (Baker-Herman et al., 2004), vascular endothelial growth factor (Dale-Nagle et al., 2011) or erythropoietin (Dale et al., 2012). Similar to metabotropic receptor activation, pMF can be elicited by direct activation of spinal protein kinases. For example, intrathecal injections of a cAMP analog over the phrenic motor nucleus activate protein kinase A and elicits pMF and partial recovery of respiratory function after cervical spinal injury (Kajana and Goshgarian, 2008). Similar studies concerning the impact of cervical spinal PKC activation have not been done.

Since multiple PKC isoforms may contribute to moderate AIH-induced pLTF, we tested the hypothesis that spinal PKC activation elicits pMF in anesthetized rats, and that this pMF does not require PKC θ or atypical PKC activity. We delivered the conventional/novel PKC activator phorbol 12-myristate 13-acetate (PMA) intrathecally over the phrenic motor

nucleus while recording phrenic nerve activity. Intrathecal injections of two PKC inhibitors with distinct isoform selectivity (Bisindolymaleimide I; NPC-15437) enabled us to confirm that PMA-induced pMF is PKC-dependent, but acts via isoforms distinct from PKC θ or PKC ζ . We conclude that spinal activation of distinct PKC isoforms elicits distinct mechanisms of pMF.

2. Methods

2.1. Animals

Adult (12–17 week old) male Sprague-Dawley rats weighing 280–500 g (Harlan Colony 211, Houston, TX; 218a, Indianapolis, IN) were used in all experiments. Rats were housed two per cage with food and water *ad libitum* and kept in a 12 h light/dark cycle. The University of Wisconsin Animal Care and Use committee approved all experimental protocols.

2.2. Surgical preparation

Rats were anesthetized in a closed chamber with isoflurane and then placed on a heated surgical table where anesthesia was maintained *via* nose cone (3.5% isoflurane; 50% O₂). Rats were then pump-ventilated through a tracheal cannula (Rodent Ventilator 683, Harvard Apparatus; tidal volume 2.2–2.7 ml) with gas mixture (50% O₂, 50% N₂) and 3.5% isoflurane for anesthetic maintenance. End-tidal CO₂ was maintained at 40–44 mmHg by adding CO₂ to the inspired gas mixture; ventilator frequency was adjusted if necessary, but was typically ~75 breaths/min. A catheter was placed in the tail vein to administer intravenous fluids (~1.5 ml/hr of 75% lactated ringer's solution, 10% HCO₃, and 15% hetastarch (Hespan, 6% hetastarch in 0.9% NaCl) to maintain arterial base excess between –4 to +4 mEq/L at baseline, with less than 1.5 mEq/L change throughout experiment. A catheter was placed in the femoral artery to monitor blood pressure and draw arterial blood samples for analysis (PaO₂, PaCO₂, pH, base excess). Using a ventral approach, bilateral vagotomies were performed, and mechanical ventilation was sufficient to prevent non-synchronous respiratory effort. Using a dorsal approach, the left phrenic nerve was isolated, cut, desheathed and covered with saline-soaked cotton. For intrathecal drug delivery, a C₂ laminectomy was performed to expose the dura, and a small incision was made at C₂ to enable insertion of a silicone catheter (2 Fr; Access Technologies, Skokie, IL) attached to a 50 μ l Hamilton syringe above the C₄ spinal segment. Conversion to urethane anesthesia was performed by slowly withdrawing inhaled isoflurane while administering intravenous urethane (1.7–1.9 mg/kg) over a 20-min period. Approximately 1 h after conversion, pancuronium bromide (2.5 mg/kg, i.v.) was administered to prevent counterproductive respiratory efforts. Body temperature was maintained at 37.5 $\pm$ 1 $^{\circ}$ C (rectal thermometer; Fisher Scientific, Pittsburgh, PA). Blood pressure was monitored to ensure physiological stability (80–150 mmHg baseline, <30 mmHg change from baseline at 90 min). Adequate anesthesia was verified by toe pinch induced blood pressure and nerve responses.

2.3. Neurophysiology procedures

Approximately 1 h post-conversion to urethane anesthesia, the phrenic nerve was desheathed, covered with mineral oil and placed on bipolar silver electrodes for recording.

Nerve activity was amplified (10,000X) band-pass filtered (300–10,000 Hz Model 1800, A-M Systems, Carlsborg, WA), rectified and integrated with a continuous moving averager (time constant: 50 ms; CWE Inc., MA-821 filter; Ardmore, PA). The integrated signal was digitized and analyzed with a data acquisition system (WINDAQ, DATAQ Instruments, Akron, OH). The CO₂ apneic and recruitment thresholds were determined by lowering inspired CO₂ and/or increasing ventilator rate until phrenic nerve bursting ceased, and then slowly raising CO₂ until phrenic nerve activity resumed. Baseline end-tidal CO₂ was set 2–3 mmHg above the recruitment threshold, and baseline nerve activity as recorded for ~20 min. Blood samples were drawn ~5 and 20 min after establishing baseline conditions; adequate regulation of blood gases and acid-base balance was confirmed in blood samples drawn at 15, 30, 60 and 90 min post PMA injections (PaO₂ > 180 mmHg, PaCO₂ ±1.5 mmHg baseline, base excess ±1.5 mEq/L baseline). Maximal CO₂ responses were elicited at the end of each experiment to verify adequate dynamic range of nerve responses and general preparation stability. On rare occasions, the phrenic response to chemoreflex activation for unknown reasons; to reduce the influence of these factors, including a limited dynamic range of phrenic nerve activity which may obscure pMF, rats with 50% maximal hypercapnic response were eliminated from analysis.

2.4. Intrathecal injections

The PKC activator phorbol 12-myristate 13-acetate (PMA) and PKC inhibitors bisindolymaleimide I (BIS) and NPC-15437 (NPC) were delivered at doses consistent with literature reports (Coderre, 1992, Ferguson et al., 2008, Hua et al., 1999, Laferrière et al., 2011, Yashpal et al., 1995). Stock solutions were diluted in aCSF (vehicle, 12 µl aCSF; in mM 120 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 23 NaHCO₃, 10 glucose, bubbled in 95% O₂, 5% CO₂ for 15 min) to their final concentration with a DMSO concentration of 1% (v/v) for PMA, 20% for BIS and 0% for NPC. All rats received three 7 µl injections (2 µl bolus/30s) through their intrathecal catheters of either PMA (9 µM) or vehicle (1% DMSO in aCSF), delivered in 5-min intervals. For rats pretreated with PKC inhibitors, 12 µl of either BIS (280 µM) or NPC (5 mM) was delivered (2 µl bolus/30s) 10–15 min prior to PMA or vehicle injections. There was no significant effect of either PKC inhibitor on phrenic nerve burst amplitude in vehicle-injected rats ($p = 0.948$).

2.5. Data analysis

Integrated phrenic burst amplitude and burst frequency was averaged in 60 s bins at baseline and 15, 30, 60, and 90 min after PMA or vehicle injections. Average burst amplitude values were expressed as a percent change from baseline; frequency was expressed as an absolute change from baseline value (burst/min). A two-way ANOVA with repeated measures design was used to analyze phrenic burst amplitude and frequency at 15, 30, 60 and 90 min post-injection. *Post-hoc* comparisons were made with Fisher's LSD test (SigmaPlot version 12.0; Systat Software, San Jose, CA) to compare differences between individual groups. In Fig. 4, vehicle-injected controls pretreated with either BIS or NPC were grouped with rats injected only with vehicle (vehicle controls) because no significant differences were found between the three groups. All differences between groups were considered significant if $p < 0.05$. All values are expressed as mean ± SEM.

3. Results

3.1. Spinal injections of PMA cause phrenic motor facilitation

We tested the hypothesis that spinal PKC activation would induce phrenic motor facilitation via intrathecal PMA injections (black arrows, Fig. 1A). PMA significantly increased ($p < 0.001$) phrenic nerve burst amplitude (ie. pMF) at 30 ($27 \pm 7\%$), 60 ($37 \pm 5\%$) and 90 min ($49 \pm 7\%$) post-injection versus vehicle-injected rats (Fig. 1B; $7 \pm 7\%$ at 90 min; $n = 6$). In vehicle treated rats, there were no significant changes in phrenic nerve burst amplitude from baseline at 90 min post-injection ($p = 0.208$); in contrast, phrenic nerve burst amplitude was significantly elevated from baseline at this same time post-PMA ($p < 0.001$). No significant differences in phrenic burst frequency were observed at any time when comparing PMA versus vehicle treated rats (Fig. 1C, $p = 0.129$).

3.2. PMA-induced pMF requires PKC activation

To confirm that PMA injections caused pMF through spinal PKC activation, we tested the hypothesis that spinal PKC inhibition prevents PMA-induced pMF. In rats pretreated with broad-spectrum PKC inhibitor, bisindolylmaleimide I (BIS; Fig. 2A, open arrow), PMA injections (black arrows, Fig. 2A) failed to cause significant pMF ($9 \pm 8\%$ at 90 min, $n = 6$) versus rats vehicle treated rats ($3 \pm 2\%$ at 90 min, $n = 4$; $p = 0.321$, Fig. 2B). In these rats, there were no significant changes from baseline in phrenic nerve amplitude at 90 min in either PMA ($p = 0.118$) or vehicle injected rats pretreated with BIS ($p = 0.644$). No significant changes in phrenic burst frequency were found at any time in PMA or vehicle injected rats pretreated with BIS (Fig. 2C, $p = 0.129$).

Since pharmacological PKC inhibition with BIS inhibits PKC θ -dependent pMF (Devinney et al., 2015), and BIS may have off-target effects, such as inhibition of p90rsk (Roberts et al., 2005), p70S6 K (Alessi, 1997, Roberts et al., 2004), or 5-HT₃ receptors (Coultrap et al., 1999), we utilized another PKC inhibitor with a distinct isoform profile, NPC-15437 (NPC). NPC inhibits PKC by preventing diacylglycerol-mediated activation of the PKC regulatory domain (Sullivan et al., 1991, Sullivan et al., 1992); in contrast, BIS prevents substrate phosphorylation through active-site inhibition (Gould et al., 2011). In rats pretreated with NPC (Fig. 3A, open arrow), PMA injections (black arrows, Fig. 3A) no longer caused significant pMF ($15 \pm 9\%$ at 90 min; $n = 5$) versus vehicle-injected rats ($5 \pm 9\%$ at 90 min; $n = 3$; $p = 0.343$; Fig 3B). However, there might have been some small, residual pMF since PMA-injected rats pretreated with NPC had slightly higher phrenic nerve burst amplitudes versus baseline ($p = 0.017$); vehicle treated rats did not ($p = 0.484$). No significant changes in phrenic burst frequency were found at any point when comparing PMA or vehicle injected rats pretreated with NPC (Fig. 3C, $p = 0.129$).

4. Discussion

Spinal PKC activation via intrathecal PMA elicits pMF (Fig. 4), an effect prevented by pretreatment with two PKC inhibitors that differ in their mechanism of action and isoform specificity: BIS and NPC (Fig. 4; $p < 0.001$). These findings rule out contributions from either PKC θ or the atypical PKCs since NPC does not block PKC θ -dependent pMF

(Devinney et al., 2015), and PMA does not activate atypical PKC isoforms. Although the specific PKC isoforms involved are not certain based on our studies, they must represent conventional or novel PKC isoforms that have not previously been implicated in phrenic motor plasticity.

4.1. Spinal PKC activation is sufficient to cause pMF

Distinct PKC isoforms are required for different forms of spinal, phrenic motor plasticity, such as PKC θ for AIH-induced pLTF (Devinney et al., 2015), and PKC ζ for inactivity-induced pMF (Strey et al., 2012). Although these studies demonstrate that specific PKC isoforms are necessary for these forms of plasticity, they do not demonstrate that PKC activation is sufficient to elicit phrenic motor plasticity. Here we demonstrate that spinal PKC activation with a phorbol ester elicits PKC-dependent pMF that is phenotypically similar to AIH- and inactivity-induced pMF.

4.2. PMA elicits pMF by activating classical/novel PKC isoforms

Phorbol esters, including PMA, are C1 domain-ligands that activate PKC and other proteins containing a functional C1 domain, such as chimaerins (Rac GTPase activating proteins), RasGRP1 (guanine nucleotide exchange factor enzyme), and Munc13 (scaffolding proteins necessary for exocytosis; Kazanietz, 2002, Marland et al., 2011). Here, we ruled out these other PMA activated molecules by using two PKC inhibitors at doses consistent with inhibition of select PKC isoforms from all three classes (classical, novel and atypical) (Felber et al., 2007, Gschwendt et al., 1996, Martiny-Baron et al., 1993, Saraiva et al., 2003, Uberall et al., 1997). Atypical PKC isoforms, including PKC ζ , contain C1 domains which lack affinity for DAG or phorbol esters, and are therefore unresponsive to PMA (Colón-González and Kazanietz, 2006, Kazanietz et al., 1994, Pu et al., 2006). Thus, our results suggest that spinal PMA induces pMF via stimulation of classical and/or novel PKC isoforms.

4.3. Different isoforms of PKC are involved in different forms of spinal plasticity

Different forms of spinal respiratory motor plasticity require different PKC isoforms. Whereas AIH-induced pLTF requires spinal PKC θ activity, but not atypical PKCs (Devinney et al., 2015, Strey et al., 2012), inactivity-induced pMF requires the atypical PKC, PKC ζ (Strey et al., 2012), but not PKC θ (Strey et al., 2012). PMA activates multiple classical and novel PKC isoforms, it does not activate atypical PKCs. Thus the PMA-induced pMF reported here resulted from a classical/novel isoform. On the other hand, we can rule out a mandatory role for PKC θ in PMA-induced pMF since NPC blocked PMA-induced pMF (Fig. 3), yet has no effect on AIH-induced pLTF (Devinney et al., 2015). Minimal PKC θ inhibition by NPC is supported by structural studies demonstrating that the C1 domains of PKC θ differ from all other PKC isoforms, and C1 B (not C1A) is the dominant DAG binding domain (Melowic et al., 2007, Steinberg, 2008). Moreover, a recent study found that the C1 B phorbol ester-binding surface in PKC θ C1 B crystal structures is narrowed versus that of a similar PKC isoform, PKC δ , (Rahman et al., 2013). Thus, NPC may be unable to bind to the DAG/phorbol ester binding sites on the C1 B domain of PKC θ , explaining the lack of effect on AIH-induced pLTF. On the other hand, since NPC blocked PMA-induced pMF, other spinal classical/novel PKC isoforms must be involved. Minimal

residual facilitation (15%) in NPC-treated rats (versus baseline only) could be due to limited PMA-induced PKC θ activation, or may be a statistical anomaly since it was not different from the vehicle time controls. Regardless, PMA-induced pMF must represent a form of pMF that is PKC θ independent, acting via a different classical/novel PKC isoform(s).

4.4. Spinal PKC activation has no effect on respiratory frequency

Protocols associated with phrenic motor plasticity, such as AIH (Baker-Herman and Mitchell, 2008), spinal serotonin injections (MacFarlane and Mitchell, 2009), or spinal BDNF (Baker-Herman et al., 2004), VEGF (Dale-Nagle et al., 2011) or erythropoietin injections (Dale et al., 2012) induce small but significant increases in phrenic burst frequency, known as frequency long-term facilitation (Baker-Herman and Mitchell, 2008, Dale-Nagle et al., 2011, MacFarlane and Mitchell, 2009). While changes in respiratory frequency are often thought to occur from effects on the medullary respiratory rhythm generator (eg. the pre-Bötzinger complex), spinal mechanisms could indirectly influence rhythmogenesis via plasticity in ascending sensory systems. In the present study, no significant frequency effects were observed, suggesting that spinal PMA induced PKC activation has minimal impact on spinal afferent pathways and frequency long-term facilitation.

5. Conclusions

These studies reveal unique kinases (conventional or novel PKCs) sufficient to elicit spinal, respiratory motor plasticity. Greater understanding of cellular mechanisms giving rise to such plasticity advances our understanding of respiratory motor plasticity, and plasticity in other, non-respiratory motor systems. We are currently working to harness respiratory and non-respiratory motor plasticity to treat motor deficits associated with clinical disorders that compromise movement, including spinal cord injury (Gonzalez-Rothi et al., 2015), motor neuron disease (Nichols et al., 2013) or obstructive sleep apnea (Mahamed and Mitchell, 2007). Greater understanding of mechanisms giving rise to such plasticity may help advance our efforts to treat these devastating clinical disorders.

Acknowledgements

Support provided by NIH Grants HL080209 and HL111598 (GSM).

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Fig. 1.

Spinal PKC activation with intrathecal injections of PMA causes phrenic motor facilitation. A. Representative phrenic neurograms of rats injected (3 injections separated by 5 mins, ↑) with PMA (9 μ M) or vehicle (1% DMSO in ACSF). Dotted line delineates baseline; increases from baseline (pMF) are shown in gray. B. Summary data for change in phrenic amplitude (%baseline) following PMA (n = 6, ∇ -) or vehicle (n = 6, $\bullet$ -) injections. C. Summary data for change in phrenic burst frequency (bursts/min) following PMA (∇ -) or vehicle ($\bullet$ -) injections. (*) denotes significantly increased compared to vehicle injected rats ($p < 0.001$). (#) denotes significantly increased compared to baseline ($p < 0.001$).

Fig. 2.

Spinal PKC inhibition with BIS prevents PMA-induced phrenic motor facilitation. A. Representative phrenic neurograms from rats pretreated with BIS (12 μ l, 280 μ M) and injected (3 injections separated by 5 mins, $\uparrow$) with PMA (9 μ M) or vehicle (1% DMSO in ACSF). Dotted line delineates baseline; Increases from baseline (pMF) are shown in gray. B. Summary data for change in phrenic amplitude (%baseline) following PMA (n = 6, ∇ -) or vehicle (n = 4, $\bullet$ -) injections. C. Summary data for change in phrenic burst frequency (bursts/min) following PMA (∇ -) or vehicle ($\bullet$ -) injections. (#) denotes significantly increased compared to baseline (p < 0.05). No significant differences were noted in phrenic amplitude compared in rats pretreated with BIS and injected with PMA compared to vehicle-injected rats. No significant differences were noted in phrenic burst frequency at any time point.

Fig. 3. Spinal PKC inhibition with NPC prevents PMA-induced phrenic motor facilitation. **A.** Representative phrenic neurograms from rats pretreated with NPC (12 μ l, 5 mM) and injected (3 injections separated by 5 mins, $\uparrow$) with PMA (9 μ M) or vehicle (1% DMSO in ACSF). Dotted line delineates baseline; Increases from baseline (pMF) are shown in gray. **B.** Summary data for change in phrenic amplitude (%baseline) following PMA (n = 6, - ∇ -) or vehicle (n = 4, - $\bullet$ -) injections. **C.** Summary data for change in phrenic burst frequency (bursts/min) following PMA (- ∇ -) or vehicle (- $\bullet$ -) injections. (#) denotes significantly increased compared to baseline (p < 0.05). No significant differences were noted in phrenic frequency at any time point. No significant differences were noted in phrenic amplitude compared in rats pretreated with NPC and injected with PMA compared to vehicle-injected rats. No significant differences were noted in phrenic burst frequency at any time point.

Fig. 4.

Spinal PMA injections cause PKC-dependent phrenic motor facilitation. Summary data from rats given intrathecal injections of PMA or vehicle and PKC antagonists. Rats given intrathecal injections of PMA alone exhibit significant phrenic motor facilitation ($49 \pm 7\%$) compared to rats pretreated with BIS and given PMA injections ($9 \pm 8\%$, * $p < 0.001$) or rats pretreated with NPC and given PMA injections ($15 \pm 10\%$, # $p < 0.001$) or rats given vehicle injections instead of PMA ($5 \pm 5\%$, & $p < 0.001$).