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Spinal Activation of the cAMP-PKA Pathway Induces Respiratory Motor Recovery Following High Cervical Spinal Cord Injury

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Abstract

The present study investigated the involvement of the adenosine 3'5'-cyclic monophosphate-dependent protein kinase A (cAMP-PKA) pathway in the activation of the crossed phrenic pathways after left C2 spinal cord hemisection. Experiments were conducted on left C2 spinal cord hemisected, anesthetized, vagotomized, pancuronium paralyzed, and artificially ventilated male Sprague-Dawley rats. One week post-injury, the ipsilateral phrenic nerve exhibited no respiratory-related activity indicating a functionally complete hemisection. Intrathecal spinal cord administration of the cAMP analog, 8-Br-cAMP at the level of the phrenic nucleus resulted in an enhancement of contralateral phrenic nerve output and a restoration of respiratory-related activity in the phrenic nerve ipsilateral to the hemisection. Furthermore, pretreatment with Rp-8-Br-cAMP, a PKA inhibitor, abolished the effects of 8-Br-cAMP. These results suggest that PKA activation is necessary for the cAMP-mediated respiratory recovery following high cervical spinal cord injury and that activation of intracellular signaling cascades may represent an important strategy for improving respiratory function after spinal cord injury.

Keywords

Cervical spinal cord injury; Respiration; Crossed phrenic pathway; cAMP; PKA

Introduction

Traumatic spinal cord injury most often occurs in young adults and results in significant physical impairment and dysfunction. In particular, respiratory complications are common in spinal cord injured patients and can have catastrophic impact on the quality and the length of their lives. High cervical spinal cord hemisection injury at the second cervical level eliminates bulbospinal projections to the ipsilateral phrenic motoneurons and paralyzes the ipsilateral hemidiaphragm (Aserinsky, 1961; Deason, 1911; Goshgarian, 1981; Guth, 1976; Lewis and Brookhart, 1951; Rosenbaum and Renshaw, 1949; Rosenblueth A, 1936). There are latent axonal pathways known as the crossed phrenic pathways that originate from the contralateral bulbospinal projections and cross the spinal cord midline below the site of injury (Moreno et al., 1992). Although these latent crossed phrenic pathways are ineffective in inducing ipsilateral phrenic motor output following a spinal hemisection, they can be stimulated via a variety of

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pharmacological treatments to restore ipsilateral phrenic output (Alilain and Goshgarian, 2007; Nantwi et al., 1996; Nantwi and Goshgarian, 2002; Zimmer and Goshgarian, 2006). For example, after a left C2 spinal cord hemisection, both theophylline and MK-801 administered chronically have been shown to restore respiratory function (Nantwi and Goshgarian, 1998; Alilain and Goshgarian, 2007). Theophylline-mediated recovery is thought to be induced by the antagonism of adenosine receptors as well as the inhibition of phosphodiesterase enzymes, while it is suggested that MK-801 promotes respiratory recovery after spinal cord injury via the upregulation of specific NMDA receptor subunits (Alilain and Goshgarian, 2007; Kajana, and Goshgarian, 2008; Nantwi and Goshgarian, 2005).

Numerous studies have shown that theophylline (Hussein et al., 2005; Liu et al., 2003) and NMDA receptors (Banko et al., 2004; Waltereit and Weller, 2003) can modulate the adenosine 3'5'-cyclic monophosphate-dependent protein kinase A (cAMP-PKA) pathway. The cAMP-PKA signaling cascade has been shown to augment the excitability of many types of motoneurons (Arata et al., 1993; Inan et al., 2006; Lee et al., 2000; Tronson et al., 2006). The effect of the cAMP-PKA signaling pathway on the excitability of phrenic motoneurons, however, is still not known. Neurotransmitters and neuromodulators that have the potential to increase cAMP levels, including NMDA receptors, dopamine and adenosine have implicated the cAMP-PKA system in respiratory plasticity (Fujii et al., 2004; Waltereit and Weller, 2003).

In our C2 hemisection model of spinal cord injury, systemically blocking adenosine A1 receptors or inhibiting phosphodiesterases, both of which generate increases in the basal levels of cAMP, have been shown to enhance respiratory output and induce recovery (Kajana and Goshgarian, 2008; Nantwi et al., 1996; Nantwi and Goshgarian, 2002). We hypothesize that stimulation of cAMP-PKA signaling cascade will augment phrenic nerve output and moreover, the activation of cAMP-PKA signaling will bring about phrenic nerve recovery following left C2 hemisection. In order to test this hypothesis, we examined the effect of direct spinal application of a cAMP analog and a PKA inhibitor (Rp-8-Br-cAMP) on phrenic nerve output in uninjured and left C2 hemisected rats.

Methods

60 adult male Sprague-Dawley rats (Harlan, weight: 325–450g, age: 3–4 months) were divided equally into 6 groups. Three of the six groups were subjected to a left C2 spinal cord hemisection one week prior to the electrophysiological experiments. The remaining three groups served as uninjured controls. Animals subjected to left C2 spinal cord hemisection were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (20 mg/kg). Following a C2 laminectomy and durotomy the left side of the spinal cord was hemisected caudal to the dorsal roots of the second cervical segment using microscissors. A fine tip surgical probe was used to scrape the floor and walls of the vertebral canal adjacent to the lesion to ensure the hemisection extended from the midline to the most lateral extent of the spinal cord. After hemisection, the overlying muscles were sutured (3-0 vicryl) and the skin closed with wound clips. Following surgery, all animals were allowed to recover from anesthesia on a heated water blanket and were given an analgesic (buprenorphine, 0.01 mg/kg, subcutaneously). The rats also received a subcutaneous injection of saline (10 ml) to prevent dehydration before placing them in individual cages to recover for one week. All animal care and experimental procedures were approved by the Wayne State University Animal Investigation Committee.

Electrophysiology

One week after hemisection, both hemisected and uninjured control rats were injected intramuscularly with atropine sulphate (0.01 mg/kg) approximately 10–15 minutes prior to

being anesthetized with urethane (1.6 g/kg, i.p.). Catheters (PE-50) were inserted into the femoral artery and vein of the right hindlimb, in order to monitor blood pressure and administer fluids, respectively. Body temperature was monitored using a rectal probe throughout the experiment and maintained at 37 ± 1 °C using a heating pad. Following a tracheotomy, a cannula (PE-240) was inserted into the trachea and rats were mechanically ventilated with a rodent ventilator (Harvard Apparatus, model 683). The ventilator was supplied with an equal mixture of air and oxygen. The right and left vagus nerves were transected to prevent mechanoreceptor feedback and intravenous injection of pancuronium bromide (0.5 mg/kg) was administered to paralyze the rats. End-tidal CO₂ was continuously monitored throughout the experimental protocol using a CO₂ monitor (Capnogard, Novamatrix, Model 1265, Wallingford, CT).

For intrathecal injections, laminectomy was performed to remove the cervical vertebrae between C3–C5. The animal was placed on a stereotaxic frame in the prone position and the left and right phrenic nerves were approached dorsally in the neck, isolated, transected and placed on a bipolar silver recording electrodes. Phrenic nerve activity was amplified (10K), band pass filtered (0.1–3 kHz) using a Tektronix 502 AM amplifier (Beaverton, OR). The neural activity was then digitized, recorded and analyzed using the Cambridge Electronic Design data acquisition system and a Spike 2 computer program (v 4.13, CED Cambridge, UK).

The phrenic nerve activity was allowed to stabilize for approximately 30 minutes before determining the CO₂-apneic threshold. The CO₂-apneic threshold was obtained by slowly increasing the rate and volume of the ventilator until inspiratory bursting ceased to occur and then the ventilator rate and volume were slowly decreased until the reappearance of inspiratory bursting. The end-tidal CO₂ value at which phrenic inspiratory bursting resumed was considered to be the CO₂-apneic threshold. Baseline phrenic nerve activity was then stabilized for approximately 20 minutes at 3–4 mmHg above the CO₂-apneic threshold before commencing the experimental protocol.

After recording bilateral phrenic nerve activity for about 20 minutes, a Hamilton syringe was used to slowly inject 10 µl of drug or vehicle solution intrathecally at the C4 level. Care was taken to administer the drug into the subarachnoid space. The drug was prevented from diffusing upward to the medulla through the CSF by placing the rat on a stereotaxic frame with the head of the rat tilted upward throughout the experimental protocol. Thus, gravity and the position of the rat helped to insure that the effects of the drug were mediated only at the spinal cord level.

Drugs

To assess the effects cAMP on phrenic nerve output, a group of uninjured rats were injected intrathecally with a 10 mM solution of the cAMP analog, 8-Br-cAMP (Sigma, n=10), in 10 µl. Phrenic nerve activity was monitored before drug administration and one hour post drug administration. A second group of uninjured rats served as saline treated controls and received intrathecal injections of saline (10 µL, n=10). In order to determine if PKA activation is involved in cAMP-mediated respiratory effects, a third group of non-hemisected rats were pretreated with an intrathecal injection of a PKA inhibitor, Rp-8-Br-cAMP (Sigma, 20 µg in 10 µL, n=10), thirty minutes prior to the 8-Br-cAMP administration.

To characterize the influence of the cAMP-PKA pathway on its ability to activate the crossed-phrenic pathway, three groups of left C2-hemisected rats were subjected to the same drug protocol as in the uninjured rats. Briefly, a group of left C2 hemisected rats (n=10) received a 10 µL intrathecal injection of 10 mM 8-Br-cAMP while another group (n=10) received the same volume of saline. A last group of left C2 hemisected rats were injected with 20 µg of Rp-

Br-cAMP in 10 μ L and this was followed thirty minutes later by an administration of 10mM 8-Br-cAMP (10 μ L). As in the first three groups, phrenic nerve activity was monitored before drug administration and one hour following the last drug administration in the three groups of hemisectioned rats.

Data analysis

Spike 2 data analysis software (v 4.13, CED Cambridge, UK) was used to rectify, integrate and quantify the left and right phrenic nerve activity. A 20 minute period of integrated phrenic nerve activity was assessed before drug application during baseline recording and again over the first, middle and last 20 minute periods after the drug application. In the two groups of rats that were injected with Rp-Br-cAMP, the 30 minutes prior to the administration of 8-Br-cAMP served as the control for the effects of the PKA inhibitor alone. In the above group of rats, phrenic nerve activity was analyzed over the last 20 minute period before the administration of 8-Br-cAMP.

Phrenic neurograms were assessed for respiratory frequency, burst area and burst amplitude. In uninjured control rats burst area and peak burst amplitude were expressed as a percentage of pre-drug baseline values. In the left C2 hemisectioned rats, the magnitude of respiratory recovery was quantified by expressing the recovered left phrenic nerve activity as a percentage of the pre-drug right phrenic nerve activity.

One way ANOVAs were used to determine the effect of drug on right and left phrenic nerve activity. In hemisectioned rats, statistical analysis of saline treated controls and drug treated groups were performed using the nonparametric Wilcoxon test for assessment of phrenic motor recovery. All data are expressed as mean $\pm$ SE with significance set at $p < 0.05$. All statistics were performed using the commercial statistics software (Systat software, Version 10.2).

Results

Integrated phrenic inspiratory burst area and peak amplitude of uninjured control rats were significantly increased following the administration of the cAMP analog, 8-Br-cAMP in 8 of the 10 animals examined (Fig. 1). Inspiratory burst area and peak burst amplitude in the first, middle and last 20 minute segments of the hour after drug application are presented as a percentage of the 20 minute period of baseline value taken immediately before drug application (Fig. 2). Assessment of phrenic nerve activity in the first 20 minutes after 8-Br-cAMP administration failed to reveal any significant increase in phrenic burst amplitude or burst area ($p > 0.05$). However, there was a significant increase in both phrenic burst amplitude and burst area in the middle and last 20 minute segments ($p < 0.05$). Respiratory burst frequency following the application of 8-Br-cAMP was not significantly altered ($p > 0.05$). In order to demonstrate a role for PKA in the cAMP-induced enhancement in respiratory-related phrenic nerve output, Rp-8-Br-cAMP, a PKA inhibitor was applied to the cervical spinal cord 30 minutes prior to the application of 8-Br-cAMP. Uninjured rats that were pretreated with Rp-8-Br-cAMP alone did not show a significant change in inspiratory burst area ($p > 0.05$), peak burst amplitude ($p > 0.05$) or burst frequency ($p > 0.05$) (Fig 2 and 4). Furthermore, 7 of the 10 rats that were pretreated with Rp-8-Br-cAMP failed to show significant increases in either phrenic burst area ($p > 0.05$), peak burst amplitude ($p > 0.05$) or burst frequency ($p > 0.05$) in response to the subsequent intrathecal application of 8-Br-cAMP at all three of the time segments tested.

Left C2 spinal cord hemisection resulted in the paralysis of the left hemidiaphragm, thus the left phrenic nerve was devoid of any respiratory-related motor output one week post injury in all hemisectioned rats. In 7 of 10 left C2 hemisectioned rats, 8-Br-cAMP administration significantly increased the contralateral phrenic nerve output above pre-drug baseline values as determined

by peak burst amplitude ($p < 0.05$) and burst area ($p < 0.05$) measurements in the middle and last 20 minute periods of the hour after the drug application. In addition, respiratory-related output was restored in the previously quiescent ipsilateral phrenic nerve of these 7 rats. Ipsilateral phrenic nerve burst amplitude and burst area were significantly increased in all three time segments following 8-Br-cAMP application. Representative neurogram tracings showing the qualitative effects of the drug treatments on the ipsilateral and contralateral phrenic nerves are illustrated in figure 3. In addition, the effect of intrathecal application of 8-Br-cAMP on the inspiratory burst area and peak burst amplitude of the ipsilateral and contralateral phrenic nerves were expressed as a percentage of the pre-drug contralateral phrenic nerve value and presented in Figure 4.

To determine if PKA activation was necessary for increased phrenic nerve output and the restoration of ipsilateral phrenic nerve activity following 8-Br-cAMP administration, 8-Br-cAMP administration was repeated in a separate group of rats pre-treated with Rp-8-Br-cAMP. Neither the ipsilateral ($p > 0.05$) nor the contralateral ($p > 0.05$) phrenic nerve outputs were significantly changed by Rp-8-Br-cAMP alone (Fig. 3 and Fig. 4). Furthermore, 8 of the 10 left C2 hemisected rats that were pretreated with Rp-8-Br-cAMP, before the application of 8-Br-cAMP failed to exhibit ipsilateral phrenic nerve recovery or significant enhancement of contralateral phrenic nerve output (Fig. 3) ($p > 0.05$). Rats that had undergone a left C2 hemisection surgery had a higher breathing frequency during baseline and after the drug treatments (Fig. 5). However, the difference in breathing frequency in uninjured and hemisected rats did not reach significance ($p > 0.05$). Furthermore, the cAMP analog, 8-Br-cAMP, the PKA inhibitor, Rp-8-Br-cAMP, or the combination of the inhibitor and the analog applied to the cervical spinal cord did not significantly affect the respiratory frequency in either uninjured control rats or left C2 hemisected rats (Fig. 5).

Discussion

The results of the present study demonstrate that intrathecal application of 8-Br-cAMP, a cAMP analog, to the cervical spinal cord causes a significant increase in phrenic nerve output in uninjured control rats and induces ipsilateral phrenic nerve recovery in left C2 spinal cord hemisected rats. Furthermore, the above effects were blocked when Rp-8-Br-cAMP, a PKA inhibitor, was applied to the cervical spinal cord 30 minutes prior to the application of 8-Br-cAMP. In the present study, we did not observe any significant changes in respiratory frequency in response to the above pharmacological manipulations, suggesting that the drugs were acting at the level of the cervical spinal cord rather than the medulla. Thus, these results strongly suggest that the cAMP-induced respiratory recovery following high cervical spinal cord hemisection is mediated via the activation of the PKA signaling pathway at the level of the cervical spinal cord.

Previously in our lab we have demonstrated that ipsilateral phrenic nerve recovery can be induced by elevating cAMP levels via phosphodiesterase inhibition, or via the antagonism of adenosine receptors (Kajana and Goshgarian, 2008; Nantwi and Goshgarian, 2002). The above studies indirectly implicated the cAMP signaling pathway in the bilateral enhancement of phrenic nerve activity following spinal cord injury. In addition, in another recent study from our lab, a one time systemic administration of rolipram, a phosphodiesterase-IV specific inhibitor, after a left C2 spinal cord hemisection was able to significantly increase spinal (ventral C3–C6) and medullary cAMP levels. Moreover, these increases in cAMP levels were correlated with enhanced ipsilateral and contralateral phrenic nerve output.

Numerous studies conducted over the years have implicated cAMP in both short-and long-term synaptic plasticity. The cAMP signaling pathway can potentially become activated by two mechanisms. Firstly, stimulation of the calcium and calmodulin (Ca/CaM)-sensitive

adenylyl cyclase can lead to the synthesis of cAMP (Eliot et al., 1989). Ca/CaM-sensitive adenylyl cyclases can be activated by the influx of calcium via NMDA receptors. Secondly, neurotransmitters bound to their receptors can stimulate adenylyl cyclase to increase the production of cAMP (Beebe, 1994; Skälhegg, 2000). Once activated, cAMP can increase the strength of synaptic connections via the following three main molecular pathways: the GTP-exchange protein EPAC, the cyclic nucleotide-gated ion channels and the PKA pathway (Schwartz, 2001). Of the above mentioned targets of cAMP, the PKA-dependent pathway plays a key role in the regulation of many forms of synaptic plasticity (Huang and Kandel, 1998; Munno et al., 2003; Nguyen and Woo, 2003). Furthermore, PKA-dependent modulation of motoneuronal excitability has been clearly demonstrated in the respiratory system (Bocchiaro et al., 2003; Lalley et al., 1997; Munno et al., 2003). Since the above mentioned studies demonstrate a pivotal role for PKA in both hippocampal and respiratory plasticity, in the current study, we examined the role of PKA in the cAMP-induced phrenic nerve recovery following spinal cord injury.

The cAMP-dependent PKA is a serine-threonine kinase that is involved in the regulation of many cellular processes including cellular metabolism, development, gene expression and neuronal excitability (Nguyen and Woo, 2003). The role of the PKA pathway in the regulation of hippocampal synaptic plasticity has been well studied (Greengard et al., 1991; Nguyen and Woo, 2003; Skeberdis et al., 2006; Yamamoto et al., 2005). The PKA pathway can modulate both short- and long- term synaptic plasticity (Huang and Kandel, 1998; Lee et al., 2000; Yamamoto et al., 2005). PKA may regulate short-term effects of synaptic plasticity by modulating ion channels. The cAMP-PKA system can increase excitatory glutamate neurotransmission by increasing cationic currents through the NMDA and AMPA receptors (Blackstone et al., 1994; Greengard et al., 1991; Shao et al., 2003). PKA has also been shown to phosphorylate AMPA receptor subunits and increase the open probability of AMPA receptors (Banke et al., 2000). In addition to modulating short-term synaptic strength, PKA activation can also mediate long lasting changes in synaptic transmission by regulating new protein synthesis. New protein synthesis can potentiate synaptic transmission by remodeling synaptic morphology. PKA signaling has been shown to increase dendritic spine density (Murphy and Segal, 1997) and the number of functional presynaptic release sites (Tominaga-Yoshino et al., 2002; Yamamoto et al., 2005). Thus, PKA regulated changes in synaptic morphology can transform less active synapses into a more functionally effective state.

Studies have also examined the influence of cAMP-dependent PKA signaling on respiratory neurons. The activation of the PKA pathway in the pre-Botzinger complex, group of neurons in the brainstem that generate respiratory rhythm, has been shown to increase respiratory frequency (Shao et al., 2003). The fact that we did not see any change in respiratory frequency following drug treatment in the present study suggests that the effects of the drug were not being mediated at the medullary level. Studies have also shown a critical role for cAMP-PKA signaling cascade in the modulation of medullary expiratory neurons (Lalley et al., 1997; Saywell and Feldman, 2004). In addition, PKA has been shown to potentiate inspiratory drive currents (Bocchiaro et al., 2003). Glutamate is the major excitatory neurotransmitter in the respiratory system (McCrimmon et al., 1989). Interestingly, PKA mediated enhancement of respiratory function has also been linked to the phosphorylation of AMPA receptors (Shao et al., 2003). Therefore, it is likely that PKA activation is involved in the modulation of expiratory and inspiratory currents that facilitate respiratory output.

As mentioned earlier, one of the main intracellular effectors of cAMP is PKA, but cAMP can mediate cellular events directly independent of PKA activation. In this study, using Rp-8-Br-cAMP we have directly implicated the cAMP-PKA signaling cascade in the synaptic plasticity leading to the enhancement of phrenic nerve output. In the current study, we have only examined the short-term changes induced by cAMP elevation. As such, we cannot rule out the

possibility that the other two cAMP-dependent mechanisms may be involved in long-term modifications in synaptic strength resulting in permanent enhancement of phrenic nerve output. Furthermore, at present the downstream cellular mechanisms underlying the effects of cAMP-PKA mediated enhancement of phrenic nerve output are not known.

Respiratory insufficiency and other respiratory related complications are a common occurrence following high cervical spinal cord injury (Winslow and Rozovsky, 2003). Furthermore, approximately 50% of the spinal cord injuries occur at the level of the cervical spinal cord. As such, understanding pharmacological interventions that promote synaptic strengthening and or regeneration leading to respiratory recovery is vital. Pharmacological results of the present study indicate a key role for the cAMP-PKA system in the enhancement of phrenic nerve output, however additional studies will be required to explore the downstream cellular events responsible for these effects. Although direct application of 8-Br-cAMP to the spinal cord may not be a viable therapeutic strategy, determining the underlying mechanisms of cAMP-PKA mediated synaptic strengthening may be useful in identifying potential drugs for the strengthening of existing but ineffective pathways following spinal cord injury.

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Figure 1.

A. Representative tracings of rectified and integrated phrenic neurograms taken before and after the intrathecal application of a cAMP analog, 8-Br-cAMP, to the cervical spinal cord of an uninjured control rat. B. Representative tracings from an uninjured control rat before drug treatment, after treatment with a PKA inhibitor, Rp-8-Br-cAMP, and after 8-Br-cAMP treatment 30 minutes after Rp-8-Br-cAMP administration. 8-Br-cAMP administration increased phrenic nerve output (A) and pretreatment with Rp-8-Br-cAMP prevented this increase (B).

Figure 2.

Quantitative data illustrating the changes in phrenic burst area and peak amplitude after the intrathecal application of 8-Br-cAMP alone to the cervical spinal cord of uninjured control rats (A and B) as well as changes in burst area and peak amplitude following pretreatment with Rp-8-Br-cAMP 30 minutes before 8-Br-cAMP (C and D). The changes in phrenic burst area and peak amplitude during the first (0–20 min), middle (20–40 min) and last (40–60 min) segments of the hour after the drug applications are expressed as a percentage of pre-drug baseline values in each graph. There was a significant increase in burst area (A) and peak amplitude (B) at the later two time intervals following treatment with 8-Br-cAMP alone, but the increase in burst area (C) and peak amplitude (D) was blocked by pretreatment with Rp-8-Br-cAMP. Rp-8-Br-cAMP had no effect on baseline values. Values are expressed as mean $\pm$ S.E. *, $p < 0.05$. N=10

Figure 3.

A. Representative tracings of right (R) and left (L) phrenic nerve recordings taken before and after the intrathecal application of 8-Br-cAMP to the cervical spinal cord of a left C2 spinal cord hemisected rat. B. Neurograms of right and left phrenic nerve output before drug treatment, after Rp-8-Br-cAMP treatment alone and 8-Br-cAMP treatment 30 minutes after Rp-8-Br-cAMP administration. 8-Br-cAMP administration increased phrenic nerve output bilaterally (A) and pretreatment with Rp-8-Br-cAMP prevented this increase (B).

Figure 4.

Graph illustrating the effect of 8-Br-cAMP on burst area and peak amplitude of the contralateral (right) and ipsilateral (left) phrenic nerves in left C2 hemisected rats. The effect of 8-Br-cAMP on right and left phrenic nerve activity during the first (0–20 min), middle (20–40 min) and last (40–60 min) segments of the hour after the drug application is expressed as a percent of pre-drug right phrenic nerve activity. 8-Br-cAMP significantly increased burst area and peak amplitude bilaterally in left C2 hemisected rats. Data is expressed as mean $\pm$ S.E. *, $p < 0.05$

Figure 5.

Summary of the effects of 8-Br-cAMP, Rp-8-Br-cAMP and Rp-8-Br-cAMPBr-cAMP on respiratory frequency of uninjured control rats and left C2 hemisected rats during baseline and first (0–20 min), middle (20–40 min) and last (40–60 min) segments of the hour after the drug application. Respiratory frequency was not significantly different from baseline in both uninjured control and left C2 hemisected rats ($P > 0.05$).