Cloud Publications
International Journal of Advanced Physiology and Allied Sciences
2015, Volume 2, Issue 1, pp. 69-77, Article ID Med-235

Research Article | Open Access

The Effect of Hyperosmotic Stress and Protein Kinase A Manipulation on Ciliary Beat Frequency at 20°C

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Publication Date: 8 August 2015

Article Link: http://medical.cloud-journals.com/index.php/IJAPAS/article/view/Med-235

Abstract Mannitol is sometimes administered as an aerosol to increase mucociliary clearance. The aim of the study was to investigate the role of mannitol on regulation of ciliary beat frequency, a component of mucociliary clearance; and to investigate the interplay between mannitol induced hyperosmotic stress and protein kinase A. Ciliated nasal epithelial cells were collected from ten healthy volunteers undergoing surgery. Using video microscopy, ciliary beat frequency was measured at 20°C in the presence of protein kinase A modulators with or without 400mM mannitol in medium 199. Protein kinase A activation with db-cAMP (1mM) in isosmotic medium significantly increased ciliary beat frequency ($p<0.05$) but kinase inhibition was without effect and similar to control. A reversible 75% reduction in ciliary beat frequency occurred when cells were exposed to a hyperosmotic solution of 400mM mannitol in normal culture medium. The ciliary beat frequency under hyperosmotic conditions was slightly lowered further when PKA was activated whereas PKA inhibition had a slight stabilizing effect; almost the opposite of what was observed under isosmotic conditions. In conclusion protein kinase activation in isosmotic medium increases ciliary beat frequency at 20°C and its inhibition has no effect. Mannitol induced hyperosmotic stress reduces ciliary beat frequency and this reduction is affected by PKA manipulation in a complex manner.
Keywords *Ciliary Beat Frequency; Hyperosmotic Stress; Mannitol*

1. Introduction

Mucociliary clearance is the movement of the mucous covering of the respiratory epithelium by the beating of cilia: rapid, forward (effective) stroke and slow, return (recovery) stroke. The underlying ciliary beat frequency (CBF) of the airway epithelial cells drives mucociliary clearance (MCC) and small changes in CBF can have large effects on MCC. Mucus expulsion from the lung results from a complex interaction between the tips of respiratory cilia, the periciliary fluid and the visco-elastic properties of the mucus (Ross and Corrsin, 1974; Duchateau et al., 1985; Sleigh et al., 1988; Inglis et al., 1997; Salathe et al., 1997). A number of regulatory factors including protein phosphorylation and physical factors such as temperature and osmolyte composition in the fluid bathing the cilia modulate

CBF (Luk and Dulfano, 1983; Di Benedetto et al., 1991; Mwimbi et al., 2003). Although β_2 agonists such as terbutaline are commonly used bronchial dilators and their effects on CBF are predicted to be stimulatory, when such drugs are administered to the osmotically challenged airway, as found in mucus-laden airways in asthma for example, the interaction between hyperosmotic stress and cAMP-dependent protein kinase or protein kinase A (PKA) manipulation remains unclear. PKA is an enzyme which is activated by second messenger cAMP; and when it is activated, it phosphorylates other proteins to bring about desired physiological responses. Understanding this interaction is important for conditions such as chronic obstructive airway disease (COAD) where mannitol aerosols in combination with a β agonist may often be co-administered. The terbutaline effect is mediated through a β_2-adrenoceptor which leads to the activation of the cAMP-PKA signal transduction pathway (Sakuma et al., 2000). The cAMP signal transduction system is one of several second messenger-dependent pathways that generate intracellular responses to extracellular signals. The primary element in this cascade is PKA, which mediates most cAMP actions by phosphorylation of other proteins (Beebe, 1994). The end result is that PKA activation can either augment or inhibit a physiological response in cells depending on regulatory factors at play (Freitas et al., 2010). An appropriate response and adaptation to hyperosmolarity, i.e., an external osmolarity that is higher than the physiological range, is vital for survival of cells. Hyperosmotic stress results from increased osmolarity of the extracellular environment resulting in movement of water from cells outward. Cells have been shown to initiate complex measures to adapt and counter the effects of stress and the response is thought to be through the activation of stress kinases which phosphorylate other proteins in the process (Saito and Posas, 2012)

Here, we model this interaction *in vitro* using human nasal airway epithelium. To determine whether hyperosmotic stress affected CBF responses to PKA manipulation, experiments were carried out using ciliated human nasal epithelial cells treated with PKA modulators in iso- and hyper-osmolar mannitol solution at 20^{o}C.

2. Materials and Methods

CBF Measurements The measurements were carried out as described before (Smith et al., 1996). Briefly, human nasal epithelial cells were collected in medium 199 (M199) and placed in a chamber and this chamber was placed on an inverted microscope connected to a video monitor. The changes in interphase as a result of ciliary motion were digitized and displayed on the screen as numerical values of the rate at which cilia were beating. The time zero reading was the baseline and other readings which followed were recorded as percentages of the baseline.

Experimental Design All measurements were carried out at 20^{o}C. In all series CBF measurements were initially done in M199 at 5 min intervals for 20 min followed by a test solution perfusion for 20 min at a rate of 0.125 ml per min. After perfusion, measurements were continued at the same interval for an hour. A second perfusion with urea test solution at 0.125 ml per min for 20 min was carried out at 100 min followed by CBF measurements for 20 min.

The test solutions perfused at 20 min were M199 the normal tissue medium, 400mM mannitol, PKA activator 1mM dibutyryl cyclic AMP (dbcAMP), 100nM cell permeant PKA inhibitor peptide (MyrPKAI), 1mM dbcAMP + 400mM mannitol, 100nM MyrPKAI + 400mM mannitol. The PKA activator acts like cAMP in activating protein kinase A (Kultgen et al., 2002). *Mannitol* is a carbohydrate with does not easily cross cell membranes and as a result it has capacity to exert considerable osmotic stress on cells if it is in high concentration in extracellular fluid which results in movement of water from cells into the extracellular space (Malek et al., 1998).
Equimolar urea test solution (400mM) was perfused at 100 min for 20 min, as a washout, and this was followed by 20 min of CBF measurements. Urea easily crosses cell membranes and hence has capacity to reverse osmotic stress created by mannitol. The urea perfusion was necessary to

demonstrate reversal of effects of osmotic stress and to rule out any irreversible damage to cells (Sato et al., 1990). Mannitol, dbcAMP and Urea were obtained from Sigma, Poole, Dorset BH12 4QH UK. MyrPKAI was obtained from Calbiochem-Nottingham UK. All the above reagents were constituted to their experimental concentrations in M199.

The pH of the solutions used was between 7.2 and 7.4.

The experiments were carried out at 20^{o}C, the ambient laboratory temperature. In addition our preliminary results showed that PKA activity was maximal around 20^{o}C. In summary, CBF was first measured in normal culture media followed by treatment of nasal epithelial cells with PKA modulators in the presence or absence of hyperosmotic stress. This was followed by CBF measurement to determine the effect of this treatment. To rule out cell damage and demonstrate the reversal of hyperosmotic stress effects, a wash-out was carried out with equimolar urea solution followed by CBF measurement.

In order to carry out PKA and adenosine monophosphate activated protein kinase (AMPK) activity assays, cells were pooled from three donors and assays were carried out on cell extracts at time 0 and after 20 min of incubation at 20^{o}C and 32^{o}C under iso-and hyperosmotic stress. AMPK and PKA activities were assayed in immunoprecipitates by measuring the incorporation of ^{32}P from 500 nM [γ^{32}P] ATP into 1mM of the synthetic peptide substrate "SAMS" [HMRSAMSGLHLVKRR] or PKA – selective kemptide respectively as previously described (Sullivan et al., 1994). Briefly samples were suspended to a final volume of 25μl in standard assay buffer (50mM Hepes, pH 7.0, 50mM NaF, 1mM EGTA, 1mM EDTA, 2 % Tween-20, 10 % glycerol) and assays were carried out at 20^{o}C and 30^{o}C for 10 min and terminated by spotting a 15 μl aliquot onto a 1 cm^{2} piece of P-81 phosphocellulose paper and washing 3 x 5 min in 1 % phosphoric acid. Samples were then air-dried and incorporation of γ^{32}P was quantified using a Packard Instant Imager. The enzyme assay component was necessary in order to determine the temperature of maximum activity for PKA. AMPK (AMP-activated protein kinase) is one of the key players in maintaining intracellular homoeostasis. AMPK is well known as an energy sensor and can be activated by increased intracellular AMP or cellular stress (Wang et al., 2012). The activity of AMPK was therefore determined to confirm that cells were indeed under stress.

Differences in CBF at specific points were determined by the nonparametric Wilcoxon test and data were considered to be statistically significant at $p < 0.05$.

Nasal epithelial cells were collected from patients who were having minor surgical procedures immediately after induction of anesthesia. Informed consent was obtained from them a day before surgery. Approval for the study was granted by the Tayside Committee on Medical Research Ethics

3. Results

3.1. PKA Manipulation at 20^{o}C in the Absence of Hyperosmotic Stress

At room temperature, *in vitro* CBF lies around 5-7 Hz. Figure 1 shows that at 20˚C, there was a quasi-linear, non-stepped reduction in CBF following perfusion with M199 (n=5, baseline CBF 5.6±0.1, squares with crosshairs). CBF was 101.6 ± 5.0 and 82.2 ± 5.9 % of baseline at 40 min and 100 min respectively. After urea perfusion there was little change in CBF. Perfusion of 1mM db-cAMP (n=5, baseline CBF 6.0 ± 0.2; small closed circles) raised CBF to 115.9 ± 3.0 and 124.7 ± 3.8 % of the baseline at 40 and 100min respectively). This rise was significantly higher than the CBF observed following M199 perfusion (p=0.03 and 0.0001 at 40 and 100 min respectively). Following perfusion with 400mM urea there was a further slight elevation of CBF to 130.2 ± 6.6 and 127.9 ± 4.5 % of the baseline at 120 and 140 min respectively (p< 0.05 as compared to M199).

Perfusion of 100nM MyrPKAI (n=6, baseline CBF 5.9 ± 0.2; small open circles) induced no significant effect on the decline in CBF (p=0.4 at 40 and 100 min as compared to M199). Perfusion with 400mM urea at 100 min had no effect CBF which was 85.3 ± 3.9 % and 79.9 ± 3.3 % of the baseline at 120 min and at 140 min respectively (p>0.05 as compared to M199).

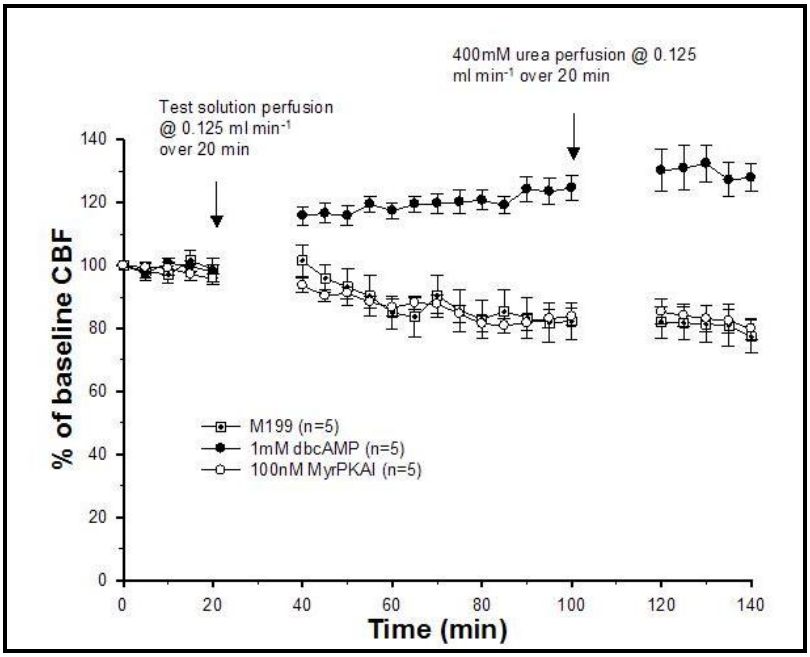

Figure 1: *Shows the Effect of PKA Manipulation on CBF under iso-osmotic Conditions at 20°C*
PKA Activation with dbcAMP in iso-osmotic Conditions Increased CBF above Baseline where as its Inhibition had no Significant Effect on CBF as Compared to M199

3.2. Hyperosmotic Stress with PKA Manipulation at 20°C

Treatment of cells with 400mM mannitol (n=5 baseline CBF 5.7 ± 0.1; closed diamonds) dropped the CBF abruptly to 24.2 ± 3.3 % of baseline at 40 min and after an hour CBF was 16.9 ± 2.5 % of the baseline (p<0.05, as compared to M199) (Figure 1). When PKA was inhibited in 400mM mannitol (n=5, baseline CBF 6.6 ± 0.2; large open circles) the CBF dropped almost to the same magnitude as in mannitol alone. The CBF was 15.7 ± 3.2 and 18.3 ± 3.0 % of baseline at 40 and 100 min respectively (Figure 2).

When PKA was activated with db-cAMP in the presence of 400mM mannitol (n=5, baseline CBF 6.2 ± 0.1; large closed circles) once again, the net result was a significantly lower frequency than in mannitol alone or mannitol with PKA inhibited at both 40 and 100min (p<0.05 at 40 min and p >0.05 at 100min as compared to mannitol alone). The CBF went down to 6.9 ± 2.0 and 15.0 ± 2.0 % of baseline at 40 and 100 min respectively, approaching ciliary arrest values (Table 1).

Following 400mM urea washout, cells that were perfused with 400mM mannitol recovered their CBF from 16.9 ± 2.5 to 83.9 ± 3.8 and 73.0 ± 2.4 % of the baseline at 120 and 140 min respectively. In cells that were in 400mM mannitol with PKA activated, CBF rose from 15.0 ± 2.0 to 102.2 ± 2.6 and 103.3 ± 3.1 % of baseline while when PKA was inhibited CBF rose from 18.3 ± 3.0 to 71.5 ± 3.1 and 67.1 ± 3.2 % of the baseline at 120 and 140 min respectively. After urea washout, cells that had PKA inhibited had poor CBF recovery (See Table 1 for CBF summary). An observation that was made was the shrinking of cells under hyperosmotic stress and upon washout with equimolar urea solution cells increased in volume.

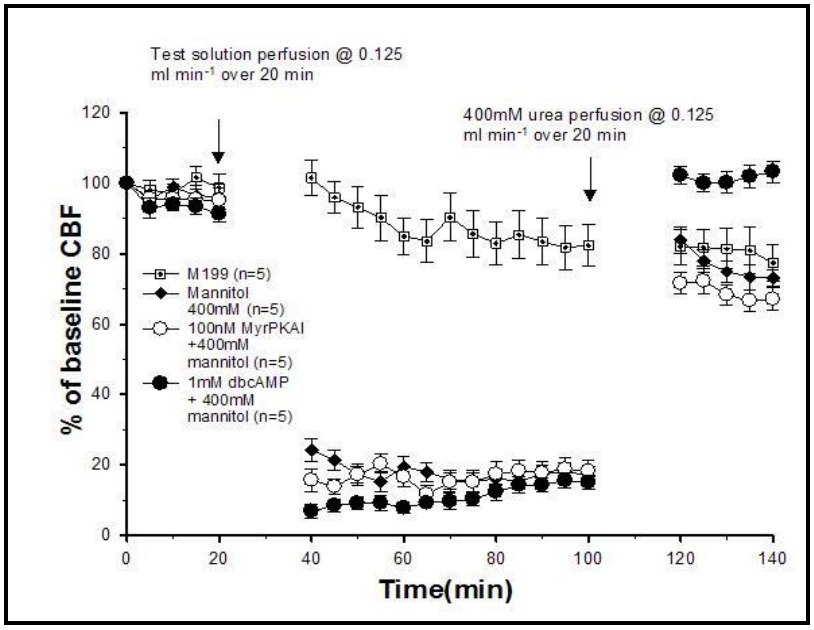

Figure 2: *Shows the Effect of Mannitol Induced Hyperosmotic Stress on CBF with and without PKA Manipulation. Under Hyperosmotic Stress Mannitol Significantly Lowered CBF and PKA Activation Had an Additive Effect on Mannitol Induced Reduction in CBF but PKA Inhibition had a Slight Stabilising Effect. After Urea Washout there was a Strong CBF Recovery in Cells that were treated with Mannitol when PKA was activated. Cells that were treated with Mannitol when PKA was Inhibited Resulted in the Lowest CBF Recovery*

Table 1 *Shows CBF Responses in Hz (Bold) and Percentages of the Baseline to PKA Modulators under iso and Hyperosmotic Conditions at 20°C*

Reagent Time (min)	M199	Mannitol	dbcAMP	MyrPKAI	MyrPKAI + Mannitol	dbcAMP + Mannitol
0	**5.6±0.1** 100%	**5.7±0.1** 100%	**5.6±0.1** 100%	**5.9±0.2** 100%	**6.6±0.2** 100%	**6.2±0.1** 100%
40	**5.6±0.2** 101.6 ± 5.0%	**1.4±0.2** 24.2 ± 3.3%	**6.9±0.2** 115.9 ± 3.0%	**5.5±0.2** 93.7 ± 2.3%	**1.1±0.2** 15.7 ± 3.2%	**0.4±0.1** 6.9 ± 2.0%
100	**4.5±0.2** 82.2 ± 5.9%	**1.0±0.2** 16.9 ± 2.5%	**7.4±0.2** 124.7 ± 3.8%	**4.9±0.2** 83.8 ± 3.8%	**1.2±0.2** 18.3 ± 3.0%	**0.9±0.1** 15.0 ± 2.0%
120	**4.5±0.2** 82.0 ± 5.0%	**4.8±0.2** 83.9 ± 3.8%	**7.5±0.3** 130.2 ± 6.6%	**5.0±0.2** 85.3 ± 3.9%	**4.7±0.3** 71.5 ± 3.1%	**6.3±0.2** 102.2 ± 2.6%
140	**4.2±0.2** 77.3 ± 5.2%	**4.2±0.2** 73.0 ± 2.4%	**7.4±0.2** 127.9 ± 4.5%	**4.7±0.2** 79.9 ± 3.3%	**4.4±0.2** 67.1 ± 3.2%	**6.4±0.3** 103.3 ± 3.1%

Baseline (0 and 20 min) and mannitol treated (0 and 20 min) assays were carried out to determine PKA and AMPK activity. PKA was more active at 20°C than 32°C and this activity was not sensitive to mannitol treatment. AMPK activity was increased after mannitol treatment in a temperature sensitive manner (Figure 3).

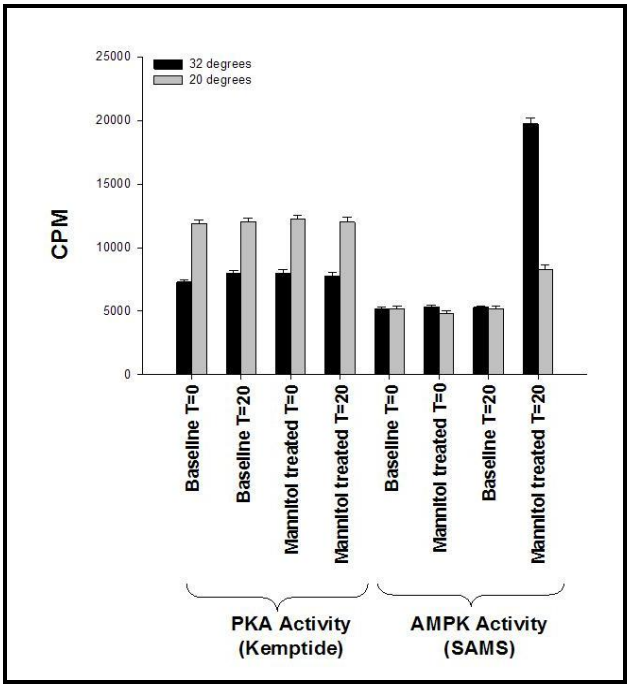

Figure 3: *PKA and AMPK Activity Studies using Specific Peptides*

4. Discussion

According to our findings in normal tissue culture medium there is gradual decline in CBF over a couple of hours and this has been observed in other studies (Sommer et al., 2010). PKA activation increased CBF above baseline but inhibition had no significant effect in CBF in normal medium. Our findings have corroborated other studies where it was shown that activation of PKA increased CBF (Sakuma et al., 2000). The fact that there is no significant difference in CBF between ciliated cells in normal medium and ciliated cells in normal medium with PKA inhibited shows that at 20^{o}C PKA activity may not be a significant factor in baseline CBF. Work done so far seems to suggest that PKA stimulates CBF by phosphorylating a structural protein of cilia, probably dynein light chain (Hamasaki et al., 1991).

Under hyperosmotic stress cells activate intracellular measures to counter the effects of acute reduction in volume. Appropriate sensors detect the stress and provide information to the cell about the ambient conditions and these signals activate responses to deal with the situation. The mitogen-activated protein kinase (MAPK) cascades is one important intracellular signal-transduction pathway activated in response to changes in osmolarity (Kultz and Burg, 1998). Osmotic stress may damage cellular macromolecules and impair cell function and if this is not corrected by the repair processes irreversible cell death occurs (Schwartz et al., 1985).

The reduction in cell volume induce a later compensatory regulatory volume increase (RVI) by initiating a series of intracellular biochemical events (Daviskas et al., 1997). The RVI response is partly brought about by active uptake of ions via the Na-K-2Cl co-transport or by the coupled activities of Na^{+}/H^{+} and Cl^{-}/ HCO_3^{-}exchangers (Jiang et al., 1997; Lytle, 1997; Lang et al., 1998; Lytle, 1998; Goss et al., 2001). The relationship between phosphorylation and osmotic stress is best illustrated after cell shrinkage which stimulates serine and threonine phosphorylation of the Na-K-2Cl co-transport (Lytle and Forbush, 1992; Torchia et al., 1992).

There was a substantial drop in CBF after mannitol perfusion and this decline was not affected greatly by simultaneous PKA inhibition. In contrast, perfusion of mannitol when PKA was co-activated

reduced CBF to almost ciliostasis despite the clear function of this kinase towards increasing CBF when mannitol was absent. After urea washout the full effects of PKA modulators under isosmotic conditions were unmasked; that is, cells that were in mannitol with PKA activated had higher CBF recovery, almost to baseline levels than cells that were in mannitol with PKA inhibited. The inhibitory effect of mannitol on CBF could not have been due to osmolarity per se as an equimolar urea solution, reversed mannitol induced reduction in CBF. It could also not have been due to viscosity because our control experiments (data not shown) showed that a dextran solution with the same viscosity as 400mM mannitol had no significant effect on CBF. One possible explanation for the decline in CBF under hyperosmotic stress is that cellular energy is diverted from less vital physiological functions to very important functions for survival of cells such as maintenance of ion channels in order to reverse the reduction in cell volume. For instance the Na^+-K^+-$2Cl^-$ co-transport requires a sodium gradient to move Na^+, K^+ and $2Cl^-$ into the cell and the movement of these ions is accompanied by an influx of water. This gradient is established by energy from ATP through activity of Na^+K^+ ATPase (McManus et al., 1995). Therefore under hyperosmotic stress extra ATP for powering ion channel channels can only be achieved if energy is diverted. This could be the reason why activation of PKA under hyperosmotic conditions gives the unexpected result of lowering CBF further as more energy gets shunted to support urgent physiological processes leaving less for CBF. The immediate survival of the cell depends on this homeostatic readjustment of RVI supported by energy redistribution (Olz et al., 1993; Varela et al., 2004). Enzyme assays carried out showed that PKA was more active at 20^{o}C than at 32^{o}C and this activity was not affected by mannitol treatment which may explain in part why there wasn't much difference in CBF in hyperosmotic mannitol when PKA was activated. However AMPK an enzyme known to be activated by stressful conditions showed increased activity under mannitol induced hyperosmotic stress.

It is important though to stress that our findings were purely experimental and may not reflect comprehensively the situation in the nasal airways; and changes in nasal epithelial cell volume due to hyperosmotic stress were merely observed, as equipment to measure cell volume changes was not available.

In conclusion, hyperosmotic stress decreases CBF and activation of PKA under hyperosmotic stress had an additive effect on reduction of CBF. AMPK is activated by hyperosmotic stress whereas PKA activity is not activated by hyperosmotic stress.

Acknowledgements

We would like to thank the ENT patients at Ninewells Hospital and Medical School, Dundee for willingly allowing us to collect nasal epithelial cells from them. We also thank the ENT surgeons, the anesthetists and nursing staff at Ninewells Hospital and Medical School for allowing us to interact and collect samples from their patients.

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