PACAP27 regulates ciliary function in primary cultures of rat brain ependymal cells

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Abstract

Ependymal cells line the brain ventricles and separate the CSF from the underlying neuronal tissue. The function of ependymal cilia is largely unclear however they are reported to be involved in the regulation of CSF homeostasis and host defence against pathogens. Here we present data that implicates a role of pituitary adenylate cyclase-activating polypeptide (PACAP) in the inhibition of ependymal ciliary function, and also that the PACAP effects are not entirely dependent on adenylyl cyclase activation. Primary ependymal cultures were treated with increasing doses of PACAP27 or adenylyl cyclase toxin (ACT), and ciliary beating was recorded using high-speed digital video imaging. Ciliary beat frequency (CBF) and amplitude were determined from the videos. Ependymal CBF and ciliary amplitude were attenuated by PACAP27 in a concentration- and time-dependent manner. The peptide antagonist PACAP6-27 blocked PACAP27-induced decreases in amplitude and CBF. Treatment with ACT caused a decrease in amplitude but had no effect on CBF, this suggests that the inhibition of CBF and amplitude seen with PACAP27 may not be completely explained by G_s-AC-cAMP pathway. We present here the first observational study to show that activation of PAC1 receptors with PACAP27 has an important role to play in the regulation of ependymal ciliary function.

Key words: Ependymal cilia, ciliary function, pituitary adenylate cyclase-activating polypeptide, PACAP, PAC1 receptor

1. Introduction

Ependymal cells lining the ventricular cavities form a protective interface between brain parenchyma and the circulating cerebrospinal fluid (CSF). Ependymal cells bear approximately 40 motile cilia per cell which beat in the coordinated pattern at approximately 40 Hz (Del Bigio, 1995; O'Callaghan et al., 1999) that rapidly move CSF adjacent to the ventricular walls. The precise functional roles of ependymal cilia are not fully understood, however, it is known that abnormal ciliary function (Banizs et al., 2005; Mönkkönen et al., 2007; Shimizu and Koto, 1992), associated with an inherited condition known as Primary Ciliary Dyskinesia (PCD) can result in hydrocephalus (Ibañez-Tallon et al., 2004). Ependymal cilia also have been shown to play an important role in host defence against pathogens (Hirst et al., 2003; Hirst et al., 2000; Mohammed et al., 1999). In addition, a recent study (Sawamoto et al., 2006) has shown that ependymal cilia are responsible for the formation of gradients of CSF guidance molecules that direct neuroblast movement in the brain parenchyma. However, few studies have addressed the question of hormonal regulation of the ciliary function.

There is previous evidence that rat ependymal ciliary function could be inhibited by an excess activity of the adenylyl cyclase (AC) – cAMP pathway (Mönkkönen et al., 2007). The dual balance between G protein families G_i and G_s in the regulation of AC is well established, but G protein-coupled receptors have not been identified in CBF inhibition. Interestingly, a recent study showed that mice overexpressing PAC1 receptors developed hydrocephalus with excess activity of PKA pathway (Lang et al., 2006). PAC1 receptors are activated by pituitary adenylate cyclase-activating polypeptide (PACAP) and coupled to G_s protein family. This

suggests that endogenous neuropeptide, PACAP, may have a receptor-dependent role in the regulation of ciliary function.

During evolution, PACAP has been remarkably well conserved, and it shares a striking sequence similarity between species (Arimura, 1998) and suggests a vital physiological role. PACAP is a pleiotropic peptide, involved in the regulation of hormone release from the pituitary and adrenal gland. PACAP has been implicated in a variety of biological processes such as reproduction, development, growth, immune responses, cardiovascular, gastrointestinal and respiratory functions (Arimura, 1998; Vaudry et al., 2000). In the CNS, PACAP acts as a neurotrophic factor during brain development and as a neuroprotective factor during adulthood (Arimura, 1998; Vaudry et al., 2000; Watanabe et al., 2007). Further, it is a neurotransmitter and/or a neuromodulator in the PNS and a preganglionic neurotransmitter in the CNS (Arimura, 1998). Physiological concentrations of PACAP in CSF and brain tissue are in the range of 0.1-2 nM (Ohno et al., 2005; Wilderman and Armstead, 1997). In rat brain, high levels of PACAP are mainly found in the hypothalamus. PACAP acts as the transmitter of the monosynaptic neuronal pathway (the retinohypothalamic tract) mediating light information to the master biological clock, the hypothalamic suprachiasmatic nuclei (Hannibal, 2006). In addition, regions adjacent to the ventricles, like septal, paraventricular and periventricular nuclei, have high PACAP concentrations as well as high density of PACAP immunopositive cells and fibers in both rat and human (Arimura et al., 1991; Ghatei et al., 1993; Köves et al., 1991; Masuo et al., 1993; Palkovits et al., 1995; Vigh et al., 1991).

We have previously described a primary rat ependymal culture model that allows ciliary function to be studied over time (Hirst et al., 2000). An often ignored fact regarding the

ciliary function is that following certain topical toxic insults the CBF remains unchanged whilst the ciliary amplitude, and hence function, may be disturbed (Chilvers and O'Callaghan, 2000). The present study allowed us to use our methodology to determine the role of PACAP in both ependymal CBF and amplitude.

2. Materials and methods

2.1. Ependymal Cell Culture

Ciliated rat ependymal cells were grown using a method adapted from Wiebel and colleagues (Weibel et al., 1986) and described previously (Hirst et al., 2000).

2.2. Compound addition

All experiments were performed in Hepes-buffered minimum essential media (MEM) without additives. PACAP27 (Tocris, Bristol, UK), PACAP6-27 (Sigma-Aldrich, Poole, UK) and adenylyl cyclase toxin (Sigma-Aldrich) were diluted to their final concentrations in 1 ml MEM. Negative controls were incubated in 1 ml of MEM alone. The compounds were added on the cells at 0 min (all compounds used), 120 min (PACAP27, PACAP27 combined with PACAP6-27, ACT) and 240 min (PACAP27) in increasing concentrations. In the case of PACAP27 and peptide antagonist PACAP6-27 combination, the cells were pre-treated for 60 min with the peptide antagonist.

2.3. CBF and amplitude

To determine CBF and amplitude the cells were placed in a humidified (70-90%) incubation (37^{o}C) chamber and observed from above using an inverted microscope system (Diphot,

Nikon, UK). In order to prevent cilia-to-cilia variation, we selected a long strip of mature cilia for each recording, and followed the function of the same cilia throughout the experiment. From each recording, 5 cilia were randomly selected for CBF and amplitude analysis. To avoid any influence of potentially varying degree of beat pattern on the results, only the mature cilia which displayed their full beat cycle when viewed from above were included. Beating cilia were recorded using a digital high-speed video camera (Troubleshooter, Lake Image Systems, UK) at a rate of 500 frames per second, using a shutter speed of 1 in 2000. The camera allows video sequences to be recorded and played back at reduced frame rates or frame by frame. CBF was determined by timing a given number of individual cilia beat cycles. Basal CBF was measured at 0 minutes, before addition of compounds. Calculation of CBF (Hz): Frequency (Hz) = Number of frames recorded per second / frames elapsed for 5 ciliary beat cycles X 5 (conversion per beat cycle). Ciliary amplitude was measured using a similar method as previously described (O'Callaghan et al., 2008). The captured video sequences were played back at a slow rate which allowed determination of the distance travelled by the cilia tips within the power stroke of the beat cycle.

2.4. Data analysis

Data are presented as mean ± SEM of CBF and amplitude measurements from 4-11 separate cell cultures. The mean reading of each individual culture was obtained from 5 randomly chosen cilia. Statistical comparisons were made using one-way analysis of variance with Tukey's multiple comparison test and Student’s unpaired two-tailed t-test. All analysis was made using GraphPad Prism for Windows (GraphPad Software, Inc., San Diego, CA). Statistical significance was achieved when $p<0.05$.

3. Results

3.1. PACAP27 caused a decrease in CBF and amplitude.

Primary ependymal cultures, which had basal CBF of approximately 40 Hz and basal amplitude of approximately 9 μm, were treated with increasing concentrations of PACAP27. At time points indicated by arrows (Figure 1), the media was completely replaced both in control cells (with fresh culture media) and in PACAP27 cells (with media containing 10 nM, 100 nM or 1μM of PACAP27). The arrows thus denote the arrival of the agonist at the receptors. Beating cilia were recorded using a digital high-speed video camera at 30 min intervals in PACAP27 treated and at 60 min intervals in control cells.

Our data showed that PACAP27 caused a decrease in CBF (Figure 1A) and amplitude (Figure 1B), and that this decrease was statistically significant (* $p<0.05$, *** $p<0.001$; Student's unpaired t test). This effect was both concentration- and time-dependent. After addition of 10 nM, 100 nM and 1 μM PACAP27, CBF was approximately 93 %, 86 % and 73 % of the basal CBF, while average amplitude was 83 %, 60 % and 37 % of the basal amplitude, respectively. Based on these data, we concluded that PACAP27 effects on CBF and amplitude were linear in the concentration range used. In the control cells, CBF and amplitude remained at the baseline level (approximately 35 Hz and 7 μm) during the whole experiment. 24 hours after PACAP27 was removed from the ependymal cells by media replacement, the inhibition of CBF and amplitude was reversed (data not shown).

3.2. PACAP27 mediated decrease in CBF and amplitude was inhibited by the competitive peptide antagonist PACAP6-27.

In order to clarify whether the PACAP27 effects on CBF and amplitude were receptor-mediated, we used 3 µM concentration of the peptide antagonist PACAP6-27 in combination with 1 µM PACAP27 (Figure 2). The baseline CBF and amplitude levels in control cells, PACAP27 cells and in cells treated with combination of PACAP27 and the antagonist PACAP6-27, were 34 Hz, 38 Hz and 44 Hz, respectively. The corresponding baseline amplitude levels were 6 µm, 8 µm and 8 µm. Compounds were added on cells at the time points indicated by arrows.

Figure 2 shows that PACAP27 addition inhibited both CBF and amplitude and this effect was negated by pre-incubation with the antagonist. At the 120 minutes time point mean CBF (Figure 2A) and mean amplitude (Figure 2B) of the PACAP27 treated ependymal cells were significantly different from the control (* $p<0.05$; One-way ANOVA with Tukey's Multiple Comparison test). In the control cells and in cells treated with combination of PACAP27 and PACAP6-27, the CBF and amplitude remained at their baseline levels throughout the whole experiment. 3 µM PACAP6-27 administered alone did not change CBF or amplitude (data not shown).

3.3. Adenylyl cyclase toxin caused a decrease in ciliary amplitude but not CBF.

In order to simulate the intracellular rise in cAMP following receptor stimulation by PACAP27, we used ACT treatment on the ependymal cells (Figure 3). ACT containing media was added on cells in increasing concentrations of 10ng/ml and 30ng/ml at time points indicated by arrows. Media was replaced on control cells at the same time points. Baseline CBF was approximately 40 Hz and amplitude 8 µm.

ACT caused a statistically significant decrease in the ciliary amplitude (Figure 3B, ** $p<0.01$; Student's unpaired t test) that was similar to the action of PACAP27. However, during the 240 min experiment it did not significantly change CBF (Figure 3A). CBF and amplitude in the control cells remained at their baseline levels of approximately 35 Hz and 7 μm during the whole experiment.

4. Discussion

This study aimed to clarify whether PACAP has a role in controlling ependymal ciliary function. Our results showed that PACAP27 reduced CBF and amplitude in cultured rat ependymal cilia in a receptor-mediated manner. Furthermore, our results suggest that the effects of PACAP27 may not be completely explained by effect on the AC-cAMP pathway. Increasing the cAMP using low dose ACT treatment inhibited ependymal ciliary amplitude but did not affect the CBF over the course of the study.

The role of ependymal ciliary beating and the control of CSF directional flow has been well described by others (Cathcart and Worthington, 1964; Yamadori and Nara, 1979). However, a recent study showed that ependymal ciliary beating was crucial for the formation of concentration gradients of CSF guidance molecules directing proper neuroblast migration (Sawamoto et al., 2006). The connection between ciliary function and CSF homeostasis has been clearly demonstrated, as ciliary dysfunction or immotility (Nakamura and Sato, 1993; Shimizu and Koto, 1992) as well as defects in ciliary structure components (Ibañez-Tallon et al., 2002; Ibañez-Tallon et al., 2004; Sapiro et al., 2002) or defects in proteins involved in ciliogenesis (Chen et al., 1998; Kobayashi et al., 2002; Taulman et al., 2001) may all result in

hydrocephalus. Further, in patients with PCD, there are reports of associated enlarged ventricles and hydrocephalus (Ibañez-Tallon et al., 2002). All of the cilia of PCD patients are either immotile or beat in a dyskinetic fashion (Chodhari et al., 2004). Recently, the connection between dysfunctional cilia and the development of hydrocephalus was clarified further, as it was demonstrated, that severely malformed and dysfunctional cilia in choroid plexus *in vivo* results in disturbed cAMP-regulated chloride transport, leading to excess CSF production and hydrocephalus (Banizs et al., 2005).

There is evidence of the involvement of AC-cAMP pathway in inhibition of ependymal ciliary function. Our previous study showed that attenuation of the inhibitory G protein subunit $G\alpha_{i2}$ resulted in ventricular dilatation and ciliary stasis *in vitro* (Mönkkönen et al., 2007). This can be explained by a disturbed balance of AC regulation by G_s-G_i. The present study has confirmed the involvement of cAMP in the control of ciliary function, as ACT was shown to significantly decrease ciliary amplitude. In line with these findings, mice overexpressing PAC1 receptor have been shown to have elevated PKA and PKC activity, ciliary defects and increased apoptosis, resulting in development of hydrocephalus (Lang et al., 2006).

While there are yet few reports on regulation of ciliary beat in the brain, tracheal ciliary function has been a focus of many studies. Although many structural and functional features seems well conserved between different ciliated tissues (Satir and Christensen, 2007), the ciliary beat-regulating pathways seems not to be shared. In contrast, ciliary beat regulatory molecules may cause completely reverse effects on the CBF in different tissues. Activation of the PKA pathway by cAMP or ATP increases tracheal CBF (Di Benedetto et al., 1991; Morse et al., 2001), whereas the same pathway is associated with CBF inhibition in the ependymal

cilia (Mönkkönen et al., 2007). In general, outer dynein arms regulate the power stroke and thus the CBF, and inner dynein arms regulate bending of the cilium (Brokaw and Kamiya, 1987; Habermacher and Sale, 1997; Satir et al., 1995). In tracheal cilia, the underlying mechanism behind the CBF increase has been proposed to be cAMP-dependent activation of axonemal PKA, which causes dynein phosphorylation (Salathe, 2007). Studies on *Tetrahymena* have shown that the ciliary amplitude changes as a result of direct Ca^{2+} binding to inner dynein arms (Guerra et al., 2003). Potentially, the amplitude decrease that we observed in the present study by PACAP27 and ACT could be explained by altered inner dynein arm activity, evoked by a cAMP-dependent pathway.

PACAP exists in two forms, PACAP38 and PACAP27 (Miyata et al., 1989; Miyata et al., 1990). Both originate from a single gene and have a common 175-176-amino acid precursor (Arimura, 1998; Arimura and Shioda, 1995). Although PACAP38 is the predominant form found in tissues, the N-terminal amino acids 1-27, which correspond to PACAP27, account for biological activity (Arimura and Shioda, 1995; Vaudry et al., 2000). PACAP functions are mediated through three G protein-coupled receptors, PAC1, VPAC1 and VPAC2. While only PACAP has high affinity to PAC1 receptor, the latter two can be activated by vasoactive intestinal peptide (VIP) and PACAP with equal affinity (Harmar et al., 1998). PAC1 has at least eight splice variants, which differ in their cell or tissue expression, and in their ability to activate the alternative signalling pathway, phospholipase C (PLC). VPAC1 and VPAC2 and seven of the PAC1 variants couple to G_s family and stimulate AC. One PAC1 variant activates solely L-type Ca^{2+} channels (Chatterjee et al., 1996; Pantaloni et al., 1996; Spengler et al., 1993).

The two PACAP forms PACAP27 and PACAP38 are equipotent in stimulation of the G_s-AC-cAMP pathway, but only PACAP38 can potently stimulate PLC via PAC1 activation (Basille et al., 1995; Spengler et al., 1993). In the present study, we selected PACAP27 in order to clarify the AC-stimulating effects of PACAP in the regulation of ciliary function. PACAP27 has a high affinity to PAC1 and VPAC1, and slightly lower affinity to VPAC2 receptors. Inactive peptide PACAP6-27 acts as antagonist on PAC1 and VPAC2, but not on VPAC1. Since our results showed that PACAP27-induced inhibitory effects on ciliary function were fully inhibited by PACAP6-27, VPAC1 receptor seems not to have a role in mediating the observed ciliary inhibition.

Concerning the receptor(s) mediating the inhibitory effects of PACAP27 on CBF and amplitude, the earlier study on PAC1 overexpressing mice strongly supports involvement of PAC1 receptors. Namely, PAC1 overexpressing mice showed a higher level of PAC1 expression especially in ventricle-surrounding regions, when compared to wild type (Lang et al., 2006). In the developing brain, PAC1 mRNA expression showed more intense subventricular localization (Basille et al., 2000; Jaworski and Proctor, 2000; Zhou et al., 1999) than in the adult (Hashimoto et al., 1996; Jaworski and Proctor, 2000; Shioda et al., 1997). There is evidence that PAC1 mRNA intensity in ventricular regions remains high during the first postnatal week (Jaworski and Proctor, 2000), which corresponds to the age of the ependymal cell cultures used in the present study. Unlike PAC1, VPAC2 is not present in subventricular regions during brain development (Basille et al., 2000), and *in situ* hybridisation studies have not provided evidence of its presence in the rat ventricle and surrounding regions (Basille et al., 2000; Sheward et al., 1995; Usdin et al., 1994). This suggests that PAC1 receptor might account for PACAP-mediated regulation of ciliary function.

Interestingly, while classical intracellular responses to G protein-coupled receptor activation are expected to be rapid, the regulatory effects of PACAP27 on ependymal ciliary function observed in the present study emerged with time and were progressive. The observed delay strongly suggests that ependymal ciliary function might be regulated in a more complicated manner than via simple receptor activation. Potentially, regulation might involve changes in the energy metabolism, or participation of the various PAC1 isoforms, which could mediate the signal further into different downstream pathways.

Regarding the role of neuropeptide PACAP in CSF homeostasis, patients with gene multiplications at regions where PACAP and PAC1 genes reside (18p11 and 7p15, respectively) (Hosoya et al., 1992; Ogi et al., 1993), have been reported to develop hydrocephalus (Miller et al., 1979; Takeda et al., 1989). Further, PAC1 overexpression in mice results in hydrocephalus (Lang et al., 2006). Consistent with these findings, we have shown for the first time, a receptor-dependent regulation of ciliary beat pattern by PACAP27.

Statement of conflict of interest

None

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Figures and figure legends

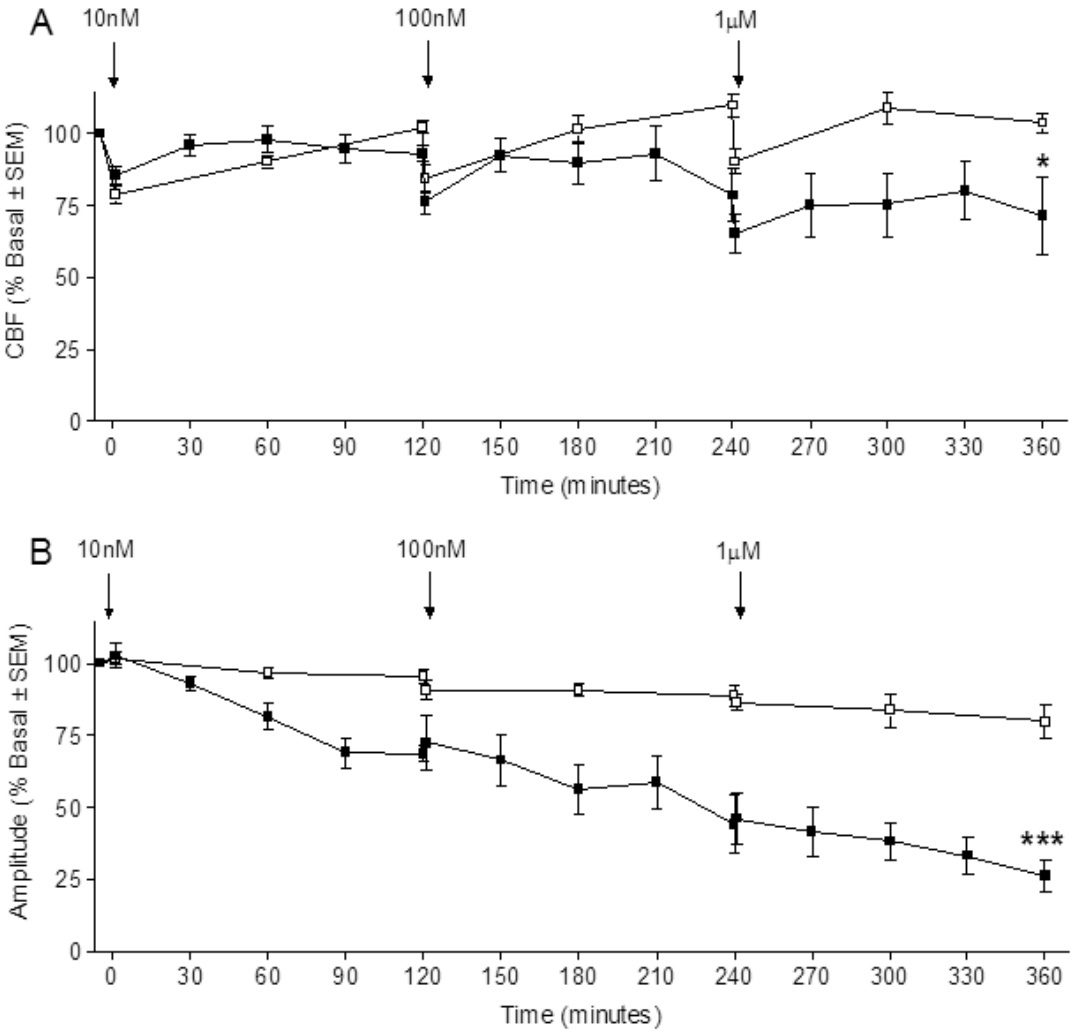

Figure 1. The effect of increasing concentrations of PACAP27 with time on CBF (A) and ciliary beat amplitude (B) in rat ependymal cilia. PACAP27 (■) caused a significant decrease in both CBF (A) and ciliary beat amplitude (B) compared to control (A and B □). Arrows show time points of PACAP27 addition in increasing concentrations (10 nM, 100 nM and 1 µM) or where media was replaced in the controls. CBF and ciliary beat amplitude in the PACAP27 treatment group were significantly different (* $p<0.05$, *** $p<0.001$; Student's unpaired t test) from the control.

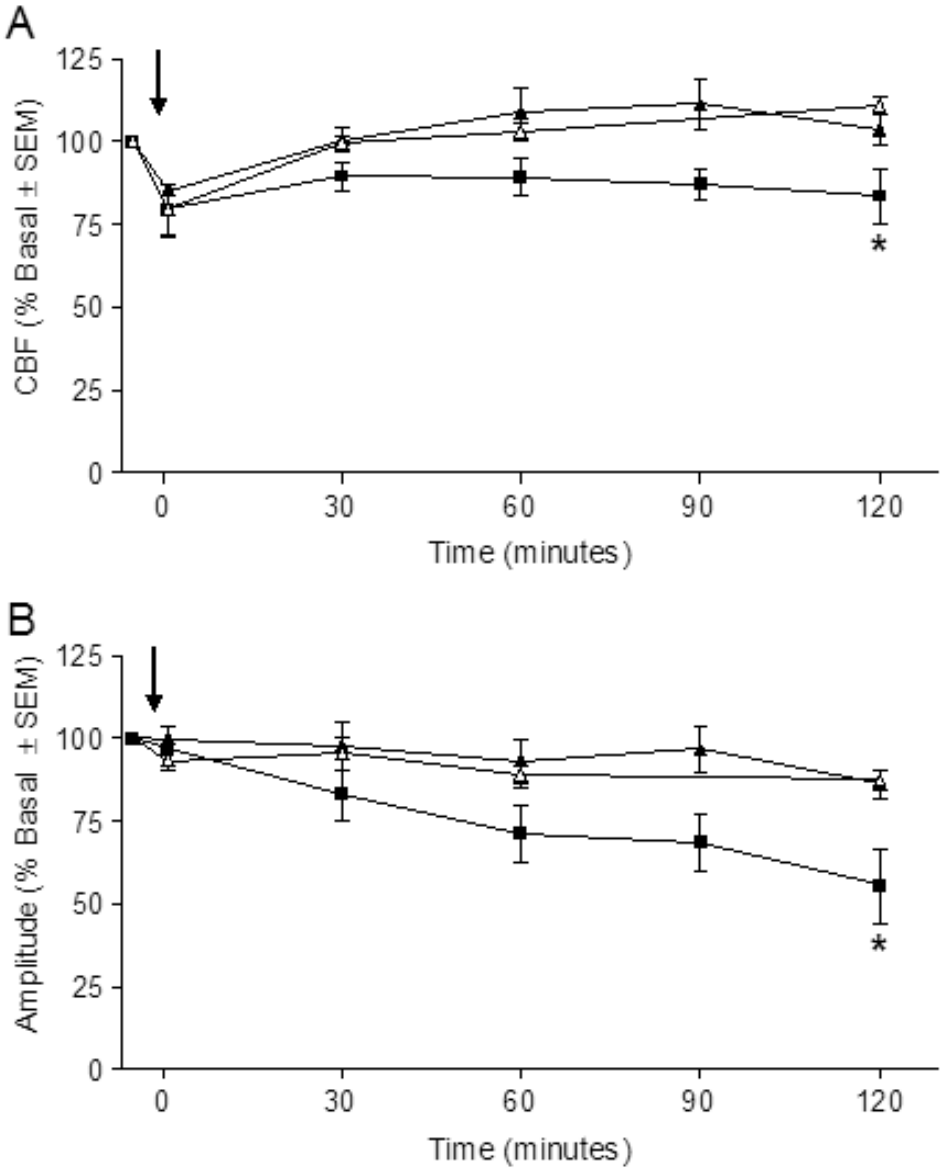

Figure 2. The effect of 1 µM PACAP27 alone (■) and in combination with 3 µM peptide antagonist PACAP6-27 (▲) on CBF (A) and ciliary beat amplitude (B) in rat ependymal cilia. The PACAP27 effects on amplitude were inhibited by receptor blockade with the antagonist PACAP6-27. Arrows show time points of PACAP27 additions and replacement of control media. At the 120 minutes time point mean amplitude and mean CBF of the PACAP27 treated ependymal cells were significantly (* $p<0.05$; One-way ANOVA with Tukey's Multiple Comparison test) different from the control (Δ).

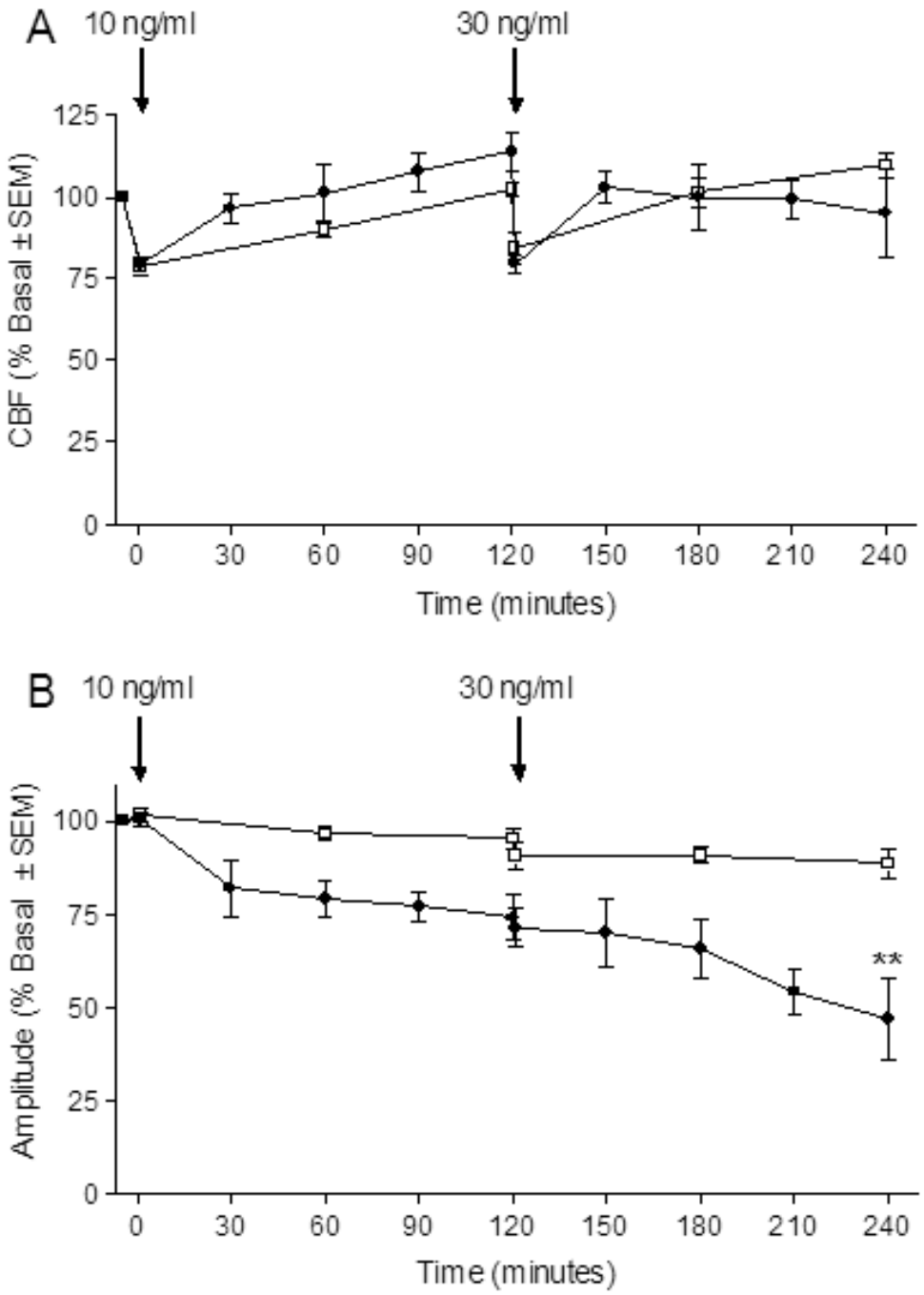

Figure 3. The effect of adenylyl cyclase toxin (ACT) on CBF (A) and ciliary beat amplitude (B) in rat ependymal cilia. ACT (●) caused a significant (** $p<0.01$; Student's unpaired t test) decrease in ciliary beat amplitude in rat ependymal cells compared with control (□). Arrows show the time points of ACT additions in increasing concentrations (10 ng/ml and 30 ng/ml) or the time of media replacement in the controls.