Research Article

Differential Role of cAMP, cGMP, and Ca^{2+} and Involvement of Kinases and CBP-CREB CRE Pathway in Regulation of Arylalkylamine N-acetyltransferase 2 mRNA Levels in the Pineal Organ of an Air-Breathing Catfish, *Clarias gariepinus*

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ABSTRACT

Transcription of arylalkylamine N-acetyltransferase 2 (*aanat2*) gene leads to the formation of AANAT2 - the rate-limiting enzyme in melatonin synthesis in the photosensitive fish pineal. Unlike in mammalian pineal, there is practically no information on signal transduction pathway(s) involved in the regulation of *aanat2* gene transcription in the fish pineal. Therefore, we investigated the role of important molecular components of signalling via cAMP, cGMP, and Ca^{2+} involving cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), Ca^{2+}-dependent protein kinase (PKC), mitogen-activated protein kinase (MAPK) kinase (MEK) and p38 MAPK (p38 MAPK) as well as the possible role of serine/threonine phosphatases (PPs), cAMP response element binding protein (CREB) and CREB binding protein (CBP) using their specific inhibitors in regulation of *aanat2* transcripts in the fish pineal under *in vitro* conditions. db-cAMP and db-cGMP increased AANAT2 mRNA levels. db-cAMP- and db-cGMP-induced increase in AANAT2 mRNA levels was significantly reduced in the presence of H-89 (PKA inhibitor), KT5823 (PKG inhibitor), chelerythrine chloride (PKC inhibitor), U0126 (MEK inhibitor), and SB 202190 (p38 MAP kinase inhibitor). Inhibitors of PP1 and PP2A significantly increased AANAT2 mRNA as well as significantly reduced db-cAMP and db-cGMP induced increase in AANAT2 mRNA levels. Inhibitors of both CREB and CBP-CREB interaction completely blocked the cAMP-induced increase in AANAT2 mRNA levels. Based on these findings, we suggest that cAMP, cGMP, and Ca^{2+} increase AANAT2 mRNA levels via PKA, PKG, and PKC, respectively. Further, protein phosphatases and the CBP-CREB-CRE pathway are actively involved in the regulation of AANAT2 mRNA levels in the fish pineal.

Key words: *Aanat2* gene, Ca^{2+}, cAMP, cGMP, CREB-CBP, Pineal organ.

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INTRODUCTION

Arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) acts as the rate-limiting enzyme and controls the rhythmic synthesis of melatonin in the pineal gland of vertebrates.[1,2] Teleosts express three isoforms of *aanat* gene, namely *aanat1a*, *aanat1b* (which are expressed in the retina, the nervous system, and other peripheral tissues), and *aanat2*, which is specifically expressed in the pineal organ.[3,4] In the mammalian pineal gland, activation of adrenergic receptors by norepinephrine stimulates transcription of *aanat* gene via cyclic adenosine monophosphate (cAMP)-cAMP-dependent protein kinase (PKA)-cAMP response element binding protein (CREB)-cAMP response element (CRE)

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pathway.[1,2] AANAT was phosphorylated by Ca^{2+}-dependent protein kinase (PKC) activation through the α_1-adrenergic receptor, and its phosphorylation might contribute to the stability and the activity of AANAT in rat pineal glands.[5] In rat pinealocytes, the effect of norepinephrine on MAPK phosphorylation is due to the integration of multiple signalling mechanisms, and activation of PKA and PKG has been reported to produce opposite effects on phosphorylation of MAPK.[6] PKG is the main kinase involved in the activation of MAPK, which acts upstream of MEK in activation of MAPK, while activated PKA has been reported to inhibit MAPK.[6] Furthermore, activation of PKA effectively abolished MAPK phosphorylation stimulated by an activator of PKC or a Ca^{2+} elevating agent.[6] Thus, the cAMP/PKA pathway antagonizes the stimulatory effect of both cGMP and Ca^{2+} on MAPK phosphorylation. In mammalian pinealocytes, protein phosphatases have also been reported to control the activity of the AANAT enzyme by dephosphorylating pCREB that down-regulates the induction of the *aanat* gene, and hence AANAT protein and melatonin synthesis.[1,7] Dephosphorylation of pCREB by protein serine/threonine phosphatases (PSPs) is an essential mechanism for down regulating *aanat* gene expression in the rat pineal gland.[8] Further, the inhibitors of serine/threonine phosphatases (calyculin A, microcystin-LR, and okadaic acid) have been reported to attenuate dephosphorylation of pCREB, and to block the decline in *aanat* mRNA levels, AANAT protein amount, and melatonin biosynthesis.[8] Cyclic AMP-dependent protein kinase has been reported to phosphorylate cAMP-responsive element (CRE)-binding protein (CREB) in the rat pineal gland.[9] High levels of cAMP at dawn and dusk and low levels at noon and midnight have been reported in pike pineal organs maintained under LD in static culture.[10] Further, variations in pineal cGMP content were not statistically significant, however, in the perfusates, the levels were higher during the night than during the day suggesting that nocturnal synthesis of cGMP is higher than its catabolism. Cyclic AMP-dependent phosphorylation of CREB in trout pineal organs has been reported to be accompanied by increased melatonin synthesis.[11] CREB binding protein (CBP) is also reportedly involved in *aanat* gene expression in the mammalian pineal gland.[12]

However, there is practically no information on the role of molecular components of the signalling pathway(s) involved in the transcription of *the aanat2* gene in the pineal organ of any fish species. Therefore, we investigated the role of major second messengers (cAMP, cGMP, and Ca^{2+}), protein kinases (PKA, PKG and PKC, MEK and MAPK), PSPs (PP1, PP2A, and PP2B), and CBP-CREB complex in the transcription of *aanat2* gene in the pineal organ of the catfish, *Clarias gariepinus*. Our findings suggest a differential role of cyclic nucleotides, Ca^{2+} and protein kinases, and involvement of the CBP-CREB complex in *aanat2* gene transcription.

MATERIALS AND METHODS

Ethics statement

All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals after obtaining institutional ethical clearance from the Institutional Ethics Committee, North-Eastern Hill University, Shillong, India.

Experimental animals

All experiments were conducted on the pineal organ of sexually mature male *Clarias gariepinus*, (length: 23-27 cm; body weight: 95-100 g). The fish was acclimatized for 15 days in a fish room under 12 hr light: 12 hr dark cycle (lights 'on' at 06:00 h, lights 'off' at 18:00 h) and water temperature 25 ± 2°C at NEHU Campus, Shillong.

Chemicals

Chemicals were purchased from the following sources: Dulbecco's Modified Eagle Medium: Gibco, Thermo Fisher Scientific, USA; MS-222, Bovine Serum Albumin, Ascorbic acid, Penicillin-Streptomycin, HEPES salt, H-89 dihydrochloride hydrate, KT5823, Chelerythrine chloride, U0126 ethanolate, SB 202190 monohydrochloride hydrate, Calyculin A, and TRI reagent: Sigma Chemicals, USA; db-cAMP and db-cGMP: Enzo, USA; Calcium carbonate: HIMEDIA, India; CREB inhibitor (666-15-Calbiochem): Merck, Germany, CBP-CREB interaction inhibitor (CAS 92-78-4-Calbiochem): Calbiochem®:Merck, Germany; Verso cDNA Synthesis Kit: Thermo Scientific, USA; Power SYBR® Green Master Mix: Applied Biosystems, USA.

Pineal organ culture and *in vitro* treatments

To collect the pineal organ, the acclimatized fish was anesthetized with MS-222, decapitated, the pineal window was exposed, the pineal organ was rapidly removed, washed, and placed in the sterile DMEM culture medium supplemented with BSA (1mg/ml), $CaCO_3$ (0.125 mg/ml), Ascorbic acid (0.1 mg/ml), Penicillin-Streptomycin (10 µl/ml) and HEPES salt (4.77 mg/ml) in a 24-well culture plate (Nunclon TM Delta Surface, Thermo Scientific, USA). All dissections were carried out between 10:30 h – 11:30 h. Pineals were

pre-incubated at 25 ± 2°C for 1 h in an atmosphere of 85% O_2, 5% CO_2, and 95% relative humidity in a gas (O_2-CO_2) incubator. After pre-incubation, the culture medium was replaced with a culture medium containing (10^{-6} M) of desired drugs separately or in combinations. Pineals incubated in sterile DMEM supplemented with BSA, $CaCO_3$, Ascorbic acid, Penicillin-Streptomycin, and HEPES salt were taken as the control group. After treatment with desired drugs, the pineal organs (three per sample) were removed and collected in micro-centrifuge tubes containing TRI reagent (500 µl), and immediately processed for isolating total RNA.

Experimental Design

The following experiments were conducted

Experiment	No. of pineals and samples	Treatments	Concentration of drugs and incubation period
1) *In vitro* effect of H-89 on AANAT2 mRNA levels	36 pineal organs (12 samples of 4 groups)	Control, H-89, db-cAMP & H-89 + db-cAMP	10^{-6} M each for 30 min
2) *In vitro* effect of KT5823 on AANAT2 mRNA levels	36 pineal organs (12 samples of 4 groups)	Control, KT5823, db-cGMP, & KT5823 + db-cGMP	10^{-6} M each for 30 min
3) *In vitro* effect of db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence and absence of chelerythrine chloride (Che)	72 pineal organs (24 samples of 8 groups)	Control, db-cAMP, db-cGMP, db-cAMP + db-cGMP, Che, Che + db-cAMP, Che + db-cGMP, & Che + db-cAMP + db-cGMP	10^{-6} M each for 30 min
4) *In vitro* effect of db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence and absence of U0126 ethanolate (UE)	72 pineal organs (24 samples of 8 groups)	Control, db-cAMP, db-cGMP, db-cAMP + db-cGMP, UE, UE + db-cAMP, UE + db-cGMP, & UE + db-cAMP + db-cGMP	10^{-6} M each for 30 min
5) *In vitro* effect of db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence and absence of SB 202190 monohydrochloride hydrate (SB)	72 pineal organs (24 samples of 8 groups)	Control, db-cAMP, db-cGMP, db-cAMP + db-cGMP, SB, SB + db-cAMP, SB + db-cGMP, & SB + db-cAMP + db-cGMP	10^{-6} M each for 30 min
6) *In vitro* effect of db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence and absence of calyculin A (CA)	72 pineal organs (24 samples of 8 groups)	Control, db-cAMP, db-cGMP, db-cAMP + db-cGMP, CA, CA + db-cAMP, CA + db-cGMP, & CA + db-cAMP + db-cGMP	10^{-6} M each for 30 min
7) *In vitro* effect of db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence and absence of cypermethrin (Cyp)	72 pineal organs (24 samples of 8 groups)	Control, db-cAMP, db-cGMP, db-cAMP + db-cGMP, Cyp, Cyp + db-cAMP, Cyp + db-cGMP, & Cyp + db-cAMP + db-cGMP	10^{-6} M each for 30 min
8) *In vitro* effect of db-cAMP in the presence and absence of a CREB inhibitor (CI) and a CBP-CREB interaction inhibitor (CCII)	54 pineal organs (18 samples of 6 groups)	Control, db-cAMP, CI, CCII, CI + db-cAMP, CCII + db-cAMP	10^{-6} M each for 30 min

Total RNA extraction, cDNA synthesis, and analysis by Quantitative Real-Time PCR

Total RNA was extracted from the pineal organs using TRI reagent (Sigma, USA) by following the manufacturer's protocol. To maintain the good quality of the samples, RNA samples with a ratio of sample absorbance at 260 and 280 nm between 1.8 and 2.1 were used for all experiments. Synthesis of cDNA was performed using 300 ng of total RNA using Verso cDNA Synthesis Kit (Thermo Scientific, USA) in a total reaction volume of 20 µl containing 5X cDNA synthesis buffer (4 µl, 1X final concentration), dNTP mix (2 µl, 500 µM each), anchored oligo-dt (1 µl, 500 ng/µl), 1 µl RT Enhancer, 1 µl Verso Enzyme mix, template RNA and nuclease-free water was added to make the volume of reaction mixture 20 µl and then incubated at 42°C for 60 min and then at 95°C for 2 min. qPCR was performed in triplicate reactions along with non-template control and negative control in each plate using a 7500 Fast Real-Time PCR system (Applied Biosystems, USA). The reactions were carried out in a total volume of 10 µl containing power SYBR® Green Master Mix, gene-specific primers, RNase-free water, and cDNA. The glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was used as the reference gene. Following primers were used:- *aanat2* gene-specific primers: CGNAT-F; 5'-ACT GGA CGA GGT GCT

GAA CT-3', CGNAT-R; 5'-CGT GGA TGT GGA CAG TAG GA-3', and *gapdh* gene-specific primers: CGGAP-F; 5'-ACC GGA GTC TTC CTC AGC A-3', CGGAP-R; 5'-TCA TGT TGG AGG GGT CGT A-3'.[13] Relative expressions of AANAT2 mRNA of all samples were calculated using the $2^{-\Delta\Delta}C_T$ (threshold cycle) method.[14]

Statistical analysis

All data were presented as the mean of three independent experimental results ± standard error of the mean. The data for AANAT2 mRNA levels were analyzed using One-way ANOVA followed by Tukey's Multiple Comparison Test by using GraphPad Prism V4.03.

RESULTS

Each histogram represents Mean ± Standard Error of the mean; n = 3. [a] and [c] Represent different levels of significance as compared with the control group: $p<0.05$ and $p<0.001$, respectively. In Figure (A), [f] Represents level of significance as compared with the db-cAMP treated group: $p<0.001$. [i] Represents level of significance as compared with H-89 treated group: $p<0.001$. In Figure (B), [d] and [e] Represent different levels of significance as compared with db-cGMP treated group: $p<0.05$ and $p<0.01$, respectively; In Figure (C), [f] Represents the level of significance as compared with db-cAMP treated group: $p<0.001$. [i] Represents the level of significance as compared with db-cGMP treated group: $p<0.001$. [j] Represents the level of significance as compared with chelerythrine chloride treated group: $p<0.05$; In Figure (D) [d] and [f] Represent different levels of significance as compared with db-cAMP treated group: $p<0.05$ and $p<0.001$, respectively. [g] Represents the level of significance as compared with db-cGMP treated group: $p<0.05$; and in Figure (E) [f] Represents the level of significance as compared with db-cAMP treated group: $p<0.001$. [i] Represents the level of significance as compared with db-cGMP treated group: $p<0.001$. db-cAMP significantly increased AANAT2 mRNA levels ($F_{3,11}$ = 268.8, $p<0.001$), H-89 had no significant effect on the levels of AANAT2 mRNA, but the stimulatory effect of db-cAMP on AANAT2 mRNA levels was significantly reduced in the presence of H-89 ($F_{3,11}$ = 268.8, $p<0.001$) (Figure 1A). db-cGMP significantly increased the levels of AANAT2 mRNA ($F_{3,11}$ = 15.09, $p<0.001$), KT5823 did not affect levels of AANAT2 mRNA, but the stimulatory effect of db-cGMP on AANAT2 mRNA levels was significantly reduced in the presence of KT5823 ($F_{3,11}$ = 15.09, $p<0.01$) (Figure 1B). Chelerythrine chloride did not affect AANAT2 mRNA levels, but the stimulatory effect of both cAMP and cGMP, separately as well as in combination, on AANAT2 mRNA levels was completely blocked in the presence of the inhibitor (Figure 1C).db-cAMP ($F_{7,23}$ =12.32, $p<0.001$) and db-cGMP ($F_{7,23}$ =12.32, $p<0.001$) significantly increased AANAT2 mRNA levels, U0126 ethanolate did not affect AANAT2 mRNA levels. The stimulatory effect of db-cAMP and db-cGMP on AANAT2 mRNA levels, when administered separately, was completely blocked in the presence of U0126 ethanolate, which did not affect the stimulatory effect of combined treatment with db-cAMP + db-cGMP on AANAT2 mRNA levels ($F_{7,23}$ =12.32, $p<0.05$) (Figure 1D). SB 202190 monohydrochloride hydrate did not affect AANAT2 mRNA levels. However, the stimulatory effect of db-cAMP ($F_{7,23}$ = 18.93, $p<0.001$), db-cGMP ($F_{7,23}$ = 18.93, $p<0.001$), and db-cAMP + db-cGMP on AANAT2 mRNA levels was completely blocked in the presence of the inhibitor (Figure 1E).

Each histogram represents Mean ± Standard Error of the mean; n = 3. [a] and [c] Represent different levels of significance as compared with the control group: $p<0.05$ and $p<0.001$, respectively. In Figure (A), [f] Represents the level of significance as compared with db-cAMP treated group: $p<0.001$. [i] Represents the level

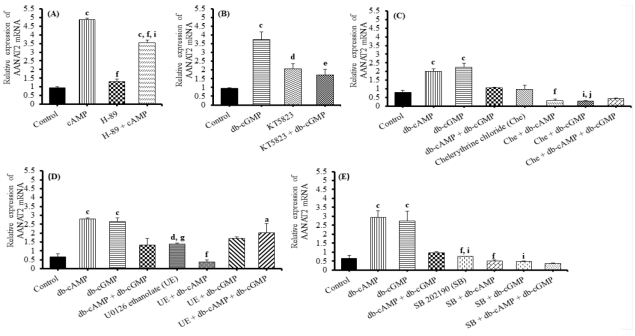

Figure 1: *In vitro* effect of (A) H-89 (B) KT5823 as well as db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence and absence of (C) chelerythrine chloride (D) U0126 ethanolate (E) SB 202190 monohydrochloride hydrate in the fish pineal organ.

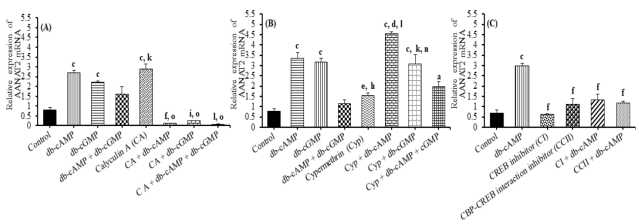

Figure 2: *In vitro* effect of db-cAMP and db-cGMP on AANAT2 mRNA levels in the presence or absence of (A) calyculin A and (B) cypermethrin as well as *in vitro* effect of (C) db-cAMP in the presence and absence of a CREB inhibitor (CI) and a CBP-CREB interaction inhibitor (CCII) in the fish pineal organ.

of significance as compared with db-cGMP treated group: $p<0.001$. [k] and [l] Represent different levels of significance as compared with db-cAMP + db-cGMP treated group: $p<0.01$ and $p<0.001$. [o] Represents the level of significance as compared with calyculin A treated group: $p<0.001$; In Figure (B), [d] and [e] Represent different levels of significance as compared with db-cAMP group: $p<0.05$ and $p<0.01$, respectively. [h] Represents the level of significance as compared with db-cGMP treated group: $p<0.01$. [k] and [l] Represent different levels of significance as compared with cypermethrin group: $p<0.01$ and $p<0.001$, respectively. [n] Represents the level of significance as compared with cypermethrin + db-cAMP treated group: $p<0.01$ and in Figure (C) [f] Represents the level of significance as compared with db-cAMP treated group: $p<0.001$. db-cAMP, db-cGMP, and calyculin A ($F_{7,23}$ = 44.03, $p<0.001$) significantly increased AANAT2 mRNA levels However, the stimulatory effect of db-cAMP ($F_{7,23}$ = 44.03, $p<0.001$), db-cGMP ($F_{7,23}$ =44.03, $p<0.001$) and cAMP + db-cGMP (Figure 2A) on AANAT2 mRNA levels was completely blocked in the presence of calyculin A. Incubation of the pineal organ with cypermethrin had no effect on AANAT2 mRNA levels or on the stimulatory effect of db-cAMP and db-cGMP on the transcript levels (Figure 2B).

CREB inhibitor (CI) and CBP-CREB interaction inhibitor (CCII) did not affect AANAT2 mRNA levels, but the presence of CI and CCII significantly inhibited the db-cAMP-induced increase in AANAT2 mRNA levels in the fish pineal organ ($F_{5,17}$ = 23.79, $p<0.001$) (Figure 2C).

DISCUSSION

The AANAT2 mRNA levels were significantly increased following *in vitro* treatment of the fish pineal organ with db-cAMP, and the db-cAMP-induced increase in AANAT2 mRNA levels was significantly decreased in the presence of H-89 (a specific inhibitor of PKA) suggesting that the stimulatory effect of cAMP on *aanat2* gene transcription is mediated by PKA (Figure 1A). A partial but significant reduction in cAMP-induced increase in AANAT2 mRNA levels in the presence of H-89 seems to suggest that besides its action via PKA, cAMP might also be stimulating *aanat2* gene transcription via additional pathway(s). Similar to db-cAMP, db-cGMP also significantly increased AANAT2 mRNA levels suggesting a direct involvement of cGMP in *aanat2* gene transcription. Further, it is important to mention that *in vitro* treatment with KT5823 (a specific inhibitor of PKG) significantly decreased the basal levels of AANAT2 mRNA, and completely blocked the cGMP-induced AANAT2 mRNA levels suggesting that cGMP stimulates *aanat2* gene transcription in the fish pineal only via PKG (Figure 1B). However, simultaneous treatment of the pineal organ with db-cAMP and cGMP did not affect the levels of AANAT2 mRNA. These findings seem to suggest that, in contrast to the mammalian pineal, both cAMP and cGMP are capable of stimulating *aanat2* gene transcription alone but in the absence of each other (Figure 1C). Based on our present findings, we suggest that both cAMP and cGMP generating pathways are separately involved in the stimulation of *aanat2* gene transcription as simultaneous treatment of the fish pineal organ with db-cAMP + db-cGMP did not affect *aanat2* gene transcription. Therefore, there is a possibility that simultaneous increases in cAMP and cGMP levels might be resulting in higher levels of the cyclic nucleotides leading to activation of the cyclic nucleotide-dependent phosphodiesterase enzyme, which converts cAMP and cGMP to AMP and GMP, respectively, and switches 'off' the downstream signaling for *aanat2* gene regulation.[1] This suggestion is supported by the report that effects of light on AANAT activity were negated by inhibitors of phosphodiesterase 6 (PDE6), the nitric oxide donor sodium nitroprusside and C-type natriuretic peptide (both increase cGMP levels), and by the calcium channel agonist Bay K 8644 (which prevents the cGMP-decrease-induced closure of cGMP-gated calcium channels) in the isolated chick pineal gland.[15] These findings also suggest that the stimulatory role of cAMP and cGMP in *aanat2* gene transcription in the fish pineal organ is in contrast to the reported effects of the cyclic nucleotides on *aanat* gene transcription in the mammalian pineal. Complete inhibition of the stimulatory effect of db-cAMP- and db-cGMP on AANAT2 mRNA levels in the presence of chelerythrine chloride (a specific inhibitor of PKC) indicates that PKC is a prerequisite for the stimulatory action of both cAMP and cGMP on AANAT2 mRNA levels (Figure 1C). Since increased intracellular levels of Ca^{2+} are essential for activation of PKC,[16] both the cyclic nucleotides seem to stimulate *aanat2* gene transcription by increasing intracellular Ca^{2+} followed by PKC activation. Inhibitors of calciproteins W7, and calmidazolium have been reported to inhibit melatonin secretion and cAMP levels in cultured trout pineal photoreceptors.[17] In trout pineal organs, voltage-gated L-type calcium channels have been reported to play a major role in the regulation of intracellular Ca^{2+} and melatonin.[11,18] Further, in the chick pineal photoreceptor cells, an increase in cAMP levels has been

reported to cause mobilization of internal Ca^{2+} stores.[19] Therefore, there is a possibility that cAMP stimulates *aanat2* gene transcription via both PKA and Ca^{2+}. The stimulatory effect of db-cAMP on AANAT2 mRNA levels was blocked completely, and of cGMP partially but significantly in the presence of U0126 ethanolate (a specific inhibitor of MEK). Further, the stimulatory effect of both the cyclic nucleotides on the levels of AANAT2 mRNA was fully blocked in the presence of SB 202190 (a specific inhibitor of p38 MAPK). These findings suggest that while MEK differentially mediates the stimulatory effect of cAMP- and cGMP-induced pathways involved in AANAT2 mRNA regulation, while normal MAPK activity is essential for the stimulatory effect of both the nucleotides on AANAT2 mRNA levels (Figure 1D and Figure 1E). In the rat pinealocytes, the effect of NE on MAPK phosphorylation represents the integration of two signalling mechanisms via PKA and PKG, each having an opposite effect on MAPK phosphorylation.[6] It has also been reported that p38 MAPK (but not p42/44MAPK) activation is tightly coupled to protein synthesis and degradation in the rat pineal gland.[20] Our findings also suggest that p38 MAPK plays an important role in the regulation of *aanat2* transcript levels in the photoreceptive fish pineal organ by cAMP- and cGMP-forming pathways.

A significant increase in AANAT2 mRNA levels in the fish pineal organ treated with calyculin A (a specific inhibitor of serine/threonine phosphatase 1 and 2A) indicates that both Ser/Thr phosphatase 1 and 2A are involved in the regulation of AANAT2 mRNA levels via cAMP and cGMP (Figure 2A). Unlike calyculin A, treatment of the fish pineal organ with cypermethrin (a specific inhibitor of serine/threonine phosphatase 2B) had no effect on AANAT2 mRNA levels or on db-cAMP- and db-cGMP-induced increase in AANAT2 mRNA levels in the fish pineal organ (Figure 2B). These findings rule out any role of serine/threonine phosphatase 2B in the regulation of AANAT2 mRNA levels. Significant inhibition of the stimulatory effect of db-cAMP on the levels of AANAT2 mRNA in the fish pineal organ in the presence of CREB inhibitor and CBP-CREB interaction inhibitor separately suggest that CREB and CREB-CBP interaction seem to be essential for increasing AANAT2 mRNA levels by cAMP-mediated pathway (Figure 2C).

CONCLUSION

Based on our findings, we suggest that AANAT2 mRNA levels in the photosensitive fish pineal organ are regulated by cAMP, cGMP, and Ca^{2+} as second messengers via a complex intracellular signaling machinery involving cAMP-PKA-CREB/CBP-CRE, cGMP-PKG/MEK-MAPK, and Ca^{2+}-PKC pathway. Further, serine/threonine phosphatases also seem to play a complex role in the up- and down-regulation of AANAT2 mRNA levels in the fish pineal organ.

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Authors' Contributions

Dr. Braj Bansh Prasad Gupta prepared the project, planned the experiments, provided financial support and scientific infrastructure, supervised the execution of the experiments and preparation of the manuscript. Ms. Hijam Nonibala executed the experiments, collected the experimental data, analyzed, plotted, and interpreted the data, and prepared the manuscript and Ms. Pynnehlang Warjri helped in executing the experiments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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