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**Cyclic AMP-dependent activation of rhodopsin gene transcription in
cultured retinal precursor cells of chicken embryo.**

Pierre Voisin and Marianne Bernard

Institut de Physiologie et Biologie Cellulaires, Université de Poitiers, CNRS ; 40 avenue du
recteur Pineau, Poitiers, F-86022, France.

Corresponding author: Pierre Voisin

email: pierre.voisin@univ-poitiers.fr

Phone: 05.49.45.39.78

Fax: 05.49.45.40.51

ABSTRACT

The present study describes a robust 50-fold increase in rhodopsin gene transcription by cAMP in cultured retinal precursor cells of chicken embryo. Retinal cells isolated at embryonic day 8 (E8) and cultured for 3 days in serum-supplemented medium differentiated mostly into red-sensitive cones and to a lesser degree into green-sensitive cones, as indicated by real time RT-PCR quantification of each specific opsin mRNA. In contrast, both rhodopsin mRNA concentration and rhodopsin gene promoter activity required the presence of cAMP-increasing agents (forskolin and IBMX) to reach significant levels. This response was rod-specific and was sufficient to activate rhodopsin gene transcription in serum-free medium. The increase in rhodopsin mRNA levels evoked by a series of cAMP analogs suggested the response was mediated by PKA, not by EPAC. Membrane depolarization by high KCl concentration also increased rhodopsin mRNA levels and this response was strongly potentiated by IBMX. The rhodopsin gene response to cAMP-increasing agents was developmentally gated between E6 and E7. Rod-specific transducin α subunit mRNA levels also increased up to 50-fold in response to forskolin and IBMX, while rod-specific phosphodiesterase-VI and rod arrestin transcripts increased 3- to 10-fold. These results suggest a cAMP-mediated signaling pathway may play a role in rod differentiation.

Keywords : retina, rhodopsin, transcriptional regulation, cyclic AMP

Running Title : cyclic AMP-dependent rhodopsin gene transcription

INTRODUCTION

During retinal development, the neuroblasts generated in the inner leaflet of the optic cup give rise to all retinal neurons (photoreceptors, horizontal cells, bipolar cells, amacrine cells, ganglion cells) and to Müller glial cells (Cepko *et al.*, 1996 ; Hatakeyama and Kageyama, 2004). In all vertebrate classes, the retinal cell types are generated in a conserved chronological order that may be reminiscent of conserved molecular determinants in retinogenesis (Yang, 2004). Ganglion cells are the first to differentiate, from a cohort of neuroblasts that exit mitosis early in development. Then horizontal cells, amacrine cells, photoreceptors, bipolar cells and Müller glial cells are generated, in that order, from neuroblasts that accomplish their final mitosis increasingly late in development (Levine *et al.*, 2000 ; Yang, 2004 ; Adler and Raymond, 2008). There is however considerable overlapping in this chronological sequence and the contextual signals that neuroblasts encounter at the exit of mitosis appear to play a major role in cell type determination. This is demonstrated by cell lineage studies indicating that the two sister cells of a final mitosis often differentiate into distinct cell types (Cepko *et al.*, 1996 ; Belecky-Adams *et al.*, 1996 ; Yang, 2004). A better understanding of this developmental signaling has been gained with the identification of diffusible molecules that regulate photoreceptor differentiation (*i. e.* taurine, activin, ciliary neurotrophic factor, basic fibroblast growth factor, sonic hedgehog ; Levine *et al.*, 2000, for review) and even the balance between rods and cones (*i.e.* retinoic acid and thyroid hormone ; Kelley *et al.*, 1995). Some of these signals would appear to act early-on in the cell fate decision cascade (*i. e.* sonic hedgehog ; Levine *et al.*, 1997) while others may act at terminal steps of photoreceptor differentiation, for example to implement the spectacular amplification of opsin gene expression that characterizes all photoreceptor subtypes (*i. e.* retinoic acid ; Kelley *et al.*, 1994). There is however considerable interspecies variability in the activity of

the identified factors and a unifying model of photoreceptor differentiation signaling is still wanted (Levine et al., 2000 ; Adler and Raymond, 2008).

In the chicken retina, photoreceptors differentiate from neuroblasts that exit mitosis between embryonic day 4 (E4) and E7, with a peak at E5 and E6 (Belecky-Adams *et al.*, 1996). In this species, the diversity of photoreceptors, based on opsin gene expression, comprises rods and four types of cones : red-sensitive, green-sensitive, blue-sensitive and violet-sensitive (Bruhn and Cepko, 1996 ; Adler and Raymond, 2008). Red-sensitive and green-sensitive cones are the first to express their respective opsins, at embryonic day 14 (E14) ; followed by rods at E15, blue-sensitive cones at E17 and violet-sensitive cones at E18 (Bruhn and Cepko, 1996 ; Cailleau et al. 2005). The long time lag between exit of mitosis and opsin gene expression would appear to give contextual signals plenty of time to act upon photoreceptor differentiation. As an experimental approach to this question, *in vitro* differentiation of photoreceptors can be obtained from retinal precursor cells isolated from chicken embryos around E7 (Adler, 2000). This experimental system has yielded valuable information on the regulation of photoreceptor-specific gene expression, including phototransduction proteins, melatonin-synthesizing enzymes and circadian transcription factors (Iuvone et al., 1990 ; Adler et al., 2001 ; Cailleau et al., 2005 ; Chaurasia et al., 2006). The photoreceptors that differentiate *in vitro* from chicken retinal progenitors are mostly red-sensitive cones, with about 1/10th green-sensitive cones (Bradford *et al.*, 2005). Red-sensitive opsin gene expression is actually accelerated in this culture system as compared to the *in vivo* situation (Adler et al., 2001), apparently in response to an unidentified serum factor (Cailleau *et al.*, 2005). In addition, the proportion of green-sensitive cones can be increased by ciliary neurotrophic factor, and that of rods by staurosporine (Xie and Adler, 2000). A better understanding of rod differentiation may be of special interest because this cell type appears particularly prone to degeneration, either in response to excessive light exposure (LaVail *et*

al., 1992), or as a result of mutations affecting either the structure or the expression levels of rhodopsin and other phototransduction proteins (Farrar *et al.*, 2002). Therefore, identifying the signaling pathways that favor rhodopsin gene expression may identify targets for future therapies. The mode of action of staurosporine being difficult to explore, due to its inhibiting a large number of protein kinases (Meggio *et al.*, 1995), we sought to identify a more physiological means of activating rhodopsin gene expression in developing chicken rods. Previous studies had indicated that cAMP-dependent protein kinase A (PKA) increased rhodopsin gene promoter activity in transgenic zebrafish (Yu *et al.*, 2007), while other studies had shown that adenylate cyclase-coupled receptors regulated rhodopsin mRNA levels in salamander rods in vitro (Alfinito and Townes-Anderson, 2001 ; Alfinito *et al.*, 2002). The observed effects were of modest amplitude (30-50%), however, we reasoned that these studies had been performed on mature rods, where rhodopsin gene transcription is set at a very high rate, so that cAMP had little chance to further increase this rate. Studies on embryonic retinal cells with low levels of rhodopsin gene expression had a better potential to unmask its regulation by cAMP. In the present report, we describe a strong stimulation of rhodopsin gene transcription by cAMP in retinal precursor cells of chicken embryo.

MATERIALS AND METHODS

Animals

Fertilized chicken eggs (*Gallus domesticus*) were purchased from Rumulo C^{ie} (Quinçay, France). The eggs were incubated at 37°C in an atmosphere of 70% humidity. Embryos were staged according to Hamburger and Hamilton (1951). The embryos were sacrificed by decapitation at different Hamburger and Hamilton's (HH) stages: HH28 (embryonic day 5.5 : E5.5), HH29 (E6), HH30 (E7) or HH34 (E8). Embryonic retinas were immediately transferred in culture medium, and processed for cell dissociation and culture, as described below. Adult chickens (postnatal day 15: P15) were purchased from Lecorne C^{ie} (Lencloître, France). They were anesthetized by an intraperitoneal injection of chloral hydrate and sacrificed by decapitation. Tissues from P15 chickens were frozen in liquid nitrogen and processed for RNA extraction. Animal care was conducted in accordance with the standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals; and French Agriculture and Forestry Ministry guidelines for handling animals, decree 87848, license E 86-040) and approved by the local ethical committee.

Embryonic retinal cell cultures

Primary cultures of embryonic chicken retinal cells were prepared as previously described (Adler, 1990), with slight modifications (Bernard and Voisin, 2008). Neural retinas, free of pigment epithelium, were dissociated by trypsin-DNase digestion and multiple pipetting. Unless otherwise indicated in the figure legends, $12 \cdot 10^6$ cells were seeded at high density ($1.25 \times 10^6/\text{cm}^2$) in non-coated 6-well plastic plates (NUNC, Illkirch, France) and cultured for 3 days (37°C, 5% CO₂, constant darkness) in medium 199 supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L glutamine (all from Sigma-Aldrich, Saint Quentin-

Fallavier, France), 0.1% bovine serum albumin (BSA) (Sigma-Aldrich) and 10% newborn calf serum (NCS) (Invitrogen, Cergy Pontoise, France).

Pharmacological treatments

Cells were allowed to adhere to culture dishes for 3 h prior to the addition of drugs. Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were dissolved in dimethyl sulfoxide (DMSO) (all from Sigma-Aldrich) and an equal volume of DMSO was added to the controls. N⁶-monobutyryl cyclic AMP (mB-cAMP), N⁶,2'-O-dibutyryl cyclic AMP (dB-cAMP), 8-(4-Chlorophenylthio) cyclic AMP (8CPT-cAMP), 8-Bromo cyclic AMP (8Br-cAMP), 8-Bromo cyclic GMP (8Br-cGMP), sodium butyrate (all from Sigma-Aldrich), 8-Bromo-2'-O-methyl cyclic AMP (Me-cAMP) and N⁶-Benzoyl cyclic AMP (Bz-cAMP) (both from BioLog, Bremen, Germany) were dissolved in double-distilled water and controls received an equal volume of water. Me-cAMP and Bz-cAMP were chosen as selective activators of the guanine nucleotide exchange protein directly activated by cAMP (EPAC) and of protein kinase A (PKA), respectively, based on their previously published pharmacological profiles (Christensen *et al.* 2003). Plasma membrane depolarization was induced by replacing 1/3rd the volume of culture medium by 150 mmol/L KCl and controls received an equal volume of 150 mmol/L NaCl.

Cell viability assays

Cell viability under different culture conditions and pharmacological treatments was assessed using the CellTiter96[®] reagent (Promega, Charbonnières, France) and confirmed by trypan blue exclusion. Data are expressed as percent of the viability measured 6 h after plating.

RNA extraction

Total RNA from cultured embryonic retinal cells and from adult chicken tissues was extracted as previously described (Cailleau *et al.*, 2005). RNA pellets were resuspended in diethyl pyrocarbonate (DEPC)-treated water (Invitrogen). RNA concentrations were measured by the optical density at 260 nm (OD₂₆₀) and adjusted to 0.25 µg/µL with DEPC-water. We verified that the RNA yield (mean ± sem, n = 48) was similar in the control cultures (12.06 ± 0.57 µg/dish) and in the forskolin-treated cultures (12.64 ± 0.72 µg/dish), thus confirming that pharmacological treatment did not affect cell viability.

Real time RT-PCR

Total RNA (1µg) was reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) and random hexamers (GE Healthcare Europe, Saclay, France), at 37°C for 2 h. The RT reaction was terminated on ice by a 1/5 dilution in double-distilled water. Real time PCR was performed on a LightCycler® (Roche Applied Science, Meylan, France) as previously described (Cailleau *et al.*, 2005). The primer pairs used in this study are listed in Table S1 and were validated as follows: they gave a single PCR product as verified by melting curve analysis, agarose gel electrophoresis and DNA sequencing, and the distribution of the PCR sigmoids was linear ($r \geq 0.99$) over 5 logs of template concentration with an efficiency of 1.85 to 1.98. We verified that 2- to 10-fold dilutions of the RNA introduced into an RT-PCR reaction caused the expected critical cycle increments. Reverse transcripts copy numbers were calculated from standard curves established with known quantities of each template. Results are expressed as number of copies of a target template per 10⁶ copies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) template. Congruency of the results obtained by real time RT-PCR and by northern blot was verified (Fig. 1). Statistical analyses were performed with the two-tailed Student's *t*-test.

Northern blot analysis

Total RNA (10 µg) from cultured embryonic retinal cells was electrophoresed and northern blotted as previously described (Bernard et al., 1993). Rhodopsin and GAPDH cDNA probes were produced by RT-PCR (using the primer pairs indicated in Table S1) and labelled by random priming (Feinberg and Vogelstein, 1983) with [α -³²P]dCTP (3000 Ci/mmol). Following hybridization, the northern blot was exposed overnight on the screen of a Storm 820 (GE Healthcare, Saclay, France) and densitometric analysis was performed with the ImageQuant software (GE Healthcare).

Promoter-reporter assay

The chicken rhodopsin promoter (Sheshberadaran and Takahashi, 1994) was PCR amplified from chicken genomic DNA with the primers listed in Table S1 and cloned upstream of the firefly luciferase reporter gene, in the pGL3-Basic plasmid (Promega). Recombinant clones were verified by DNA sequencing (Big Dye reagent and ABI 3130 Genetic Analyzer, Applied Biosystems, Courtaboeuf, France).

Before transfection, retinal cells from E8 embryos were seeded at high density in polyornithine-coated plates and cultured for 24h either in control conditions or in the presence of forskolin (10^{-5} mol/L) and IBMX (10^{-4} mol/L). They were then cotransfected by the CaPO₄ method, with 150 ng of the normalizing plasmid pRL-TK (Promega) and either with 1.5 µg of pGL3-pRhodo1.6kb or with 1.5 µg of a promoterless, negative control plasmid (pGL3-Basic), as previously described (Bernard and Voisin, 2008) and further cultured for 48h with or without forskolin and IBMX. Firefly and *Renilla* luciferase activities, were assayed using the Dual-Glo™ luciferase assay system (Promega). Light emission was measured on a

GloMaxTM20/20 luminometer (Promega). Transfection efficiency was normalized to the firefly/*Renilla* luciferase activity ratio.

RESULTS

1) Regulation of rhodopsin gene transcription by cAMP-increasing agents

Embryonic chicken retinal cells dissociated at E8 expressed very low levels of rhodopsin mRNA (400 to 2000 cDNA copies per million copies of GAPDH cDNA), similar to the concentrations observed in non-photosensitive tissues (Fig. 1A). The concentration of rhodopsin mRNA remained at the same low values after 3 days of culture in control medium (Fig. 1A). Addition of IBMX (100 $\mu\text{mol/L}$) or forskolin (10 $\mu\text{mol/L}$) to the culture medium increased rhodopsin mRNA levels 5-fold and 20-fold, respectively (Fig. 1A and Table S2). Addition of both drugs together caused a 50-fold rise in rhodopsin mRNA concentration (Fig. 1A and Table S2). This response was highly reproducible, although its amplitude could vary from 10- to 50-fold, as illustrated in Table 1. The rise in rhodopsin mRNA levels elicited by forskolin and IBMX was directly detectable on northern blot. Rhodopsin mRNA was detected as a major band of ~ 1.6 kb and a minor band of ~ 3 kb, in agreement with previous reports (Okano *et al.*, 1992 ; Adler *et al.*, 2001 ; Cailleau *et al.*, 2005). Densitometric analysis of the northern blot confirmed a 60-fold increase in rhodopsin mRNA levels (Fig. 1 B). Cell viability was not consistently affected by the cAMP-elevating agents (Fig. 1C). Transient transfection of a rhodopsin promoter-reporter construct into embryonic chicken retinal cells indicated that the rhodopsin promoter was very weak in control cultures (only 3-fold above a promoterless plasmid) and its activity increased 16-fold in the presence of forskolin and IBMX (Fig.2). A result confirming that rhodopsin mRNA levels reflect cAMP-dependent activation of transcription.

2) cAMP-dependent activation of photopigment gene transcription is rod-specific.

As reported earlier (Adler et al., 2001 ; Cailleau et al., 2005), cultured retinal cells contained high levels of red cone opsin mRNA, nearing half the concentration of GAPDH mRNA (Table 1). The concentration of green cone opsin mRNA was about 1/10th that of red cone opsin (Table 1). The mRNA concentrations for blue cone opsin and violet cone opsin were in the very low range (Table 1). In contrast to rhodopsin, the concentrations of transcripts encoding the different cone opsins were not increased by forskolin and IBMX, as confirmed in five independent experiments (Table 1). Depending on experiments, the cone opsins either failed to respond to cAMP-increasing agents or responded with a 50-80 % decrease in mRNA levels (Table 1). These results point to a rod-specific effect of cAMP.

3) Rhodopsin gene response to cAMP-increasing agents in different culture conditions

Previous studies have indicated that *in vitro* differentiation of retinal precursor cells can be altered by changes in cell density, plastic coating, or serum supplementation (Levine et al., 2000 ; Haque et al., 2003 ; Cailleau et al., 2005). This prompted us to investigate how these changes in culture conditions might affect the rhodopsin gene response to cAMP-increasing agents. As illustrated in Fig. 3A, forskolin increased rhodopsin mRNA levels to the same extent in retinal cells cultured at either high or low cell-density, on coated or uncoated plastic, as well as in serum-free or serum-supplemented medium (see Table S2 for details). A result confirming the robustness of the rhodopsin gene response. As illustrated in Fig. 3B, cell viability was not consistently affected by either forskolin or culture conditions.

4) Time course and dose-response to forskolin

Forskolin caused a significant 4-fold increase in rhodopsin mRNA levels after 12h (Fig. 4A). The concentration of transcript continued to increase at 24h and 48h, then plateaued-out 30-

fold above control values after 72h (Fig. 4A). In a 72h experiment, a significant 8-fold increase in rhodopsin mRNA levels could be observed at 1 μ mol/L forskolin and the stimulation reached 45-fold at 100 μ mol/L forskolin (Fig. 4B and Table S2).

5) Effect of membrane depolarization

Plasma membrane depolarization induced by 50 mmol/L KCl caused a 5-fold increase in rhodopsin mRNA concentration (Fig. 5). IBMX, which caused a 5-fold increase in rhodopsin mRNA levels by itself, strongly potentiated the effect of KCl to reach rhodopsin mRNA levels 45-fold above control values (Fig. 5 and Table S2).

6) Effect of cyclic nucleotide analogs through protein kinase A

The effects of cyclic nucleotide analogs confirmed a cAMP-dependent increase in rhodopsin mRNA levels. The order of potency was mB-cAMP > dB-cAMP \approx 8Br-cAMP > 8CPT-cAMP (Fig. 6A). This order of potency appeared to match the degree of specificity of these analogs for PKA (Christensen et al., 2003). This prompted us to examine the effect of analogs specifically designed to activate either PKA or EPAC. The PKA-selective analog N⁶-Bnz-cAMP efficiently increased rhodopsin mRNA levels, whereas the EPAC-selective analog 8Br-2'O-Me-cAMP was without effect (Fig. 6B and Table S2). Similar results were obtained in 3 independent experiments. We verified that neither 8Br-cGMP (1 mmol/L), nor sodium butyrate (1 mmol/L) had any effect on rhodopsin mRNA levels (data not shown). It was also verified that a representative cAMP analog did not affect cell viability (Fig. 6C).

7) Development of the rhodopsin gene response to cAMP-increasing agents

Previous studies have indicated that the developmental stage of donor embryos had a strong impact on the differentiation of retinal cells *in vitro* (Belecky-Adams et al., 1996 ; Ivanova

and Iuvone, 2003). In accordance with this, we observed that cells obtained at Hamburger and Hamilton (H.H.) stages 28 and 29 (E5.5 and E6) gave very weak responses of the rhodopsin gene to forskolin (Fig. 7), whereas cells obtained at H.H. stages 30 and 34 (E7 and E8) displayed full responses (Fig. 7 and Table S2). A result indicating that a critical event in the regulation of the rhodopsin gene takes place between E6 and E7.

8) cAMP-dependent regulation of transcripts related to rod-specific phototransduction

To investigate a possible role of cAMP as a general activator of rod differentiation, we examined the regulation of other genes of the rod-specific phototransduction cascade. Rod-specific transducin alpha subunit (Tar) mRNA levels were increased by forskolin and IBMX to the same extent (10- to 50-fold) as rhodopsin mRNA levels (Table 2). The concentration of mRNA encoding the rod-specific regulatory subunit of phosphodiesterase-6 (P γ r) was reproducibly increased 4- to 10-fold by forskolin and IBMX (Table 2). The levels of transcripts encoding the rod-specific catalytic subunit of phosphodiesterase-6 (P β) and rod-specific arrestin, displayed less reproducible responses to forskolin and IBMX (Table 2). Nevertheless, a 3- to 10-fold rise in mRNA concentration could be observed for these genes, in 5 out of 7 experiments (Table 2). These results suggest a pleiotropic effect of cAMP on rod-specific transcription.

DISCUSSION

The present study describes a robust cAMP-dependent regulation of rhodopsin gene transcription in cultured retinal cells from chicken embryo. The potency of this cAMP-dependent mechanism is underscored by the fact that it proved sufficient to activate rhodopsin gene transcription in the absence of serum-derived growth factors.

Despite many studies on photoreceptor differentiation in this model system, cAMP-dependent regulation of the rhodopsin gene had eluded detection until now. This response does not appear to reflect an accidental dysregulation of the rhodopsin gene in unspecified cells, because it was accompanied by the activation of four other genes of the rod-specific phototransduction cascade, with a strong correlation between rhodopsin and *Tor* regarding the amplitude of the response. The different amplitudes observed for the other rod-specific gene responses argue against a diffuse effect of cAMP on rod survival, as this would have resulted in an equal bias for all the rod-specific markers. Similar cell viabilities and RNA yields in control and forskolin-treated cultures also argue against pro- or anti-apoptotic effects of cAMP, in contrast to previous studies on cardiomyocytes and hippocampus (Ding et al., 2005 ; Mioduszevska et al., 2008). In addition, the cAMP effect appeared highly cell-type specific, as it failed to stimulate transcription of the cone opsin genes. Therefore, our data would rather suggest that cAMP is a significant second messenger in a signaling pathway that specifically activates rod differentiation. This is in keeping with multiple examples of a role of cAMP at specific steps of neuronal differentiation, including dopamine beta-hydroxylase gene activation in catecholaminergic neurons (Chen et al., 2005), morphological maturation of hippocampal neurons and cerebellar granule cells (Fujioka et al., 2004 ; Obara et al., 2007), neurite outgrowth in pheochromocytoma cell lines (Shi et al., 2006 and refs therein), upregulation of neuronal genes in stem cells (Tondreau et al., 2008).

Because previous studies aimed at identifying signals that stimulate rod differentiation have yielded contrasted results in different species (Levine *et al.*, 2000), it will be important to determine whether the cAMP effect described herein can be extended to other species. Previous studies on cAMP-dependent regulation of the rhodopsin gene in zebrafish, salamander and mouse cannot directly compare with the data presented herein, because they were performed on cells that already expressed appreciable levels of rhodopsin (Alfinito and Townes-Anderson, 2001 ; Alfinito *et al.*, 2002 ; Yu *et al.*, 2007 ; da Silva *et al.* 2008). Further studies in different species should be performed at adequate embryonic stages to determine whether cAMP-dependent activation of rhodopsin gene transcription is a conserved mechanism of rod differentiation in vertebrates .

Previous attempts to stimulate rod differentiation in cultured retinal cells from chicken embryo have indicated that staurosporine increased rhodopsin mRNA levels, as well as the number of rhodopsin mRNA-expressing cells (Xie and Adler, 2000 ; Bradford *et al.*, 2005). The lack of specificity of staurosporine, which is known to act as a general inhibitor of protein kinases (Meggio *et al.*, 1995) and as an apoptosis-inducing agent (Posmantur *et al.*, 1997) makes it difficult to relate its effect with that of cAMP. However, this possibility cannot be ruled out, because one report on peripheral blood mononuclear cells indicated that staurosporine enhanced both receptor-mediated (prostaglandin) and direct (forskolin) activation of adenylate cyclase (Buc *et al.*, 1998).

Because cAMP appears potentially relevant as a physiological regulator of rod differentiation, one obvious challenge raised by our findings is to elucidate the signaling pathway upstream and downstream of this second messenger. As a first step downstream of cAMP, it appeared important to determine whether this response was mediated by PKA or by EPAC, because both pathways have been shown to regulate gene expression and neuronal differentiation (Kim *et al.*, 2002 ; Shi *et al.*, 2006 ; Obara *et al.*, 2007 ; Gakhar-Koppole *et al.*, 2008). Our

experiments clearly decided in favour of PKA, based on the effects of PKA-selective versus EPAC-selective analogs and on the potency order of the more commonly used analogs (Christensen *et al.*, 2003 ; Connolly *et al.* 1992).

A further step down the signaling pathway will be to examine whether the rhodopsin gene promoter is directly activated by the phosphorylated form of the cAMP response element-binding protein (phospho-CREB). We already obtained evidence in promoter-reporter experiments that a 1.6 kb promoter of the chicken rhodopsin gene is sufficient to drive a 16-fold cAMP-dependent increase in transcription. Analysis of this sequence with the TESS program (Schug, 2003) revealed three incomplete matches to the cAMP response element that will deserve further analysis.

The coordinated effect of cAMP on five rod-specific transcripts is of interest as it suggests a pleiotropic effect on rod differentiation. Preliminary *in silico* investigations revealed putative cAMP response elements in the 5'-flanking regions of chicken P γ r, P β and Tar genes, but not in the rod arrestin promoter. The possibility that coordinated activation of rod-specific transcription reflects the action of an intermediate regulatory gene should also be considered. In contrast to mammals, where Nrl has been identified as a potent activator of rod differentiation (Kumar *et al.*, 1996 ; Mears *et al.*, 2001), a rod-specific transcription factor remains elusive in chicken. In the absence of Nrl, a possible candidate in chicken would L-Maf, whose timing of expression in rod precursor cells appears compatible with the activation of rhodopsin gene transcription (Ochi *et al.*, 2004). Regulation of L-Maf expression by cAMP has not been reported so far and would be worth studying.

Analysis of the signaling pathway, upstream of cAMP, warrants further studies aimed at identifying the adenylate cyclase-coupled receptors that regulate rhodopsin mRNA levels. Hopefully, this may open the way to pharmacological treatment of retinopathies characterized by low levels of rhodopsin gene expression (Farrar *et al.*, 2002). Another interesting

possibility, however, is that a rise in cAMP levels might be driven *in vivo* by depolarization of rod precursor cells, as was observed experimentally with high KCl concentration. Recently, retinal waves of depolarization spreading through the population of developing retinal ganglion cells have been shown to increase cAMP levels via activation of the Ca⁺⁺/calmodulin-activated adenylate cyclase, leading to cAMP-dependent regulation of ephrin-A5 signaling and refinement of axonal projections (Ravary et al., 2003 ; Rodger et al., 2005 ; Dunn et al., 2006 ; Nicol et al., 2007). The possibility of a similar mechanism taking place in rod precursors to trigger the cAMP-dependent activation of phototransduction genes would appear both attractive and testable.

It is generally agreed that photoreceptor differentiation occurs in two chronologically well separated steps: first, acquisition of competence soon after the exit of mitosis (from E4 to E7 in chicken) ; then, about one week later, activation of photoreceptor-specific gene transcription (Belecky-Adams et al., 1996 ; Bruhn and Cepko, 1996, Levine et al., 2000 ; Adler and Raymond, 2008). Previous studies have shown that retinal precursors isolated at E6 are capable of activating the red opsin gene when cultured for 4 days *in vitro* (Adler et al., 2001). A result indicating that red cone precursors are competent for red opsin gene activation as early as E6. In contrast, the results presented herein clearly show that rod precursors are not competent for cAMP-dependent rhodopsin gene activation at E6 and that they acquire this competence about one day later. The molecular mechanism of this developmental switch in competence is open to further analysis. One attractive hypothesis is that it might reflect chromatin remodeling at the rhodopsin locus. This would deserve investigation by means of chromatin immunoprecipitation protocols. Together, our data appear compatible with a scenario in which some retinoblasts become competent for rod differentiation as early as E7, provided they encountered a signal that raised cAMP levels. *In vivo*, this signal (receptor ligand or membrane depolarization) would be delivered only around E15, to activate

rhodopsin gene transcription (and maybe other rod-specific gene transcription). This new point of view on rod differentiation opens perspectives of research aimed at elucidating the mechanism of competence acquisition, the nature of the signal that raises cAMP levels in rod precursor cells and the relevance of this scenario to the *in vivo* situation.

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Table 1. cAMP-dependent activation of photopigment gene transcription is rod-specific.

Retinal cells from E8 chicken embryos were cultured either in control conditions (0.2% DMSO) or with 10^{-5} mol/L forskolin (Fsk) and 10^{-4} mol/L IBMX. mRNA levels were quantified by real time RT-PCR. The table summarizes the results of 5 individual experiments on triplicate samples. Results are expressed as number of copies of a given template per million copies of GAPDH template (mean \pm SEM, n = 15 ; **p<0.0001, *p<0.05, Student's *t*-test). The column on the right shows the fold-change observed in each individual experiment, with the median value underlined.

	Control (copies/ 10^6 GAPDH) mean \pm sem (n = 15)	Forskolin+IBMX (copies/ 10^6 GAPDH) mean \pm sem (n = 15)	Fold-changes in 5 individual experiments (median underlined)
Rhodopsin	785 \pm 106	19 264 \pm 1 290 **	10x ; 23x ; <u>25x</u> ; 32x ; 50x
Red Opsin	502 169 \pm 49 220	326 880 \pm 70 461	0.15x ; 0.58x ; <u>0.82x</u> ; 0.84x ; 1.2x
Green Opsin	40 157 \pm 7 260	27 240 \pm 6 322	0.38x ; 0.57x ; <u>0.58x</u> ; 0.65x ; 1.2x
Blue Opsin	79 \pm 20	50 \pm 14	0.3x ; 0.6x ; <u>0.68x</u> ; 0.94x ; 1x
Violet Opsin	2 385 \pm 595	857 \pm 238 *	0.18x ; 0.33x ; <u>0.35x</u> ; 0.47x ; 0.58x

Table 2. cAMP-dependent regulation of transcripts related to rod-specific phototransduction. Retinal cells from E8 chicken embryos were cultured either in control conditions (0.2% DMSO) or with 10^{-5} mol/L forskolin and 10^{-4} mol/L IBMX (F+I). The transcripts encoding GAPDH, rod-specific transducin alpha subunit (T α rod), rod-specific phosphodiesterase-6 gamma subunit (PDE γ rod), rod-specific phosphodiesterase-6 beta subunit (PDE β rod) and rod-specific arrestin were quantified by real time RT-PCR. The table summarizes the results of 7 individual experiments on triplicate samples. Results are expressed as number of copies of a given template per million copies of GAPDH template (mean \pm SEM, n = 21 ; **p<0.001, *p<0.01, Student's *t*-test). The column on the right shows the fold-increase observed in each individual experiment, with the median value underlined.

	Control (copies/10 ⁶ GAPDH) mean \pm sem (n = 21)	F+I (copies/10 ⁶ GAPDH) mean \pm sem (n = 21)	Fold-increases in 7 individual experiments (median underlined)
Tα (rod)	487 \pm 56	12123 \pm 1560 **	9x ; 13x ; 20x ; <u>34x</u> ; 41x ; 42x ; 48x
PDEγ (rod)	784 \pm 59	4465 \pm 473 **	3.7x ; 3.8x ; 4.5x ; <u>4.8x</u> ; 4.9x ; 6.9x ; 12x
PDEβ (rod)	671 \pm 183	1616 \pm 259 *	1.5x ; 1.7x ; 2.8x ; <u>3.5x</u> ; 4x ; 4x ; 5.6x
Arrestin (rod)	278 \pm 30	1626 \pm 202 **	1.7x ; 2.3x ; 4x ; <u>6.7x</u> ; 10x ; 10x ; 11x

FIGURE LEGENDS

Figure 1. Rhodopsin mRNA levels in adult chicken tissues and in embryonic chicken retinal cells cultured with cAMP-elevating agents.

A.) Real time RT-PCR detection of rhodopsin mRNA in adult (P15) chicken tissues (Mu, *muscle* ; Me, *mesencephalon*) and embryonic (E8) chicken retina (Re), and in E8 chicken retinal cells cultured either in control conditions (C: 0.2% DMSO), or with 10^{-4} mol/L IBMX (I), 10^{-5} mol/L forskolin (F), or both (F+I). Results are expressed as thousand copies of rhodopsin (Rh) template per million copies of GAPDH template (mean \pm SEM, n = 3). B.) Northern blot analysis of total RNA (10 μ g per lane) from E8 chicken retinal cells cultured as described in A (n = 5 in each group). The northern blot was hybridized successively with 32 P-labelled rhodopsin and GAPDH cDNA probes and exposed overnight on the screen of a Storm 820. Rhodopsin mRNA is detected as a major band of \sim 1.6 kb (large arrowhead) and a minor band of \sim 3 kb (small arrow). Densitometric analysis was performed with the ImageQuant software. C.) Cell viability as measured with CellTiter96[®].

Figure 2. Cyclic-AMP-dependent activation of the chicken rhodopsin gene promoter.

E8 chicken retinal cells were cultured either in control conditions (0.2% DMSO) or with 10^{-5} mol/L forskolin and 10^{-4} mol/L IBMX (F+I). The cells were transiently transfected with 1.5 μ g of a plasmid containing 1.6 kb of chicken rhodopsin promoter upstream of the firefly luciferase reporter gene (pGL3-pRhodo 1.6kb) or with 1.5 μ g of a promoterless plasmid (pGL3-Basic). Corrections for variations in transfection efficiency were performed by cotransfecting the cells with 150 ng of pRL-TK. Results are expressed as normalized firefly/*Renilla* luciferase activity ratio (mean \pm SEM, n = 4).

Figure 3. Influence of culture conditions on the rhodopsin gene response to forskolin.

E8 chicken retinal cells were seeded at low density ($0.3 \times 10^6/\text{cm}^2$) or at high density ($1.25 \times 10^6/\text{cm}^2$) in plastic plates that were either non-coated (NC), or coated with poly-D-lysine (PL, $5 \mu\text{g}/\text{cm}^2$), or with poly-ornithine (PO, $20 \mu\text{g}/\text{cm}^2$). Cells were cultured for 3 days in serum-supplemented medium or in serum-free medium supplemented with 0.5% BSA (no serum), either in control conditions (C: 0.1% DMSO) or with 10^{-5} M forskolin (F). A.) Rhodopsin and GAPDH mRNA levels were quantified by real time RT-PCR and expressed as thousand copies of rhodopsin (Rh) template per million copies of GAPDH template (mean \pm SEM n = 3). B.) Cell viability as measured with CellTiter96[®].

Figure 4. Time-course and dose-dependence of the rhodopsin response to forskolin.

A.) E8 chicken retinal cells were cultured for 12 to 72 h with 10^{-5} mol/L forskolin (Fsk: full circles) or with 0.1% DMSO (control: open circles). B.) E8 chicken retinal cells were cultured for 3 days with increasing concentrations of forskolin (10^{-6} mol/L to 10^{-4} mol/L: full circles) or with 1% DMSO (control: open circle). Rhodopsin and GAPDH mRNA levels were quantified by real time RT-PCR and expressed as thousand copies of rhodopsin (Rh) template per million copies of GAPDH template (mean \pm SEM, n = 3).

Figure 5. Effect of membrane depolarization.

Retinal cells from E8 chicken embryos were cultured either under control conditions (C: 50 mmol/L NaCl), or with 50 mmol/L KCl, with or without 10^{-4} mol/L IBMX. Rhodopsin and GAPDH mRNA levels were quantified by real time RT-PCR and expressed as thousand copies of rhodopsin (Rh) template per million copies of GAPDH template (mean \pm SEM, n = 3).

Figure 6. Effects of cAMP analogs on rhodopsin mRNA levels.

Retinal cells from E8 chicken embryos were cultured with the indicated concentrations of cAMP analogs and controls received an equal volume of water. A.) Cells were cultured with increasing concentrations of monobutyl cAMP (mB-cA), 8-Bromo cAMP (8Br-cA), N⁶,2'-O-dibutyl cAMP (dB-cA) or 8-(4-Chlorophenylthio) cAMP (CPT-cA). B.) Cells were cultured with 10⁻³ mol/L N⁶-Bnz-cAMP (BzcA), or 10⁻³ mol/L 8Br-2'O-Me-cAMP (MecA). Rhodopsin and GAPDH mRNA levels were quantified by real time RT-PCR and expressed as thousand copies of rhodopsin (Rh) template per million copies of GAPDH template (mean ± SEM, n = 3). C.) Cell viability as measured with CellTiter96[®] for control cells and for cells treated with 3.10⁻³ mol/L mB-cA.

Figure 7. Development of the rhodopsin gene response to cAMP-increasing agents.

Chicken embryos were staged according to Hamburger and Hamilton and their retinal cells were cultured either under control conditions (C: 0.2% DMSO) or with 10⁻⁵ mol/L forskolin plus 10⁻⁴ mol/L IBMX (F+I). Rhodopsin and GAPDH mRNA levels were quantified by real time RT-PCR and expressed as thousand copies of rhodopsin (Rh) template per million copies of GAPDH template (mean ± SEM, n = 3). Note that the HH29 and HH30 cultures were performed in the same experiment. Similar results were obtained in 3 independent experiments.

Figure 1

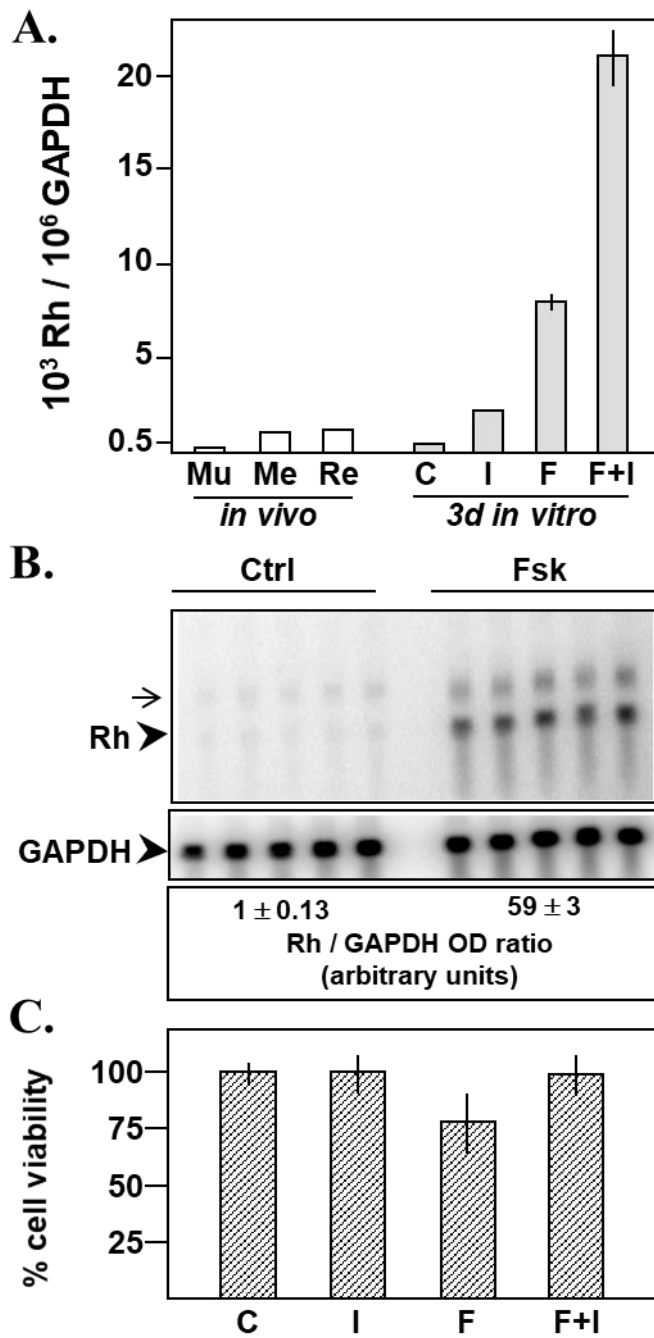


Figure 2

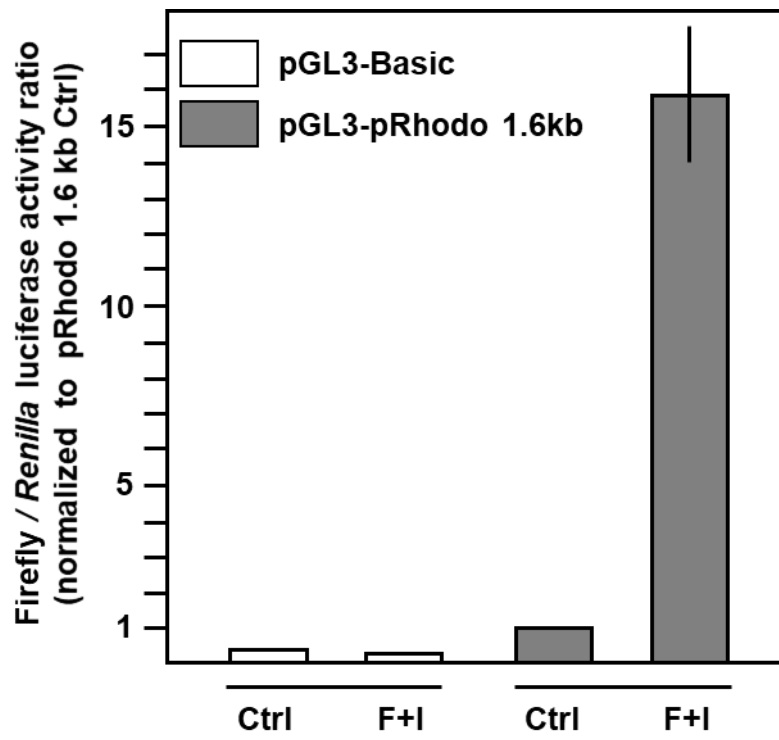


Figure 3

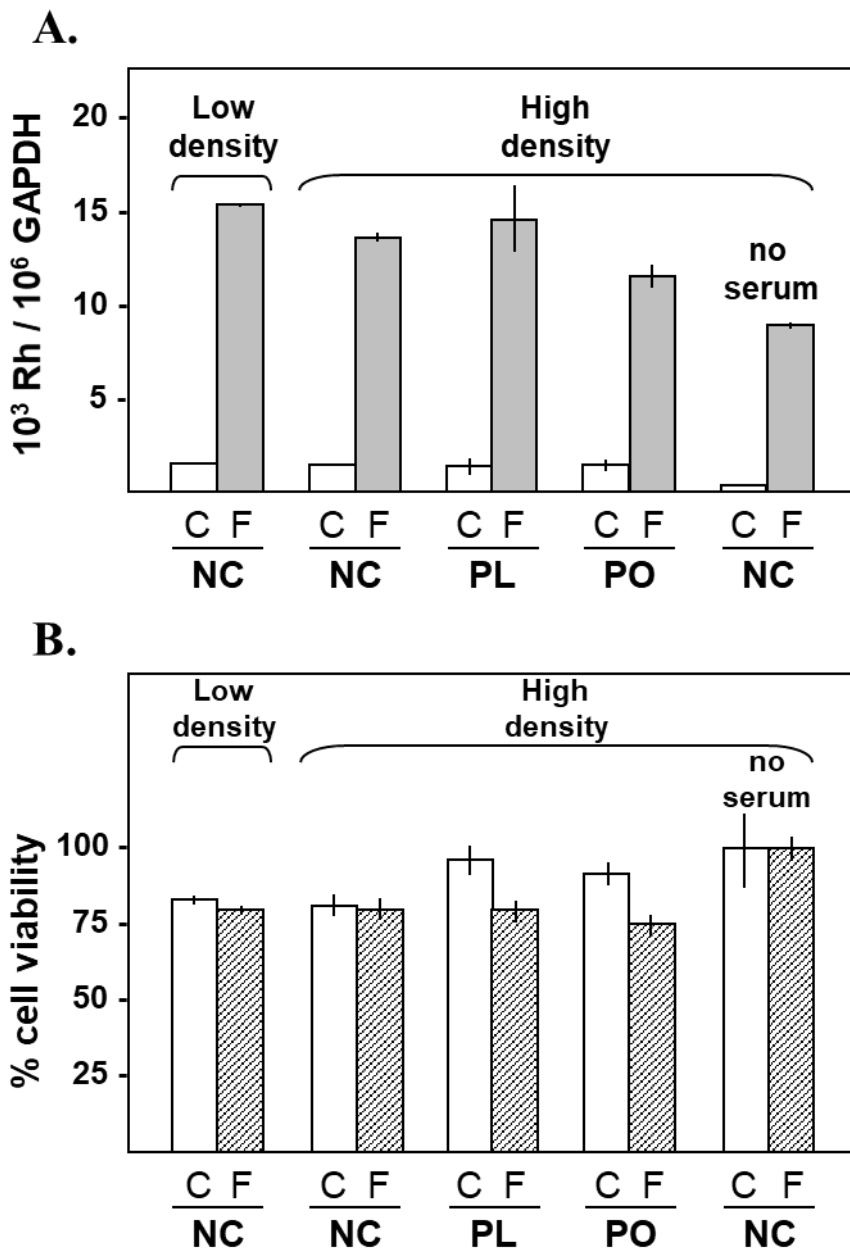


Figure 4

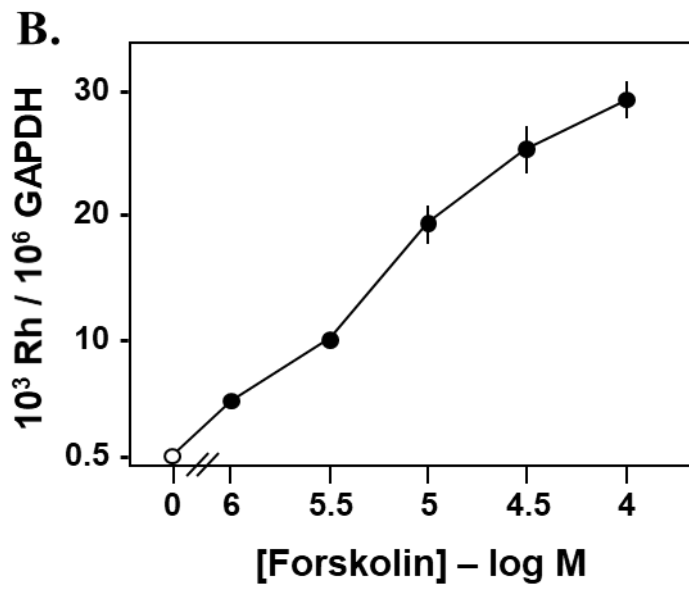
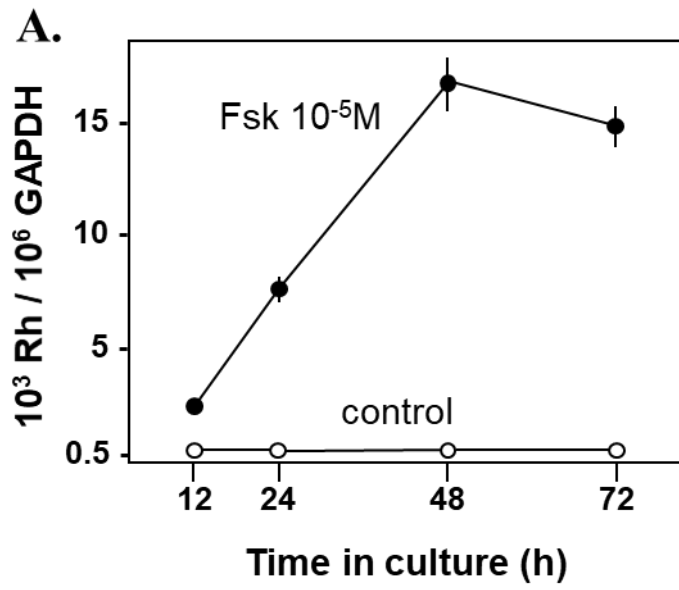


Figure 5

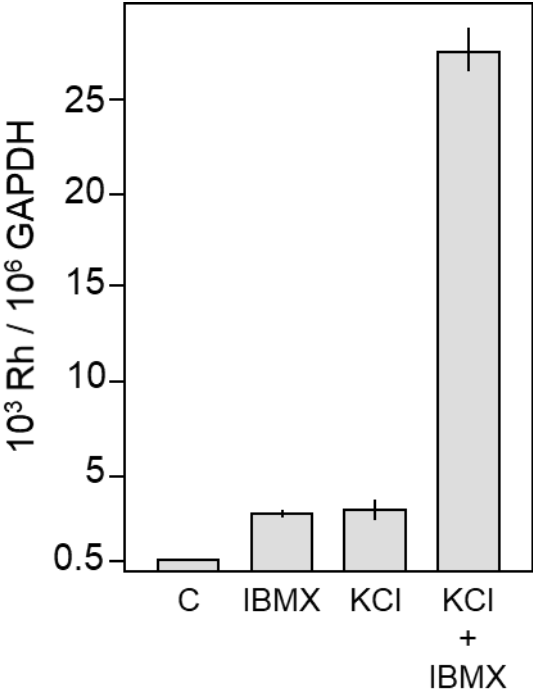


Figure 6

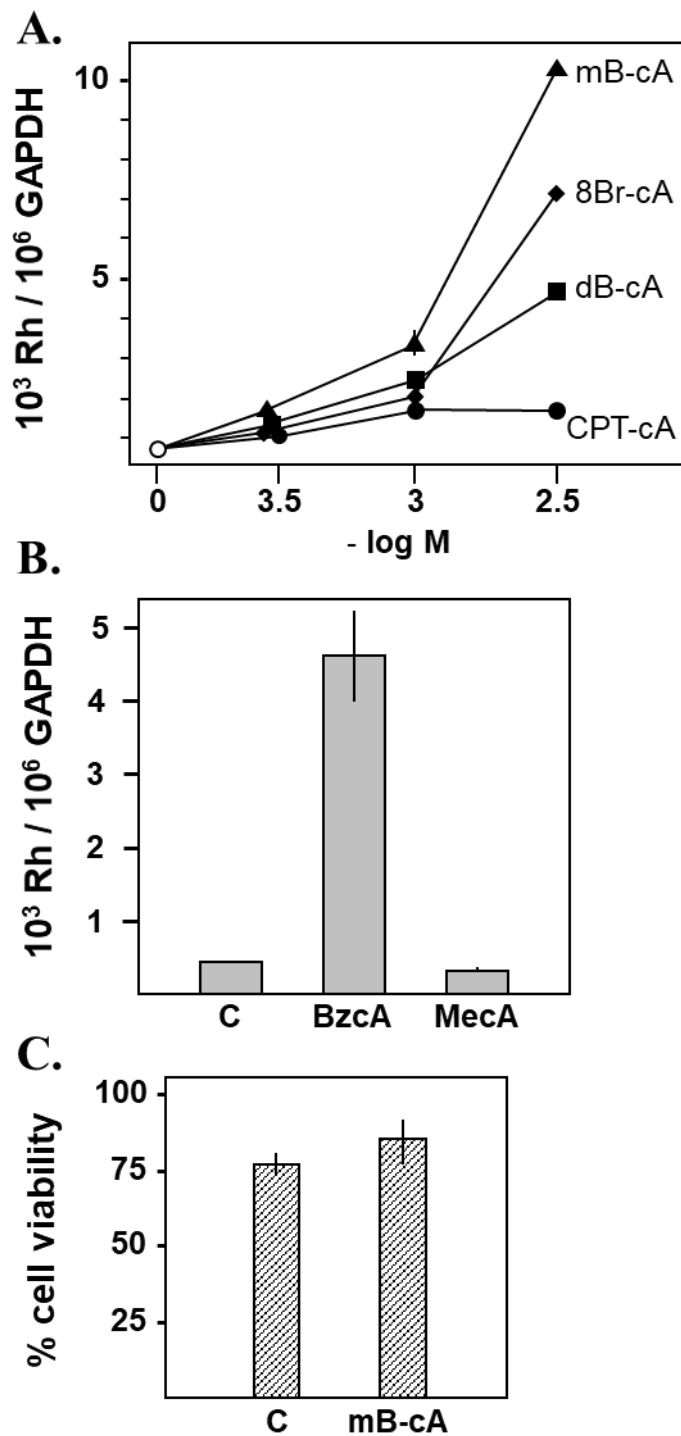


Figure 7

