Interaction Between Arachidonic Acid and cAMP Signaling Pathways Enhances Steroidogenesis and StAR Gene Expression in MA-10 Leydig Tumor Cells

(Revised version)

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Abstract

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Previous studies have demonstrated that trophic hormone stimulation induced cyclic AMP (cAMP) formation and arachidonic acid (AA) release from phospholipids and that both these compounds were required for steroid biosynthesis and steroidogenic acute regulatory (StAR) gene expression in MA-10 mouse Leydig tumor cells. The present study further investigates the synergistic effects of the AA and cAMP interaction on steroidogenesis. To demonstrate cAMP-induced AA release, MA-10 cells were pre-loaded with ^{3}H-AA and subsequently treated with dibutyryl cyclic AMP (dbcAMP). Stimulation with dbcAMP significantly induced AA release in MA-10 cells to a level 145.7% higher than that of controls. Lowering intracellular cAMP concentration by expressing a cAMP-phosphodiesterase significantly reduced human chorionic gonadotrophin-induced AA release. The dbcAMP-induced AA release was inhibited significantly by the phospholipase A_2 (PLA_2) inhibitor dexamethasone and also by the protein kinase A (PKA) inhibitor H89, suggesting the involvement of PKA phosphorylation and/or PLA_2 activation in cAMP-induced AA release. The effect of the interaction between AA and cAMP on StAR gene expression and steroid production was also investigated. While 0.2 mM dbcAMP induced only very low levels of StAR protein, StAR mRNA, StAR promoter activity and steroid production, all of these parameters increased dramatically as AA concentration in the culture medium was increased from 0 to 200 μM. Importantly, AA was not able to induce a significant increase in steroidogenesis at any concentration when used in the absence of dbcAMP. However, when used in concert with submaximal concentrations of dbcAMP (0.05 mM to 0.5 mM), AA was capable of stimulating StAR gene expression and increasing steroid production significantly. The results from this study demonstrate that AA and cAMP act in a highly synergistic manner to increase the

sensitivity of steroid production to trophic hormone stimulation and probably do so by increasing StAR gene expression.

Introduction

Cyclic AMP (cAMP) is a well-known second messenger in trophic hormone-stimulated steroidogenesis. However, trophic hormone stimulation not only induces cAMP formation, but also results in the release of arachidonic acid (AA) from intracellular phospholipids. Earlier observations in rat Leydig cells indicated that following luteinizing hormone (LH) stimulation, AA is released within one minute (Cooke et al., 1991). It was also reported that in human chorionic gonadotrophin (hCG)-stimulated rat Leydig cells AA release occurred in a concentration- and time-dependent manner with the amount of AA released being dependent upon hormone-receptor interaction and on the concentration of LH/hCG binding sites on the cell surface (Moraga et al., 1997). The inhibition of AA release using inhibitors of phospholipase A_2 (PLA_2), an enzyme catalyzing AA release from phospholipids, inhibited LH-stimulated steroid hormone production, but did not change the intracellular concentration of cAMP (Abayasekara et al., 1990). In addition, studies over the past two decades have demonstrated an essential role for AA in steroidogenesis in different steroidogenic cells (Band et al., 1986; Nishimura et al., 1989; Romanelli et al., 1995; Yamazaki et al., 1996), but the mechanism for the role of AA is not clear.

Those early studies also suggested that AA and its metabolites acted by regulating the rate-limiting step of steroidogenesis, cholesterol transfer to the mitochondrial inner membrane (Lopez-Ruiz et al., 1992; Mele et al; 1997). Indeed, our previous studies showed that AA acted at this step through its regulation of steroidogenic acute regulatory (StAR) protein expression (Wang et al., 1999; Wang et al., 2000). The StAR protein has been demonstrated to play a critical function in trophic hormone-stimulated steroidogenesis by facilitating cholesterol transfer to the mitochondrial inner membrane where the P450 side chain cleavage enzyme resides (Clark et al., 1994; Lin et al., 1995; Wang et al., 1998). Inhibition of AA release using PLA_2 inhibitors reduced LH or dbcAMP-induced StAR protein, StAR mRNA, StAR promoter activity and

concomitantly reduced steroid hormone production in MA-10 mouse Leydig cells (Wang et al., 1999; Wang et al., 2000). Importantly, the inhibitory effects of the PLA_2 inhibitors on StAR gene expression and steroid production were reversed by the addition of exogenous AA to the culture medium, indicating the necessity of AA in StAR gene expression and steroidogenesis. These studies further indicated that AA transduces the signal from the trophic hormone/receptor interaction to the nucleus through a pathway separate from the reported PKA-phosphorylation pathway and that both pathways are required, with neither alone being sufficient for trophic hormone-induced StAR gene expression and steroid production (Wang and Stocco, 1999; Wang et al., 2000). The mechanism(s) involved in the two-pathway co-regulation of StAR gene expression and steroid production remains unknown. The present study represents a continuation of the investigation into the mechanisms involving the two signaling pathways and demonstrates that dbcAMP stimulation directly induces AA release in MA-10 mouse Leydig cells and that the interaction between these two pathways enhances both steroidogenesis and StAR gene expression.

2. Materials and methods

2.1 Chemicals:

Arachidonic acid, N6,2-O-dibutyryladenosine-3:5-cyclic monophosphate (dbcAMP) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). H89 was obtained from Calbiochem (La Jolla, CA). Waymouth's MB/752 medium, horse serum, trypsin-EDTA, antibiotics and PBS were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). RO 20-1724 was purchased from Biomol (Plymouth Meeting, PA). Rabbit antisera generated

against N-62 StAR protein was a generous gift from Dr. W. L. Miller (Dept. of Pediatrics, University of California, San Francisco, CA). Donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Amersham (Arlington Heights, IL). ^{3}H-arachidonic acid was purchased from Dupont-New England Nuclear (Boston, MA). North2South Biotin Random Prime kits and Chemiluminescent Nucleic Acid Hybridization and Detection kits were obtained from Pierce (Rockford, IL). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Other common chemicals used in these studies were obtained from either Sigma or Fisher Chemicals.

2.2 Cell culture:

The MA-10 mouse Leydig tumor cells and MA-10(P29) cells, a stable transfectant which constitutively overexpresses cAMP-phosphodiesterase (Swinnen et al., 1991), were generous gifts from Dr. Mario Ascoli (Department of Pharmacology, University of Iowa College of Medicine, Iowa City, Iowa). MA-10 cells were grown in Waymouth's MB/752 medium containing 15% horse serum. MA-10(P29) cells were grown in the same medium which also contained 200 µg/ml G418 and 5µM RO 20-1724, a cAMP-phosphodiesterase inhibitor (Stocco and Ascoli, 1993). Prior to experiments, MA-10(P29) cells were cultured in medium containing 200 µg/ml G418 with or without 50µM RO 20-1724. The cells were cultured in 6-well tissue culture plates in an incubator at 37^{o}C and 5% CO_2.

2.3 Arachidonic acid release

The cells were cultured with 0.375 µCi ^{3}H-AA/ml in Waymouth's medium containing 1.5% horse serum for 4 hours, washed twice with 2 ml serum-free Waymouth's medium

containing 0.5% fatty acid-free BSA, and incubated in the same medium for 1 additional hour (Cooke et al.,1991; Moraga et al., 1997). The cells were then incubated in Waymouth's medium and stimulated with hCG or dbcAMP for one hour. The stimulation was terminated by the addition of an equal volume of cold methanol. The cells and medium were collected on ice, extracted (Powell, 1987) and centrifuged for 30 minutes at 2000 x g at 4°C. The supernatant was collected and analyzed for ^{3}H-AA (Yao et al., 1999), by reverse phase HPLC on a Waters Symmetry C18 column (4.6 x 75m, 3.5 µm particle size, Waters Corporation, Milford, MA) using a flow rate of 1 ml/min. The column eluate was collected in 1 ml fractions and the radioactivities of the fractions were measured using a Beckman LS 6500 scintillation counter (Beckman, Fullerton, CA). The total radioactivity in the fractions containing AA were determined and used for all further calculations. The methods described above were validated by characterizing the uptake of ^{3}H-AA into MA-10 cells. In these experiments we determined that on average 63.18% of the added ^{3}H-AA was incorporated into MA-10 cells. Of this total, it was found that 1.86% of the incorporated radioactivity could be extracted with this approach. Following HPLC separation, 0.44% of the incorporated ^{3}H-AA was detected in the AA fraction of the control group while 0.81% of the total was present in the dbcAMP-stimulated group.

2.4 Steroid production:

MA-10 cells were cultured for 30 min in serum-free Waymouth's medium containing AA (as described in the Figure Legends) and then stimulated with dbcAMP for 6 hours. The medium was collected at the end of each experiment and stored at -80°C. The cells were washed with cold PBS and stored at -80°C. Progesterone concentrations in the medium were determined by RIA (Resko et al., 1974).

2.5 MA-10 cell transfections:

MA-10 cells (0.5×10^6 per well) were cultured in 6-well plates overnight. The cells in each plate were transfected with 6 μg DNA of the StAR promoter/luciferase plasmid expressing firefly luciferase driven by the -966bp sequence of the StAR promoter (Caron et al., 1997). Transfections also included 75 ng of the pRL-SV40 vector DNA (a plasmid which constitutively expresses Renilla luciferase, a control reporter under the control of the SV40 promoter, Promega, Madison, WI). Transfections were performed using FuGENE Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. After 48 hours in culture, the cells were used for experiments.

2.6 Luciferase assay:

Following the experiments, the cells were washed three times with ice-cold PBS and lysed with Passive Lysis Buffer (Promega, Madison, WI). The supernatants were used for luciferase assays using a Dual Luciferase Reporter Assay System following the manufacturer's instructions (Promega, Madison, WI). The Relative Light Units (expressed as the reading from the StAR promoter/luciferase divided by the reading from Renilla luciferase) were measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

2.7 Northern analyses:

In experiments designed to determine StAR expression at the mRNA level, cells were washed three times with cold PBS and used for total RNA purification using TRIzol reagent following the manufacturer's instructions (GibcoBRL, Grand Island, NY). The RNA was

separated by electrophoresis in an agarose/formaldehyde gel (1%/6%) and blotted onto a Hybond-N^+ membrane (Amersham, Alington Heights, IL). StAR mRNA on the membrane was probed with biotin-labeled mouse StAR cDNA and detected using the North2South Chemiluminescent Nucleic Acid Hybridization and Detection kit following the manufacturer's instructions (Pierce, Rockford, IL). The membrane was stripped with a buffer containing 15 mM NaCl, 15 mM sodium citrate and 1% SDS, pH7.0 for 30 min at 55^{o}C. 18S rRNA on the membrane was probed to adjust for the RNA loading in each lane.

2.8 Western blot analysis:

StAR protein expression in MA-10 cells was detected by Western blot analysis as described previously (Wang, et al., 1998). Western analysis data was obtained from experiments that were performed at least three times and the results of one representative experiment are shown.

2.9 Statistical analyses:

Each experiment was repeated at least three times. Statistical analysis of the data was performed with ANOVA using the Stat View SE system (Abacus Concepts, Berkeley, CA). The data were expressed as mean ± standard error.

3. Results

3.1. dbcAMP-induced arachidonic release

To study the effect of cAMP on AA release, MA-10 cells were pre-incubated with ^{3}H-AA and then stimulated with dbcAMP. ^{3}H-AA released from the cells was extracted and separated by HPLC. Stimulation with dbcAMP significantly increased AA release from MA-10 cells by 145.7% over that of control cells (Fig. 1A). Co-incubation with the PLA_2 inhibitor dexamethasone almost completely inhibited dbcAMP-induced AA release, there being no significant difference in AA release between control and dexamethasone-treated groups. Treatment of MA-10 cells with 30 μM of the PKA inhibitor H89 also resulted in a significant inhibition of ^{3}H-AA release from the cells.

To verify the role of cAMP in AA release, MA-10(P29) cells, a stable transfectant of MA-10 cells which constitutively overexpress the enzyme cAMP-phosphodiesterase, were used in the analysis of AA release. Following pre-loading with ^{3}H-AA, MA-10(P29) cells showed little response to the trophic hormone stimulation, with 50 ng/ml hCG resulting in a 17.4% increase in ^{3}H-AA release over controls (Fig. 1B). However, in the presence of RO 20-1724, an inhibitor of cAMP-phosphodiesterase, the same dosage of hCG induced a 50.4% increase in ^{3}H-AA release. RO 20-1724 alone did not significantly affect the AA release.

3.2. Synergistic effects of arachidonic acid on StAR protein expression and steroid production

To determine the effects of AA on dbcAMP-stimulated StAR protein expression and steroidogenesis, increasing concentrations of exogenous AA were added to MA-10 cell cultures containing 0.2 mM dbcAMP. As shown in Fig. 2A, 0.2 mM dbcAMP resulted in a low level of StAR protein expression. However, the addition of AA to the cell culture increased StAR protein significantly beyond control levels. This stimulatory effect of AA occurred in a concentration-dependent manner, with StAR protein increasing as the concentration of AA in the media

increased. The addition of 200 µM AA to the MA-10 cell cultures increased StAR protein mass by 2.8 fold when compared to cells stimulated with 0.2 mM dbcAMP alone.

As StAR protein mass increased, a concomitant increase in steroid production was also detected in the groups treated with AA (Fig. 2B), an increase that was positively correlated with AA concentration in the medium. Steroid production increased by 3.6 fold when AA was elevated from 0 µM to 200 µM in the culture medium containing 0.2 mM dbcAMP.

AA alone did not affect StAR protein expression and steroid production. In the absence of dbcAMP but in the presence of 175 µM AA, neither StAR protein nor steroid production were significantly increased over that seen in control cells (Fig.3). However, the synergistic effect of AA was once again clearly evident when dbcAMP was present in the culture medium. The addition of 175 µM exogenous AA to the MA-10 cell culture containing 0.25 mM dbcAMP resulted in a 4.5 fold increase in StAR protein and a significant 1.8 fold increase in steroid production compared to stimulation with 0.25 mM dbcAMP alone ($P < 0.0001$).

3.3. Synergistic effects of arachidonic acid on StAR gene transcription

To study the possible mechanism involved in the effect of AA on dbcAMP-stimulated StAR protein expression and steroidogenesis, total RNA was isolated from MA-10 cells treated with 0.2 mM dbcAMP and increasing concentrations of AA and then assayed by Northern analysis for StAR mRNA content. Two major bands of 1.6 and 3.4 kilobases were detected and shown in Fig. 4A. A low level of StAR mRNA was detected in the MA-10 cells stimulated with 0.2 mM dbcAMP but containing no exogenous AA. As also seen in this figure, StAR mRNA levels increased significantly as the concentration of AA in the culture medium was increased. As AA concentration increased from 1 µM to 200 µM, total StAR mRNA levels increased to levels 3.5 fold of those detected in the MA-10 cells treated with 0.2 mM dbcAMP alone.

Similar results were obtained in studies utilizing StAR promoter activity assays (Fig. 4B). Stimulation with 0.2 mM dbcAMP alone did not significantly increase StAR promoter activity, however, promoter activity was enhanced by the addition of increasing concentrations of AA. The addition of 100 µM AA resulted in a significant 1.6 fold increase in StAR promoter activity which was further increased to 1.9 fold with the addition of 200 µM AA ($P<0.0001$).

The presence of dbcAMP in the culture medium is critical for the observed effect of AA on StAR gene transcription. In both the absence of dbcAMP and when the concentration of dbcAMP was below 0.1 mM, 175 µM AA had no statistically significant effect on the stimulation of either StAR mRNA levels or StAR promoter activity (Fig. 5). However, as the level of dbcAMP was increased to 0.1 mM and above, this same dose of AA induced a significant ($P<0.001$) increase in both StAR mRNA and StAR promoter activity as seen in Fig. 5.

4. Discussion

Trophic hormone-stimulation of steroidogenic tissues induces the expression of StAR protein, an event which has been demonstrated to play a critical role in steroidogenesis by facilitating the transfer of cholesterol to the mitochondrial inner membrane where it is converted to pregnenolone by the P450 side chain cleavage enzyme (Stocco, 2001). However, the manner in which trophic hormones regulate StAR gene expression is not completely clear. It has been proposed that trophic hormone-stimulation induces cAMP formation followed by activation of PKA which in turn phosphorylates transcription factors involved in StAR gene expression (Reinhart et al., 1999). Moreover, in previous studies it has also been demonstrated that trophic hormone stimulation results in the release of arachidonic acid (AA) from intracellular phospholipids and that this release was a requirement for obtaining normal levels of steroidogenesis (Abayasekara et al., 1990; Romanelli et al., 1995; Cooke, 1999). In a more

recent study, our laboratory demonstrated that inhibition of AA release in MA-10 cells blocked steroid biosynthesis and did so by blocking the expression of StAR protein in spite of high intracellular levels of PKA activity (Wang et al., 2000). This present study has further demonstrated that AA and cAMP positively interact with each other resulting in a synergistic effect on StAR gene expression and steroid production in MA-10 Leydig cells.

One aspect of this interaction is the stimulatory effect of cAMP on AA release in MA-10 mouse Leydig cells. This was demonstrated by two separate observations obtained in the present study (Fig. 1). First, the incubation of MA-10 cells with a cAMP analog significantly increased AA release. Second, the expression of cAMP-phosphodiesterase, which lowers the level of endogenous cAMP in hCG-stimulated MA-10 cells, significantly reduced AA release, and the subsequent inhibition of this enzyme reversed this reduction. The decrease in AA release in response to trophic hormone stimulation of the MA-10(P29) cells is well in agreement with the reported cAMP levels found in previous studies in these cells following similar treatments. Swinnen et al., (1991), demonstated that upon stimulation, wild type MA-10 cells produce significantly more cAMP than do MA-10(P29) cells, thus coroborating the observations obtained in the present studies. While dbcAMP stimulated AA release, the AA release was significantly inhibited by the PLA_2 inhibitor dexamethasone and also by the PKA inhibitor H89 (Fig. 1A). This strongly suggests the involvement of cAMP-activated PKA phosphorylation and PLA_2 activation in AA release from phospholipids. Further studies will be needed to elucidate the role of PKA phosphorylation in PLA_2 activation. Another possible mechanism for cAMP-dependent AA release was reported by Finkielstein et al. who described a cAMP-induced 43-kDa phosphoprotein which they named arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt). ARTISt shows the highest substrate specificity with arachidonyl-CoA and plays a role in steroidogenesis by regulating AA release from this substrate (Finkielstein et al., 1998). Also, an earlier study reported an hCG-induced AA release that was

dependent upon hormone-receptor interaction or the concentration of LH/hCG binding sites on the cell surface (Moraga et al., 1997). In that study, stimulation with low dosages of hCG induced significant levels of AA release while cAMP levels remained unchanged. This suggested a cAMP-independent AA release involving the direct activation of PLA_2, possibly occurring through an interaction of the hormone-receptor complex with a G protein. Our results and the data from this earlier study (Moraga et al., 1997) indicate that trophic hormone stimulation may induce AA release through both cAMP dependent and independent mechanisms. The functional relationship, if any, between these two different modes of AA release remains unknown.

Another aspect of this interaction is the synergistic effect of AA on cAMP-induced StAR protein expression and steroid production. This is clearly indicated by the results shown in Fig. 2 and Fig. 3. This observation is supported by previous studies that reported the stimulatory effects of AA on both basal and hormone-stimulated testosterone production in the testes of rat and goldfish (Wade and Van der Kraak, 1993; Romanelli et al., 1995). The synergistic effect of AA on dbcAMP-stimulated StAR protein expression and steroidogenesis was concentration-dependent, being highly dependent upon the levels of AA in the cell culture medium. In the presence of AA in the culture medium, 0.25 mM dbcAMP was able to increase StAR protein to a level higher than that obtained by treatment with 0.5 mM dbcAMP alone (Figure 3). These results indicate that the synergistic interaction between these two signaling pathways may act to significantly enhance the sensitivity and/or efficiency of trophic hormone action, a concept that could have very important physiological implications for our understanding of the function of the trophic hormones. Also, this observation may help to explain the reports of others documenting the stimulatory effects of some growth factors on steroidogenesis in cases where intracellular cAMP levels remained unchanged while AA release was enhanced (Majercik and Puett, 1991).

Our results further demonstrate that the presence of cAMP in the culture medium is essential for observing the synergistic effects of AA on both steroid biosynthesis and StAR gene expression. In the absence of dbcAMP, AA alone did not increase StAR protein and steroid production significantly. However, with AA present in the culture medium, we observed a concentration-dependent effect of dbcAMP on this synergistic effect of AA (Fig. 3). These results suggest that a critical threshold level of cAMP, or perhaps PKA activity activated by cAMP, is required for the synergistic effect of AA on StAR protein expression and steroidogenesis. Continuing to increase the concentration of dbcAMP above 0.5 mM did not induce a proportional increase in the synergistic effect possibly indicating that ability of AA to produce its subsequent effects was almost maximal at this concentration.

The mechanism for the synergistic effect of AA on dbcAMP-stimulated StAR protein expression and steroid production is unknown. The data shown in Figures 4 and 5 illustrate that AA enhanced dbcAMP-stimulated StAR gene expression at the level of transcription, as reflected by our findings that AA significantly increased dbcAMP-induced StAR promoter activity and StAR mRNA levels. As discussed earlier, this effect was also cAMP-dependent. Similarly, the synergistic effect of AA on StAR gene transcription was positively correlated with AA concentrations, with StAR promoter activity and StAR mRNA increasing as AA in the culture medium increased. To date there is not sufficient information to explain the mechanism for how this two-pathway interaction enhances StAR gene transcription. Recently, preliminary results obtained in our laboratory showed that the interaction of AA with dbcAMP increased the binding of a transcription factor(s) to a StAR promoter region located between -68 and –96bp, a location that is essential for the StAR promoter activity (unpublished data). It is possible that signals transduced through these pathways act to co-regulate the activation or expression of transcription factors regulating StAR promoter activity.

In summary, the observations obtained in the present study demonstrate a synergistic interaction between AA and cAMP which results in significant increases in StAR gene expression and steroid production in MA-10 mouse Leydig cells. In this interaction, cAMP has a stimulatory effect upon AA release from intracellular phospholipids and the AA released can in turn enhance cAMP-induced StAR gene expression and steroid biosynthesis. The interaction between these two pathways may serve to increase the sensitivity of trophic hormone action and as such could represent an important physiological phenomenon.

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Figure Legends

Fig. 1. Stimulatory effect of dbcAMP on arachidonic acid release in MA-10 mouse Leydig cells. The cells were pre-loaded with ^{3}H-AA and then treated as indicated in the figure. After treatments, the ^{3}H-AA release was analyzed by HPLC as described in the Materials and Methods. The ^{3}H-AA release was calculated and expressed as percentages of control. The average total ^{3}H-AA release of the control group was 7.59 pg for MA-10 cells and 4.56 pg for MA-10(P29) cells. (A), MA-10 cells were cultured in medium containing 2.5 μM of the PLA_2 inhibitor dexamethasone (Dex) or 30 μM of the PKA inhibitor H89 for 30 minutes and then stimulated with dbcAMP for one hour. The ^{3}H-AA release was analyzed. *, significantly different from control or dexamethasone-inhibited group ($P<0.005$). **, highly significantly different from control ($P<0.001$). ***, very highly significantly different from control ($P<0.0001$). (B), MA-10(P29) cells were cultured in medium containing 200 μg/ml G418 and 50 μM RO 20-1724, a cAMP-phosphodiesterase inhibitor for one hour. Then the cells were cultured in the same medium and stimulated with 50 ng/ml hCG for one hour. The ^{3}H-AA release was analyzed. *, significantly different from control ($P<0.05$). **, highly significantly different from hCG stimulation ($P<0.0005$)

Fig. 2. Arachidonic acid enhanced dbcAMP-stimulated StAR protein expression and steroid production. MA-10 cells were cultured for 30 min in serum-free Waymouth's MB/752 medium with increasing concentrations of AA as indicated in the figure and then stimulated with 0.2 mM dbcAMP for 6 hours. (A), the cells were collected and 25 μg of cell lysate protein was used to analyze StAR protein by Western blot. Each StAR specific band was quantitated using the BioImage Visage 2000 and expressed as integrated optical density (IOD). (B), progesterone production in the medium was analyzed by RIA and expressed as percentages of the highest

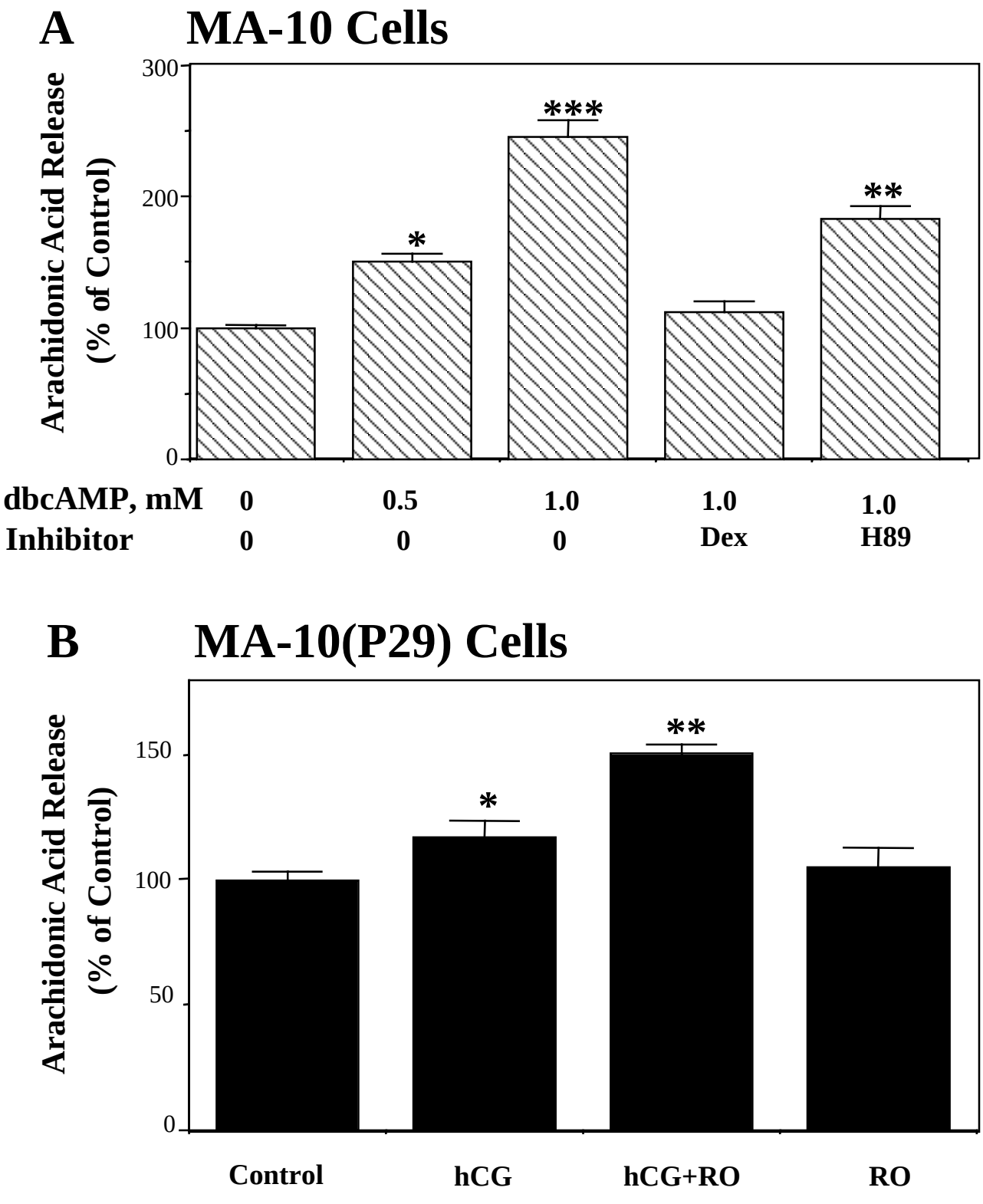
production (the average of the highest progesterone production was 442.58 ng/ml culture medium). *, significantly different from 0.2 mM dbcAMP stimulation (P<0.005). **, highly significantly different from 0.2 mM dbcAMP stimulation (P<0.0001).

Fig. 3. Role of dbcAMP in the synergistic effect of arachidonic acid on StAR protein expression and steroid production. MA-10 cells were cultured for 30 min in serum-free Waymouth's MB/752 medium with 175 μM AA and stimulated with increasing concentrations of dbcAMP for 6 hours. (A), the cells were collected and 25 μg of cell lysate protein was used to analyze StAR protein by Western blot. Each StAR specific band was quantitated using the BioImage Visage 2000 and expressed as integrated optical density (IOD). (B), progesterone production in the medium was analyzed by RIA and expressed as percentages of the highest production (the average of the highest progesterone production was 88.65 ng/ml culture medium). *, significantly different from the paired group without AA (P<0.05). **, highly significantly different from the paired group without AA (P<0.0001).

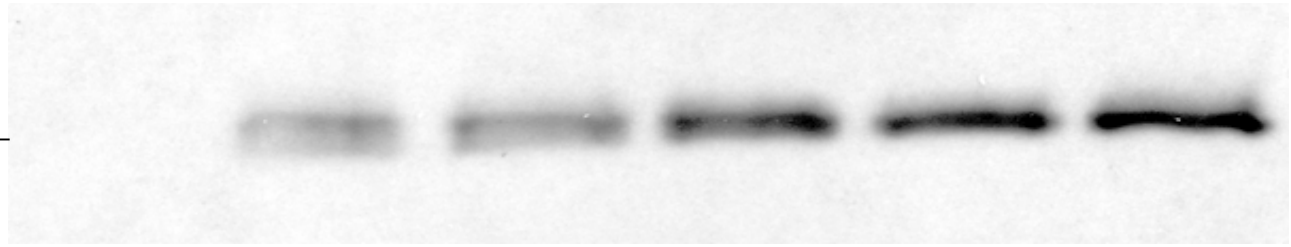
Fig. 4. Arachidonic acid enhanced dbcAMP-stimulated StAR mRNA levels and StAR promoter activity. (A), MA-10 cells were cultured for 30 min in serum-free Waymouth's MB/752 medium with increasing concentrations of AA as indicated in the figure, and stimulated with 0.2 mM dbcAMP for 6 hours. Cells were collected for total RNA purification. StAR mRNA was analyzed by Northern blot and StAR specific bands were quantitated using the BioImage Visage 2000 and expressed as total integrated optical density (IOD). (B), MA-10 cells were transfected with a StAR promoter/luciferase plasmid expressing firefly luciferase driven by -966 bp of the StAR promoter and pRL-SV40 vector DNA, a plasmid which constitutively expresses Renilla luciferase. After 48 hours in culture, the cells were treated as described above. The cell lysate was used for the luciferase assay using a Dual Luciferase Reporter Assay System as described in

Materials and Methods. StAR promoter activities were expressed as percentages of the highest activity (the average of the highest activity was 0.132 Relative Light Units). *, significantly different from 0.2 mM dbcAMP stimulation ($P<0.0001$).

Fig. 5. Role of dbcAMP in the synergistic effect of arachidonic acid on StAR gene transcription. (A), MA-10 cells were cultured for 30 min in serum-free Waymouth's MB/752 medium with 175 μM AA, and stimulated with increasing concentrations of dbcAMP for 6 hours. Cells were collected for total RNA purification. StAR mRNA was analyzed by Northern blot and StAR specific bands were quantitated using the BioImage Visage 2000 and expressed as total integrated optical density (IOD). (B), cells were transfected with a StAR promoter/luciferase plasmid expressing firefly luciferase driven by -966 bp of the StAR promoter and pRL-SV40 vector DNA, a plasmid which constitutively expresses Renilla luciferase. After 48 hours in culture, the cells were treated as described above. The cell lysate was used for the luciferase assay using a Dual Luciferase Reporter Assay System as described in Materials and Methods. StAR promoter activities were expressed as percentages of the highest activity (the average of the highest activity was 0.335 Relative Light Units). *, significantly different from the paired group without AA ($P<0.0001$).

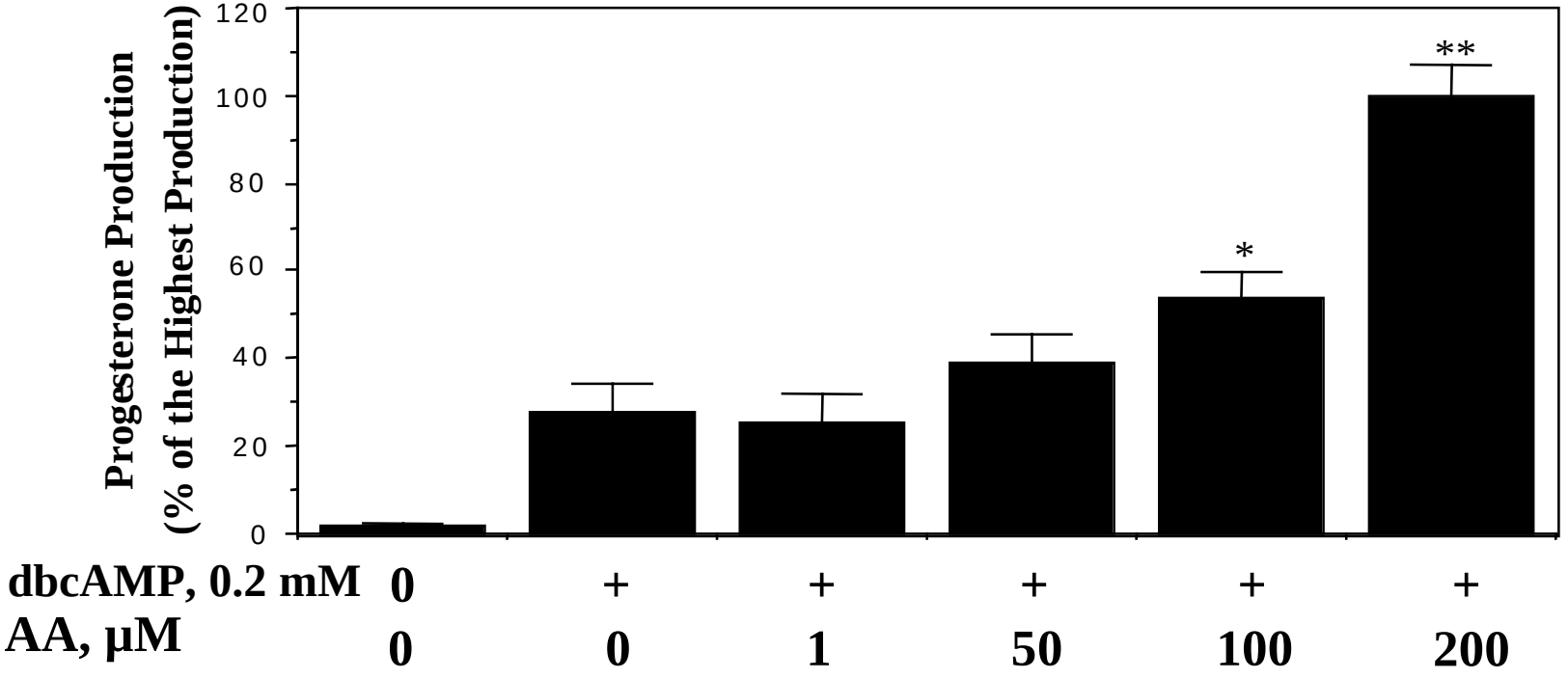

A
MA-10 Cells
Arachidonic Acid Release
(% of Control)
300
200
100
0
*
***
**
dbcAMP, mM 0 0.5 1.0 1.0 1.0
Inhibitor 0 0 0 Dex H89
B
MA-10(P29) Cells
Arachidonic Acid Release
(% of Control)
150
100
50
0
*
**
Control
hCG
hCG+RO
RO

A StAR Protein

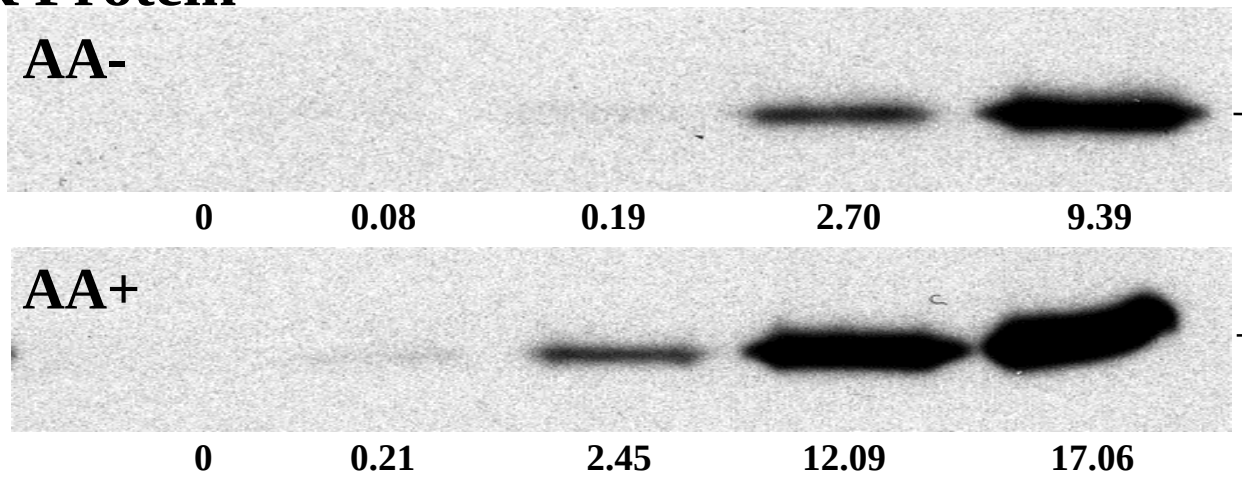


IOD	0	1.27	1.78	3.07	3.11	3.53

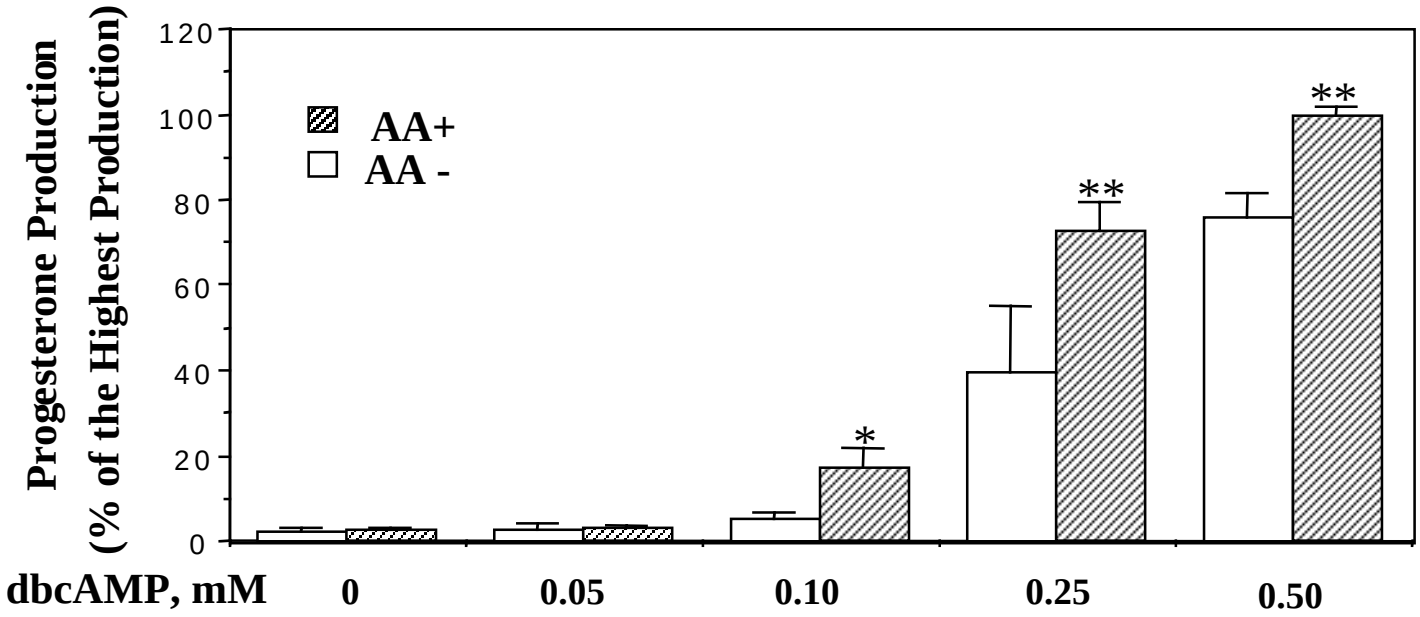
B Progesterone Production



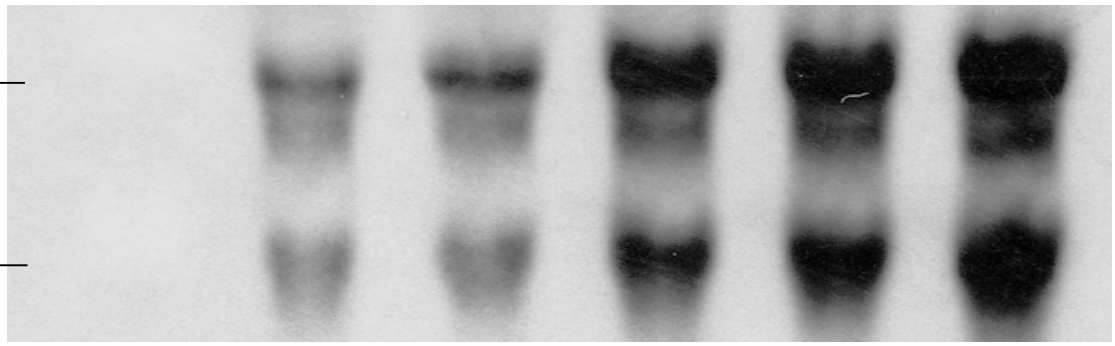
A StAR Protein



B Progesterone Production

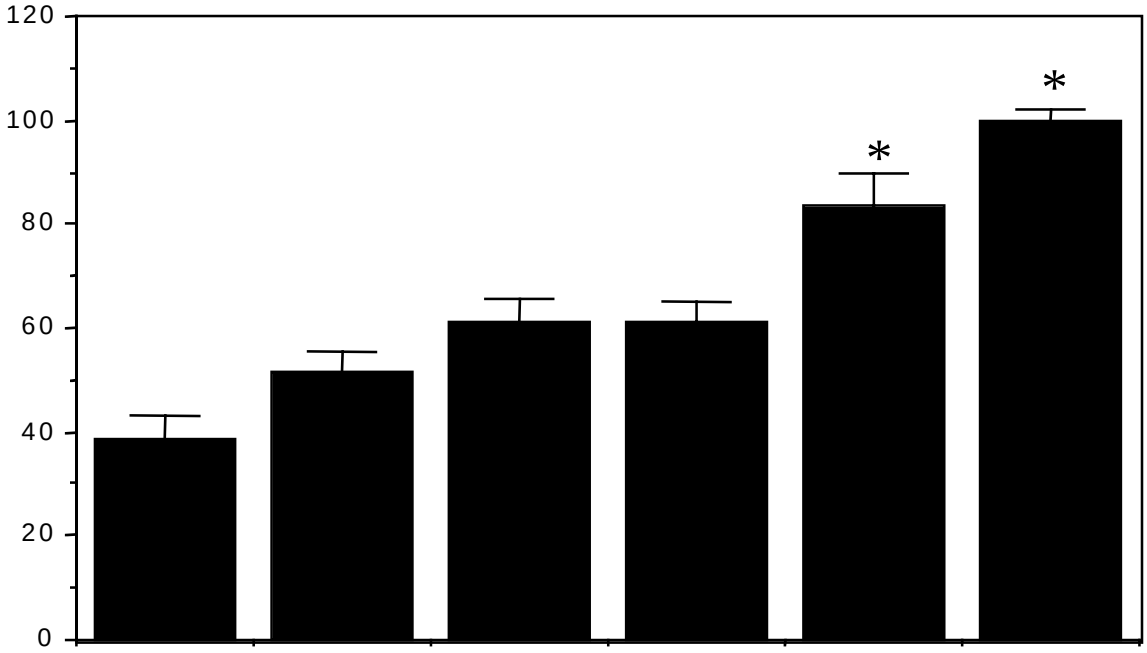


A StAR mRNA



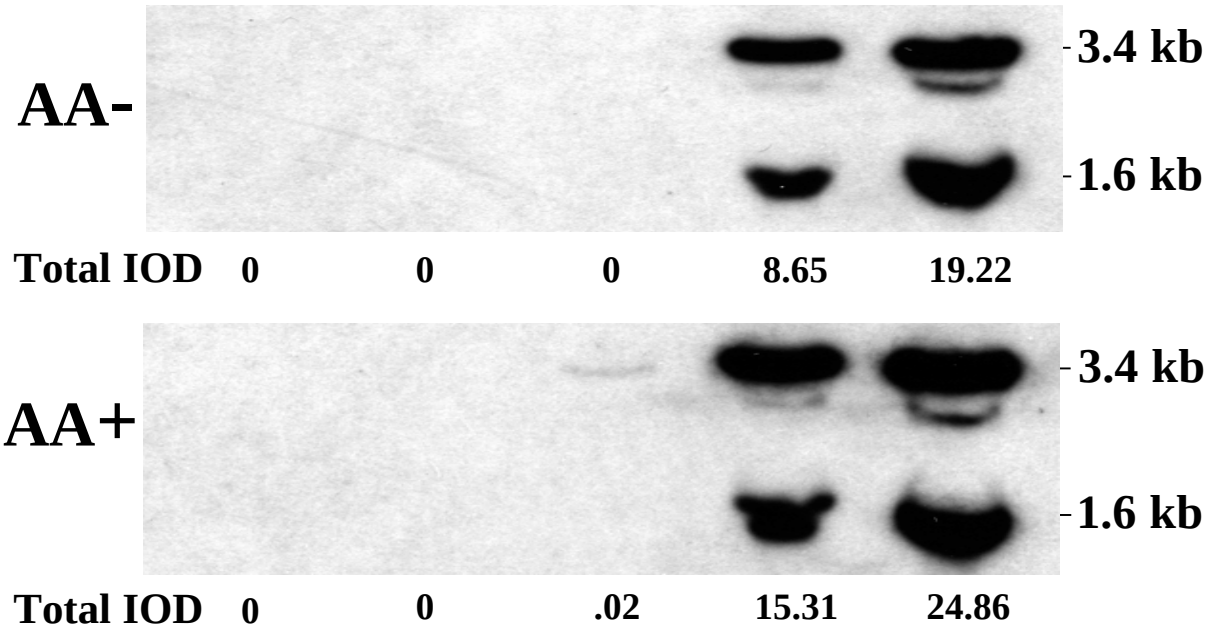
Total IOD	0	11.39	15.53	29.10	32.63	40.38

B StAR Promoter Activity



dbcAMP, 0.2mM	0	+	+	+	+	+
AA, µM	0	0	1	50	100	200

A StAR mRNA



B StAR Promoter Activity