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Production and Application of a Polyclonal Peptide Antiserum for Universal Detection of StAR Protein

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Abstract

We report production of a polyclonal antibody against the StAR (steroidogenic acute regulatory) protein of steroidogenic cells and immunohistochemistry (IHC) staining of bovine adrenal gland tissue available in paraffin block. The epitope-specific polyclonal antibody was produced in a rabbit immunized against a synthetic 26 amino acid peptide (82AMQRALGILKDQEGWKKESRANGDE107) derived from the coding sequences reported for the bovine StAR gene (Gene Bank Accession No. Q28918). Western blots were developed using the StAR-specific peptide antiserum with an alkaline-phosphatase-conjugated anti-rabbit IgG second antibody chromogenic system. The antiserum was found to be highly specific for StAR, which exhibited an estimated molecular weight of about 30 KDa for all species analyzed. Finally, the peptide antiserum was successfully employed to localize StAR protein by immunohistochemical staining of thin sections prepared from bovine adrenal gland tissue. This study is the first to report a polyclonal peptide antiserum that apparently recognizes native StAR protein, regardless of the species of origin. The successful production of the antibody has provided a useful tool for studying regulation of StAR protein.

Key Words: StAR, peptide, antibody, steroidogenesis, immunohistochemistry

Introduction

Steroidogenesis is the process in which specialized cells in specific tissues synthesize steroid hormones. An example of an important class of steroid hormones is the adrenal glucocorticoids which regulate carbohydrate metabolism and manage the stress response. Other examples of steroid hormones include the ovarian progesterones and estrogens, which regulate reproductive function and secondary sex characteristics in the female. The testicular androgens are essential for fertility and secondary sex characteristics in the male. Although the steroid hormones have distinct functions, their biosynthetic pathways are identical in the initial stages (11, 25).

The biosynthesis of steroid hormones is regulated by such pituitary trophic hormones as adrenocorticotropin (ACTH) and luteinizing hormone (LH). In all steroidogenic tissues, the initial step in steroidogenesis is the conversion of cholesterol to the first steroid, pregnenolone (14, 18). The rate-limiting step controlling steroidogenesis is the steroidogenic acute regulatory protein (StAR) protein-mediated translocation of cholesterol from the cholesterol-rich outer mitochondria membrane to the cholesterol-poor inner membrane, which is where the cholesterol side cleavage enzyme (P450scc) is located (7).

StAR, a phosphoprotein expressed in steroidogenic cells, is essential for this cholesterol translocation process. Since the cloning of StAR in 1994, the importance of

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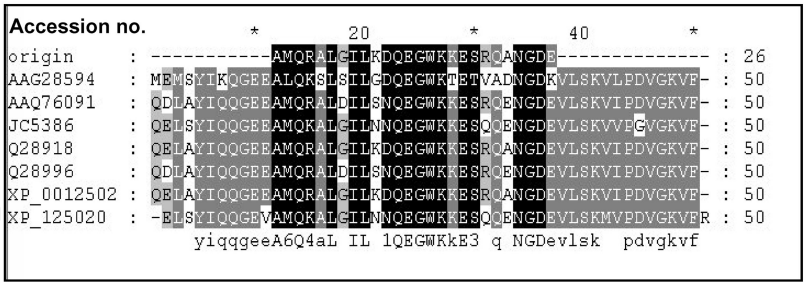

Fig. 1. Amino acid sequences comparison for chicken (C), pig (P), rat (R), bovine (B), and mouse (M) StAR protein. Amino acid sequences were derived from gene coding sequence data deposited in Gene Bank. Shaded boxes indicate contiguous; totally conserved regions of amino acid sequence within the protein.

this protein in hormonally regulated steroid biosynthesis has been confirmed by many investigators (15).

Orem-Johnson and his colleagues identified a group of mitochondrial 30 KDa phosphoprotein that appeared in adrenal cells stimulated with ACTH and gonad cells stimulated with LH (20, 23). The induction of this protein could be blocked by protein synthesis inhibitors, and its presence was directly correlated with steroidogenesis. The identical protein was found in 8-dibutyryl-cAMP-stimulated MA-10 mouse Leydig cells by Stocco and his colleagues. Indeed, the role of StAR in cholesterol translocation has been the subject of several reviews that can be examined for a more comprehensive summary of studies performed to date (21-24).

The adrenal cortex produces the glucocorticoid cortisol and the mineralocorticoid aldosterone under the control of regulatory systems that largely function independently. The cortex is divided into 3 distinct zones-the outer zona glomerulosa, the middle zona fasciculata, and the inner zona reticularis-defined by different cellular arrangements. These zones are functionally distinct, *i.e.*, mineralocorticoids are synthesized in the zona glomerulosa, glucocorticoids are produced by the zona fasciculata reticularis, and androgens are synthesized in the zona reticularis (18, 19, 27, 28). The steroidogenic acute regulatory protein shuttles cholesterol to the inner mitochondrial membrane. The production of cortisol in the zona fasciculata occurs in 5 steps: cleavage of the cholesterol side chain by the cholesterol desmolase enzyme cytochrome CYP11A1 (P450scc) to yield pregnenolone; conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase with accompanying D5,4-isomerization; and successive hydroxylations at the 17α, 21, and 11β positions, each mediated by a distinct cytochrome P450, resulting in cortisol. Cortisol is synthesized under the trophic control of ACTH and in turn regulates ACTH synthesis in the pituitary gland by means of a negative feedback loop (10, 17).

The main methodologies for the assay of StAR protein expression in steroidogenic tissue or cells are Western blotting and immunohistochemistry. This is necessary primarily for the determination of rodent or mammal StAR protein. However, the antisera and antibodies employed in these assays exhibit varying degrees of species-specificity that constrains their application to accurately measure StAR protein in samples obtained from a broader range of animal species. The purpose of this work was to produce an antiserum that would be useful in the immunological detection of StAR protein without concerning its species of origin. To do this we compared the published amino acid sequences derived from the coding sequences for all reported StAR genes and determined unique of conservation (Fig. 1). We selected a specific sequence of bovine StAR protein and synthesized a 26 amino acid peptide against which a polyclonal antiserum was produced by immunizing rabbits with a KLH-conjugated peptide antigen. Our results, reported here, detail the application of this polyclonal peptide antiserum in the detection of native StAR protein from the expression response in adrenocortical cells stimulated by ACTH or cAMP and the different animal species.

Materials and Methods

Peptide Synthesis and Antiserum Production

Peptide synthesis and antiserum production were conducted according to standard and proprietary protocols. A peptide corresponding to a portion of the bovine StAR protein sequence (82AMQRALGILKDQEGWKKESRANGDE107) was synthesized using a solid phase support and FMOC chemistry. The C-terminal of this peptide was conjugated to 8 branches of multiple antigenic peptide (MAP). The MAP-peptide conjugate was purified by gel filtration

chromatography and freeze-dried. After reconstitution in deionized water, the conjugate was mixed with either complete (Sigma F-5881) or incomplete Freund adjuvant (Sigma F-5506) just before immunizing the 2 New Zealand white rabbits. Twelve separate injections of the peptide-MAP conjugate were made for each rabbit at 2-week intervals. Each injection was divided among multiple subcutaneous sites and delivered a total of 0.1 mg of peptide. The initial immunization was given in Freund complete adjuvant and all subsequent (booster) immunizations were given in Freund incomplete adjuvant. Blood (yielding 25-30 ml of serum per rabbit) was collected prior to immunization and at 2-week intervals commencing on the seventh week following the initial immunization. Serum antibody titer (determined by ELISA using the peptide antigen) rapidly rose to 1:10,000 and remained at that level throughout the ensuing immunization period (12).

Immunohistochemistry

Adrenal gland samples obtained from bovine tissue was fixed overnight in 10% formalin solution, embedded in paraffin, sectioned at 5 μm, and the sections were mounted on poly-L-lysin coated glass slides. Following deparaffinization in xylene, tissue sections was rehydrated by passing them through decreasing concentrations of ethyl alcohol to Tris-buffer saline. Endogenous peroxidase activity was eliminated by incubating the sections for 15 min in methanolic H_2O_2. Nonspecific binding was blocked with normal goat serum, followed by 3 hours, incubation with anti-StAR peptide antiserum (1:1000 dilutions). Immunoreactive StAR protein was visualized with an avidin-biotin-enhanced horseradish peroxidase method (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine as the substrate, followed by a light nuclear counter-stain with Gill hematoxylin.

Culture of Adrenocortical Cells

Bovine adrenocortical tissue was prepared from adrenal gland of cow collected at the local ablator. Primary zona fasciculata reticularis (ZFR) cells were isolated from sliced bovine adrenocortical tissue as previously described and were plated at density of 1×10^6 per plate on a 100-mm diameter cell culture dish (Corning® 430167) for both protein and cortisol assay studies. After plating, cells were maintained for 48 hours in M199 medium containing 5% v/v fetal bovine serum (Life Technologies, Inc. Carlsbad, CA, USA), 100 IU penicillin, 100 μg/ml streptomycin, 5 mg/ml insulin and 200 mM L-glutamine at 37°C under 95% air/5% CO_2, with one medium change after 24 hours, and then for a further 0, 1, 4, and 24 hours in M199 medium without serum and antibiotics as above, using a different dose of ACTH (donated from the NIDDK) in the same medium.

Gel Electrophoresis and Western Blotting for StAR Protein Expression

Total protein of cell lysates were denatured by boiling for 5 min in loading dye (0.125 M Tris-base, 4% SDS, 0.001% bromophenol blue, 12% sucrose, and 0.15 M dithiothreitol). Total protein (20 μg) in the sample was separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 60 V for 15 min and then at 100 V for 1 h using the running buffer. The proteins in the gel were electrophoretically transferred to polyvinylidene difuoride (PVDF) membrane (NEN Life Science Products, Inc., Boston, MA, USA). Using a Trans-Blot SD semi-dry transfer cell (170-3490, Bio-Rad, Hercules, CA, USA) at 64 mA (for 8 mm × 10 mm membrane) for 45 min in a blotting solution.

The membranes was washed in PBS-T buffer (0.8% NaCl, 0.02% KCl, 0.14% Na-HPO_4, 0.024% KH_2PO_4, and 0.1% Tween-20, pH 7.4) for 5 min and then blocked by blocking buffer (PBS-T buffer containing 10% nonfat milk powder) at 4°C overnight. Then the membranes were incubated with anti-StAR protein antiserum (1:5,000), β-actin monoclonal antibodies (1: 10,000) and 10% nonfat dry milk in PBS-T buffer for 2 hours at room temperature. After one 15 min wash and three 5 min washes with PBS-T buffer, the membranes were incubated for 1 hour in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilutions) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000 dilutions) in 10% nonfat dry milk of PBS-T buffer. The membrane was washed 4 to 5 times with PBS-T buffer, and then the bands for StAR protein and β-actin were visualized by chemiluminescence (ECL, Western blotting detection reagent, Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

Analysis of Chemiluminescence Western Blot Data

Quantification of chemiluminescence signal data on X-ray film (Kodak) were performed as follows: chemiluminescence pseudoautogradiograms were photographed by means of a digital camera (Kodak DC290 zoom). Quantification of images was performed according to the 1D limited edition software, and the StAR protein signals were normalized to the β-actin signal.

Enzyme Immunoassay for Cortisol

A valid competitive enzyme immunoassay for cortisol was established in our laboratory using polyclonal antibody (6). Briefly, 50 μl of diluted test medium and 150 μl of cortisol-horseradish peroxidase

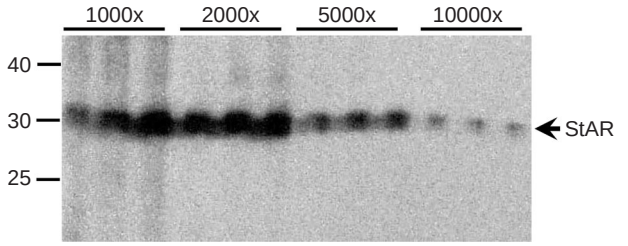

Fig. 2. Representative Western blotting for serous dilution (1000×, 2000×, 5000× and 10000×) of anti-StAR polyclone antiserum after 4th immunization. Screening well was loaded with 20 µg total protein of bovine luteal cell lysates.

were added to 96 wells of an EIA plate (Costar 9018) coated with polyclonal antibody. After incubation at room temperature for 15 min and washing with phosphate buffer saline (PBS) twice, the color was developed in 200 µl of 2.2 mM o-phenylenediamine in 0.003% H_2O_2 at room temperature for 30 min. The reaction was stopped by 50 µl of 8 N sulphuric acid. Absorbance at 490 nm was compared with a cortisol standard curve. The variation coefficient within and between assay were approximately 7% and 10%, respectively, and the sensitivity was 0.5 ng/ml.

Statistical Analysis

Each experiment was repeated for a minimum of three times and the mean and standard error (SE) of one experiment with identical results was used to express the concentration for each determination. Measurements of cortisol were expressed as ng/ml/10^5 cells. The effect of hormonal stimulation and dose response of ACTH or 8-Br-cAMP on cortisol content were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple comparison. $P < 0.05$ values greater than this significant.

Results

High Titer of Polyclonal Antibody

A high-titer polyclonal peptide antiserum was produced in rabbits. This antiserum recognized an epitope containing a specific amino acid sequence (82 to 107) found in the StAR protein. After the fourth immunization, the antiserum was obtained from rabbit. By using serous dilution of antiserum, we detected the titer of polyclonal antiserum. With the Mini-PROTEAN II (Bio-Rad) multiscreen apparatus, we screened different dilution times of antiserum. We found that when the antiserum is diluted 10,000 times, it still can clearly detect the major band in 30 KDa, which is shown in Fig. 2. Although raised against a

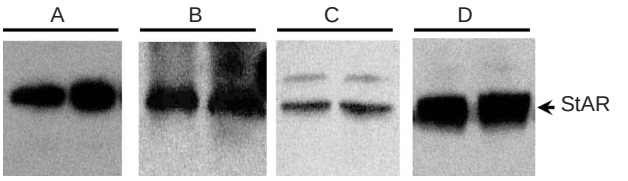

Fig. 3. Anti-StAR polyclonal antiserum specifically recognized a major band in 30 KDa StAR proteins in different species of steroidogenic cells. A: bovine adrenal cells; B: porcine adrenal cells; C: caprine luteal cells; D: duck granulosa cells.

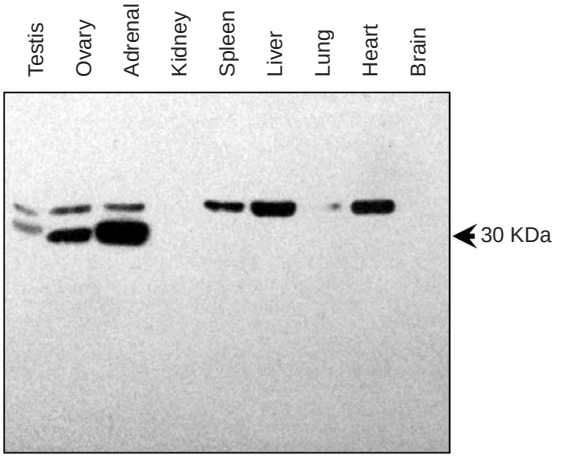

Fig. 4. Anti-StAR polyclonal antiserum specifically recognized an about 30 KDa to 37 KDa StAR proteins in different tissues of rat from the steroidogenic tissues from testis, ovary, and adrenal gland or another non-steroidogenic tissue such as kidney, spleen, liver, lung, heart, and brain.

MAP-conjugated peptide antigen, this antiserum recognizes StAR protein in Western blot assay and immunohistochemistry staining for a number of different species tested to date, including bovine, pig, goat, duck, and rat (shown in Fig. 3), and the specific major band was recognized in 30 KDa.

Specific Reorganization of StAR Protein in Cellular Lysates from Different Species

A single protein band with an apparent molecular weight of 30 KDa was detected in cell extracts from bovine adrenal cells (positive control), pig adrenal cells, goat luteal cells and duck granulosa cells (Fig. 3). Incubation of a bovine adrenal cell sample with antiserum that had been pre-absorbed with the immunizing peptide did not result in detection of any protein (data not shown), which indirectly demonstrates that the antibody was specific and no proteins were detected by the second antibody. In other experiments, a protein with identical mobility was present in different cellular extracts from adult male rat testis and female rat ovary (Fig. 4).

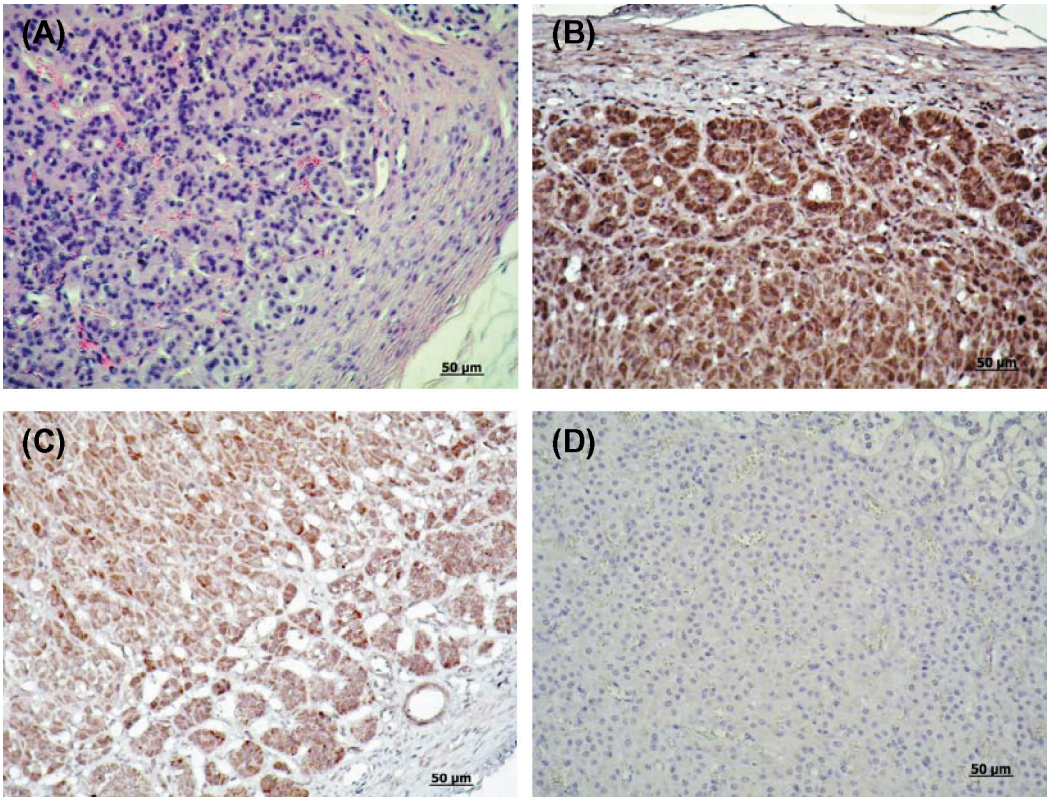

Fig. 5. H&E and immunohistochemistry staining of StAR protein in bovine adrenal tissue. All slides were photographed at 200× magnification. (A) H&E staining, (B) and (C): the StAR protein located in the cortex, (D) counter staining.

StAR Protein Expression in Different Tissues of Rat

The major band of StAR protein shown in Fig. 4 used the StAR polyclone antiserum that were hybridized to different tissues of the protein from the rat. The rat testis, ovary, and adrenal cellular lysates displayed messages similar in size in these steroidogenic tissues. No specific major hybridization could be detected in kidney, spleen, liver, lung, heart, and brain. The antibody for StAR hybridized with the expected major band of rat testis, ovary, and adrenal gland at 30 KDa, but this message was not present in kidney, spleen, liver, lung, heart, and brain (1, 16). However, the minor band message was considerably weaker than what was found in spleen, liver, and heart. StAR protein in the same 2 isoforms was present in rat testis, ovary, and adrenal gland. The minor band was slighly larger, and when present, it was less than 10% of the major band. It is highly likely that this larger-molecular-mass band is the approximate 37 KDa precursor protein of StAR previously observed and described in MA-10 and rat adrenal cortex cells. We estimate that the major band migrated at 30 KDa and the minor band at approximate 37 KDa. The 30 KDa proteins were shown to be derived from a 37 KDa precursor synthesized in the cytoplasm and then imported into mitochondria and processed to the 30 KDa forms (2, 4, 5).

Immunohistochemistry on Bovine Adrenal Gland

In order to know the mechanism of steroid hormone biosynthesis, the localization of StAR protein was studied in bovine adrenal gland by light as well as electron microscopic immunohistochemistry. In this study we performed immunohistochemistry staining of bovine adrenal gland sections with our anti-StAR antibody. Samples of the staining of various cells are shown in Fig. 5. IHC for all of these studies was done by using standard protocol as described in the Materials and Methods section. With light microcopy, the cytoplasm of the glomerulosa and fasciculata-reticularis cells were intensely immunostained by anti-StAR antibody, but the capsular connective tissue cells were entirely negative for this reaction. The present results indicate that the StAR protein is present in the cytoplasm of bovine adrenal cortical cells (8, 9, 28).

Effect of ACTH or cAMP on the Protein Expression of StAR in Bovine Adrenal Cells

Total cellular protein was also prepared from similar cultures of non-stimulated and stimulated cells. The cells were subjected to Western blotting using a

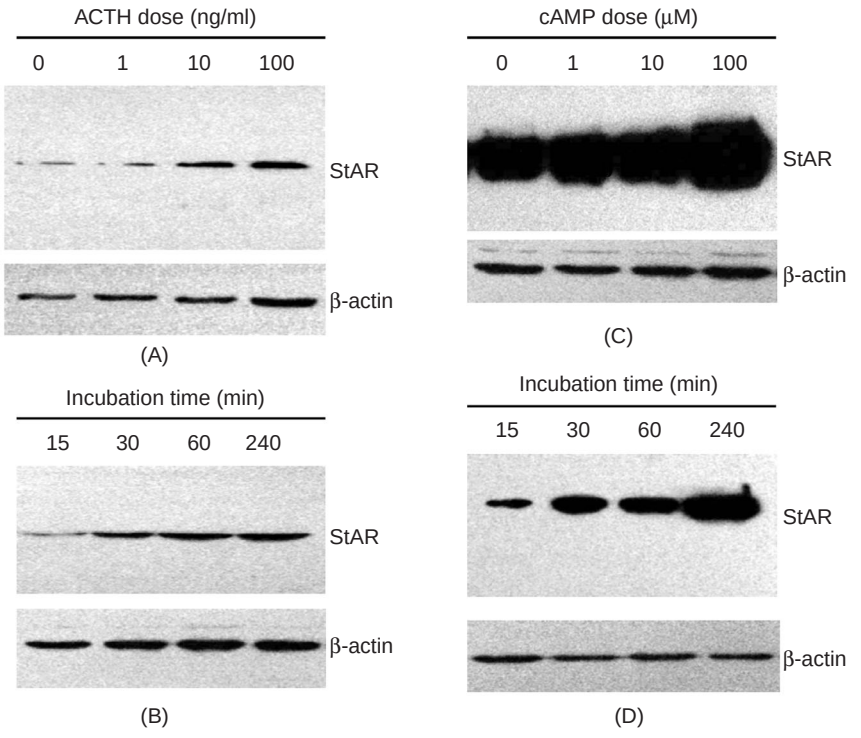

Fig. 6. Dose response or time course of ACTH or cAMP on the StAR protein expression in bovine adrenal cells. Bovine adrenal cells were incubated with different doses (0, 1, 10 and 100 ng/ml) of ACTH or (0, 1, 10 and 100 μM) of 8-Br-cAMP at 37°C for 30 min (A, B). Bovine adrenal cells were incubated with ACTH (10 ng/ml) or cAMP (10 μM) at 37°C for 15, 30, 60 and 240 min (C, D). Then the cells were collected and analyzed by Western blotting. Each well was loaded with 20 μg total protein of sample.

newly developed anti-StAR polyclonal antibody. The endogenous StAR protein in bovine adrenocortical cells was expressed as translocations one size, as shown previously for bovine luteal cells and adrenocortical cells. After treatment of adrenocortical cells with a different dose of 8-Br-cAMP and ACTH, it displayed a rapid increase in the relative amount of the StAR protein in this cell culture system. This increase was already detectable after 10 ng/ml ACTH and 10 μM 8-Br-cAMP of stimulation, compared with the untreated control, and reached a 3-fold increase at 100 ng/ml ACTH and 100 μM cAMP of treatments. Additional, the increase was already detectable after 30 min of 10 ng/ml ACTH treatment. The time course of changes in StAR protein levels in the 2 cell systems stimulated with ACTH and cAMP were similar, with a first detectable increase at 30 min, and maximum levels being maintained throughout 240 min or somewhat longer (Fig. 6).

Cortisol Secretion of Bovine Adrenal Cells by ACTH or cAMP

The cortisol secretion by bovine adrenal cells at 30 min of incubation with a different dose of ACTH or 8-Br-cAMP is shown in Fig. 7. In the absence of ACTH or cAMP stimulation (basal), the basal cortisol concentration was very low. However, either ACTH or cAMP stimulated cortisol secretion above basal levels, yet there was a dose response. This means that the bovine adrenal cells culture system was useful in this situation. In this culture system there was a dose response and progression in cortisol synthesis in response to ACTH and cAMP. The effect of ACTH or cAMP on cortisol synthesis by bovine adrenal cells is shown in Figs 7A and 7B. The addition of ACTH at 10 ng/ml and 100 ng/ml induced a 3- and 4-fold increase versus basal concentration, in cortisol synthesis, respectively, after 30 min of incubation. The effect of the added cAMP as second messenger to stimulate cortisol synthesis by bovine adrenal cells is shown in Fig. 7B. cAMP stimulation at 10 μM and 100 μM induced 10- and 30-fold increases versus basal concentration, in cortisol synthesis, respectively after 30 min of incubation. The time-course of the addition of ACTH 10 ng/ml or cAMP 10 μM to stimulate cortisol secretion displayed a significant increase at 30 min. These results are shown in Fig. 7.

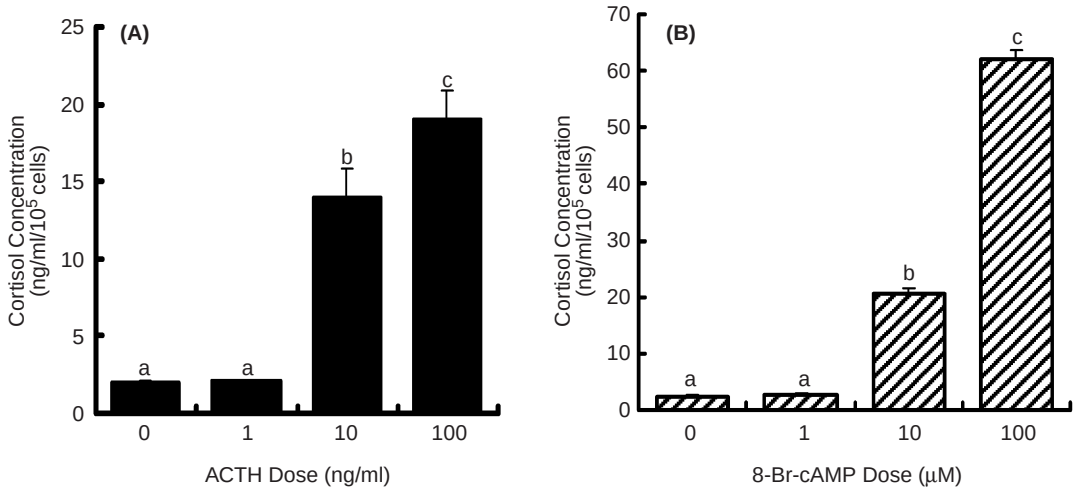

Fig. 7. The stimulation effects of (A) ACTH (0, 1, 10 and 100 ng/ml) or (B) cAMP (0, 1, 10 and 100 μM) on cortisol secretion by bovine adrenal cells. Bovine adrenal cells were incubated with ACTH or cAMP at 37°C for 30 min. Bars with different letters were significantly different compared with the value at basal = 0 ($P < 0.05$). Each bar represents mean ± standard error (S.E.).

Discussion

Using standard techniques for antiserum production, we successfully produced a polyclonal antibody against the StAR protein. The specificity of this StAR antibody was tested *via* immunoblot with whole cell lysates from different steroidogenic cells of different species. More importantly, we have demonstrated that this antibody can be used in the immunoblot and immunohistochemistry staining of many different steroidogenic cells or tissues (2, 10). By choosing a peptide within StAR amino acid sequences conserved between species, we have produced a polyclonal antibody that recognizes the protein in different species.

The antibody was used successfully for both Western analysis and to localize the StAR protein in fixed bovine adrenal gland section. The detection of a single or double band with an apparent molecular size of 30 KDa to 37 KDa in steroidogenic tissue extracts of testis, ovary, and adrenal gland is consistent with the reported size of the protein (26). We were also able to examine adrenal specimens from bovine adrenal gland. The intensity of the immunopositive signal in adrenocortical cells was marked, and endothelial cells lining the blood vessels and other connection cells were immunonegative (13, 26). The StAR protein expression is high in the adrenal gland, which makes it the tissue of choice for the advanced study of StAR regulated function (3).

In the present study we examined ACTH or cAMP stimulation on the expression of StAR protein; its expression level displayed dose and time-course response upon treatment with ACTH or cAMP. We found that the adrenal cells became strongly immunopositive 30 min after stimulation with ACTH (10 ng/ml) or cAMP (10 μM), and accumulated StAR protein by increased incubation time. The adrenal cells isolated from bovine adrenal gland also secreted cortisol in response to ACTH or cAMP treatment (17).

In conclusion, we have developed a new polyclonal antibody, specific for StAR protein, which gives clear immunoblot band with low background on Western blotting from a wide range of species including tissues from bovine, porcine, caprine, duck, and rodent specimens. This antibody enhances our understanding of the regulated function of StAR protein in steroidogenesis research.

Acknowledgments

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References

1. Arakane, F., Kallen, C.B., Watari, H., Stayrook, S.E., Lewis, M. and Strauss, J.F. 3rd. Steroidogenic acute regulatory protein (StAR) acts on the outside of mitochondria to stimulate steroidogenesis. *Endocr. Res.* 24: 463-468, 1998.
2. Bauer, M.P., Bridgham, J.T., Langenau, D.M., Johnson, A.L. and Goetz, F.W. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. *Mol. Cell. Endocrinol.* 168: 119-125, 2000.
3. Bornstein, S.R. and Ehrhart-Bornstein, M. Basic and clinical aspects of intraadrenal regulation of steroidogenesis. *Z, Rheumatol,* 59 Suppl 2: II/12-17, 2000.

4. Burns, C.J., Gyles, S.L., Persaud, S.J., Sugden, D., Whitehouse, B.J. and Jones, P.M. Phosphoprotein phosphatases regulate steroidogenesis by influencing StAR gene transcription. *Biochem. Biophys. Res. Commun*. 273: 35-39, 2000.
5. Caiola, S., Stigliano, A., Maroccia, E. and Toscano, V. [Molecular analysis of the gene of steroidogenic acute regulatory protein (StAR) in adrenal incidentaloma]. *Ann. Ist. Super. Sanita*. 36: 111-115, 2000.
6. Chen, H.L., Chen, Y.L., Wu, L.S., Kaphle, K. and Lin, J.H. Establishment and application of enzyme immunoassay for saliva cortisol in Taiwanese context. *J. Immunoassay Immunochem*. 27: 239-249, 2006.
7. Christenson, L.K. and Strauss, J.F. 3rd. Steroidogenic acute regulatory protein: An update on its regulation and mechanism of action. *Arch. Med. Res*. 32: 576-586, 2001.
8. Coulter, C.L., Ross, J.T., Salkeld, M.D., Bennett, H.P., James, S. and McMillen, I.C. N-proopiomelanocortin (1-77) suppresses expression of steroidogenic acute regulatory protein (StAR) mRNA in the adrenal gland of the fetal sheep. *Endocr. Res*. 26: 523-529, 2000.
9. Haidan, A., Bornstein, S.R., Liu, Z., Walsh, L.P., Stocco, D.M. and Ehrhart-Bornstein, M. Expression of adrenocortical steroidogenic acute regulatory (StAR) protein is influenced by chromaffin cells. *Mol. Cell. Endocrinol*. 165: 25-32, 2000.
10. Hauet, T., Liu, J., Li, H., Gazouli, M., Culty, M. and Papadopoulos, V. PBR, StAR, and PKA: Partners in cholesterol transport in steroidogenic cells. *Endocr. Res*. 28: 395-401, 2002.
11. Hsu, H.J., Hsu, N.C., Hu, M.C. and Chung B.C. Steroidogenesis in zebrafish and mouse models. *Mol. Cell. Endocrinol*. 248: 160-163, 2006.
12. Kobayashi, N., Sun, P., Fujimaki, Y., Niwa, T., Nishio, T., Goto, J. and Hosoda H. Generation of a novel monoclonal antibody against cortisol-[c-4]-bovine serum albumin conjugate: Application to enzyme-linked immunosorbent assay for urinary and serum cortisol. *Anal. Sci*. 18: 1309-1314, 2002.
13. Lo, Y.C., Brett, L., Kenyon, C.J., Morley, S.D., Mason, J.I. and Williams, B.C. StAR protein is expressed in both medulla and cortex of the bovine and rat adrenal gland. *Endocr. Res*. 24: 559-563, 1998.
14. Manna, P.R., Dyson, M.T., Eubank, D.W., Clark, B.J., Lalli, E., Sassone-Corsi, P., Zeleznik, A.J. and Stocco DM. Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Mol. Endocrinol.* 16: 184-199, 2002.
15. Miller, WL. Mitochondrial specificity of the early steps in steroidogenesis. *J. Steroid Biochem. Mol. Biol*. 55: 607-616, 1995.
16. Peters, B., Clausmeyer, S., Obermuller, N., Woyth, A., Kranzlin, B., Gretz, N. and Peters, J. Specific regulation of StAR expression in the rat adrenal zona glomerulosa. An *in situ* hybridization study. *J. Histochem. Cytochem*. 46: 1215-1221, 1998.
17. Poderoso, C., Maciel, F.C., Gorostizaga, A., Bey, P., Paz, C. and Podesta, E.J. The obligatory action of protein tyrosine phosphatases in ACTH-stimulated steroidogenesis is exerted at the level of StAR protein. *Endocr. Res*. 28: 413-417, 2002.
18. Rehman, K.S., Carr, B.R. and Rainey, W.E. Profiling the steroidogenic pathway in human fetal and adult adrenals. *J. Soc. Gynecol. Investig*. 10: 372-380, 2003.
19. Shen, W.J., Patel, S., Natu, V., Hong, R., Wang, J., Azhar, S. and Kraemer, F.B. Interaction of hormone-sensitive lipase with steroidogenic acute regulatory protein: Facilitation of cholesterol transfer in adrenal. *J. Biol. Chem*. 278: 43870-43876, 2003.
20. Stocco, D.M. and Clark, B.J. Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochem. Pharmacol.* 51: 197-205, 1996.
21. Stocco, D.M. Steroidogenic acute regulatory (StAR) protein: What's new? *Bioessays* 21: 768-775, 1999.
22. Stocco, D.M. The role of the StAR protein in steroidogenesis: Challenges for the future. *J. Endocrinol.* 164: 247-253, 2000.
23. Strauss, J.F. 3rd, Kishida, T., Christenson, L.K., Fujimoto, T. and Hiroi, H. START domain proteins and the intracellular trafficking of cholesterol in steroidogenic cells. *Mol. Cell. Endocrinol*. 202: 59-65, 2003.
24. Sugawara, T. and Fujimoto, S. The potential function of steroid sulphatase activity in steroid production and steroidogenic acute regulatory protein expression. *Biochem. J.* 380: 153-160, 2004.
25. Thompson, C.J., Ross, S.M., Hensley, J., Liu, K., Heinze, S.C., Young, S.S. and Gaido K.W. Differential steroidogenic gene expression in the fetal adrenal gland versus the testis and rapid and dynamic response of the fetal testis to di(*n*-butyl) phthalate. *Biol. Reprod*. 73: 908-917, 2005.
26. Walsh, L.P., Kuratko, C.N. and Stocco, D.M. Econazole and miconazole inhibit steroidogenesis and disrupt steroidogenic acute regulatory (StAR) protein expression post-transcriptionally. *J. Steroid Biochem. Mol. Biol.* 75: 229-236, 2000.
27. Yamazaki, T., Matsuoka, C., Gendou, M., Izumi, S., Zhao, D., Artemenko, I., Jefcoate, C.R. and Kominami, S. Mitochondrial processing of bovine adrenal steroidogenic acute regulatory protein. *Biochim. Biophys. Acta* 1764: 1561-1567, 2006.
28. Zilz, A., Li, H., Castello, R., Papadopoulos, V. and Widmaier, E.P. Developmental expression of the peripheral-type benzodiazepine receptor and the advent of steroidogenesis in rat adrenal glands. *Endocrinology* 140: 859-864, 1999.