Rod Outer Segment Membrane Guanylate Cyclase Type 1-Linked Stimulatory and Inhibitory Calcium Signaling Systems in the Pineal Gland: Biochemical, Molecular, and Immunohistochemical Evidence†

Venkateswar Venkataraman,‡ Robert Nagele,§ Teresa Duda,‡ and Rameshwar K. Sharma*,‡

The Unit of Regulatory and Molecular Biology, Departments of Cell Biology and Ophthalmology, and Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084

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ABSTRACT: Recent evidence indicates the presence of a novel $\alpha_{2D/A}$-adrenergic receptor ($\alpha_{2D/A}$-AR) linked membrane guanylate cyclase signal transduction system in the pineal gland. This system operates via a Ca^{2+}-driven rod outer segment membrane guanylate cyclase (ROS-GC). In the present study, this transduction system has been characterized via molecular, immunohistochemical, and biochemical approaches. The two main components of the system are ROS-GC1 and its Ca^{2+} regulator, S100B. Both components coexist in pinealocytes where the signaling component $\alpha_{2D/A}$-AR also resides. The presence of ROS-GC2 was not detected in the pineal gland. Thus, transduction components involved in processing $\alpha_{2D/A}$-AR-mediated signals are Ca^{2+}, S100B, and ROS-GC1. During this investigation, an intriguing observation was made. In certain pinealocytes, ROS-GC1 coexisted with its other Ca^{2+} modulator, guanylate cyclase activating protein type 1 (GCAP1). In these pinealocytes, S100B was not present. The other GCAP protein, GCAP2, which is also a known modulator of ROS-GC in photoreceptors, was not present in the pineal gland. The results establish the identity of an $\alpha_{2D/A}$-AR-linked ROS-GC1 transduction system in pinealocytes. Furthermore, the findings show that ROS-GC1, in a separate subpopulation of pinealocytes, is associated with an opposite Ca^{2+} signaling pathway, which is similar to phototransduction in retina. Thus, like photoreceptors, pinealocytes sense both positive and negative Ca^{2+} signals, where ROS-GC1 plays a pivotal role; however, unlike photoreceptors, the pinealocyte is devoid of the ROS-GC2/GCAP2 signal transduction system.

In a recent study, the existence of a novel epinephrine signal transduction mechanism in the bovine pineal gland was demonstrated (*1*). In this mechanism, epinephrine, through its $\alpha_{2D/A}$-adrenergic receptor, signals the activation of a membrane guanylate cyclase (mGC).[1] The activation process envisions at least three intervening sequential steps: (1) there is a rise in intracellular Ca^{2+}; (2) Ca^{2+} binds to an EF-hand Ca^{2+}-binding protein, S100B; (3) S100B activates a guanylate cyclase belonging to the ROS-GC subfamily of mGCs. Consistent with this hypothesis, the expression of transcripts for S100B and a rod outer segment membrane guanylate cyclase (ROS-GC) has been detected in the pineal gland.

To date, the ROS-GC transduction system has been assumed to be the exclusive domain of neurosensory cells of the retina linked with phototransduction and, presumably, with the retinal synaptic activity (reviewed in ref *2*). There are two members of the ROS-GC subfamily. They have been termed ROS-GC1 and ROS-GC2 (reviewed in refs *2* and *3*). Both ROS-GCs reside in photoreceptors and mimic the native photoreceptor cyclase activities under conditions of phototransduction; i.e., their cyclase activities are cooperatively activated by Ca^{2+}, with $K_{1/2}$ near 100 nM, and are progressively inhibited with increasing Ca^{2+} concentrations (*4*−*9*). Hence both cyclases meet the criterion of being linked with phototransduction. In line with the mGC family trait, both ROS-GCs are single transmembrane-spanning proteins, with an extracellular segment and an intracellular segment of almost equal length; a 26 amino acid transmembrane domain divides the two segments (*5*, *10*−*12*). In contrast to the peptide receptor subfamily, ROS-GC subfamily members are not stimulated by peptide hormones (*10*). Instead, they appear to be specifically designed to transduce intracellularly generated Ca^{2+} signals in retinal neurons. Regulation of ROS-GCs by Ca^{2+} is indirect, via Ca^{2+}-binding proteins. Two isoforms of guanylate cyclase activating protein (GCAP), GCAP1 and GCAP2, activate ROS-GC at 100 nM free Ca^{2+} or below (*4*, *6*−*9*, *13*, *14*). S100B activates ROS-GC at micromolar concentrations of free Ca^{2+} (*15*−*19*). Thus, ROS-

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* To whom all correspondence should be addressed. Phone: 856-566-6976. Fax: 856-566-7057. E-mail: sharmark@umdnj.edu.

‡ The Unit of Regulatory and Molecular Biology, Departments of Cell Biology and Ophthalmology.

§ Department of Molecular Biology.

[1] Abbreviations: $\alpha_{2D/A}$-AR, $\alpha_{2D/A}$-adrenergic receptor; DD, double deletion; FITC, fluorescein isothiocyanate; GCAP, guanylate cyclase activating protein; HSF, heat stable factor; mGC, membrane guanylate cyclase; ROS-GC, rod outer segment membrane guanylate cyclase; RT-PCR, reverse transcription−polymerase chain reaction.

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GC is a double Ca^{2+} switch, both stimulated and inhibited by Ca^{2+} signals. The inhibitory switching component is linked with phototransduction (*4*, *6*−*8*, *14*) and the stimulatory component presumably with retinal synaptic activity (*15*−*18*; reviewed in ref *2*).

The objectives of this study were to (1) identify the ROS-GC present in the pineal gland; (2) determine its cellular localization; (3) scrutinize its cellular copresence with its modulator, S100B; and (4) from the findings assemble the functional components of the $\alpha_{2D/A}$-AR-linked ROS-GC signal transduction model. These objectives were expanded when a surprising discovery was made; i.e., a certain population of pinealocytes contained the paired presence of GCAP1 and ROS-GC1, but was lacking S100B. In view of these findings, the study was expanded to investigate role of GCAP1 in ROS-GC1 regulation. These studies revealed that ROS-GC1 is associated with two opposing Ca^{2+} signaling pathways in pinealocytes. It is anticipated that these findings will have general applications in explaining Ca^{2+}-dependent neurosensory processes.

EXPERIMENTAL PROCEDURES

ROS-GC1 Mutants. Deletion mutants of ROS-GC1 were constructed as described earlier (*7*, *17*, *20*). Mutations were verified by sequencing, and mutant derivatives were ligated into the pcDNA3 expression vector for expression studies.

Expression Studies. COS7 cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with the expression constructs by the calcium phosphate coprecipitation technique. Sixty hours after transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ buffer, scraped into 2 mL of cold buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed several times with the same buffer. The resulting pellet represented crude membranes.

Isolation of Membrane and Soluble Fractions. Membrane and heat-stable soluble fractions from the pineal gland were isolated exactly as described previously (*1*). A similar procedure was adapted to isolate membrane and soluble fractions from the retina, which served as positive controls for Western blot analyses.

Guanylate Cyclase Assay. The crude membrane fraction was assayed for GC activity as described previously (*21*). Briefly, membranes were preincubated on an ice bath with or without GCAP1, GCAP2, or S100B in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5) adjusted to the appropriate free Ca^{2+} concentrations with precalibrated Ca^{2+}/EGTA solutions (Molecular Probes). The total assay volume was 25 μL. The reaction was initiated by addition of the substrate solution containing 4 mM $MgCl_2$ and 1 mM GTP and maintained by incubation at 37 °C for 10 min. Termination was effected by addition of 225 μL of 50 mM sodium acetate buffer (pH 6.2), followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by radioimmunoassay (*22*).

Western Blot. After being boiled in gel-loading buffer [62.5 mM Tris-HCl (pH 7.5), 2% SDS, 5% glycerol, 1 mM β-mercaptoethanol (BME), and 0.005% bromophenol blue], 20 μg of membrane protein was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in a buffer (pH 8.3) containing 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS. The proteins were transferred to Immobilon membranes (Millipore) in the same buffer but containing 5% methanol. The blot was incubated in Tris-buffered saline (TBS, pH 7.5) containing 100 mM Tris-HCl, 0.9% NaCl, and 0.05% Tween-20 (TBS-T) with 5% powdered nonfat Carnation milk (blocking buffer) overnight at 4 °C and rinsed with TBS-T. The primary antibodies against ROS-GC1 or GCAP1 were added at appropriate dilutions in the blocking buffer, and the incubation was continued for 1 h. After the solution was rinsed with TBS-T, incubation was continued with the secondary antibody conjugated with horseradish peroxidase in blocking buffer (1:10 000) for another hour. Finally, the blot was developed with diaminobenzoate and hydrogen peroxide according to manufacturer's (Vector labs) protocol. The immunoreactive band was detected visually.

Detection of S100B requires modifications to obtain reproducible results (*15*, *23*). This modified protocol was followed for the detection of S100B in the pineal soluble fraction.

Expression and Purification of Recombinant GCAP1 and GCAP2. A 649 bp fragment containing the GCAP1 coding region was amplified from pineal RNA through reverse transcription followed by polymerase chain reaction (PCR). The procedure was as described previously (*1*) except the primers specific for GCAP1—5′CGGAATTCCTGAGCGATGGGGAACATT3′ (forward; *Eco*RI adapter underlined) and 5′GTACAGAAAGAGTAGGCAGT3′ (reverse)—were used for PCR at an annealing temperature of 54 °C. The amplified fragment was cloned into pSV-Sport1 (Gibco-BRL) initially. The GCAP1 coding region was subsequently cloned into the bacterial expression vector pET-30a+ and expressed in *Escherichia coli* ER2566 strain carrying the plasmid pBB131 (a kind gift of Dr. J. Gordon) encoding yeast *N*-myristoyl transferase. The protein was purified as described previously (*24*), and this pineal GCAP1 was identical with the retinal GCAP1 in all respects.

GCAP2 was cloned from the retina, expressed, and purified as described earlier (*8*).

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). The procedure for RT-PCR analysis of retinal and pineal RNA was as described before except that a 4-fold excess of pineal cDNA was used for the PCR reaction (*1*). The primers and conditions for detection of GCAP2 mRNA have also been described previously (*8*).

Antibodies. GCAP1 expressed in bacterial cells and purified was used to raise an antibody against this protein in rabbits. A monospecific antibody against GCAP2 was raised in rabbits using the peptide corresponding to ^{187}DLNPSSWISQQR^{198} of bovine GCAP2 as an antigen. The antisera against both GCAPs were tested for their specificity through enzyme-linked immunosorbent assay (ELISA) and Western blotting according to standard protocols. After the specificity of the reaction was established, antibodies were enriched by precipitating the immunoglobulin fraction using ammonium sulfate. ELISA and Western blots were used to determine the titer of the enriched antibodies. A dilution of 1:10 000 was generally used for colorimetric detection of the antigen on Western blots. The ROS-GC1 antibody used

for immunohistochemical studies was a kind gift from Dr. Akio Yamazaki (Wayne State University, Detroit, MI), and the one used for Western blot analyses was generously supplied by Dr. Karl-Wilhelm Koch (Institut für Biologische Informationverarbeitung, Jülich, Germany). A monoclonal antibody against S100B (clone no. SH-B1) was purchased from Sigma Chemical Co.

Immunohistochemical Analyses. Fresh bovine pineal glands were obtained from a slaughterhouse, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and cryosectioned. The sections were refixed in 4% paraformaldehyde in PBS, washed in PBS, and used for detecting ROS-GC1, GCAP1, and S100B. After being blocked in PBS containing 4% BSA and 0.1% Tween-20, sections were incubated with primary antibodies in the same solution for 60 min at 37 °C in a humid atmosphere and washed for 60 min at room temperature with PBS containing 0.1% Tween-20. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit and CY3-conjugated anti-mouse antibodies were used for detection. Incubation and washing conditions were as described for the primary antibodies. Specimens were then mounted in Vectashield mounting solution (Vector Labs) containing 4′,6-diamidino-2-phenylindole (DAPI) as a DNA counterstain. Immunostaining was visualized with a Nikon FXA microscope equipped with epifluorescence optics. Separate digital images of the FITC, CY3, and DAPI staining were acquired using appropriate wavelength filters and a Princeton Instruments cooled CCD camera. Images were processed using commercially available software (ImagePro Plus, Phase3 Imaging Systems). Controls included detection reactions carried out under identical conditions except that either the primary antibody was not added or was replaced by nonimmune serum.

In Situ Hybridization. A 564 bp fragment from ROS-GC1 cDNA was amplified using primers and conditions described before (*1*), except that digoxigenin-labeled dUTP was used in the reaction mix. The $\alpha_{2D/A}$-AR probe was generated by PCR amplification from the bovine $\alpha_{2D/A}$-AR gene in the presence of CY3-tagged dCTP. A similar probe was generated from the human $\alpha_{2D/A}$-AR gene; because its target is absent in the bovine genome, this probe was used as control. Pineal cryosections were obtained and prepared as described above and washed in 2× SSC with 0.1% SDS. Hybridization was performed in Hybrisol VI overnight at 37 °C in a humid atmosphere. ROS-GC1 transcripts were detected with an FITC-conjugated anti-digoxigenin antibody, while the $\alpha_{2D/A}$-AR transcripts were directly visualized. Imaging was carried out as described above. No significant fluorescent signal was detected in the control.

RESULTS

Pineal Cell Membranes Express a Guanylate Cyclase with the Biochemical Attributes of ROS-GC1. A hallmark feature of the ROS-GC subfamily members is their Ca^{2+}-dependent modulation by the GCAPs (reviewed in refs *2* and *3*). The pineal gland contains the ROS-GC1 transcript (*1*). However, the direct functional or structural presence of the enzyme in the gland has not been shown. To determine the functional presence of ROS-GC1 and/or ROS-GC2, advantage was taken of the selectivity of Ca^{2+} probes for these guanylate cyclases. In the presence of 20 nM or lower free Ca^{2+}, both GCAPs stimulate ROS-GC1; GCAP1, however, is about an order of magnitude more selective for ROS-GC1 (*8*); its EC_{50} value is 1 μM (*7*−*9*). Similar to GCAP1, S100B is also about an order of magnitude more selective for ROS-GC1; its EC_{50} value is about 0.8 μM (*15*, *18*, *25*). In contrast to GCAPs, S100B elicits its effect in the presence of micromolar (or higher) ranges of free Ca^{2+} (*15*, *17*, *18*). At nanomolar concentrations of free Ca^{2+}, ROS-GC2 responds only to GCAP2 with an EC_{50} of about 1 μM (*9*) and is about 10-fold less responsive to S100B (*25*); the EC_{50} value of S100B for ROS-GC2 is about 8 μM (*25*).

To assess if ROS-GC is functionally expressed in the pineal gland membranes, individual aliquots of membrane fractions were incubated with saturating GCAP concentrations in the presence of 10 nM Ca^{2+}. Both GCAPs stimulated pineal membrane guanylate cyclase activity about 3-fold above control levels (Figure 1A). These results show the presence of a ROS-GC in the pinealocytes. To determine the type(s) of ROS-GC present in the membranes, they were incubated with individual GCAPs at 10 nM Ca^{2+} and with S100B at 1 mM Ca^{2+}.

GCAP1 stimulated the guanylate cyclase in a dose-dependent manner by about 3-fold with an EC_{50} of about 1 μM (Figure 1B). GCAP2 stimulated the cyclase activity by about the same level, but with an EC_{50} of ∼8 μM (Figure 1C). S100B also stimulated the pineal ROS-GC in a dose-dependent fashion with an EC_{50} of approximately 1 μM (Figure 1D). Thus, the pineal ROS-GC behaves as a typical ROS-GC1, not like ROS-GC2.

To directly establish the presence of ROS-GC1 on pineal membranes, Western blot analysis was carried out. Retinal membranes were used as a positive control for the experiment. Results demonstrate that a specific antibody against ROS-GC1 reacted with a protein of identical mobility in both the retinal and pineal membranes (Figure 1E). The estimated molecular size, ∼118 kDa, corresponded to that of ROS-GC1.

Thus, the results demonstrate that the sole ROS-GC expressed in the pineal gland is ROS-GC1, in agreement with the earlier report showing the absence of ROS-GC2 expression in the pineal gland (*12*).

Expression of ROS-GC1 Is Localized in the Pinealocytes. The pineal gland is composed of two cell types: pinealocytes and glial. To precisely determine the cell specificity of ROS-GC1 expression, its transcript and the encoded protein were localized via in situ hybridization and immunohistochemistry, respectively. In these experiments, the pinealocytes were readily identified on the basis of their distinct nuclear morphology: their nuclei are larger and more diffusely packed when compared to glial cells. The nuclei were stained with DAPI and are shown in blue (Figure 2). The ROS-GC1 transcripts, in green, were detected after hybridization to a digoxigenin-labeled DNA probe and staining with an FITC-conjugated anti-digoxigenin antibody (Figure 2A). In situ hybridization analysis showed that there is a widespread, cytoplasmic staining of ROS-GC1 transcripts (green; Figure 2A) around the larger nuclei (blue; Figure 2A). Therefore, ROS-GC1 transcripts are expressed in pinealocytes. The staining among pinealocytes, however, was not uniform, indicating that the level of ROS-GC1 expression is not the same among all pinealocytes. No staining was observed around smaller nuclei, indicating that ROS-GC1 transcripts

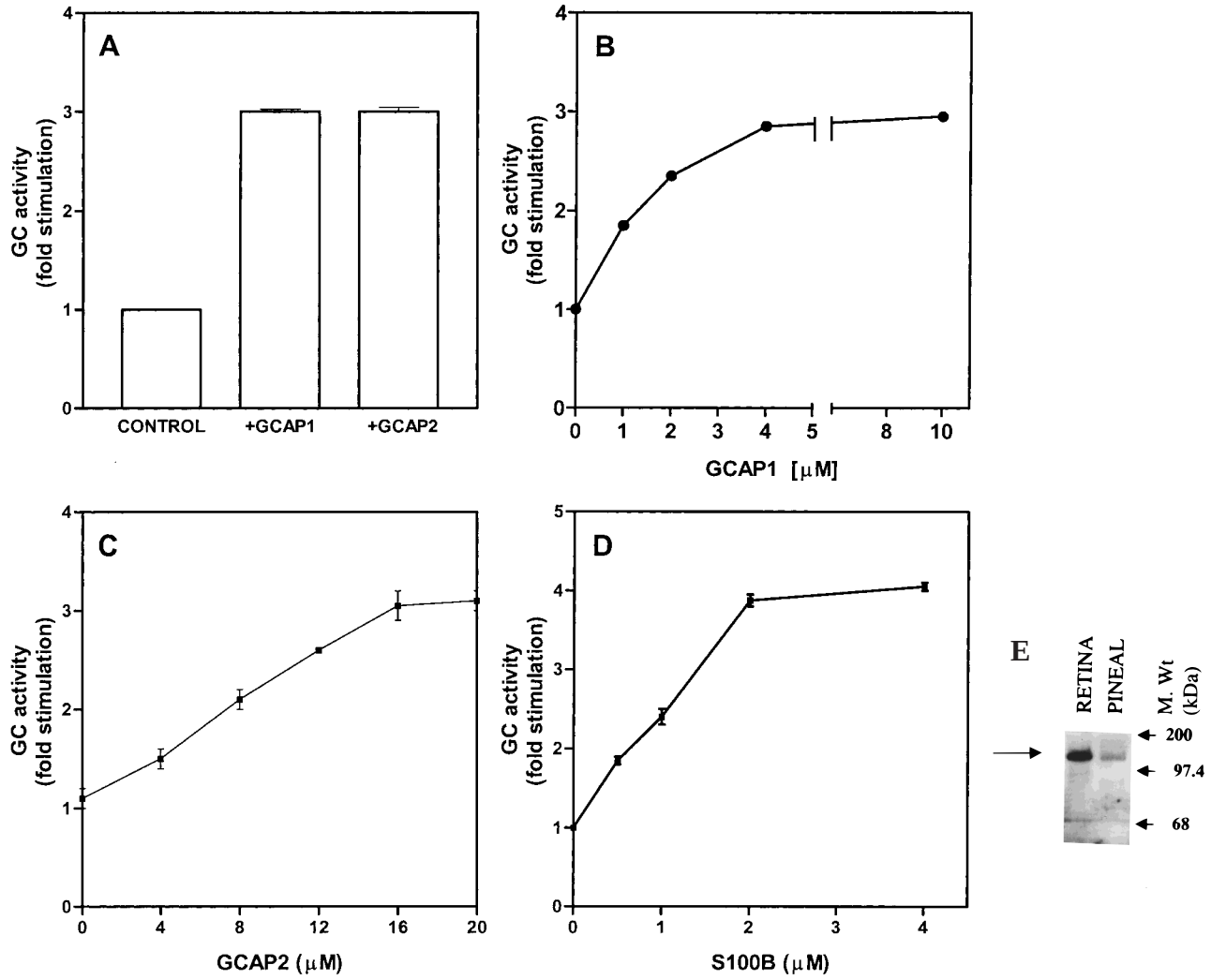

FIGURE 1: Biochemical characterization of pineal ROS-GC. Stimulation of pineal mGC by GCAP1, GCAP2, and S100B: Pineal membranes were isolated as described in Experimental Procedures and assayed for guanylate cyclase (GC) activity in the presence or absence of (A) 4 μM GCAP1 or 16 μM GCAP2 and 10 nM Ca^{2+}, (B) incremental concentrations of GCAP1 and 10 nM Ca^{2+}, (C) increasing concentrations of GCAP2 and 10 nM Ca^{2+}, and (D) incremental concentrations of S100B and 1 mM Ca^{2+}. Each experiment was done in triplicate and repeated twice. (E) Western blot: Retinal and pineal membrane fractions were isolated as described in Experimental Procedures. Equal amounts of protein were loaded onto an 8% SDS−polyacrylamide gel and electrophoresed. After the proteins were transferred to Immobilon membranes, the blot was incubated with anti-ROS-GC1 antibody and secondary antibody as described in Experimental Procedures. The immunoreactive bands were visualized after development with the peroxidase staining kit according to the manufacturer's protocol. The position of ROS-GC1 is indicated by an arrow. Positions of molecular size markers (kDa) are provided alongside.

are not expressed in glial cells. In situ reactions with a control probe did not show any significant staining (Figure 2B), demonstrating the specificity of the reaction. Thus, the ROS-GC1 transcript is specifically localized in the pinealocytes.

To determine if the ROS-GC1 protein was also localized in pinealocytes, pineal sections were probed with a ROS-GC1 monospecific antibody, followed by detection through an FITC-conjugated anti-rabbit antibody. Positive reaction is indicated in green (Figure 2C), while the DAPI-stained nuclei are in blue (Figure 2). Results show that ROS-GC1 protein (green; Figure 2C) is present in pinealocytes, whereas the control showed no reaction (Figure 2D). It is also evident that the staining is not uniform among pinealocytes, indicating varying levels of ROS-GC1 expression among these cells. This pattern of the protein expression is similar to that of its transcripts (Figure 2A). No staining was observed within glial cells. Thus, both the ROS-GC1 transcript and the encoded protein exist in pinealocytes; their expression is pinealocyte-specific and does not occur in glial cells.

Pinealocytes That Express ROS-GC1 Also Express $\alpha_{2D/A}$*-AR.* In a previous study it was established that a ROS-GC transduction system in the pineal gland is linked with the $\alpha_{2D/A}$-AR-mediated signals (*1*). With the information from the present study that the system is exclusively present in pinealocytes and is solely represented by ROS-GC1, the question was raised: Are the receptor and the transduction components colocalized in the pinealocyte? This issue was addressed through the technique of in situ hybridization. The expression of the ROS-GC1 transcript was probed with a digoxigenin-labeled ROS-GC1 cDNA fragment as described in the previous section. The $\alpha_{2D/A}$-AR transcript was detected through the use of a CY3-tagged DNA probe (in red; Figure 2E,F), generated from the bovine $\alpha_{2D/A}$-AR gene. In Figure 2F, the transcripts for ROS-GC1 (green; Figure 2A) have been superimposed on those for $\alpha_{2D/A}$-AR (in red; Figure 2F). Both transcripts are detected around the larger pinealocyte nuclei visible in the plane of sectioning (in blue; Figure 2). Superimposition of the two transcripts (green and red

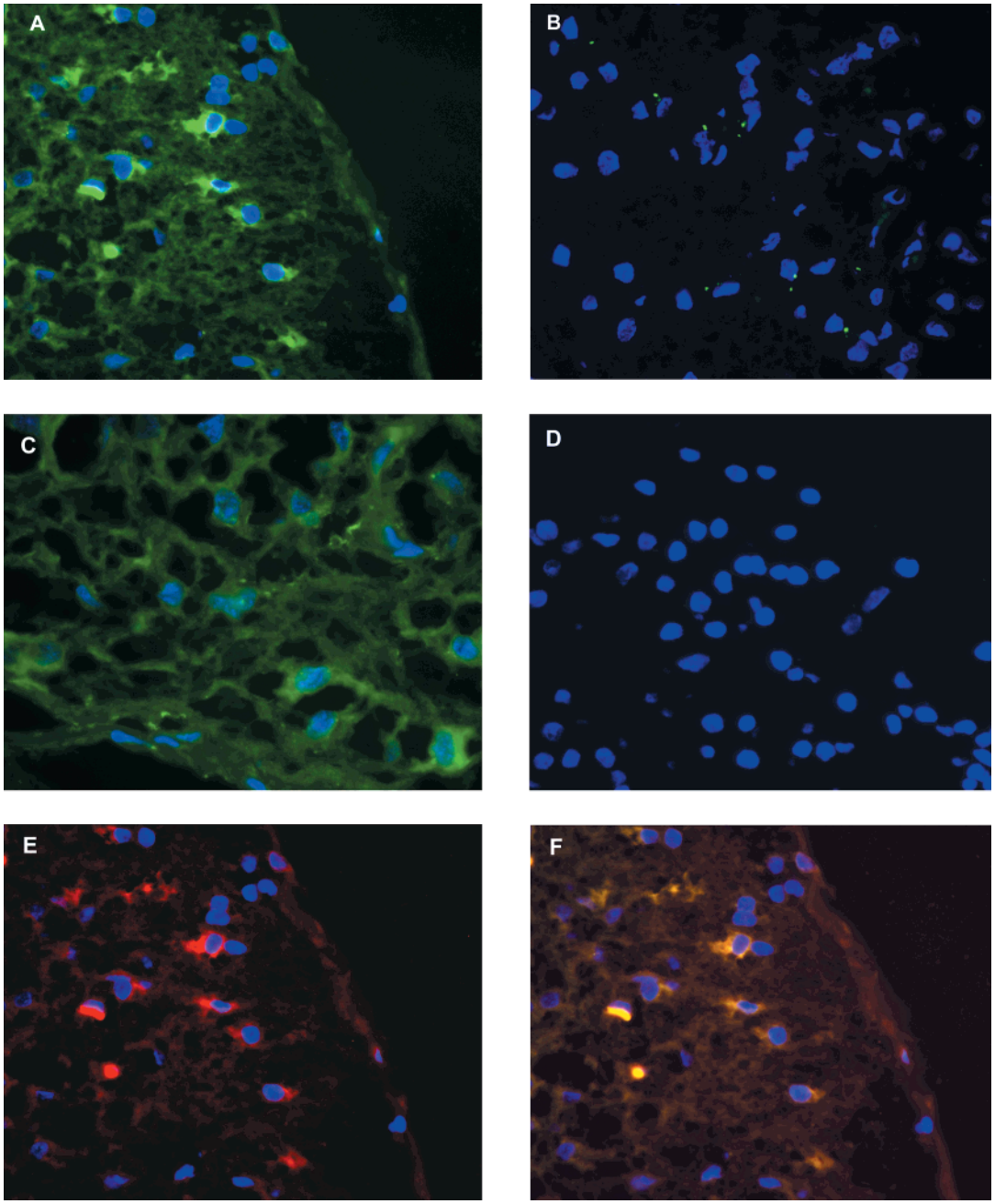

FIGURE 2: Molecular presence of ROS-GC1 and $\alpha_{2D/A}$-AR in pinealocytes. ROS-GC1 transcript: Pineal cryosections were hybridized to the digoxigenin-labeled ROS-GC1 probe and visualized as described in Experimental Procedures. (A) Staining for the ROS-GC1 transcript is in green. Pinealocyte nuclei in the plane of sectioning are in blue. (B) Control: A CY3-tagged probe generated from the human $\alpha_{2D/A}$-AR gene, whose target is absent in the bovine genome, was used as control. Detection and visualization were as described in Experimental Procedures. Nuclei are in blue. ROS-GC1 protein: A ROS-GC1 monospecific antibody was used to detect ROS-GC1 on pineal cryosections. (C) The presence of ROS-GC1 is indicated in green, while the nuclei are in blue. (D) Control: The procedure was exactly as above, except that nonimmune serum was used instead of ROS-GC1 antibody. The nuclei are in blue. Colocalization of ROS-GC1 and $\alpha_{2D/A}$-AR transcripts: Dual immunostaining for the ROS-GC1 and $\alpha_{2D/A}$-AR transcripts was carried out with digoxigenin- and CY3-tagged probes, respectively. Detection and visualization were as described in Experimental Procedures. (E) The presence of $\alpha_{2D/A}$-AR transcripts is indicated in red, and nuclei are in blue. (F) Images for the two transcripts were merged. Green denotes ROS-GC1, red denotes $\alpha_{2D/A}$-AR, and superimposition of the two results in a yellowish color. The nuclei are in blue.

colors) results in a yellowish color, which is seen in almost all the pinealocytes because the staining of the two transcripts overlaps (Figure 2F). Therefore, the pinealocytes that express ROS-GC1 transcripts also express $\alpha_{2D/A}$-AR. Thus, the signal initiator $\alpha_{2D/A}$-AR and the responsive transducer ROS-GC1 are colocalized in pinealocytes, consistent with the concept that the signaling $\alpha_{2D/A}$-AR and the transducing ROS-GC1 components occur in the same cell.

ROS-GC1 Modulators GCAP1 and S100B, but Not GCAP2, Are Expressed in the Pineal Gland. In photoreceptors, ROS-GC1 is modulated by intracellular Ca^{2+} levels through the Ca^{2+}-binding proteins GCAP1, GCAP2, and S100B (reviewed in ref *2*). Modulation through GCAPs is linked with phototransduction and through S100B, presumably, with synaptic activity (reviewed in ref *2*). Because ROS-GC1 is expressed in bovine pinealocytes (Figure 2), the presence

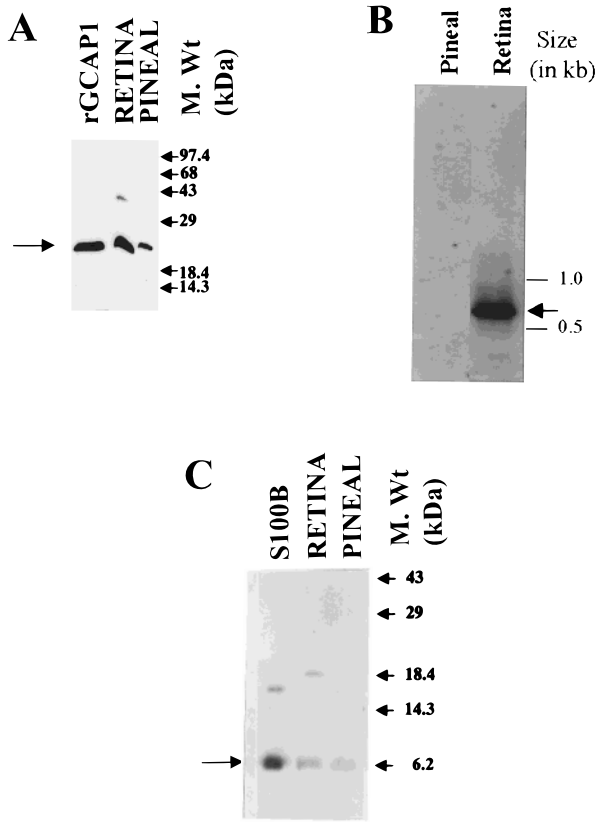

FIGURE 3: Expression of GCAP1 and S100B but not GCAP2 in the pineal gland. (A) GCAP1: Retinal and pineal membranes were isolated and subjected to Western analyses as described under Experimental Procedures. Equal amounts of proteins were compared. Molecular size markers are provided alongside, and the position of recombinant (r) GCAP1 is indicated by an arrow. (B) GCAP2: Pineal and retinal RNA were isolated and analyzed through RT-PCR as described before (*1*, *12*) using primers specific for GCAP2 (*8*). One-fifth of the reaction volume was electrophoresed on 1% agarose. A 4-fold excess of pineal, as compared to the retinal, template was used. Molecular size markers are provided alongside, and the position of the amplified fragment in the retinal lane is indicated by an arrow. This fragment was sequenced to confirm its identity. (C) S100B: The heat-stable pineal-soluble fraction was isolated as described previously (*1*). Electrophoresis was carried out under nonreducing conditions as described in ref *23*. Detection was carried out as described in ref *18*. The position of S100B is indicated by an arrow, and molecular size markers are provided.

of its Ca^{2+}-dependent modulators in the pineal gland was examined.

Highly specific antibodies against GCAP1, GCAP2, and S100B were used for Western blotting analyses. A single band in the lane containing the pineal membrane fraction reacted with GCAP1 antibody (Figure 3A, pineal); the band was of identical mobility to that from the retinal membrane fraction (Figure 3A, retina) and to that of purified r-GCAP1 (Figure 3A, r-GCAP1; position indicated by the arrow). This establishes the expression of GCAP1 in the pineal gland. This result formed the basis of directly cloning it from this tissue. The pineal GCAP1 has been expressed and has been used for several GCAP1-related studies (*26*), including this one. No GCAP2 protein was detected in the pineal gland by Western analyses (data not shown). Expression of GCAP2 in this gland was further investigated through RT-PCR. No GCAP2 expression was detected (Figure 3B, pineal). A 649 bp fragment of GCAP2 cDNA could be amplified from the retinal RNA pool (used as a positive control) under conditions that detect low-abundance mRNAs (Figure 3B, retina; position indicated by the arrow). However, under similar conditions no amplified product was obtained from pineal RNA even at a 4-fold excess of template (Figure 3B, pineal). These results provide compelling evidence for the absence of GCAP2 expression in the pineal gland. Thus, the pineal gland expresses GCAP1 and does not express GCAP2.

The previous report showed that a heat-stable factor (HSF) in the pineal soluble fraction stimulates mGC (*1*). Clued by the observation that the S100B transcript is present in the pineal gland, the presence of the S100B protein in this fraction was tested. Western analyses with antibody against S100B showed a single band of identical mobility (indicated by the arrow) from the retinal- and pineal-soluble fractions (Figure 3C; lanes retina and pineal). The migration was identical with that of purified S100B (Figure 3C; lane S100B). Authentic S100B and the retinal-soluble fraction show a higher molecular weight species that reacts with the antibody. This is a multimeric form of the protein observable when the electrophoresis is carried out under nonreducing conditions (*18*). Thus, the S100B protein is present in the pineal gland, as is its transcript (*1*).

These findings show that the ROS-GC1 Ca^{2+} modulators, GCAP1 and S100B, are expressed in the pineal gland, whereas GCAP2 is not present. This is consistent with the idea that Ca^{2+}-dependent modulation of ROS-GC1 in the pineal gland is mediated by GCAP1, that the HSF stimulating mGC is S100B, and that GCAP2 is not involved in pineal ROS-GC1 modulation.

The HSF That Stimulates Pineal mGC Is S100B. To determine if the pineal HSF mimics S100B in regulating recombinant ROS-GC1 (rROS-GC1), the functional features of HSF and S100B were compared. Increasing concentrations of HSF or S100B were incubated with COS cell membranes expressing rROS-GC1. Both HSF and S100B stimulated the cyclase activity in a dose-dependent fashion with almost identical patterns (Figure 4A). Hence, HSF mimics S100B in regulating ROS-GC1.

The S100B modulatory domain on ROS-GC1 has been localized to a region between amino acids (aa) 731 and 1054 (*17*, *25*). Thus, a question arises as to whether the same domain is involved in the modulation of ROS-GC1 by HSF. To answer this question, the following deletion mutants of ROS-GC1 were used: Δ8−412, Δ447−730, and Δ8−412, Δ447−730 [double deletion (DD)]. These mutants are graphically represented in Figure 4B, and the positions of aa residues have been indicated. They were transiently expressed in COS cells, and their membrane fractions were incubated with the maximally stimulating concentrations of S100B (4 μM) or HSF (20 μg) in the presence or absence of 1 mM Ca^{2+}. The results show that HSF, like S100B, modulates ROS-GC1 via aa 731−1054 in a Ca^{2+}-dependent fashion.

Finally, additive experiments were performed to demonstrate that the HSF is, indeed, S100B. Saturating amounts of S100B, HSF, or S100B and HSF together were tested for the stimulation of pineal GC and rROS-GC1. COS cell membranes expressing rROS-GC1 were exposed to the combined saturating concentrations of HSF and S100B (20 μg and 4 μM, respectively). The resulting stimulation was the same as that caused by HSF or S100B alone (Figure 4C).

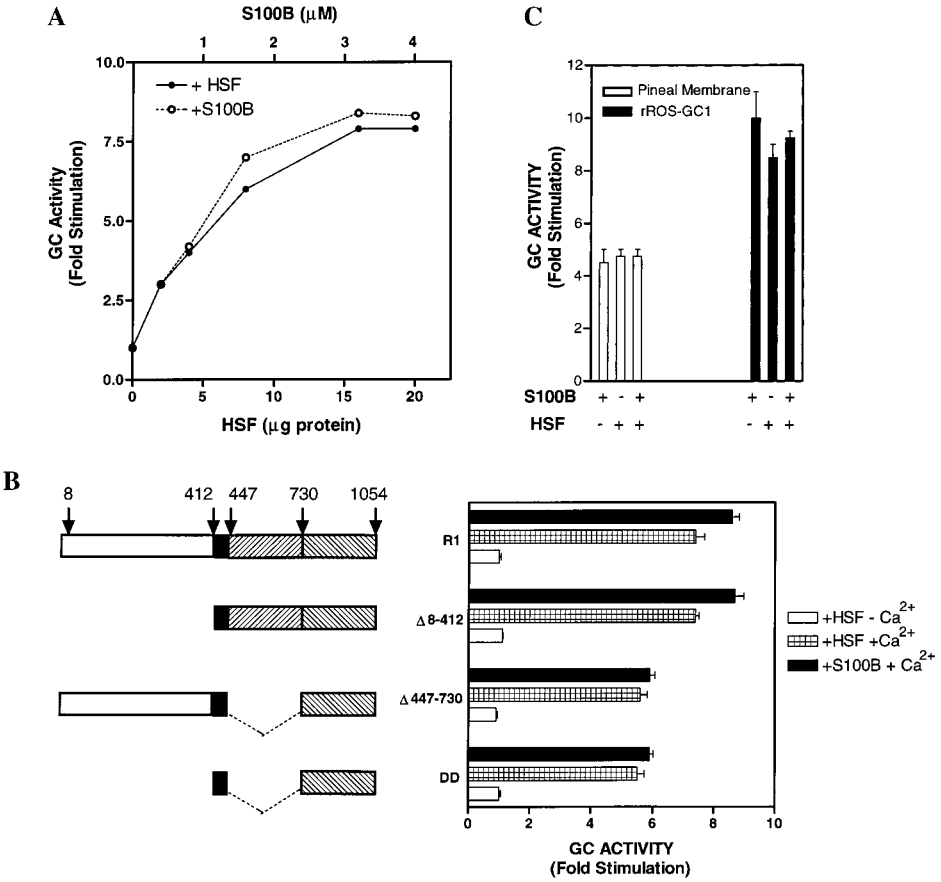

FIGURE 4: Stimulation of pineal mGC and rROS-GC1 by HSF. COS7 cells were transfected with wild-type recombinant ROS-GC1 (wt rROS-GC1) or its deletion mutants, and the membranes were prepared as described in Experimental Procedures. These were assayed for guanylate cyclase activity. The pineal-soluble fraction was used as the source of HSF. (A) Incremental concentrations of HSF or S100B in the presence of 1 mM Ca^{2+} were incubated with wt rROS-GC1. (B) wt rROS-GC1 and its deletion mutants were incubated with 20 μg of PSF or 4 μM S100B in the presence or absence of 1 mM Ca^{2+}. ROS-GC1 and its deletion derivatives are graphically depicted in the left panel. The different domains of ROS-GC1 have been indicated by amino acid positions. The parent full-length ROS-GC1 is denoted by R1. The mutants which are deleted for the extracellular domain alone (Δ8−412), the juxtamembrane domain alone (Δ447−730), or both (DD) have also been indicated. (C) Isolated pineal membranes (pineal membrane) or cell membranes from COS7 cells expressing rROS-GC1 were incubated with S100B alone (4 μM), HSF alone (20 μg), or the two together in the presence of 1 mM Ca^{2+}. All experiments were carried out in triplicate and repeated for reproducibility.

Similar results were obtained with pineal mGC (Figure 4C). Therefore, the HSF that stimulates pineal mGC in a Ca^{2+}-dependent fashion is S100B.

Pinealocytes Express ROS-GC1 with GCAP1 or S100B, but Not Both. To address the issue of whether the components of the ROS-GC1 transduction system occur in the same pinealocyte, highly specific antibodies against ROS-GC1, GCAP1, and S100B were used. The relative localization patterns of the three proteins were analyzed. The technique involved dual immunostaining for GCAP1 and S100B on one set of specimens and for ROS-GC1 and S100B on another. The results are presented in Figure 5. Boxed regions of panels A, C, and E have been magnified and presented as panels B, D, and F, respectively. A consistent and distinct localization pattern for each protein was observed. Panels A and B (green/yellowish color) show the localization of ROS-GC1 in the pinealocytes; the nuclei in the plane of sectioning are shown in blue. All the pinealocytes were found to express ROS-GC1, and this expression was largely perinuclear with a higher level of expression occurring in the juxtanuclear region (Figure 5A,B, green/yellowish color). Panels C−F (green color) show that pinealocytes also express GCAP1. The staining, as for ROS-GC1, was perinuclear, with a fairly uniform distribution pattern (Figure 5C−F; green color) around the nucleus (Figure 5C−F; blue color). These results support the conclusion that ROS-GC1 and GCAP1 are colocalized in the majority of the pinealocytes.

Results related to the S100B localization are intriguing. Panels A−E (yellowish color, A and B; red color, C−F) of Figure 5 show that the distribution pattern of S100B was found to be restricted to a small population of pinealocytes. Unlike the more diffuse staining observed within cells expressing GCAP1 or ROS-GC1, staining for S100B was localized to discrete regions of the cell (Figure 5: yellowish color, A and B; red color, C−F). The appearance and pattern of S100B staining were consistent with a cell-surface association of this protein. The pinealocytes expressing S100B were also positive for ROS-GC1 (yellowish color; Figure 5A,B). These findings show that ROS-GC1 and S100B are colocalized in a small population of pinealocytes.

A striking difference was observed in the relative distributions of GCAP1 and S100B—they were nearly mutually exclusive. A large number of cells expressed GCAP1 alone (hollow straight arrows, Figure 5C−F). A smaller population of cells expressed S100B, and in these cells, GCAP1 staining was not detected (filled bent arrows, Figure 5C−F). Hence, in the bovine pineal gland ROS-GC1 and GCAP1 are colocalized within a large number of pinealocytes; ROS-

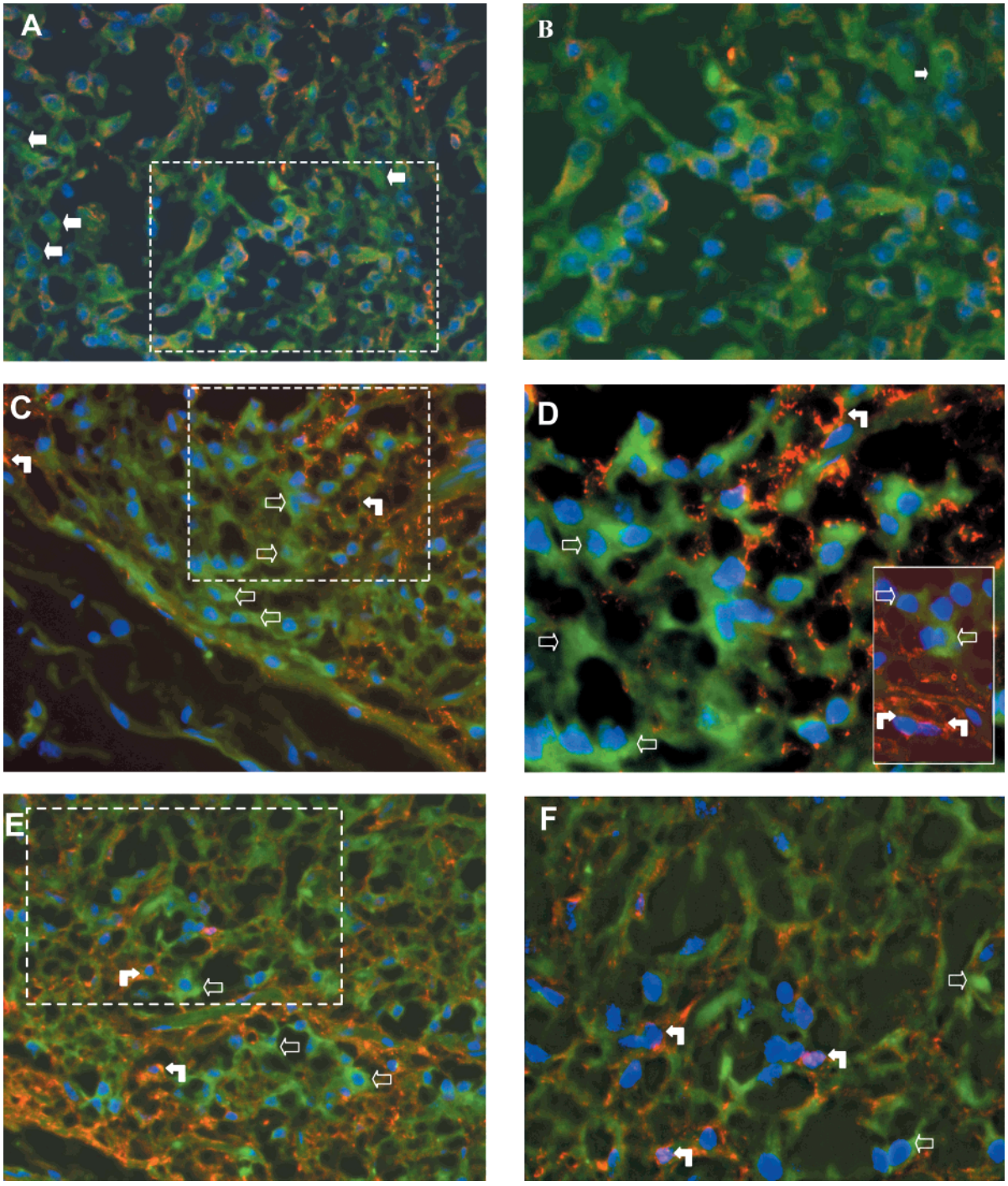

FIGURE 5: Relative distribution of ROS-GC1, GCAP1, and S100B. Immunohistochemical analyses: Pineal cryosections were probed for the localization of ROS-GC1 (A, B), GCAP1 (C−F), or S100B (A−F) as described in Experimental Procedures. Fields containing positively staining cells were chosen. Regions boxed in panels A, C, and E have been magnified in panels B, D, and F, respectively. ROS-GC1 (A, B) and GCAP1 (C−F) have been colored green. S100B has been colored red (A−F). Superimposition of green (ROS-GC1) and red (S100B) results in a yellowish color where the two overlap (A, B). The nuclei appear in blue (A−F). Filled arrows indicate pinealocytes that express ROS-GC1 but not S100B (A, B). Hollow arrows indicate cells that express GCAP1 but not S100B (C−F). Filled, bent arrows indicate cells that express S100B but not GCAP1 (C−F). A magnified inset in panel D shows juxtaposition of cells that exclusively express GCAP1 or S100B.

GC1 and S100B are colocalized in a small population of pinealocytes. This leads to the fascinating conclusion that the two ROS-GC1 transduction systems are not present in the same pinealocyte.

DISCUSSION

Almost 3 decades ago the existence of an α-AR-coupled mGC transduction system in the rat adrenocortical carcinoma was described (*27*, *28*). This was an ectopically developed signal transduction system, occurring in the carcinoma cells but nonexistent in the normal adrenal cortex (reviewed in ref *29*). At the time the heterogeneity of α-AR and guanylate cyclase was not defined. Hence, the biochemical linkage between the signaling receptor and the transduction component remained baffling. About 15 years ago, the receptor was purified, biochemically characterized (*30*), cloned (*31*, *32*),

and shown to belong to a new pharmacologically defined α_{2D}-AR subtype (*32*, *33*). Thus, the original carcinoma receptor subtype linked to the mGC was α_{2D}-AR. Yet, the identity of the mGC remained elusive, although it was recognized that the cyclase was not a surface receptor (reviewed in ref *29*). The carcinoma cyclase was Ca^{2+}-sensitive (*34*), and the surface receptor subfamily of mGCs is Ca^{2+}-insensitive (reviewed in ref *2*). It has now been established that the α_{2D}-AR receptor subtype is a species variant of the pharmacologically distinct α_{2A}-AR (*32*, *33*, *35*) and, therefore, is referred to as $\alpha_{2D/A}$-AR (*36*).

Recently, an $\alpha_{2D/A}$-AR-linked mGC transduction system similar or identical to that in the carcinoma cells was discovered in the pineal gland (*1*). This mGC responds specifically to $\alpha_{2D/A}$-AR agonists, and the linkage between the receptor and the cyclase is via Ca^{2+} (*1*). Predicted features of the transduction system are that (1) it responds to the receptor signal via an intracellular rise of Ca^{2+}; (2) the response is indirect, occurring via a pineal HSF; and (3) the responsive cyclase is a ROS-GC, which is a new subfamily of membrane guanylate cyclases. This subfamily is specifically designed to transduce the intracellularly generated neurosensory Ca^{2+} signals (reviewed in ref *2*). In the present study, components of this transduction system have been characterized in the pinealocyte. Furthermore, in a distinct population of pinealocytes, another Ca^{2+}-modulatory component, GCAP1, has been discovered. GCAP1 also colocalizes with ROS-GC1 and $\alpha_{2D/A}$-AR, and it is localized in the majority of pinealocytes (Figures 2 and 5). A striking feature of the new transduction component is that it regulates ROS-GC1 in a fashion opposite to that of S100B. Essential features of these two transduction systems under the subheadings, *Original* and *New*, are discussed below.

The Original Transduction System Operates through ROS-GC1, Is Accelerated by Ca^{2+} Signaling, and Is Restricted in Pinealocytes. An earlier study shows that the pineal ROS-GC is stimulated by a pineal HSF (*1*). In the present study, extensive biochemical, molecular, immunological, and, finally, reconstitutional analyses using the rROS-GC1 system show that this factor is S100B. The pineal gland is known to be composed of two cell types: pinealocytes and glial. Immunocytochemical analysis reveals that S100B resides in the pinealocytes (yellowish color, Figure 5A,B; red color, Figure 5C−F).

Studies with the retinal photoreceptor system have established the existence of two ROS-GCs: 1 and 2 (reviewed in ref *2*). Scanning of ROS-GC by biochemical, molecular, immunochemical, and reconstitutional means shows that the pineal gland contains only one form of ROS-GC, which is ROS-GC1, and that it is solely confined to the pinealocytes (Figure 2, green color; Figure 5A,B, green/yellowish color). Thus, both S100B and ROS-GC1 reside in the pinealocytes (Figure 5A,B, yellowish color).

Careful examination of the pinealocyte regions shows that the residence of $\alpha_{2D/A}$-AR is also pinealocyte-specific (Figure 2E,F, red color), and all three signal transduction molecules—the receptor, the cyclase, and S100B—are colocalized in the same region of the pinealocyte. These findings are consistent with the model that the $\alpha_{2D/A}$-AR-generated signal activates a ROS-GC (1), that the ROS-GC is ROS-GC1, that Ca^{2+} signals the activation of ROS-GC1 via S100B, and that the S100B regulatory domain in ROS-GC1 is at the C-terminus, between aa 731 and 1054.

An intriguing observation related to this model is the finding that although $\alpha_{2D/A}$-AR and ROS-GC1 molecules are present in almost all the pinealocytes, S100B is present in only a few, where the receptor and the cyclase also reside (Figure 5A,B, green vs yellowish color; Figure 2A,E,F, green vs yellowish color). This indicates that the S100B-dependent Ca^{2+} signaling of ROS-GC1 is restrictive, occurring only in a minority of the pinealocyte population. Thus, $\alpha_{2D/A}$-AR signals the activation of ROS-GC1 only in selective pinealocytes. On a functional basis, this finding also divides the pinealocytes in two classes, only one of which responds to the $\alpha_{2D/A}$-AR signaling in the generation of cyclic GMP.

Hence, the pineal gland contains a ROS-GC1-linked $\alpha_{2D/A}$-AR signaling system, this system is restricted to a set of pinealocytes, and the system is Ca^{2+}-modulated by S100B. Upon receptor activation, ROS-GC1 is stimulated to produce cyclic GMP.

What is the physiological relevance of this signal transduction system? Because the system is epinephrine/norepinephrine-regulated, an attractive speculation will be that it might have a bearing in explaining the light-regulated feature of melatonin secretion. The pineal gland is light-regulated. Light inhibits the synthesis of melatonin, and melatonin secretion is regulated by epinephrine (reviewed in ref *37*). At midnight, norepinephrine levels in the pineal gland significantly rise, and the sympathetic nerves in the pineal gland are capable of producing norepinephrine in the pineal gland (*38*). In the current model, the generated norepinephrine, via its $\alpha_{2D/A}$-AR, will cause a rise in intracellular Ca^{2+}, which will bind to S100B, and S100B, in turn, through the ROS-GC1 domain localized in the aa 731−1054 segment will activate the cyclase. Cyclic GMP, thus produced, will be a mediator of epinephrine/norepinephrine modulation of melatonin synthesis and secretion. The role of epinephrine in regulating melatonin synthesis via the cAMP pathway is already well-established (*39*, *40*; reviewed in ref *41*).

A New Transduction System That Also Operates through ROS-GC1, Is Inhibited by Ca^{2+} Signaling, and Is Predominant in Pinealocytes. Studies with the retinal photoreceptor system have established the existence of two GCAPs, 1 and 2, which regulate ROS-GC1. The present study shows that the pinealocyte contains a GCAP, GCAP1 and not GCAP2 (Figure 3A,B), and that GCAP1 resides in the region where ROS-GC1 and $\alpha_{2D/A}$-AR also occur (Figure 5C−E, green color). There are three notable aspects of GCAP1's residence: (1) along with the receptor and ROS-GC1, it occurs in the majority of the pinealocytes (Figure 5C−E, green color); (2) in those pinealocytes expressing GCAP1, S100B does not occur (Figure 5C−E, green vs red color); and (3) in the pinealocytes where S100B occurs, GCAP1 does not occur (Figure 5C−E, red vs green color). Thus, S100B- and GCAP1-regulated pathways are segregated in pinealocytes, the GCAP1-regulated pathway is dominant, and both pathways operate through ROS-GC1. Studies in the photoreceptor system have shown that Ca^{2+} regulates ROS-GC1 in opposing fashions via these two pathways. In contrast to S100B, elevated Ca^{2+} levels in the presence of GCAP1 inhibit ROS-GC1. The site(s) on ROS-GC1 that interact with GCAP1 is (are) also distinct from that which interacts with S100B (*25*, *42*). In a recent study, two GCAP1 modulatory domains of

the cyclase have been mapped: M445−L456, the transduction domain, and L503−I522, the binding domain (*42*). The GCAP1-linked ROS-GC1 transduction pathway is linked with phototransduction. The present findings indicate that this pathway also exists in the pinealocytes.

Current operational principles of phototransduction involve regulation of dark and light states of photoreceptors by ROS-GC1 via its Ca^{2+}-sensing modulator GCAP1. In the dark state, when Ca^{2+} levels are around 500 nM, GCAP1 bound to ROS-GC1 keeps the cyclase in its basal state. In the light state, Ca^{2+} levels decrease to 50 nM due to closure of cGMP-gated channels resulting from amplified hydrolysis of cGMP. Under these conditions, GCAP1 bound to ROS-GC1 stimulates the cyclase activity, resulting in elevation of cGMP leading to the recovery state. Thus, in photoreceptors, Ca^{2+} via GCAP1 oscillates ROS-GC1 activity between basal (inhibited) and stimulated states, depending on a light stimulus (reviewed in ref *2*). In the mammalian pineal gland, where there is no direct light stimulus, it is possible that this role is taken over by photosensitive oscillations of epinephrine/norepinephrine. Elevation of cellular Ca^{2+} from norepinephrine signaling would cause a dark-like state in pinealocytes and the removal of the signal to a light-like state. Thus, in this hypothetical scheme, cycling between dark and light in the pineal gland is mediated by photosensitive oscillations of epinephrine/norepinephrine levels. This hypothetical scheme proposes a plausible sensing mechanism of light by the pineal gland. If this mechanism is confirmed, it may explain a mysterious feature of the pineal gland termed photoentrainment (*43*). It has been proposed that light for this process is sensed through cryptic photoreceptors in retina. These photoreceptors are distinct from those involved in phototransduction because their removal does not affect the rhythmic behavior of mice (*44*, *45*). It is noteworthy that the postganglionic nerve fibers that innervate the pineal gland and release norepinephrine in a circadian fashion arise from the superior cervical ganglion, which receives input from the ganglionic cells in the retina through the suprachiasmatic nucleus, paraventricular nucleus, and intermediolateral column (reviewed in ref *37*). It is conceivable that the GCAP1-modulated ROS-GC1 machinery might be the one that is propelled by photoentrainment.

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