The $\alpha_{2D/A}$-adrenergic receptor-linked membrane guanylate cyclase: a new signal transduction system in the pineal gland

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Abstract In the pineal gland, the membrane guanylate cyclase activity was specifically stimulated by $\alpha_{2D/A}$-adrenergic receptor ($\alpha_{2D/A}$-AR) agonists. The agonists, however, did not stimulate the cyclase activity in the cell-free membranes. It was possible to stimulate the cyclase in cell-free membranes by the addition of the pineal soluble fraction, but this stimulation was Ca^{2+}-dependent and $\alpha_{2D/A}$-agonist-independent. It was also possible to achieve Ca^{2+}-dependent stimulation of the cyclase by the direct addition of CD-GCAP to the isolated pineal membranes. CD-GCAP is a Ca^{2+}-binding protein and is a specific activator of one of the two members of the ROS-GC subfamily of membrane guanylate cyclases, ROS-GC1. The soluble fraction of the pineal gland stimulated recombinant ROS-GC1 in a Ca^{2+}-dependent fashion. The direct presence of both ROS-GC1 and CD-GCAP in the pineal was established by molecular cloning/PCR studies. The findings demonstrate the existence of a novel signal transduction mechanism – the linkage of the $\alpha_{2D/A}$-AR signaling system with ROS-GC1 transduction system, occurring through intracellular Ca^{2+} via CD-GCAP.

Key words: $\alpha_{2D/A}$-Adrenergic receptor; ROS-GC1; CD-GCAP; Guanylate cyclase

1. Introduction

The original investigations in the late 1970s demonstrated the presence of an epinephrine-dependent membrane guanylate cyclase in rat carcinoma cells and showed that the cyclase activation occurs via the α-adrenergic receptor (α-AR), thereby establishing the existence of an α-AR-coupled membrane guanylate cyclase transduction system in these cells [1,2]. At the time, neither the heterogeneity of α-AR nor that of guanylate cyclase was defined. Hence, the biochemical and molecular nature of the interactions between the signaling receptor and the transduction component of the cyclase remained elusive. The rat receptor was then purified and biochemically characterized [3], cloned [4,5], and shown to belong to a new pharmacologically defined α_{2D}-AR subtype [5,6]. This implies that the original carcinoma receptor subtype linked to the membrane guanylate cyclase was α_{2D}-AR. It has now been established that the α_{2D}-AR subtype is a species variant of the pharmacologically distinct α_{2A}-AR [6,7] and, therefore, is now referred to as $\alpha_{2D/A}$-AR [8].

The present study shows the presence of a membrane guanylate cyclase in the pineal gland that is also linked to the $\alpha_{2D/A}$-AR signaling: (1) the stimulation of the cyclase is specifically mediated by $\alpha_{2D/A}$-AR and involves Ca^{2+}; (2) the signaling is indirect; (3) the stimulation of the cyclase can be independently mimicked by the pineal soluble fraction in a Ca^{2+}-dependent fashion; and (4) the reconstitution and molecular cloning/PCR studies indicate that the cyclase is ROS-GC1 and that its activator is CD-GCAP. Recent studies have shown that the CD-GCAP/ROS-GC1 transduction system is specifically regulated by intracellular Ca^{2+}, and may be linked to the retinal synaptic activity (reviewed in [9]). This report, therefore, demonstrates a new catecholamine signaling mechanism in the pineal gland, that includes components which mimic the retinal CD-GCAP/ROS-GC1 transduction system.

2. Materials and methods

2.1. Treatment of pineal glands and isolation of membranes

Bovine pineal glands were obtained fresh from a slaughterhouse (Moyer Packaging Co., Souderton, PA), washed in buffer A [0.32 M sucrose, 20 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol (DTT), 0.5 mM ethylene diamine tetraacetate (EDTA), and protease inhibitors: 1 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and minced carefully in the same buffer. The pineal pieces were divided into portions of equal weights for incubation in Dulbecco's modified Eagle's medium in 6-well dishes at 37°C in atmosphere containing 5% CO_2 with or without added reagents. After 15 min of incubation, the tissue was washed twice in buffer A and homogenized with a Teflon pestle. The homogenates were initially centrifuged at $10\,000\times g$, followed by centrifugation at $100\,000\times g$. The pellet constituted the membrane fraction and the supernatant was labeled as the 'soluble fraction'. The soluble fraction was incubated at 75°C for 3 min in the presence of 5 mM $CaCl_2$ (heat inactivation) and centrifuged at $10\,000\times g$. The supernatant was dialyzed against buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$ and protease inhibitors benzamidine (1 mM) and PMSF (0.5 mM).

2.2. Expression of membrane guanylate cyclases in COS cells

COS7 cells maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected according to previously described protocols with the atrial natriuretic factor-receptor guanylate cyclase (ANF-RGC) [10], C-type natriuretic factor-receptor guanylate cyclase (CNP-RGC) [11], or ROS-GC1 [12] expression constructs. At 60 h after transfection, cells were washed with buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$, scraped into 2 ml of cold buffer, homogenized, centrifuged for 15 min at $5000\times g$, and washed several times with the same buffer. The pellet represented the crude membranes.

2.3. Guanylate cyclase assay

Membranes isolated from the pineal gland or COS7 cells transfected as above were assayed for guanylate cyclase activity [13]. All experiments were carried out in triplicate and repeated at least three times for reproducibility. The results are expressed as mean ± standard deviation.

2.4. Isolation of RNA and reverse transcriptase-polymerase chain reaction

RNA was isolated from the pineal gland through acid-phenol extraction procedure [14] and reverse transcriptase reaction was carried out as described earlier [15]. Briefly, 10 μg of RNA was treated with

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RNase-free DNase (Promega Corp) and converted into cDNA with Superscript II reverse transcriptase (Life Technology). A 200 bp fragment corresponding to the CD-GCAP/S100B mRNA was amplified as described previously [16] using primers 5′-GTTGCCCTCATTGAYGTNTTYCA-3′ (corresponding to nt 145–157 of bovine CD-GCAP) and 5′-ATAAACTCCTGGAARTCRAYTC-3′ (complementary to nt 322–344). Amplification of ROS-GC1 (548 bp fragment) and L30 (238 bp fragment) for quantitative RT-PCR was exactly as described in [17]. The identity of each amplified fragment was confirmed by sequencing using the fmol sequencing method (Promega). The amplified products were electrophoresed, transferred to nytran membranes and hybridized to radioactively labeled probes generated from the respective cDNA clones. The blots were washed to remove non-specific hybridization and subjected to autoradiography. The signals were quantified using the Imagemaster VDS system (Pharmacia).

2.5. Cloning of ROS-GC1 from bovine pineal gland

A pineal cDNA library was constructed exactly as described in [12] for retinal cDNA library. The library was screened with multiple probes derived from the retinal ROS-GC1 cDNA which were radioactively labeled and used for hybridization as described in [12]. A single positive clone was isolated and sequenced to determine its identity.

3. Results and discussion

3.1. The $\alpha_{2D/A}$-AR-mediated signal activates membrane guanylate cyclase

A general characteristic of all members of α_2-AR and β-AR families is that epinephrine is their natural ligand (reviewed in [18,19]). The members of these two families, however, are linked in opposing fashions to its transducer adenylate cyclase: the α_2-AR signaling inactivates and the β-AR signaling activates the adenylate cyclase [18,19]. Early studies with the rat adrenocortical carcinoma cells showed that the epinephrine signal mediated by the $\alpha_{2D/A}$-AR not only inhibits adenylate cyclase activity, but also stimulates the membrane guanylate cyclase activity in an indirect fashion (reviewed in [20]). To determine if an epinephrine-responsive membrane guanylate cyclase existed in the pineal gland, the glands were treated with the incremental concentrations of epinephrine, and the membrane fractions were scrutinized for the guanylate cyclase activity. Epinephrine caused a concentration-dependent increase in the membrane guanylate cyclase activity (Fig. 1A). The concentration causing half-maximal response (EC_{50}) was 80 nM. The enzyme saturation was achieved at 1 μM with more than three-fold stimulation and significant stimulation was observed with as little as 1 nM of epinephrine (Fig. 1A). Rauwolscine, an α_2-AR antagonist [8], caused a near-total loss of the epinephrine-dependent cyclase stimulation (Fig. 1B). Thus, the pineal gland contains a membrane guanylate cyclase that is epinephrine-dependent and epinephrine acts through α_2-AR.

Among the α_2-ARs, the bovine pineal gland is exclusively populated by the $\alpha_{2D/A}$-AR subtype as demonstrated by extensive pharmacological analyses [21] suggesting the functional linkage of this receptor subtype with the membrane guanylate cyclase. To determine if this was indeed the case, the effect of specific $\alpha_{2D/A}$-AR agonists and antagonists on this linkage was investigated. The membrane guanylate cyclase activity of the pineal gland was measured after its treatment with *p*-aminoclonidine (PAC), a specific agonist. PAC yielded a stimulatory pattern similar to that of epinephrine, with an EC_{50} of 95 nM and the maximal enzyme saturation was achieved at 1μM PAC (Fig. 1A). The cyclase stimulation was completely blocked by two specific $\alpha_{2D/A}$-AR antagonists, rauwolscine and yohimbine, but not by the α_1-AR antagonist, prazosin (Fig. 1B). Similarly, UK14304, another specific $\alpha_{2D/A}$-AR agonist, elicited a dose-dependent stimulation of membrane guanylate activity (Fig. 1A) with EC_{50} value identical to PAC and again the stimulation was blocked by the antagonist, yohimbine (Fig. 1B). Thus, the epinephrine effect was mimicked by $\alpha_{2D/A}$-AR agonists and the effect could be blocked by specific $\alpha_{2D/A}$-AR antagonists but not the α_1-AR

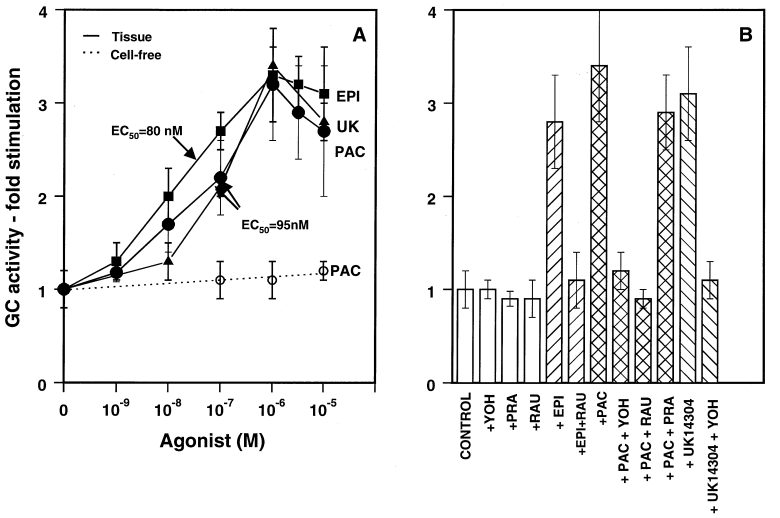

Fig. 1. Epinephrine activation of pineal guanylate cyclase through $\alpha_{2D/A}$-AR. Treatment of pineal glands, isolation of membranes and guanylate cyclase assay was performed as described in Section 2. A: Epinephrine and $\alpha_{2D/A}$-AR agonists stimulate pineal guanylate cyclase indirectly. Pineal glands were treated with indicated concentrations of epinephrine (EPI) or $\alpha_{2D/A}$-AR agonists (*p*-aminoclonidine (PAC) or UK 14304 (UK)) as described in Section 2. Membranes were then isolated and assayed for guanylate cyclase activity (solid line). The effect of PAC addition on isolated membranes (dotted line) was also investigated. Values presented are mean ± standard deviation. B: Pineal membrane guanylate cyclase is stimulated upon $\alpha_{2D/A}$-AR activation specifically. All agents used for treatment were at a concentration of 1 μM and the treatment was for 15 min at 37°C. Experiments were carried out in triplicate and repeated thrice for reproducibility. Values presented are mean ± standard deviation. EPI, epinephrine; PAC, *p*-aminoclonidine; PRA, prazosin; RAU, rauwolscine; UK, UK 14304; YOH, yohimbine.

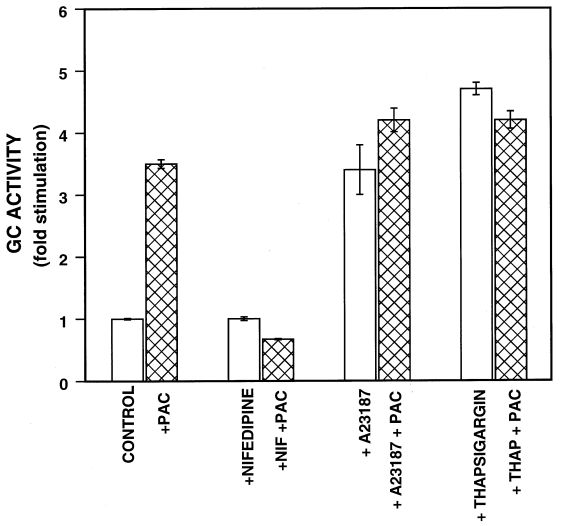

Fig. 2. $\alpha_{2D/A}$-AR guanylate cyclase signaling involves calcium. Treatment of pineal glands, isolation of membranes and guanylate cyclase assay was performed as described in Section 2. All agents used for treatment were at a concentration of 1 μM. Experiments were carried out in triplicate and repeated thrice for reproducibility. Values presented are mean ± standard deviation.

antagonist. Hence, activation of the $\alpha_{2D/A}$-AR subtype signals the stimulation of pineal membrane guanylate cyclase and there is no involvement of any other α_2-AR subtype or α_1-AR.

3.2. The $\alpha_{2D/A}$-AR-mediated membrane guanylate cyclase signaling occurs indirectly and involves calcium

To determine if the $\alpha_{2D/A}$-AR-mediated cyclase signaling was direct or indirect, membranes isolated from untreated pineal gland were tested directly for the PAC-dependent guanylate cyclase activity. PAC up to 1 μM concentration, in contrast to results obtained with intact gland, did not stimulate the guanylate cyclase activity (Fig. 1A). Thus, the $\alpha_{2D/A}$-AR signaling of the membrane guanylate cyclase is indirect.

At present, two types of membrane guanylate cyclases have been characterized (reviewed in [9,22]). One type constitutes a group of peptide receptors and is directly activated by the peptide hormones. The other type distinguishes itself from the peptide receptors in that its members are not regulated by extracellular peptides. This group consists of two members of the ROS-GC subfamily, ROS-GC1 and ROS-GC2, which are modulated by intracellular Ca^{2+} signals. This suggested a possible mechanism of the $\alpha_{2D/A}$-AR signaling in which activation of the receptor causes a rise in intracellular Ca^{2+}, which activates the pineal ROS-GC via CD-GCAP.

With this hypothesis, experiments were conducted to determine the possible role of calcium in mediating the $\alpha_{2D/A}$-AR signaling in the pineal gland. Three different agents which affect intracellular Ca^{2+} in different ways were used: A23187, a Ca^{2+} ionophore; thapsigargin, which mobilizes intracellular Ca^{2+} [23]; and nifedipine, a Ca^{2+}-channel blocker [24]. The results presented in Fig. 2 demonstrate that: (1) PAC-dependent stimulation of the membrane guanylate cyclase is blocked by nifedipine, which has no effect by itself; (2) treatment of the pineal gland with A23187 and thapsigargin causes a stimulation of the membrane guanylate cyclase activity, which mimics the cyclase stimulation by PAC; (3) concomitant addition of PAC does not cause significant added stimulation of the membrane guanylate cyclase. Taken together, these results demonstrate the role of intracellular Ca^{2+} in stimulating the pineal membrane guanylate cyclase activity and suggest that the $\alpha_{2D/A}$-AR stimulates guanylate cyclase through a rise in intracellular Ca^{2+}, possibly through increased influx. This suggestion is consistent with the studies in other reports, where activation of $\alpha_{2D/A}$-AR resulted in stimulation of G_i, which in turn, opened the Ca^{2+} channels, causing a rise in intracellular Ca^{2+} [25,26].

3.3. A heat-stable soluble factor in the pineal gland directly stimulates the membrane guanylate cyclase in a Ca^{2+}-dependent manner

Clued by the observation that the $\alpha_{2D/A}$-AR stimulation of the pineal gland membrane guanylate cyclase is mediated by calcium and with the knowledge that, specifically, ROS-GC1 is intracellularly regulated by Ca^{2+} through a heat-stable Ca^{2+}-binding protein CD-GCAP [16,27], the possibility was considered that the pineal gland cyclase might be regulated by Ca^{2+} through CD-GCAP or a CD-GCAP-like protein. To test this possibility, the soluble fraction of the pineal gland was heat-inactivated and the guanylate cyclase activity in the isolated membranes of pineal glands was measured in the presence and absence of 1 mM Ca^{2+}. The results indicated that the soluble factor stimulated the membrane guanylate cyclase activity more than three fold, but this effect was lost if EGTA (a known Ca^{2+} chelator) was added (Fig. 3). Thus, the pineal gland membrane guanylate cyclase is stimulated by a pineal soluble factor, which behaves like CD-GCAP.

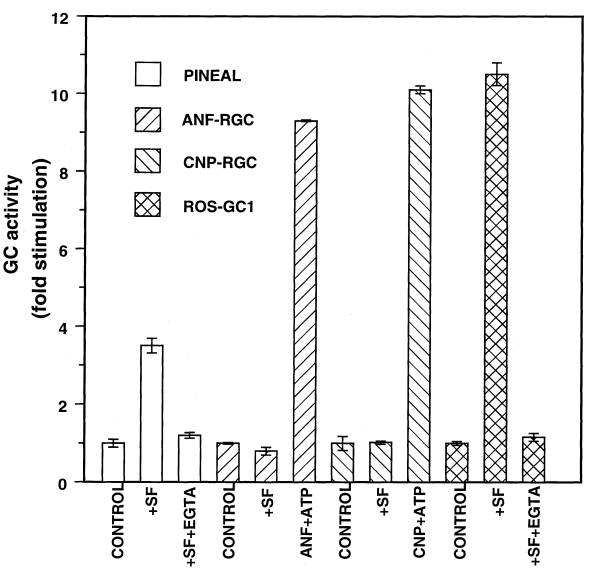

Fig. 3. Pineal soluble fraction stimulates ROS-GC1 in a Ca^{2+}-dependent fashion. Pineal membranes or membranes from COS cells expressing ANF-RGC, CNP-RGC, or ROS-GC1 were isolated as described in Section 2. ANF-RGC, CNP-RGC, and ROS-GC1 denote membranes from COS cells transfected with constructs encoding the respective cyclase. The membranes were incubated with 20 μg of protein equivalents of pineal soluble fraction (SF) and assayed for guanylate cyclase activity. ANF-RGC and CNP-RGC were also incubated with ANF and CNP (0.1 μM) respectively in the presence of 0.8 mM ATP. Experiments were carried out in triplicate and repeated thrice for reproducibility. Values presented are mean ± standard deviation.

3.4. The heat-stable soluble factor mimics CD-GCAP in the Ca^{2+}-dependent stimulation of recombinant ROS-GC1, and CD-GCAP stimulates the pineal gland membrane guanylate cyclase activity in a Ca^{2+}-dependent manner

Biochemical and molecular analyses have established that CD-GCAP is S100B (β,β), a member of the S100 proteins family [28]. A unique functional feature of CD-GCAP is that it is a positive regulator of ROS-GC1 when bound to Ca^{2+} [16,27]. Since CD-GCAP specifically activates ROS-GC1 [27,28], the possible presence of ROS-GC1 in the pineal membranes was investigated by the addition of CD-GCAP followed by assay for guanylate cyclase activity. Fig. 4A shows that in the presence of 1 mM Ca^{2+}, CD-GCAP stimulates the pineal gland membrane guanylate cyclase activity in a dose-dependent fashion with a half-maximal activation at ~2 μM and saturation at ~5 μM. In the absence of Ca^{2+}, CD-GCAP had no effect on the cyclase activity. This pattern of the pineal cyclase stimulation is very similar to that established earlier for the recombinant ROS-GC1 which is stimulated by CD-GCAP with an EC_{50} of 1.1 μM and saturation at ~5 μM [16], thus indicating the presence of ROS-GC1 in the pineal membranes.

The effect of the pineal soluble fraction on the recombinant ROS-GC1 activity was also tested. Membranes were isolated from COS cells expressing ROS-GC1, and were incubated with the pineal soluble fraction at a fixed (1 mM) Ca^{2+} concentration. The pineal soluble fraction stimulated recombinant ROS-GC1 more than 10-fold above its basal level in the presence of 1 mM Ca^{2+}; addition of EGTA resulted in loss of cyclase stimulation (Fig. 3).

Since the presence of two additional membrane guanylate cyclases, ANF-RGC and CNP-RGC, has been reported in the pineal gland [29], the effect of pineal soluble fraction on these cyclases expressed in COS cells, was investigated and was found to be insignificant (Fig. 3), consistent with the fact that ANF-RGC and CNP-RGC are not regulated by intracellular Ca^{2+} [9]. However, the ligands – ANF and CNP – elicited a stimulatory response from the respective receptors, ANF-RGC and CNP-RGC, indicating the fidelity of expression of these receptors (Fig. 3). Thus, the stimulation was specific for ROS-GC1, which is characterized by Ca^{2+}-dependent regulation [16,27,28], and establishes that the pineal soluble fraction contains a factor that is functionally similar to CD-GCAP.

At present, only three proteins – GCAP1, GCAP2 and CD-GCAP [9] – are known to regulate ROS-GC1, while other calcium-binding proteins such as calmodulin and troponin C have no effect on its activity [16]. Among GCAP1, GCAP2 and CD-GCAP, only CD-GCAP stimulates ROS-GC1 activity in response to high Ca^{2+} and is heat stable [16,27,28] – features shared by the pineal soluble factor. The remote possibility that additional heat-sensitive proteins are present in the pineal gland that may regulate ROS-GC1 in response to elevated Ca^{2+} levels, like CD-GCAP, cannot be ruled out.

3.5. Molecular probes directly demonstrate the presence of CD-GCAP and ROS-GC1 in the pineal gland

Guided by the earlier observations that ROS-GC1 exclusively resides (among the tissues tested) in the retina [12], where CD-GCAP is also present and is a specific regulator of ROS-GC1 [16,27,28], and the present findings that the pineal soluble factor stimulates the recombinant ROS-GC1 and also the native guanylate cyclase present in the pineal membranes (Fig. 3), the direct molecular presence and the relative abundance of ROS-GC1 and CD-GCAP in the pineal gland was scrutinized. Results from quantitative RT-PCR analyses presented in Fig. 4B show that both ROS-GC1 and CD-GCAP are expressed in this tissue and at levels 2–3 times lower when compared to the retina. The expression of L30, measured as a control, remained the same between the two

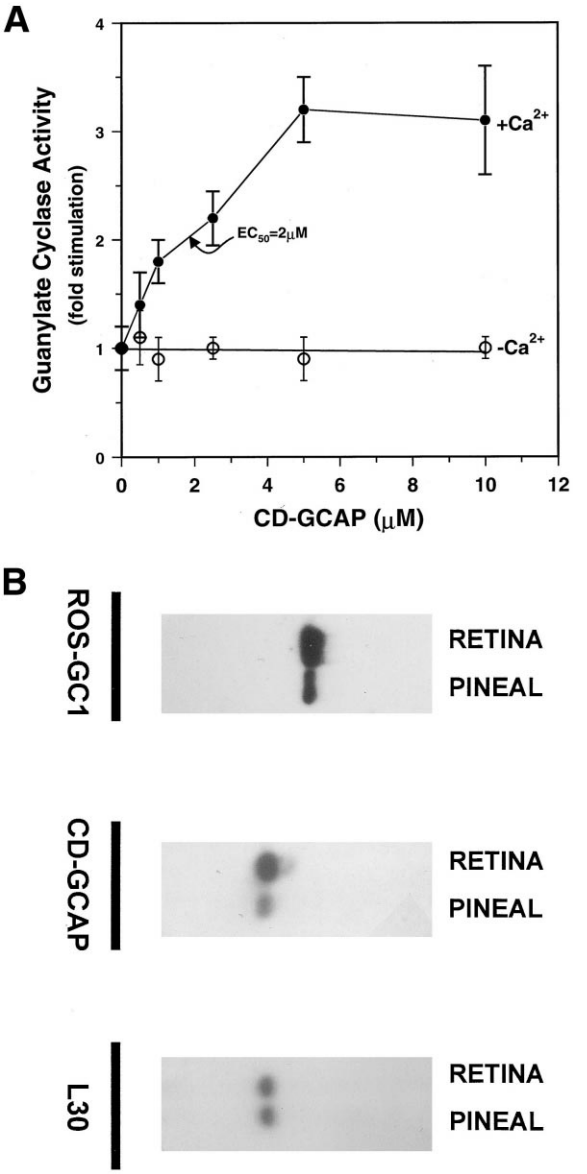

Fig. 4. A: CD-GCAP activation of pineal membrane guanylate cyclase. Isolation of membranes from pineal glands was carried out as described in Section 2. Assay for guanylate cyclase activity was performed in the absence of Ca^{2+} ($-Ca^{2+}$) or in the presence of 1 mM Ca^{2+} ($+Ca^{2+}$). Values presented are mean ± standard deviation. B: Relative abundance of ROS-GC1 vs. CD-GCAP in the retina and the pineal gland. Quantitative RT-PCR was performed as described in Section 2. Fragments corresponding to ROS-GC1, CD-GCAP and L30 (30 kDa protein from the large subunit of the ribosome) were amplified from equal amounts of RNA isolated from the retina and the pineal gland. After electrophoresis, the products were transferred onto nytran membranes and hybridized to probes generated from the respective cDNA clones. The blots were washed, exposed and subjected to autoradiography. The signals were quantified using Imagemaster VDS (Pharmacia).

tissues indicating that equivalent amounts of cDNA were used for the analyses. The results indicate that the relative amounts of ROS-GC1 and CD-GCAP remains the same in the retina and the pineal gland. To further establish the identity of CD-GCAP and ROS-GC1 in the pineal gland at the molecular level, a cDNA library from the pineal gland was screened for the presence of ROS-GC1 and a single positive clone was obtained and sequenced; the amplified fragments of CD-GCAP mRNA was also sequenced. The amplified fragment of CD-GCAP was identical to the region between nt 145 and 344 of bovine CD-GCAP sequence [16,27]. Sequencing of the isolated ROS-GC1 cDNA clone revealed that it contained regions corresponding to the catalytic domain and over 3 kb of the 3′ non-coding region with the sequence identical to the ROS-GC1 cDNA cloned earlier from the bovine retina [12]. The presence of the second member of ROS-GC subfamily, ROS-GC2, was not detected through RT-PCR in the pineal gland [30]. Thus both biochemical and molecular analyses indicate that the high Ca^{2+} signal transduction pathway is operational in the pineal gland.

3.6. Concluding remarks

By the molecular, biochemical and functional criteria, the identity of the transduction components of the $\alpha_{2D/A}$-AR linked guanylate cyclase signaling system have been revealed. Epinephrine signal originates at the extracellular receptor portion of the $\alpha_{2D/A}$-AR subtype and the signal transduction occurs within the interior of the cell, resulting in the stimulation of ROS-GC1.

It is noteworthy that a series of careful investigations have demonstrated another linkage of epinephrine signaling with the elevation of cellular cGMP [31,32], which occurs through a mechanism different from the one reported in the present study. This linkage [31,32] (i) is through conjoint activation of α_1- and β-AR, while activation of the individual receptor has no effect; and (ii) results in the activation of a soluble guanylate cyclase, whose identity is unknown. Thus, epinephrine, in the pineal gland, signals the formation of cGMP via two distinct pathways: one pathway activates the soluble guanylate cyclase, and the other, activates ROS-GC1 – a membrane guanylate cyclase.

A model is envisioned for the $\alpha_{2D/A}$-AR guanylate cyclase signaling where (i) the signaling system is turned on by the binding of epinephrine to its $\alpha_{2D/A}$-AR, (ii) this causes a rise of intracellular Ca^{2+}, (iii) Ca^{2+} then binds to CD-GCAP, and (iv) CD-GCAP activates the catalytic domain of ROS-GC1, enhancing the production of cGMP. Thus, Ca^{2+} couples the 7-transmembrane $\alpha_{2D/A}$-AR signaling system with the single transmembrane ROS-GC1 transduction system. Prior to this study, the CD-GCAP-ROS-GC1 interaction had been considered the exclusive domain of the retina. The findings of the present report extend the boundary of the system and suggest that the $\alpha_{2D/A}$-AR linked ROS-GC1 signal transduction system might represent a unified feature of neurosensory cells.

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References

[1] Perchellet, J.-P. and Sharma, R.K. (1980) Endocrinology 106, 1589–1593.
[2] Shanker, G. and Sharma, R.K. (1980) Endocrinology 106, 1594–1598.
[3] Jaiswal, R.K. and Sharma, R.K. (1985) Biochem. Biophys. Res. Commun. 130, 58–64.
[4] Chalberg, S.C., Duda, T., Rhine, J.A. and Sharma, R.K. (1990) Mol. Cell. Biochem. 97, 161–172.
[5] Lanier, S.M., Downing, S., Duzic, E. and Homcy, C.J. (1991) J. Biol. Chem. 266, 10470–10478.
[6] Wypijewski, K., Duda, T. and Sharma, R.K. (1995) Mol. Cell. Biochem. 144, 181–190.
[7] O'Rourke, M.F., Iversen, L.J., Lomasney, J.W. and Bylund, D.B. (1994) J. Pharmacol. Exp. Ther. 271, 735–740.
[8] Bylund, D.B., Eikenberg, D.C., Hieble, J.P., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Molinoff, P.B., Ruffolo Jr., R.R. and Trendelenburg, U. (1994) Pharmacol. Rev. 46, 121–136.
[9] Pugh Jr., E.N., Duda, T., Sitaramayya, A. and Sharma, R.K. (1997) Biosci. Rep. 17, 429–473.
[10] Duda, T., Goraczniak, R. and Sharma, R.K. (1991) Proc. Natl. Acad. Sci. USA 88, 7882–7886.
[11] Duda, T., Goraczniak, R. and Sharma, R.K. (1993) Biochemistry 32, 1391–1395.
[12] Goraczniak, R.M., Duda, T., Sitaramayya, A. and Sharma, R.K. (1994) Biochem. J. 302, 455–461.
[13] Paul, A.K., Marala, R.B., Jaiswal, R.K. and Sharma, R.K. (1987) Science 235, 1224–1226.
[14] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[15] Venkataraman, V., Duda, T. and Sharma, R.K. (1997) Mol. Cell. Biochem. 177, 113–123.
[16] Duda, T., Goraczniak, R.M. and Sharma, R.K. (1996) Biochemistry 35, 6263–6266.
[17] Duda, T., Venkataraman, V., Krishnan, A. and Sharma, R.K. (1998) Mol. Cell. Biochem. (in press).
[18] Hein, L. and Kobilka, B.K. (1995) Neuropharmacology 34, 357–366.
[19] Lefkowitz, R.J., Cotecchia, S., Kjelsberg, M.A., Pitcher, J., Koch, W.J., Inglese, J. and Caron, M.G. (1993) Adv. Second Messenger Phos. Res. 28, 1–9.
[20] Sharma, R.K. (1990) in: Nutrients and Cancer Prevention (Prasad, K.N. and Meyskens, F.L. Jr., Eds.), pp. 3–18, Humana Press, Englewood Cliffs, NJ.
[21] Simmonneaux, V., Ebadi, M. and Bylund, D.B. (1991) Mol. Pharmacol. 40, 235–241.
[22] Sharma, R.K., Duda, T., Goraczniak, R.M. and Sitaramayya, A. (1997) Indian J. Biochem. Biophys. 34, 40–49.
[23] Sagara, Y., Fernandez-Belda, F., Meis, L. and Inesi, G. (1992) J. Biol. Chem. 267, 12606–12613.
[24] Waters, D. (1997) Can. J. Cardiol. 13, 757–766.
[25] Nebigil, C. and Malik, K.U. (1992) J. Pharmacol. Exp. Ther. 206, 1113–1124.
[26] Lepretre, N., Mironneau, J. and Morel, J.L. (1994) J. Biol. Chem. 269, 29546–29552.
[27] Duda, T.M., Goraczniak, R.M., Pozdnyakov, N., Sitaramayya, A. and Sharma, R.K. (1998) Biochem. Biophys. Res. Commun. 242, 118–122.
[28] Pozdnyakov, N., Goraczniak, R.M., Margulis, A., Duda, T., Sharma, R.K. and Sitaramayya, A. (1997) Biochemistry 36, 14159–14166.
[29] Olcese, J., Muller, D., Munker, M. and Schmidt, C. (1994) Mol. Cell. Endocrinol. 103, 95–100.
[30] Goraczniak, R.M., Duda, T.M. and Sharma, R.K. (1997) Biochem. Biophys. Res. Commun. 234, 666–670.
[31] Sugden, A.L., Sugden, D. and Klein, D.C. (1987) J. Biol. Chem. 262, 741–745.
[32] White, B.H. and Klein, D.C. (1995) J. Neurochem. 64, 711–717.