BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 150–156 (1996)
ARTICLE NO. 0197

The Mineralocorticoid Hormone Receptor and Action in the Eye

M. Mirshahi, C. Nicolas, A. Mirshahi, C. Hecquet, F. d'Hermies, J. P. Faure, and M. K. Agarwal

INSERM U-86 and Hormone Laboratory, Centre Universitaire des Cordeliers, 15 rue de l'Ecole de Médecine, 75270 Paris 06, France

Received November 15, 1995

Immunoblotting with a polyclonal antibody, directed against the mineralocorticoid receptor protein purified from rat kidney in presence of the receptor-specific ligand RU 26752, labeled a single 98–102 kDa band in soluble extracts from bovine retina and from cultured bovine retinal pigment epithelial cells, identical to the receptor in several other tissues from the rat. This antibody also immunoprecipitated the receptor-^{3}H-RU 26752 complex in bovine retinal extract. The growth of the isolated pigment epithelial cells was inhibited by RU 26752 and ZK91587, two ligands specific to the mineralocorticoid receptor. Successive passages in culture led to the disappearance of immunoreactivity in Western blots, concurrently with the refractoriness of the cells to growth inhibition by the two antagonists. On sections of the human eye, mineralocorticoid receptor-specific immunofluorescence was observed in retinal cone cells, pigment epithelium, epithelium of ciliary body, iris, cornea and lens. To our knowledge, this is the first ever demonstration of the mineralocorticoid receptor in ocular tissues.

Steroid hormones influence the development, differentiation and homeostasis of a large number of mammalian tissues by binding to receptors that are members of a superfamily of proteins involved in the transcription-regulation of cell-specific genes (1,2). Mineralotropic hormones regulate the hydrosodic balance in many cell types of mesodermal and ectodermal origins (3,4) via appropriate ion channels in the cell membrane (4,5). Ion and fluid transport regulation mechanisms play a prominent role in the maintenance of several physiological processes in the eye that include aqueous humor secretion and intraocular pressure (6), hydration and transparency of the cornea (7) and the lens (8), adhesion of the retina and photoreceptor function (9). The intracellular and extracellular ionic environment is regulated by Na^+/K^+/ATPase channels located in the membrane of several cell types such as the non pigmented ciliary epithelium (10), retinal pigment epithelium (RPE) (11), lens epithelium (8), and corneal endothelium (7).

The regulation of ion transport in ocular cells may conceivably proceed via the receptor mediated action of mineralocorticoids. Consequently, we investigated the possible presence of the mineralocorticoid receptor (MCR) in the retina since this tissue is a target for other classes of steroids (12,13) and responds to light by changes in sodium concentration (14,15). The availability of a polyclonal antibody directed against the rat renal MCR (16,17) permitted us to demonstrate the presence of the MCR in selected areas of the eye. Furthermore, the growth response of RPE cells was altered by two steroids RU 26752 and ZK 91587 that are specific antagonists of the MCR action (3,18).

MATERIALS AND METHODS

Bovine enucleated eyes, obtained from the local slaughter house, were stored for 24 h at 4°C to reduce adhesion between the neural retina and the pigmented epithelium, and bisected posterior to the ora serrata. After removal of the vitreous body and separation of the retina, the eyecup was rinsed with RPMI-1640 (Gibco) and 1 ml trypsine (0.25%, Gibco) was introduced into the eyecup, followed by incubation for 1 h at 37°C. The RPE cells were pipetted out of the Bruch's membrane, washed with RPMI by centrifugation (10 min, 1500 × g) and finally resuspended in RPMI 1640 supplemented with 5 mM glutamine, 100 U/ml penicilline, 100 mg/ml streptomycine and 20% foetal bovine serum. The cells were seeded at 5×10^4 cells/cm^2 in tissue culture flasks and incubated at 37°C in 5% CO_2 atmosphere. The influence of mineralocorticoid antagonists (RU 26752 and ZK 91587) on the proliferation of RPE cells was assessed for 3 days in complete cell culture

0006-291X/96 $18.00

medium and expressed as % of ethanol control. Such cell survival studies were performed on RPE cells after one or several passages of three days each.

Bovine retina, myocardium and RPE cells were homogenized in phosphate buffered saline (PBS) and centrifuged at 105,000 × g to obtain a supernatant fraction. Cytosol proteins were denatured in Tris-HCl pH 6.8 containing 2% SDS + 15% glycerol and separated by electrophoresis for 3–4 h on 15% SDS-polyacrylamide gels (SDS-PAGE) calibrated with markers of known molecular weight and stained with Coomassie brilliant blue. For Western blots, proteins separated by SDS-PAGE were electrotransferred to PVDF membranes (Millipore), blocked for 1 h at 37°C with 15% fat-free dry milk, incubated with rabbit anti-rat renal MCR immune serum for 2 h at 4°C and finally saturated with sheep anti-rabbit biotinylated antibody for 90 min, followed by 90 min in presence of streptavidin-biotinylated horseradish peroxidase complex (Amersham). The membranes were washed repeatedly with PBS between each of these steps. The blots were finally developed in dark with hydrogen peroxide-4-chloro-1-naphthol-methanol (16,17).

For immunoprecipitation, 0.5 ml aliquots of bovine retinal extract were incubated (2 h 4° C) with 20 nM of the MCR-specific ligand ^{3}H-RU 26752 alone or in presence of 1000 fold excess of the homologous radioinert molecule and charcoal treated to remove free steroids. The solution was brought to 0.3 M KCl and incubated (3 h, 4°C) with 5 μg of the preimmune or anti-MCR IgG. Finally, 100 ml of goat anti-rabbit IgG-agarose (Sigma) was added to each sample and the incubation continued for additional 3 h. The precipitates at 3000 g were washed thrice with PBS and eluted once with 1 ml of 0.1 M glycine-0.15 M NaCl, pH 2.4, all at 4°C. The radioactivity in the precipitate was quantitated in 10 ml Picofluor (16,17).

Enucleated eyes of two patients with intraocular tumors were fixed in Bouin's solution for 18 h followed by standard paraffin embedding. After removal of paraffin with toluene, eye sections were hydrated in graded series of ethanol (100% to 70%) and washed with PBS. Thereafter, the slides were covered sequentially with anti MCR antiserum (1/100), sheep anti-rabbit IgG biotinylated antibody (1/200), and streptavidin-FITC, all diluted in PBS + 1% BSA. Each incubation step (60 min at room temperature) was followed by extensive washing with PBS and the slides were finally mounted in Fluoprep. Sections were photographed on a Kodak 400 ASA film under a Nikon fluorescent microscope (16).

^{3}H-RU 26752 (lot X3025A) and the corresponding radioinert molecule were a gift from Roussel-Uclaf, Romainville, France; ZK 91587 was kindly supplied by Schering AG, Germany. Goat anti-rabbit IgG-agarose (lot 77F-8985) was purchased from Sigma Chemie, France. All other chemicals were of high purity reagent grade.

RESULTS

Western blot analysis of bovine retinal extract revaled a major band of 98–102 kDa (Fig 1, lane D) in good agreement both with the theoretical value of 107 kDa estimated for the MCR cloned from human kidney (2) and with the biochemically purified antigen (18). Similar results were obtained with a control bovine myocardium cytosol (Fig 1, lane E). Data in Fig. 2 show a 98–102 kDa band (lane A) in Western blot of cytosol prepared from freshly isolated RPE. Immunoreactivity was lost after successive passages (5 and 7) of RPE cells in vitro (lanes B and C, respectively). Therefore, a functional appraisal of the MCR in RPE cells was attempted.

Both RU 26752 and ZK 91587 inhibited ($p < 0.001$ vs control) RPE cell proliferation in the first passage but not after 5 or 7 passages in culture. Microscopic examination did not reveal any difference in cell morphology between controls and steroid treated cells, so the action of the

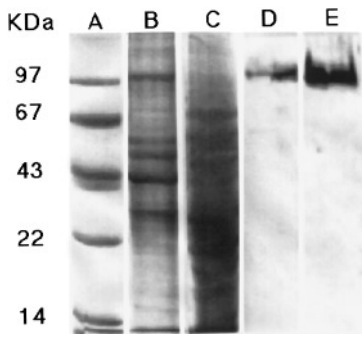

FIG. 1. Western blot analysis of the mineralocorticoid receptor in bovine retina. Standard molecular weight markers (A), 60 μg of retinal extract (B) and cardiac cytosol (C) were stained by the Coomassie reagent. Western blot analysis was performed on 60 μg protein of bovine retina (D) and heart cytosol (E).

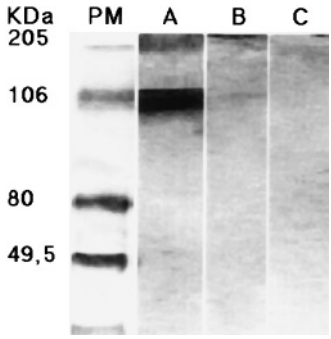

FIG. 2. The mineralocorticoid receptor is lost upon in vitro culture of the retinal pigment epithelial cells from bovine eye. Western blot analysis was performed on 60 μg protein from RPE cells after 1st (A), 3rd (B) and 7th (C) passages of three days each.

antimineralocorticoids appears to be cytostatic. Thus, the loss of MCR-immunoreactivity in passages 5 and 7 (Fig. 2) correlates well with the refractoriness to the action of MCR-specific antagonists (Fig. 3).

The anti-receptor IgG precipitated the ^{3}H-RU 26752-MCR complex in bovine retinal extract. The precipitation was limited to <50% of the total cellular MCR, as with other organs and receptor classes, and the amount precipitated suggests a concentration of <5 fmol/mg retinal protein, in keeping with studies in classical MCR targets (3,16,17).

The intracellular distribution of the MCR was assessed by immunofluorescence staining in sections of human eyes obtained from the ophthalmic pathology laboratory. In the retina, the labelling was limited to the perinuclear region of selected photoreceptor cells situated in the outer portion of the external granular layer (Fig. 5a–c). The morphology of these MCR-positive photoreceptors suggested that they are probably cones (Fig. 5a,b, arrows). Limited staining of outer segments of rod photoreceptors and of the RPE (Fig. 5a, b, double arrows) was also observed. The residual cone cells from a profoundly damaged retina after chronic glaucoma due to a tumor were strongly MCR-positive (Fig. 5c). Intense immunoreactivity was also observed in the epithelium of

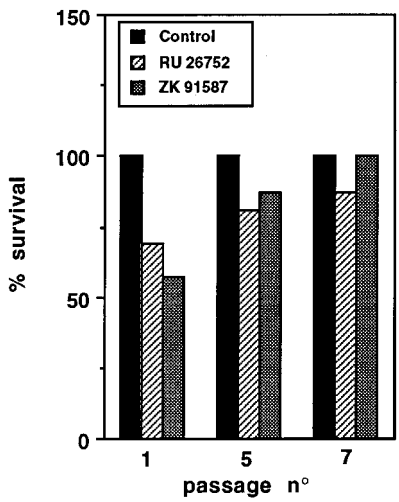

FIG. 3. Mineralocorticoid antagonists inhibit the growth of retinal pigment epithelial cells. The cells were cultivated in presence of ethanol control alone, 100 nM RU 26752 or 100 nM ZK 91587 after the 1st, 5th and 7th passages in vitro. The number of surviving cells is presented as % of control on six separate determinations. The Student's *t* test was used to determine the level of statistical significance between different groups.

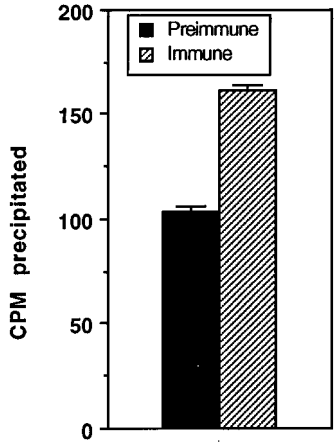

FIG. 4. Immunoprecipitation of the mineralocorticoid receptor from bovine retina. Bovine retina extract was equilibrated with 20 nM radiolabelled RU 26752 for precipitation with either the preimmune or immune IgG. The results are expressed as cpm/mg protein obtained on three separate determinations ± the standard error.

the ciliary body. In the pars plana (Fig. 5d) and the pars plicata of the ciliary body (Fig. 6a,b), both the pigmented (arrow) and the unpigmented (double arrow) epithelia exhibited MCR-specific labelling. The pigmented epithelium at the posterior face (Fig. 6c) and the pupillary margin of the iris (Fig. 6d) were similarly MCR-positive. In these epithelia, the MCR-specific immunoreactivity was present both in the cytoplasm and the nucleus. In control sections, the non-immune serum did not stain any region at all (data not shown).

DISCUSSION

Ocular tissues have generally escaped analysis as possible targets for transcription regulation by steroid hormones despite the fact that the local production of steroids may lead to paracrine and/or autocrine effects (19). Earlier work has been restricted to the demonstration of glucocorticoid receptors by radioligand binding in the lens (12,13) and the induction of glutamine synthetase by such hormones (20,21). This may partially stem from the limited quantity of retinal tissues available in animals generally used for laboratory investigation.

The studies reported here suggest that ion regulation in ocular tissues may well be under the control of mineralocorticoid hormones, much as in other organs (3–5). The antiserum generated against the rat renal receptor (16,17), purified in presence of the MCR-specific ligand RU 26752 (3,18), revealed a 98–102 kDa band in Western blots from cytosol of the whole retina and isolated RPE cells. The band disappeared concurrently with the development of refractoriness of cultured RPE cells to the growth-inhibiting action of two MCR-specific antagonists. The MCR-mediated events may therefore be required for the growth of these cells in vivo. Further, the localization of the MCR-specific immunofluorescent labelling in situ in different ocular epithelia (Figures 5 and 6) corresponds well with the distribution of Na^+/K^+/ATPase channels (10,11). So, the regulation of sodium in the interphotoreceptor matrix (22,23) may be under the control of mineralocorticoid hormone-dependent pump activity in the RPE, much as in other epithelial cells (3–5).

On the other hand, the physiological role of the MCR in the nervous tissue of the retina remains speculative. The restricted distribution in the photoreceptor cells (Fig. 5a–c) is reminiscent of the situation in the brain (24) such that the overall concentration in vivo, as evident from immunoprecipitation (Fig. 4), appears to be <5 fmol/mg protein, much as in other MCR-targets (3). Growth, development, differentiation, neonatal imprinting are some of the functions assigned to the cortical hormones in the nervous tissue (25) and these may also be true for the retina. Thus, our demon-

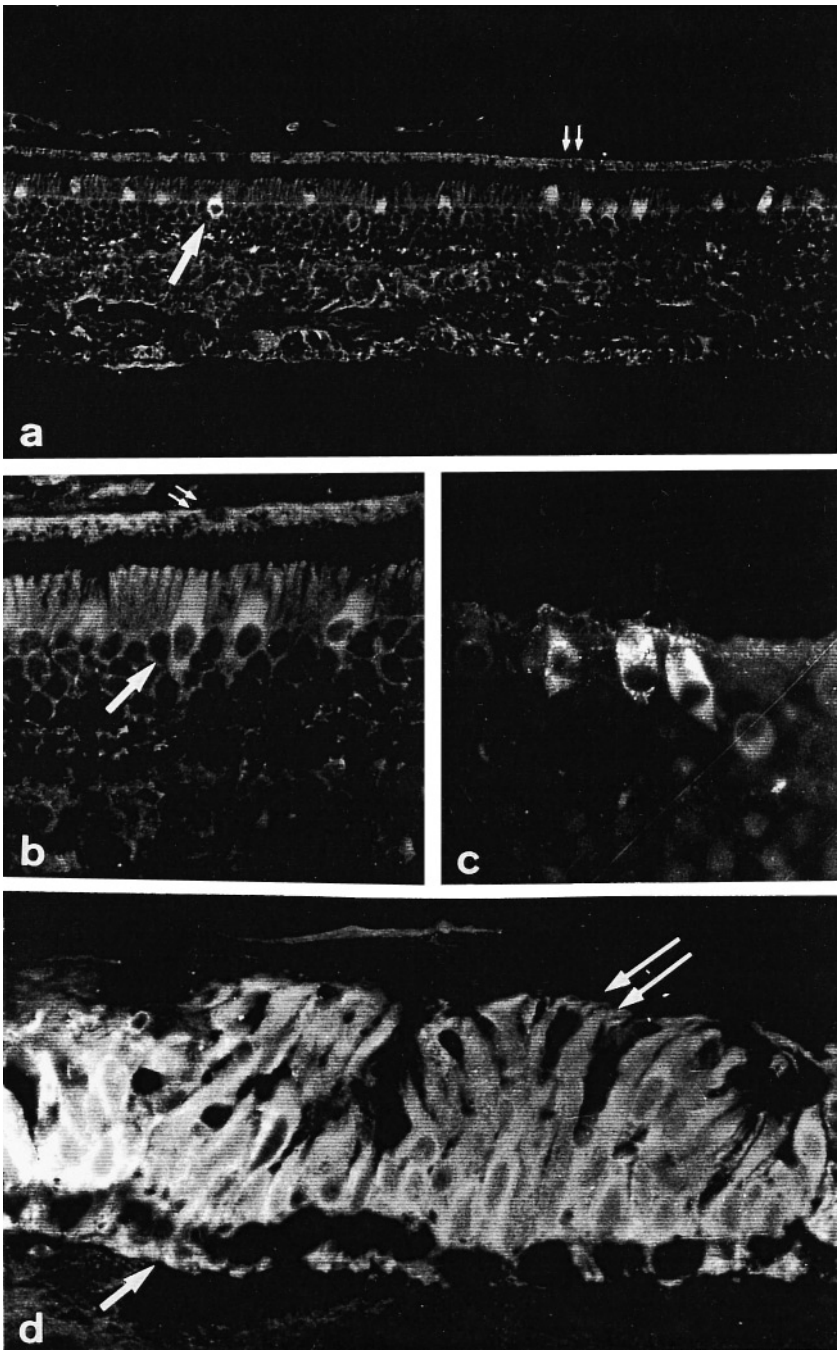

FIG. 5. Demonstration of the mineralocorticoid receptor by immunofluorescence in the retina and pars plana of the human eye. In the retina (a–c), cone cells (arrow), pigment epithelium (double arrow) and the outer segments of rods were labelled. The immunoreactivity was intense in the perinuclear cytoplasm of cones, including the residual cones of a damaged retina (c). In the pars plana of the ciliary body, the fluorescence was present in the pigmented (arrow) and the nonpigmented (double arrow) epithelia (d). In all cells, the cytoplasmic region exhibited stronger immunofluorescence than the nucleus (original magnification: a, ×200; b–d, ×500).

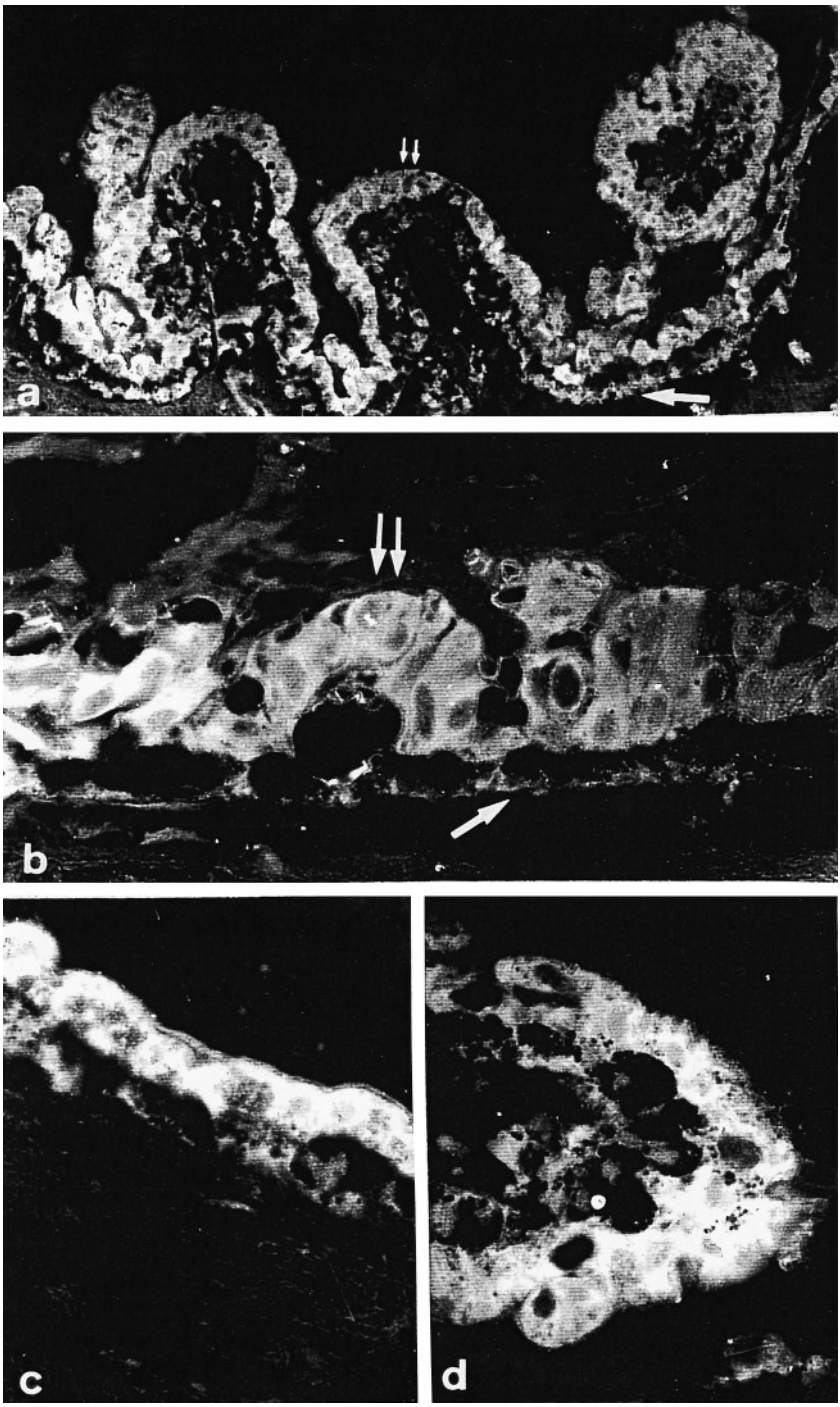

FIG. 6. Immunofluorescent staining of pars plicata of the ciliary body and iris of the human eye. The pigmented (arrow) and unpigmented (double arrow) ciliary epithelia exhibited MCR-specific immunofluorescence (a,b). The epithelium at the posterior side (c) and the pupillary margin (d) of the iris were also MCR-positive. The MCR-specific immunoreactivity was intense in the cytoplasm and variable in the nucleus (original magnification: a, ×200; b–d, ×500).

stration here of the presence of the MCR in ocular tissues identifies new targets to delineate the mechanism of action of mineralotropic hormones.

REFERENCES

1. Glass, C. K. (1994) *Endocr. Rev.* **15,** 391–407.
2. Evans, R. M. (1988) *Science* **240,** 889–895.
3. Agarwal, M. K. (1994) *Pharmacol. Rev.* **46,** 67–87.
4. Schafer, J. A., and Hawk, C. T. (1992) *Kidney Intnl.* **41,** 255–268.
5. Ewart, H. S., and Klip, A. (1995) *Am. J. Physiol.* **269,** C295–C311.
6. Usukura, J., Fain, J. L., and Bok, D. (1988) *Invest. Ophthalmol Vis. Sci.* **29,** 606–614.
7. Brown, S., and Hedbys, B. (1965) *Invest. Ophthalmol Vis. Sci.* **4,** 216–221.
8. Bonting, S. L. (1965) *Invest. Ophtalmol Vis. Sci.* **4,** 723–738.
9. Schneider, B. (1992) *Internat. Rev. Cytol.* **133,** 151–185.
10. Cole, D. F. (1964) *Exp. Eye Res.* **3,** 72–77.
11. Quinn, R. H., and Miller, S. S. (1992) *Invest. Ophthalmol Vis. Sci.* **33,** 3513–3537.
12. Southern, A. L., Gordon, G. G., Yeh, H. S., Dunn, M. W., and Weinstein, B. I. (1978) *Science* **200,** 1177–1178.
13. Ono, S., Hirano, H., and Obara, K. (1972) *Ophthal. Res.* **3,** 233–240.
14. Botchkin, L. M., and Mathews, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4564–4568.
15. Hodson, S., Armstrong, I., and Wigham, C. (1994) *Experientia* **50,** 438–441.
16. Mirshahi, M., Pagano, M., Mirshahi, A., and Agarwal, M. K. (1992) *Biochim. Biophys. Acta* **1120,** 17–23.
17. Agarwal, M. K., and Mirshahi, M. (1992) *Biochem. Biophys. Res. Commun.* **183,** 405–410.
18. Lazar, G., Pagano, M., and Agarwal, M. K. (1990) *Biochim. Biophys. Acta* **1033,** 41–48.
19. Guarneri, P., Guarneri, R., and Casio, C. et al. (1994) *J. Neurochem.* **63,** 86–96.
20. Chader, G. J., and Lehrer, L. R. (1972) *Biochim. Biophys. Acta* **264,** 186–196.
21. Dror, I. B., Havazelet, N., and Vardimon, L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1117–1121.
22. Zhang, J. J., Jacob, T. J. C., and Valverde, M. A. et al. (1994) *J. Clin. Invest.* **94,** 1690–1697.
23. Strauss, O., Richard, G., and Wienrich, M. (1993) *Biochem. Biophys. Res. Commun.* **191,** 775–778.
24. Agarwal, M. K., Mirshahi, F., Mirshahi, M., and Rostene, W. (1993) *Neuroendocrinology* **58,** 575–580.
25. Meyer, J. S. (1985) *Physiol. Rev.* **65,** 946–1021.