Photoreceptor-specific proteins in the mammalian pineal organ: immunocytochemical data and functional considerations

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Abstract. The mammalian pineal organ contains photoreceptor-specific proteins, whose distribution shows conspicuous variation among different species of mammals. Nevertheless, the following general conclusions can be drawn: immunoreactions for S-antigen and recoverin labeled more pinealocytes than the rod-opsin immunoreaction. The intensity of the recoverin- and S-antigen immunoreactions varied from cell to cell. α-Transducin immunoreaction was absent from the pineal organ of all mammals investigated with the exception of the blind mole rat. Immunoreaction for the cyclic GMP-gated cation channel was undetectable in the pineal organ of all mammals investigated. The functional significance of photoreceptor-specific proteins in the mammalian pineal organ remains unknown. It has been speculated that the S-antigen might be involved in adrenergic transduction mechanisms. To test this assumption, we have started to analyze calcium responses of single rat pinealocytes to norepinephrine stimulation using the Fura-2 technique. The cells were subsequently labeled by means of S-antigen immunocytochemistry. These combined investigations showed that variation in S-antigen immunoreactivity is not correlated with differences in the rapid calcium response to stimulation with norepinephrine. It remains to be determined whether cells displaying different intensities of the S-antigen immunoreaction show different cyclic AMP responses to noradrenergic stimulation. Investigations along this line should help to clarify further whether there is indeed a relation between the expression of S-antigen and noradrenergic transduction mechanisms in the mammalian pineal organ.

Key words: calcium, Fura-2, norepinephrine, pineal organ, recoverin, rod-opsin, S-antigen

INTRODUCTION

The retina and the pineal complex of vertebrates share several common characteristics: (1) Both organs have developed as evaginations from the diencephalon; (2) they primarily serve photoreceptive functions, and (3) they are apparently capable of producing melatonin in a light-dependent manner. However, in contrast to the retina, the pineal organ has undergone a conspicuous transformation during phylogeny and as a result, the pinealocytes differ in their fine structure and function among vertebrates (Collin 1971, Oksche 1971). Three principal types of parenchymal cells can be distinguished: classical pineal photoreceptors, modified pineal photoreceptors and neuroendocrine pinealocytes (Collin and Oksche 1981, Korf and Oksche 1986). Classical pineal photoreceptors are the characteristic feature of pineal sense organs in anamniotes. The cells display inner and regularly lamellated outer segments and establish *via* basal processes synaptic contacts with second-order neurones which are located within the pineal organ and project to various regions of the di- and mesencephalon. Classical pineal photoreceptors hyperpolarize in response to illumination. Modified photoreceptors occur in the pineal organ of reptiles and birds; they closely resemble classical pineal photoreceptors with respect to their segmental organization. However, their outer segments are less regularly organized; some cells have only a bulbous cilium lacking membrane discs. The basal processes do not establish synaptic contacts, but terminate at the basal lamina. They contain numerous dense-core granules pointing toward the secretory nature of these cells. According to Deguchi (1981), these cells respond to light stimuli with an inhibition of melatonin production and release. Neuroendocrine pinealocytes establish the main cellular component of the mammalian pineal organ. They have lost outer and inner segments, but may bear a cilium of the 9+0 type. They have also lost the direct photosensitivity and produce melatonin under noradrenergic control (Vollrath 1981). Irrespective of these phylogenetic changes, the nonphotosensitive mammalian pineal organ has retained the capability to express molecules of the phototransduction cascade.

This cascade has been well characterized in retinal rods and comprises the visual pigment rhodopsin as the membrane-bound receptor protein, the heterotrimeric GTP-binding protein transducin, cyclic GMP phosphodiesterase, cyclic GMP-gated cation channels, rhodopsin kinase, S-antigen (arrestin) and recoverin (Stryer and Bourne 1986, Cook et al. 1987, Dizhoor et al. 1991). As is characteristic for all vertebrate photopigments, rhodopsin is composed of a protein group, the apoprotein called rod-opsin and a prosthetic group (11-cis retinal) covalently bound to the apoprotein. Light stimuli cause stereoisomerization of 11-cis retinal into all-trans retinal (Wald 1968). This process is accompanied by conformational changes of the apoprotein initiating activation of transducin. Upon activation, transducin couples to the rhodopsin molecule, the GDP molecule bound to the α-subunit of transducin is exchanged by GTP, the α-subunit is released from the ß-γ subunit and catalyzes the activation of the cGMP phosphodiesterase which hydrolyzes cyclic GMP. Light stimuli also lead to a drop of free calcium within 0.5 s after light onset. The decreasing concentrations of intracellular cyclic GMP causes closure of cyclic GMP-gated cation channels and hyperpolarization of the photoreceptor membrane. After exchange of GDP by GTP, the affinity of α-transducin to rod-opsin is lowered and the latter is free to interact with other transducin molecules. Hence, one stimulated rod-opsin molecule can interact with approximately 500 transducin molecules. Desensitization is mediated by rhodopsin kinase which phosphorylates the activated rhodopsin molecule (Kühn and Dreyer 1972) and S-antigen (arrestin) which binds to phosphorylated rod-opsin and catalyzes the inhibition of the cyclic GMP-phosphodiesterase (Pfister et al. 1985). Recoverin is a 26 kDa calcium-binding protein originally thought to activate guanylate cyclase (Dizhoor et al. 1991, Lambrecht and Koch 1991) when free calcium decreases after light onset. Recent evidence, however, undermines a role of re-

coverin in Ca^{2+}-dependent cGMP synthesis (Gray-Keller et al. 1993), and its actual function remains to be determined. Perhaps recoverin accelerates deactivation of the light-stimulated rhodopsin molecule (Kawamura 1993).

The aim of this contribution is to review immunocytochemical data on the distribution of photoreceptor-cascade proteins in the pineal organ of various mammalian species and to present an *in vitro* model allowing to test hypotheses on the functional significance of these proteins in the mammalian pineal organ.

IMMUNOCYTOCHEMICAL DEMONSTRATION OF PHOTORECEPTOR-SPECIFIC PROTEINS IN THE MAMMALIAN PINEAL ORGAN *IN SITU*

Rod-opsin

The problem whether rod-opsin, the protein moiety of the visual pigment rhodopsin is present in the mammalian pineal organ has been discussed controversially for a decade. Vigh and Vigh-Teichmann (1981) were unable to find immunostaining in the mammalian pineal organ by use of an anti-bovine rhodopsin serum which labeled outer segments of retinal rods in all classes of vertebrates and also reacted with outer segments of numerous classical pineal photoreceptors in lower vertebrates. In contrast, studies with the use of another polyclonal antiserum against bovine rod-opsin (CERN-JS 858) have revealed positive immunoreactions in the pineal organ of various mammalian species including man (Korf et al. 1985a, Korf and Ekström 1987, Korf and Wicht 1992, Huang et al. 1992, Kramm et al. 1993). The specificity of these reactions was initially doubted by some investigators (Araki et al. 1988), but several results strongly suggest that the immunoreaction elicited by the antibody CERN-JS 858 in the mammalian pineal organ indeed reflects the presence of the authentic protein group of the visual pigment rhodopsin.

(1) Preabsorption of the antibody with purified bovine rhodopsin abolishes the immunoreaction in the mammalian pineal organ (Korf et al. 1985a). (2) Apart from pinealocytes, the antibody labels only photoreceptor cells in the retina clearly shown to be rods (Müller et al. 1989). (3) Immunoblotting experiments have shown that CERN-JS 859 detects three protein bands of identical relative molecular weight in mouse pineal and retinal homogenates, which obviously correspond to the mono-, di- and trimeric form of the rod-opsin molecule. (4) The same bands were labeled in retinal and pineal preparations by a monoclonal rod-opsin antibody (Kramm et al. 1993), previously characterized as specific for rod-opsin (Adamus et al. 1991). (5) When this monoclonal antibody was used in immunocytochemistry, it produced virtually the same staining pattern as did CERN-JS 859 (Kramm et al. 1993). (6) Rod-opsin transcripts were found in low, but detectable levels in adult rat pineal organs by means of the polymerase chain reaction (Araki and Taketani 1992).

Immunocytochemical investigations showed that the number of pinealocytes containing immunoreactive rod-opsin varies considerably among different species of mammals. Virtually no rod-opsin immunoreactive cells were found in the pineal organ of adult albino rodents (Korf et al. 1985a, Korf and Ekström 1987, Araki et al. 1988). Very few, but intensely labeled pinealocytes were observed in the pineal organ of cat (Korf et al. 1985a) and Djungarian hamster (Foster et al. 1989). In the pigmented mouse, approximately 25-30% of all pinealocytes displayed rod-opsin immunoreaction in perikarya and/or processes. These immunocytochemical results correspond well with quantitative estimations of the rod-opsin content in the pineal organ of albino rat, hamster and pigmented mouse: 0.075 pmoles immunoreactive rod-opsin was found per pineal gland of albino rat (Palczewski et al. 1990); one hamster pineal was shown to contain 0.34 pmoles (Foster et al. 1989). Very similar values (0.3 pmoles rod-opsin/gland: Kramm et al. 1993) were found in one pineal organ of the pigmented mouse which, however, is about tenfold smaller than the hamster pineal organ. The most

conspicuous rod-opsin immunoreaction was seen in the pineal organ of the "blind" mole rat, *Cryptomys damarensis:* approximately 50% of all pinealocytes were heavily labeled (Fig. 1a). Interestingly, this animal possesses a characteristic patch of white hairs in the parietal region of the skull and rudimentary eyes which, however, still contain numerous rod-opsin immunoreactive cells in the photoreceptor layer. In the pineal organ of adult man 3-5% of the pinealocytes were rod-opsin immunoreactive.

S-antigen (arrestin)

Long before the function of the S-antigen has been established for retinal photoreceptors, immunocytochemical studies have shown that this protein occurs selectively in the retina and pineal organ of the guinea pig (Kalsow and Wacker 1973, 1977). Thus, the S-antigen is the first "photoreceptor-specific" protein which has been shown to be expressed by retinal photoreceptors and mammalian pinealocytes. The distribution of this protein in the retina and pineal organ has been widely investigated in various mammalian species. Immunocytochemical studies with a polyclonal antiserum against bovine S-antigen (NEI 04111083) have revealed that the S-antigen immunoreaction is not restricted to retinal rods (as has been shown for the rod-opsin immunoreaction) , but labels in addition a subpopulation of cones (Müller et al. 1989). Also in the pineal organ, the number of S-antigen immunoreactive pinealocytes consistently exceeded that of rod-opsin immunoreactive cells in any mammalian species investigated to date. In hedgehog, albino or pigmented mouse and rat, hamster, blind mole rat, and cat numerous pinealocytes were S-antigen immunoreactive (Korf et al. 1985b, 1986, van Veen et al. 1986a, Fig. 1b), but the intensity of the S-antigen immunoreaction varied from cell to cell. In the pineal organ of adult man 5-10% of all pinealocytes displayed S-antigen immunoreaction.

There is good evidence that the S-antigen immunoreaction in retina and pineal organ of mammals is elicited by the same protein. As shown for the rat, the S-antigen has the same sequence in retina and pineal (Abe and Shinohara 1990). Accordingly, immunoblots from the mouse retina and pineal organ showed that the S-antigen antibody used for immunocytochemical studies labeled in both organs a single protein band of approximately 50 kDa (Korf et al. 1990).

α-Transducin

Immunoreaction for α-transducin was found in retinal photoreceptors, true and modified pineal photoreceptors, but could not be demonstrated in the bovine and rat pineal organ (van Veen et al. 1986b). This led to the assumption that transducin is absent in the mammalian pinealocytes. However, there seems to be one exception: several pinealocytes displayed immunoreactive transducin in *Cryptomys damarensis* (Fig. 1c), the species which also contained the highest number of rod-opsin immunoreactive pinealocytes.

Recoverin

In the retina, the recoverin immunoreaction appeared to label both rod- and cone type photoreceptors (Dizhoor et al. 1991, Korf et al. 1992). Moreover, a regularly arranged subpopulation of bipolar cells (Milam et al. 1993) and scattered neurons in the ganglion cell layer (Wiechmann and Hammarback 1994) showed strong recoverin immunoreaction in several mammalian species (Fig. 1g). Recoverin immunoreaction occurred also in true pineal photoreceptors of *Xenopus* and in modified pineal photoreceptors of the pigeon (Korf et al. 1992). In mammals, the number of recoverin-immunoreactive pinealocytes showed conspicuous interspecific variation. Very few cells were stained in the pineal of the albino rat whereas in rabbit, sheep, bovine (Fig. 1d), cat (Fig. 1e) and man several cells contained recoverin immunoreaction (Korf et al. 1992). As shown by immunoblotting techniques, the recoverin antibody reacts with a 26 kDa protein in both retina and mammalian pineal organ (Korf et al. 1992) thus suggesting that the molecule may be identical in retina and pineal.
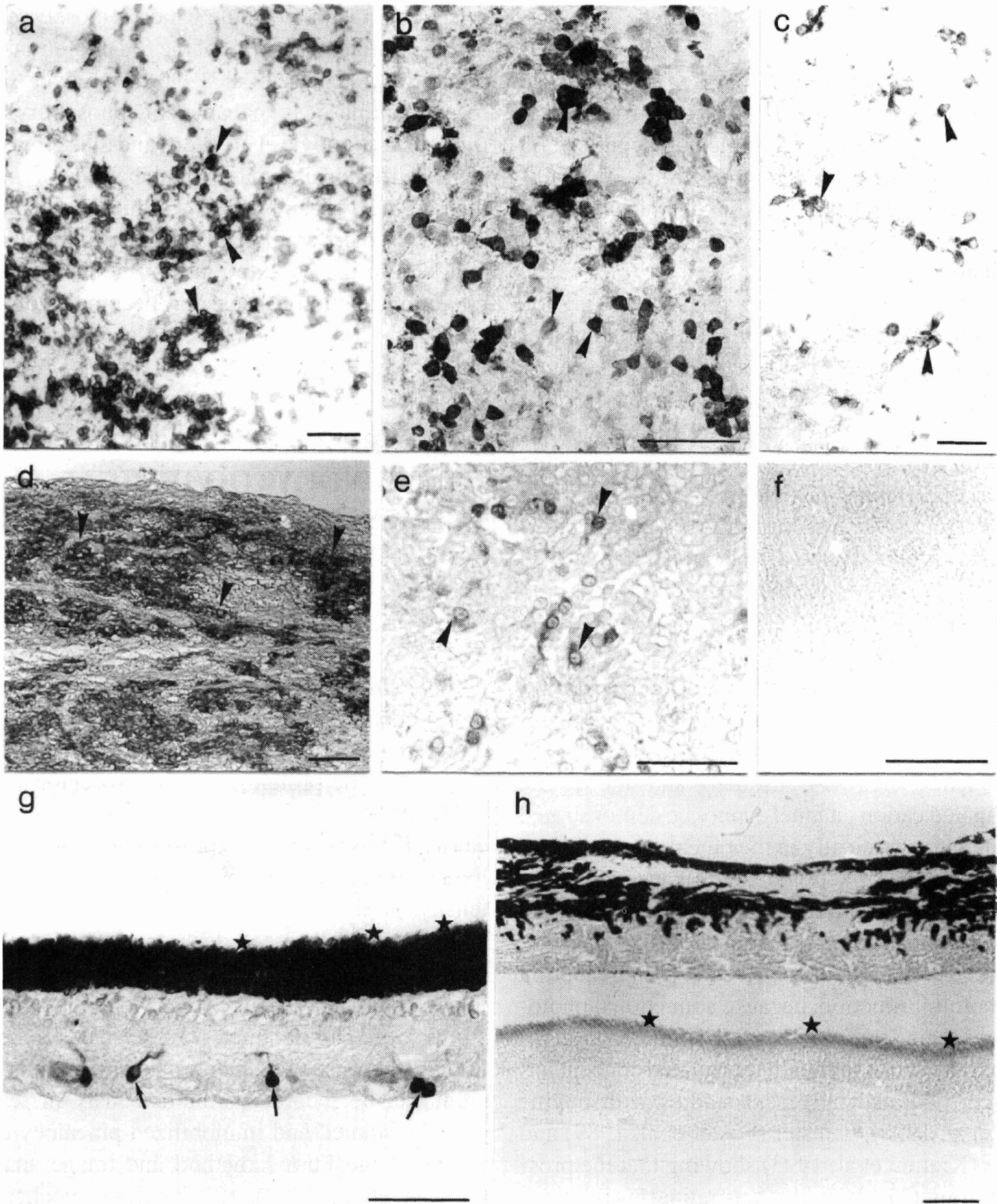
Fig. 1. Immunocytochemical demonstration of photoreceptor-specific proteins in pineal organ and retina of mammals. a-c, pineal organ of the "blind" mole rat, *Cryptomys damarensis*. a, rod-opsin immunoreaction (for methods, see Korf et al. 1985a); b, S-antigen immunoreaction (for methods, see Korf et al. 1985b); c , α-transducin immunoreaction (for methods, see van Veen et al. 1986b); d, bovine pineal organ. Recoverin immunoreaction (for methods, see Korf et al. 1992); e, cat pineal organ. Recoverin immunoreaction; f, bovine pineal organ. No immunoreaction for the cyclic GMP-gated cation channel is found (for methods, see Wässle et al. 1992); g, bovine retina. Recoverin immunoreaction decorates photoreceptor cell layer (stars) and scattered neurons in the ganglion cell layer (arrows); h, bovine retina. Immunoreaction for the cyclic GMP-gated cation channel decorates photoreceptor outer segments (stars). Arrowheads in a-e, indicate immunoreactive pinealocytes. Bar = 50 μm.

Cyclic GMP-gated cation channel

This channel has been identified, purified and functionally reconstituted from retinal rods (Cook et al. 1987). It consists of a single polypeptide of 63 kDa which probably functions as a homo-oligomeric complex. Immunocytochemical studies with the use of a monoclonal antibody have located the channel to the rod outer segment membrane (Cook et al. 1989, Wässle et al. 1992). Using the same antibody, we found immunoreaction in outer segments of rat and bovine retinal photoreceptors (Fig. 1h) thus confirming these previous results. However, no immunoreactivity was detected in the pineal organ of these species (Fig. 1f), suggesting that rat and bovine pinealocytes either lack this channel or express it at very low levels which escape immunocytochemical detection.

FUNCTIONAL CONSIDERATIONS

The above-mentioned data show that photoreceptor-specific proteins are expressed in the mammalian pineal organ to a varying degree. Some components, e.g., α-transducin and the cyclic GMP-gated cation channel cannot be demonstrated immunocytochemically in the pineal organ of most mammals. The functional significance of those photoreceptor-specific proteins that are expressed in the mammalian pineal organ remains obscure. Apparently they are not involved in photoreception and phototransduction, because a functional photopigment seems to be lacking in the mammalian pineal organ. This previously formulated concept has been corroborated by recent studies with bovine (Tsin et al. 1990), hamster (Foster et al. 1989) and mouse (Kramm et al. 1993) showing that the prosthetic group (11-cis or all-trans retinal) was absent from the pineal organ. The prosthetic group, however, is required for photoreceptive function (Wald 1968).

In many aspects, the phototransduction cascade resembles the adrenergic transduction cascade shown to play a dominant role for the regulation of pineal function in mammals. For example, it comprises an arrestin molecule which is closely related to the S-antigen (arrestin) of the rod phototransduction cascade (Lohse et al. 1990). Moreover, the ß-receptor kinase is capable of phosphorylating rhodopsin (Benovic et al. 1986) and thus closely resembles rhodopsin kinase which has been found in the rat pineal organ by means of biochemical techniques (Somers and Klein 1984). Such similarities have led to the assumption that molecules of the phototransduction cascade may become involved in adrenergic transduction mechanisms in the mammalian pineal organ.

COMBINED FUNCTIONAL AND IMMUNOCYTOCHEMICAL INVESTIGATIONS OF CULTURED RAT PINEALOCYTES

To test this attractive hypothesis we have started to analyze responses of single rat pinealocytes to norepinephrine stimulation and to characterize these functionally identified cells further by means of immunocytochemical demonstration of photoreceptor-specific proteins. As photoreceptor marker we analyzed the S-antigen immunoreaction, because (1) this protein is expressed in comparatively high amounts in the rat pineal organ and (2) the intensity of the S-antigen immunoreaction shows conspicuous cell-to-cell differences. As functional parameter we investigated the changes of intracellular calcium $[Ca^{2+}]_i$ in response to norepinephrine stimulation. This parameter was selected for the following reasons: (1) Changes in intracellular calcium can be recorded simultaneously in several (20-40) isolated and immobilized pinealocytes by means of the Fura-2 method and image analysis (Schaad et al. 1993). (2) Previous biochemical studies with suspended rat pinealocytes have shown that norepinephrine via activation of α_1-adrenergic receptors causes an elevation of $[Ca^{2+}]_i$ by increasing net influx (Sugden et al. 1987), and it is now well established that Ca^{2+} plays an important role in regulation of pineal function. Calcium obviously potentiates cyclic AMP stimulation of pineal sero-
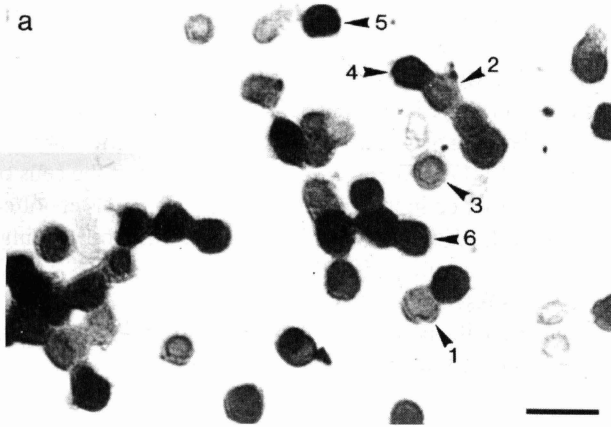
Fig. 2. Combined immunocytochemical and functional analyses of isolated rat pinealocytes. a, immunocytochemical demonstration of S-antigen in isolated, immobilized rat pinealocytes (for methods, see Wicht et al. 1993). Weakly immunoreactive pinealocytes (1,2,3) can be distinguished from strongly immunoreactive cells (4,5,6). The calcium responses of the indicated cells to norepinephrine stimulation were recorded prior to immunostaining and are shown in b and c; b, calcium response of weakly S-antigen immunoreactive cells (1,2,3); c, calcium response of strongly S-antigen immunoreactive cells (4,5,6). Variations in the intracellular calcium level are presented as changes of the ratio of Fura-2 fluorescence intensity at 340 nm and 380 nm. Arrows indicate the addition of norepinephrine (final concentration: 1 μM). Note that no differences in the calcium responses can be detected between weakly and strongly immunoreactive pinealocytes. Bar = 20 μm.

tonin-N-acetyltransferase (Yu et al. 1993), which is mediated by activation of ß-adrenergic receptors (Klein 1985).

Our investigations were performed with cells that were isolated from the pineal organ of two month old, male pigmented and albino rats by papain digestion and plated on coated coverslips with an internal grid (for methodological details, see Schaad et al. 1993). The plated cells were incubated for 20 h, loaded in growth medium with 2.5 μM Fura-2/AM for 15 min, placed in a perfusion chamber and perfused with Hank's Balanced Salt Solution (HBSS) for at least 5 min to allow complete deesterification of the dye. For stimulation, the perfusion was stopped and norepinephrine was added to the perfusion chamber at a final concentration of 1 μM. After 10 s norepinephrine was washed out by starting the perfusion again. The changes of $[Ca^{2+}]_i$
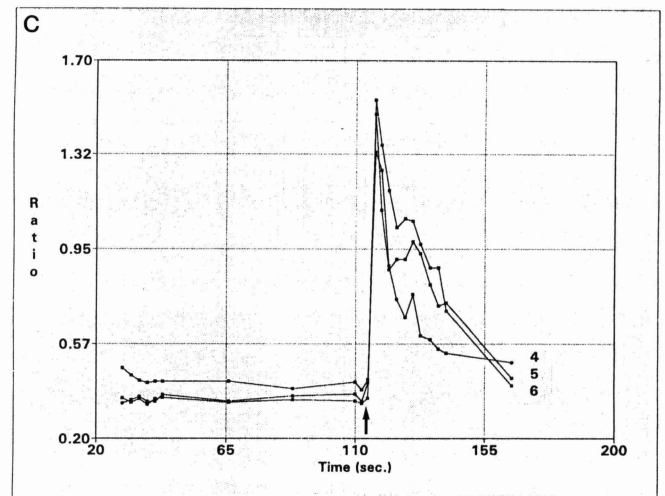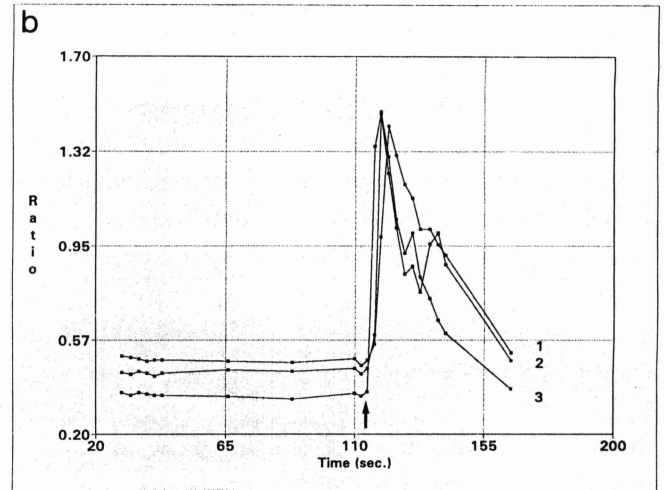
were analyzed with an imaging system (Attofluor, Zeiss, Germany). Under these experimental conditions, the majority of the plated cells responded to norepinephrine stimulation rather uniformly with a rapid and transient increase in $[Ca^{2+}]_i$ followed by a slow decrease to a plateau well above basal values (Fig. 2b and c). This plateau persisted until norepinephrine was washed out.

Subsequent to the Fura-analysis, the cells were fixed on the coverslips with 4% paraformaldehyde and processed for immunocytochemical demonstration of S-antigen as described (Wicht et al. 1993). As found for rat pinealocytes fixed in situ (see above), the intensity of the S-antigen immunoreaction varied considerably among rat pinealocytes fixed in vitro (Fig. 2a).

With the aid of the internal grid in the coverslip, 38 cells displaying a rather uniform calcium re-

sponse to norepinephrine stimulation were identified in the immunocytochemical preparations and analyzed for their S-antigen immunoreactivity. Out of these 38 cells, 18 were strongly S-antigen immunoreactive, whereas 20 showed a moderate or very weak immunoreaction. These combined analyses support the notion that the variation in S-antigen immunoreactivity is apparently not correlated with differences in the rapid calcium response to short term (10 s) stimulation with norepinephrine. It remains to be determined whether pinealocytes displaying strong or weak S-antigen immunoreactivity differ in their response to long term (up to 1 h) stimulation with norepinephrine. Moreover, it is necessary to examine whether there is any correlation between the intensity of the S-antigen immunoreaction and the cyclic AMP response elicited by norepinephrine via stimulation of ß-adrenergic receptors. Such investigations can be performed with isolated pinealocytes kept *in vitro*, as soon as a fluorescent indicator for changes in intracellular cyclic AMP becomes available. All these investigations will help to clarify whether there is a relation between the S-antigen molecule and noradrenergic transduction mechanisms in the mammalian pineal organ.

ACKNOWLEDGEMENTS

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