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Neuropeptide Y (NPY)-like immunoreactive amacrine cells in retinas of frog and goldfish

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Summary. The distribution of neuropeptide Y (NPY)-like immunoreactivity in rat, rabbit, chick, frog and goldfish retinas was investigated by immunohistochemistry. Positive results were observed only in the frog and goldfish retinas. NPY immunoreactivity was associated with a small population of amacrine cell bodies in the inner nuclear layer and cell processes in the inner plexiform layer of both retinas. In the frog retina, three distinct layers containing immunoreactivity were observed in the inner plexiform layer. In contrast, the immunoreactivity in the same area of the goldfish retina was more or less separated into two layers. Convincing evidence could not be found for the co-existence of NPY-like material with other putative transmitter-like substances in the two retinas.

Radioimmunoassay revealed the presence of small amounts of NPY-like immunoreactivity in the rabbit retina; the goldfish and frog retinas contained significantly more immunoreactive material. High performance liquid chromatography of the immunoreactive material in frog and goldfish retinas showed each retina containing different molecular forms of NPY-like proteins, neither of which resembled porcine NPY or PYY.

The endogenous NPY-like material of the frog retina can be released by potassium depolarisation in a calcium-dependent way. In view of all these data an NPY-like protein must now be considered a potential retinal transmitter.

Key words: Neuropeptide Y – Retina – Immunocytochemistry – Radioimmunoassay

Neuropeptide Y (NPY), a thirty-six amino acid peptide with a characteristic carboxyterminal tyrosine amide group, is widely distributed. In the CNS it is found in neurones in the cerebral cortex to spinal cord, occurring in cerebral, cortical and basal ganglion interneurones, hypothalamic neurones and certain central adrenergic groups of cells (see Allen et al. 1983a; Emson and Quidt 1984). Outside the CNS, NPY-like neurones are associated with the enteric nervous system (Furness et al. 1983; Ferri et al. 1984), iris-ciliary body (Allen et al. 1983b; Terenghi et al. 1983) and heart (Gu et al. 1983, 1984). There is some evidence that NPY and noradrenaline may be released together to cause the vasoconstrictor, hypertensive effects of sympathetic nerve stimulation (Emson and Quidt 1984; Allen et al. 1982, 1983c), although the function of most NPY-containing neurones is completely unknown.

The purpose of this communication is to provide data on the occurrence of NPY-immunoreactive amacrine neurones in the frog and goldfish retina. The retina, which is embryologically derived from the same tissue as the brain, is part of the CNS and has previously been shown to contain a variety of other neuropeptide-containing amacrine cells (see Brecha et al. 1984). We also report the characterisation of the NPY-immunoreactivity in the retina and that the peptide can be released from the tissue as a result of exposure to an elevated level of potassium chloride.

Materials and methods

Immunohistochemistry

Retinas from the following animals were dissected: goldfish, frog, rabbit, chick and rat. They were immediately placed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 2–3 h and kept in the same buffer containing 30% sucrose for 3 h. Ten-micrometer frozen sections were obtained at −20° C and recovered on gelatine-coated glass slides. The sections were incubated in an antibody raised in rabbit to natural porcine NPY conjugated to bovine serum albumin with carbodiiamide (for details see Terenghi et al. 1983), diluted 1:400 at room temperature for 2 h and then developed with goat antirabbit IgG conjugated to fluorescein (Miles) (diluted 1:20) for 40 min at room temperature. The primary and secondary antibodies were made up with phosphate-buffered saline (PBS) containing 0.2% Triton X-100. The glycerol/PBS-mounted sections were observed with a microscope equipped with epifluorescence optics and the photographs were taken with Kodak Tri-X-film (ASA 400).

In double labelling experiments sections were incubated first with NPY antibody plus either monoclonal antibody to serotonin (Sera Lab.) (diluted 1:100) or monoclonal antibody to substance P (Sera Lab.) (diluted 1:40). In these experiments the developing antibodies were goat anti-rabbit IgG conjugated to fluorescein plus goat anti-rat IgG conjugated to rhodamine (Dynatech) (diluted 1:20).

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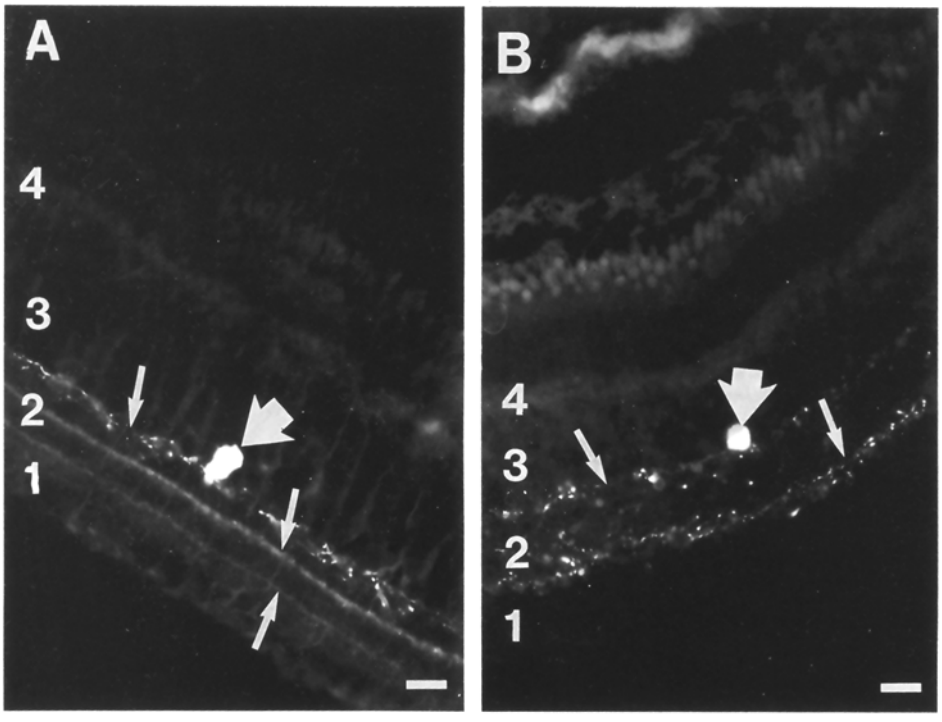

Fig. 1 A, B. Immunochemical localisation of NPY-immunoreactivity in retinas of frog (**A**) and goldfish (**B**). In each instance there are some cell bodies (*large arrows*) situated in the inner nuclear layer (*3*) and some processes (*small arrows*) situated in the inner plexiform layer (*2*) which contain immunoreactivty; *1* ganglion cell layer; *2* outer plexiform layer. Scale bar = 20 μm

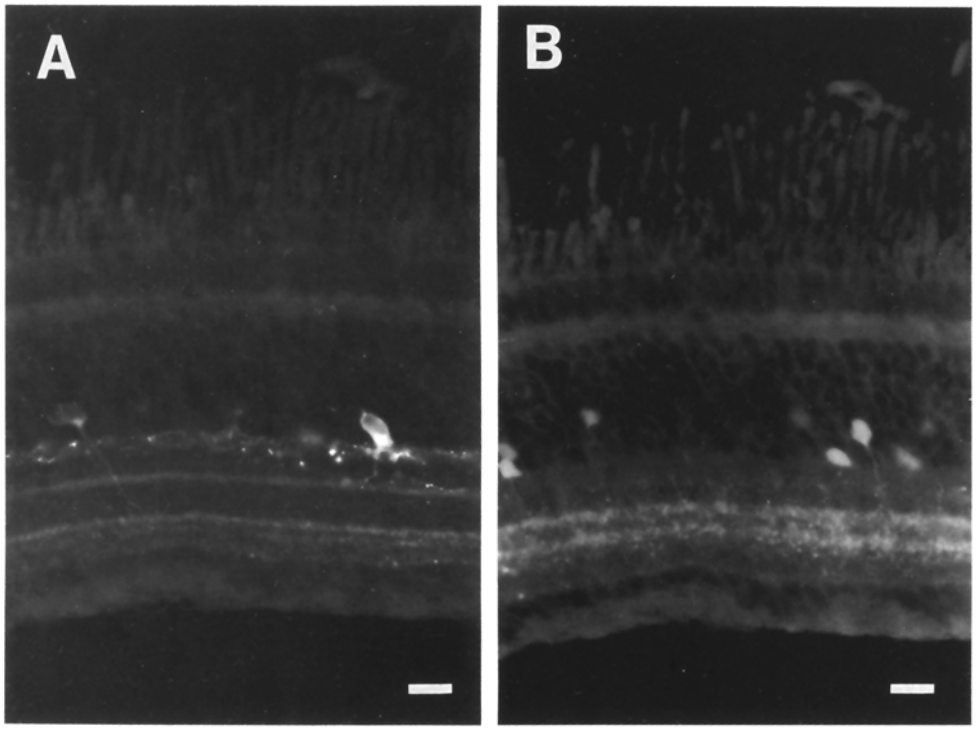

Fig. 2 A, B. Immunochemical localisation of NPY (**A**) and substance P (**B**) immunoreactivities in the same section of frog retina. Scale bar = 20 μm

Release experiments

Fresh frog retinas were either placed in a large volume of physiological saline (NaCl, 0.65 g, KCl, 0.025 g; $CaCl_2$ $2H_2O$, 0.04 g; $NaHCO_3$, 0.02 g; glucose; 0.2 g per 100 ml) or physiological saline containing 30 mM KCl or physiological solution containing 30 mM KCl plus 15 mM $CoCl_2$ for 15 min at room temperature. Thereafter, the retinas were fixed in 4% paraformaldehyde and processed for the immunohistochemical detection of NPY as described.

Radioimmunoassay and chromatography

For radioimmunoassay (RIA), frog, goldfish and rabbit retinas (n = 6) were dissected, weighed rapidly and placed in 20 vol. of boiling 0.5 M acetic acid for 10 min. The solutions were then centrifuged and aliquots of the supernatants were assayed in duplicate using an antiserum YN10 as described by Allen et al. (1984b). The assay was shown to detect changes between adjacent assay tubes of 2 fmol of NPY with 95% confidence. The intraassay variation is 7%

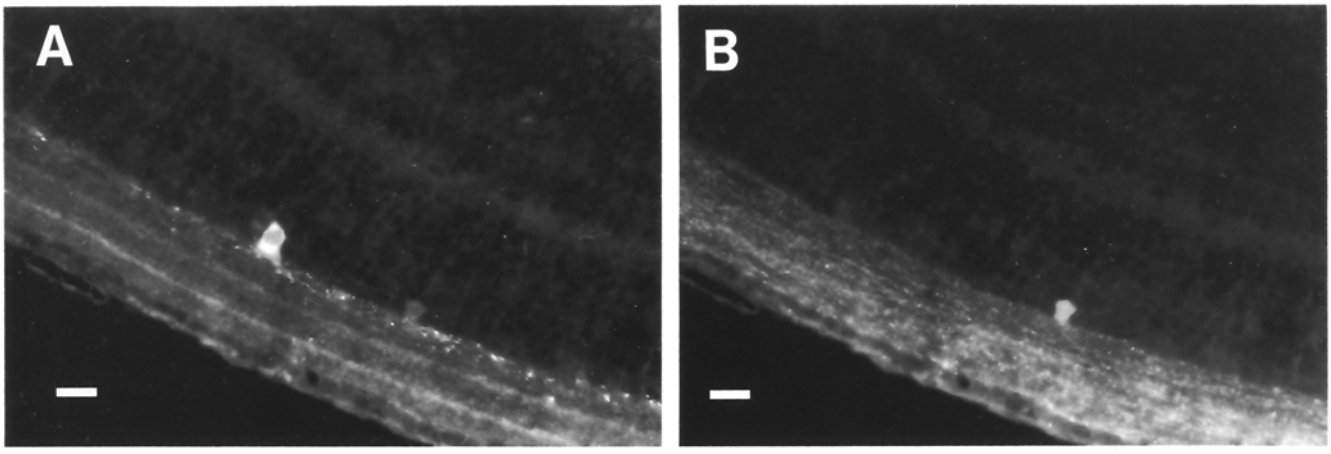

Fig. 3. Immunochemical localisation of NPY (**A**) and serotonin (**B**) immunoreactivities in the same section of frog retina. Scale bar is 20 μm

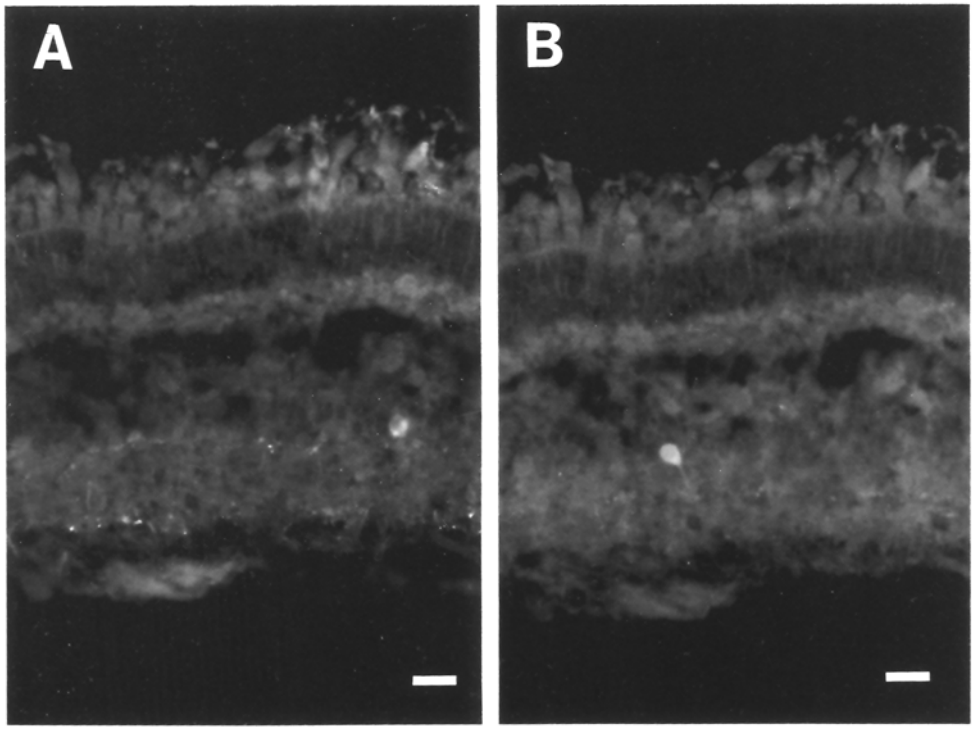

Fig. 4 A, B. Immunochemical localisation of NPY (**A**) and substance P (**B**) immunoreactivities in the same section of goldfish retina. Scale bar = 20 μm

and the interassay variation is 4%. 30% cross-reaction was seen with the related peptide PYY and 10% with avian PP (APP). Pure porcine NPY was used as standard.

Fractionation of NPY-like immunoreactivity from pooled extracts of goldfish and frog retinas was carried out by high performance chromatography by means of a reverse phase column (Waters Bondapak C-18). The column was eluted at a flow rate of 2 ml per minute and equilibrated with 35% acetonitrile in water containing 0.2% trifluoracetic acid. After the addition of sample, a linear gradient was used from 35% to 45% acetonitrile in water containing 0.2% trifluoracetic acid. Porcine NPY and PYY were used as standards (see Allen et al. 1984b).

Results

In two of the five animal retinas examined (goldfish and frog) for the immunohistochemical localisation of NPY-immunoreactivity, amacrine cell bodies situated in the inner nuclear layer and terminal processes in the inner plexiform layer were found to contain immunoreactivity (Fig. 1). No immunoreactive material was observed in the outer nuclear layer or in the ganglion cells, nor was immunoreactivity associated with Müller cells. In the frog retina three layers of immunoreactivity could be seen in the inner plexiform layer (Fig. 1 A). The layer on the border of the inner plexiform and nuclear layer was more intense than the other two layers situated in the middle and innermost areas of the inner plexiform layer. The imunofluorescence in the inner plexiform layer of the goldfish retina was, in contrast, less pronounced and more or less restricted to two layers (Fig. 1 B). Immunoreactivity for NPY in frog and goldfish retinas was observed in peripheral and central retinal regions and a limited analysis could not distinguish obvious differences in the distribution of immunoreactive positive amacrine cells in the various areas of a specific retina.

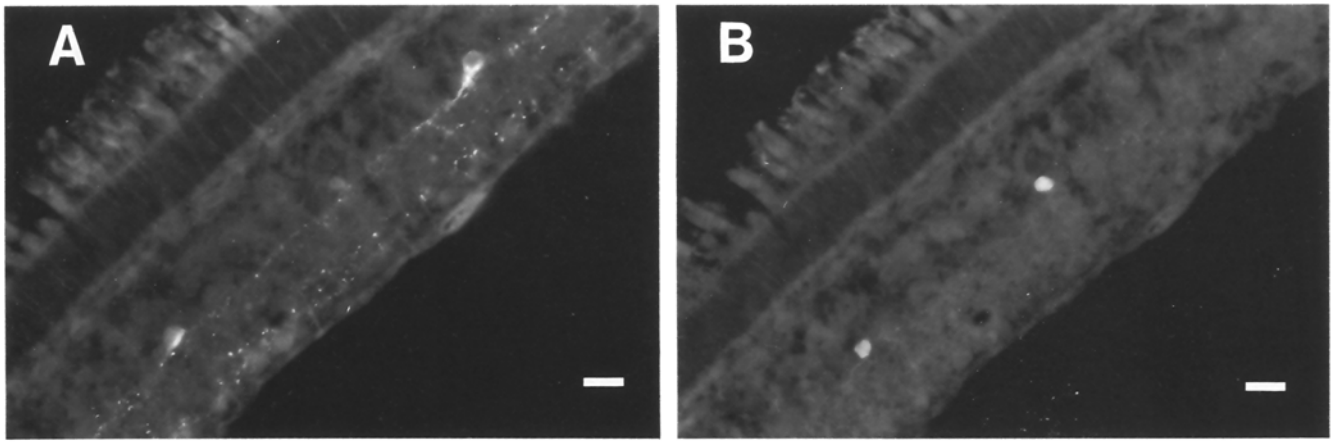

Fig. 5. Immunochemical localisation of NYP (**A**) and serotonin (**B**) immunoreactivities in the same section of gold fish retinas. Scale bar is 20 μm

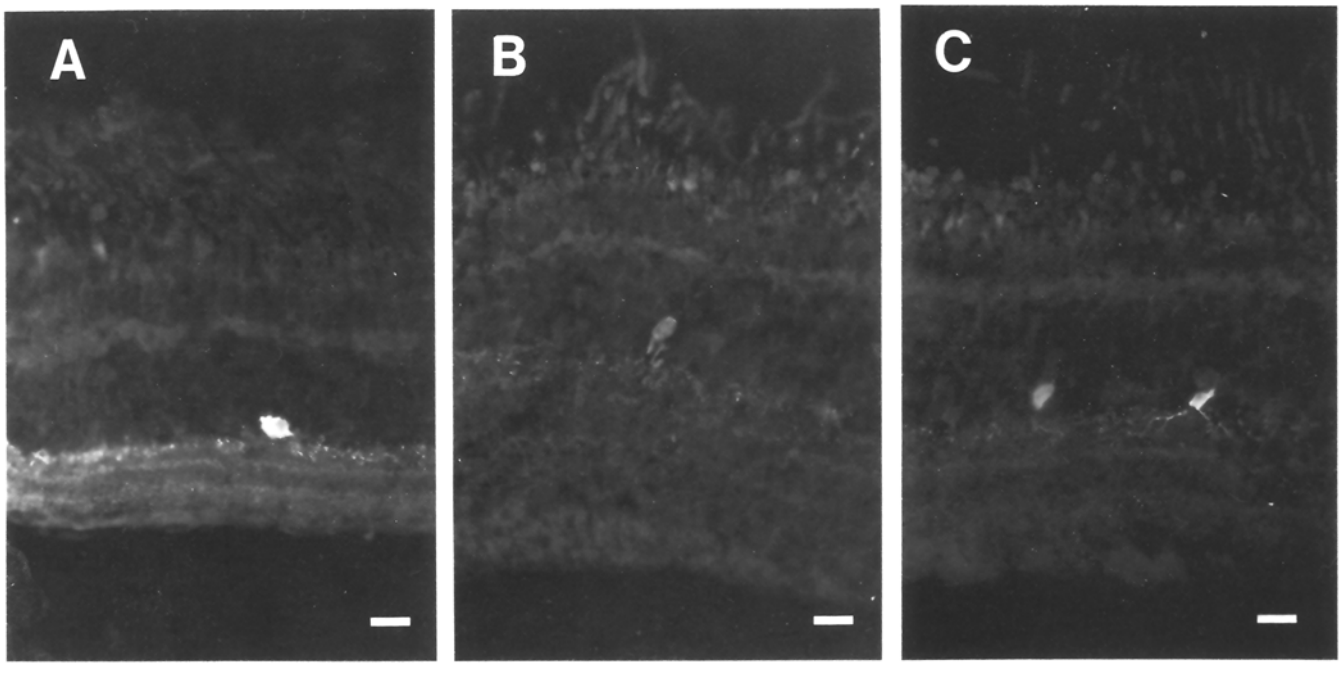

Fig. 6 A–C. Immunohistochemical localisation of NPY immunoreactivity in frog retina following incubation in physiological saline (**A**), physiological saline containing 30 mM KCl (**B**), and physiological saline containing 30 mM KCl and 15 mM $CoCl_2$ (**C**). Scale bar = 20 μm

Convincing evidence could not be found for the co-existence of NPY immunoreactivity with other putative neurotransmitters known to occur in amacrine neurones of goldfish or frog retina. It was possible to carry out double labelling experiments for NPY and either serotonin or substance P immunoreactivity because of the availability of monoclonal antibodies to the last two compounds. These results are shown in Figs. 2–5 where it can be seen that NPY immunoreactive amacrine cells have different morphologies from substance P- and serotonin-immunoreactive neurones in each retina type. An examination of many sections revealed no evidence for the co-existence of NPY with either of the other two substances in the frog retina. The same conclusion was reached for the goldfish retina, although on two occasions a neurone was found to contain both substance P and NPY immunoreactivity.

A comparison of the morphologies of tyrosine-hydroxylase (frog and goldfish), glucagon (frog and goldfish), enkephalin (frog) and somatostatin (goldfish)-immunoreactive amacrine neurones with NPY-immunoreactive neurones also showed that NPY cells have a morphology distinct from that of amacrine neurones containing the other substances (results not shown). In these studies double labelling experiments were not possible because all the available antibodies were raised in rabbits.

Evidence for a potassium-induced release of NPY-immunoreactivity from frog retinas is shown in Fig. 6. It can be seen that, after exposure of retinal tissue to 30 mM KCl for 10 min, the normally intense NPY-immunoreactivity observed in histochemical sections is dramatically reduced. The release of the immunoreactive material by KCl is counteracted by adding cobalt chloride to the incubation solution (Fig. 6).

The analysis of extracts of frog, rabbit and goldfish

Table 1. Concentrations of NPY (pmole/g wet.wt.) in three different retinas. Values are means ± S.E.M. where $n = 6$

Species	Concentrations
Frog	102.5 ± 11.0
Goldfish	14.8 ± 5.7
Rabbit	2.5 ± 0.4

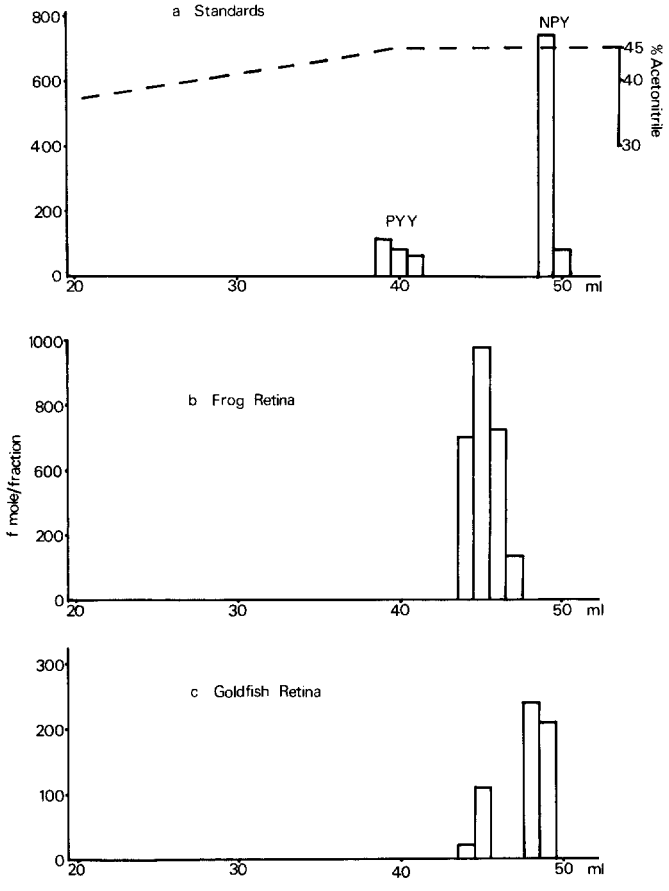

Fig. 7a–c. Radioimmunoassay of 1 ml fraction of (**a**) porcine NPY and PYY standards, (**b**) frog retinal extract, and (**c**) gold fish retinal extract

retinas by radioimmunoassay using a c-terminally directed antiserum (Allen et al. 1984b) provided a general confirmation of the immunohistochemical data. These results are shown in Table 1 where it can be seen that the amount of NPY-like immunoreactivity was most pronounced in the frog retina, which is consistent with immunohistochemical data. The rabbit retina did record a low level of NPY-like material, even though no positive results were achieved at the histochemical level.

HPLC analysis of NPY-material in goldfish and frog retina is shown in Fig. 7. The upper graph shows the separation of the two structurally related peptides PYY and NPY. PYY eluted from the column in fractions 39, 40 and 41, whereas NPY eluted at positions 49 and 50. The NPY-immunoreactivity from the frog retina eluted in a distinct peak between the two standards at positions 44, 45 and 46, whereas the NPY-immunoreactivity of the goldfish appears in two distinct peaks at positions 44 and 45, and 48 and 49.

Discussion

The results show that a population of amacrine cells in goldfish and frog retina contains a peptide(s) with characteristics similar to those of NPY. While detailed biochemical studies have not been conducted on the immunoreactive material, the HPLC data show that they are unlike true porcine NPY. Two distinct species occur in the goldfish retina and one in the frog; they are immunologically similar to NPY but of different hydrophobicity. Thus, an NPY-like peptide is present in the retina of frog and goldfish, but is not identical to porcine NPY. The absence of NPY-immunoreactivity from rabbit, rat and chick retinas which have been subjected to immunohistochemistry signifies that if true NPY exists in these retinas, the level is very low, or the antigenic site remains unrecognised by the antibodies used in the present study. The small amount of NPY-immunoreactivity actually associated with the rabbit retina and only detectable by radioimmunoassay was not analysed by HPLC.

The co-existence of neuropeptides with neurotransmitter-like compounds in one neurone has been reported in a variety of situations (see Osborne 1983; Chan-Palay and Palay 1984). In spite of the finding that amacrine neurones in different retinas contain thyrotropin-releasing hormone-like (Jackson and Reichlin 1977), enkephalin-like (Brecha et al. 1979), somatostatin-like (Yamada et al. 1980), substance P-like (Karten and Brecha 1980), neurotensin-like (Brecha et al. 1981), VIP-like (Brecha et al. 1980), β-endorphin-like (Jackson et al. 1980), CCK-like (Osborne et al. 1982) and glucagon-like (Brecha et al. 1980), peptides, as well as tyrosine-hydroxylase (Ballesta et al. 1984; Osborne et al. 1984), serotonin (Osborne et al. 1983), GABA (see Voaden 1976) and acetylcholine (see Neal 1983), only two studies have shown the co-occurrence of two transmitter-like molecules (viz., GABA with enkephalin-like immunoreactivity and glycine with neurotensin-like immunoreactivity) in the same amacrine neurones (see Watt et al. 1984; Weiler and Ball 1984). In view of this rarity, a fairly detailed immunohistochemical analysis was undertaken to see whether NPY-immunoreactive neurones in either frog or goldfish retina contained another transmitter-like compound. As described above, no persuasive evidence could be found for the co-occurrence of NPY-like material and a variety of other compounds known to be associated with specific populations of amacrine neurones. The double labelling experiments, which were limited to serotonin and substance P, were particularly convincing because of the obvious advantages of such an analysis. In the case of the goldfish retina one cell on two different occasions stained positively for both NPY and substance P immunoreactivities. Because of this rarity in this detailed study it is difficult to assess the significance of the observation. It is possible that it is an artefact caused by two different cells giving the appearance of a single neurone in the plane of section. It is noteworthy that NPY has been shown to occur in both central and peripheral neurones known to contain catecholamines (Lundberg et al. 1982a, b).

The results also demonstrate that the endogenous NPY-like immunoreactivity associated with specific amacrine neurones in the frog retina is released by potassium depolar-

isation. This release is shown to be calcium-dependent as it is inhibited by the presence of cobalt ions in the medium. It is thought that cobalt ions interfere with the entry of calcium into synaptic endings resulting in a reduction in, or abolition of, transmitter release (Weakly 1973). These data thus provide an indication that the NPY-like material could be functioning as a transmitter in the frog retina.

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