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0014-4754/84/070763-03$1.50 + 0.20/0

Neuropeptide Y in the guinea-pig biliary tract

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Summary. High concentrations of neuropeptide Y (NPY) have been demonstrated in the gall bladder (16.7 ± 5.4 pmol/g), cystic duct (25.4 ± 9.2 pmol/g) and common bile duct (54.7 ± 11.5 pmol/g) of the guinea-pig using a recently developed radioimmunoassay. Immunoreactive NPY containing nerves were demonstrated in all layers of the biliary tree using immunocytochemistry, being particularly dense in the myenteric and mucosal plexuses.

Neuropeptide Y (NPY) has recently been isolated from porcine brain. It consists of 36 amino acids and is characterized by an N-terminal tyrosine (Y) and a C terminal tyrosine amide[3,4]. This peptide has been identified within neurones in the brain[5] and gastrointestinal tract[6,7]. The presence of this peptide has been determined in the biliary tract of the guinea pig using radioimmunoassay and immunocytochemistry.

Methods. For radioimmunoassay four Dunkin-Hartley guinea-pigs were sacrificed by cervical dislocation. The gall bladder, cystic duct and common bile duct were dissected and the peptides extracted from these tissues by boiling in 0.5 M acetic acid (10% weight/volume) for 10 min.

NPY concentrations in these extracts were measured using an antiserum raised in a rabbit by conjugating porcine NPY to bovine serum albumin with carbodiimide. The third and subsequent monthly boosts of the conjugate used keyhole limpet hemocyanin as carrier. Natural porcine NPY was iodinated using chloramine T and purified on a G-50 superfine column providing a tracer with a specific activity of 70 Bq/fmol. The antiserum was used in a final dilution of 1:10,000 in an assay volume of 600 µl, 0.06 M sodium phosphate buffer, pH 7.2 containing 0.05 M EDTA and 1% bovine serum albumin. The assay could detect with 95% confidence, a minimum of 2 fmol of NPY per assay tube, and 20 fmol of peptide YY per assay tube. No crossreaction was observed with human or porcine pancreatic polypeptide up to 100 pmol/tube. Pure porcine NPY was used as standard. Results are expressed as mean and SEM.

Chromatography. Tissue extracts were fractionated on a HPLC Bondapak C18 reverse phase column (3.9 × 300 mm) using a linear gradient elution system from 35% to 45% acetonitrile in water containing 0.2% trifluoracetic acid over 10 min at a flow rate of 1 ml per min. 1-ml fractions were collected for subsequent radioimmunoassay. Pure porcine NPY and PYY were used as standards.

Immunocytochemistry. 6 adult Dunkin-Hartley guinea-pigs of both sexes were sacrificed by cervical dislocation. The gall bladders, cystic ducts, hepatic ducts, common bile ducts and the sphincters were removed and fixed in 0.4% benzoquinone in 0.01 M phosphate buffered saline (pH 7.2) (PBS) for 1 h at room temperature[8]. The samples were then washed in 7% sucrose in PBS overnight. 5-µm-thick cryostat sections were cut and mounted on poly-L-lysine-coated[9] glass slides. To investigate the complete distribution pattern of the NPY-containing nerves, whole-mount stretch preparations of the gall bladder were made according to a procedure described previously[10].

Immunocytochemistry was carried out on both cryostat sections and whole mount stretch preparations following modified indirect immunofluorescence procedures described previously[11,12]. Antiserum to NPY was raised in rabbit against natural procine NPY conjugated to bovine serum albumin with bis-diazobenzidine. The antiserum was used in a dilution of 1:800 in PBS. The controls included pre-absorption of the first layer antiserum with pure natural NPY (5.0 nmol/ml of

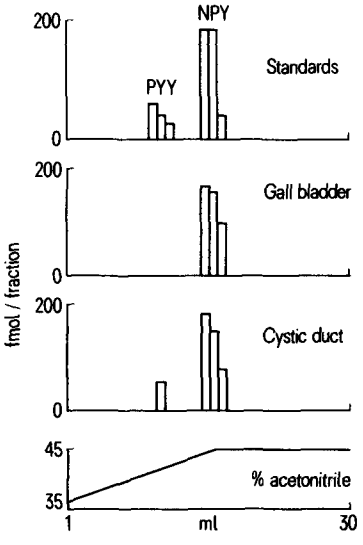

Figure 1. Fractionation of extracts of gallbladder and cystic duct on an HPLC C18 Bondapak reverse phase column using a linear gradient elution of 35–45% acetonitrile in water containing 0.2% trifluoracetic acid. Fractions of 1 ml were collected for subsequent radioimmunoassay. The positions of porcine NPY and PYY standards are shown in the top panel for comparison. Recoveries from the columns ranged from 85 to 98%.

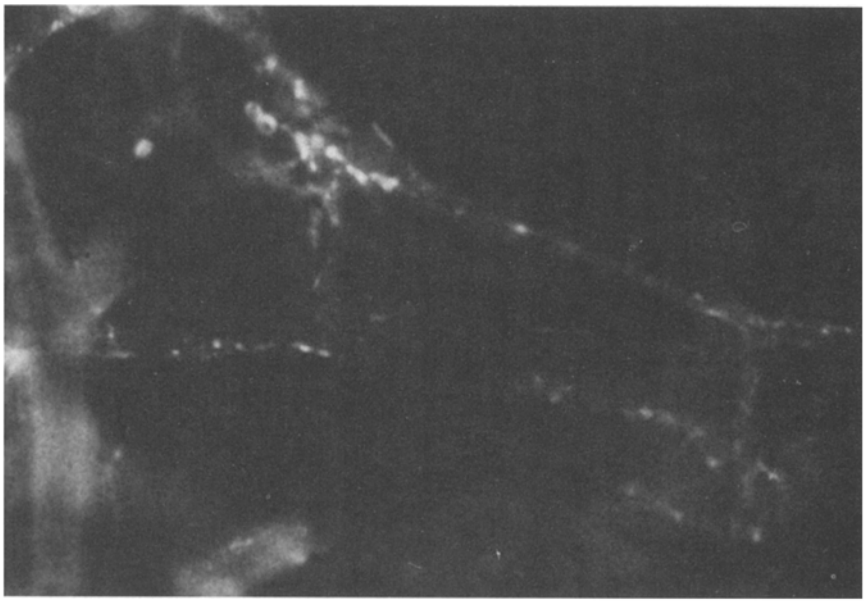

Figure 2. Network of NPY-immunoreactive nerves in the ganglionic plexus of the gallbladder (whole mount stretch preparation). × 900.

Figure 3. NPY-immunoreactive nerves associated with the smooth muscle in the sphinter of Oddi. × 650.

diluted antiserum) and using normal rabbit serum or PBS as the first layer.

Results. All regions of the biliary tract contained NPY, the highest concentration at 54.7 ± 11.5 pmol/g being found in the common bile duct. The cystic duct, including the gall bladder neck contained 25.4 ± 9.2 pmol/g and the gall bladder contained 16.7 ± 5.4 pmol/g.

Chromatographic analysis (fig. 1) confirmed that the NPY like immunoreactivity coeluted in the same position as porcine NPY standard. Analysis of the extract of the cystic duct revealed the majority to coelute at the position of the NPY standard but a small proportion eluted earlier from the column at 36% acetonitrile in the position of porcine PYY standard.

In both the stretch preparations and the cut sections of the gall bladder, the common bile duct, the cystic duct and the sphincter of Oddi, NPY-immunoreactivity was found in varicose nerves in all the layers. They were particularly dense in the 2 plexuses, i.e. the myenteric and the mucosal plexuses (fig. 2). These nerves were sometimes found to surround non-immunoreactive ganglia and occurred with less varicosities in the connecting strands which linked different ganglia. No ganglia were found to be reactive to NPY antiserum. Only a small number of NPY-immunoreactive nerves were found in the muscle layer of the gall bladder. However, more such nerves were seen in the smooth muscle from the middle portion of the common bile duct down to the ampulla. In the sphincter of Oddi, NPY-immunoreactive nerves ran parallel to the longitudinal and circular musculatures (fig. 3) and were denser in the latter. The cystic duct blood vessels, particularly the arteries, were surrounded by NPY-immunoreactive nerves. The specificity of the immunostaining was validated by negative results in the control slides.

Discussion. High concentrations of NPY have been demonstrated in the biliary tract of the guinea-pig. Preliminary work links NPY neurones anatomically with sympathetic neurones[13] and NPY functionally appears to interact with noradrenergic transmission[13,14]. Noradrenaline containing neurones have been demonstrated in the fibromuscularis layer of the biliary tract[15] and in keeping with the distribution of NPY found in this study, Baumgarten and Lange[16] demonstrated a higher

content of noradrenaline in the gall bladder neck than in the body or fundus. Immunocytochemical studies have shown a similar distribution pattern of NPY immunoreactive nerves to that reported for adrenergic nerves[11] and this is distinct from nerves containing vasoactive intestinal polypeptide, substance P, somatostatin, enkephalin and bombesin-like immunoreactivity[11]. The distribution was also different from cholinergic nerves, mainly in that no NPY-immunoreactive ganglia were found in the tissues[11].

Sympathetic function appears to play an important role in neural control of biliary function as stimulation of beta adrenergic receptors results in smooth muscle relaxation[15] and stimulation of thoracic splanchnic nerves anatagonises cholecystokinin-induced gall bladder contraction[17]. In view of the close links of NPY with sympathetic function, it appears that this peptide may play an important role in the control of gall bladder motility, especially as the structurally related peptide, porcine pancreatic polypeptide[13] has already been shown to relax biliary smooth muscle[18]. The presence of high concentrations of NPY within the biliary tract should stimulate further investigation of its physiological role.

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2 Acknowledgments. JMA is a recipient of a Wellcome Trust Training Fellowship. JG is a visiting scholar from the Department of Pathology, Peking Medical College, Peking, China.

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0014-4754/84/070765-03$1.50 + 0.20/0

Arousal and fright responses and their habituation in the slippery dick, *Halichoeres bivittatus*

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Summary. Behavioral arousal and fright responses of *Halichoeres bivittatus*, occurred in aquaria to a moving shadow and a 'tap' stimulus. Arousal was characterized by changes in the beat of pectoral fins, dorsal fin erection and eye movements, whereas in fright, adduction of pectoral and dorsal fins and rapid forward movement occurred. Serial stimulus presentation caused the fright response to be replaced by arousal which habituated in that the proportion of behavioral components exhibited decreased during the process.

Behavioral arousal and fright and their physiological correlates have been extensively studied in domesticated cyprinids[4]. In these fish arousal is associated with slight changes in orientation or an increase in stabilizing movements. Thus, changes in pectoral and/or tail fin movement and erection of the dorsal fin often occur. Novel stimuli may elicit arousal or the more violent response of fright. This escape response consists in cyprinids of a rapid lateral flexion of the tail or tail-flip. Both arousal and fright habituate in cyprinids, and if the initial responses are of fright then these habituate to be replaced by arousal, which itself habituates[5]. *Halichoeres bivittatus* is a diandric protogynous hermaphrodite, and with the other labrids its reproductive biology has been extensively studied[6]. The labrids are extremely active and alert fish and this makes them ideal subjects for testing the generality of descriptions of behavioral arousal and fright, and the motor components of these responses.

Material and methods. Adult (phase 4) *Halichoeres bivittatus* were collected for aquarium observations using minnow traps baited with squid. Individuals were placed for 36 h in a 0.7 × 0.3 × 0.3 m outdoor aquarium, shaded from direct sunlight, filled with fresh aerated seawater and enclosed in brown paper. Stimuli (or equivalent pre-experimental non-stimulus control observations) were presented at 30-sec intervals and changes in the movement or position of eyes, head, body, tail, pectoral fins and dorsal fin were recorded from the prestimulus 5 sec to the 5 sec after stimulus initiation. Overall responses of the fish were considered to fall into 2 categories: realignment or reorientation and rapid flight. The former was interpreted as arousal, the latter as fright. The stimuli were: serial presentation of a 10 × 20 cm black card moved over the aquarium at ≅ 8 cm/sec for 4 sec and serial presentation of a consistent sharp tap on the end of the aquarium. Waning of the

Table 1. Components of behavioral arousal and fright in *Halichoeres bivittatus:* mean ± SEM of proportion of responses with each component

Component	Arousal	n	Fright	n
Head movement	39.8 ± 10	7	97 ± 2.5	4
Eye movement	62.5 ± 8.6	7	54.2 ± 21	4
Change in pectoral beat	57.3 ± 10.2	7		4
Adduction of pectorals		7	90.6 ± 8.1	4
Dorsal fin erection	50.3 ± 12.2	7		4
Adduction of dorsal fin		7	100 ± 0	4
Tail movement	21.1 ± 7.9	7	97.0 ± 2.5	4
Body movement	18.8 ± 5.4	7	100 ± 0	4