Peptidergic hormones and neuropeptides, and aminergic neurotransmitters of the pancreatic islets of the Houbara bustard (*Chlamydotis undulata*)

E. P. K. MENSAH-BROWN[1], T. A. BAILEY[2], D. J. PALLOT[1] AND A. GARNER[3]

[1] *Department of Anatomy, Faculty of Medicine and Health Sciences, UAE University, Al Ain, UAE,* [2] *Wild life Veterinary Research Institute, Environment and Wildlife Development Agency, Abu Dhabi, UAE,* [3] *Clinical Sciences, University of Manchester, School of Medicine, Hope Hospital, Salford, UK*

(*Accepted 20 September 1999*)

ABSTRACT

Immunoreactivity to insulin (Ins), somatostatin (Som), glucagon (Glu) and pancreatic polypeptide (PP) was found in 70%, 22%, 15% and 11% respectively of Houbara pancreatic endocrine islet cells. Whilst Ins occurred centrally and SOM was observed both in peripherally and centrally located islets, the other hormones were localised in peripheral islet cells; Som was also observed in neuronal cell bodies and nerve fibres. In addition, the islet cells contained substance P (SP) (65%) in the centre and vasoactive intestinal polypeptide (VIP) (2%) at the periphery. Immunoreactivity to choline acetyltransferase (ChAT), VIP and galanin (Gal) occurred in the walls of blood vessels located mainly at the periphery of islets. Occasionally, VIP and Gal immunoreactive varicose nerve terminals and ChAT immunoreactive cell bodies were also observed in the centre of islets. SP neuronal cell bodies were not observed but prominent SP immunoreactive varicose terminals were discernible in capillary walls within the islets. Neuropeptide Y (NPY) immunoreactive neurons were detected in neuronal cell bodies located mainly peripherally. Neuronal nitric oxide synthase (nNOS) immunoreactivity occurred in neuronal cell bodies and nerve fibres mainly at the periphery and also in centrally located islet endocrine cells. Immunoreactivity to tyrosine hydroxylase (TH) was similar in distribution to that of ChAT. In comparison with other avian species, the islets of the dorsal pancreatic lobe of the bustard contain all the peptidergic hormones normally present in the islets of other avian species, but are not segregated into dark A and light B cells. Many of the insulin containing cells also contained SP. The islets also contained several neuropeptides which are probably involved in their regulation.

Key words: Innervation; varicosities; pancreatic islets; endocrine cells.

INTRODUCTION

In all vertebrates, the islets of the pancreas function as a part of the gastro-entero-pancreatic endocrine system. Four hormonal peptides, insulin, glucagon, somatostatin and pancreatic polypeptide produced by light type B, dark type A, D and PP islet cells (Falkmer, 1985) are under the control of the autonomic system (Richens, 1945). In addition to acetylcholine (Van der Zee et al. 1992), various neuropeptides including vasoactive intestinal polypeptide (VIP) (Holst et al. 1984) and galanin (Verchere et al. 1996) have also been implicated as parasympathetic postganglionic neurotransmitters.

Postganglionic sympathetic nerves terminate in the pancreas and release the neurotransmitter noradrenaline which acts via α or β receptors to produce either inhibitory or stimulatory effects on pancreatic function (Stagner & Samols, 1986; Dunning et al. 1991). In addition to these classical neurotransmitters and their coexisting neuropeptides, peptidergic neurons containing the neuropeptides VIP (Larsson et al. 1978; Sundler et al. 1978; Bishop et al. 1980), neuropeptide Y (NPY) (Su et al. 1987), galanin (Gal)

Correspondence to Dr E. P. K. Mensah-Brown, Department of Anatomy, Faculty of Medicine and Health Sciences, UAE University, P.O. Box 17666, Al Ain, United Arab Emirates. Tel. +971-3-5039495; fax +971-3-672033; email: ericb@uaeu.ac.ae

(Lindskog et al. 1991; McDonald et al. 1992) and the neurotransmitter nitric oxide (Ekblad et al. 1994; De Giorgio et al. 1992) have also been identified in the pancreatic parenchyma of various species; they are believed to interact with the neurotransmitters acetylcholine and noradrenaline in regulating islet secretion (see review by Brunicardi et al. 1995).

Studies on the distribution and role of peptidergic hormones neuropeptides and neurotransmitters have been performed on the domestic fowl (Falkmer, 1985; Tomita et al. 1985; Liu et al. 1994, Hiramatsu & Oshima, 1997), turkey (Valiant et al. 1980) the Australian wedge-tailed eagle, *Aquila audax* (Edwin & Leigh, 1993) and the duck (Lucini et al. 1996), but there is no study in any Houbara species. Bustards belong to the Otittidae family and are medium-sized to very large terrestrial birds mainly inhabiting open plains and semi-desert regions of the world. The pancreas of the Houbara bustard comprises a smaller ventral lobe and a larger dorsal lobe lying on the ventral and dorsal aspects of the 2 limbs of the duodenum (Bailey et al. 1997). The aim of this study was to determine the distribution of peptidergic hormones, peptidergic and aminergic neurotransmitters within specific endocrine cells, neurons and nerve fibres in pancreatic islets of captive-bred Houbara bustards (*Chlamydotis undulata*). The study also aimed at ascertaining whether their distribution is similar to those of other avian species.

MATERIALS AND METHODS

The 4 dorsal lobes of the pancreas of 2–3 y old male Houbara bustards were collected. Those birds used in the study had sustained severe injuries during flight in captivity that required euthanasia on humanitarian grounds by injection of pentobarbital. The dorsal lobe has been used in these studies because it was easier to identify and could be excised rapidly. The weight and length of each pancreas was measured to the nearest 0.01 g after which the gland was divided into approximately 4 equal cubes. Each cube was weighed again and the specimens placed in Zamboni's fixative solution overnight at 4 °C. The fixed tissue was then routinely embedded in paraffin wax. Serial sections (5 μm) were cut with a Shandon A325 retraction microtome and consecutive sections immunostained by the indirect PAP-diaminobenzidene method using the LSAB staining kit for monoclonal and polyclonal antibodies (DAKO, Copenhagen). Two series of sections from each tissue cube were studied (i.e. a total of 8 sections) for each antiserum. The specimens were deparaffinised and incubated in 3.3% hydrogen peroxide in absolute methanol for 30 min to block endogenous peroxidase. After washing in 0.05 mM Tris-BSA buffer, pH 7.4, the slides were incubated with a blocking agent (PBS containing carrier protein and 15 mM sodium azide provided by the manufacturer) for 30 min followed by overnight incubation in the primary antiserum. Sets of 13 consecutive sections were immunostained using antisera to insulin (Ins), glucagon (Glu), somatostatin (Som), and pancreatic polypeptide (PP), cholecystokinin-8 (CCK-8), galanin (Gal), gastrin releasing peptide (GRP), neuropeptide Y (NPY), substance P (SP), vasoactive intestinal polypeptide (VIP), nitric oxide synthase (nNOS), choline acetyl transferase (ChAT) and tyrosine hydroxylase (TH) (Table). These were followed by a series of 16 sections for controls. The sections were allowed to warm to room temperature and then incubated with biotinylated antirabbit IgG in PBS for 30 min. After three 5 min washes in 0.05 mM Tris-BSA buffer, pH 7.4, the sections were treated with peroxidase labelled-streptavidin tertiary antibody for 1 h. The specimens were washed in 0.05 M Tris-BSA buffer (3 steps, 5 min each). Peroxidase activity was demonstrated by incubation for 3–5 min in filtered diaminobenzidene (10 mg diaminobenzidene hydrochloride [Sigma, St Louis, USA] in 15 ml of 0.05 M Tris buffer, pH 7.4) containing 12 μl of 30% hydrogen peroxide. The light brown solution was placed on the sections for 3–5 min. Sections were then counterstained with haematoxylin, coverslipped using Cytoseal 60 mounting medium (Stephens Scientific, Riversdale, USA) and examined on a Zeiss Axiophot microscope. The antisera used, their sources, dilutions and specificities are shown in the Table.

The control experiments consisted of the following: (1) omission of primary antiserum, (2) substitution of primary antibody with 0.05 M Tris-BSA buffer, (3) substitution of primary antibody with antiserum readsorbed with the various hormones at a concentration 10^{-6} M and constitutive nitric oxide synthase (Sigma, USA) at a concentration of 50 μg/ml. These controls were carried out on sections at the same time as the treatment with the primary antibody.

Distribution of islet endocrine cell immunoreactivity

Quantitative studies of islet cells immunoreactive to Ins, Glu, PP, SP, and VIP were performed using a VIDS V image analyser. The sections of pancreas were analysed at a calibrated magnification of $\times 400$, employing a camera lucida. The system determined

Table 1. *Details of antisera used in the study*

Antiserum	Type	Host	Source	Working dilution	Specificity
ChAT	Rat	Mouse	Incstar STR, USA	1:100	Reacts specifically with ChAT
CCK-8	Synthetic	Rabbit	Sigma, USA	1:3000	Specific for CCK 8 containing cells
GAL	Human	Rabbit	Peninsula Lab., USA	1: 2000	Specific for galanin containing nerves
GRP	Human	Rabbit	Sigma, USA	1:1500	Specific for GRP containing cells
Glu	Synthetic	Rabbit	Harlan Seralab, UK	1:2000	Specific for glucagon containing cells
Ins	Human	Guinea pig	DAKO, Copenhagen	Prediluted	Specific for Ins containing cells
nNOS	Rat	Rabbit	Transduction Labs, USA	1.0 µg/l	Specific for nNOS containing neurons
NPY	Porcine	Rabbit	Sigma, USA	1:4000	Specific for NPY containing cells
PP	Synthetic porcine	Rabbit	Sigma, USA	1:2000	Specific for PP containing cells
SOM	Synthetic AES 313	Rabbit	Harlan Seralab, UK	1:1000	No known cross reactivities
SP	Synthetic AES 320	Rabbit	Peninsula Labs., USA	1:1000	No known cross reactivities
TH	Rat	Mouse	Sigma, USA	1:8000	No known cross reactivities
VIP	Synthetic porcine	Rabbit	Sigma, USA	1:3000	No known cross reactivities

* Information provided by manufacturers.

the total area of each islet and excluded the area occupied by immunoreactive cells. It also recorded the number of islets analysed. From the data, the relative percentage of area $\pm$S.E.M. occupied by the immunoreactive cells was calculated. The total area occupied by immunoreactive cells was determined for every islet present in each section. Significance of differences was determined using the Student's *t* test.

RESULTS

Immunolabelling was absent in all control experiments. Preadsorption of antiserum with the peptidergic hormone, neuropeptide, or neurotransmitter completely abolished staining.

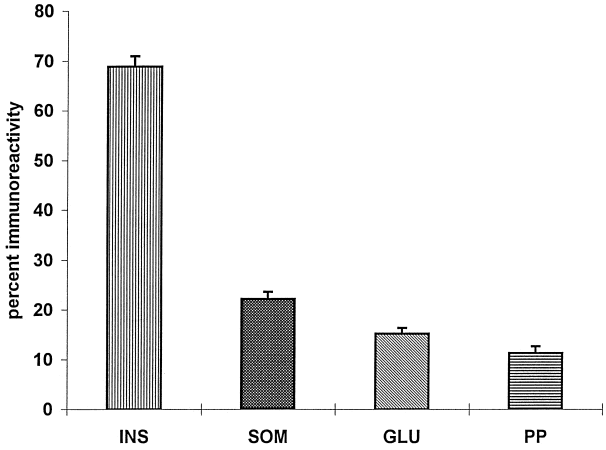

Fig. 1. Bar graph showing percentage frequency of peptidergic hormones in the islets of Langerhans of the Houbara bustard.

Pancreatic islets

The mean weight and length of the pancreata were 0.96 ± 0.006 g and 38.7 ± 2.67 mm respectively. The mean length of the cubes was 9.88 ± 0.56 mm and the mean weights were 0.20 ± 0.02, 0.29 ± 0.03, 0.31 ± 0.02, 0.22 ± 0.02 g respectively. The total number of islets analysed for each peptidergic hormone ranged from 75 to 125 with means of 75 ± 8.02, 118 ± 9.12, 125 ± 6.22 and 85 ± 7.56 in the 1st, 2nd, 3rd and 4th quarters of the dorsal lobe respectively. For each series of sections obtained from each piece of pancreatic tissue, there was no significant difference between the immunoreactive areas computed for the different peptidergic hormones. Thus the number of islets ranged 3.7–4.1 per mg of pancreatic tissue throughout the dorsal lobe. The size of the islets however varied greatly, hence the use of the relative percentage of area occupied by the peptidergic hormone in calculations.

Peptidergic hormones. Ins containing cells were the most numerous, forming approximately 70% of all endocrine cells. They were located towards the centre of the islet leaving only a peripheral rim of nonreactive cells (Fig. 2*a*). Whilst immunoreactivity to Glu (15%), and PP (10%), was discernible in the endocrine cells located at the periphery of the islet, Som immunoreactive cells were discernible at the periphery and extending towards the centre (Fig. 2*b–d*). Cell bodies of neurons immunoreactive to Som were also observed towards the centre of islets (Fig. 2*c*). 20% of all the Glu immunoreactive cells also contained PP.

Peptidergic neurotransmitters. Immunoreactivity to ChAT occurred as varicose nerve terminals in the wall of capillaries at the periphery of islets (Fig. 3*a*). Very occasionally, neuronal cell bodies immunoreactive to

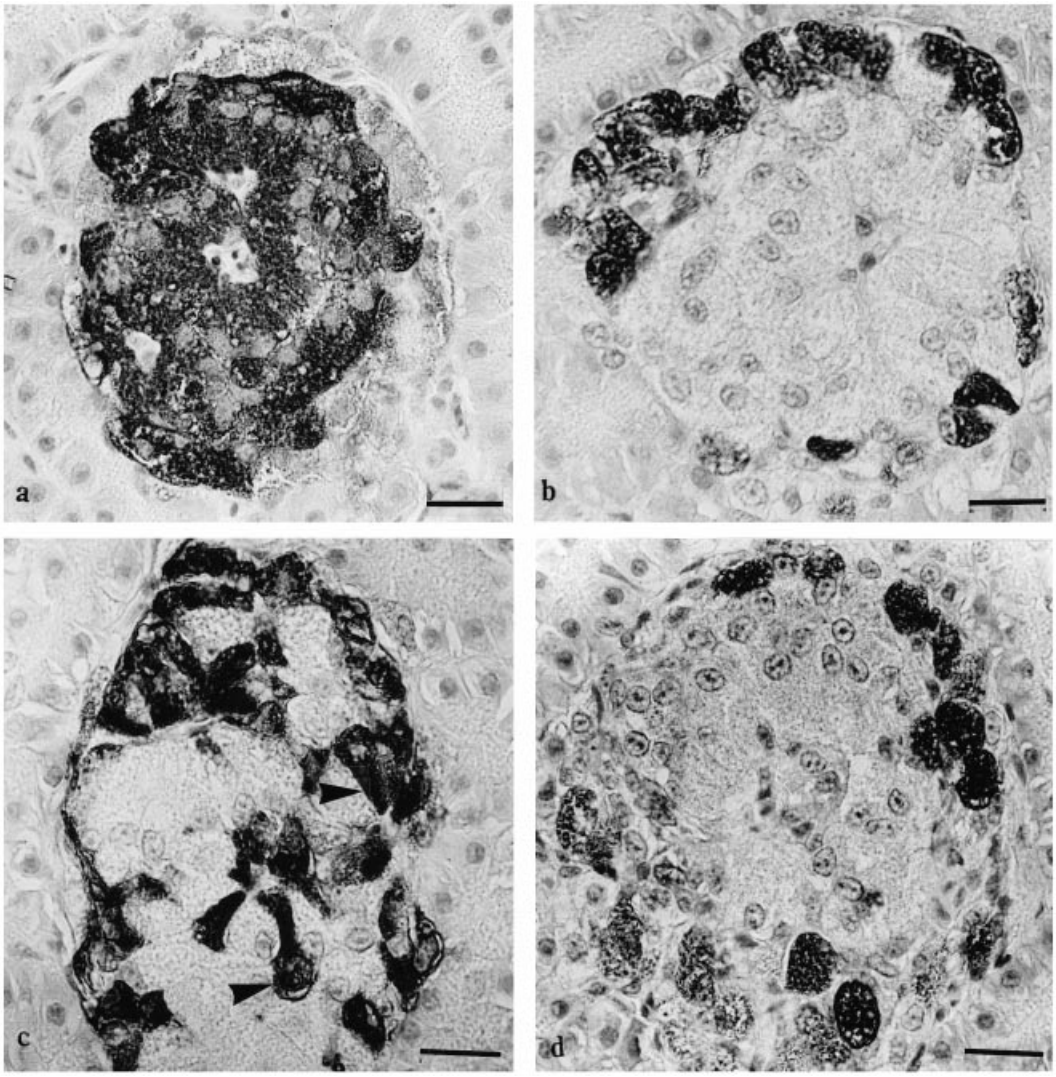

Fig. 2. Light micrographs showing pancreatic islet cells of the Houbara bustard immunoreactive to (*a*) insulin (*b*) glucagon, (*c*) somatostatin and (*d*) pancreatic polypeptide. Note that whilst cells immunoreactive to insulin are located in the centre all the others occur at the periphery of islets. Note also the presence of neurons (arrowhead) immunoreactive to somatostatin in the centre of islets. Bars, 6 μm.

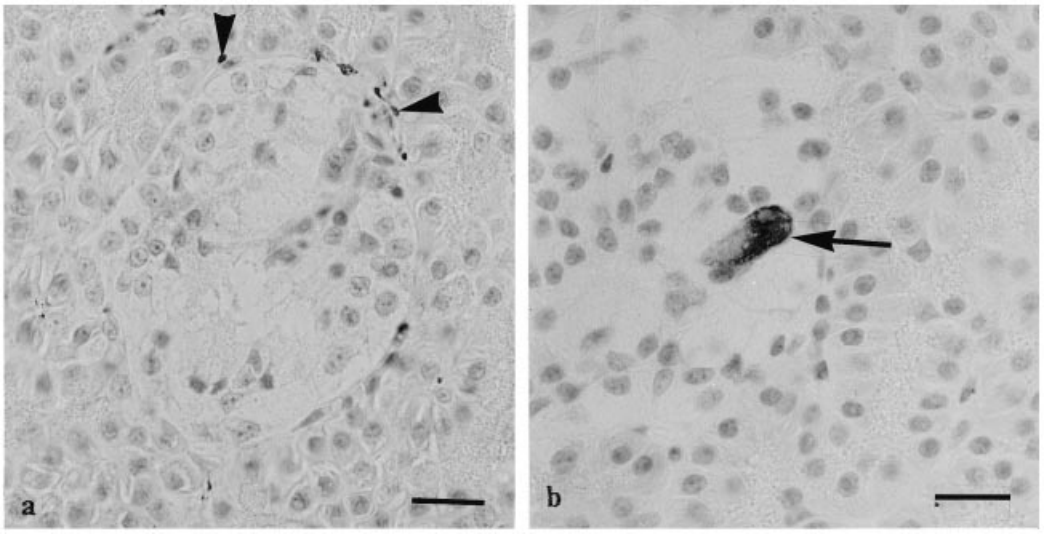

Fig. 3. Light micrographs showing choline acetyltransferase immunoreactive (*a*) varicose terminals (arrowheads) at the periphery and (*b*) a solitary immunoreactive neuron cell body (arrow) in the centre of an islet. Bars, 6 μm.

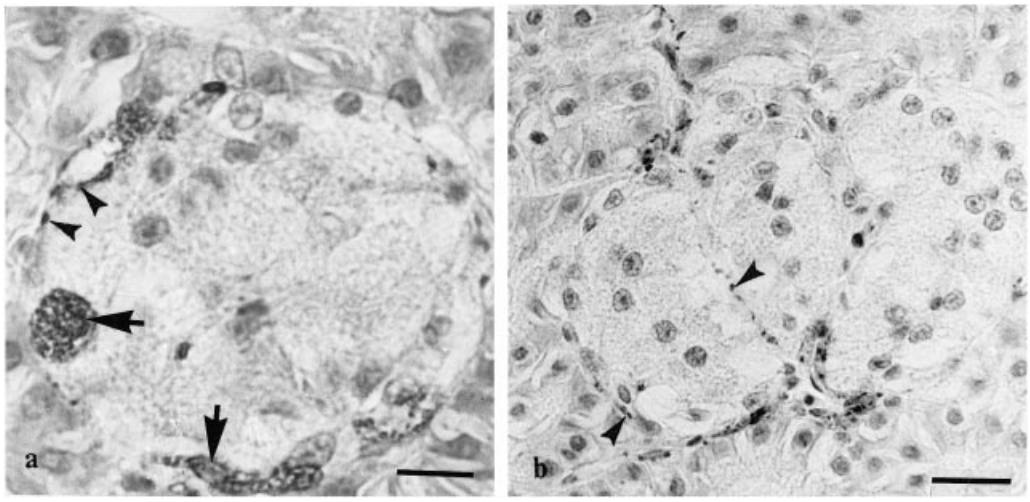

Fig. 4. Light micrographs of pancreatic islets showing immunoreactivity to (*a*) VIP and (*b*) galanin. Note the presence of peripherally located immunoreactive islet cells in (*a*, arrows) and varicose terminals in the walls of vessels in *a* and *b* (arrowheads). Punctate sites of immunoreactivity believed to be varicose terminals are seen in the centres of both *a* and *b*. Bars: *a*, 4 μm; *b*, 8 μm.

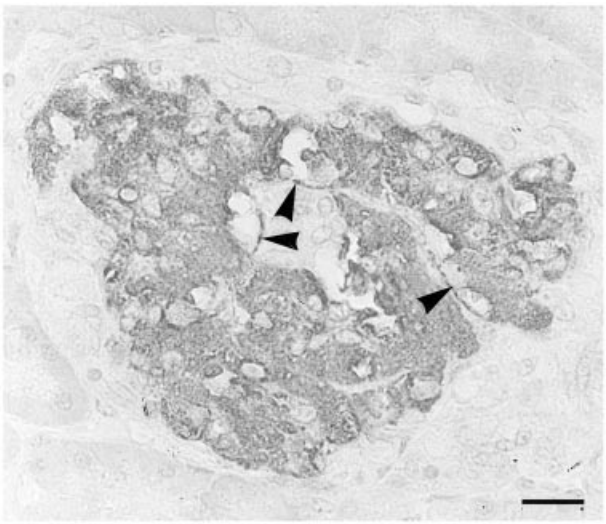

Fig. 5. Light micrograph showing centrally located islet cells immunoreactive to substance P. Note the presence of varicose terminals (arrowheads) in the walls of capillaries in the centre of the islet. Bar, 6 μm.

ChAT were also detected in the centre of the islet (Fig. 3*b*). Immunoreactivity to VIP and Gal occurred in varicose terminals in the walls of capillaries at the periphery of the islet; less commonly, centrally located nerve terminals were also seen (Fig. 4*a*, *b*). In addition, 2.8% islet cells located peripherally were immunoreactive to VIP (Fig. 4*a*). Immunoreactivity to SP was observed in 65% of all islet cells. These cells, located in the centre of the islet, were also immunoreactive to insulin. SP also occurred in varicose nerve terminals in the wall of capillaries in the centre of the islet (Fig. 5). SP and insulin were colocalised in the same islet cells (Fig. 6*a*, *b*). Immunoreactivity to NPY was discernible in neurons mainly at the periphery but nerve cells were also found towards the centre of islets (Fig. 7). CCK-8 and GRP immunoreactive cells were not observed within the islets.

Aminergic neurotransmitters. Immunoreactivity to nNOS was encountered in endocrine cells located in the centre of islets. In addition, neuronal cell bodies, nerve fibres and varicose nerve terminals immunoreactive to nNOS occurred mainly at the periphery. Centrally located neurons were also observed (Fig. 8*a*). Colocalisation of nNOS and Som in the same cells was also observed at the periphery of the islet (Fig. 8*b*, *c*). The distribution of TH immunoreactivity was similar to that of ChAT (Fig. 9*a*, *b*).

DISCUSSION

It has been demonstrated by immunohistochemistry that the pancreas of the Houbara bustard possesses peptidergic hormones and neurotransmitters as well as aminergic neurotransmitters which are known to be involved in an array of regulatory mechanisms controlling pancreatic function in other avian and mammalian species.

The distribution and relative density of the peptidergic hormones and neurotransmitters in the dorsal lobe of the pancreas of the Houbara bustard are similar to that in mammalian and other avian pancreata but the segregation of islets into dark A and light B cells observed in other avian species (Falkmer, 1985; Lucini et al. 1996) is not evident. The concentration of glucagon confirmed the observation

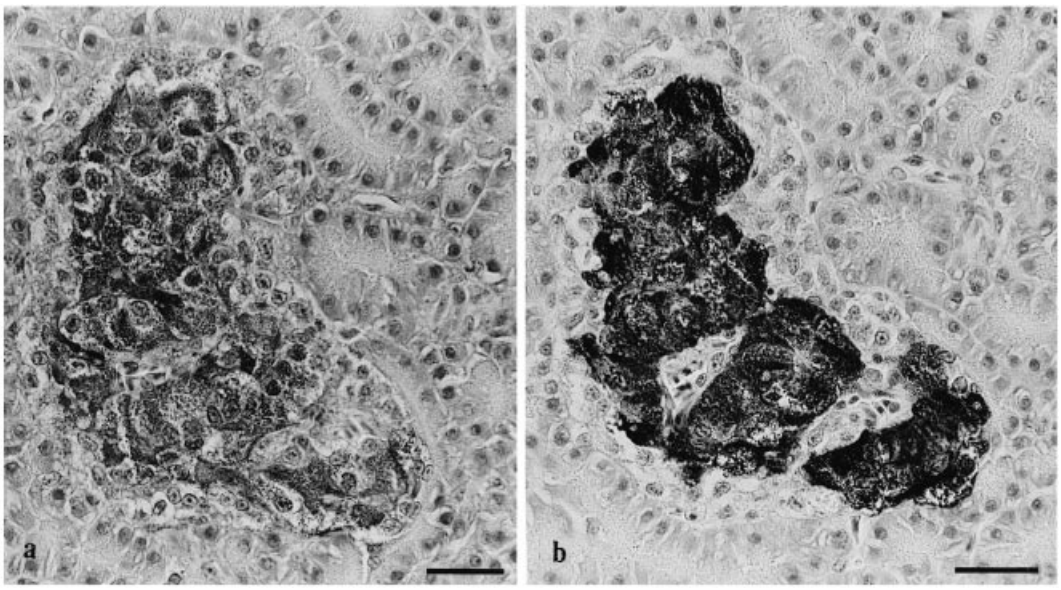

Fig. 6. Light micrographs showing centrally located islet cells in which (*a*) substance P (*b*) insulin are colocalised. Bar, 7 μm.

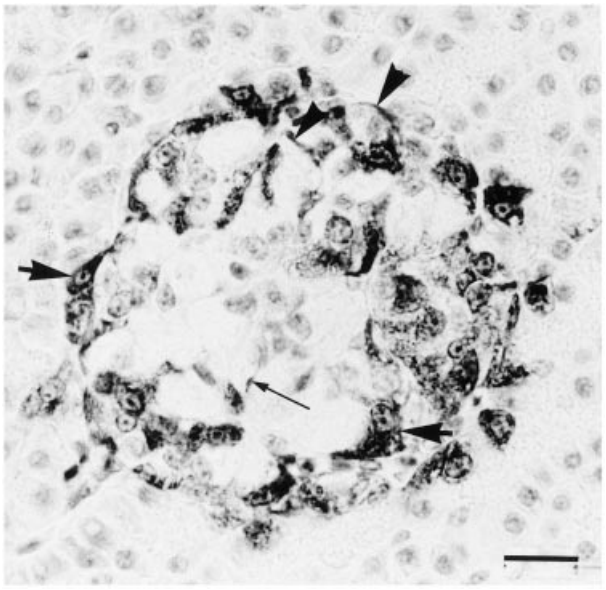

Fig. 7. Light micrograph showing neuron cell bodies (large arrows) and nerve fibres (arrowheads) immunoreactive to neuropeptide Y located mainly at the periphery of the islet. Some of the immunoreactive cell bodies are present towards the centre of the islet. Note also the presence of a rare varicose terminal at the centre of the islet (small arrow). Bar, 6 μm.

that glucagon levels are low in the dorsal lobe of the pancreas of birds (Weir et al. 1976). Weir et al. also reported a very high concentration of somatostatin in the chicken pancreas which might explain the higher density of endocrine cells immunoreactive to somatostatin compared with glucagon in this avian species. Insulin is the most abundant peptidergic hormone in the dorsal lobe of the Houbara bustard as in the chicken pancreas (Tomita et al. 1985). The bustard possesses both endocrine and exocrine PP cells unlike the domestic fowl that possesses predominantly parenchymal exocrine PP cells (Alumets et al. 1978). In addition, not only did PP and glucagon not exhibit a reciprocal distribution as observed in the chicken pancreas (Tomita et al. 1985), but 20% of glucagon immunoreactive cells also contained PP. Whilst glucagon is recognised as a major pancreatic hormone involved in glucoregulation in birds (Epple et al. 1980; Falkmer & Van Noorden, 1983; Hazelwood, 1984), the dominant factor in homeostasis is the insulin/glucagon (I/G) ratio. The I/G ratio of 1.8–2.2 established in birds suggests that birds are normally in a catabolic mode. The concentrations of insulin and glucagon in the dorsal pancreas of the Houbara bustard are more like those of mammals and further studies involving the ventral lobe will be required to determine whether the I/G ratio of this bird is also in a catabolic mode or not. Somatostatin inhibits the release of all pancreatic hormones with the greatest inhibitory effect being against glucagon. It has least effect on PP secretion (Hazelwood, 1984). The Houbara pancreatic islet also contains NPY immunoreactive cells which as in other avian species occur only in neuronal structures of the pancreas (El-Salhy et al. 1987) and mammals (Su et al. 1987; De Giorgio et al. 1992). Our study has demonstrated colocalisation of insulin with substance P in centrally located islet endocrine cells. Whilst its functional significance remains to be demonstrated, substance P could be acting as a neuromodulator of insulin secretion. Studies on the distribution of neurotransmitters in other avian species for example, the chicken

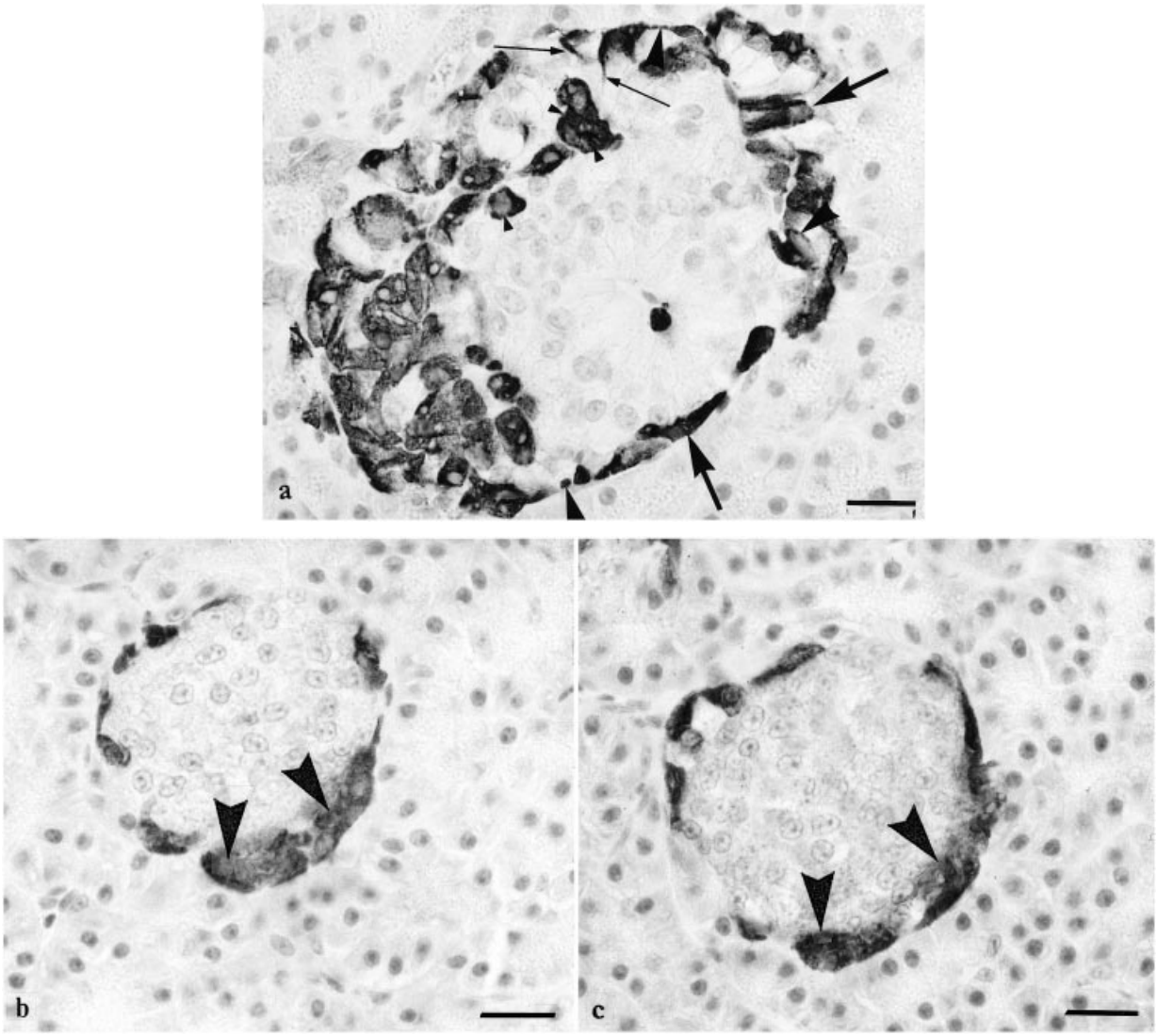

Fig. 8. Light micrographs showing neuron cell bodies (large arrows) and nerve fibres (small arrows) immunoreactive to polyclonal nNOS at the periphery of islets. Note the presence of immunoreactive varicose terminals at the periphery and towards the centre (large arrowheads) of islets, and also the immunoreaction of centrally located islet cells (small arrowheads) to NOS. Peripherally located islet cells (arrow) show colocalisation of nNOS (*b*) and somatostatin (*c*). Bar, 8 μm.

have revealed VIP immunoreactive nerves around B cells (Hiramatsu & Oshima, 1997) and galanin immunoreactive nerves in the walls of blood vessels, ducts and acinar cells (Hiramatsu & Oshima, 1995). The present data have demonstrated the presence of VIP and galanin immunoreactive varicose terminals both in the centre and the periphery of the islet, with nerves at the periphery being the most common. VIP was also present in peripherally located islet endocrine cells. The present study confirms the observation that GRP immunoreactive cells do not occur in the avian pancreas (Campbell et al. 1991). CCK-8 immunoreactive cells were also absent in the islets of the Houbara pancreas. The reason for this distribution is unknown, particularly as physiological studies have shown that CCK-8 is not a major regulator in the digestive system of birds (Satoh et al. 1994).

The distribution of the neurotransmitters nNOS, ChAT and tyrosine hydroxylase was similar to that reported in mammals and other avian species (Oomori et al. 1994; see review by Brunicardi et al. 1995). In the islets, as in other parts of the digestive system of the Houbara bustard (Mensah-Brown, unpublished observations), nNOS is colocalised with somatostatin and not with VIP as observed in the digestive systems of humans and monkeys (De Giorgio et al. 1994).

The role of these neuropeptides and neurotransmitters in the regulation of islet hormone secretion is not clear, but several studies have shown that both glucagon and insulin release are affected by stimu-

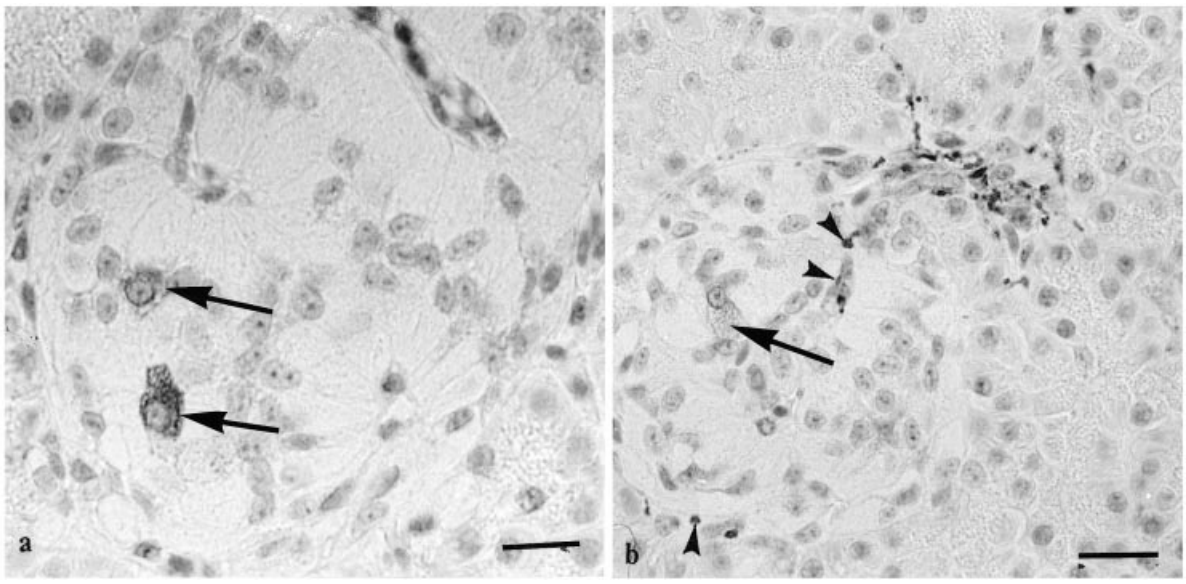

Fig. 9. Light micrographs showing tyrosine hydroxylase immunoreactive (*a*) neuron cell bodies (*a*, arrows) at the centre and varicose terminals (b, arrowheads) mainly at the periphery of islets. Bars: *a*, 5 μm; *b*, 6 μm.

lation of adrenergic and cholinergic nerves. Generally, it is known that cholinergic fibres strongly stimulate whilst α-adrenergic fibres inhibit insulin, glucagon and pancreatic polypeptide secretion (Brunicardi et al. 1995). It is also known that VIP (Jensen et al. 1978) stimulates insulin secretion, whereas galanin (Dunning & Taborsky, 1988; Messell et al. 1990) inhibits insulin and somatostatin but stimulates glucagon secretion. These neuropeptides may have similar functions in the Houbara bustard. Nitric oxide has been associated with the regulation of pancreatic blood flow and insulin release. The synthesising enzyme nNOS has been found in β-cells of rats (Schmidt et al. 1992) and chickens (Ekblad et al. 1994; Liu et al. 1994) and NO may therefore play a regulatory role in islet hormone secretion (Jansson & Sandler, 1991; Ekblad et al. 1994).

In conclusion, the dorsal pancreatic lobe of the Houbara bustard contains all the peptidergic hormones normally present in the islets of other avian species. It is also characterised by a high concentration of substance P-containing endocrine cells within its B cells. It has been proposed that neuropeptides and neurotransmitters present in endocrine cells, neuronal cell bodies and nerves of the pancreas regulate the secretion of these hormones.

ACKNOWLEDGEMENTS

Mr Ashok of the Medical Illustration unit is acknowledged for help with photography. This work was supported by a grant from the Faculty of Medicine, UAE University.

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