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Glucagon of Caviomorphs and Other Tetrapods Immunohistochemically Investigated with Two Antisera against the N- and C-Terminal Portions of the Molecule

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Pancreatic sections from diverse tetrapods, including various species of caviomorph rodents, were immunohistochemically investigated using two antisera which reacted with the N- and C-terminal portions of the glucagon molecule. While the antiserum against the N-terminal portion stained α cells in all the species studied, the antiserum against the C-portion failed to stain α cells in two caviomorphs of the Caviidae family (guinea pig and cuis) and in one of the Octodontidae family (degu). The observations in guinea pig and degu were expected, since their glucagons differ from those of many other tetrapods in the C-terminal portion of the molecule. In this paper, the cuis was added to these two species. It is noteworthy that among the caviomorphs studied herein (nine species), immunohistochemical differences were detected only in the three above-mentioned species and did not involve higher taxa, thus suggesting that these modifications are relatively recent in the evolution of this group of rodents.

INTRODUCTION

The order Rodentia comprises two suborders, Sciurognathi and Hystricognathi. In the neotropical region, the latter is represented by a widely diversified and endemic assemblage of peculiar rodents which form the infraorder Caviomorpha (Mares and Ojeda, 1982). Two members of this infraorder, the guinea pig and the degu, are widely used species in research. Interestingly, both animals show the peculiarity of elaborating a glucagon whose composition differs from that of many other tetrapods in the C-terminal portion of the molecule (Sundby, 1976; Huang *et al.*, 1986). This peculiarity is also shared by another caviomorph, the chinchilla (Eng *et al.*, 1990).

Two commercially available anti-glucagon sera have different properties, namely one of them stained α cells in many tetrapods, whereas the other did not stain these cells in the guinea pig and in the degu. Considering the above-mentioned differences in the molecule, we hypothesized that the first serum might recognize the N-terminal portion, while the other could react with the C-terminal portion of the hormone. In order to prove this assumption, we decided to immunostain specimens of pancreases from different tetrapods, including various species of caviomorphs, with both antisera. Our aim was to establish whether variations in immunological reactions to the two antisera might reveal differences in the molecular composition of glucagon among the various animals. Of special interest was whether the caviomorph species mentioned above shared the differences in the molecular structure of their glucagon with other members of the infraorder Caviomorpha.

MATERIALS AND METHODS

Pancreases from nine species that belong to three of the four caviomorph superfamilies were studied (see Table 1). Comparative control studies were performed using pancreases from noncaviomorphs including *mammals:* humans, dogs, cats, cows, pigs, horses, sheep, rabbits, hamsters, rats, and mice; *birds:* turkeys, ducks, pigeons, and chickens; *rep-*

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tiles: snakes (*Xenodon merremii*), lizards (*Tupinambis teguixin* and *Podarcis s. sicula*), and turtles (*Chelonoidis chilensis* and *Trachemys scripta*); and *amphibians:* toads (*Bufo arenarum*) and salamanders (*Ambystoma mexicanum*).

Pancreases were fixed in Bouin's fluid; sections were embedded in paraffin and then treated with two kinds of primary polyclonal antisera, one assumed to be against the N-terminal portion of the glucacon molecule (N-serum) and the other to be against the C-terminal portion (C-serum). To test this assumption aliquots of these antibodies were preincubated overnight at 4° with a 0.5-mg/ml solution of porcine glucagon 1-29 (Novo Nordisk A/S, Denmark), glucagon 19-29 (Peninsula Laboratories, Inc., Belmont), or secretin (Sigma Co., St. Louis, MO). Sections of rat pancreas were used to test the effect of this preincubation on the ability of the antibodies to stain α cells.

N-serum was a kind gift of Peninsula Laboratories, Inc., Belmont (Cat. No. RAS7165N, Lot 022953-2) and C-serum was purchased from BioGenex Laboratories, San Ramon (Cat. No. PA039-5P, Lot RHO39071S). Both antibodies were raised in rabbits, using as antigens human and pig glucagon, respectively, which are known to have identical amino acid sequences. None of the antisera were characterized by the producing laboratories.

N-serum was diluted 1:100, whereas C-serum was diluted 1:5. Primary antibodies reacted for 1 hr at room temperature. Whenever α cells in a given pancreas were negatively stained with the C-serum, new sections either were allowed to react with the first antibody for 24 hr at 4° or were previously digested with trypsin to unmask antigens, as described by González and Rodríguez (1980). Antibodies were demonstrated with the avidin–biotin method following a protocol published elsewhere (Iturriza and Thibault, 1993). Diaminobenzidine was used as oxygen acceptor. To test the specificity of the immunohistochemical reaction, parallel control sections were run, omitting the specific antibody or replacing it with other rabbit hyperimmune sera. Under these conditions, no immunostaining of islet cells was observed (data not shown).

TABLE 1

CAVIOMORPH SPECIES INCLUDED IN THIS STUDY, CLASSIFIED ACCORDING TO REIG (1986)

Infraorder Caviomorpha
Superfamily Octodontoidea
Family *Octodontidae*
Subfamily Octodontinae
Octodon degus (degu)*
Subfamily Ctenomyinae
Ctenomys talarum (tuco-tuco)
Family Myocastoridae
Myocastor coypus (coypu)
Superfamily Cavioidea
Family Caviidae
Subfamily Dolichotinae
Dolichotis patagonum (mara)
Subfamily Caviinae
Cavia porcellus (guinea pig)*
Cavia aperea (cuis)*
Family Hydrochoeridae
Hydrochoerus hydrochaeris (capybara)
Superfamily Chinchilloidea
Family Chinchillidae
Subfamily Chinchillinae
Chinchilla sp. (chinchilla)
Subfamily Lagostominae
Lagostomus maximus (plains viscacha)

Note. Asterisks show the species which did not react with the C-serum.

RESULTS

Some morphological differences could be observed in the endocrine pancreas of the animals studied. In the rat (Figs. 1d and 1f; Fig. 2a) the characteristic peripheral location of α cells was clearly noticed. In ducks (Fig. 2b) the islets appeared irregular in shape. α cells were numerous and distributed all over the islet section. In snakes (Fig. 2c) α cells were relatively scarce and irregularly distributed. In toads (Fig. 2d) a large number of α cells were observed dispersed through the exocrine pancreas, other than those composing the islets.

In the three species of caviomorphs studied (Fig. 3, top), the size and shape of the pancreatic islet did not differ much from those of other laboratory mammals such as rats and mice. However, α cells, which were moderately abundant, did not show a peripheral distribution but appeared irregularly located covering different areas of the islet.

Positively immunostained rat islet α cells were observed when fresh samples of both antisera (C- and N-terminal glucagon) were used. This reaction was completely abolished when both antisera were previously incubated with the intact glucagon molecule (1-29 glucagon) (Figs. 1a and 1b). Different results were obtained when the so-called N- and C-sera were preincubated with the C-terminal glucagon moiety (19-29 glucagon) or with secretin; the latter is similar though not identical to glucagon in the N-terminal portion. The immunostaining obtained with the N-serum was completely abol-

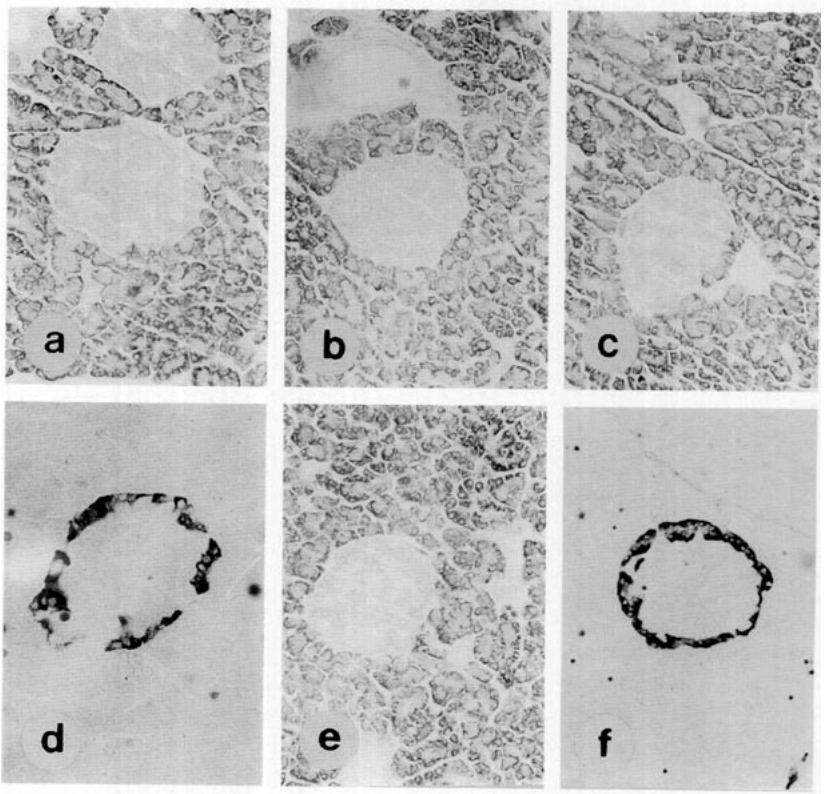

FIG. 1. Pancreatic rat islets. The immune reaction to reveal α cells using C-terminal (a) and N-terminal (b) antisera was abolished when previous incubation with the intact glucagon molecule (19-29 glucagon) was performed. Immunostaining with the N-serum was abolished with preincubation with secretin (c) and it remained unchanged with preincubation with 19-29 glucagon (d). Immunostaining with the C-serum was blocked after preincubation with 19-29 glucagon (e) while it remained unchanged with preincubation with secretin (f). Sections showing negative reactions were contrasted with toluidine blue. ×110.

ished when preincubated with secretin (Fig. 1c) but it remained unchanged when preincubated with 19-29 glucagon (Fig. 1d). The opposite effect was observed with the C-serum; while preincubation with 19-29 glucagon completely blocked its immunostaining capacity (Fig. 1e), it remained unchanged when preincubated with secretin (Fig. 1f).

α cells in the islets of all specimens investigated gave a positive reaction with the two antisera used, excepting *Cavia porcellus, Cavia aperea,* and *Octodon degus* (Figs. 2 and 3). These three species—the only ones described because of their peculiar staining properties—showed positively stained α cells after using the N-serum but they did not when the C-serum was employed. Neither prolonging the exposure to the first antibody nor treating the sections with trypsin rendered positive reactions for the C-serum in those specimens which had primarily been negative for it.

DISCUSSION

Glucagon is a 29-amino-acid peptide whose molecular structure has been conserved fairly well along evolution. Its molecule has been sequenced in mammals (see references in Nishi and Steiner, 1990), and it has been reported that pig, cow, man, rabbit, rat, and hamster glucagon molecules have identical amino acid sequences. Avian glucagon was studied in the turkey (Markussen *et al.*, 1972) and in the duck (Sundby *et al.*, 1972); in the former, glucagon

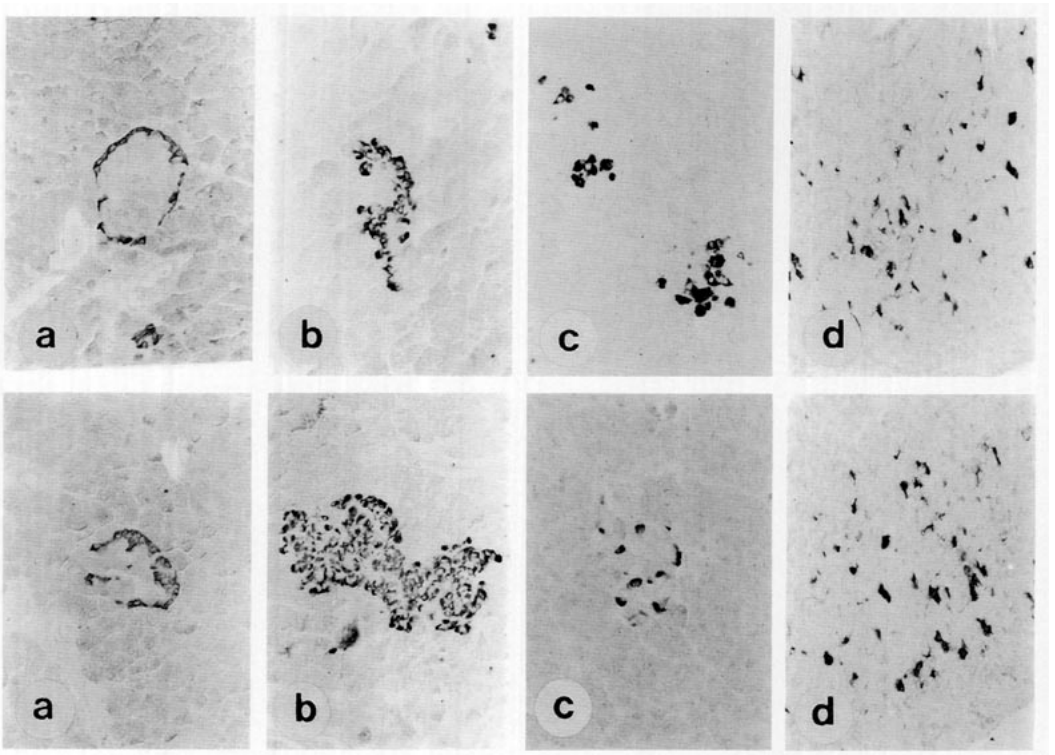

FIG. 2. Pictures of species arbitrarily chosen from the four classes of tetrapods to show that both N-serum (top) and C-serum (bottom) stained pancreatic α cells. (a) Mammalian (rat), (b) avian (duck), (c) reptilian (snake), (d) amphibian (toad). ×110.

differs from the mammalian hormone in that asparagine is substituted by serine at residue 28. In addition to this change, in ducks serine is substituted by threonine at residue 16. Interestingly, duck glucagon has the same composition as turtle glucagon (Conlon and Hicks, 1990), as is the case with the opossum (Yu *et al.*, 1989) and the turkey (Markussen *et al.*, 1972). Pollock *et al.* (1988) have reported that amphibian glucagon is the same as pig glucagon except for a single threonine-for-serine substitution at position 29.

Caviomorph glucagon has been sequenced only in three species: *C. porcellus* (Huang *et al.*, 1986), *O. degus* (Nishi and Steiner, 1990) and *Chinchilla* sp. (Eng *et al.*, 1990). These reports showed that in *C. porcellus* the molecule differs from other mammalian glucagons by five amino acids, all located in the C-terminal portion at positions 21, 23, 24, 27, and 29, whereas in *O. degus* the differing residues at the C-terminal end are three, at positions 23, 24, and 27. However, the replacing amino acids in the C-terminal portion at positions 24 and 27 are not the same as in *C. porcellus* (Table 2).

The current results confirm our preliminary observations that the C-serum did not stain the α cells in *O. degus* or in *C. porcellus* while the N-serum did; in comparing these results with those obtained by molecular sequencing it can be concluded that the first serum recognizes the C-terminal portion, whereas the second one reacts with the N-portion.

Our results clearly show that the minor differences among mammalian, avian, reptilian, and amphibian glucagon molecules did not preclude the staining of α cells with either N- or C-sera.

The caviomorphs *C. porcellus, O. degus,* and *C. aperea* were the only species among the

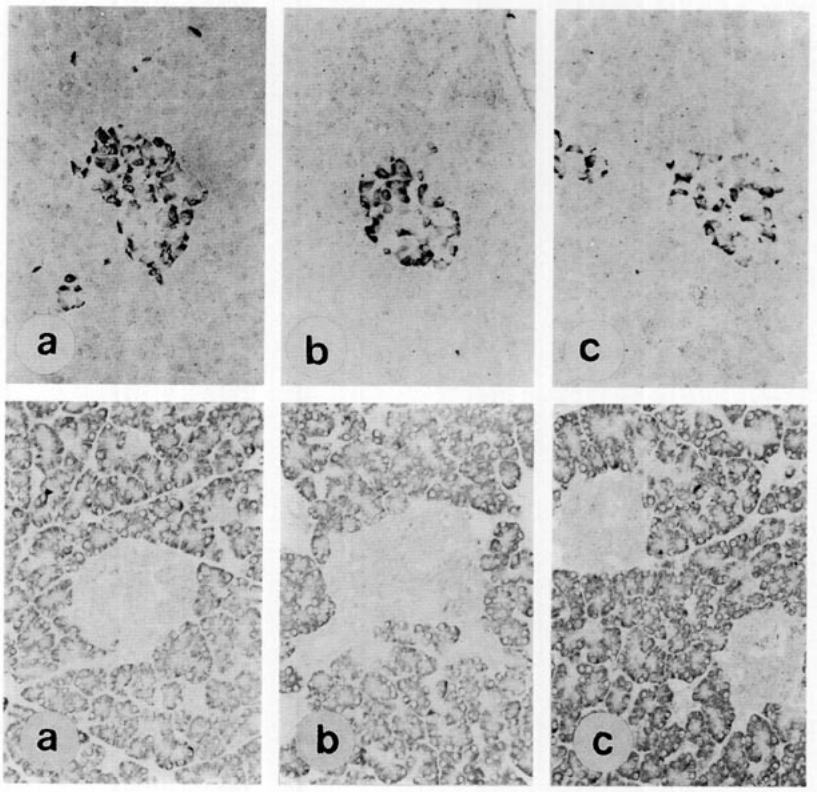

FIG. 3. Sections from (a) *C. porcellus,* (b) *C. aperea,* and (c) *O. degus* pancreases. Note that α cells stained with the N-serum (top) but they did not stain with the C-serum (bottom). Sections showing no α cells were contrasted with toluidine blue. ×110.

many studied herein which did not react with the C-serum. This lack of reaction is in line with biochemical investigations (Huang *et al.,* 1986; Nishi and Steiner, 1990) undertaken in the first two species reporting differences in the C-terminal portion. Interestingly, glucagon of *Chinchilla* sp. also has differences at the C-portion; however, substitutions are located at positions

TABLE 2
SEQUENCE OF AMINO ACIDS IN THE C-TERMINAL PORTION OF THE GLUCAGON MOLECULE IN VARIOUS ANIMALS

	Residues														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	References
Non-caviomorph mammals	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	Various
Opossum													S		Yu *et al.,* 1989
Caviomorphs															
C. porcellus						Q		L	K			L		V	Huang *et al.,* 1986
Chinchilla sp.			Y			E									Eng *et al.,* 1990
O. degus	T							L	D			K			Nishi and Steiner, 1990
Duck	T												S		Sundby *et al.,* 1972
Turkey													S		Markussen *et al.,* 1972
Turtle	T												S		Conlon and Hicks, 1990
Bullfrog														S	Pollock *et al.,* 1988

Note. Unfilled spaces correspond to the same symbols as expressed for non-caviomorph mammals.

18 and 21. Thus, the results obtained in these three caviomorphs would suggest that amino acids 23, 24, and 27 form epitopes which are essential for the binding of the C-serum to the glucagon molecule.

The close phylogenetic relationship between *C. porcellus* and *C. aperea* suggests that the modification in the glucagon molecule could have occurred in a common ancestor. *Hydrochoerus hydrochaeris* and *Dolichotis patagonum* are related to *Cavia* at superfamily and family levels, respectively; however, both species show a similar glucagon immunoreactivity to that of other tetrapods.

O. degus is another species whose glucagon displays modifications in the C-terminal portion; however, the amino acid substitutions are not the same as in *C. porcellus*. Both species are only slightly related and they belong to different superfamilies (Table 1). On the other hand, *Ctenomys talarum* and *Myocastor coypus*, related to *O. degus* at family and superfamily levels, respectively, show an immunohistochemical glucagon identity (Table 1).

In view of the aforementioned evidence, it is clear that in caviomorphs the immunohistochemical changes detectable with our antibodies in the C-terminal portion of the glucagon molecule do not involve higher taxa (i.e., families or superfamilies). This implies that modifications are relatively recent in the evolution of these peculiar rodents.

Based on the anomalous molecular features detected mainly in *Cavia*, it has been suggested that Caviomorpha (or Hystricomorpha) might represent a separate mammalian order (Graur *et al.*, 1991, 1992). However, Luckett and Hartenberger (1993) have demonstrated that such a hypothesis is the result of "an inadequate (poor) sampling of taxa." According to these authors, the available information shows that Hystricognathi present variation in their molecular composition and that *Cavia*, in this regard, is highly derived. Our results support this claim.

The C-serum used in this paper reacts exclusively with epitopes of the C-terminal portion. Probably, diversities in the carrier molecules used to conjugate the antigen, the conjugation method, or the immunization protocol could account for this property. C-serum could constitute a useful tool when used simultaneously with an antibody which recognizes the glucagon N-terminal portion for fast screening of evolutionary or other changes in this hormone molecule.

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