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THE IMMUNOCYTOCHEMICAL DETECTION OF GAMMA-AMINOBUTYRIC ACID (GABA) IN THE PANCREAS OF AMPHIBIAN RANA ESCULENTA; LIGHT- AND ELECTRON-MICROSCOPIC OBSERVATIONS

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ABSTRACT. The immunolabelled structures for gamma-aminobutyric acid (GABA) and their topographic distributions in the pancreas of the frog Rana esculenta are described for the first time in light and electron microscopy. At light microscopic level, the immunoreaction was detected both in the islets of Langerhans and the nerve fibbers supplying the exocrine tissue. At submicroscopic level, this multifunctional amino acid was identified in cytosol, secretory granules matrix and nuclear chromatin of insulin (INS)-, stomatostatin (SOM)-, glucagon (GLUC)- and pancreatic polypeptide (PP)- producing cells. Other reactive sites for this amino acid included the zymogen granules, mitochondria and nuclei of acinar cells. The above results and their functional significances entirely supporting the concept of a good conservation of this amino acid during phylogeny are discussed in connection with the findings previously reported in human and mammalian organs.

Keywords: gamma-aminobutyric acid, GABA, *Rana esculenta*, pancreas, immunocytochemistry

INTRODUCTION

Since the initial localization of the gamma-amino butyric acid (GABA) in the mammalian brain (Awapara et al., 1950; Roberts and Frankel, 1950), there have been gathered numerous evidence indicating that this aminoacid is a strong inhibitor of the neurotransmission in the central nervous system (Roberts and Hammershlag, 1972; Krnjevic, 1976; Fekete et al., 2002; Kalsbeek et al., 2008). In addition, the biochemical and immunohistochemical studies revealed the existence of several significant quantities of aminoacid in the fibbers and ganglions of the autonomous nervous system (Iwasa et al., 1998, 1999; Gladkevich et al., 2006; Wiens and Trudeau, 2006) and in the endocrine and peripheral tissues such as ovaries and oviducts (Schaeffer and Hsueh, 1982; Laszló et al., 1989; Erdö et al., 1989), testes (Hu and Yan, 2002; Geigerseder et al., 2003), surrenal glands (Akinci and Schofield, 1999; Metzeler et al., 2004), endocrine (Garry et al., 1987b; Adeghate and Ponery, 2002; Gammelsaeter et al., 2004; Gladkevich et al., 2006) and exocrine pancreas (Garry et al., 1988; Park et et al., 2006), adenohypophysis (End et al., 2005; Gladkevich et al., 2006), thyroid gland (Akinci and Schofield, 1999; Wiens and Trudeau, 2006), the diffuse neuroendocrine gastrointestinal system (Akinci and Schofield, 1999; Hardt et al., 2000), uterus (Akinci and Schofield, 1999; Gladkevich et al., 2006) and salivary glands (Kosuge et al., 2009). The fact that in the endocrine pancreas have been revealed significantly major quantities of GABA in contrast with other peripheral glands (Taniguchi et al., 1979; Michalik and Erecinska, 1992) has lead to the present status of B cells as a major source of this aminoacid. Taking into account the above results regarding the various GABA localizations in the peripheral and glandular mammalian and human organs, the purpose of this study was to detect immunohistochemicaly in light- and electron microscopy the distribution of this functional aminoacid in the pancreas of the anuran Rana esculenta. This, so much the more the multiple implications of the aminoacid in the central nervous system (CNS) and in the peripheral mammalian organs pledge in the favour of its bradycardic and hypotensive effects (Roberts and Kuriyama, 1968; Krnjevic, 1976).

MATERIALS AND METHODS

The investigations were performed on 5 adult specimens, of both sexes, belonging to the anuran amphibian *Rana esculenta* L., all specimens purchased from a commercial source.

For light microscopic observations, the pancreas pieces from two individuals, were fixed by immersion in a mixture of previously chilled 2% paraformaldehedida + 15% saturated picric acid, prepared in 0.1M phosphate buffer (pH 7.4) (Zamboni and De Martino, 1967). Further on, the organ' fragments were embedded in paraffin (Mőller, 1976) and the resulted 7 µ-thick sections were processed according to the peroxidase anti-peroxidase (PAP) procedure initiated by Sternberger in 1974.

The anti-GABA polyclonal serum was provided by Biotrend (Kőln, Germany), goat anti-rabbit IgG from

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Behring Werke (Marburg, Germany), and the PAP complex from Dakko (Copenhagen, Denmark).

The conditions for optimal immunostaining were achieved by increasing the dilutions of the primary antibody (1:400; 1:500; 1:700) prepared in saline phosphate buffer (0.1M PBS, pH 7.4) containing 0.25% Triton X-100. The 1:400 concentration which maximally stains the immunoreactive structures without any other unspecific reaction was chosen. The goat anti-rabbit IgG (dilution 1:40) and the PAP complex (dilution 1:80) were used in excess.

The specificity of the immunoreaction was tested by replacing the primary antiserum, goat anti-rabbit IgG or PAP complex with phosphate saline or with Tris-saline buffers. In addition, the anti-GABA serum was tested by pre-adsorption with various quantities of specific antigens or with structural related antigens.

For ultrastructural observations, small fragments of pancreas ≤ 1mm3 were embedded in Lowicryl K4M

RESULTS AND DISCUSSIONS

The optimal immunostaining was obtained in light microscopy by using the dilution 1:400 of the primary serum, which didn't allow an unspecific reaction (background). With this dilution the immunoreactivity appeared localized in the periacinar nerve fibers and in the pancreatic islets, mostly in the insulin producing cells (B cells), but also in the other types of endocrine cells (Fig. 1).

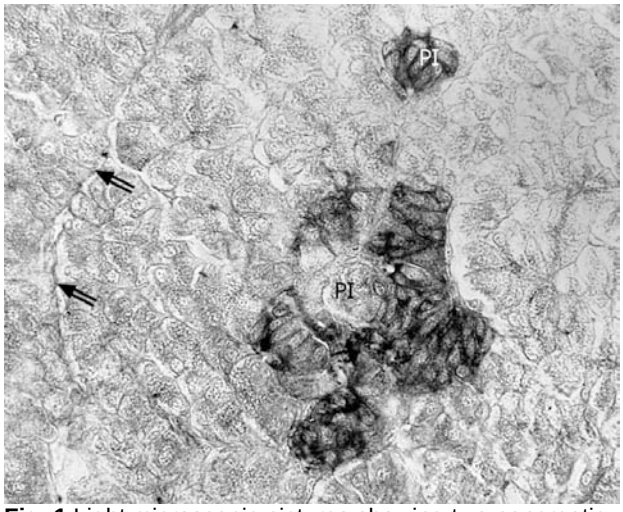


Fig. 1 Light microscopic pictures showing two pancreatic islets of Langerhans (PI) in frog immunostained for GABA. The interacinar profile (double arrows) of an immunostained nerve fibber could be also seen. PAP-procedure, x245

The immunolabelling at ultrastructural level, using constantly the dilution 1:200 of the primary antibody, revealed many reactive sites not only in the endocrine and exocrine pancreas but also in the intrinsic nerve fibers of this gland (Figures 2-10).

Therefore, irrespective of the endocrine cell type, the colloidal gold particles appeared distributed in (Carlemalm et al., 1982) and then processed accordingly to the postinclusion method. The ultrathin sections, prepared on a LKB ultramicrotome, were collected on polioform-coated Ni-grids and directly exposed to primary antibody containing 0.25% serum albumin. After an incubation of 4 h at room temperature followed by a proper rising, the grids were transferred to a protein A-gold solution (dilution 1:25) for 1 h, prepared according to the method of Roth (1982) and Bendayan (1984). The diameter of gold particles was of 1nm. Subsequently, the sections were stained in accordance with the protocol of Reynolds (1963) and observed with a Philips 201 electron microscope.

The controls for the specificity of the immunoreaction at electron-microscopic level were obtained by using adequate saline buffer (PBS or TBS) instead of anti-GABA serum, goat anti-rabbit IgG or PAP-complex, respectively.

cytosol, in the matrix of secretory granules (Figures. 2-8), on the nuclei surfaces (Figures. 6, 7) and rarely on the mitochondrial cristae.

As the acinar tissue of the gland is concerned the immunoreactivity was associated with mitochondria (Fig. 9), zymogen granules (Fig. 10) and only occasionally with the nuclear chromatin (Figures. 6, 7).

Our results referring to the variety of immunoreactive localizations for GABA in the pancreas of the anuran amphibian *Rana esculenta*, seem to correspond in the main to the previously reported localizations for this amino acid and its regulating enzymes (GABA-T, GAD) in the same human gland (Okada et al., 1976; Michalik and Erecinska, 1992) and in that of other mammalian species (Taniguchi et al., 1982 a,b; Tanaka, 1985; Garry et al., 1986; 1987a,b, 1988; Michalik and Erecinska, 1992). Taking into account the above, the singular evidence up to date of the GABA microscopic and ultrastructural distributions in the pancreas of a poikilotherm vertebrates, enriches not only our present knowledge regarding the phylogenetic perenity of the topographic distribution and of the hormonal and non-hormonal co-localizations of this aminoacid, but also its functional meanings in this mixed gland. All these, so much the more a series of biochemical and immunohistochemical investigations on invertebrates have indicated that GABA has both excitatory and inhibitory effects on neuromuscular transmission (McIntire et al., 1993; Ukena et al., 1995; Telkes et al., 1996) at the level of intestinal tract and its derivatives like the Arthropods and Gastropods' hepatopancreas. Considering the above, a special attention should be paid to the immunocytochemical expression of this functional amino acid in the pancreas and gastrointestinal tract of amphibians and of other systematic groups of poikilotherm vertebrates (fishes, reptiles).

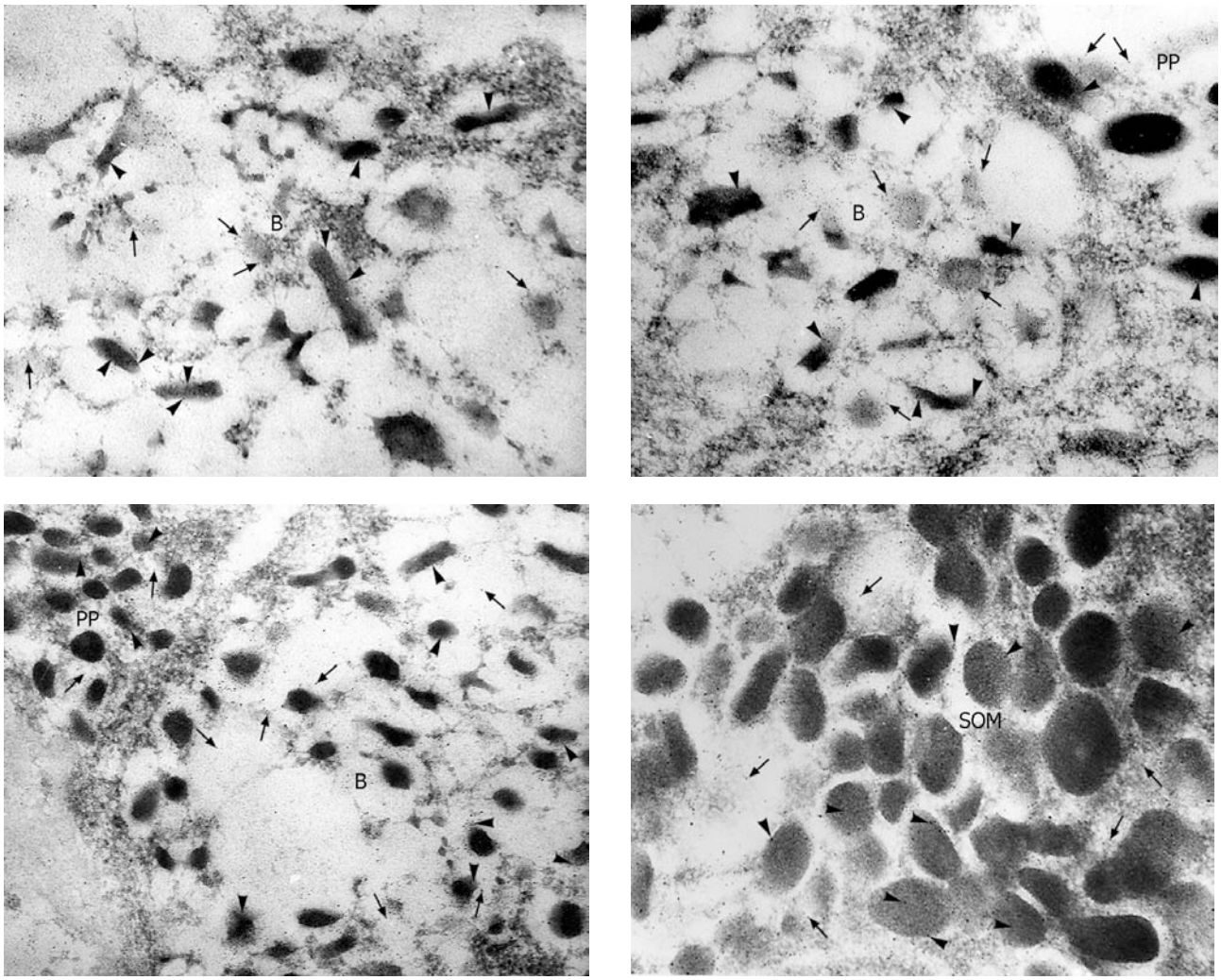


Fig. 2-5 Electron microscopic pictures displaying the distribution of 1nm gold particles in the cytoplasm (arrows) and the matrix of secretory granules (arrowheads) of insulin (B), pancreatic polypeptide (PP) and somatostatin (SOM) producing cells. Protein A-gold procedure. Fig. 2: x41000; Fig. 3: x43100; Figs. 4,5: x44800

Referring to the insular organ of *Rana esculenta*, our light-microscopic observations have confirmed the previous investigations regarding the co-localization of GABA and insulin in the rat (Garry et al., 1986; Sakaue et al., 1987) and other mammalian species (Michalik and Erecinska, 1992), as well as its periacinar distribution in nerve fibers of the gland exocrine tissue (Gerber and Hare, 1980; Garry et al., 1988). As regard the accumulation of GABA in the other cell types of the pancreatic islets observed by us in *Rana esculenta* and also by other authors in several mammalian species (Garry et al., 1987a,b, 1988; Michalik and Erecinska, 1992) it seems to be related to the trophic role of this multifunctional amino acid in non-neuronal peripheral cells (Gladkevich et al., 2006). In their turn, our submicroscopic investigations revealed a large variety of immunoreactive sites not only in the islets, but also in the exocrine pancreatic tissue. With some minor exceptions, the distribution of these localizations conformed itself with the topographic model described in mammals.

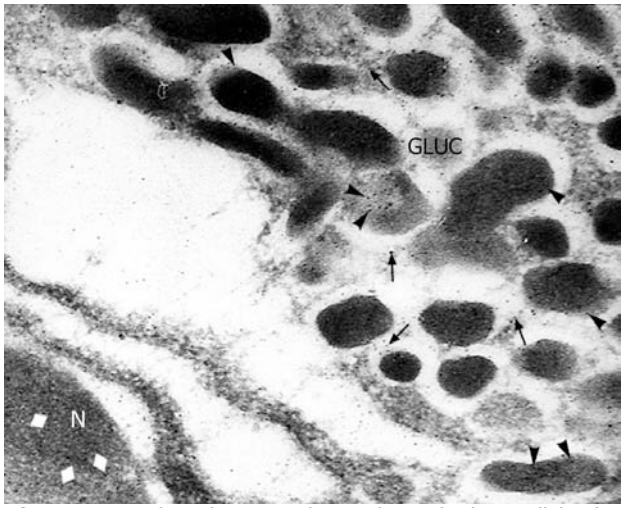
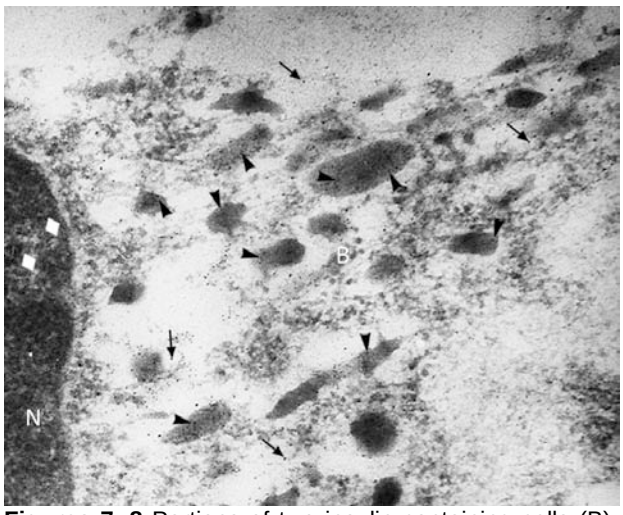
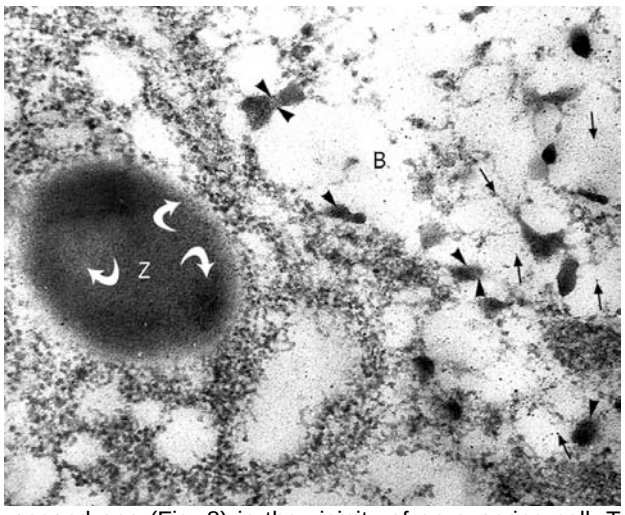


Fig. 6 Area of a glucagon (GLUC) producing cell in the pancreas of *Rana esculenta*. To note the immunolabelling of the secretory granules core (arrowheads), of cytoplasm (arrows) and of the chromatin (rhombheads) by 1nm gold particles. Protein A-gold procedure; x44800

Among these exceptions are to be mentioned the reduced density and even the absence of immunolabellings for GABA in the secretory granules matrix of rat B cells and also the lack of mitochondrial labellings in the same endocrine cell type (Garry et al., 1987a, b). Contrary, the cytosol, the core of the secretory granules and the nuclear chromatin of insulin (B cells), somatostatin (SOM cells), glucagon (GLUC cells) and pancreatic polypeptide (PP cells) producing cells, appeared as a rule constantly marked with colloidal gold particles. In addition, in the exocrine pancreatic tissue of the same amphibian species, the mitochondrial immunolabellings and those of zymogen granules, similar to the ones reported in the rat pancreas (Garry et al., 1988), were frequently seen.

The multiple functional meanings ascribed to the pancreatic GABA are mostly due to the investigations carried out on untreated mammals (Garry et al., 1987a, b; Garry et al., 1988), or on mammals exposed to a large variety of experimental conditions (Smismans et al., 1997; Adeghate and Ponery, 2002; Park et al., 2006; Ligon et al., 2007; Kalsbeek et al., 2008). They suggest the involvement of the amino acid as a mediator of B-cell activity (Garry et al., 1987a), its release by a nongranular mechanism (Garry et al., 1987 b), the decrease of B-cells number in animals pre-treated with streptozotocin (Sakaue et al., 1987), the nutrient regulation of this amino acid release (Smismans et al., 1997), the significant reduction of GABA containing cells in diabetes (Adeghate and Ponery, 2002) and its modulating role of exocrine secretion (Park et al., 2006), probably function of glucose level (Dong et al., 2006), the function as an endogenous co-regulator of B-cell mass (Ligon et al., 2007), its implication in the circadian control of the daily plasma glucose rhythm (Kalsbeek et al., 2008), the intrainsular role of GABA as an endocrine or paracrine factor (Gilon et al., 1991) and finally its uptake by pancreatic polypeptide- and glucagon-producing cells in the stomach and pancreas (Gilon and Remacle, 1989).





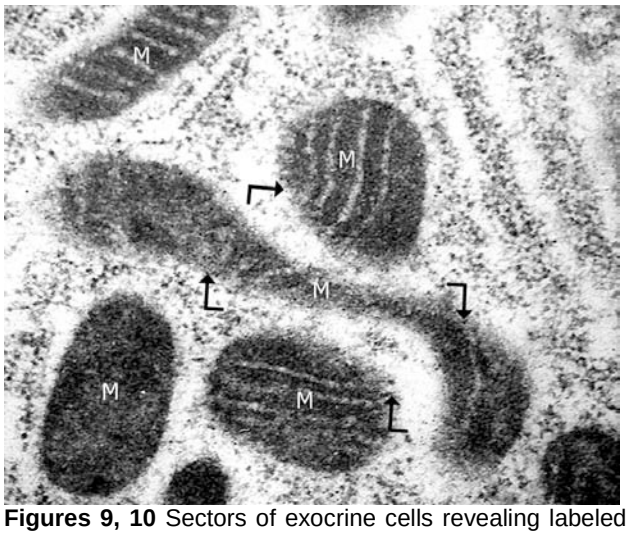
Figures 7, 8 Portions of two insulin containing cells (B), the second one (Fig. 8) in the vicinity of an exocrine cell. To observe the gold particles associated with the cytoplasm (arrows), secretory granules core (arrowheads), nuclear (N) chromatin (rhomheads) and the zymogen (Z) granule (curved arrows). Protein A-gold procedure. Fig. 7: x53900; Fig.8: x36100

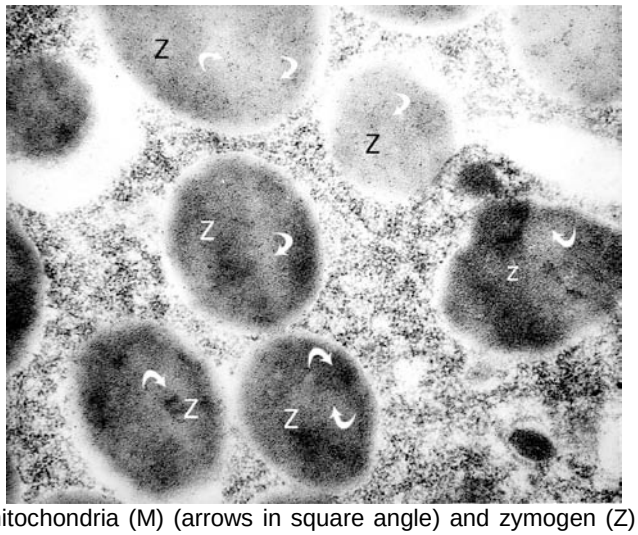
CONCLUSIONS

This paper represents the first immunocytochemical demonstration of the presence and topographic distribution of GABA in the pancreas of a poikilotherm vertebrate. Therefore it has the purpose to enrich our present knowledge on the presumed phylogenetic perenity of this multifunctional aminoacid in such a glandular organ.

The main expression of GABA in the insulin producing cells suggest both its role as fuel generated metabolically as an alternative way to glycolysis, or rather its involvement in the secretory events within in the islets. In the favour of the second hypothesis plead not only the distribution of the aminoacid in all the endocrine cell types, but also in the acinar cells and nerve fibbers of the frog pancreas. Nevertheless, a series of questions regarding the releasing mechanism of GABA from B cells or from other types of endocrine cells, the way in which the aminoacid controls the secretion of diverse pancreatic hormones and, the last but not the least, its function in exocrine cells claimed answer in the future.

Considering the aforesaid, detailed investigations on the presence and distribution of this multifunctional aminoacid in the pancreas of other species and taxa of poikilotherm vertebrates are imposed.





Figures 9, 10 Sectors of exocrine cells revealing labeled mitochondria (M) (arrows in square angle) and zymogen (Z) granules (curved arrows). Protein A-gold procedure. Fig. 9: x45300; Fig. 10: x31800

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