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Immunocytochemical localization of metabotropic (mGluR2/3 and mGluR4a) and ionotropic (GluR2/3) glutamate receptors in adrenal medullary ganglion cells

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Summary. The localization of metabotropic glutamate receptors of groups II (mGluR2/3) and III (mGluR4a) and the subunits 2 and 3 of alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors (GluR2/3) was investigated with immunocytochemical methods in the rat adrenal gland. MGluR2/3, mGluR4a and GluR2/3 immunoreactivities were observed in large-sized, centrally located type I adrenal medullary ganglion neurons. Furthermore, the small-sized type II adrenal ganglion neurons identified by their immunoreactivity to brain nitric oxide synthase (bNOS), also expressed mGluR2/3, mGluR4a and GluR2/3. These cells were disposed in the peripheral portion of the adrenal medulla. None of the type I neurons were positively labeled for bNOS. These morphological observations suggest that activation of glutamate receptors in ganglion neurons may be instrumental in the control of adrenal endocrine systems as well as blood regulation.

Key words: Peripheral nervous system, Excitatory amino acids, Neurotransmission, Preembedding immunocytochemistry, Rat

Introduction

Several bioactive signaling molecules such as acetylcholine, norepinephrine, nitric oxide (NO) and peptides like enkephalin or pituitary adenylate cyclase-activating peptide (PACAP) are the principal transmitters of the presynaptic fibers to the adrenal medullary ganglion neurons (Ginda et al., 1999; Holgert et al., 2003). In addition, some evidence points out that excitatory amino acids, such as glutamate, can also have a transmitter role in adrenal cells (Carlton et al., 1998; Hinoi et al., 2004).

The effect of L-glutamate is through the activation of ionotropic and metabotropic glutamate receptors (Nakanishi and Masu, 1994). The ionotropic glutamate receptors are subdivided into N-methyl-D-aspartate (NMDA) and non NMDA receptors. In this last group, the best characterized members are the family of alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors with four known subunits (GluR1-4) (Monaghan et al., 1989; Sommer and Seeburg, 1992). The metabotropic glutamate receptors (mGluRs) comprise eight different subtypes classified into group I: mGluR1, mGluR5; group II: mGluR2, mGluR3; and group III: mGluR4, mGluR6, mGluR7, mGluR8, on the basis of their DNA sequence similarities, pharmacological properties and intracellular signal transduction mechanisms (Knöpfel et al., 1995; Fagni et al., 2000).

The adrenal medulla contains chromaffin cells producing epinephrine and norepinephrine, and at least two types of intrinsic ganglion neurons (type I and II) characterized by their size, location and neurotransmitter phenotype (Holgert et al, 1996, 1998). These two populations of ganglion cells innervate chromaffin cells, adrenal glomerular cortical cells as well as vascular structures, both, in the adrenal medulla and cortex, participating the neuroactive substances contained in these adrenal ganglion cells in adrenal cellular functions and blood flow regulation (Afework and Burnstock, 1985; Kondo, 1985; Pelko-Huikko, 1989; Afework et al., 1994).

Radioligang binding studies performed in adrenal gland membranous preparations or slide-mounted sections of adrenal glands, confirmed a binding of [^{3}H] glutamate to adrenal medulla cells (Yoneda and Ogita, 1986; O'Shea et al., 1992). Although, reverse

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transcription-polymerase chain reaction (RT-PCR) analyses revealed constitutive expression of mRNA for GluR1, GluR3, GluR5, KA1, KA2 and the NMDA receptor subunits NR-1, NR-2C and NR-2D in the rat adrenal gland, Western blotting assays have failed to confirm the expression of receptor proteins for GluR1, GluR2/3, GluR4, NR-2C and NR-2D subunits in adrenal medulla (Hinoi and Yoneda, 2001; Hinoi et al., 2002). Anatomical studies using *in situ* hybridization techniques have previously demonstrated an intense expression of the four GluR1-4 mRNAs in adrenal medullary ganglion cells (Kristensen, 1993). Also, in vitro experiments have shown that glutamate and glutamate receptor agonists increase both basal and nicotine-evoked catecholamine secretion from cultured bovine chromaffin cells, effect that is mediated by ionotropic and metabotropic glutamate receptors (Gonzalez et al., 1998; Arce et al., 2004).

Taken together, although there are indications of the presence of ionotropic and metabotropic glutamate receptors in the adrenal medullary cells, there is a lack of information about the adrenal medullary cell types where glutamate receptors are localized. The present study was undertaken to determine the localization of groups II (mGluR2/3) and III (mGluR4a) metabotropic glutamate receptors and the AMPA receptor subunits 2 and 3 (GluR2/3) in the rat adrenal ganglion neurons using specific antisera combined with immunocytochemical methods. Furthermore, we applied an antiserum to brain nitric oxide synthase (bNOS) to identify type II adrenal neurons and to distinguish them from type I. Part of the results were published previously in abstract form (Sarria et al., 2003).

Materials and methods

The protocols for animal care and use were designed according to the European Communities Council Directive (8857609/ EEC).

Six adult albino rats (Sprague-Dawley, 200-220g) were anaesthetized with sodium pentobarbital (60 mg/Kg body weight, intraperitoneal injection) and transcardially perfused for 20 seconds with phosphate buffered saline (PBS, pH 7.4 at room temperature) followed by 1 liter of fixative containing 4% formaldehyde and 0.2% picric acid in ice-cold 0.1M phosphate buffer (PB pH 7.4) for 10-15 minutes.

Adrenal glands were removed and transferred first to a 15% (wt/vol) sucrose solution in 0.1M PB (pH 7.4) and after the tissue had sunk to a 30% sucrose solution. Serial 30 µm-thickness sections were cut on a cryotome and rapidly placed in plastic vials containing PBS and subsequently preincubated in 1.5% normal goat serum in PBS for 1 hour at room temperature.

Sets of sections were incubated with specific polyclonal antisera to mGluR2/3 (0.35 µg/ml) (Chemicon International Inc, Temecula CA, USA), mGluR4a (0.25 µg/ml) (Mateos et al., 1999; Benitez et al., 2000; Azkue et al., 2001) and GluR2/3 (1 µg/ml) (Chemicon International Inc, Temecula CA, USA) for 24 hours at 4°C. The tissue was processed for a conventional avidin-biotin horseradish peroxidase complex method (ABC Elite; Vector Laboratories, Burlingame, CA, USA). In brief, sections were incubated sequentially with a biotinylated secondary antibody and with an avidin-biotin complex, each for 1 hour at room temperature. Next, sections were preincubated for 5 minutes with 0.05% 3.3'-diaminobenzidine (DAB) and subsequently incubated for 5 minutes by adding 0.01% hydrogen peroxide to the same solution. Stained sections were mounted, air-dried, dehydrated and coverslipped with DPX (Fluka Chemie AG, Switzerland).

A double-staining immunofluorescence method was used to identify cells expressing glutamate receptors (mGluR2/3, mGluR4a and GluR2/3) and bNOS. A simultaneous incubation of each of the above mentioned polyclonal antibodies for the glutamate receptors with a monoclonal anti-bNOS antibody(1:200) (Sigma Chemical CO, St Louis MO, Los Angeles, CA, USA) was performed in the adrenal sections. Following this, the tissue was incubated with a Cy3-conjugated goat anti-rabbit (1:100; Jackson Immunoreasearch Laboratories, Inc. PA, USA) and fluoresceine (DTAF)-conjugated goat anti-mouse (1:100; Jackson Immunoreasearch Laboratories, Inc. PA, USA) in PBS for 1 hour at room temperature. Then, sections were mounted, dried and coverslipped with an antifading medium (Vectashield; Vector Laboratories, Burlingame, CA, USA).

The specificity of the immunocytochemical staining was confirmed by replacing the primary antiserum with normal horse serum and saline buffer. No immunostaining was observed under these conditions.

Light microscopy immunostaining was registered in greyscale film photographs and double labelling immunocytochemistry was evidenced in consecutive colour slides. Images were scanned, smoothly improved by adjusting brightness/contrast intensity and finally labeled and saved as tiff format.

Results

Large-sized type I ganglion cells, located preferentially in the middle portion of the adrenal medulla among chromaffin cells, formed clusters of various cellular elements strongly immunoreactive for mGluR2/3 (Fig. 1A, 1B). In addition, several type II small-sized ganglion cells disposed in the peripheral portion of the adrenal medulla were also mGluR2/3 immunoreactive (Fig. 1A).

MGluR4a in adrenal tissue was observed in numerous type I polygonal-shaped cells in the center of the medulla, sometimes in close relation with blood vessels. MGluR4a immunoreactive nerve fibers arising from these large ganglion cells were visible around medullary blood vessels and running among chromaffin cells (Fig. 2A). Immunoreactivity for mGluR4a was also

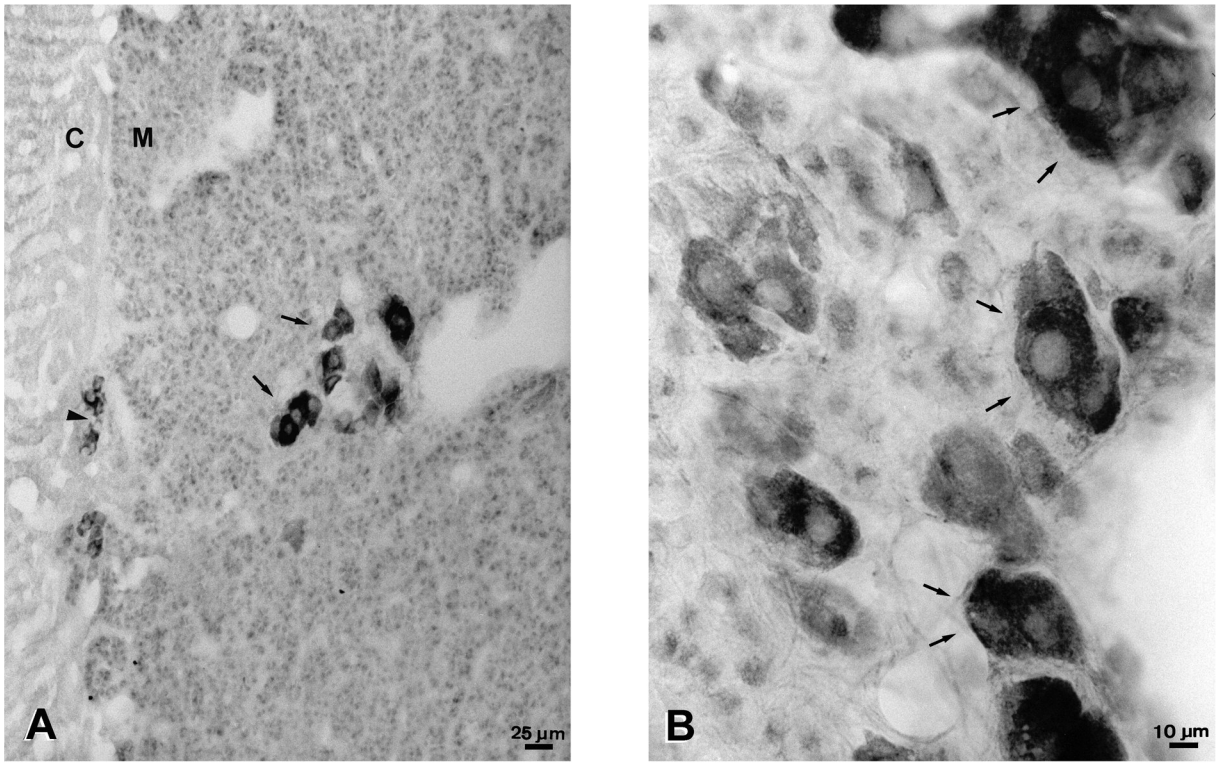

Fig. 1. Immunocytochemical localization of the metabotropic glutamate receptor 2/3 (mGluR2/3) in the rat adrenal gland. Large-sized mGluR2/3 immunoreactive ganglion cells appear in the middle portion of the adrenal medulla (arrows). Less numerous and smaller immunoreactive cells are preferentially distributed in the peripheral portion of the medulla (arrowhead) **(A).** C: Cortex; M: Medulla. Scale bar: 25 µm. In the center of the medulla, cells show a strong mGluR2/3 immunoreaction and are generally disposed in small clusters (arrows) **(B).** Scale bar: 10 µm.

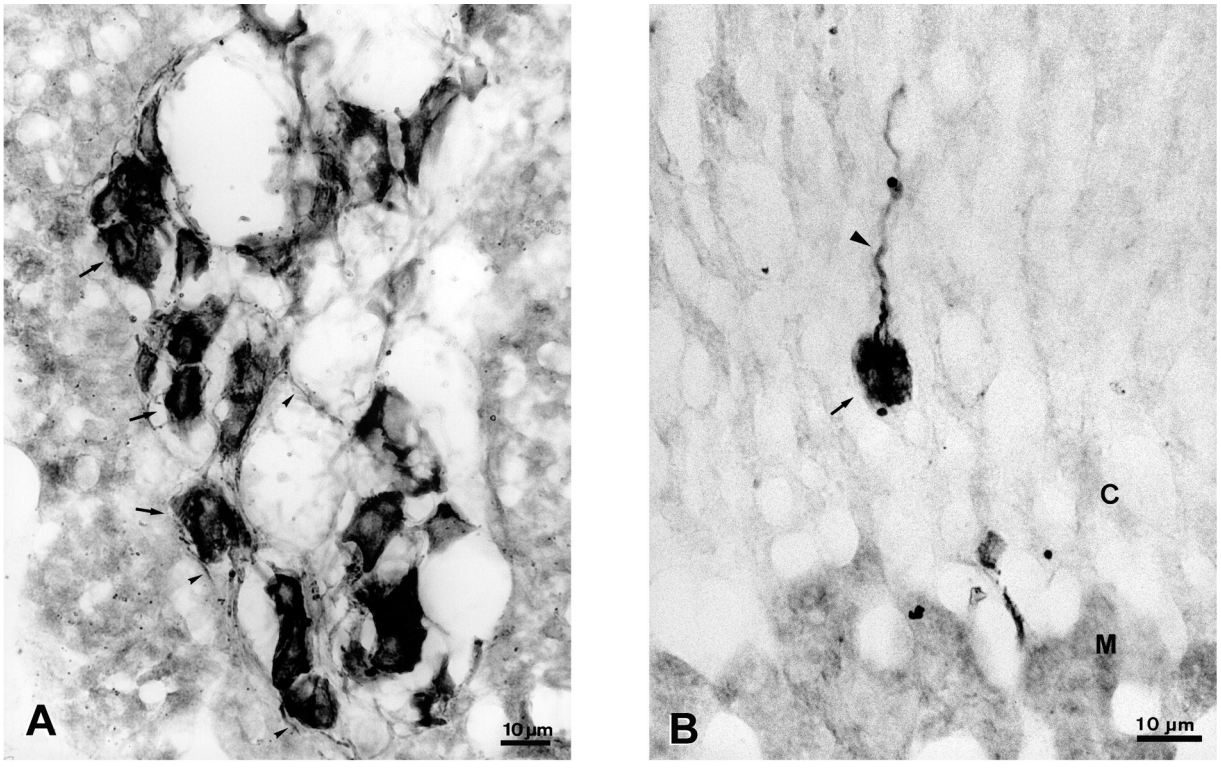

Fig. 2. Immunocytochemical localization of the metabotropic glutamate receptor 4a (mGluR4a) in the rat adrenal gland. Polygonal-shaped mGluR4a immunoreactive cells are numerous in the center of the adrenal medulla sometimes in close relation with blood vessels (arrows). MGluR4a immunoreactive nerve fibers (arrowheads) arising from these ganglion cells are visible around medulla blood vessels and between chromaffin cells **(A)**. Scale bar: 10 µm. Sometimes mGluR4a immunopositive cells (arrow) are located in the juxtamedullar cortex (*zona reticularis*). Note the long neuronal process (arrowhead) from one of the immunoreactive cells extending towards the periphery of the adrenal cortex **(B)**. C: Cortex; M: Medulla. Scale bar: 10 µm.

in isolated or 3-4 grouped type II ganglion cells in the periphery of the medulla (Fig. 4C). Labeling was also observed in single cells in the juxtamedullar cortex/*zona reticularis*. Nerve fibers arising from the immunopositive ganglion cells were found to extend among adrenocortical cells towards the periphery of the adrenal cortex (Fig. 2B).

The labeling obtained with the GluR2/3 antiserum revealed the presence of immunopositive ganglion neurons both in the center and the peripheral portions of the adrenal medulla. Type I cells localized in the central portion of the gland, sometimes in close relation with blood vessels, were strongly stained (Fig. 3A). Individual GluR2/3 immunopositive type II ganglion neurons were also found in the outermost portion of the medulla (Fig. 3B).

In double-labeling experiments with the antisera used above for the localization of glutamate receptors and a monoclonal antibody for bNOS, only the small peripheral type II ganglion cells that were immunoreactive for mGluR2/3, mGluR4a or GluR2/3 were also positively labeled for bNOS (Fig. 4). These small-sized bNOS immunoreactive cells were always found in the outermost portion of the medulla among chromaffin cells (Fig. 4B,D,F). Sometimes, nerve fibers emerging from them were observed running between chromaffin cells and penetrating into the adrenal cortex (Fig. 4A-D). The large ganglion type I mGluR2/3, mGluR4a and GluR2/3 immunopositive cells placed in the central portion of the medulla did not contain bNOS immunoreactivity in any case (Fig. 5).

Discussion

Our present findings provide immunocytochemical evidence for the localization of mGluR2/3, mGluR4a and GluR2/3 in ganglion cells of the rat adrenal medulla.

Previous immunocytochemical reports demonstrated that the rat adrenal medulla contains two different populations of ganglion neurons. Type I large-sized ganglion cells are preferentially located in the central portion of the gland, contain noradrenaline and neuropeptide Y (NPY), and are postganglionic sympathetic neurons (Dagerlind et al., 1990; Holgert et al., 1996, 1998). We have observed in this study that type I neurons are also equipped with the glutamate receptors mGluR2/3, mGluR4 and GluR2/3. Postganglionic sympathetic neurons of the superior cervical ganglion, with a similar phenotype to those of the type I adrenal medullary ganglion cells, have been

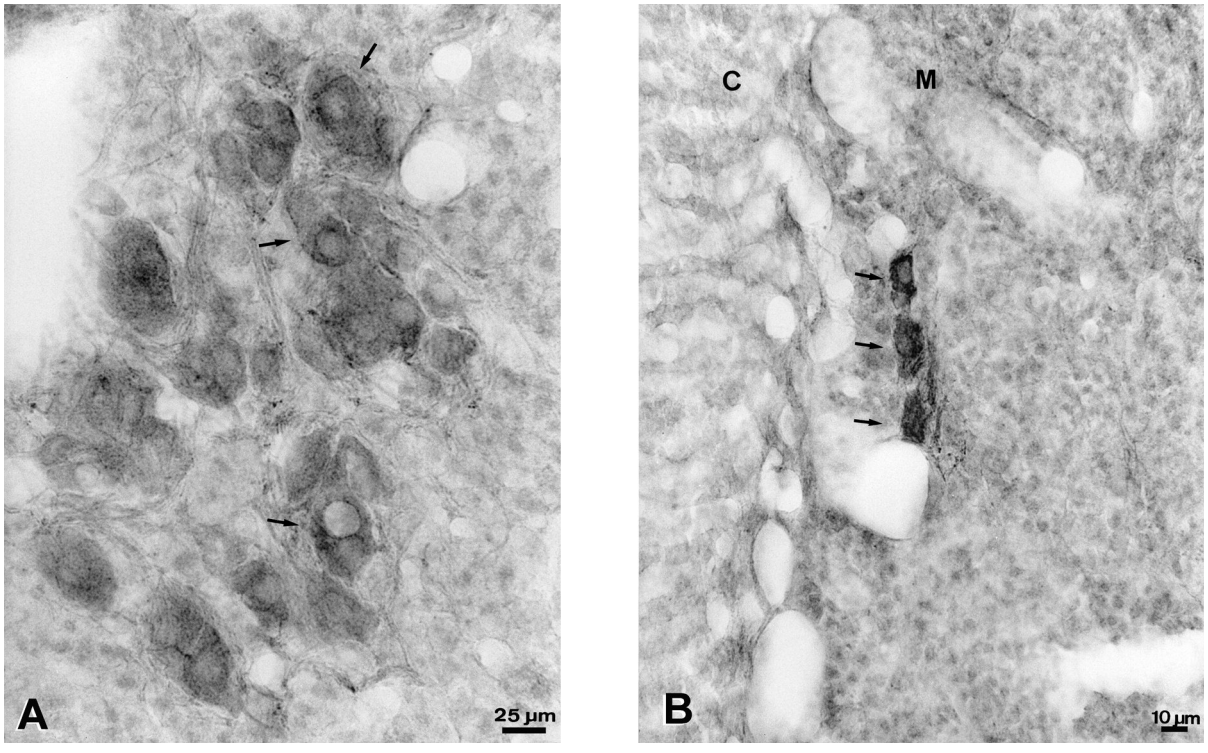

Fig. 3. Immunocytochemical localization of the AMPA glutamate receptor 2/3 (GluR2/3) in the rat adrenal gland. GluR2/3 immunoreactive ganglion cells in the center of the medulla are large in size and display a big and often centrally located nucleus (arrows) **(A)**. Scale bar: 25 µm. Intensely stained small immunoreactive cells are also localized in the peripheral portion of the medulla (arrows) **(B)**. C: Cortex; M: Medulla. Scale bar: 10 µm.

reported to be immunoreactive for the mGluR7 that, similarly to mGluR2/3 and mGluR4a, inhibit the cyclic AMP cascade (Ohishi et al., 1995; Li et al., 1996; Mateos et al., 2000). Furthermore, intense signals for GluR1, 2, 3 and 4 of the AMPA receptors have been observed by in situ hybridization in adrenal ganglion cells, as well as in other adrenal cells (Kristensen, 1993).

Type II ganglion cells are known to express AChE and NOS. Although these more peripherally-located neurons initially were considered cholinergic, the observation that only a single type II ganglion cell expressed choline acetyltransferase (ChAT) has led to consider that thay may also belong to the so-called nonadrenergic-noncholnergic neurons (NANC) immunorecative to NOS and VIP (Oomori et al., 1994; Holgert et al., 1996, 1998). These latter observations prompted us to make double labeling experiments with an antibody to bNOS in order to identify unequivocally the type II cell population and to know whether or not they express glutamate receptors. Indeed, bNOS

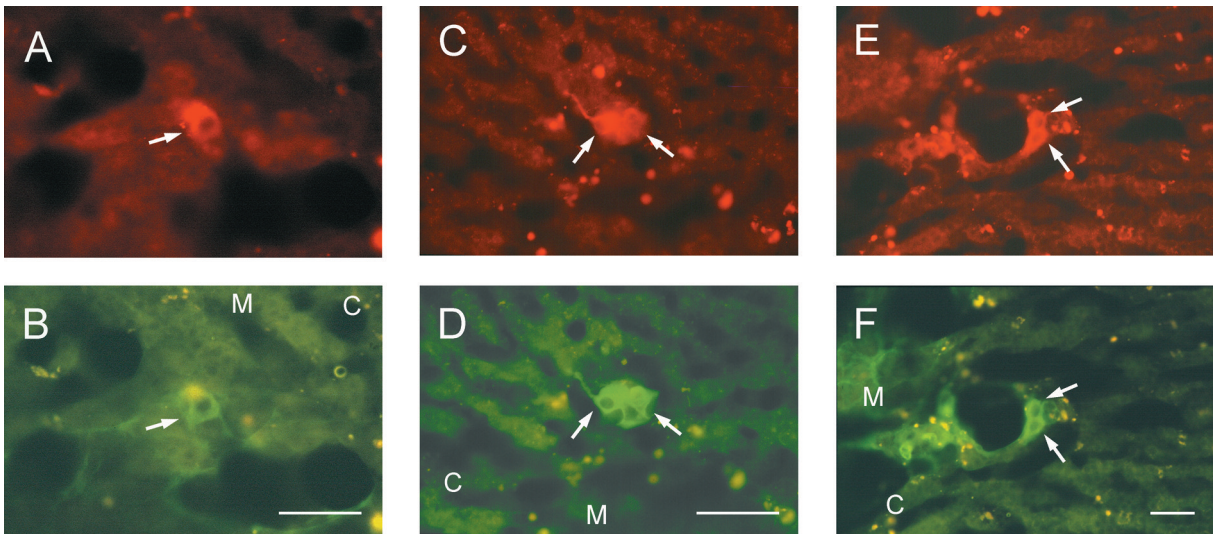

Fig. 4. Double immunofluorescence with mGluR2/3, mGluR4a, GluR2/3 and bNOS. Co-localizations of mGluR2/3 (arrow) **(A)** and bNOS **(B)**, mGluR4a (arrows) **(C)** and bNOS (**D)** and GluR2/3 (arrows) **(E)** and bNOS **(F)** are observed in small sized type II ganglion neurons located in the peripheral portion of the adrenal medulla. C: Cortex; M: Medulla Scale bar: 10 µm.

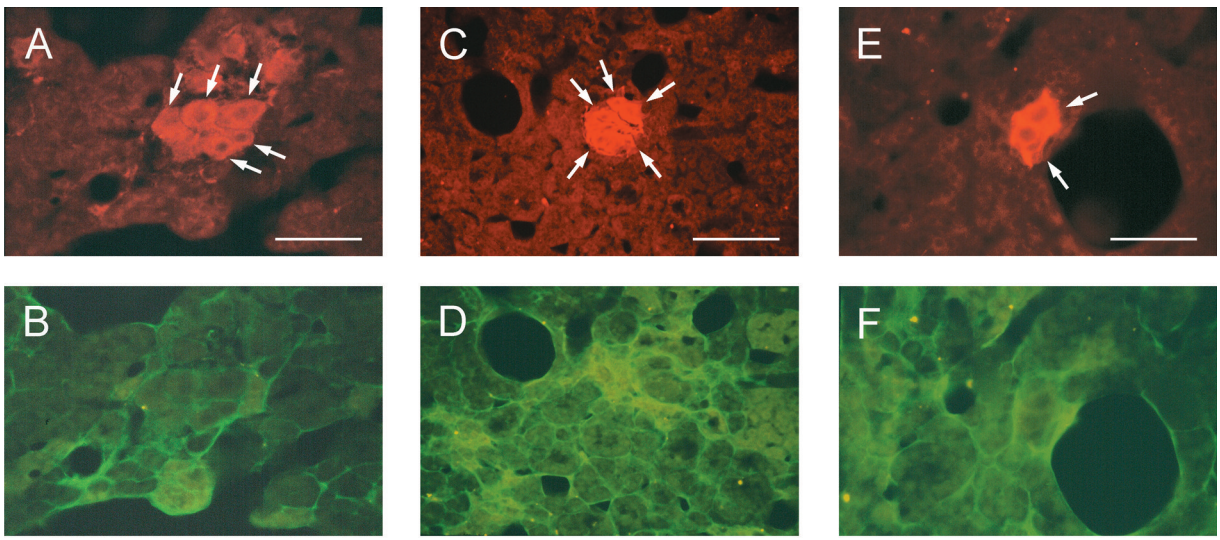

Fig. 5. Double immunofluorescence with mGluR2/3, mGluR4a, GluR2/3 and bNOS. Large sized and centrally disposed type I adrenal medullary ganglion neurons immunoreactive for mGluR2/3 (arrows) **(A)**, mGluR4a (arrows) **(C)** and GluR2/3 (arrows) **(E)** are bNOS immunonegative **(B, D, F)**. Scale bar: 10 µm.

expressing type II ganglion neurons also localize mGluR2/3, mGluR4a and GluR2/3. Interestingly, NANC neurons of the gastrointestinal tract containing NOS, VIP and NPY (Moffatt et al., 1998) also expressed mGluR2/3 immunoreactivity (Larzabal et al., 1999). The observation that only type II adrenal ganglion cells immunopositive for mGluR2/3, mGluR4a and GluR2/3, also express bNOS corroborates many immunohistochemical reports demonstrating that this enzyme is characteristic of type II ganglion neurons but it is not in type I ganglion cells (Afework and Burnstock, 1985; Afework et al., 1994; Cracco et al., 1996; Holgert et al., 1996, 1998).

The observed presence of bNOS immunoreactive fibers in the adrenal cortex may have functional implications in the steroidogenic process that takes place in the adrenal cortex, either by a direct participation in the control of adrenal cell functions or by an indirect participation regulating the blood flow to the gland (Afework and Burnstock, 1985; Afework et al., 1994).

Nerve fibers immunoreactive for glutamate provide most of the excitatory input to sympathetic preganglionic neurons that innervate adrenal medulla ganglion cells (Llewellyn-Smith et al., 1997). At present there is no available information about the presence of a glutamatergic innervation of the adrenal neuronal ganglion cells. However, the localization of the adrenal medulla outside the blood-brain barrier (Jezova et al., 1995a), the presence of glutamate in the blood of several species including man (Gonzalez et al., 1998; Tsai and Huang, 1999) or in chromaffin cells (Romero et al., 2003), suggest the possibility of an activation of the glutamate receptors distributed in adrenal medulla cells. Thus, mGluR2/3, mGluR4a and GluR2/3 in type I and II intraadrenal ganglion cells would modulate the activity of these cells, which in turn would affect the physiology of the chromaffin cells and of the cells in the cortical portion of the gland. Accordingly, a direct relation between the administration of high doses of glutamate and the increase of plasmatic levels of norepinephrine and cortisol has been detected (Jezova et al., 1995a). Elevated epinephrine levels in plasma have also been observed under stress conditions, a situation in which glutamate receptors have been implicated (Jezova et al., 1995b).

In conclusion, the expression of mGluR2/3, mGluR4a and GluR2/3 on both adrenal postganglionic type I and type II cells indicates that glutamate might be involved in the modulation of the activity of these neurons. Moreover, the colocalization of the studied glutamate receptors with bNOS in type II adrenal medullary ganglion neurons could provide the anatomical substrate for a possible interaction between the glutamatergic input and the release of NO, a gaseous messenger molecule that may participate in the control of adrenal cell functions and blood regulation. Furher studies will be necessary to elucidate the precise origin of glutamate as well as its effects on adrenal cell functions.

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