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Immunocytochemical localization of glutamate immunoreactivity in the guinea pig cochlea

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The localization of glutamate immunoreactivity was examined in the guinea pig cochlea using affinity purified polyclonal antibodies to glutamate and immunoperoxidase post-embedding staining techniques on one micron plastic sections. Glutamate immunoreactive staining was seen in both inner and outer hair cells and in spiral ganglion cells and auditory nerve fibers. These results support the hypothesis that glutamate may function as the hair cell transmitter or as a precursor to the transmitter and add further support for an excitatory amino acid as the transmitter of the auditory nerve.

Glutamate; Cochlea; Hair cell; Neurotransmitters; Immunocytochemistry

Introduction

Excitatory amino acids are now believed to be major neurotransmitters in the nervous system, with glutamate suggested as an important transmitter in many areas of the central nervous system areas including the cochlear nucleus (see Wenthold et al., 1986; Wenthold, 1989 for review). While many neurotransmitter candidates have been suggested as the transmitter of cochlear hair cells (Bledsoe et al., 1988; Bobbin et al., 1984; Guth et al., 1988; Klinke et al., 1986 for reviews), the substances with the most evidence supporting their candidacy are the excitatory amino acids. This evidence is predominantly pharmacological/biochemical and includes K⁺ evoked Ca⁺⁺ dependent release (Jenison et al., 1985), sound evoked release (Bledsoe et al., 1981; Drescher and Drescher, 1985), uptake mechanisms (Eybalin and Pujol, 1983; Gulley et al., 1979; Schwartz and

Ryan 1983; Ryan and Schwartz, 1984) post-synaptic action on the auditory nerve (Bobbin, 1979; Bobbin and Thompson, 1978; Comis and Leng, 1979; Klinke and Oertel, 1977) and activation by agonists (Bledsoe et al., 1981; Jenison and Bobbin, 1985; Jenison et al., 1986; Kusakari et al., 1984) and blockage by antagonists (Bobbin et al., 1981; Bobbin and Cesar, 1987; Littman et al. in press). The studies with agonists and antagonists suggest that the action of excitatory amino acids is on the quisqualate or kainic acid receptors. Levels of glutamate have been shown to be elevated in the organ of Corti (Godfrey et al., 1976). Kainic acid excitotoxicity is believed to be related to presence of kainic acid receptors and Bledsoe et al. (1981) showed irreversible depression of afferent activity. Pujol et al. (1985) showed kainic acid induced swelling of auditory nerve dendrites in guinea pig and Juiz et al. (1989) demonstrated kainic acid induced loss of spiral ganglion cells in rat. There is also evidence against glutamate as the cochlear hair cell transmitter although much of the negative evidence is only against the NMDA receptor being involved in hair cell transmission (Fex and Martin, 1980; Bobbin et al., 1981; Bobbin and Cesar,

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1987; Jenison et al., 1986). The concentration of glutamate necessary for excitation of single afferents has been questioned (Klinke, 1986) although a EC_{50} of 1.41 mM (Jenison et al., 1986) is not out of line considering fluid barriers and uptake systems. There is data both for (Bledsoe et al., 1981; Drescher and Drescher, 1985) and against (Drescher et al., 1983; Medina et al., 1981; Melamed et al., 1982) sound evoked release into perilymph and sound evoked release has not been shown to be Ca^{++} dependent. There is additional evidence both for (see Bledsoe et al., 1988 for review) and against (see Guth et al., 1988) glutamate as a hair cell transmitter based on studies in non-mammalian hair cells.

In studies examining immunoreactivity for glutaminase, an enzyme we have proposed as a marker for excitatory amino acid neurons (Altschuler et al., 1984), a light labelling of inner and outer hair cells was seen, but this was not considered strongly positive (Fex et al., 1984). Development of antibodies to aspartate and glutamate made possible the direct immunocytochemical localization of these amino acids, however when we applied such antibodies using our routine pre-embedding staining techniques (Fex and Altschuler, 1985) we did not see positivity in any cochlear cells or fibers (Fex and Altschuler, unpublished observations). It may be that the neurotransmitter pool of glutamate and aspartate is sequestered in such a way as to be less accessible to antibodies than other neurotransmitter candidates and post-embedding staining techniques, in which cells are sliced open during sectioning may provide better accessibility of antibody to antigen (Ottersen et al., 1987). We therefore used polyclonal affinity purified antiserum to glutamate with post-embedding staining techniques on one micron plastic sections through the guinea pig cochlea. With these techniques glutamate immunoreactivity was seen in inner and outer hair cells as well as spiral ganglion cells and auditory nerve fibers.

Materials and Methods

Twelve NIH strain guinea pigs were heavily anesthetized with chloral hydrate and perfused through the heart with 0.15 M sodium cacodylate buffer, pH 7.3 followed by fixative containing

either 4% paraformaldehyde and 0.1–0.2% glutaraldehyde or 2% paraformaldehyde and 1% glutaraldehyde in the same buffer. This was followed immediately by intrascalar perfusion of the same fixative. The cochlea was rinsed in buffer for 12–14 h and the bony shell removed while in the buffer rinse. The cochlea was then placed in 0.1% osmium in 0.15 M sodium cacodylate buffer for 1 h, followed by rinse in the same buffer. Segments of the cochlear spiral were removed or the whole cochlea was decalcified in 2% EDTA in phosphate buffered saline (PBS) for 1–2 days. Segments or the decalcified whole cochlea were then dehydrated through graded alcohols, infiltrated and embedded in EMbed 812 resin (Electron Microscopy Sciences).

One – two micron thick sections through the segments or whole cochlea were cut and sections mounted on glass slides. Sections were then etched for 11 min in 1:1 sodium ethoxide/ethanol, followed by bleaching consisting of two 5 min immersions in 1% periodic acid separated by a PBS wash. Three percent normal goat serum in PBS was then placed on sections for 1 hour, followed by incubation in affinity purified antibodies to glutamate. Antiserum against glutamate conjugated to bovine serum albumin (BSA) was made as described previously for making antibodies to GABA (Wenthold et al., 1986). Antibodies were purified by applying antiserum to a column of glutamate conjugated to ovalbumin with glutaraldehyde and attached to cyanogen bromide-activated Sepharose. Bound antibodies were eluted with 0.1 M acetic acid and immediately neutralized. This fraction was passed through a column of aspartate conjugated to cyanogen bromide activated Sepharose. The unretained fraction was used for immunocytochemical localizations. Immunoblot analysis of the original serum and the final fraction is shown in Fig. 1. The affinity purified antiserum was applied at a dilution of 1:1000–1:2000 in PBS at 4°C for 16–40 h in a moist environment. Our routine immunoperoxidase procedures (Altschuler et al., 1984, 1986) were then followed using a modification of the avidin biotin peroxidase complex (ABC) method of Hsu (1981) and reagents from Vector Laboratories. The peroxidase complex was visualized using DAB as a chromogen. Sections were then examined

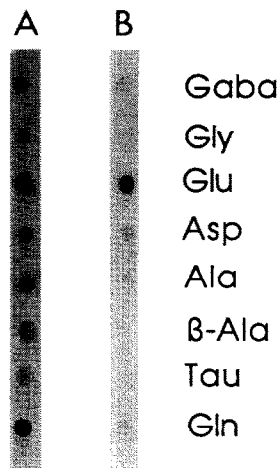


Fig. 1. Immunoblot analysis of glutamate antibody preparations. Ovalbumin conjugates of amino acids (listed on right) were applied to nitrocellulose paper and reacted with the original (A) or affinity purified (B) glutamate antibody preparations at dilutions of 1:1000. Bound antibody was determined using HRP conjugated goat anti-rabbit immunoglobulins with 4-chloro-1-naphthol as a chromogen.

using bright-field and differential interference contrast optics through a Leitz Dialux photomicroscope.

Antibodies to GABA and to aspartate were applied on semi-adjacent sections to compare and contrast immunostaining.

Results

In cochleae fixed with 4% paraformaldehyde and 0.1–0.2% glutaraldehyde, glutamate immunoreactive staining was seen in both inner and outer hair cells (Figs. 2–4). Staining in inner hair cells was often more intense than that in outer hair cells. In most animals the immunostaining was uniform throughout the hair cells, however in several the staining at the base was more intense. Glutamate immunoreactive staining was also present in spiral ganglion cells and auditory nerve fibers (Fig. 5). In some animals staining was also seen in the inner and tunnel spiral bundles suggesting staining of lateral efferents (Figs. 2 and 3). Immunostaining of interdental cells and scattered supporting cells was also occasionally seen. Glutamate immunoreactive labeling was not observed in stria vascularis.

With the higher glutaraldehyde fixative no specific staining in the cochlea was seen with antibodies to glutamate, all cells showed equivalent light label.

Antibodies to GABA showed no immunostaining of hair cells or spiral ganglion cells. Antibodies to aspartate showed immunoreactive staining that had a similar pattern to that of glutamate but was weaker so that it was less clearly above background staining.

Discussion

The results of the present study show selective immunoreactive staining for glutamate in inner and outer hair cells as well as spiral ganglion cells and auditory nerve fibers suggesting elevated levels of glutamate in these elements. One explanation for the increased levels is that it reflects a neurotransmitter pool and that glutamate is the neurotransmitter of cochlear hair cells and the auditory nerve. However, increased levels of glutamate could, alternatively, reflect its role as a precursor to the real transmitter or a role in general metabolism. Our results cannot therefore by themselves establish glutamate as the hair cell or auditory nerve transmitter. When taken as part of a larger body of work, however, our results add another crucial piece of evidence to the growing list supporting an excitatory amino acid or related peptide as the transmitter of the auditory nerve (see Wenthold 1986; Wenthold, in press; Caspary et al., 1986 for reviews) and as the transmitter of cochlear hair cells (see Bledsoe et al., 1988; Bobbin et al 1984 for reviews).

GABA and GAD immunoreactivities have recently been reported in chick hair cells (Usami et al., 1987a,b). Neither GABA nor GAD immunoreactivities were found in mammalian organ of Corti hair cells using pre-embedding techniques (Eybalin, 1988; Fex and Altschuler 1984; Fex et al., 1986; Thompson et al., 1986, Usami et al., 1988), GABA levels in guinea pig cochlea are low (Godfrey, 1976) and we did not see GABA immunoreactive labeling of guinea pig cochlear hair cells with post-embedding staining. This along with previous physiological studies (Klinke 1986; Bledsoe et al., 1988, for reviews) suggests that GABA is not a cochlear hair cell neurotransmitter.

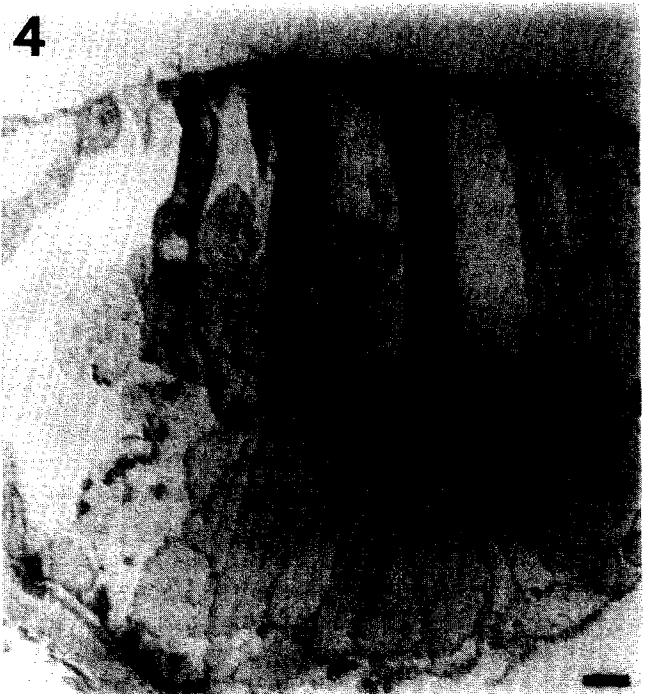
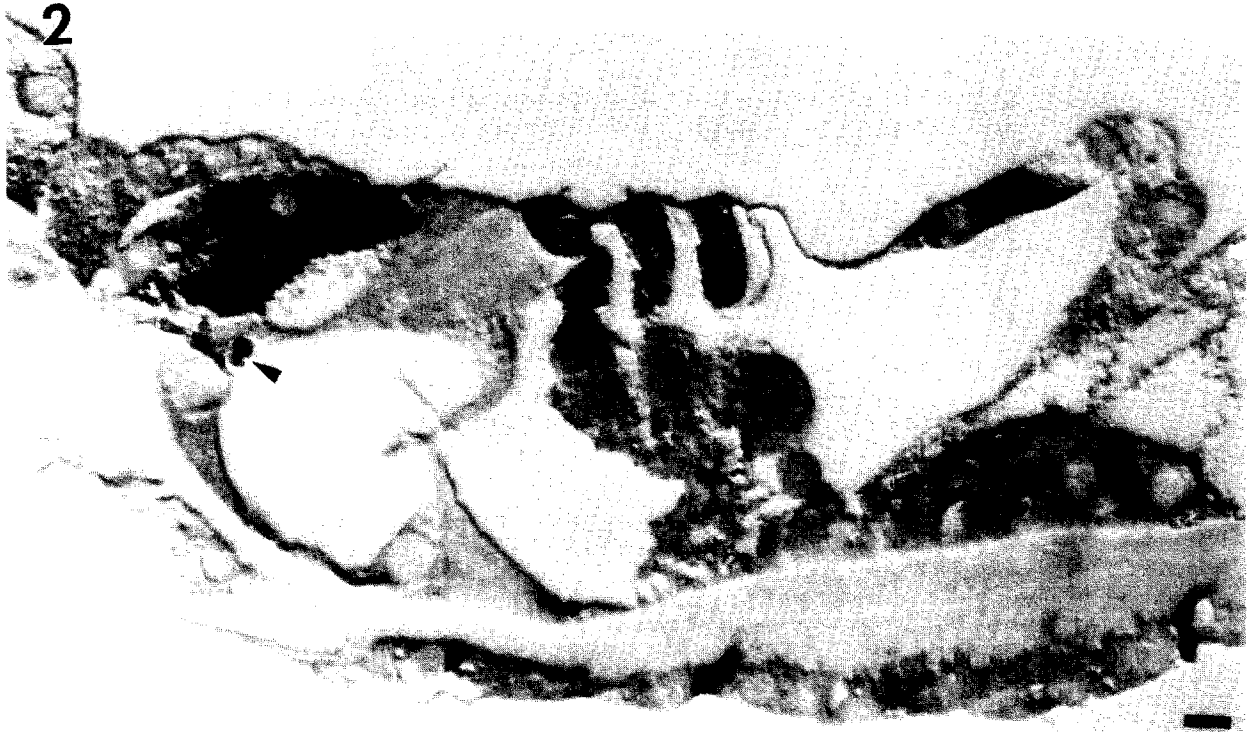


Fig. 2. Glutamate-like immunoreactive staining of inner and outer hair cells is seen in a one micron section through the first turn of the organ of Corti. Immunoreactive staining is also seen in the tunnel spiral bundle (arrow). Scale bar = 10 μ m.

Fig. 3. Glutamate immunoreactive staining of an inner hair cell, in the region below the inner hair cell and in the tunnel spiral bundle (arrow) is seen in a one micron plastic section through the first turn of the organ of Corti. Scale bar 10 μ m.

Fig. 4. Glutamate immunoreactive staining of outer hair cells is seen in a one micron section through the second turn of the organ of Corti. Scale bar 10 μ m.

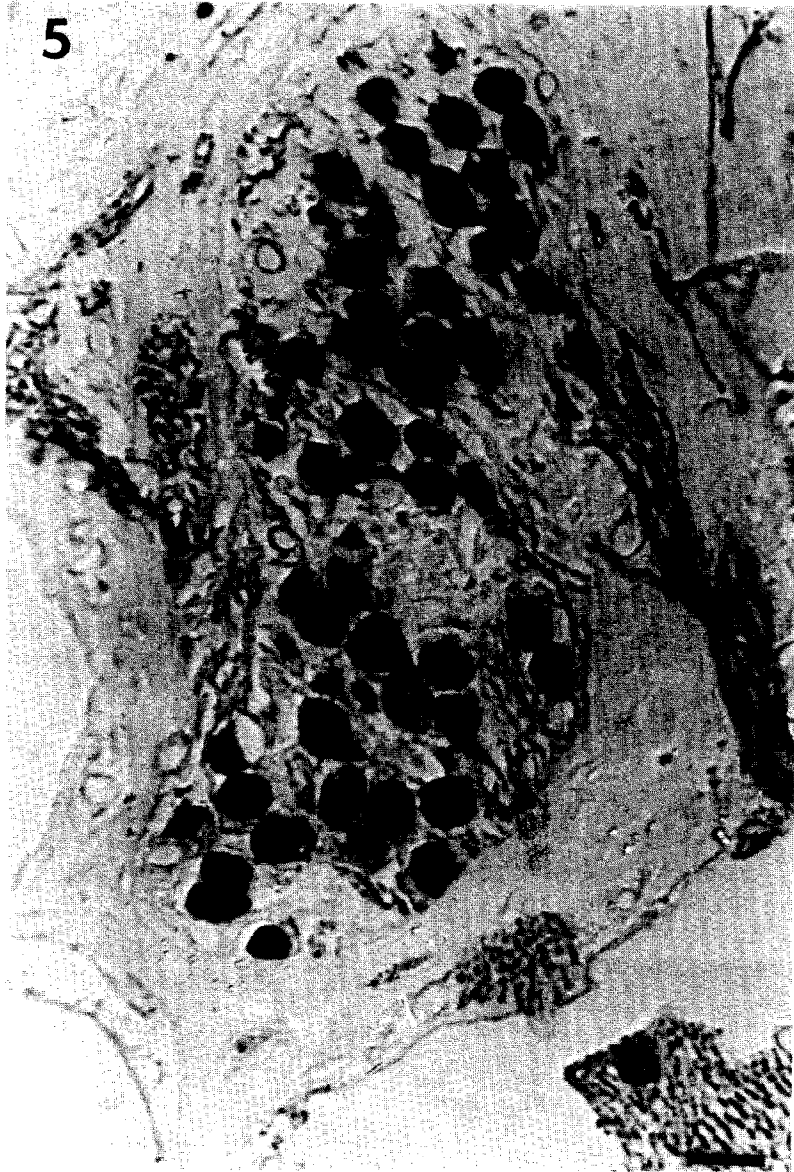


Fig. 5. Glutamate immunoreactive staining of spiral ganglion cells is seen in a one micron para-modiolar plastic section through Rosenthal's canal near the second turn of the organ of Corti. Scale bar 20 μm .

Glutamate immunoreactive staining was often seen in lateral efferent fibers and puncta. This is consistent with uptake studies (Gulley et al., 1979; Eybalin and Pujol 1983; Schwartz and Ryan 1983; Ryan and Schwartz, 1984) which showed uptake of glutamate into lateral efferents. Uptake of glutamate into hair cells was not seen in these studies, however uptake of glutamine was seen in

inner and outer hair cells (Eybalin and Pujol 1983; Ryan and Schwartz, 1984). This suggests that at the inner hair cell the sequence of events following putative release of glutamate may be similar to events in the central nervous system, but with efferents substituting for glial elements. In the central nervous system it is believed that glial elements clear glutamate by uptake from the syn-

aptic area before it reaches toxic levels (Hertz, 1979; Fonnum et al., 1980 for reviews). Glutamate is then converted into glutamine, released from the glia and taken up by the neuronal terminal(s). Glutamine within these terminals is then reconverted into glutamate (by an enzyme such as glutaminase) and rejoins the neurotransmitter pool. At the hair cell – auditory nerve synapses there are no corresponding glial elements, however, it is just as important that glutamate be rapidly cleared before reaching toxic levels. Glutamate might therefore be cleared and taken up by efferents, converted to glutamine, released, taken up by hair cells and reconverted to glutamate. While a glutamate-glutamine-glutamate cycle in the cochlea has been proposed by Eybalin et al. (1983) based on their uptake studies of glutamate and glutamine and is supported by presence of light glutaminase immunoreactivity in hair cells and glutamate immunoreactive staining in hair cells and lateral efferents, additional evidence is necessary to address whether efferents may have an additional ‘vacuum cleaner-like’ function.

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