Brain Research, 555 (1991) 75–83

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ADONIS 0006899391168268

BRES 16826

Kainate and NMDA toxicity for cultured developing and adult rat spiral ganglion neurons: further evidence for a glutamatergic excitatory neurotransmission at the inner hair cell synapse

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(Accepted 26 February 1991)

Key words: Afferent auditory neuron; Excitotoxin; Glutamate; Cochlea

In the inner ear, the excitatory amino acid glutamate is a proposed neurotransmitter acting at the synapse between hair cells and afferent auditory neurons. Using cultures of 5-day-old rat auditory neurons, we show that the afferent auditory neuronal population can be divided, on the basis of its sensitivity to the neuronotoxic effect of glutamate and its analogs, in at least 3 subpopulations, one responding to *N*-methyl-D-aspartate (NMDA), one responding to kainate and a third minor one unresponsive to NMDA, kainic acid and glutamate. No toxic effect of quisqualate is observed. The use of specific antagonists (kynurenate and 2-amino-5-phosphonovalerate (DAP-5) demonstrates the specificity of the receptors to the excitatory amino acids on the afferent auditory neurons. Afferent auditory neurons from adult rats can also be cultured and in these preparations only the large neurons are sensitive to glutamate, kainate and NMDA while the small neurons are not responsive, suggesting that a glutamatergic neurotransmission occurs only at this synapse between the inner hair cells and the large radial afferent auditory neurons. We also show that, in vitro, the organ of Corti releases, in response to an increased potassium concentration and in the presence of calcium, a toxic activity for the afferent auditory neurons that is antagonized by kynurenate and DAP-5. Pathophysiological implications are discussed.

INTRODUCTION

In the auditory system, afferent neurons of the acoustic ganglion transfer information from hair cells of Corti's organ to the cochlear nuclei of the central nervous system. The neuronal population of the acoustic (spiral) ganglion can be divided in two subpopulations: the large type I neurons which represent approximately 95% of the afferent auditory neurons and the small type II neurons which include the remaining 5% of the neuronal population[29,39]. Type I auditory neurons innervate primarily inner hair cells (IHC) while type II neurons establish contacts with the outer hair cells (OHC)[23,35]. The nature of the neurotransmitter(s) released at the synapses between cochlear sensory hair cells and the dendrites of the first auditory neurons remains unknown. Circumstantial evidence, however, suggests that glutamate and/or aspartate might be the involved neurotransmitter. Indeed, (i) a higher endogenous concentration of these amino acids has been measured in the hair cell region as compared to other parts of the cochlea[18]; (ii) glutamate-like immunoreactivity is localized in the hair cells, mostly in the IHC[1,15], only a weak labeling being however observed in the OHC; (iii) the glutamate concentration in perilymph increases under conditions that mimic a synaptic release, i.e. in a potassium-stimulated and calcium-dependent manner[9,20]; (iv) Eybalin and Pujol (1985)[14] have indicated that only the inner hair cells (IHCs) are involved in the glutamate-glutamine cycle, which is proposed for glutamate metabolism and compartmentation in the central nervous system; (v) in electrophysiological experiments, application of glutamate increases the frequency of firing of the VIII cranial nerve fibers[5–8,24]; (vi) kainic acid (KA), a glutamate analog, or the activation of the NMDA subtype of glutamate receptors selectively alters the auditory dendrites connected with cochlear inner hair cells[33,45] and affect the compound action potential, which reflects the synchronous activity of the primary auditory fibers[4,30].

Glutamatergic transmission is generally associated with the well established phenomenon of excitotoxicity[41]

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in which excessive stimulation of glutamate receptors can lead to neuronal lesion and death. This process is thought to involve glutamate receptor mediated massive influxes of Na^+ ions and most importantly Ca^{2+} ions leading to calcium-related cell damage. These neuronal lesions are induced by the excitotoxins, a group of related compounds able to interact with glutamate receptors to produce specific dendrosomatic, axon-sparing lesions in the central nervous system[13]. Glutamate receptors are classified in three subtypes characterized by their preferred sensitivity to *N*-methyl-D-aspartate (NMDA), kainate and quisqualate[16]. Excitotoxicity seems to be mainly dependent on NMDA and kainate receptor activation.

The possible occurrence of glutamatergic transmission between cochlear hair cells and afferent neurons raises the possibility for the involvement of an excitotoxic mechanism in some pathologies affecting the sensory afferent neurons of the cochlea. We therefore undertook the present study to evaluate the possibility that glutamate and various excitotoxins exert a cytopathic effect on cultured afferent auditory neurons obtained from 5-day-old and adult rats and to investigate a possible release of endogenous excitotoxic activity within the organ of Corti.

MATERIALS AND METHODS

Culture of 5-day-old rat afferent auditory neurons

Five-day-old Wistar rats were killed by cervical transection. The mandible was sectioned and removed. The bullae were exposed, the temporal bones were removed and transferred into a glass Petri dish (60 mm diameter) containing warmed phosphate-buffered saline (PBS). Under the dissecting stereomicroscope, using watchmaker forceps, the cochlea was dissected. After having discarded the bony cochlea, the cochlear duct was removed and the spiral ganglion was dissected. The ganglia were then incubated for 25 min at 37 °C in calcium-magnesium-free saline solution containing 0.1% trypsin, 0.1% collagenase and 0.01% DNAse. Enzymatic dissociation was terminated by adding 10% fetal calf serum and the ganglia were carefully washed through 5 sedimentation-resuspension cycles in Dulbecco's modified Eagle's medium supplemented with glucose (final concentration 6 g/l) and the N_1 cocktail[11] (DMEM-g-N_1). Dissociation was then achieved by up and down aspirations using a flamed Pasteur pipette. The resulting cell suspension was inoculated in multiwells (17 mm, Nunc, Roskilde, Denmark, 001-43982A) previously coated sequentially with polyornithine (0.1 mg/ml in borate buffer, for 1 h at RT) and laminin (10 μg/ml in PBS, overnight at 37 °C). Seeding density was 800,000 cells per well (of which 5000 cells were neurons) in 250 μl DMEM-g-N_1, a density that allows the survival and regeneration of 5% of the seeded neurons (i.e. 250 neurons per well) in the absence of added exogenous trophic support. Medium was renewed on day 3.

Culture of adult rat afferent auditory neurons

Adult Wistar rats, 200–250 g, were anesthetized with Nembutal (0.1 mg/g) and decapitated. The head was skinned, the calvarium opened and the brain removed. Both temporal bones were dissected and transferred to a glass Petri dish (60 mm diameter) containing PBS. The cochlea was removed from the temporal bones. The bony modiolus which encases the neurons of the acoustic ganglion was extracted and cleaned as much as possible from the surrounding tissue. The ganglia were then incubated for 20 min in a saline solution containing 5% collagenase. A 0.25% trypsin solution was then added to the collagenase solution to achieve a final concentration of 0.1% trypsin and enzymatic dissociation was carried on for an additional 17 min at 37 °C and terminated by adding 10% FCS. The cellular dissociation is completed mechanically as described for the 5-day-old rat neurons. The cellular suspension resulting from the dissociation of 2 acoustic ganglia is seeded in DMEM-g-N_1 (100 μl/well) in 3 wells of a microwell plate (NUNC 1-69620) previously coated sequentially with polyornithine (0.1 mg/ml in borate buffer for 1 h at room temperature), laminin (10 μg/ml in PBS overnight at 37 °C) and finally, astrocyte-conditioned medium for 24 h at 37 °C. Cultures are grown for 3 days before use. Under these conditions, about 350 neurons are seeded in each well of which about 140 survive after 4 days in vitro.

Medium conditioned by organs of Corti

The cochlear ducts from 5-day-old rats were dissected as described in the previous sections and the organs of Corti were isolated with sharp forceps. They were then incubated (10 organs of Corti per ml of medium) first for 24 h in the presence of DMEM-glucose supplemented with 10% fetal calf serum and subsequently for another 24 h in normal (5.4 mM) or high (110 mM) potassium-containing DMEM-glucose supplemented with the N_1 cocktail. Conditioned medium was collected, ultrafiltrated on a Amicon PM10 membrane (cut off: 10,000 Da) and sterilized by filtration. The organs of Corti were also incubated for 24 h in high potassium (110 mM)-containing DMEM-g-N_1 with (1.8 mM) or without calcium. This conditioned medium was collected, ultrafiltrated as described above and sterilized. The concentration of calcium in the calcium-free conditioned medium was normalized to 1.8 mM. The ultrafiltrates were stored at –20 °C until use.

Medium conditioned by astrocytes

Cultures of newborn rat cerebral cortex astroglial cells were prepared according to Booher and Sensenbrenner[10] and cultured in DMEM-10% FCS. When subconfluent (after 8–10 days in vitro), the cultures which contain more than 95% glial fibrillary acidic protein (GFA-P)-positive cells were washed and further cultured for 24 h in DMEM without serum. This conditioned medium was collected and stored at –20 °C until use.

Bioassay of cell survival

After 5 days (for the 5-day-old rat neurons) or 3 days (for the adult rat neurons) in dissociated culture, the medium was discarded and replaced by the experimental medium for 24 h. At that time, the cultures were fixed with an acetone-methanol solution (50/50 v/v) at –20 °C for 15 min and processed for neurofilament immunolabelling. The number of surviving neurons identified by neurofilament immunostaining as described below, was then counted using an inverted microscope. Comparison of phase-contrast and bright-field illumination showed that all surviving neurite-bearing neurons were labelled after neurofilament immunostaining. The whole culture surface was examined. Three culture dishes were used for each experimental condition. Results are expressed in % of controls ± S.D.

Immunohistochemistry

Neurofilaments (NF) were detected by the immunoperoxidase method. Washed cultures were fixed as described in the previous section. They were then dehydrated in alcoholic solutions. Endogenous peroxidases were inhibited using a solution of 2% H_2O_2 in methanol for 20 min at 37 °C. The preparations were rehydrated and incubated in fetal calf serum for 30 min at 37 °C to prevent non-specific binding of antibodies. The cultures were subsequently incubated overnight at 20 °C in anti-neurofilament antibodies (mouse monoclonal antibody RT97, dilution 1:1,000 in PBS supplemented with 10% fetal calf serum). The cultures were then rinsed 3 times in PBS and incubated in a peroxidase-conjugated antimouse Ig antibody solution (dilution 1:20 in PBS supplemented with 10% fetal calf serum) for 30 min at 37 °C. The resulting

peroxidase was then revealed with diaminobenzidine-tetrahydrochloride (0.05%) and H_2O_2 (0.005%) in PBS for 5–10 min.

Culture media and reagents

DMEM and FCS were obtained from Gibco (Gent, Belgium). In all experiments, DMEM was supplemented with the N_1 cocktail (bovine insulin 5 μg/ml, progesterone 10^{-8} M, putrescine 100 μM, transferrin 100 μg/ml and selenium $3 \cdot 10^{-8}$ M)[11] and the glucose concentration was increased to 6 g/l (DMEM-g-N_1). In high potassium-containing DMEM, potassium concentration was increased to 110 mM and osmolarity was maintained by equimolar decrease of sodium concentration. Laminin was purchased from Collaborative Research (Bedford, MA, U.S.A., 2002963). Trypsin was from Gibco, collagenase from Boehringer Mannheim (Brussels, Belgium) and DNAse from Sigma (St. Louis, MO, U.S.A., D5025). Antineurofilament antibodies were a gift from M. Manthorpe (University of California at San Diego, La Jolla, U.S.A.). Other antibodies used for immunostaining were obtained from Dakopatts (Glostrup, Denmark, P-260).

Statistical analysis

Dose–response curves were fitted by logit regression analysis and statistical significance of slopes was assessed by a unilateral *t*-test at the 5% critical level. The statistical significance of the data presented in Figs. 2, 5B, 6, 8A and 8B was assessed by a *t*-test at the 5% critical level comparing control and experimental data in

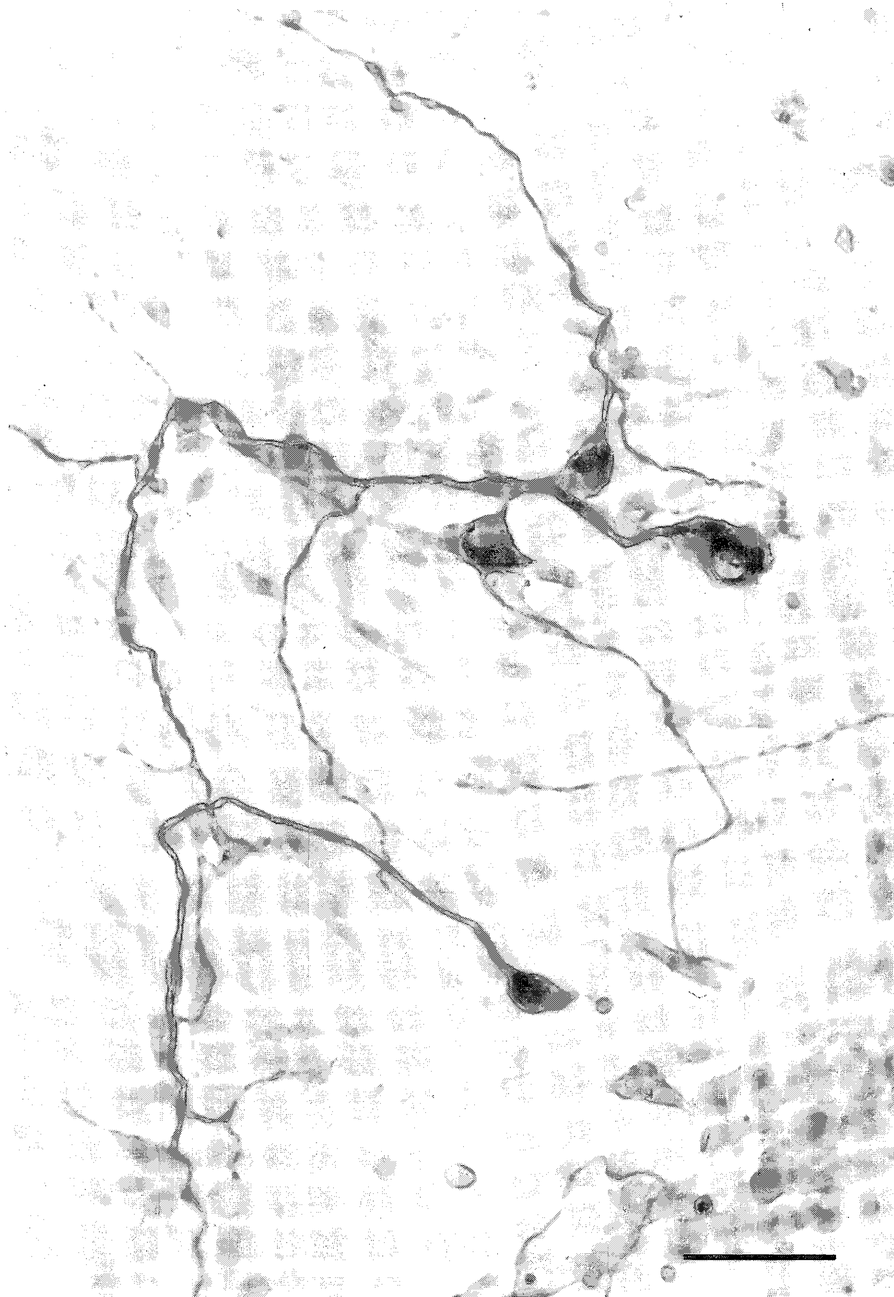

Fig. 1. Five-day-old rat afferent auditory neurons cultured for 5 days in serum-free medium. Immunohistochemical demonstration of neurofilaments. Bar = 70 μm.

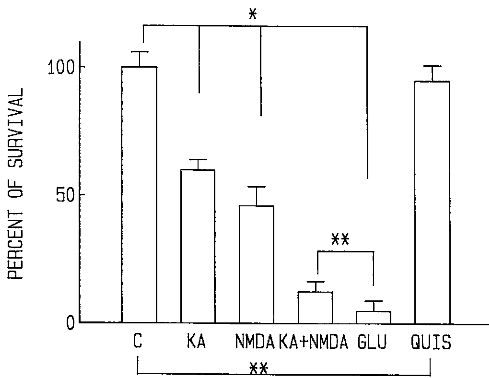

Fig. 2. Five-day-old rat afferent auditory neurons' survival after 24 h in the presence of 10^{-3} M glutamate (GLU), 10^{-4} M KA, 10^{-4} M NMDA, 10^{-4} M quisqualate (QUIS), both 10^{-4} M kainate and 10^{-4} M NMDA (KA + NMDA), and in control condition (C). Survival is measured by direct counting of all the neurons in 3 culture dishes per experimental condition after immunolabelling of the neurofilaments. The results are expressed as % of the number of neurons surviving in control conditions. *, Statistical significance as compared to control at the $P < 0.05$ level; **, Non-significance at the $P < 0.05$ level.

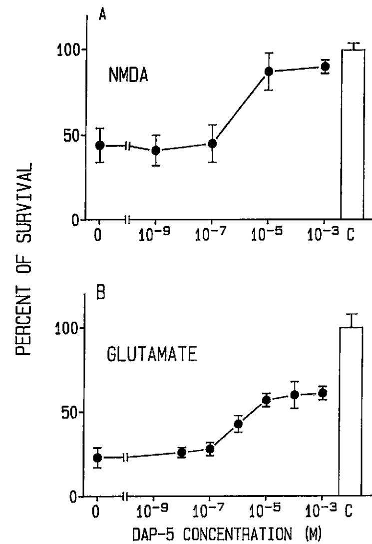

Fig. 4. Effect of DAP-5 on the toxicity induced by 10^{-4} M NMDA (A) or 10^{-3} glutamate (B) on 5-day-old rat afferent auditory neurons after 24 h. C indicates the survival in control condition. Survival is measured and expressed as in Fig. 2. Significance after logit regression analysis at the 5% critical level for A and B.

most instances, or sometimes, as indicated comparing two sets of experimental data.

RESULTS

When 5-day-old rat auditory neurons are cultured for 5 days, 5% (i.e. 250 neurons/well) of the neurons present in vivo in the spiral ganglion survive and generate long neurites (Fig. 1). The cultures were subsequently incubated in the presence of 1 mM glutamate for 24 h in DMEM-g-N_1. As shown in Fig. 2, a toxicity of glutamate for these auditory neurons is obvious: less than 10% of the neuronal population survive after 24 h in the presence

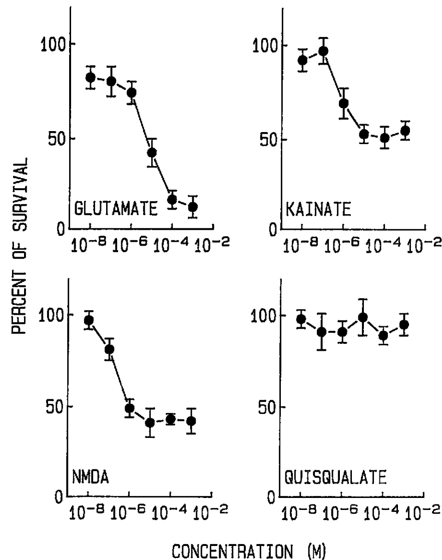

Fig. 3. Five-day-old rat afferent auditory neurons' survival after 24 h as a function of the concentration of glutamate, kainate, NMDA or quisqualate in the culture medium. Survival is measured and expressed as in Fig. 2. Significance after logit regression analysis at the 5% critical level for glutamate, kainate and NMDA. Non-significance for quisqualate.

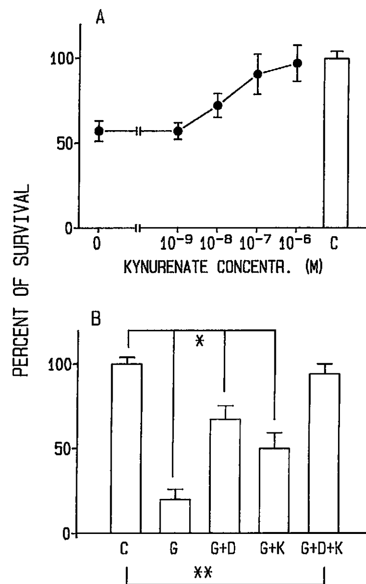

Fig. 5. A: effect of kynurenate on the toxicity induced by kainate (10^{-4} M) on 5-day-old rat afferent auditory neurons after 24 h. C indicates the survival in control condition. Survival is measured and expressed as in Fig. 2. Significance after logit regression analysis at the 5% critical level. B: effect of 10^{-6} M kynurenate (K), 10^{-5} M DAP-5 (D) and both 10^{-6} M kynurenate and 10^{-5} M DAP-5 (D + K) on the toxicity induced by 10^{-4} M glutamate (G). C indicates the survival in control condition. Survival is measured and expressed as in Fig. 2. *, Statistical significance as compared to control at the $P < 0.05$ level; **, Non-significance at the $P < 0.05$ level.

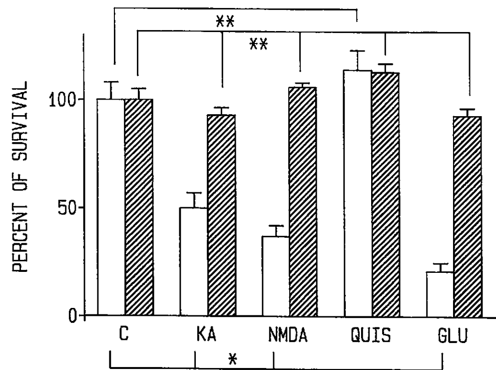

Fig. 6. Adult rat afferent auditory neurons' survival after 24 h in the presence of 10^{-3} M glutamate (GLU), 10^{-4} M kainate (KA), 10^{-4} M *N*-methyl-D-aspartate (NMDA), 10^{-4} M quisqualate (QUIS) and in control condition (C). Survival is measured as in Fig. 2. The results are expressed as % of the number of neurons of both classes (>12 μm, empty bars, and <12 μm, dashed bars) surviving in control conditions. Notice, however, that in control condition small neurons represent only 15% of the total neuronal population (see text). *, Statistical significance as compared to control at the $P < 0.05$ level; **, Non-significance at the $P < 0.05$ level.

of glutamate (100% being the number of neurons surviving in control conditions). Other excitotoxins were also studied: when incubated in the presence of NMDA (10^{-4} M) or kainate (10^{-4} M), respectively 54 ± 7% and

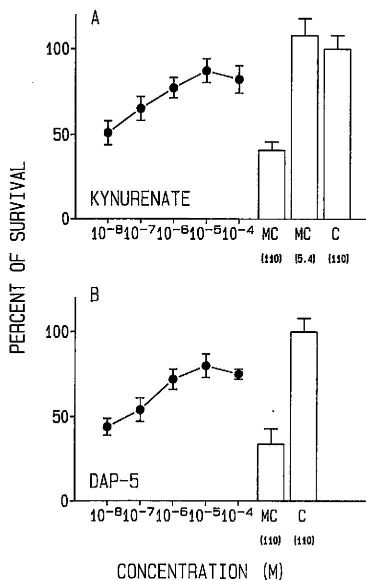

Fig. 7. The neurotoxic effect on 5-day-old rat afferent auditory neurons, of high potassium containing medium conditioned by organs of Corti (see Materials and Methods) has been measured in the absence or the presence of various concentrations of kynurenate or DAP-5. Survival is measured and expressed as in Fig. 2. A: MC (110) and MC (5.4) indicate respectively the survival observed in a medium conditioned by organs of Corti at 110 mM and 5.4 mM potassium. C (110) indicates the survival measured in unconditioned medium containing 110 mM potassium. B: MC and C indicate respectively the survival measured for conditioned and unconditioned medium containing 110 mM potassium. Significance after logit regression analysis at the 5% critical level for A and B.

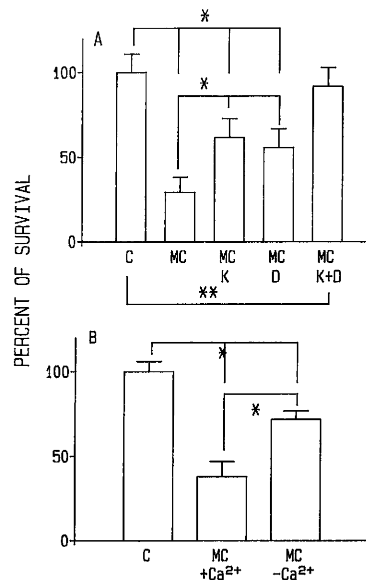

Fig. 8. A: effect of 10^{-4} M kynurenate (K), 10^{-4} M DAP-5 (D) and both 10^{-4} M kynurenate and 10^{-4} M DAP-5 (K+D) on the toxicity induced by high potassium (110 mM) containing medium conditioned by organ of Corti (MC) (see Materials and Methods). Survival is measured and expressed as in Fig. 2. B: effect of extracellular calcium on high potassium induced release of the toxic activity. $+Ca^{2+}$ and $-Ca^{2+}$ indicate respectively the medium that has been conditioned in the presence or in the absence of calcium. Notice that during the bioassay of neurotoxic activity, the calcium concentration was the same in all conditions. Survival is measured and expressed as in Fig. 2. For both A and B, * represents statistical significance as compared to control at the $P < 0.05$ level, ** non-significance at the $P < 0.05$ level.

40 ± 4% of the neurons die. Quisqualate has no significant effect on the survival of these cultured neurons (Figs. 2 and 3). Dose–response curves show that the maximum toxic effect of glutamate, NMDA or kainate is obtained at concentrations of about 10^{-4} M for glutamate and 10^{-5} M for NMDA and KA with the half-maximum toxic concentration respectively around 10^{-5} (for glutamate) and 10^{-6} M (for NMDA and KA) (Fig. 3). When the auditory neurons are incubated in the presence of both kainate and NMDA, an additive toxic effect (survival: 15%) is observed, reaching the level of toxicity produced by glutamate alone (Fig. 2). Thus, considering excitotoxin sensitivity, these experiments suggest that the population of afferent auditory neurons obtained from 5-day-old rats and cultured for 5 days can be divided in three subpopulations: one sensitive to NMDA (± 55%), one sensitive to kainate (± 40%) and a third minor, repeatedly observed, component (± 10%) which is insensitive to excitotoxins. A slight overlap between the NMDA- and KA-sensitive populations is observed. The use of specific antagonists of the NMDA and KA receptors confirms such a view. Indeed, the toxicity induced by NMDA and part of the toxicity induced by glutamate are antagonized by 2-amino-5-phosphono-

valerate (DAP-5; concentration 10^{-5} M), a specific NMDA receptor competitive antagonist (Fig. 4). The proportion of neurons surviving in the presence of glutamate (10^{-3} M) + DAP-5 (10^{-5} M) corresponds to the proportion of neurons surviving in the presence of kainate, suggesting that the residual glutamate toxicity is mediated by a kainate receptor. The toxicity induced by kainate is suppressed by kynurenate which acts as an antagonist of the KA receptor (Fig. 5A). Moreover, the toxicity induced by glutamate (10^{-4} M) is completely abolished by the combination of both DAP-5 (10^{-5} M) and kynurenate (10^{-6} M) (Fig. 5B).

NMDA receptors have been suggested to play a role during the maturation of the central nervous system[27,28]. Therefore, our finding of the presence of NMDA receptors on the neurons from 5-day-old rat cochlea could be interpreted as relevant to the maturation of neuronal structures in the developing cochlea in which case the expression of NMDA receptors should be transient. We have therefore studied the effect of the various excitotoxins on cultured adult rat auditory neurons. About 2–3% of the total neuronal population of a spiral ganglion (i.e. 140 neurons per well) can survive and regenerate in vitro for at least 4–5 days in serum-free medium on a substrate that has been coated sequentially by polyornithine, laminin and astrocyte-conditioned medium (Lefebvre et al., in preparation). We have differentially analyzed the effects of various excitotoxins on large (cell body diameter >12 μm) and small (cell body diameter <12 μm) neurons which represent respectively 85 and 15% of the neurons surviving after 3 days in culture. When incubated in the presence of NMDA or kainate, respectively 62% and 50% of the large neuronal population die, while no apparent cytopathic effect could be observed on the small neuronal component (Fig. 6). No effect of quisqualate is observed whatever the neuronal subpopulation considered.

In the cochlea, elevation of the extracellular potassium is known to induce the release of various amino acids[9,20]. Normal (5.4 mM) of high (110 mM) potassium-containing media were conditioned by isolated organs of Corti for 24 h (see Materials and Methods section) and subsequently added for 24 h on dissociated 5-day-old rat afferent auditory neurons previously cultured for 5 days. A toxicity of the high K^+-conditioned medium is observed, that can be partly antagonized by either DAP-5 or kynurenate in a dose-dependent fashion (Fig. 7). Furthermore, as illustrated in Fig. 8A, the protective effect of DAP-5 and kynurenate are partly additive. No significant toxic activity is found in the medium conditioned at normal potassium concentration (Fig. 7A). High (110 mM) potassium-containing media with (1.8 mM) or without calcium were conditioned by isolated organs of Corti for 24 h. The calcium-free conditioned medium was, after the conditioning, supplemented with the appropriate amount of $CaCl_2$ to achieve a final concentration of 1.8 mM. Both conditioned media were then added on dissociated 5-day-old rat afferent auditory neurons previously cultured for 5 days. In the absence of calcium during conditioning, there is a significant decrease but not a complete inhibition of the potassium-induced release of toxic activity by the organ of Corti, demonstrating that part of this release occurs in synaptic-like conditions, i.e. most likely from hair cells (Fig. 8B). Thus these experiments suggest that the organ of Corti is able, upon appropriate stimulation, to release diffusible agents acting on kainate- or NMDA-subtypes of receptors present on the surface of afferent auditory neurons.

DISCUSSION

The data presented in this paper can be summarized as follows: (i) Of the dissociated and cultivated 5-day-old rat afferent auditory neurons, 85–90% are sensitive to the neuronotoxic effect of glutamate. (ii) A residual subpopulation of these cultured neurons (10–15%) is repeatedly found to be unsensitive to the excitatory neurotransmitters. (iii) NMDA and KA are toxic for respectively 54 ± 7% and 40 ± 4% of these neurons while quisqualate has no toxic effect. (iv) The neuronotoxic effect of NMDA and kainic acid is suppressed in a dose-dependent fashion by their respective antagonists DAP-5 and kynurenate. (v) If one separates cultured adult auditory neurons on the basis of the diameter of the soma into a large (>12 μm) and a small (<12 μm) subpopulation, the large afferent adult auditory neurons are sensitive to the neuronotoxic effects of NMDA and kainate in the same proportion as the 5-day-old rat neurons, while the small afferent neurons are unresponsive to excitotoxins. Quisqualate has no effect whatever the neuronal subtype, large or small. (vi) When incubated in high potassium-containing medium, organs of Corti from 5-day-old rat release a neuronotoxic activity for these cultured spiral ganglia neurons. This neuronotoxic effect is suppressed partly by DAP-5 and kynurenate and completely when both antagonists are used together. (vii) The K^+-induced release of neuronotoxic activity by organs of Corti from 5-day-old rats is significantly decreased but not abolished, if the K^+ stimulation is made in Ca^{2+}-free condition.

It has first to be stressed that all these data were obtained using cell cultures in which only a small percentage of the original neuronal population survives. To what extent the surviving neurons are representative of the whole acoustic ganglion neuronal population is not known. Interestingly, however, if one separates the adult cultured neurons on the basis of size, the proportion of

large vs small neurons is close to the proportion of type I vs type II neurons as described in vivo[3,23,46]. Still, extrapolation from cultured neurons to in vivo neurons has to be cautious[38]. Acoustic ganglia derived from 5-day-old rats contain developing neurons. To what extent the data obtained from developing neurons apply to adult ones is not known. This question is particularly relevant in the case of excitatory amino acid receptors since a developmental regulation of the subtype of these receptors has been demonstrated in several instances[27]. More precisely, a developmental role for the intracellular Ca^{2+} increase triggered by activation of NMDA receptor-Ca^{2+} channel complex has been suggested[28]. This is the reason why we turned to adult animal-derived cultures. However, cultures generated from adult acoustic ganglion actually contain regenerating neurons which might be, and probably are, different from in situ neurons. Again, to what extent these differences apply to the excitatory amino acid receptors is not known at the present time.

In the cochlea, the two afferent neuronal systems, radial (consisting of large type I ganglion neurons) and spiral (consisting of small type II neurons) are connected respectively to inner and outer hair cells[3,23,46]. As reviewed in the Introduction, glutamate is a candidate neurotransmitter at the hair cell-afferent endings synapse in both systems[8]. This view is further supported by the presence of glutamate receptors on a substantial proportion of the cultured neurons and the release of excitotoxic activity from isolated organs of Corti. Furthermore, that release occurs at least partly in synaptic-like conditions, i.e. K^+-stimulated and Ca^{2+}-dependent. However, the acoustic ganglion neuronal population is heterogeneous if one considers not only the presence or absence of glutamate receptors but also the distribution of glutamate receptor subtypes. The proportion of glutamate receptor-negative neurons in vitro is close to the proportion of type II neurons found in vivo, raising the possibility that glutamate neurotransmission is restricted to the inner hair cell system. Since in 5-day-old rats, type I and type II neurons cannot be distinguished on morphological basis[43], we tried to approach that question using cultured auditory neurons obtained from adult rats. The excitotoxin sensitivity was differentially quantified for both large and small neurons assuming that, during the 4-day period of culture, cell body size could remain, in the absence of known specific immunological marker, a useful criterion to differentiate type I from type II neurons. Our demonstration that the small (presumably type II) component is not sensitive to excitotoxins, argues against a role for glutamate as transmitter at the outer hair cell-afferent ending synapse. A kainate receptor seems to be present on type I dendrites, since Pujol et al. (1985) described an important swelling of auditory dendrites below the inner hair cells in rat cochlea injected with kainate through the round window[33]. In the 6-day-old rat cochlea, but not in the adult rat cochlea, kainate induces also a swelling below the outer hair cells[36], an observation that could be explained by the presence of a transient radial branching to outer hair cells[26,31,32,34,35]. Furthermore, the proportion of cultured neurons sensitive to the cytopathic effect of KA fits with the number reported by Juiz et al.[22] in an in vivo study on the adult rat auditory neurons' sensitivity to KA[22]. In our experiments, NMDA induces a neuronotoxic effect on 50–60% of the afferent neuronal population in dissociated culture. Although some electrophysiological studies using NMDA and selective NMDA antagonists have shown no effect on cochlear potentials leading to the proposal that the cochlea lacks NMDA receptors[7,8,17,21]. Puel et al. demonstrated very recently that NMDA and DAP-5, perfused through the perilymphatic scalae modify the amplitude of the compound action potential and the N_1 latency under their stimulation conditions. These authors suggest that the NMDA receptors are located on the auditory nerve dendrites contacting the inner hair cells since there was no effect of NMDA antagonists on the cochlear microphonic potential[30]. This hypothesis of the presence of the NMDA receptor subtype in the radial auditory system is also supported by the finding that the activation of NMDA receptors causes swelling of radial afferent dendrites in isolated cochlea[45]. These arguments support further the hypothesis of a glutamate neurotransmission at the inner hair cell synapses as has been proposed by Eybalin and Pujol[14] in a study of the glutamate-glutamine cycle[14]. Several arguments also argue against glutamate being a neurotransmitter at the outer hair cell synapse: outer hair cells contrary to inner hair cells are never[14] or only weakly[42] labelled by glutamate in uptake experiments and show a weak labelling for glutamate in immunohistochemical studies[1,15]. Recent electrophysiological data also support such a view[30].

Pathophysiological implications

For many years, glutamate neurotransmission has been associated with excitotoxicity and excitotoxic neuronal death has been implicated in various neurological diseases, both acute, such as trauma, hypoglycemia or ischemia[12,19,37,40], and chronic such as neurodegenerative diseases[28]. This is indeed the reason why, in this paper, we focused our attention on the neurotoxic effect of glutamate and related substances and used neuronal death as a criterion to monitor the various glutamate receptors. Since glutamate receptors are present on auditory neurons both during development and in adult-

hood, such excitotoxic mechanisms could be involved in the pathophysiology of various acute or chronic deafnesses. Our demonstration that excitotoxins are released by the organ of Corti from synaptic (calcium-dependent) as well as non-synaptic (calcium-independent) stores (see also refs. 9 and 20) further supports such a view and opens the prospect of a neuroprotective pharmacological approach using glutamate antagonists[2,44]. It should be clear, however, that the situation is likely to be more complex. Indeed, glutamate and its analogs have no cytopathic effect on hair cells themselves (data not shown). However, incubating the organ of Corti in high potassium induces the release of a toxic activity for hair cells that is pharmacologically unrelated to excitotoxins[25]. The relative pathophysiological importance of these toxic activities, as well as other endogenous cytotoxic agents such as free radicals remains to be determined as well as their possible involvement in human inner ear pathology.

Acknowledgements. P.L. is Research Associate and B.R. is Research Assistant of the Belgian National Fund for Scientific Research (Belgium). This work was supported by grants from the Belgian 'Fonds de la Recherche Scientifique Médicale and Fonds Médical Reine Elisabeth'. We thank Mrs. P. Ernst and A. Brose for their expert technical assistance. P.P.L. is supported by 'La Communauté Française de Belgique'.

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