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Glutamate excitatory effects on ampullar receptors of the frog

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Summary. The action of glutamate on frog ampullar receptors was investigated to assess the potential role of this excitatory amino acid as an afferent transmitter in the hair cell system. Intracellular recordings from single afferent units in the isolated labyrinth revealed that glutamate and the glutamate receptor agonists, N-methyl-D-aspartic acid, quisqualic acid and kainic acid increase dose-dependently the frequency of the resting afferent discharge of EPSPs and spikes and produce long lasting depolarizations. After blocking synaptic transmission by using 5 mM Co^{2+}, the same compounds elicited only depolarizations of amplitude comparable to those observed in normal saline. Quisqualic acid and kainic acid were much more potent than N-methyl-D-aspartic acid in increasing the frequency of afferent discharge and in causing axonal depolarizations. The depolarization caused by glutamate was reduced dose-dependently by the competitive non-NMDA receptor antagonist 6-cyano-7-nitroquinaxoline-2,3 dione and disappeared almost completely in Na^+-free Ringer solution. These results are consistent with the hypothesis that glutamate is the afferent transmitter in vestibular organs and indicate that receptors mainly of the non-NMDA type are present not only at postsynaptic level but also in hair cells. Presynaptic glutamate receptors may function as autoreceptors controlling by a positive feed-back mechanism the release of the afferent transmitter.

Keywords: Amino acids – Vestibular receptors – Semicircular canals – Intra-axonal recordings – Afferent transmission – Glutamate receptors

Introduction

The sensory organs of the inner ear contain highly specialized hair cells which act as mechanoreceptors to detect angular and linear acceleration and sound waves. These receptors transduce the natural stimuli and transmit the sensory information through chemical synapses to primary afferent neurons. There is general agreement that transmission is fast and involves calcium dependent release of a chemical substance at the basal pole of the hair cell. However, the mechanism of transmission in inner ear synapses has not been sufficiently

investigated and the identity of the afferent transmitter, in particular, has not been conclusively established. Although many substances can act as modulators of afferent transmission at inner ear receptors, a large body of pharmacological evidence suggests that acetylcholine, norepinephrine, dopamine, substance P or 5-hydroxytryptamine are not the primary afferent transmitter in the hair cell system (Bledsoe et al., 1988). The most plausible candidate for such a role is an excitatory amino acid, such as glutamate (Glu) or a related substance (Bledsoe et al., 1988).

In this article, we describe the effects of glutamate and Glu receptor agonists and antagonists (Watkins and Olvermann, 1987), on isolated preparations of frog semicircular canals. The action of these substances were evaluated by recording the resting discharge of EPSPs and spikes from single afferent units of the posterior semicircular canal. Evidence is presented that Glu actions are more complex than those described in other hair cell systems and involve at least two separate effects: a presynaptic effect on hair cells, which is responsible for enhanced afferent activity, and a postsynaptic effect on afferent neurons resulting in membrane depolarization. It is of interest that both these effects appear to be predominantly mediated by activation of non-NMDA receptors.

Materials and methods

Whole labyrinth preparations were isolated from the right half head of adult frogs (Rana esculenta), by using a dissection procedure which has been described in detail elsewhere (Prigioni et al., 1983). The preparation was pinned at the bottom of a chamber filled with Ringer solution having the following composition (mM): NaCl 117, KCl 2.5, $CaCl_2$ 1.8, D-glucose 5, Tris-HCl 5.0 (pH 7.3).

Glu and the Glu receptor agonists N-methyl-D-aspartic acid (NMDA), quisqualic acid (Qu) and Kainic acid (Ka) were applied to the preparation by using a microinjection procedure which allowed the precise delivery of streams of buffered solutions (pH 7.3) containing the compounds of interest (Prigioni et al., 1990). In this procedure, a small glass pipette with an internal diameter of 0.5 mm, connected to a motor-driven 1 ml syringe, was positioned on the ampulla close to the sensory organ. Injections were usually made at a rate of 60 μl/min for 10 sec. In all experiments a control injection of normal Ringer solution was also performed to ascertain that no artifacts were introduced by the method of application.

Responses of the sensory organ to Glu and Glu receptor agonists were quantified by measuring changes in membrane potential and resting discharge of EPSPs and spikes in single afferent neurons, by using glass microelettrodes (3M KCl filled; 20–30 Mohm impedence) inserted into the posterior nerve close to the ampulla. Spike discharge was measured by using a window discriminator and a frequency-to-voltage converter. EPSP discharge frequency was evaluated by counting at appropriate time intervals the number of subthreshold potentials in photographic reproductions of the recordings. Changes in membrane potentials were assessed by measuring directly the baseline shift in the recording. In order to evaluate accurately changes in EPSP frequency following application of the test compounds, recordings were made in afferent units showing relatively low discharge frequencies at rest. Recordings were amplified by a conventional DC amplifier, monitored on a storage oscilloscope and stored on FM magnetic tape.

In experiments designed to assess specifically the postsynaptic action of the test compounds, afferent transmission was blocked by adding 5 mM Co^{2+} to the normal Ringer solution and by adjusting accordingly the Na^+ concentration. Under these condi-

tions, impalement of the afferent axon was assessed by evoking antidromic spikes through electrical stimulation of the posterior ampullar nerve sucked up in a fluid electrode. The Glu receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and kynurenic acid, dissolved in Ringer solution, were applied to the preparation by perfusion. When the concentration of Na^+ and Ca^{2+} in the bath was reduced, the osmolality of the medium was kept constant by adding choline to the Ringer solution. Glu and Glu receptor agonists and antagonists were purchased from Tocris-Neuroamin and Sigma Chemicals Co. For each set of experiments, number of sensory units impaled (n) and means ± SE are reported.

Results

In preliminary experiments designed to assess whether Glu has any action on afferent fibres, a stream of Glu-containing Ringer solution was applied to preparations in which the two ends of the ampullar nerve of the posterior canal were cut close to the ampulla. In these experiments, no evidence of excitatory effects was detected in impaled fibres at Glu concentrations as high as 10 mM.

When applied to normal preparations, 5 mM Glu elicited in the impaled fibres (12 units in 4 preparations) a clear enhancement of the resting afferent discharge, consisting in an increase of EPSPs and spike frequency. These effects were invariably accompanied by a prominent depolarization (Fig. 1). The increase of the afferent discharge was fast in onset and of brief duration (30–50 s), while axonal depolarization developed slowly and was longer lasting (4–5 min). The enhancement of afferent discharge was usually followed by a period of reduced activity whose time course was roughly parallel to that of axonal depolarization. When depolarization attained its maximum, the discharge of both EPSPs and spikes decreased below its resting level, and a return of the discharge to baseline was noted when depolarization disappeared. Glu effects were promptly reversible after washing with normal saline.

When synaptic transmission was blocked by using 5 mM Co^{2+}, 5 mM Glu produced only long lasting depolarizations (Fig. 2A), which were similar to those observed in normal saline. Glu-induced depolarizations were dose-dependent (Fig. 2B) and concentrations between 0.01 and 10 mM were usually found to produce a full range of minimal to maximal responses. Although depolarizations elicited by these concentrations of Glu were fully reversible within few minutes after washing with normal saline, higher Glu concentrations produced prominent depolarizations which could be restored only partially by washing and therefore were not considered in the construction of dose-response curves.

In order to determine the nature of the receptors involved in Glu-induced depolarizations, the action of the Glu receptor agonists Qu, Ka and NMDA, and the Glu receptor antagonists CNQX and kynurenic acid was tested. Like Glu, all three agonists determined consistently a depolarization response in afferent units. As seen in the dose-response curves shown in Fig. 2B, however, Qu and Ka were much more potent than NMDA. In fact, clear depolarizations could be observed at concentrations of Qu and Ka of about 10^{-6} M, whereas NMDA concentrations about 1,000-fold higher were re-

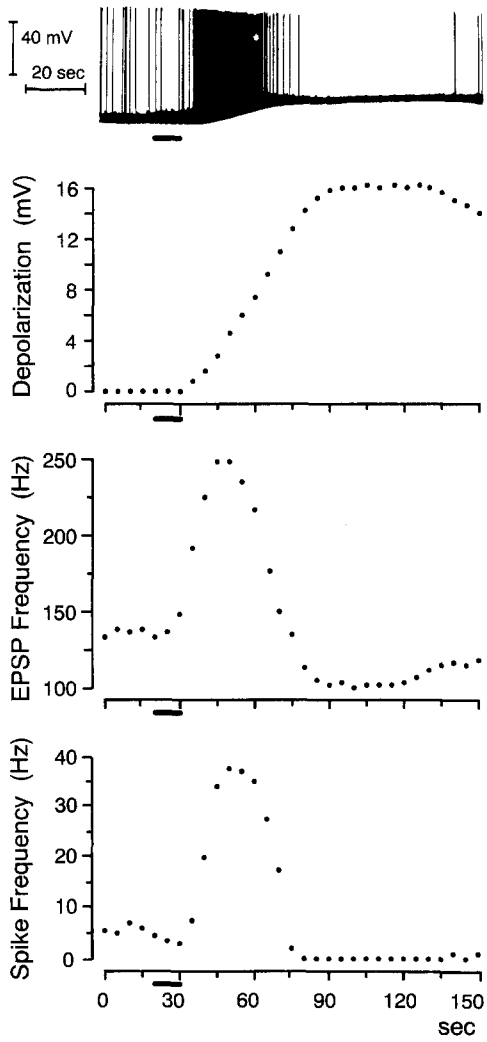

Fig. 1. Effect of 5 mM Glu on the resting discharge recorded intra-axonally from an afferent sensory unit of the posterior ampullar nerve. The graphs below the tracing show the quantitative evaluation of the axonal depolarization and the frequency of the discharge of EPSPs and spikes. Solid bars indicate the time of Glu injection

quired to produce comparable effects. The excitatory effects of all Glu receptor agonists were fully reversible at concentrations up to 10^{-4} M for Qu and Ka and up to 10^{-2} M for NMDA. With respect to the action of antagonists, CNQX was effective in blocking dose-dependently the depolarization induced by 5 mM Glu (Fig. 3A). The threshold concentration of CNQX at which antagonism was observed was close to 10^{-7} M, and almost complete suppression of depolarization was achieved at a concentration of 10^{-5} M. The antagonism was reversible after washing with normal saline. Dose-dependent blockade of Glu-induced depolarization was also achieved with kynurenic acid. This antagonist, however, was less potent than CNQX: the threshold concentration at which the antagonist action of kynurenic acid was observed was close to 10^{-4} M, and almost complete blockade of Glu-induced depolari-

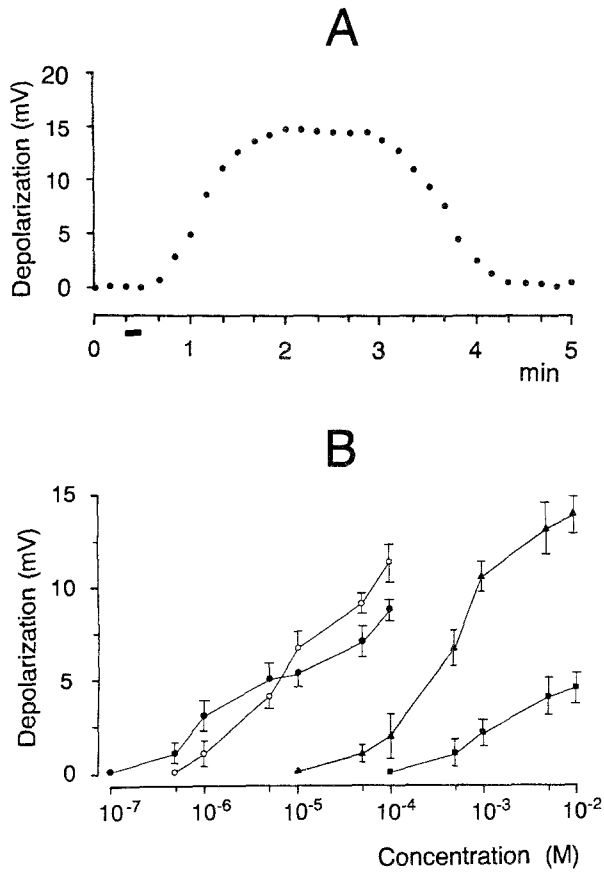

Fig. 2A,B. Typical depolarization induced by 5 mM Glu in a single afferent fibre after blocking afferent transmission with 5 mM Co^{2+}. The horizontal bar indicates the time of Glu injection. Mean dose-response curves for Qu (●) (n = 6), Ka (○) (n = 6), Glu (▲) (n = 8) and NMDA (■) (n = 6). Vertical bars indicate SE

zation was achieved at a concentration of 10^{-3} M (5 experiments, data not shown). The effect of kinurenic acid was only partially reversible after washing with normal saline.

In order to investigate the nature of the ions involved in Glu-induced depolarization, the action of 5 mM Glu was evaluated after modifying the concentration of Na^+ or Ca^{2+} in the Ringer solution. Because lowering the Na^+ concentration resulted in an increased resting membrane potential in impaled fibres (up to a level of about 5 mV in Na^+-free saline), the action of Glu was tested after the membrane had reached a steady-state level. Under these conditions, a progressive reduction of Na^+ concentration was associated with a parallel decrease in the amplitude of depolarization elicited by Glu (Fig. 3B). Depolarization disappeared almost completely when a Na^+-free solution was used. By contrast, lowering the Ca^{2+} concentration (5 experiments) did not produce any apparent effect on the resting membrane potential and Glu-induced depolarizations persisted virtually unchanged in a Ca^{2+}-free saline (data not shown).

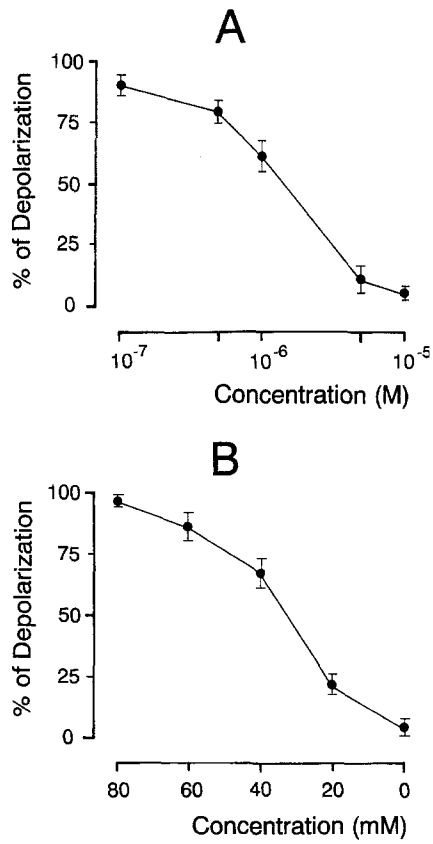

Fig. 3. A Mean dose-response curve for the non-NMDA receptor antagonist CNQX. **B** Effect of decreasing the Na^+ concentration in the bath. Data shown in the two graphs refer to percent changes in the amplitude of depolarization induced by 5 mM Glu (n = 6). Vertical bars indicate SE

The observation that in normal saline Glu produced not only axonal depolarization but also a marked increase in EPSPs discharge frequency, triggering a parallel increase in spike discharge, clearly indicates the occurrence of a presynaptic action on the canal organ. To evaluate the nature of the receptors involved in this action, the effect of Qu, Ka and NMDA was tested. In almost all impaled fibres (32 units in 8 preparations) all three Glu receptor agonists elicited effects similar to those produced by Glu. A typical increase in EPSPs and spike frequency of resting afferent discharges following application of Qu, Ka and NMDA is shown in Fig. 4. The enhancement of the afferent discharge was fast in onset and the duration of response was dependent on the concentration of the agonist. As observed with Glu, the action of Glu receptor agonists was followed by a period of reduced afferent activity, and among the three agonists Qu invariably evoked longer lasting responses.

Although all agonists produced a dose-dependent increase of EPSPs and spike discharges (Fig. 5), Qu and Ka were much more potent than NMDA. In fact, the threshold concentration for Qu and Ka was close to 10^{-6} M, whereas a NMDA concentration 500–1,000-fold higher was required to evoke compa-

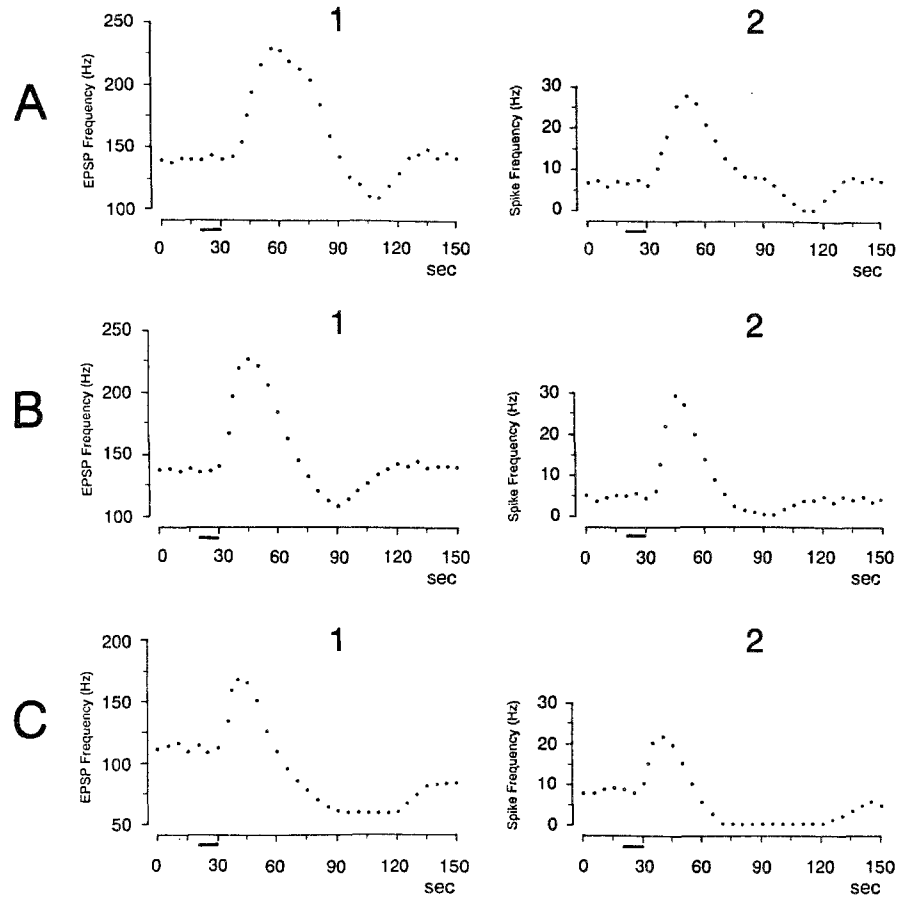

Fig. 4. Quantitative evaluation of EPSPs (*1*) and spike (*2*) frequency of discharges induced by 5 μM Qu (**A**), 10 μM Ka (**B**) and 5 mM NMDA (**C**). Solid bars indicate the time of injection of the compounds

rable effects. The excitatory effects of amino acids were fully reversible at concentrations up to 10^{-4} M for Qu and Ka and up to 10^{-2} for NMDA. Higher concentrations produced effects which were only partially reversible and therefore they were not considered in the construction of dose-response curves.

Discussion

The excitatory effects of glutamate on frog semicircular canal were more complex than those described in other acoustico-lateralis organs. In fact, Glu was able to determine in single afferent units a brief increase of resting activity accompanied by a longer lasting axonal depolarization (Valli et al., 1985). Evidence was obtained that these two effects originate at different sites: the enhancement of the afferent discharge appears to be related to a presynaptic action, while the axonal depolarization originates at postsynaptic level. The postsynaptic nature of Glu-induced depolarization is demonstrated by the

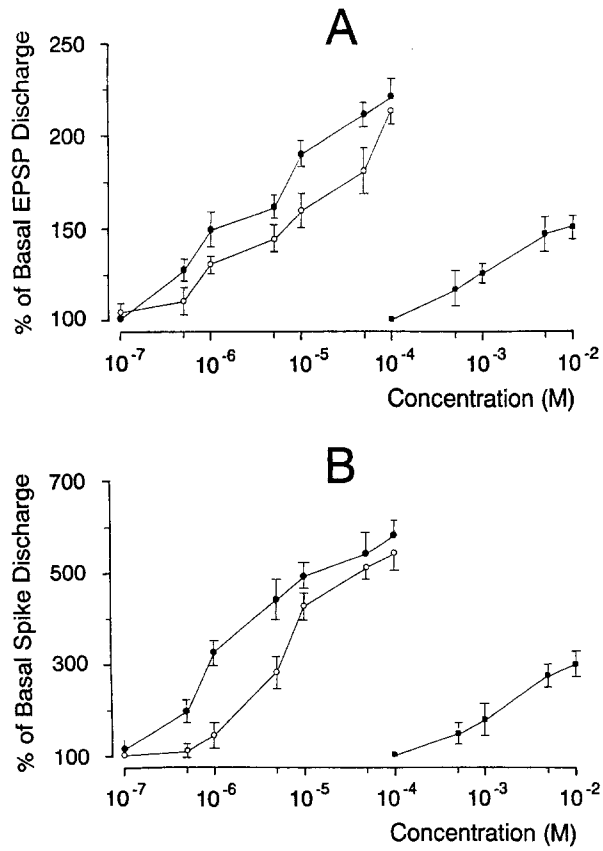

Fig. 5. Mean dose-response curves for Qu (●) (n = 10), Ka (○) (n = 12) and NMDA (■) (n = 10). Effects are expressed as percent increase in the discharge of EPSPs (**A**) and spikes (**B**) at rest. Vertical bars indicate SE

observation that this response, unlike the enhancement of afferent activity, is unaffected by manipulations that block transmitter release from hair cells, such as an increase in the concentration of Co^{2+} or Mg^{2+} in the medium (Prigioni et al., 1990). On the other hand, a presynaptic effect of Glu is clearly demonstrated by the fact that the increase in afferent discharge was associated with a marked increase in the frequency of EPSPs, an effect which cannot be explained by a direct action of Glu on postsynaptic structures. These results are consistent with the hypothesis that Glu can act directly at two separate sites: at the level of the hair cells, by increasing the release of the afferent transmitter (which in turn, is responsible for the increased propagated activity), and at the endings of afferent neurons, by evoking axonal depolarization. However, the possibility that Glu can also act, at least in part, through less selective mechanisms on other structures, such as glial or supporting cells, cannot be excluded by our observations.

Another interesting issue is the nature of the membrane receptors involved in the pre- and postsynaptic effects of Glu on semicircular canals. Our experiments have shown that the postsynaptic depolarization is blocked dose-dependently by the competitive antagonist CNQX (Prigioni et al., 1994), and

that depolarizations comparable to those elicited by Glu were produced also by very low concentrations of the non-NMDA receptor agonists Qu and Ka. Conversely, NMDA was much less potent than Qu and Ka, as indicated by the fact that it required concentrations at least 500 times as high to exert similar excitatory effects. These results indicate that the postsynaptic action of Glu involves mainly an activation of non-NMDA receptors. The ionic channels associated with these receptors have a high permeability to Na^+ and K^+, and a very low permeability to divalent cations, such as Ca^{2+} (MacDermott and Dale, 1987). Our observation that the postsynaptic depolarization elicited by Glu was impaired in Na^+-free medium but was unaffected by Ca^{2+} removal provides further support to the hypothesis that non-NMDA receptors are the main receptor type involved in postsynaptic depolarization. The same receptor type also appears to mediate the presynaptic action of Glu on semicircular canals. This conclusion is supported by the observation that the non-NMDA agonists Qu and Ka, were much more potent than NMDA in increasing the frequency of EPSPs and spike discharges at rest in almost all impaled units.

In conclusion, the present findings support the hypothesis that Glu or a related substance is the afferent transmitter in vestibular receptors. The presence of presynaptic Glu receptors in the canal organ would not conflict with this hypothesis. Presynaptic receptors may function as autoreceptors by controlling through a positive feed-back mechanism the release of the afferent transmitter from hair cells, as shown for other cyto-neural synapses (Sarantis et al., 1988). Such a mechanism may have a physiological role in increasing the gain of mechano-electrical transduction in the hair cells.

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