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GLUTAMATE AS THE NEUROTRANSMITTER OF CEREBELLAR GRANULE CELLS IN THE RAT: ELECTROPHYSIOLOGICAL EVIDENCE

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1 Glutamate and the excitatory aminoacid antagonist, α-aminoadipic acid (αAA), have been applied by microiontophoresis to Purkinje cells in the rat cerebellum.

2 Glutamate produced excitation of Purkinje cells and αAA selectively reduced that excitation without affecting responses to acetylcholine or hydrogen ions.

3 Monosynaptic spikes were evoked in Purkinje cells by stimulating the parallel fibres. αAA had little effect on these spikes when applied alone.

4 When the Purkinje cell excitability was reduced by the iontophoresis of γ-aminobutyric acid, αAA then produced failure of the monosynaptic spike on 10 of 13 Purkinje cells, in doses shown to be selectively antagonistic towards aminoacids.

5 These results support neurochemical evidence that glutamic acid may be the neurotransmitter released by granule cell parallel fibres.

Introduction

The excitatory amino acids, glutamate and aspartate, have been proposed as putative neurotransmitters in the mammalian central nervous system (Curtis & Johnston, 1974; Krnjević, 1974). Initially they were suspected of being transmitters of primary afferent neurones and spinal cord interneurones respectively (Johnson, 1972; Curtis & Johnston, 1974) and subsequently glutamate has seemed a strong candidate as the transmitter released by pyramidal tract neurones (Stone, 1973; 1976). More recent work has supported the concept that glutamate may be released as the transmitter by many cerebral corticofugal neurones (Spencer, 1976; Divan, Fonnum & Storm-Mathisen, 1977).

In all these cases a key factor in the assessment of the transmitter role of dicarboxylic amino acids has been the use of antagonist compounds. However, there now exists a good deal of indirect and circumstantial evidence favouring a transmitter role for glutamate at the synapses from granule cells via the parallel fibres onto Purkinje and other neurones in the cerebellar cortex but few direct studies on the effects of antagonists on this pathway have been made. In the present study we have therefore examined the effects on these synapses of α-aminoadipic acid, recently shown to be a potent and specific antagonist of excitatory amino acids (Hall, McLennan & Wheal, 1977; Biscoe, Evans, Francis, Martin, Watkins, Davies & Dray, 1977; McLennan & Hall, 1978).

Methods

Male Porton Wistar rats weighing 250 to 350 g were anaesthetized with urethane (1.25 g/kg. i.p.) and then placed in a stereotaxic frame. Body temperature was maintained at 37 to 38°C by means of an automatically regulated heating blanket and rectal probe. An area approximately 4 mm^2 of cerebellar cortex was exposed, the dura was removed and after positioning the electrodes the area was covered with a layer of 4% agar in saline to prevent cooling and drying and to reduce vascular and respiratory movements of the tissue.

Stimulation of parallel fibres was effected by single cathodal pulses of 0.5 ms duration applied from a stainless steel wire electrode of diameter 250 μm. An indifferent electrode was located in 0.9% w/v NaCl solution (saline) soaked cotton wool overlying the temporalis muscles. Stimuli were applied from a Grass S44 stimulator via a photically coupled stimulus isolator.

For the application of compounds by microiontophoresis, 7-barrelled micropipettes were used. The pipette tips were broken under microscopic observation to an overall diameter of 10 to 15 μm, and the barrels were filled with drug solutions immediately before use. The following solutions were used: L-glutamate sodium, 0.2 M, pH 7.0; L-aspartate potassium, 0.2 M, pH 7.0; acetylcholine chloride, 0.2 M, pH 4.5; DL-α-aminoadipic acid, 0.05 M, adjusted to pH 7.0.

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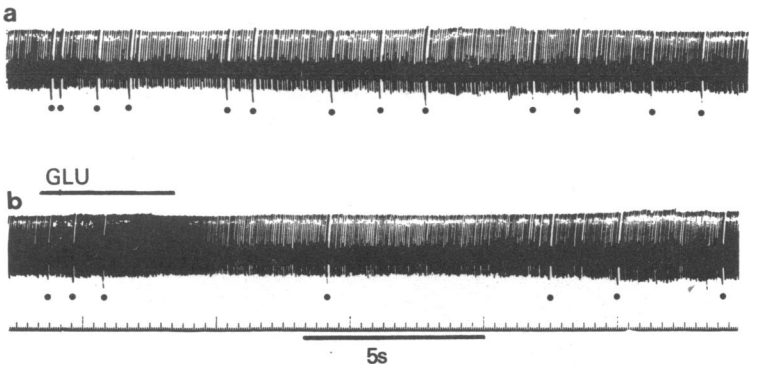

Figure 1 A spike record showing characteristics of Purkinje cells used for identification. This cell is firing at a mean frequency of 21 Hz and shows climbing fibre bursts (indicated by dot below the record) followed by a brief inactivation pause (Eccles *et al.*, 1967). In (b) a dose of 150 nA of glutamate (GLU) was applied by microiontophoresis, and produced excitation of this cell. Doses of less than 100 nA were ineffective. (a) and (b) are consecutive records. The spikes were replayed from magnetic tape onto a pen recorder.

Iontophoretic ejection was effected by a Digitimer Neurophore Unit incorporating automatic balancing at the electrode tip. In this way current flow to ground and thus current artefact was minimized. When not being ejected, all compounds were subjected to a retaining current of 10 to 15 nA.

The use of relatively large multibarrel tips of 10 to 15 μm deserves comment. It has been found that pipettes of this size will eject compounds with noticeably less electrical noise than with the more conventional 5 μm tips, presumably since drug-filled barrel resistances are reduced (e.g. about 2 to 5 MΩ for 0.2 M glutamate compared with about 20 MΩ). Furthermore, drug responses tend to be obtained at lower ejecting currents, and are generally more consistent and reproducible than with 5 μm tips. A high rate of drug efflux by diffusion does not seem to be a problem, as control experiments with 1 M glutamate or GABA solutions alone, and employing a 15 nA retaining current have not led to net changes of cell firing rate from the time cells were first encountered to the end of a recording 90 min later. As unit activity is recorded through a separate electrode, the clarity of recordings is unaffected.

Extracellular recordings of unit activity were made with single glass microelectrodes containing 1 M potassium acetate and having resistances of 1 to 8 MΩ at 1 kHz, fixed alongside the multibarrel pipettes (Stone, 1973). The tips of the recording pipettes were bent to an angle of approximately 20° during the pulling process to facilitate fixing them alongside the multibarrel. The tips were initially approximated by eye and then under microscopic control. During this stage the electrodes were held together by Plasticine. Permanent fixing was then achieved with an epoxy resin. Tip approximation was always confirmed microscopically immediately before and after each experimental penetration. The recording electrode was arranged to project 10 to 20 μm beyond the multibarrel tip.

Unit activity was amplified by a Grass P511 amplifier and the spikes were then passed through a window discriminator, the output pulses of which were counted and displayed on a Grass polygraph. Spikes were simultaneously displayed on oscilloscopes and were also available for recording on magnetic tape and for on-line generation of post-stimulus time histograms.

Results

Purkinje neurones were identified by means of their regular and usually high firing rates of 20 to 60 Hz, and the occurrence of the characteristic climbing fibre bursts consisting of 1 to 6 high frequency spikes followed by a brief period of inactivation (Eccles, Ito & Szentagothai, 1967). Such a pattern of firing is shown in Figure 1, which also illustrates the fact that in spite of the high frequency of firing, Purkinje cells can be excited by the iontophoresis of glutamate without any diminution of spike height. However, it was a characteristic observation that relatively high doses of glutamate were needed to produce excitation.

Local electrical stimulation of the cerebellar cortex was initially effected in approximately the same coronal plane as the recording electrode and not more than 1 mm distant. Small adjustments of the stimulating electrode position were sometimes needed in order to evoke single spikes in the Purkinje cells with minimum stimulus strengths. The evoked spikes had

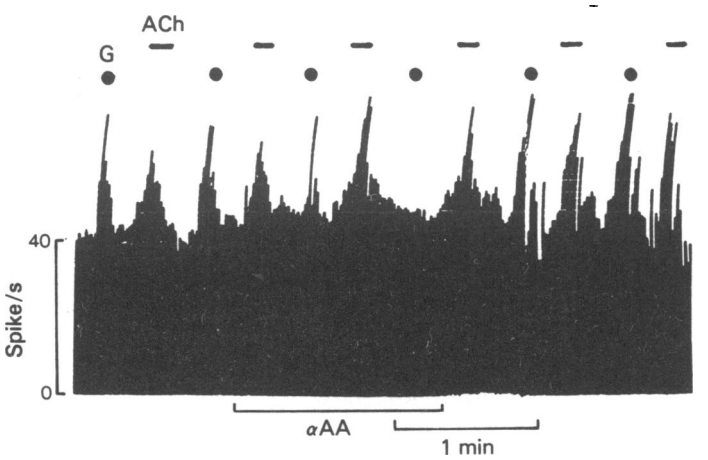

Figure 2 Records of the firing rate of a Purkinje cell showing excitatory responses to glutamate, 100 nA (G) and acetylcholine, 100 nA, (ACh). α-Aminoadipic acid, applied with a current of 60 nA (αAA) selectively reduced the response to glutamate. The slight increase of acetylcholine response was often observed and probably reflects the excitatory tendency of the L-isomer of αAA (Biscoe *et al.*, 1977). The ordinate scale is spikes per second.

latencies in the range 1.6 to 3.8 ms (Figure 4). At low stimulus strengths (up to 50 μA) these single spikes were not associated with other changes of Purkinje cell excitability. However, as stimulus strength was increased above this level an inhibitory pause succeeded the spike in about 50% of the cells studied, presumably due to stimulus spread to 'off-beam' parallel fibres with consequent activation of basket and other inhibitory neurones.

The evoked spikes were clearly monosynaptic and seemed to involve a high safety factor as the latency was almost invariant and the spikes would follow stimulation at rates of around 50 Hz. Failure of most spikes to follow 80 Hz stimuli confirmed that they were not antidromic.

Aminoadipic acid and aminoacid responses

It has been reported from several laboratories that αAA is a relatively specific antagonist of neuronal responses to excitatory aminoacids, in studies of neurones in the spinal cord (Biscoe *et al.*, 1977) thalamus (McLennan & Hall, 1978) and cerebral cortex (Stone, unpublished observations). Figure 2 confirms this to be the case for cerebellar Purkinje cells. On 30 of 38 cells studied, a dose of αAA could be found which depressed responses to glutamate and aspartate without affecting acetylcholine (8 units) or hydrogen ion (22 units) excitations (Figure 2). The effective dose of αAA ranged from 30 nA for 15 s to 50 nA for 60 s. On the remaining 8 units of those studied, αAA would produce substantial reduction of the aminoacid responses, but only at doses of 80 nA for at least 15 s which on these cells also reduced the control agonist responses (acetylcholine or hydrogen ion).

αAA itself produced small increases of basal firing rate, as may be noted in Figure 2, on 21 of 38 units, and a slight depression on 4. The increase of firing was probably due to the use of the racemate of αAA, as previous groups have noted excitation by the L-isomer (Hall *et al.*, 1977).

No preference of αAA for inhibiting aspartate rather than glutamate on cerebellar neurones has been observed, in contrast to studies in the cerebral cortex (unpublished observations).

Aminoadipic acid and synaptic activity

The spikes evoked monosynaptically in Purkinje neurones by 'on-line' parallel fibre stimulation were monitored during the antagonism of aminoacid responses by αAA. Of 16 cells studied, only one showed any effect by αAA at aminoacid selective doses, but even that one cell only showed an approximately 1 in 3 failure of the monosynaptic spike. On a further 2 cells a similar frequency of transmission failure occurred when αAA was applied at doses which also reduced acetylcholine or hydrogen ion excitations.

As the parallel fibre evoked monosynaptic spike was of short and of virtually unvarying latency, and αAA was unable to affect transmission significantly at the parallel fibre to Purkinje cell synapses, it must be considered that there is a high safety factor for transmission at these synapses.

An attempt has therefore been made to repeat the experiments under conditions in which the safety fac-

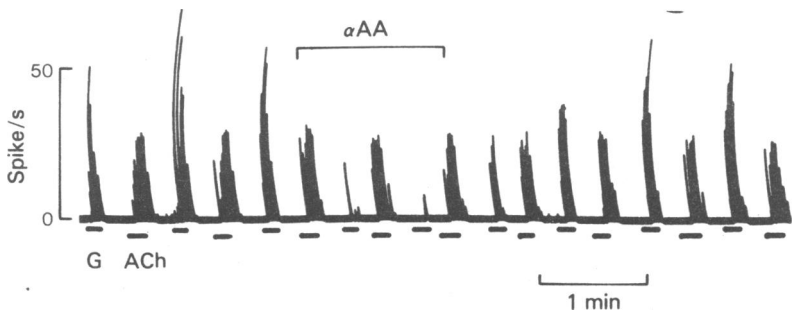

Figure 3 Records of the firing rate of a Purkinje cell which is depressed by the iontophoresis of γ-aminobutyric acid GABA, 50 nA, applied throughout this recording. The cell can still be excited by glutamate, 200 nA (G) and acetylcholine, 220 nA (ACh). Under these conditions, α-aminoadipic acid (αAA) 30 nA still exhibits selective antagonism towards glutamate. This recording was made on the same cell as Figure 4. The ordinate scale is in spikes per second.

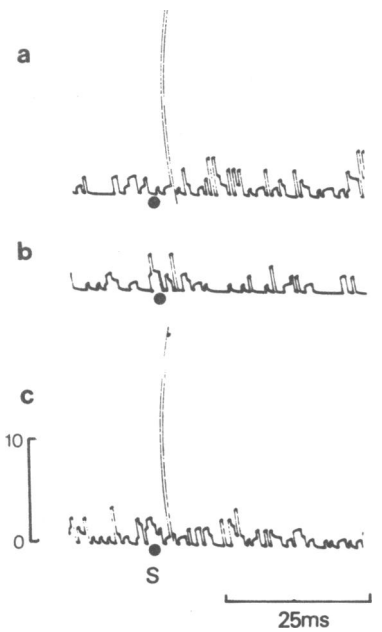

Figure 4 Poststimulus time histograms of the firing of a cerebellar Purkinje cell in response to stimulation of parallel fibres. Each record consists of 32 summated sweeps, using a bin width of 0.5 ms. The stimulus, delivered at the point indicated by a dot below each record, was approximately of threshold intensity (but see text), so that the evoked spike failed to occur after roughly one third of stimuli. The spike had a mean latency of 3.8 ms. Spontaneous firing rate is low because γ-aminobutyric acid, 50 nA was applied throughout this sequence. (a) Before, (b) and (c) after the iontophoresis of α-aminoadipate (αAA) with a current of 30 nA. Record (b) was started 2 min after beginning the αAA ejection, and record (c) was begun 4 min after ending the ejection. The ordinate scale is in spikes per address; time bar 25 ms.

tor might be reduced. The use of threshold stimuli in several experiments did not increase the success of blocking spikes with αAA, and therefore the safety factor was reduced by producing a depression of the Purkinje cell itself with doses of γ-aminobutyric acid (GABA) sufficient to inhibit regular spontaneous discharge of the Purkinje cells studied. The lack of improvement using threshold stimulation is consistent with the view that when the appropriate parallel fibres are activated, the resulting synaptic activation of the Purkinje cell occurs with a high safety factor.

Figure 3 indicates that even when cell firing was greatly depressed or abolished and high doses of the excitatory materials were needed to induce a response, αAA maintained selectivity of antagonism towards aminoacids compared with acetylcholine or hydrogen ions.

When tested against the parallel fibre induced monosynaptic spikes, αAA now induced a consistent failure of the spike on 10 of 13 units, in the same low doses that were found adequate to block the exogenous aminoacids (Figure 4).

Discussion

One of the observations in the present work has been that glutamate, applied by microiontophoresis, can excite Purkinje cells (Eccles *et al.*, 1967; Woodward, Hoffer, Siggins & Bloom, 1971). It is also clear that αAA can act as a specific antagonist of excitatory aminoacids on Purkinje cells at appropriate doses, as observed in other parts of the central nervous system (Biscoe *et al.*, 1977; McLennan & Hall, 1978; Stone, unpublished observations).

However, a difficulty of interpretation arises in considering the ability of αAA to block parallel fibre evoked activity in Purkinje cells only under circum-

stances where the excitability of the Purkinje cell is reduced by GABA.

One explanation might be that the parallel fibre to Purkinje cell synapse has a high safety factor such that high concentrations of antagonist would normally be needed to prevent depolarization of the Purkinje cells to threshold by the transmitter. By depressing the cell with GABA the safety antagonist now becomes effective.

The problem is reminiscent of that encountered by Curtis & Eccles (1958) in the study of cholinergic synapses in the spinal cord. These authors invoked a restrictive permeability barrier to account partially for the inability of dihydro-β-erythroidine to block the early spikes induced in Renshaw cells by a ventral root volley. If such a hypothetical barrier existed with respect to the synapses examined in the present study, it might also account for the need for high doses of glutamate to excite the Purkinje cells.

A different explanation of the need for high doses of glutamate is suggested by the experiments of Chujo, Yamada & Yamamoto (1975). This group observed a high sensitivity of Purkinje cells to the excitatory action of glutamate when this aminoacid was applied in the region of Purkinje cell dendrites in the molecular layer. It is therefore possible that the high doses of glutamate needed in the present study reflected the need for diffusion of the compound to receptive dendritic membrane located some distance away. For the same reasons, of course, perhaps only low concentrations of αAA were being achieved at the relevant synapses, concentrations which could only block the neurotransmitter under conditions which reduced the safety factor of activation.

As GABA is now widely acknowledged as the transmitter mediating presynaptic inhibition (Curtis & Johnston, 1974) part of the explanation of the action of GABA in the present work might be that, as well as depressing the Purkinje cell it produces a depolarization of parallel fibre boutons and thus reduces the release of transmitter. Such an action would also lead to a reduced safety factor of transmission. Given the evidence that αAA retained its antagonistic specificity to aminoacids this effect, if it occurred, should not affect the general conclusion of the present work.

The existing evidence for a transmitter role of an aminoacid from granule cells in cerebellar cortex is primarily neurochemical. In particular, it has been demonstrated that in conditions in which there is a substantially reduced number of granule cells in the cerebellum, as following certain viral infections, X-irradiation or in some mutant strains of mice (Weaver and Staggerer) there is a pronounced reduction in the levels of glutamate (Young, Oster-granite, Herndon & Snyder, 1974; McBride, Nadi, Altman & Aprison, 1976; Hudson, Valcana, Bean & Timiras, 1976; Roffler-Tarlov & Sidman, 1978). There is also, possibly more significantly, a depression of the high affinity glutamate uptake capability of cerebellar cortex in granule cell depleted animals (Young *et al.*, 1974). Evidence has also been obtained recently for a potassium-evoked and calcium-dependent release of glutamate from synaptosomes prepared from cerebellar cortex. This release is reduced in X-irradiated animals showing depletion of granule cells (Sandoval & Cotman, 1978).

There is therefore an appreciable amount of neurochemical evidence to support the implication of the present study that an aminoacid, probably glutamate, could be the neurotransmitter released by granule cell parallel fibres in the cerebellar cortex.

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