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LOCALIZATION OF SENSITIVE SITES TO TAURINE, γ-AMINOBUTYRIC ACID, GLYCINE AND β-ALANINE IN THE MOLECULAR LAYER OF GUINEA-PIG CEREBELLAR SLICES

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1 The localization, in the molecular layer of guinea-pig cerebellar slices, of the sites most sensitive to iontophoretically applied taurine, γ-aminobutyric acid (GABA), glycine and β-alanine was investigated.

2 The most sensitive sites were located (expressed as the distance from the Purkinje cell body to the pial surface): 0 μm, 60 μm and 220 μm for taurine; 0 μm and 180 μm for GABA; 80 μm and 200 μm for glycine, and 80 μm, 180 μm and 300 μm for β-alanine.

3 The sensitive site (at 0 μm) to GABA was considered to represent the basket synapses on the Purkinje cell soma, while the sites (60 to 80 μm and 200 to 300 μm) for taurine, glycine and β-alanine were tentatively assigned to the synapses of the stellate neurones on the Purkinje cell dendrites.

4 Inhibitory actions of all four amino acids tested at the most sensitive sites were antagonized by both picrotoxin and strychnine.

5 The possibility that taurine might be the neurotransmitter of the stellate neurones in guinea-pig cerebellum is discussed.

Introduction

Spontaneous spike discharge frequency in guinea-pig cerebellar slices is dose-dependently suppressed by the bath application of not only γ-aminobutyric acid (GABA) but also of glycine-like amino acids such as taurine, glycine and β-alanine (Okamoto & Quastel, 1973; 1976; Okamoto, Quastel & Quastel, 1976). Although it appears to be established that GABA is a neurotransmitter of the inhibitory synapses of the cerebellar basket interneurones on the soma of the Purkinje cell (Curtis, Duggan, Felix, Johnston & McLennan, 1971; Woodward, Hoffer, Siggins & Oliver, 1971; Bisti, Josif, Marchesi & Strata, 1971), the transmitter of the stellate interneurones is yet to be identified. However, recent investigations by Nadi, McBride & Aprison (1977) and by Frederickson, Neuss, Morzorati & McBride (1978) suggest a possible transmitter role of taurine in the stellate neuronal synapses. Since the suppression of spike discharges of the Purkinje cell, evoked by the off-beam electrical stimulation applied to the pial surface of a cerebellar slice, is antagonized by both picrotoxin and strychnine (Okamoto & Sakai, 1979), it is conceivable that one of the strychnine-sensitive amino acids might act as the inhibitory transmitter of the interneurones synapsing with the Purkinje cell. On the basis of these previous findings, we have attempted, in guinea-pig cerebellar slices, to locate the sites which are most sensitive to the iontophoretic application of the inhibitory amino acids, taurine, GABA, glycine and β-alanine. The location of the sensitive sites appears to be related to the anatomical location of the inhibitory synapses on the Purkinje cells of the cerebellum. At each of these spots the antagonistic effects of picrotoxin and strychnine on the inhibitory action of each amino acid were also investigated.

Methods

The guinea-pig (350 to 400 g, male) was killed by stunning, and a block (2 to 3 mm^3) of the cerebellar nodulus or uvula removed. The cut surface of the tissue block was glued with an adhesive (Aron Alpha, Toha Gosei Kagaku, Ltd., Tokyo) to a supporting agar block (about 4 mm^3), with the sagittal surface horizontal and the pial surface vertical. Then, the tissue was sliced horizontally by a Vibratome (Oxford Laboratories, Calif., U.S.A.) to give a slice thickness of 100 to 150 μm. The slices were placed on the bottom of a superfusion chamber (Okamoto & Quastel,

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1973) and superfused (1 ml/min) for at least 45 min at 37°C with normal glucose-Ringer medium, before the first penetration with a recording microelectrode. In slices thus prepared, the molecular layer (approx. 300 μm wide) was clearly distinguishable from the granular layer, and the cell bodies of the Purkinje cells were easily identifiable under the microscope (× 100 to × 120), using transmitted light, as bright round-shaped spots near the border of the molecular and granular layers.

Discharge frequencies of spikes (spikes/s) were counted and recorded as described previously (Okamoto & Sakai, 1979). Amino acids or convulsants to be tested were ejected from the micropipettes by use of a constant current supply unit (Model S-5125A, Nihon Kohden, Tokyo).

The most sensitive spots to each amino acid were located as follows: an extracellular recording glass microelectrode (2 to 4 MΩ, filled with 2.5M NaCl) was first placed as close as possible to a Purkinje cell body in order to record spontaneous spikes, discharging on average at a rate of 55 ± 8 spikes/s (mean $\pm$ s.e.mean, $n = 80$). A single or double barrel micropipette (1 to 2 μm tip diameter, filled with solutions of amino acids or convulsants) was placed as near as possible to the cell body. The amino acid was ejected by passing currents of 3 to 4 different intensities so as to inhibit the spike discharge rate by 30 to 80%, and the results recorded. The micropipette was then moved 20 μm horizontally from the Purkinje cell body along a line perpendicular to the pial surface and passing through the centre of the Purkinje cell body. The amino acid was ejected at this point, and the results recorded. The micropipette was then shifted laterally by 10 or 20 μm steps and the ejection was made at each of these lateral points to identify the most sensitive point. The same current intensities as those applied at the previous spot were used, as long as the percentage inhibitions were in the range of 30 to 80%. However, the current intensities usually had to be changed because of differing spot sensitivities to the amino acid. Even in such cases, in addition to a new set of current intensities, one of the intensities used at the previous spot was always applied to compare the inhibitory potencies of the amino acid at each point. These procedures were repeated at 20 μm intervals until the micropipette reached the pial surface. The entire process was then repeated in reverse at 20 μm intervals, starting from the pial surface, to verify the first results.

The most sensitive spot was determined by the following two methods: comparison of the inhibitory potencies, at each spot, of the amino acid ejected by the constant current (see Figures 1 and 3) and comparison of the current intensities required to give a 50% inhibition of spike discharge frequency at each spot. The first method was found more convenient and accurate, while the second was less precise. In the second case, the slope of current intensity vs percentage inhibition usually became steeper near the most sensitive spot and the available 3 to 4 readings did not permit accurate determination of the 50% inhibition point. Although the second method was less precise, both gave similar results.

The normal medium which was superfused continuously consisted of (mM): NaCl 125, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 24 and glucose 11, pH 7.4. The iontophoretic micropipettes were filled with solutions of GABA (0.1 M or 0.5 M, pH 3.5), taurine (0.5 M, pH 8.5), glycine (0.5 M, pH 3.5), β-alanine (0.5 M, pH 3.5), picrotoxin (5 mM + 150 mM NaCl) and strychnine (5mM plus 150 mM NaCl). All of these substances were ejected by anodic current except for picrotoxin which was ejected by cathodic current. Each of the amino acids was retained in the micropipette by applying −20 to −30nA, picrotoxin by 20nA, and strychnine by −10 to −20nA.

All amino acids and convulsants used were purchased from Wako Pure Chemicals Industries, Ltd., Tokyo.

Results

Locations of the sites sensitive to amino acids

Figure 1 shows typical distributions of sensitive spots to each of the amino acids on 4 different cells. As shown in Figure 1a, the sensitive areas to GABA on this cell were found around 0 μm (the site closest to the Purkinje cell body) and 180 μm. The former site was approximately twice as sensitive to GABA as the latter determined by comparing the iontophoretic current for equipotent doses as defined in Methods. Although the width of the sensitive spots varied from cell to cell, the spot around 0 μm was never wider than 40 μm, contrasting to a somewhat broader area around 180 μm as seen in Figure 1a. On the cell shown in Figure 1b the sensitive areas to taurine were located around 0 μm, 80 μm and 200 μm. The sensitivities of the first two spots were about twice that of the last and the width of the area around 0 μm was again the narrowest. β-Alanine had three broad sensitive areas around 80 μm, 180 μm and 280 μm on the cell shown in Figure 1c. The sensitivities of these were very similar. The sensitive spots to glycine were found at around 80 μm and 200 μm on the cell shown in Figure 1d, the first being slightly more sensitive.

Since the location of the points of maximum sensitivity varied to some extent from cell to cell, histograms showing the distributions of the most sensitive spots were prepared from about 20 cells for each amino acid (Figure 2). In Figure 2 the spots of maximum sensitivity for each of the individual cells, indi-

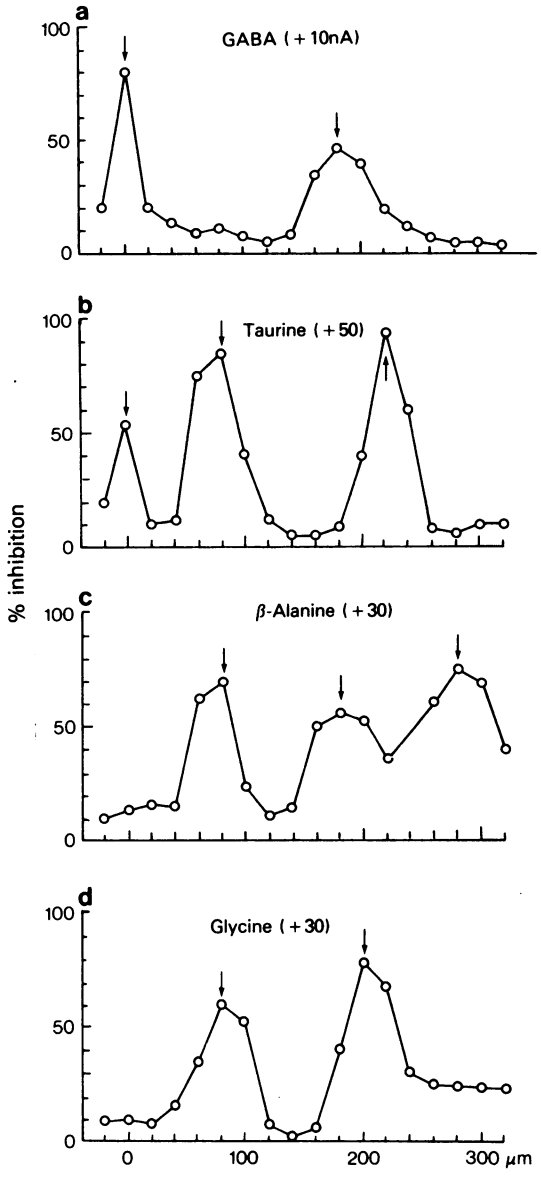

Figure 1 Typical distributions of sensitive spots to γ-aminobutyric acid (GABA, a), taurine (b), β-alanine (c) and glycine (d) on individual cells. Ordinate scales: % inhibition of discharge frequency induced by the amino acid. Abscissa scales: distance (μm) from the Purkinje cell body along a line passing through the Purkinje cell body and perpendicular to the pial surface, 0 μm being the position of the cell body and 300 μm being near the pial surface. Each of the amino acids was ejected by the current given in parentheses at all the spots tested. The most sensitive spots are indicated by the arrow. Each of these spots was counted as one and was taken, together with those obtained from other cells, to construct the histograms in Figure 2.

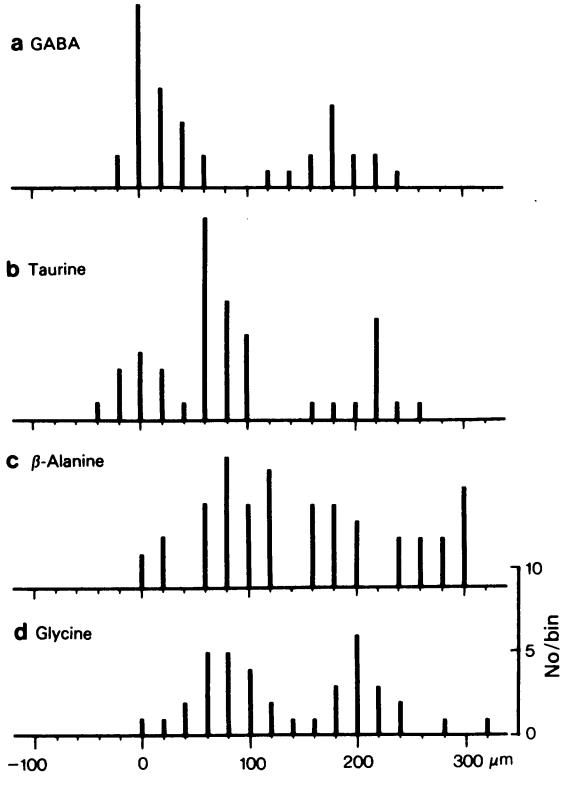

Figure 2 Histograms showing the locations of the most sensitive sites to γ-aminobutyric acid (GABA, a), taurine (b), β-alanine (c) and glycine (d) in the molecular layer of guinea-pig cerebellar slices. Vertical scale: the number of the most sensitive spots which appeared in each of the 20 μm bins; each of the most sensitive spots, as denoted by arrows in Figure 1, was counted as one. Horizontal scales: the distance (μm) from the Purkinje cell body as defined in Figure 1. The numbers of cells tested were 20 for (a), (c) and (d), and 16 for (b).

cated by arrows in Figure 1, are accumulated into the corresponding bins at 20 μm intervals (Figure 2).

In Figure 2, 20 independent observations show the most sensitive spots to GABA to be located at 0 and 180 μm, to taurine at 0, 60 and 220 μm and glycine at 80 and 200 μm. Although the sensitive spots to β-alanine were much more diffuse (Figure 2c), they appeared to occur at 80, 180 and 300 μm.

As seen in Figure 2, the most sensitive spot to GABA, at 0 μm, appeared to be quite distinct from the spots for the other three glycine-like amino acids, although there was some sensitivity for taurine at this spot (Figure 2b). However, based only on the results shown in Figure 2, it was still difficult to determine whether these three amino acids act on a common site. This specificity of the sites was then examined further.

Localization of the sensitive spots to two amino acids on the same cell

Two amino acids, from a double barrel micropipette, were alternately ejected onto a single cell, and the spots most sensitive to each of them located in the same way as in the experiments described above. The combinations of amino acids used were taurine/glycine, taurine/β-alanine and glycine/β-alanine. Typical results obtained from 3 different cells are shown in Figure 3.

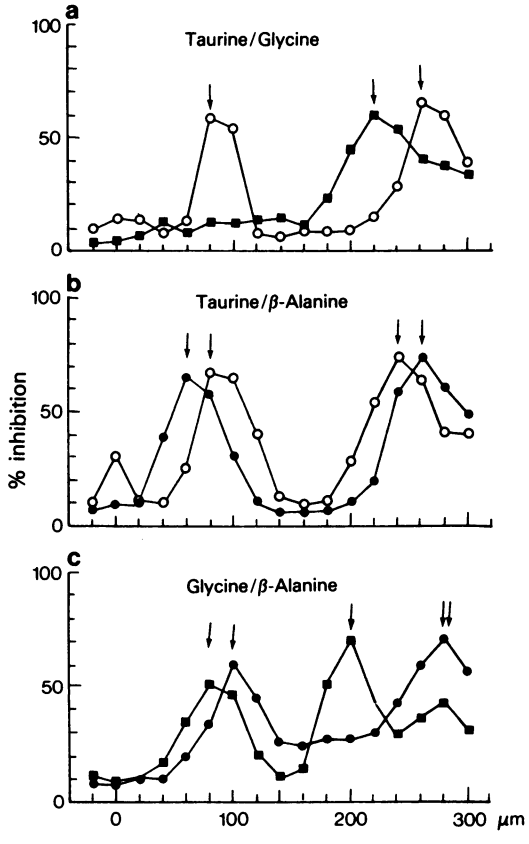

Figure 3 Locations on the same cell of the sensitive spots to two amino acids ejected from double barrel pipettes by iontophoresis. In (a) taurine (○) and glycine (■) were combined; and taurine (○) and β-alanine (●), and glycine (■) and β-alanine (●) were combined in (b) and (c) respectively. Ordinate scales: % inhibition of discharge frequency induced by the amino acid. Abscissa scales: distance (μm) from the Purkinje cell body as defined in Figure 1. The records were obtained from 3 different cells, and are typical of observations made on 6 other cells for each of the amino acid combinations. Arrows indicate the location of the most sensitive spots.

As shown in Figure 3a, there appears to be about 40 μm displacement between the peaks for taurine and glycine, and no sensitive spot to glycine is seen around 100 μm on this particular cell. Similar displacement, but only by 20 μm, is also observed for taurine and β-alanine (Figure 3b). However, as seen in Figure 3c, both glycine and β-alanine have sensitive spots at 280 μm, while around 100 μm the spots are displaced from each other by about 20 μm, and only glycine has the sensitive spot at 200 μm on this cell. Similar results, namely the displacement of the most sensitive spots, the absence of a sensitive spot to one of the paired amino acids and the sensitive spots to glycine and β-alanine being coincident, were also obtained from 6 other cells tested for each of the amino acid combinations. These results (Figure 3) seem to suggest that β-alanine and glycine might act on the same sites and taurine acts on separate sites.

Antagonistic actions of picrotoxin and strychnine

In view of well known specificity of the antagonism between GABA and picrotoxin, and glycine-like amino acids and strychnine, the selective effects of these convulsants upon the inhibitory actions of each of the four amino acids at their sensitive sites were investigated. While recording spike discharge frequencies from a Purkinje cell body, a double barrel micropipette containing separately an amino acid and a convulsant was placed onto the spots sensitive to the amino acid, and the solutions ejected. Typical results are presented in Figure 4. The inhibitory actions of all four amino acids at the sensitive sites were apparently antagonized by both picrotoxin and strychnine (Figure 4, the results for glycine and β-alanine are not shown.). However, there seemed to be some tendency for the inhibitory action of GABA to be reduced if the ejecting current for strychnine were increased, while it was completely abolished by increasing the picrotoxin ejecting current. The reverse was the case for taurine, glycine and β-alanine. It was also observed that the effect of picrotoxin on the inhibitory action of taurine was stronger on those taurine-sensitive sites closer to the Purkinje cell body (see Figure 4c). The antagonisms evoked by both picrotoxin and strychnine were reversible when the ejection of the convulsants was discontinued.

Discussion

Amino acid-sensitive sites and the neuronal structures of the cerebellum

The possible correlation of amino acid-sensitive site locations (Figure 2) with the well-established neuronal structures of the cerebellum was examined. Since the

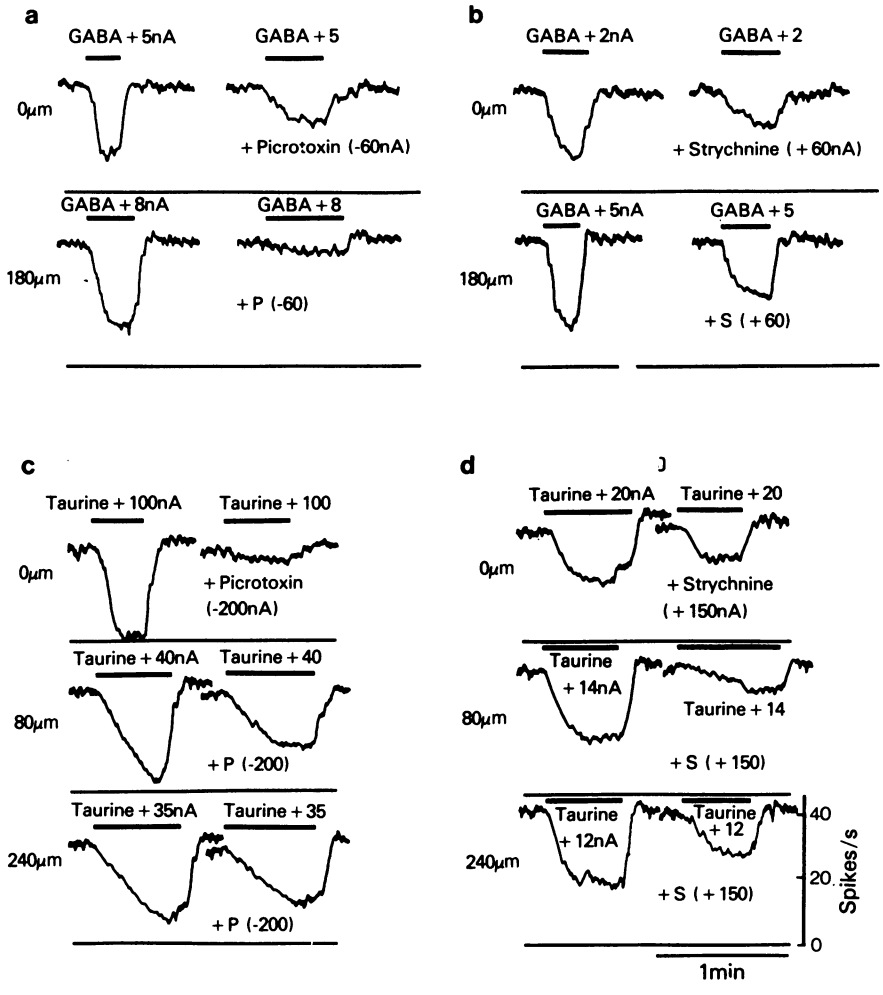

Figure 4 Antagonistic actions of picrotoxin and strychnine on the inhibitory effects of γ-aminobutyric acid (a and b) and taurine (c and d) at the most sensitive sites. Vertical scale: number of spikes per s. Horizontal scale: time, 1 min. The distances from the Purkinje cell body of the most sensitive spots to amino acid on which the amino acids and convulsants were ejected, are given on the left of each record. The amino acids were ejected, by the current indicated, for the periods denoted by the horizontal thick bars. Picrotoxin or strychnine was applied, by electrosmosis by the current given in parentheses, 3 min before and throughout the ejection of amino acids. The records are from 4 different cells and are typical of 4 other observations made with each of the combinations of the amino acid and convulsant.

detailed neuro-anatomy of guinea-pig cerebellum is not currently available, the comparison was made with the structure of cat cerebellum (Eccles, Ito & Szentágothai, 1967).

In view of the proven function of GABA as the neurotransmitter of the basket synapses on the Purkinje cell soma (Eccles *et al.*, 1967; Curtis *et al.*, 1971; Woodward *et al.*, 1971; Bisti *et al.*, 1971), the major GABA-sensitive site located at the site of the Purkinje cell body (around 0 μm, Figure 2a) is considered to represent the GABA receptor sites on the basket neuronal synapses on the Purkinje cell soma. The second less sensitive site located at around 180 μm (Figure 2a), which cannot be distinguished from the β-alanine sensitive spot around 180 μm (Figure 2c), is difficult to assign to a particular synapsis at present.

Spots sensitive to all the glycine-like amino acids tested, taurine, glycine and β-alanine, showed a rather diffused distribution (Figure 2b–2d). However, in general the two primary sensitive areas were found to be 60 to 80 μm and 200 to 300 μm from the Purkinje cell body. The locations of the synapses of the stellate

interneurones have been assigned to the outer two-thirds of the molecular layer (Eccles *et al.*, 1967), and it has been suggested that the inhibitory influence of the stellate cells is restricted to the more distal branches and spiny branchlets of the Purkinje cell dendrites (Palay & Chan-Palay, 1974). Therefore, it seems reasonable to assume that the sites sensitive to the glycine-like amino acids (Figure 2b–2d) are those of the stellate cell synapses.

The location of one of the sensitive sites to taurine, at around 60 μm, observed in guinea-pig cerebellar slices (Figure 2b) is in perfect agreement with the result reported by Frederickson *et al.*, (1978) for rat cerebellum *in vivo*. It was also found in the present study that taurine, glycine and β-alanine, all had sensitive sites in the outer molecular layer, 200 to 300 μm distant from the Purkinje cell body (Figure 2b–2d).

The excitatory action on spike discharges of taurine applied iontophoretically to the area close to the Purkinje cell body in rat cerebellum *in vivo* (Frederickson *et al.*, 1978) was not observed in the present study with guinea-pig cerebellum *in vitro*.

The sensitive site to taurine at around 0 μm (Figure 2b) is anatomically unlikely to be assigned to the stellate synapses on the Purkinje cell. Since picrotoxin blocked this taurine action fairly effectively (Figure 4c), it is conceivable that the binding sites for GABA around 0 μm (Figure 2a) are also sensitive to taurine.

Effects of picrotoxin and strychnine

Data presented in Figure 4 show the apparent lack of specificity of both picrotoxin and strychnine as antagonists of the inhibitory actions of GABA, taurine, glycine and β-alanine. In contrast Frederickson *et al.*, (1978), have shown the inhibitory actions of both GABA and taurine on rat cerebellar Purkinje cells *in vivo* not to be antagonized by strychnine, but by both bicuculline and picrotoxin. Thus, the inhibitory action of taurine on guinea-pig cerebellar Purkinje cells resembles that on the neurones in cat cerebral cortex (Curtis *et al.*, 1971) where both bicuculline and strychnine block the inhibitory action of taurine.

In our previous papers (Okamoto & Quastel, 1976; Okamoto *et al.*, 1976) it was shown that the inhibitory action of GABA is antagonized by picrotoxin, but not by strychnine, while the reverse is the case for taurine, glycine and β-alanine. The discrepancy between the previous and present results may be explained by the differences in experimental design: (a) cerebellar slices previously used were as thick as 300 to 400 μm and were cut in parallel to the pial surface, while the slices used in the present study were 100 to 150 μm thick and were cut perpendicular to the pial surface; (b) amino acids and antagonists were applied by superfusion onto the pial surface of the slices, while in the present study they were applied focally by iontophoresis. Under the previous conditions, the antagonists and amino acids might have acted primarily on sites situated immediately below the cerebellar pial surface, as antagonists applied to the pial surface by superfusion may diffuse into the tissue at different rates.

Taurine as the neurotransmitter of the stellate interneurones

Based only on this study, either taurine or glycine may be the inhibitory transmitter of the stellate neurones; β-alanine is unlikely to be the transmitter because of its low cerebellar content. The comparison of the inhibitory potency of taurine with that of glycine, at their most sensitive sites, is unreliable, because taurine was applied by electrosmosis, while glycine was applied by iontophoresis. However, considering the localized distribution of taurine in the molecular layer of rat cerebellum (Nadi *et al.*, 1977), the presence of a specific high affinity uptake system for its reuptake and the Ca^{2+}-dependence of the K^+-evoked release in guinea-pig cerebellar slices (Okamoto & Namima, 1978), it seems plausible to suggest that in the cerebellum taurine is the inhibitory neurotransmitter of the stellate interneurones.

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