K^+-STIMULATED RELEASE OF ENDOGENOUS GLUTAMATE, GABA AND OTHER AMINO ACIDS FROM NEURON- AND GLIA-ENRICHED CULTURES OF THE RAT CEREBELLUM

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1. Introduction

Of the 5 cerebellar neurons, the Purkinje, Golgi, stellate and basket cells are inhibitory and use GABA as their transmitter [1,2]. However, the identity of the transmitter of the sole excitatory neuron, the granule cell, is still in some doubt. Evidence from several lines of research has indicated that glutamate may fulfill this role [3–6]. As yet, however, glutamate release has not been demonstrated from identifiable granule cells in vivo or in vitro.

We have extensively characterized our neuron-enriched cultures of the rat cerebellum and found them to be comprised of ~95% neurons, 90% of which are thought to be granule cells. The remaining neurons (~5%) are GABAergic and are presumably stellate and basket cells [7–9]. We have used these cultures to investigate the K^+-stimulated release of the endogenous amino acids glutamate, GABA, aspartate, alanine and leucine.

These data show that of the amino acids assayed, only glutamate and GABA are released from neuron-enriched cultures in a Ca^{2+}-dependent manner in response to K^+-depolarization. Furthermore, the release is neuron specific and increases with the time the cells remain in vitro. We believe that granule cells are responsible for the glutamate release seen in these studies.

2. Methods

2.1. *Cell cultures*

Neuron- and glia-enriched cerebellar cultures from 7–8-day-old rats were prepared and maintained as in [8,10]. Cells were seeded at 2×10^4 cells/mm^2 (neuron-enriched) or 10^4 cells/mm^2 (glia-enriched) onto 22 mm^2 glass coverslips precoated with poly-(L-lysine) (50 μg/ml).

2.2. *Release experiments*

At 7 and 14 days in vitro (DIV) cultures were washed in medium and mounted in a specially designed perfusion chamber [11]. The cultures were superfused with a medium containing KCl (2 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1 mM), NaH_2PO_4 (1 mM), $NaHCO_3$ (4.2 mM), NaCl (145.3 mM), Hepes (25 mM), glucose (5.55 mM) brought to pH 7.4 at 37°C with NaOH. The superfusion medium was supplied to the chamber by a peristaltic pump at 0.4 ml/min, and four 2 min fractions were collected. The medium was then changed for one containing 50 mM KCl (balanced by reducing [NaCl]) and a further four 2 min fractions collected. In experiments designed to investigate the Ca^{2+}-dependency of released amino acids, Ca^{2+} reduced to 0.1 mM and Mg^{2+} raised to 15 mM.

2.3. *Amino acid analysis*

The amino acid content of each fraction was determined using a double isotope dansylation procedure essentially as in [12]. Briefly, known amounts of ^{14}C-labelled glutamate, GABA, aspartate, alanine and leucine were added to 50 μl aliquots of chamber effluent. The mixture was evaporated then resuspended in 10 μl 0.05 M Na_2CO_3 buffer (pH 10.2). This was then reacted with an equal volume of 5 mM [^{3}H]dansyl-chloride (spec. act. 13.3 Ci/mmol) for 60 min at 37°C in the dark. The reaction mixture was evaporated and the dansylated amino acids extracted from the salts with 5 μl 0.05 M Na_2CO_3 and 5 μl acetone:acetic acid

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(3:2). A 0.5 μl aliquot was carefully applied to the corner of a 50 mm^2 micropolyamide thin-layer chromatography plate (Schleicher and Schuell) to which cold dansylated amino acids had been applied to act as markers. The dansylated amino acids were separated from each other by ascending two-dimensional chromatography using 1.5% formic acid in the first dimension followed by benzene:acetic acid (9:1) in the second dimension. A further development in the second dimension with ethyl acetate:acetic acid:methanol (20:1:1) was necessary to separate dansyl-glutamate from dansyl-aspartate. The plates were viewed under UV light, the spots cut out and the radioactivity eluted from each spot with 1 ml butyl acetate:methanol (9:1) for 60 min prior to liquid scintillation counting. The amount in pmol amino acid in each sample was calculated from the ^3H dpm-values whilst the ^{14}C dpm channel was used as a measure of the recovery of each internal standard. The recoveries obtained in this study ranged from 49.3 ± 3.8% for aspartate to 91.4 ± 5.6% for GABA.

Baseline release was calculated as the mean of 2 fractions collected prior to depolarization. Evoked release was taken as the mean of the peak response elicited by 50 mM K^+.

3. Results and discussion

One of the major criteria used in identifying a neurotransmitter is that it should be released from a neuron in response to depolarization, the release process being dependent on Ca^{2+} and antagonized by Mg^{2+} [13]. Our results show that, of the amino acids assayed, only glutamate and GABA fulfilled this criterion.

The release characteristics of endogenous glutamate and GABA from neuron-enriched cultures of the rat cerebellum at 7 and 14 DIV are shown in fig.1. At 7 DIV, 50 mM K^+ in the presence of Ca^{2+} produced a small but significant increase in the release of both glutamate and GABA as compared to basal levels, 33% and 34%, respectively. By 14 DIV Ca^{2+}-dependent release of glutamate increased to 75% and that of GABA to 73% over control levels. The neuronal specificity of the release of these amino acids was substantiated by the fact that K^+-stimulated, Ca^{2+}-dependent release of glutamate and GABA was not seen in glia-enriched cultures (see table 1). These results suggest the development of neuronal transmitter release mechanisms, possibly linked to synapse formation, in these cultures.

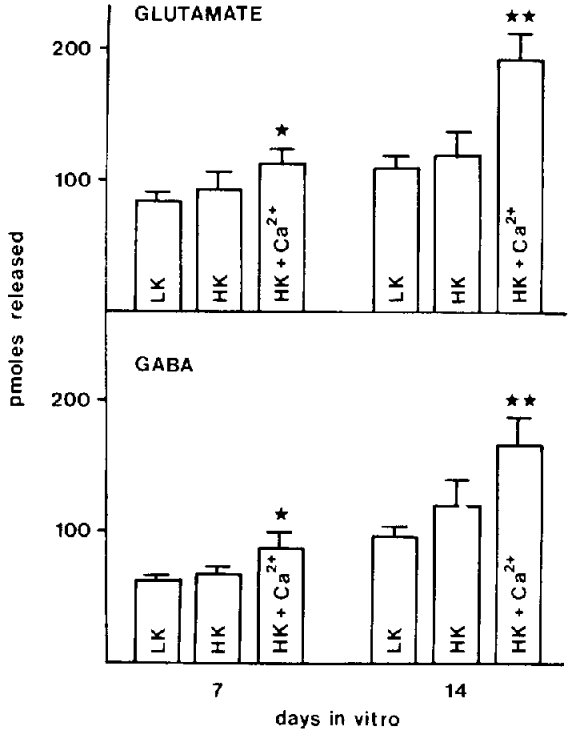

Fig.1. Release of endogenous glutamate and GABA from neuron-enriched cultures at 7 and 14 DIV: LK, spontaneous release of these amino acids in response to 2 mM K^+; HK, release evoked by 50 mM MK^+ in the presence of 0.1 mM Ca^{2+} and 15 mM Mg^{2+}; HK + Ca^{2+}, release evoked by 50 mM K^+ in the presence of 2.5 mM Ca^{2+}. Results expressed as pmol released ±SEM from 4–6 expt (data as shown in table 1). Statistical analysis by an unpaired Student's t-test gave either $^*P < 0.05$ or $^{**}P < 0.005$ for comparisons between LK and HK + Ca^{2+}

Of the other amino acids assayed, leucine and alanine are not considered to be neurotransmitters. However, aspartate may function as a transmitter in some of the cerebellar climbing fibers [14]. Our results show that, although each of these amino acids were released from cells in culture, none exhibited increased release in response to K^+-stimulation.

Interpretation of these data is made easier by the fact that the neuron-enriched cerebellar cultures have been well characterized with respect to cell type. A small population of these neurons (5–7%) possesses a high affinity uptake system for [^{3}H]GABA and is able to release [^{3}H]GABA in a Ca^{2+}-dependent manner in response to K^+-stimulation [8,9]. We have tentatively identified these neurons as stellate and basket cells, which are the likely source of the endogenous

Table 1
Release of endogenous amino acids from neuron- and glia-enriched cultures at 7 and 14 DIV

Amino acid	Neuronal		Glial
	7 DIV	14 DIV	14 DIV
Glutamate			
LK	85.25 ± 5.18	109.42 ± 10.42	295.73 ± 22.67
HK	94.37 ± 12.62	119.58 ± 18.14	300.77 ± 19.64
HK + Ca^{2+}	113.30 ± 9.65	191.40 ± 19.40	303.23 ± 2.82
GABA			
LK	63.95 ± 3.98	96.76 ± 9.39	207.66 ± 10.45
HK	67.97 ± 6.58	120.32 ± 19.40	196.74 ± 13.86
HK + Ca^{2+}	85.56 ± 13.42	167.89 ± 21.70	255.89 ± 10.93
Aapartate			
LK	129.89 ± 13.38	137.67 ± 9.93	239.33 ± 32.38
HK	158.38 ± 23.02	160.81 ± 10.60	196.49 ± 10.44
HK + Ca^{2+}	132.95 ± 23.78	152.12 ± 16.44	227.84 ± 1.93
Alanine			
LK	80.69 ± 10.28	253.07 ± 15.23	–
HK	77.04 ± 13.09	173.97 ± 13.05	–
HK + Ca^{2+}	83.94 ± 8.94	274.02 ± 56.45	–
Leucine			
LK	110.46 ± 6.82	399.62 ± 52.47	
HK	134.32 ± 26.54	350.14 ± 32.33	–
HK + Ca^{2+}	113.28 ± 14.39	348.29 ± 25.75	–

[a] Results are expressed as pmol released ±SEM from 4–6 expt; LK, HK and HK + Ca^{2+} as in fig.1

GABA release observed here. We have proposed that the majority of the neurons in these cultures are granule cells [7,8]. These are the neurons most likely to be responsible for the K^+-stimulated release of endogenous glutamate. When compared to control level, the % stimulation of endogenous glutamate and GABA release produced by depolarization was not as high as that reported from synaptosome [4] and tissue slice [5] preparations. However, it was of the same order as those in [15] for endogenous glutamate and GABA release from rat cortical cultures.

These results strongly support the proposal that glutamate is the transmitter of one of the cerebellar neurons, probably the granule cell. This is the first report of endogenous glutamate and GABA release from well-characterized neuron-enriched cultures of the rat cerebellum, demonstrating the usefulness of such cultures as a tool for studying neurotransmitter systems in vitro.

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References

[1] Curtis, D. R. and Johnson, G. A. R. (1974) Engebn. Physiol. 69, 97–188.
[2] Storm-Mathiesen, J. (1976) in: GABA in Nervous System Function (Roberts, E. et al. eds) pp. 149–168, Raven, New York.
[3] Young, A. B., Oster-Granite, M. L., Herndon, R. M. and Snyder, S. H. (1974) Brain Res. 73, 1–13.
[4] Sandoval, M. E. and Cotman, C. W. (1978) Neuroscience 3, 199–206.
[5] Foster, A. C. and Roberts, P. J. (1980) J. Neurochem. 35, 517–519.

[6] Roberts, P. J. and Sharif, N. A. (1981) in: Glutamate as a Neurotransmitter (Di Chiara, G. and Gessa, G. L. eds) pp. 295–305, Raven, New York.
[7] Currie, D. N. (1980) in: Tissue Culture in Neurobiology (Vernadakis, A. and Shahar, A. eds) pp. 75–86, Raven, New York.
[8] Currie, D. N. and Dutton, G. R. (1980) Brain Res. 199, 473–481.
[9] Piarce, B. R., Currie, D. N., Beale, R. and Dutton, G. R. (1981) Brain Res. 206, 485–489.
[10] Dutton, G. R., Currie, D. N. and Tear, K. (1981) J. Neurosci. Methods 8, 421–427.
[11] Pearce, B. R., Currie, D. N., Dutton, G. R., Hussey, R. E. G., Beale, R. and Pigott, R. (1981) J. Neurosci. Methods 3, 255–259.
[12] Clark, R. M. and Collins, G. G. S. (1976) J. Physiol. 262, 383–400.
[13] Werman, R. (1966) Comp. Biochem. Physiol. 18, 745 766.
[14] Nadi, N. S., McBride, W. J. and Aprison, M. H. (1977) J. Neurochem. 28, 453–455.
[15] Snodgrass, S. R., White, W. F., Biales, B. and Dichter, M. (1980) Brain Res. 190, 123 138.