Volume 223, number 1, 143-147 FEB 05216 October 1987

Glutamate acting on NMDA receptors stimulates neurite outgrowth from cerebellar granule cells

Ian A. Pearce, Martin A. Cambray-Deakin and Robert D. Burgoyne

MRC Secretory Control Group, The Physiological Laboratory, The University of Liverpool, PO Box 147, Liverpool L69 3BX, England

Received 2 September 1987

The effect of endogenous glutamate on neurite outgrowth from cerebellar granule cells in culture was examined. Neurite outgrowth was inhibited by enzymatic removal of endogenous glutamate from the culture medium. The broad-spectrum glutamate receptor antagonist kynurenate also inhibited neurite outgrowth from granule cells in serum-containing and serum-free cultures; the inhibition by kynurenate was reversed by exogenous glutamate. Neurite outgrowth was inhibited to the same extent by the NMDA receptor antagonist APV. These results indicate that endogenous glutamate, possibly released by granule cells themselves, stimulated neurite outgrowth through activation of the NMDA class of glutamate receptors. Activation of NMDA receptors on developing neurons may be an important mechanism for the regulation of neuronal growth and differentiation.

Neurite; Glutamate; *N*-Methyl-D-aspartate; Neuronal differentiation; (Cerebellum)

1. INTRODUCTION

A great deal of attention has been paid to the isolation of factors that regulate survival and differentiation of CNS neurons [1] in the way that NGF acts on peripheral sympathetic and sensory neurons [2]. So far, limited progress has been made in the precise identification of such molecules. One possibility is that conventional neurotransmitters could regulate neuronal differentiation. Glutamate is a major excitatory neurotransmitter in the CNS which acts on three receptor types classified on the basis of their agonists as quisqualate, kainate or *N*-methyl-D-aspartate (NMDA) receptors [3,4]. Activation of NMDA but not the other two classes of glutamate receptors results in the opening of channels that allow Ca^{2+} influx [5] and leads to a rise in cytosolic free Ca^{2+} [6,7]. It has been suggested that the growth of neurites from isolated neurons is controlled by the level of cytosolic calcium in the growing tip of the neurite [8-10] and therefore activation of NMDA receptors is a potential mechanism for the control of neurite growth.

Immature cerebellar granule cells in culture release glutamate in both a basal- and depolarisation-dependent fashion [11,12]. In addition, these cells possess NMDA receptors [13] which are linked to channels with a conductance of 50 pS [14,15]. We have examined the possibility that endogenous glutamate acting through NMDA receptors stimulates neurite outgrowth from cerebellar granule cells in culture.

Correspondence address: R.D. Burgoyne, MRC Secretory Control Group, The Physiological Laboratory, University of Liverpool, PO Box 147, Liverpool L69 3BX, England

2. MATERIALS AND METHODS

Cerebellar granule cells were dissociated from the cerebella of 4 or 5-day-old rats by trypsin treatment [16,17], resuspended in culture medium and

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

plated at a cell density of $1 \times 10^3/mm^2$ onto poly-L-lysine-coated glass coverslips in the wells of a 24-well tray. Culture medium was either serum$^+$ (Eagle's minimal essential medium containing 10% fetal calf serum, 2.5% chick embryo extract, 100 μg/ml gentamycin, 33 mM glucose, 290 μg/ml glutamine, 25 mM KCl) or serum$^-$ (Eagle's minimal essential medium containing 30 μg/ml insulin, 30 nM sodium selenite, 33 mM glucose, 290 μg/ml glutamine, 25 U/ml penicillin, 25 μg/ml streptomycin, 25 mM KCl). In the case of drug additions the drugs were present throughout the period of culture. After 8 h the cells were fixed with 4% formaldehyde in PBS, examined on an Olympus inverted IMT microscope and the percentage of granule cells with processes scored. In each well 350 cells were counted.

3. RESULTS

Cultures of dissociated cells of the early postnatal rat cerebellum consist predominantly [19,20] of granule cells (92–95% of the cell population) which can easily be distinguished from the other cell types by their small size (diameter 5–7.5 μm). Granule cells were grown in sparsely seeded cultures to minimise cellular interactions and to facilitate assessment of neurite outgrowth. When placed into culture, some granule cells begin to extend neurites within minutes [21]. By 8 h after plating $23 \pm 1\%$ ($n = 35$) of cells had extended neurites (fig.1) and were examined at this time in order to assess the rate of initiation of neurite outgrowth rather than long term neurite growth and cell survival. By 1 day in culture most granule cells have extended neurites. The neurites are axonal in nature since after 1 or more days in culture they contain synaptic vesicles [22].

Addition of low concentrations (30 μM) of NMDA to the cultures had little effect on neurite outgrowth and higher concentrations were neurotoxic (not shown). Since granule cells in culture release glutamate the possibility that endogenous glutamate was sufficient to stimulate neurite outgrowth was tested. In serum containing cultures in which glutamate receptors were blocked by the broad-spectrum glutamate receptor antagonist kynurenate [23] neurite outgrowth was inhibited

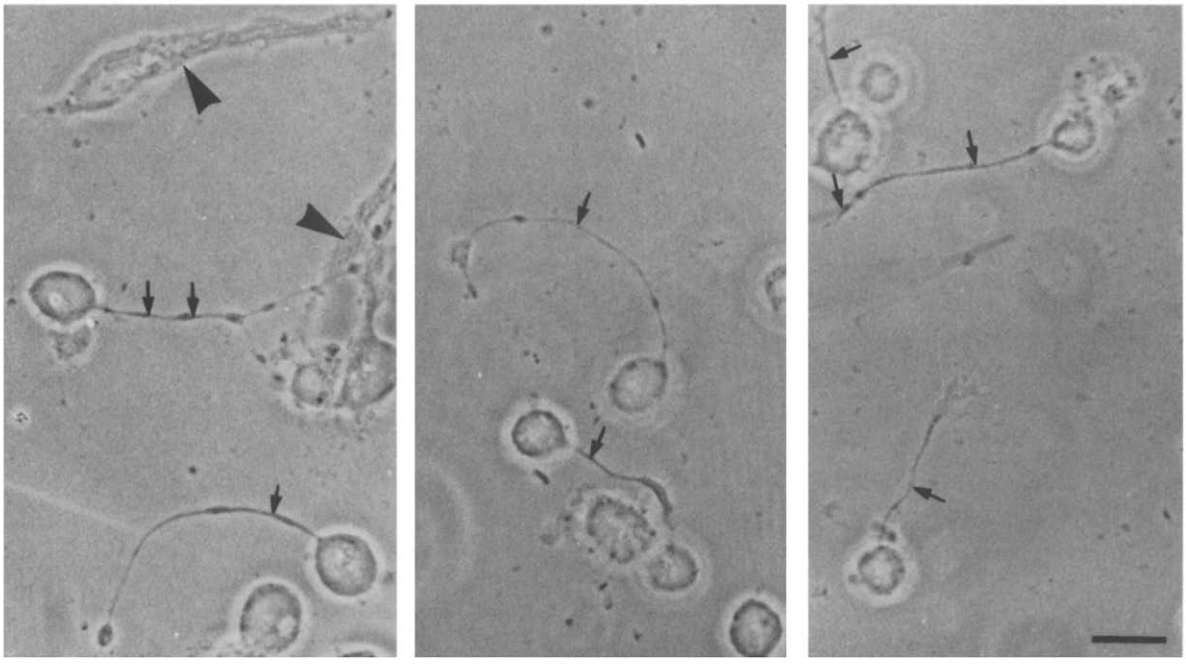

Fig.1. Phase-contrast micrographs of granule cells fixed after 8 h in culture in a serum$^+$ medium. The three panels show the range of morphologies of granule cell neurites (arrows). The granule cells can easily be distinguished from non-neuronal cells (arrowheads). Bar, 10 μm.

by 43% (fig.2). Kynurenate also inhibited neurite outgrowth in serum$^-$ cultures by 54% (fig.2) showing that serum was not the sole source of glutamate in the cultures. These results suggest that endogenous glutamate released by granule cells in culture is sufficient to stimulate neurite outgrowth.

In order to exclude the possibility that the inhibitory effect of kynurenate was a non-specific effect of the drug rather than an effect on glutamate receptors we examined the effect of addition of exogenous glutamate on the kynurenate inhibition. As shown in fig.3 exogenous glutamate prevented the inhibition of neurite outgrowth by kynurenate consistent with a direct effect of the antagonist on glutamate receptors.

The role of NMDA receptors in the stimulation of neurite outgrowth by endogenous glutamate was examined by growing the cells in the presence of the selective NMDA receptor antagonist [3,4] D-2-amino-5-phosphonovalerate (APV). APV inhibited neurite outgrowth to the same extent as kynurenate (fig.3) and the inhibition by APV was

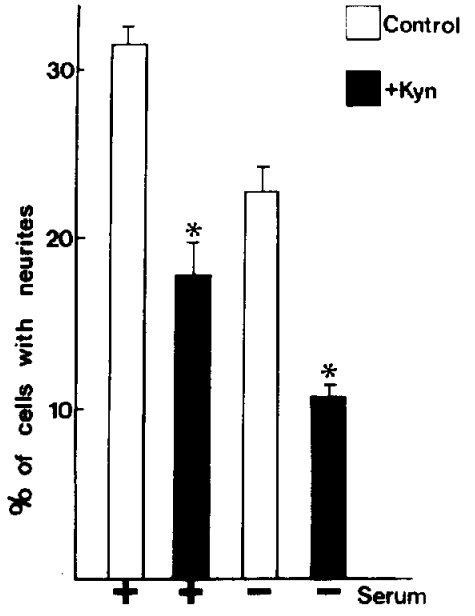

Fig.2. Effect of kynurenate on neurite outgrowth from granule cells in serum$^+$ and serum$^-$ cultures. Granule cells were grown in the indicated medium in the presence or absence of 3 mM kynurenate. Data shown are the percentage of cells with neurites from counts of 350 cells in 4 wells per treatment. * $p<0.001$ compared to the respective control.

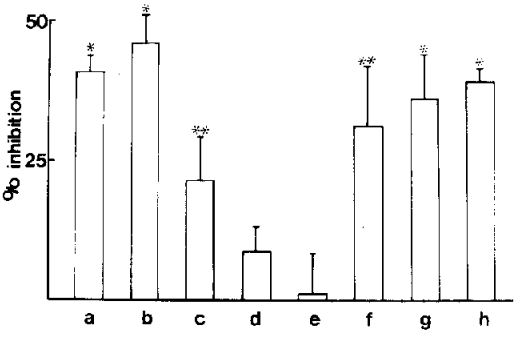

Fig.3. Effect of kynurenate and APV on neurite outgrowth from granule cells in serum$^+$ cultures. (a) 3 mM kynurenate; (b) 3 mM kynurenate + 10 μM glutamate; (c) 3 mM kynurenate + 100 μM glutamate; (d) 3 mM kynurenate + 250 μM glutamate; (e) 3 mM kynurenate + 1 mM glutamate; (f) 30 mM DL-APV; (g) 300 μM DL-APV; (h) 1 mM DL-APV. The data shown are expressed as percentage inhibition of neurite outgrowth and are taken from 4–10 wells per treatment. In control wells $25 \pm 2\%$ ($n = 16$) of granule cells had neurites. * $p<0.001$; ** $p<0.02$ compared to control.

virtually maximal at 30 μM DL-APV. The specificity of the inhibitory effect of APV was demonstrated in a separate series of experiments in which 30 μM DL-APV inhibited neurite outgrowth by $43 \pm 13\%$ ($n = 4$) but the inhibition was reduced to $0.2 \pm 0.2\%$ ($n = 4$) when 30 μM NMDA was also present.

The requirement for endogenous glutamate for normal neurite outgrowth in culture was also demonstrated by enzymatic removal of glutamate from the culture medium. Serum$^+$ cultures were maintained in the presence of 2 mM pyruvate and 1 unit/ml glutamate pyruvate transaminase which should result in conversion of endogenous glutamate to α-ketoglutarate. This treatment resulted in a $52.5 \pm 3.4\%$ ($n = 7$) inhibition of neurite outgrowth.

4. DISCUSSION

The results presented here suggest that endogenous glutamate released by granule cells in culture stimulates neurite outgrowth. This action of glutamate appears to be mediated by the NMDA class of glutamate receptors. Glutamate analogues have been shown to stimulate production of cGMP [24,25] and inositol phosphates [20]

in granule cell cultures. The production of both of these messengers was not restricted to NMDA but was seen after activation of all three glutamate receptor types. However, a major difference between NMDA and non-NMDA receptor agonists is the specific ability of NMDA to open channels which allow Ca^{2+} influx [5] and a rise in cytosolic free Ca^{2+} [6,7]. We have found that NMDA elevates cytosolic free calcium in cerebellar granule cells in culture (our unpublished observations). Cytosolic free calcium has been suggested to be involved in the control of neurite outgrowth [8–10] and so it is likely that the mode of action of glutamate in stimulating neurite outgrowth involves the NMDA-receptor mediated rise in cytosolic calcium. Consistent with this we found (unpublished) that addition of 3 mM EGTA to granule cell cultures inhibited neurite outgrowth by 80%. This effect, however, could also involve inhibition of cellular adhesion.

The NMDA receptor is partially inhibited at physiological concentrations (1 mM) of Mg^{2+} and this block is overcome by depolarisation. In our standard culture conditions with 0.8 mM Mg^{2+} in the medium the Mg^{2+}-block would be at least partially overcome by depolarisation by the 25 mM K^+ present. It is also possible that immature granule cells may respond to NMDA without additional depolarisation since it has recently been shown that while the resting membrane potential of mature granule cells in culture is -60 mV that of immature granule cells is -30 to -35 mV [27]. NMDA receptors are also regulated by glycine [28] which increases their sensitivity to agonist. The culture medium used in this study did not contain added glycine but the possibility does exist that the action of glutamate on NMDA receptors in the cultures could have been potentiated by glycine released from granule cells.

The findings of this study suggest that, at least for the cerebellar granule cell, glutamate could have a role in the stimulation of neurite outgrowth during development. The conditions under which glutamate stimulates neurite outgrowth in vivo remain to be determined as does the sensitivity of other developing neurons to this widespread neurotransmitter. It is of interest that higher levels of responsiveness to NMDA have been found for immature cerebellar granule cells, Purkinje cells [13] and visual cortex neurons [29] compared to these cell types in the adult brain. Whether these developmental changes are related to an important role of the NMDA receptor at critical stages of brain development is as yet unknown.

ACKNOWLEDGEMENTS

This work was supported by the Wellcome Trust. We thank Dr J. Garthwaite for his helpful comments during the course of this project and the reviewers for suggesting the use of glutamate pyruvate transaminase.

REFERENCES

[1] Thoenen, H. and Edgar, D. (1985) Science 229, 238–242.
[2] Levi-Montalcini, R. (1982) Annu. Rev. Neurosci. 5, 341–362.
[3] Watkins, J. and Evans, R.H. (1981) Annu. Rev. Pharmacol. Toxicol. 21, 165–204.
[4] Foster, A.C. and Fagg, G.E. (1984) Brain Res. Rev. 7, 103–164.
[5] Jahr, C.E. and Stevens, C.F. (1987) Nature 325, 522–525.
[6] MacDermott, A.B., Mayer, M.L., Westbrook, G.L., Smith, S.J. and Barker, J.L. (1986) Nature 321, 519–521.
[7] Miller, R.J. (1987) Science 235, 46–52.
[8] Anglister, L., Farber, I.C., Shahar, A. and Grinvald, A. (1982) Dev. Biol. 94, 351–365.
[9] Bolsover, S.R. and Spector, I. (1986) J. Neurosci. 6, 1934–1940.
[10] Connor, J.A. (1986) Proc. Natl. Acad. Sci. USA 83, 6179–6183.
[11] Pearce, B.R. and Dutton, G.R. (1981) FEBS Lett. 135, 215–218.
[12] Gallo, V., Ciotti, M.T., Coletti, A., Aloisi, F. and Levi, G. (1982) Proc. Natl. Acad. Sci. USA 79, 7919–7923.
[13] Garthwaite, J., Garthwaite, G. and Hajos, F. (1986) Neuroscience 18, 449–460.
[14] Cull-Candy, S.G. and Ogden, D.C. (1985) Proc. R. Soc. Lond. B. 224, 367–373.
[15] Cull-Candy, S.G., Howe, J.R. and Ogden, D.C. (1987) J. Physiol., in press.
[16] Currie, D.N., Dutton, G.R. and Cohen, J. (1979) Experientia 35, 345–347.
[17] Dutton, G.R., Currie, D.N. and Tear, K. (1981) J. Neurosci. Methods 3, 421–427.
[18] Currie, D.N. (1980) in: Tissue Culture in Neurobiology (Giacobini, E. et al. eds) pp. 75–89, Raven, New York.

[19] Nicoletti, F., Wroblewski, J.T., Novelli, A., Alho, H., Guidotti, A. and Costa, E. (1986) J. Neurosci. 6, 1905–1911.
[20] Cambray-Deakin, M.A., Morgan, A. and Burgoyne, R.D. (1987) Dev. Brain Res., in press.
[21] Cambray-Deakin, M.A., Norman, K.M. and Burgoyne, R.D. (1987) Dev. Brain Res. 34, 1–7.
[22] Quinlan, J.E. and Davis, J. (1985) Neurosci. Lett. 66, 39–46.
[23] Garthwaite, J. and Garthwaite, G. (1987) J. Neurochem. 48, 29–39.
[24] Novelli, A., Nicoletti, F., Wroblewski, J.T., Alho, H., Costa, E. and Guidotti, A. (1987) J. Neurosci. 7, 40–47.
[25] Nowak, L., Bregostovski, P., Ascher, P., Herbet, A. and Prochiantz, A. (1984) Nature 307, 462–465.
[26] Mayer, M.L., Westbrook, G.L. and Guthrie, P.B. (1984) Nature 309, 261–263.
[27] Hockberger, P.E., Tseng, H.Y. and Connor, J.A. (1987) J. Neurosci. 7, 1370–1383.
[28] Johnson, J.W. and Ascher, P. (1987) Nature 325, 529–531.
[29] Tsumoto, T., Hagihara, K., Sato, H. and Hata, Y. (1987) Nature 327, 513–514.