Acta Neuropathol (Berl) (1987) 74:183–190

Acta Neuropathologica

Selective kainic acid lesions in cultured explants of rat hippocampus*

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Summary. The influence of the excitotoxin kainic acid (KA) on cultivated explants of rat hippocampus was investigated. Addition of 3 μM KA to the culture medium over 24–48 h induced a destruction of the pyramidal cells in the CA3 region, whereas the CA1 pyramidal cells and the granule cells were left undamaged. Higher concentrations (10–100 μM) of KA destroyed also the latter cell groups. The selectivity of the KA lesion at 3 μM was further indicated by the fact that the acetylcholinesterase-positive neurons in the hippocampus were not destroyed through KA administration and that the stereoisomer dihydrokainic acid was ineffective in inducing lesions. Application of tetrodotoxin did not protect the CA3 pyramidal cells from KA lesion, whereas γ-glutamylaminomethylsulphonic acid (GAMS) only offered a very small, statistically not significant, protection. Baclofen protected the cultures slightly from KA lesions but not when added together with GAMS. Possible mechanisms responsible for the KA lesions in these cultures are discussed.

Key words: Kainic acid – Selective lesion – Cultured hippocampus

The effects in the brain of kainic acid (KA), a neuroexcitatory and neurotoxic glutamate analogue, have been extensively studied during the last decade, in situ (Köhler et al. 1978; Olney et al. 1979; Nadler et al. 1980) as well as under culture conditions (Honegger and Richelson 1977; Seil et al. 1979; Panula 1980; Whetsell and Schwarcz 1983). KA preferentially destroys the perikarya in the injected brain structure, whereas the passing fibres are mostly spared (see McGeer and McGeer 1981, for discussion). The damage following KA injection into the rat brain supposedly depends on two different mechanisms. Systemically or intraventricularly injected KA causes neuronal degeneration away from the injection site through the inducement of epileptic seizure activity (Nadler 1981; Ben-Ari et al. 1980). Locally injected KA, however, probably destroys the neurons through a receptor-mediated excitation of the cells. It was initially assumed (Olney et al. 1974) that KA exerted its neurotoxic effect through binding to and overexcitation of glutamate receptors. The discovery that KA receptors, distinct from glutamate receptors (London and Coyle 1979; Hall et al. 1978) are present on neuronal perikarya and dendrites, [in the case of the hippocampus especially on the pyramidal cells in the CA3 region (Foster et al. 1981)], led to the suggestion that endogenous excitotoxins, similar to KA in structure and action, might exist in the brain and be responsible for various brain disorders (Schwarcz et al. 1984). Quinolinic acid (Schwarcz et al. 1984; Foster et al. 1985) and glutamate and aspartate dipeptides (Bernstein et al. 1985) have been mentioned as possible candidates.

The studies of the effects of KA in the rat brain in situ have been accompanied by certain difficulties. Injection trauma (McGeer and McGeer 1981) and uneven distribution and diffusion of the drug (Zaczek et al. 1980) may impede the interpretation of the results. It has also been found that the type of anaesthesia used may influence the extent and severity of the so called "distant lesions" (Zaczek et al. 1978; Olney et al. 1986). Furthermore, secondary effects of the status epilepticus induced by KA administration, for example oxygen deprivation and impaired blood flow (Sperk et al. 1983), might cause unspecific damage to perikarya and fibres in the brain, although this has been disputed (Pinard et al. 1984). Some of

* Supported in part by a grant from the Swiss National Foundation for Scientific Research (No. 3.528.-0.83)

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these difficulties of in situ KA studies are avoided by using an in vitro preparation. Hippocampal explants cultivated according to the roller tube technique (Gähwiler 1981) have previously been shown to retain many of the morphological (Gähwiler 1984; Keller et al. 1986) and physiological (Gähwiler 1984; Gähwiler and Dreifuss 1982; Keller et al. 1983; Rimvall et al. 1985) in situ characteristics. This induced us to investigate the effects of KA on organotypic cultures of rat hippocampal explants.

Methods

Cultures were prepared as previously described (Gähwiler 1981; Rimvall et al. 1985). Briefly, hippocampal explants were dissected from 7-day-old rats (strain SIV 50) and subsequently embedded on glass cover slips (Gribi or Gold Seal) in plasma clots (plasma, Difco; thrombin, Roche). Thereafter, they were placed in roller tubes, medium was added and the tubes were incubated in a roller drum at 36°C. The medium contained 25% heat-inactivated horse serum (Seralab), 50% Eagle's basal medium (BME; Gibco) with Earle's salts, 25% Hanks' balanced salt solution (BSS; Gibco) and glucose (6 mg/ml) and was changed once a week.

The cultures used in this investigation were selected by viewing them, in their roller tubes, under a dark field illumination dissection microscope. Only explants with all cell layers intact were used. In this study the hippocampal formation, consisting of the area dentata with the granule cell layer, the hippocampus proper with the pyramidal cell layer, and parts of the subiculum (Storm-Mathisen 1977), will be referred to as the hippocampus. The pyramidal cell layer is further divided into the CA1 and CA3 regions as described by Storm-Mathisen (1977). Due to the heterogeneity of the CA4 area and of the hilus fasciae dentatae, the influence of KA on these regions was not judged separately, but together with the CA3 area.

KA (Sigma) was added to the nutrient medium in various concentrations (1 µM to 100 µM) as indicated in the legends and tables. After incubation periods ranging from 1 to 72 h (usually 48 h) the cultures were washed in 2 ml Hanks' BSS, fed 1 ml fresh medium and allowed to survive for at least 3 days. The explants were thereafter fixed in a sucrose-formalin solution (9 g NaCl, 75 g sucrose, 100 ml formalin, 900 ml distilled water) for 10–15 min and stained with either cresyl violet or neutral red.

Each Nissl stained culture was judged under the microscope by the same microscopist. Depending on the degree of destruction of the hippocampal explants, each culture was graded on a scale from 1 to 4:

Grade 1: normal culture with pyramidal cell layer and granule cell layer intact.

Grade 2: small to moderate reduction in pyramidal cell number in CA3, granule cell layer intact.

Grade 3: severe to total reduction in pyramidal cell number in CA3, granule cell layer intact.

Grade 4: total degeneration of the whole culture.

The *mean grade* for a series of cultures which had received the same treatment was defined as this group's *lesion index*.

Some cultures were also stained for acetylcholinesterase (AChE) activity according to a modified Karnovsky-staining procedure (El-Badawi and Schenk 1967). In some Karnovsky-stained cultured explants, the exact numbers of AChE-positive cells in different parts of the hippocampal explants were counted.

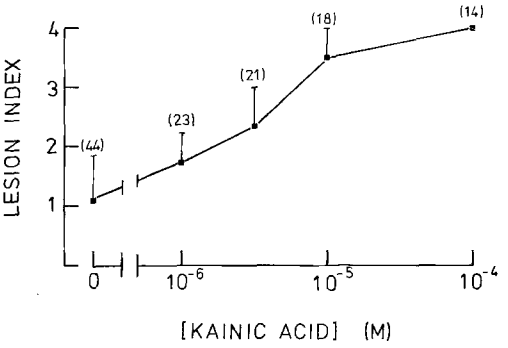

Fig. 1. Cultures of the rat hippocampus [> 8 days in vitro (DIV)] were incubated with different concentrations of kainic acid (KA) for 24–48 h. The cultures were subsequently washed, allowed to survive for 5 days in a normal nutrient medium, Nissl stained and evaluated under the light microscope. Abscissa, concentration of KA in M; Ordinate, lesion index (see Methods for explanation). Each point represent a mean lesion index and standard deviation. The number of cultures is indicated in parentheses

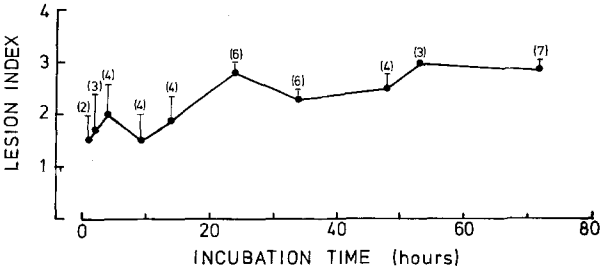

Fig. 2. Cultures of the rat hippocampus (14 DIV) were incubated with 3 µM KA for various periods of time. The cultures were subsequently washed, allowed to survive for 4 days in a normal nutrient medium, Nissl stained and evaluated under the light microscope. Abscissa, incubation time in h; Ordinate, lesion index (see Methods). Each point represent a mean lesion index and the standard error of mean. The number of cultures is indicated in parentheses

The counting was performed with camera lucida drawings of the cultures.

Radiochemical determinations of the AChE activity in the hippocampal cultures were performed using a modified version of the method of Johnson and Russel (1975) as previously described (Rimvall et al. 1985). [^{3}H]Acetylcholine (Amersham) was used as substrate and the AChE activity was expressed either on a per culture basis (pmol acetate/min per explant) or per mg protein. The protein content was determined with the Coomassie blue method described by Bradford (1976) using bovine serum albumin as a standard.

In experiments investigating the influence of different substances on KA-induced explant lesions, a preincubation period of at least 2 h with the tested drug alone preceded the actual incubation (48 h) with both KA and the tested drug. The substances employed in these experiments were the kainate receptor antagonist γ-glutamylaminomethylsulphonic acid (GAMS; 10, 100 and 300 µM; Tocris Chemicals), baclofen (10 µM; kindly donated by Ciba-Geigy, Basel) and the blocker of the voltage-dependent sodium channels tetrodotoxin (TTX; 3 and 100 µM). The stereospecificity of the KA-induced hippocampal lesions

was checked with the analogue of KA dihydrokainic acid (3 and 100 μM; Sigma).

Results

Concentration of KA

In preliminary experiments, the appropriate dose of KA for a selective destruction of hippocampal CA3 pyramidal cells was established. The effects of different concentrations of KA on cultivated hippocampal explants is shown in a dose-response curve (Fig. 1). Treatment with concentrations of KA above 10 μM during 48 h led to an almost complete destruction of the cultures (lesion index 4) as compared with controls [0 μM KA; lesion index 1.2 (= 41 undamaged cultures and three spontaneously degenerated cultures)]. At concentrations of KA of 1 μM the lesion index was only 1.7 (SD = ± 0.54). A KA dose of 3 μM was chosen for routine work as it gave a lesion index of around 2.5.

Duration of incubation with kainic acid

In Fig. 2 the effect of 3 μM KA on the cultured hippocampal explants as a function of the incubation time is depicted. As can be seen, the mean lesion indices in the cultures which had been incubated with KA for less than 20 h were under 2 and accompanied by fairly large standard errors. In cultures incubated for more than 20 h the lesion indices approached 3 and were more constant between separate cultures (lower standard errors). Routinely, the cultures were incubated with KA for 24–48 h. A post-incubational survival time of 3–5 days was found to be appropriate. Longer survival times, up to 10 and 15 days, did not enhance the lesion index for the cultures treated with KA.

Light-microscopic observations

The cultures were examined under the phase contrast microscope before being Nissl stained. In a control culture, the pyramidal cell layer was intact and myelin was visible. In cultures, treated with 3 μM KA for 48 h, one could observe degenerated myelin strands, big, swollen cell bodies and increased density in the CA3 area. These changes were also grossly visible when examining the cultures, in their roller tubes, under the dissection microscope (magnification × 4).

In a Nissl-stained hippocampal culture, not treated with KA, the pyramidal cell layer and the granule cell layer were usually intact [lesion index 1; Fig. 3A; 36 days in vitro (DIV)]. The infrapyramidal blade of the area dentata had a tendency to spread out on the cover slip (Fig. 3B) and sometimes it was destroyed

Table 1. Effects of kainic acid (KA) and dihydrokainic acid on cultured explants of hippocampus [drug administration at 14 days in vitro (DIV)]. Each lesion index value represents the mean destruction (± SEM) in the number of cultures which has received the same drug treatment (see Methods for details)

Drug	Concentration (μM)	Lesion index (± SEM)	Number of cultures
–	–	1.0±0	4
KA	3	2.6±0.17*	8
Dihydrokainic acid	3	1.3±0.22	10
Dihydrokainic acid	100	1.0±0	11

* Lesion index significantly ($P < 0.005$; U-test of Wilcoxon, Mann and Whitney; Sachs 1972) different from indeces of control and dihydrokainic acid-treated culture groups

Table 2. Effects of baclofen, GAMS and TTX on the sensitivity of the pyramidal cells in cultured explants of hippocampus for KA (drug administration at >9 DIV). GAMS: γ-Glutamylaminomethylsulphonic acid; TTX: tetrodotoxin

Drug	Concentration (μM)	Lesion index (± SEM)	Number of cultures
KA	3	2.9±0.11	16
Baclofen	10	1.3±0.25	4
KA + baclofen	3 + 10	2.4±0.18[a]	19
KA	3	2.8±0.25	4
GAMS	10	1.0±0	3
KA + GAMS	3 + 30	2.4±0.20	7
KA + GAMS	3 + 100	2.0±0.23	11
KA + GAMS	3 + 300	2.4±0.30	7
KA	3	2.7±0.17	11
KA + GAMS + baclofen	3 + 100 + 10	2.4±0.17	14
KA	3	2.6±0.25	5
TTX	3	1.2±0.20	5
KA + TTX	3 + 3	2.8±0.29	10

[a] Lesion index significantly different ($P < 0.04$) from lesion index in the culture group treated with KA

(Zimmer and Gähwiler 1984, for possible explanations). In every series of experimental cultures it was, however, checked that the number of cultures with such an "unspecific" lesion of the infrapyramidal part of the granule cell layer, was similar in the control groups and in the test groups.

Figure 3B shows a Nissl-stained hippocampal culture which received 3 μM KA at day 22 in vitro. A partial destruction of the pyramidal cell layer in the CA3 area was apparent and the CA1 cells and the granule cells were spared (= lesion index 2). A culture with a lesion index of 3 is shown in Fig. 3C (36 DIV). Here, the CA3 pyramidal cells were totally destroyed, whereas the CA1 cells and the granule cells survived.

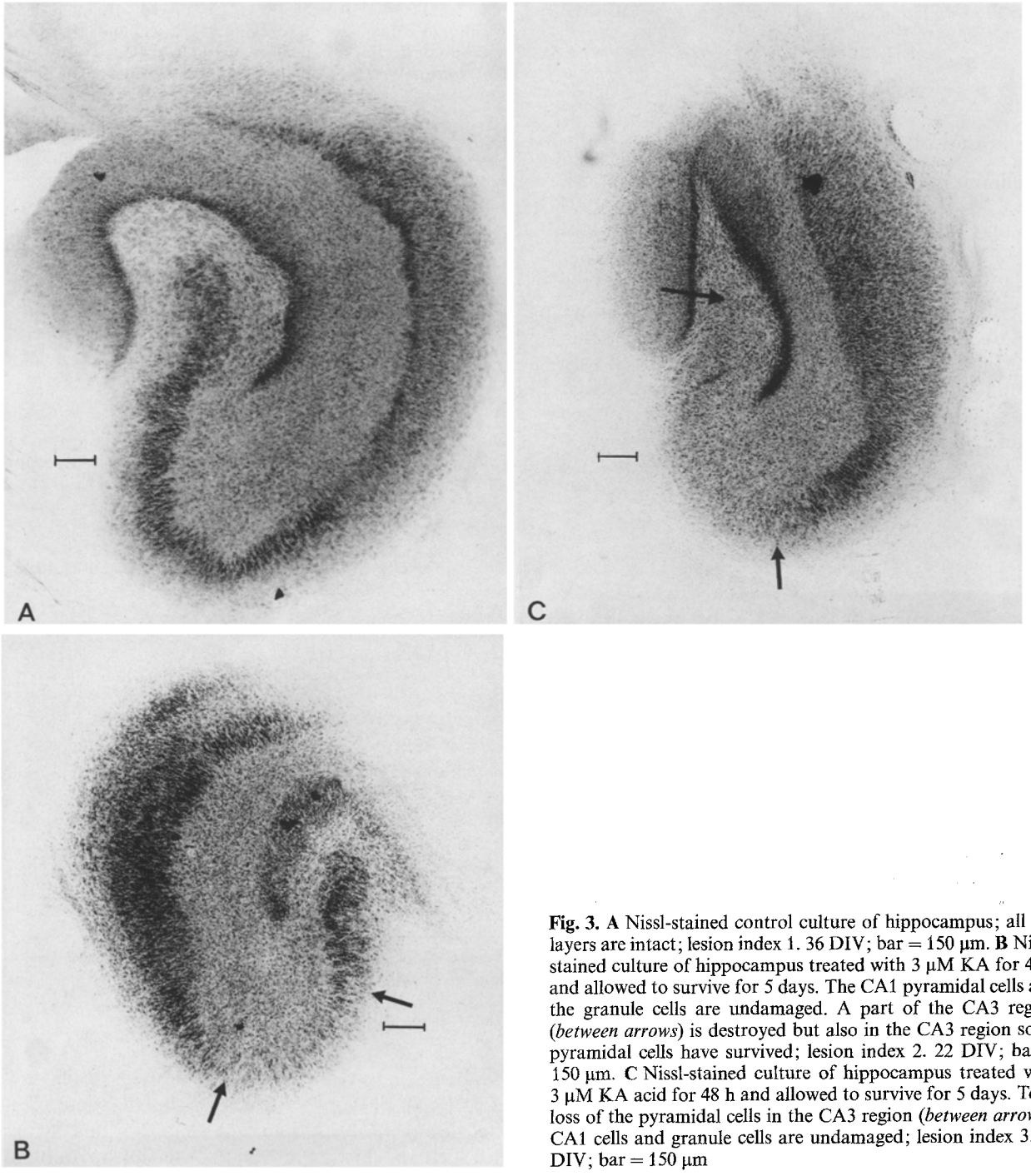

Fig. 3. **A** Nissl-stained control culture of hippocampus; all cell layers are intact; lesion index 1. 36 DIV; bar = 150 µm. **B** Nissl-stained culture of hippocampus treated with 3 µM KA for 48 h and allowed to survive for 5 days. The CA1 pyramidal cells and the granule cells are undamaged. A part of the CA3 region (*between arrows*) is destroyed but also in the CA3 region some pyramidal cells have survived; lesion index 2. 22 DIV; bar = 150 µm. **C** Nissl-stained culture of hippocampus treated with 3 µM KA acid for 48 h and allowed to survive for 5 days. Total loss of the pyramidal cells in the CA3 region (*between arrows*); CA1 cells and granule cells are undamaged; lesion index 3. 36 DIV; bar = 150 µm

Drug influence

The KA analogue dihydrokainic acid had no destructive effect on the hippocampal explants at all (Table 1). Table 2 shows the influence of some other drugs on the KA-induced hippocampal lesions. Tetrodotoxin did not protect the hippocampal explants from the specific destruction of pyramidal cells through KA. The drug GAMS had only very small, statistically not significant (two-tailed *U*-test of Wilcoxon, Mann and Whitney; Sachs 1972) protective effect. Baclofen afforded a protection against KA lesions, which just reached statistically significant levels ($p < 0.04$; Table 2). However, GAMS and baclofen given together had no additive protective effect.

AChE biochemistry and histochemistry

KA treatment of hippocampal cultures did not induce any loss in biochemically determined AChE when expressed on a per culture basis (Table 3). Due to a statistically significant (20%) protein loss after KA administration, the AChE content in these explants increased somewhat when it was expressed per mg protein. No decrease in the number of AChE-positive cells in the cultivated hippocampal explants, treated with KA and thereafter stained for AChE activity, could be observed. Rather, one observed an increase in the number of AChE-positive cells in the cultures treated with KA (Table 3). This increase was most prominent in the stratum pyramidale of the CA3 area and, thus, was probably a consequence of an increased penetration of staining medium and a decreased background secondary to the observed protein and cell loss.

Table 3. Influence of KA on acetylcholinesterase (AChE) and protein contents and on the number of AChE-positive cells in explants of hippocampus (> 20 DIV). The values represent the means and the standard errors of the number of homogenates (AChE activity and protein content) or explants (AChE-positive cells) indicated in parentheses. Each homogenate contained at least five explants

	Control cultures	KA-treated cultures (3 μM)
AChE activity		
pmol Ac^-/min/explant	2.4 ± 0.27 (4)	2.3 ± 0.16 (5)
pmol Ac^-/min/mg protein	59 ± 8.2 (4)	74 ± 8.1 (5)
Protein content		
μg protein/explant	41 ± 1.3 (4)	32 ± 1.8 (5)*
Amount of AChE-positive cells		
Number of cells/explant	246 ± 31 (6)	412 ± 50 (6)*

* Significantly different from control at $P < 0.05$ level

Culture age

Specific lesions of the hippocampal explants using 3 μM KA were apparent only in cultures older than 8 days. At days 3, 5 and 7 in vitro, no specific destruction of the CA3 area could be observed at a KA concentration of 3 μM (lesion indices 1.0–1.5). At higher KA concentrations (100 μM) these relatively immature cultures were totally destroyed (lesion indices 4). For routine work only cultures older than 8 days were used.

Discussion

We have shown that a treatment of cultured explants of the rat hippocampus with KA in a concentration of 3 μM for 48 h causes a selective degeneration of the pyramidal cells in the CA3 area, whereas the granule cells and the pyramidal cells in the CA1 area are spared. This is comparable with the findings of in situ investigations, where the CA3 and CA4 cells show the largest sensitivity for KA, whereas the CA1 and granule cells are less sensitive (Köhler et al. 1978; McGeer and McGeer 1981). Without an ultrastructural study of the treated cultures it is of course not possible to exclude a reaction of the pyramidal cells in CA1 and the granule cells to KA in these cultures. Such a reaction is, however, apparently not severe enough to cause a lasting destruction of the cell group in question at the concentration of KA used (3 μM).

The action of KA on the explants of hippocampus was found to be stereospecific, since the KA analogue dihydrokainic acid did not influence the explants at all (Table 1). A further indication for the selectivity of the KA lesion is that the number of AChE-positive cells in the cultivated explants of hippocampus was not negatively influenced by KA treatment and that no decrease in the amount of biochemically determined AChE was registered after KA treatment (Table 3). These AChE-positive cell, which in situ are normally hidden in the AChE-positive neuropil (Storm-Mathisen 1977) are visible primarily in the strata oriens and radiatum and in the hilus fasciae dentatae of the cultivated explants of hippocampus (Rimvall et al. 1985; Zimmer and Gähwiler 1984). They could be cholinoceptive and have been found to contain somatostatin (Zimmer et al. 1983). Also Köhler (1983) described a class of hippocampal interneurons, containing glutamic acid decarboxylase and different peptides, which survived KA treatment in situ. Whether these surviving neurons were AChE positive was not mentioned.

The incubation times needed to achieve a clear lesion (lesion index 3), as judged light microscopically, in a majority of cultures treated with KA, exceeded 20 h (Table 2). It can, of course, not be excluded that an ultrastructural study of the treated cultures would reveal a reaction of the CA3 pyramidal cells to KA at earlier incubation times. These reactions are, however, not grave enough to cause a permanent neuronal destruction visible on the light microscopic level.

Depending upon the localisation of the injection of KA into the brain, both so called distal and local lesions have been described (see Introduction). The particular preparation used in these investigations is a totally deafferented hippocampal explant with intrinsic connections similar to those in the in situ

hippocampus (Keller et al. 1986; Zimmer and Gähwiler 1984). Furthermore, no penetration barriers are present in these explants and thus it must be considered that the lesion under study here represents a so-called local lesion.

Baclofen is an inhibitor of the transmission of the mossy fibre system in the hippocampus (Lanthorn and Cotman 1981) and it has also been shown to protect striatal neurons against the neurotoxic effects of kainic acid (McGeer et al. 1980). It was also seen to afford a slight protection to the CA3 pyramidal cells in the hippocampal explants treated with kainic acid (Table 2). In situ, a lesion of the mossy fibre tract has been found to protect the CA3 pyramidal cells when KA is applied intraventricularly, but not when it is applied locally (Nadler et al. 1980). Similarly, striatal neurons are protected from the actions of KA when the afferents are lesioned (Biziere and Coyle 1978). Other observations dispute the need for intact afferental input in KA toxicity in situ (Bird and Gulley 1979), as well as under culture conditions (Seil and Woodward 1980). The observation that the cultures are not sensitive for KA, at 3 μM, before the age of 8 days may be due to the delayed development of the mossy fibre tract in the hippocampus [immature hippocampal explants contain very few mossy fibres, as determined with Timm's stain for heavy metals (Zimmer and Gähwiler 1984; own unpublished observations)]. However, preliminary studies of cultured explants where the mossy fibres have been cut, indicate that this fibre tract is not necessary for a lesion of the CA3 pyramidal cells caused by KA. Thus, we failed to totally implicate the mossy fibre system in the toxicity of KA in this system.

A late development of putative KA receptors in the cultures may account for the observed dependence on culture age in KA toxicity. In the in situ hippocampus the first KA receptors are detected around the tenth postnatal day (Berger et al. 1984). In the cultivated hippocampal explants the first signs of KA sensitivity appeared around day 8–9 in vitro, i.e. at an age corresponding to a 2-week-old rat. Thus, since the development of the neurons in the cultivated explant might have been temporarily stunted by the explanation procedure, the in situ appearance of kainate receptors correlates well with the first in vitro observations of sensitivity for moderate doses of KA.

The drug GAMS has been found to be a relatively specific antagonist at the kainate receptor in electrophysiological experiments (Davies et al. 1983) and it has, furthermore, been proved to inhibit sound-induced seizures in mice (Croucher et al. 1984). The kainate receptor characterised electrophysiologically seems to correspond to the receptors described in binding studies with tritiated KA (Roberts and Foster 1983) and KA receptors have been autoradiographically demonstrated to be most abundant in the stratum lucidum, i.e. in the area where the mossy fibre terminals make contact with the CA3 pyramidal cells (Foster et al. 1981). Thus, one would expect GAMS to protect the cultivated neurons in the CA3 region against KA lesions. We have, however, shown that GAMS only has a very small, statistically not significant, protective effect against the actions of KA. It might be that the putative receptors mediating neuronal destruction in these cultures are not the same as the receptors which mediate the electrophysiological KA excitation in situ and which can be antagonised by GAMS. Extended studies with other putative protective agents would be needed to clarify the properties and the role of possible receptors for KA in this preparation. In this context it must also be taken into account the mechanism through which KA causes its lesions may not be receptor mediated but more "unspecific", perhaps related to ion fluxes and degrees of depolarisation. It has, for example, been shown that the CA3 cells are more easily depolarised by KA than the CA1 cells (Robinson and Deadwyler 1981; de Montigny and Tardif 1981). The concentrations at which KA causes depolarisation of the different cell groups in this particular preparation of the hippocampus have, however, not been determined.

In conclusion, we have shown that a specific, selective, and very reproducible destruction of neurons in cultivated hippocampal explants can be induced through the incubation of the cultures (> 8 DIV) with moderate doses (3 μM) of KA for more than 20 h. The exact mode of action of KA remains to be clarified. The hippocampal explants, cultured with the roller-tube technique, provides a potentially interesting model for the study of the irreversible, chronic effects of excitotoxic agents, which are presumed to play a role in a number of human pathological conditions like Huntington's disease, epilepsia, Alzheimer's senile dementia and depression (Schwarcz et al. 1984; Stone and Connick 1985).

Acknowledgements. The authors wish to thank Dr. Gähwiler for critical reading of the manuscript and for helpful advice.

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Received February 13, 1987/Accepted April 17, 1987