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In vitro kinetics of a newborn rat astroglia-derived neuronotoxic activity

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A low-molecular weight astrocyte-derived neuronotoxic activity (ANTA) was detected, using a colorimetric bioassay of cell survival, by its effect on cultured granule cells. This neuronotoxic activity was found to be released rapidly from newborn rat astrocytes in culture upon incubation in 50 mM K^+-containing growth medium. The release by astrocytes could be induced repetitively by successive incubations in high-K^+ medium alternating with incubations in normal medium. Astrocytes were also found to inactivate rapidly isobutanol-extracted ANTA in normal K^+-containing growth medium. Kinetic studies showed that ANTA induces a slow (> 12 h) degeneration of cultured granule cells. ANTA is shown here to be an intermediate of normal astrocyte metabolism and to display appropriate kinetic characteristics compatible with its proposed role in inducing part of the delayed neuronal loss that occurs after a brain injury (secondary neuronal death).

The production and liberation in the nervous system of endogenous neuronotoxins such as the excitotoxins glutamate and its analogs is a surprising, yet well established, mechanism leading to neuronal cell death after injury [11, 15–17]. Even less expected was the recent finding that astroglia, a brain cell population considered to be devoted to the maintenance of an appropriate environment for the survival and activity of neurons, was also able to release a low-molecular weight toxic activity for neurons under physiological conditions [6]. This astroglia-derived neuronotoxic activity was found to be specifically neuronotoxic, that is to act on neurons but not on astroglial cells or meningeal fibroblasts [9]. By several criteria it is clearly distinct from the excitotoxins [10] and its output from astroglial cells in culture is enhanced when the extracellular K^+ concentration of the conditioning medium is increased [9]. Since in ischemia, hypoglycemia, trauma or status epilepticus there is an increase in brain extracellular potassium concentration, we have proposed that part of the neuronal death that is observed in such pathological conditions could result from the release by astroglia of this neuronotoxic factor. We have studied further the characteristics

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of this astroglia-derived neuronotoxin produced in vitro and we report here kinetic aspects of the release, inactivation and neuronal effects of this activity.

Cultures of newborn rat cerebral cortex astroglial cells prepared according to Booher and Sensenbrenner [3] were used as described [9, 10] to produce astroglia conditioned medium (ACM) containing the astroglia-derived neuronotoxic activity (ANTA). This neuronotoxic activity was measured by its effect on cultures of granule cells prepared, as described [10], from 7-day-old rat cerebellum by enzymatic dissociation followed by sieving through 17 μm nylon sieve. This assay involves a 20 h incubation of purified granule cells (150,000 cells/well in 96-well microplate (NUNC, Roskilde, Denmark)) with the medium to be tested, followed by measurement of the remaining living cells with the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St-Louis, MO, U.S.A.). Details concerning this assay can be found elsewhere [9, 12]. ANTA was concentrated by 3 successive extractions of ACM with water-saturated isobutanol, followed by evaporation at 80°C under vacuum and reconstitution into one-fifth of the original volume in minimal essential growth medium (MEM) (Gibco, Gent) supplemented with bovine insulin (5 μg/ml) (Sigma).

Subconfluent cultures of astroglia cells (from 8 to 10 days after dissociation) containing more than 95% glial fibrillary acidic protein (GFAP)-positive cells were used to study the kinetic of release of ANTA. Fig. 1A indicates that the release of ANTA induced by high-K^+ concentration was rapid, reaching a maximal value after about 20 min of incubation. After a short (2 h) recovery time in normal serum-containing growth medium, astrocytes were again able to release ANTA when switched to serum-free high-K^+ medium (Fig. 1B). This process could be repeated several times with comparable amounts of toxic activity produced at each stimulation. The occurrence of such a plateau in the release of toxic activity after 20–30 min can be understood either as the result of a desensitization toward the high-K^+ stimulation, as an exhaustion of a storage pool of preformed ANTA upon continuous stimulation or

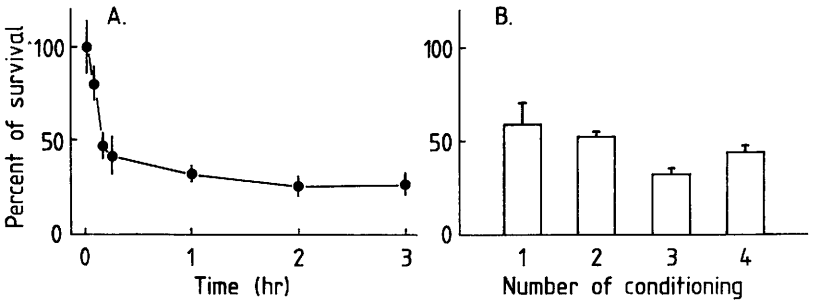

Fig. 1. Release of ANTA from astrocytic cells in culture. A: time-course of release. Subconfluent astrocyte cultures were incubated for the time shown in 50 mM K^+-containing MEM. The conditioned media were ultrafiltered, extracted in isobutanol as described [11] and their content in neuronotoxic activity was measured using granule cells and the colorimetric cell survival assay. B: repetitive conditioning. The same astrocyte cultures were incubated in 50 mM K^+-containing MEM for 30 min alternating with 2 h incubation in normal serum-containing MEM. The successive conditioned media were processed, and their content in neuronotoxic activity was measured as described above.

as the establishment of a steady-state equilibrium between decreasing ANTA release and a mechanism of inactivation by the secreting glial cells.

Such an inactivation mechanism was indeed demonstrated in the experiment described in Fig. 2A where ANTA was added in normal (3 mM) K^+-containing MEM onto confluent astroglial cells. Clearly, glial cells were highly efficient in inactivating ANTA in this medium since no more toxic activity was detected after only 1 h of incubation.

Contrasting with the rapidity of release and inactivation of ANTA by glial cells, its action on neuronal target showed rather slow time course. At least 12 h of continuous incubation with ANTA were required to observe the first sign of necrosis of cultured granule cells (Fig. 2B). Thereafter, neuronal death proceeded steadily, being almost complete after 36 h of treatment.

The molecular nature of ANTA has still to be determined. Our data suggest that it is a product of normal astrocytic metabolism, as it is released very shortly after onset of stimulation and can be inactivated rapidly by astrocytes.

In analyzing cerebrospinal fluid samples from severely head-injured patients, we found that a neuronotoxic activity, showing similarities with ANTA, was released in human brain [8]. Yet a precise identification of ANTA in mammalian brain has still to be made.

Those kinetics of release, action and inactivation of ANTA in vitro are compatible with an involvement of this agent in acute neuronal death. Indeed, brain injuries such as ischemia, hypoxia or trauma lead to an immediate primary death at the focus of injury, followed by a delayed secondary death which can spread in space [14]. Alterations of K^+ concentrations compatible with an acute release of ANTA are not uncommon after such injuries (reviewed in ref. 11), thus high amounts of this toxic activity could be released and overwhelm normal astrocytic inactivation processes. This accumulation of ANTA should induce a slow neuronal necrosis which would result into the characteristic secondary death after nervous system injury.

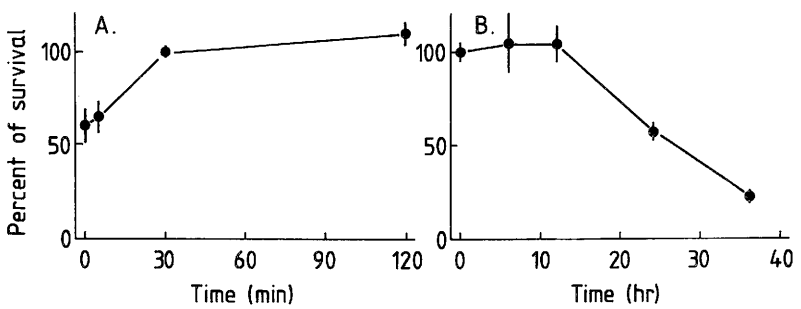

Fig. 2. A: inactivation of ANTA by astrocytes in culture. An isobutanol extract of astrocyte conditioned medium was reconstituted in normal MEM and incubated for the time shown on subconfluent cultures of astrocytes. The remaining neuronotoxic activity was measured as described in Fig. 1. B: kinetic of action of ANTA on granule cell cultures. An isobutanol extract of astrocyte conditioned medium was reconstituted in normal MEM and added for the time shown on cultures of cerebellar granule cells. The medium was then replaced with MEM and cell survival was measured at the end of a combined incubation time of 40 h.

There are many similarities between the properties of ANTA and those of members of the excitotoxin family. Excitotoxins are indeed released by neurons during excessive electrical activity (such as occurs in status epilepticus [13]), or, like ANTA, upon increase of extracellular potassium concentration following ischemia [2, 5]. Inactivation of the excitotoxins is carried out by astrocytes which possess efficient uptake systems for excitatory amino acids [7]. The glutamate uptake system is however inhibited by high external K^+ concentrations [1], a fact that could aggravate the severity of the excitotoxic damage.

Excitotoxins and ANTA present however some important dissimilarities. Excitotoxins act only on restricted populations of glutamate-sensitive neurons, while ANTA is active on all central and peripheral neurons tested, including neuronal subsets insensitive to the excitotoxins [10]. Contrasting with the mode of action of excitotoxins, ANTA does not induce specific ionic fluxes for its lethal effect and its action is not inhibited by antagonists of the excitotoxins [10].

There seems to be room for several types of neuronotoxic mechanisms in acute neuropathologies. Indeed sensitivity to excitotoxins is restricted to specific areas of the brain such as the hippocampal formation where specific glutamate receptor antagonists effectively protect against the excitotoxic damages. Yet acute neuronal death can occur in all part of the nervous tissue. Possible interactions between neuronotoxic mechanisms are likely. For example, astrocytes have recently been shown to be depolarized by agonists of the kainate type of glutamate receptor [4, 18]. This depolarization could accelerate the release of ANTA, leading to the death of glutamate-insensitive neurons.

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