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UPREGULATION OF CALBINDIN-D-28k IMMUNOREACTIVITY BY EXCITATORY AMINO ACIDS

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INTRODUCTION

Calcium in the past few years has been shown to have important signaling and regulatory roles in the cytoplasm. It is also believed that, in excess, it can be toxic (see 15). While many studies have been done on calcium and its roles, relatively little is known about how cells might regulate cytoplasmic calcium. Sequestration, metabolic pumping and exchange extrusion (see 8) are the principal ways cells are thought to keep cytoplasmic calcium at low levels. On the other hand, several proteins with high calcium binding capacities have been identified in many cell types (see 22). Calbindin D-28k (Calbindin), a calcium binding protein, vitamine D dependent, 28 kd, (42, 43) belong to the group of intracellular proteins having high affinity for calcium and are supposed to have an intracellular buffering function (see 22). Selected types of neurons of the mammalian central nervous system contain Calbindin and the immunoreactivity to this protein is generally used to identify these neurons in normal and experimental conditions as well as in degenerative diseases (27, see 9). This identification rests on the non verified presumption that "physiological" concentrations of Calbindin, detected by immunohistochemistry, remain unchanged. The cerebellum contains the highest concentration of Calbindin (42) and, in the adult cerebellar cortex fixed *in vivo*, the Purkinje cells (PCs) are exclusively Calbindin immunoreactive (Calbindin-IR) (3). They are therefore a good model to study experimental changes of the Calbindin-IR. The present experiments were designed to investigate whether changes in Calbindin-IR of the PCs could be induced by excitatory amino acids (EAA). In fact many neurodegenerative diseases recognize a pathophysiology initiated by the neurotoxic action of the EAA and it has been postulated that Calbindin may protect neurons from EAA toxicity (23). Part of these results were reported in a preliminary note (5).

METHODS

The experiments were performed in 57 Sprague Dawley rats. Cerebella were rapidly removed from the animals decapitated under ether anesthesia. Three to five cerebellar slices (400 μm

thick) were cut in the parasagittal plane with a vibratome from a trimmed block containing the vermis. The slices were kept in a sintered glass filter funnel containing perfusion solution bubbled continuously with 95% O_2 and 5% CO_2 at room temperature of 22-23°C. Some of the slices were transfered to a chamber containing bubbled perfusion solution heated at 35°C. The perfusion solution contained in mM: 124 NaCl, 5 KCl, 2.4 CaCl2, 1.24 KH2PO4, 1.3 MgSO4, 26 NaHCO3 and 10 glucose. After one hour recovery, different drugs were added at various concentrations to the perfusing solution as specified in the results.

At the end of the experiment the slices were fixed for 3-4 hours in a solution of 4 % paraformaldehyde and 0.3 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4; they were transfered and kept overnight in 30 % saccharose in 0.1 M phosphate buffer. They were then serially cut frozen at 30 to 50 μm. Alternate sections of a slice were subsequently treated for immunohistochemistry with antibodies raised in rabbit against rat kidney Calbindin (26). The sections were incubated free-floating overnight in the primary antibody diluted 1:4000 in saline phosphate buffer (PBS). The second incubation was carried out with anti-rabbit IgG serum diluted 1:200 in PBS for 1 hour. They were finally processed by the indirect peroxidase-antiperoxidase (41) or avidin biotin (24) methods. Diaminobenzidine was used as chromagen. Few control experiments were performed with mouse monoclonal anti-calbindin-D-28k antibodies (purchased from Sigma) diluted 1:200. The remaining sections were Nissl stained or, in a few experiments, they were treated immunohistochemically with antibodies raised in rabbit against glutamate (Glu) or y-aminobutyric acid (GABA) or parvalbumin (PV) using the methods described previously (4, 5).

Since graded changes in immunoreactivity cannot be objectively quantitated, only PCs intensely calbindin-IR and showing uniform dark cytoplasmic staining were counted. For each slice the count was performed in three sections and averaged. The results were somewhat variable from one animal to another. Therefore, one slice from each animal was always used as control (maintained in normal solution). In each animal the data of the experimental slices were always compared to those of the control slice. Student Test was applied and only data with $P<0,005$ were considered significant. In selected experiments (Fig. 2) the data were normalized as follows: the number of intensely calbindin-IR PCs in a slice (averaged from counts of three sections Calbindin immunostained) was expressed with respect to the total number of PCs (averaged from counts of three other sections Nissl stained) in the same slice.

In each step of this work at least six experiments were performed.

RESULTS

Calbindin immunoreactivity of the Purkinje cells at low temperature.

Knowing that the slices maintained at low temperature are less susceptible to anoxia (2), as a first approach slices incubated for two to four hours at 35°C were compared to slices incubated at 22°C for the same time. It was found that while at 35°C many of the PC show intense Calbindin-IR, at low temperature, almost all the PCs were immunonegative, or only slightly immunopositive (Fig. 1A). Only very few PCs were intensely labeled. At low temperature protein synthesis in hippocampal slices is decreased (36) and it might be expected that the low Calbindin-IR in the PCs at 22°C is also due to a lower synthesis.

Since PCs are an identifiable population of neurons, all having the mechanism to synthezise Calbindin, the hypothermic slice preparation becomes a useful tool to detect experimental changes in Calbindin-IR. All the following experiments were therefore done at room temperature.

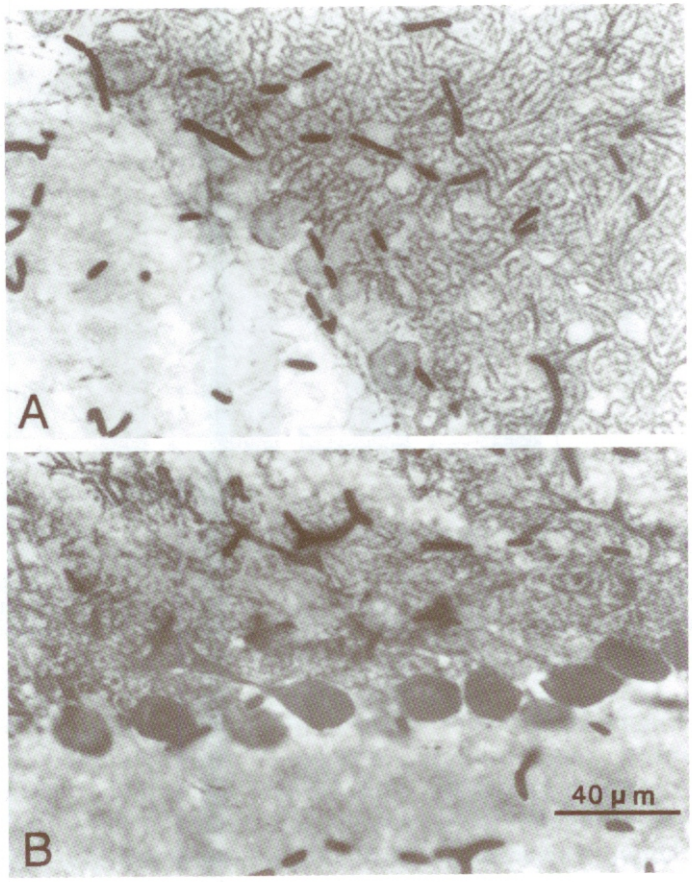

Fig. 1. - *Increased KA-induced Calbindin is a rapid process.*

Photomicrograph of the cerebellar cortex in Calbindin immunostained sections from two slices of the same animal after incubation in normal solution (A) and in solution containing 200 µM KA for 5 minutes (B). Note the intensely Calbindin-IR PCs in B. Worms-like structures are capillaries containing rbcs.

Kainic acid induces upregulation of the calbindin immunoreactivity in the Purkinje cells.

PCs are vulnerable to kainic acid (KA), a potent neurotoxic glutamate analogue (16). If Calbindin protects the neuron from the toxic effects of the EAA, it should increase in hypothermic PCs under the influence of excitotoxic doses of KA, or the hypothermic PCs should be more vulnerable.

KA was added at final concentrations of 3 µM to 1mM to the perfusion solution. Pairs of adjacent slices were alternatively incubated either in KA or in normal solution for control. Compared to controls, an upregulation of the Calbindin immunoreactivity was obtained in all slices incubated in KA, whatever the concen-

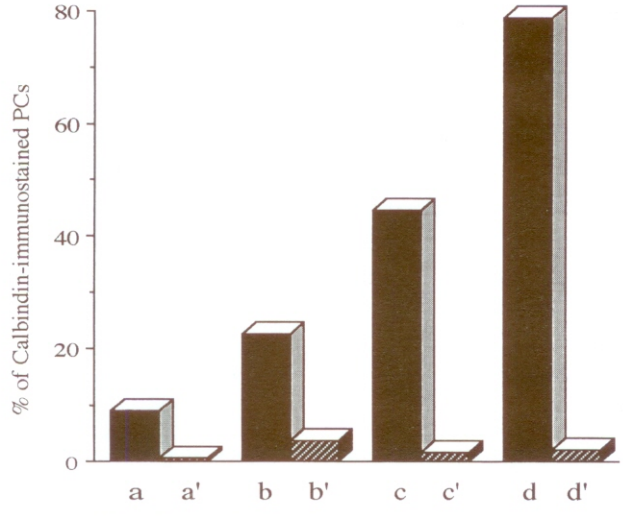

Fig. 2. - *Dose dependence of KA-induced Calbindin increase.*

Percentage of Calbindin-IR PCs (see method) in four experiments. In each experiment one slice was perfused for three hours in media containing KA at the concentration of 25 μM (a), 100 μM (b), 500 μM (c) or 1mM (d) and one was kept in normal media for control (respectively a', b', c', d').

tration of the drug used. However, while at high concentrations almost all the PCs were very intensely immunostained, at lower concentrations fewer PCs were intensely immunostained. Therefore, to quantitate the results, the number of PCs Calbindin-IR was compared in control and KA treated slices of the same experiment (see Methods). The results, shown in the normalised histograms of Fig. 2, indicate that the Calbindin upregulation under the influence of KA could be dose dependent.

As shown previously (16), KA is already toxic for PCs at 10 μM. In our experiments vacuolation of PCs was particularly important at 100 μM or higher concentrations but vacuolated PCs were also intensely immunostained. Whereas at lower concentration of KA, PCs Calbindin was detectable in the soma and in the dendrites including the most distal dendrites, at concentrations of 500 μM or higher, it was usually observed that only the somata and not the dendrites were intensely immunopositive.

The lowest concentration of 3 μM used in these experiments is not neurotoxic. Nevertheless PC Calbindin-IR was also observed, although to a lesser extent thus demonstrating that increased immunoreactivity is not due to a toxicity side effect but to the action of the agonist.

None of the other neurons of the cerebellar cortex or of the cerebellar nuclei has ever shown any Calbindin-IR under KA perfusion even at the highest concentrations used and the longest incubation time applied. The results indicate that the expression of this protein is locked to specific neurons, at least in the adult animals.

The time dependence of increased PC Calbindin-IR was also tested. Slices were incubated from 5 min to 4 h in KA containing media (100 μM or 200 μM). Compared to controls, modest but significant results were already obtained with 5 min (Fig. 1B). With 15 min a large number of PCs were intensely Calbindin-IR and remained at about the same level with longer incubation times.

Increased Calbindin-IR of the PCs is a reversible process.

In order to assess whether the increased Calbindin immunoreactivity is a reversible process, the slices were successively bathed (for 1h to 3 h) in media with and without KA (200 μM to 1mM). While the number of Calbindin-IR PCs increased in slices incubated with KA, they returned near the control level in slices reincubated without KA. Slices exposed to a second incubation with KA showed again an increased number of PC Calbindin-IR over their controls. To test the time course of the recovery from KA-induced Calbindin-IR, the cerebellar slices were exposed to KA for 30 min and then transfered to normal solution for 15, 30, 60, 90, 120 min. Compared to slices exposed to KA only, recovery started at 30 min, was near the control level at 60 min and was complete at 90 min.

These findings demonstrate that the increased Calbindin-IR to neurotoxic doses of KA is reversible and is therefore an active process. However, while the increase is very fast, recovery is a much slower process.

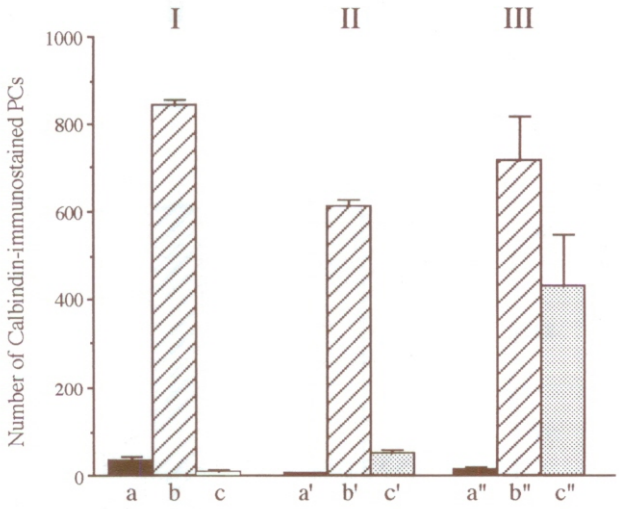

Fig. 3. - *Effect of selective competitive antagonists on KA- AMPA- and Glu-induced increase of Calbindin-IR PCs.*

Average number of Calbindin-IR PCs from three experiments (I, II, III). Control slices (a, a', a"); slices perfused with 200μM KA (b), 200μM AMPA (b') or 7mM Glu (b"); slices perfused with 200μM KA and 50μM CNQX (c), 200μM AMPA and 50μM CNQX (c') or 7mM Glu, 100μM CNQX and 1mM AP5 (c").

KA-induced increase of Calbindin-IR is blocked by the competitive KA/AMPA antagonist but is independent of calcium influx.

KA binds to specific KA receptors identified in the cerebellar cortex (7, 21). If the increased Calbindin-IR depends on the ligand-gated receptors, the effect should be suppressed by the competitive KA/AMPA (a-amino-3-hydroxy-5-metyl-4-isoxazolepropionate) specific antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione). Slices incubated in KA (200 μM) containing media were compared to slices incubated in media with the same concentration of KA but in presence of CNQX (5 μM or 50 μM) and to slices incubated in CNQX only for control. Bathing time was 30 min, 1 h or 2 h; preincubations in CNQX were performed for 30 min to 2 h before adding KA. In all the experiments increased Calbindin IR was induced in slices incubated with KA only, whereas in slices also treated with CNQX the effect of KA was deeply antagonized, the immunoreactivity remaining at the level of the controls slices (Fig. 3,I).

KA-induced calcium influx has been described in the PCs (31, 6) and could be the signal for increased Calbindin expression. To prevent Ca^{2+} influx, slices were incubated in a Ca^{2+} free media also containing 1 mM of EGTA, a potent calcium chelator, to avoid any residual calcium. Nevertheless in presence of KA (200 μM) the PCs were intensely Calbindin-IR and no appreciable difference could be observed with slices incubated in normal media with the same concentration of KA (Fig. 4,I).

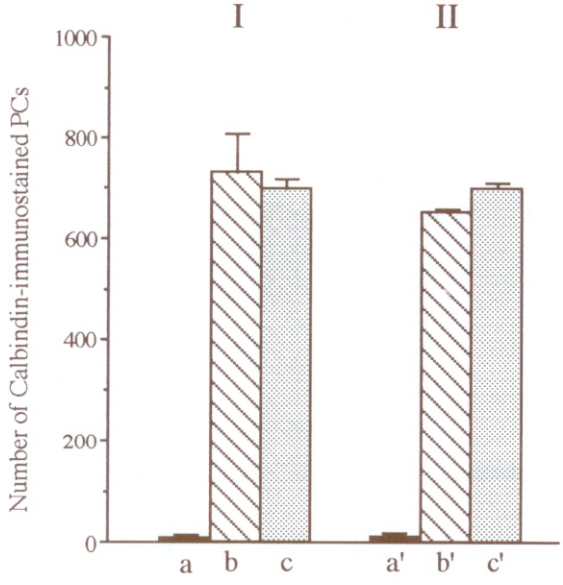

Fig. 4. - *KA- and Glu-induced increase of Calbindin-IR in PCs incubated in calcium-free solution.*

Average number of Calbindin-IR PCs from two experiments (I and II). Control slices (a, a'), slices perfused with 200μM KA (b) or 10mM Glu (b') and slices perfused in calcium-free solution with 200μM KA (c) or 10mM Glu, (c').

Therefore, increased PC Calbindin-IR with KA appears to depend on specific receptor activation by the agonist and not on the agonist activated Ca^{2+} influx.

AMPA-induced upregulation of the Calbindin-IR of the Purkinje cells.

AMPA is the EAA agonist binding on the ionotropic AMPA receptor. Like KA, AMPA is neurotoxic when applied in excess (18) although with less efficacy (17) and like KA should increase Calbindin-IR of the PCs. Slices were incubated in media containing excitatory (30 μM) or excitotoxic (200 μM) concentrations (18) of the agonist. In both cases, the PCs Calbindin-IR were significantly increased over controls (Fig. 3, II, a', b'). The effect of the specific antagonist CNQX on the excitotoxic concentrations of the agonist was also tested. In slices incubated in media with AMPA (200 μM) and CNQX (50 μM), the number of Calbindin-IR PCs was significantly decreased and approched the control level (Fig. 3, II, c'). Thus, as expected, the results were analog to those obtained in KA treated slices.

Glutamate-induced upregulation of the Calbindin-IR of the Purkinje cells.

Glutamate (Glu) is the only endogenous excitatory amino acid binding on the various specific receptors including the KA/AMPA receptors. Thus, Glu like KA and AMPA should increase the Calbindin IR of the PCs.

The experiments were performed on pairs of slices bathed in normal media and in media containing Glu at final concentrations of 300 μM to 10 mM. Compared to controls, a significant but very variable number of PCs intensely Calbindin-IR was observed from one experiment to another for concentrations of at least 5mM. A number of PCs showing an increased Calbindin-IR was however obtained at Glu concentrations of 7mM and 10 mM (Figs. 3, III, b"; 4, II, b' and 5, A, B). In addition, at 10 mM of Glu, the number of PCs intensely Calbindin-IR was already very high, almost maximal, with 15 min exposure.

In slices of adult cerebella exposed to 3mM of Glu for 2 h, the neurotoxicity on the PCs is very poor, while severe necrosis is induced with 10mM (16). However, it has also been pointed out that in adult cerebellar slices Glu penetration is very low and increases with concentration (16). Therefore, it seems difficult to know whether the results obtained in our experiments should be attributed exclusively to neurotoxic doses of Glu or to excitatory concentrations as well.

In another set of experiments, the antagonistic action of CNQX on Glu-induced Calbindin-IR was tested. AP5 (2-amino-5-phosphonopentanoic acid), the competitive antagonist binding on NMDA (N-methyl-D-aspartate) receptors was also used although PCs show poor chemosensitivity to this agonist in the adult cerebellar cortex (11, 19). Slices incubated in normal media were compared to slices incubated in media with Glu (7mM or 10mM) and to slices incubated in media containing the same amount of Glu and of CNQX (5 μM to 1mM) or AP5 (1 μM or 2mM) or both together. Neither antagonist by itself, nor both together clearly antagonize the effect of Glu whatever the concentration and the application time. A decrease in number of PCs intensely Calbindin-IR could be observed, but the results were variable from one experiment to another and usually not significant, at least not at the limit chosen in this work (Fig. 3, III).

Finally, the Glu-induced increase of PC Calbindin-IR was tested in Ca^{2+} free solution. In these experiments, the results were similar to those obtained with KA: a persistence of the upregulation of Calbindin-IR (Fig. 4, II).

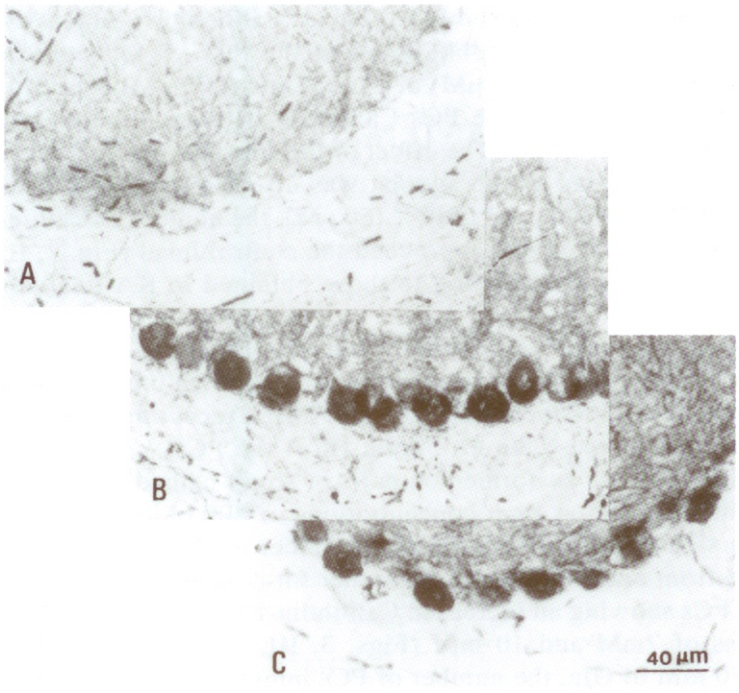

Fig. 5. - *Glu-induced Calbindin increase in PCs is independent from calcium influx.*

Photomicrograph of the cerebellar cortex in Calbindin immunostained sections from three slices of the same animal incubated for 30 minutes in normal solution (A), in solution containing 10mM Glu (B) and in free calcium solution containing 10mM Glu (C). Note the intensely Calbindin-IR PCs in B and C.

Controls

a) Using Celio monoclonal antibodies against Calbindin, increased IR of the PCs was tested in the presence of KA (200 µM for 30 min) and in presence of Glu (10 mM for 30 min). The results obtained were in agreement with those described above.

b) In sections treated for Glu and GABA immunoreactivity no difference could be appreciated in slices treated with KA (200 µM) or Glu (10 mM) over controls. In those treated for PV immunoreactivity, the immunostaining was not succesful and therefore no conclusive results could be drawn.

c) In slices treated with GABA (300 µM or 10 mM) Calbindin-IR of the PCs showed no difference from controls.

DISCUSSION

Several lines of evidence have shown that EAA induced cytotoxicity may explain the pathophysiology of acute and chronic neurodegenerative syndrome and disease (see 1, 32). The EAA agonists experimentally applied in large doses can initiate neurotoxicity operating through specific receptor subtypes leading to toxic increase in intracellular Ca^{2+} (see 10, 29). The ability of a neuron to maintain calcium homeostasis would determines its resistance to the degenerative process. One way to reduce intracellular Ca is by the buffering action of calcium binding proteins. We have shown here that Calbindin rapidly increases in P C s superperfused with Glu and the Glu analogues KA and AMPA. This effect was obtained with all the concentrations used and varied with concentration. The results strongly suggest that EAA induces an upregulation of Calbindin expression. Our control experiments indicate that the effect is specific and is not dependent on the type of Calbindin antibodies. Experiments of others (35) has shown that the antibodies bind to Calbindin whether or not the protein is loaded with calcium. We do not know however if similar results could be obtained with other calcium binding proteins. All PCs express PV but our experiments were not conclusive. The unexpected result is that Calbindin can be turned on very fast and is therefore unlikely due to *de novo* synthesis, but which is the mechanism we do not know.

PCs in slices irreversibly degenerate when incubated with 10 μM KA or 100 μM AMPA applied for at least 30 minutes but not at lower concentrations (18, 20). Therefore in our experiments, PC enhanced Calbindin-IR occurs with both, excitatory and excitotoxic concentrations of KA and AMPA and probably also Glu. However, while such distinction might be interesting for experimental purposes, it is irrelevant in pathophysiology since neurodegeneration take place not only under excessive doses of EAA but also under prolonged exposure to EAA, particularly in chronic diseases (see 1).

Signs of degeneration are observed a few hours after onset of neurotoxic concentrations of KA exposure (20) when PCs still express high levels of Calbindin in our experiments. But advanced degeneration of the PCs somata is obtained after 24 hours (20). Slices can be followed only for short times, but the time course of KA-induced Calbindin-IR assessed in *vivo* (5) confirmed the results obtained in slices and showed in addition that PCs in cerebella superfused *in vivo* with KA 24 hours before, were non immunoreactive and degenerated. Therefore, increased expression of Calbindin persists during KA exposure even if PCs are in the process of irreversible degeneration. Moreover, in the process of irreversible degeneration, the expression is still reversible. Only degenerated cells completely lack Calbindin. Neurons containing Calbindin were shown to be either more vulnerable (25, 38, 39) or more resistent (28, 44) to degeneration in certain neurodegenerative diseases. Our data indicate that the presence of Calbindin immunoreactivity in the neuron does not necessarily mean that these neurons are not degenerating. On the contrary, intensely Calbindin-IR neurons might indicate an ongoing degenerative process. This could be the case for the results reported in the hippocampus in kindling epilepsy (40).

In the adult cerebella Calbindin is only expressed in PCs, but during developement it is also expressed transiently in neurons of the cerebellar nuclei (13). Nevertheless, besides PCs, none of the other cerebellar types of neurons has ever shown any Calbindin immunoreactivity with EAA.

KA and AMPA bind to a specific receptor subtypes, the KA/AMPA receptors, whose presence has been described in the cerebellar cortex (7, 21, 34, 37). Our results demonstrate that KA- and AMPA-induced Calbindin expression is mediated by selective receptor activation since is suppressed by the antagonistic activity of CNQX. However, the Glu-induced Calbindin expression was poorly suppressed by the EAA antagonists used in the present experiments. Glu, being the general agonist, binds on all subtypes of EAA receptors, including the G-protein linked (metabotropic) receptor. The more likely hypothesis is that the Calbindin-IR is also induced by Glu activation of the metabotropic receptor. Experiments are in progress to shed light on this aspect.

Kidney and uterus calbindin expression changes with external calcium concentration (14, 33), but we do not know if this is the case for neurones as well. Calcium influx with KA and Glu in mammalian neurons has been reported (6, 12, 31). In cultured PCs, KA-induced Ca influx persists when voltage-gated Ca^{2+} channels are blocked but is suppressed by CNQX (6). Enhanced $[Ca^{2+}]i$ could be the signal for the upregulation of Calbindin expression. However, our experiments indicate that Ca influx is not the signal since KA- and Glu-induced increase of Calbindin expression in PCs is not prevented in slices incubated in a Ca^{2+} free solution, when there should be no calcium influx.

In conclusion our finding show an upregulation of the Calbindin expression in the PCs exposed to EAA at physiological as well as at excitotoxic concentrations. The effect is partially mediated by KA/AMPA receptor subtypes, is rapidly turned on, is reversible and is independent of Ca^{2+} influx.

In cultured hippocampal tissus, Mattson *et al.* (30) have shown that the Calbindin containing neurons more rapidly reduce Glu-induced transient Ca^{2+} influx than other neurons, although the initial Ca^{2+} increase is the same. These results can be explained if Calbindin levels are transiently and reversibly increased as suggested by the present data: it would be a reasonable way for the cytoplasm to have a high buffer capacity when needed. It should be also reversible since if such a capacity were constantly present, excessive buffering would alter Ca homeostasis. While this mechanism is well suited to confer, on the Calbindin containing neurons, a higher resistence to EAA toxicity it should not prevent toxicity from excessive intracellular calcium increases other then through receptor-gated chanels.

SUMMARY

Excessive or prolonged exposure to excitatory aminoacids (EAA) are thought to be neurotoxic by altering calcium homeostasis. A protective role of Calbindin-D-28k (Calbindin) has been postulated due to its capacity to buffer calcium. Calbindin is highly expressed in the Purkinje cells (PCs), of the cerebellar cortex. Changes

of the Calbindin immunoreactivity (IR) by the EAA has been here investigated in cerebellar slices maintained *in vitro*. It was found that at low temperature, PCs are very sligthly immunoreactive and therefore the experiments were done at 22°C. The results show that Calbindin-IR increases in PCs exposed to the neurotoxic agonists, Kainic acid (KA) and AMPA as well as to glutamate (Glu), the endogenous EAA. The increase is very rapid and slowly reversible; is induced by excitatory and excitotoxic concentrations of the agonists; is independent of the calcium influx. While KA- and AMPA-induced Calbindin-IR is blocked by CNQX, the KA/AMPA receptor antagonist, Glu-induced Calbindin-IR is only slightly decreased by CNQX and AP5, the NMDA receptor antagonist. It is concluded that Calbindin-containing neurons can increase their calcium buffering capacity in response to EAA binding to specific receptors, the response being independent of, but concomitant to calcium influx.

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