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INCREASED CALBINDIN-D28K IMMUNOREACTIVITY IN RAT CEREBELLAR PURKINJE CELL WITH EXCITATORY AMINO ACIDS AGONISTS IS NOT DEPENDENT ON PROTEIN SYNTHESIS

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Neurons exposed to excessive stimulation by excitatory amino acids (EAA), undergo a process of degeneration (11) which is thought to be induced by a massive calcium influx altering cytoplasmic calcium homeostasis. A high affinity calcium binding protein, Calbindin-D28K (CaBP), is supposed to protect neurons against the EAA excitotoxicity with its high calcium buffer capacity (19, 9, 10). However, a low level of CaBP is also thought to protect human hippocampal granule cells from excessive calcium influx (12). A calcium dependent promoter for the expression of CaBP mRNA has been identified (1) which suggests that CaBP levels may be transcriptionally regulated in response to cytoplasmic calcium levels. But, it was found earlier in kindled animal brains that a decrease in CaBP-immunoreactivity (CaBPIR) levels was not accompanied by a decrease of CaBP mRNA (16). These and other (see 5) diverse evidence indicate that the question of how CaBP levels are regulated by the neuron is still not clear.

The cerebellar Purkinje cells (PCs) have much higher levels of CaBP than all the other neurons in the central nervous system, perhaps consistent with the very large calcium movements in these neurons. The PCs in cerebellar slices show a rapid, dose dependent and reversible increase in CaBP-IR when exposed to glutamate, the natural EAA, and to its ionotropic analogs Kainic acid (KA) and AMPA (2, 3). These increases are blocked by the specific receptor antagonist CNQX. However with glutamate, CNQX and the specific NMDA receptor antagonist AP5, only slightly decrease CaBP-IR (2, 3). These results suggest that mGluR may also induce CaBP-IR increases. The experiments reported here examine this possibility and whether in the cerebellum, translational or transcriptional processes may be involved in the CaBP-IR increases.

Adult Sprague Dawley rats (120-150 g, males) were used for these experiments. Four slices (400 µm thick) were cut in the parasagittal plane from the vermis of cerebella and treated as described in details previously (2, 3). Individual slices were kept in the perfusion solution, then transferred to separate chambers where different drugs were added to the medium. One slice from each animal was maintained in normal solution for con-

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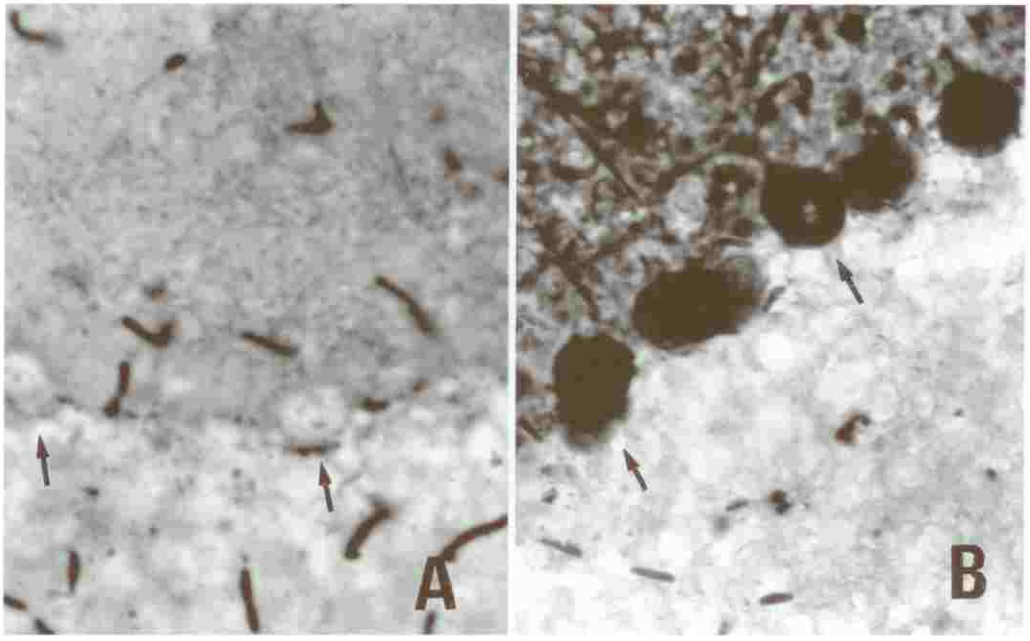

Fig. 1. - *CaBP-IR in sections from cerebellar slices.*

A: Slice incubated in normal medium for 4 hours. Arrows indicate positions of PC somata. B. Slice incubated in 200 µM KA for 1 hour after incubation in normal medium for 3 hours. Arrows point to 2 of the 6 PC somata visible in the figure. 15 mm in the figure corrisponds to 50 µm.

trol. All the slices were then fixed, serially cut frozen and alternate sections were treated for CaBP-IR or Nissl stained. The CaBP activity was evaluated by counting only those PCs showing intense CaBP-IR staining in the cytoplasm (Figure 1). For each experimental slice, the intensely stained PC were counted in three sections from each slice and averaged for the slice. The Averages for the experimental and control were compared by Student-t Test and only data with $P < 0.005$ were considered significant.

To test the effect of a mGluR agonist on CaBP-IR, in 8 experiments slices were incubated in a solution containing *t*-ACPD (trans-(±)-laminocyclopentane-1,3-dicarboxylate) at concentrations of 50 µM to 1mM for 30-60 minutes. The results were compared to those ($n = 12$) obtained with slices incubated in a solution containing 200 uM of KA inducing a constant significant increase of the CaBP-IR. At all concentrations the number of CaBP-IR was greater than in the control slices. The increase was variable not only from one experiment to another, but also from one section to another of the same slice. The inner sections of a slice had fewer intensely immunoreactive PCs than the more external sections probably due to a poor penetration of *t*-ACPD into the slice. We therefore counted PCs only in 3 of the most external sections of the slices. The increased CaBP-IR with this agonist was significant at $P < 0.005$ for 4 of the 8 experiments only and the number of intensely immunoreactive PCs was smaller for *t*-ACPD than for KA (Figure 2).

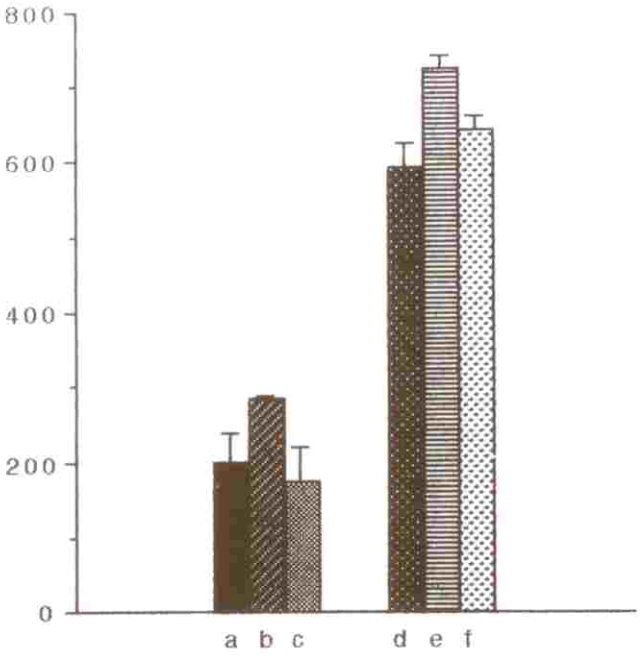

Fig. 2. - *Increased CaBP-IR induced in PCs by t-ACPD and glutamate.*

In this and the following figures, intensely CaBP-IR PCs were counted in three sections from a slice and averaged; bars are SEM. a, b and c) slices perfused with 500 µM *t*-ACPD, d, e and f) slices perfused with 200 µM KA. The incubation time was 30 minutes for each. Counts were significantly different ($P < 0.005$) from control slices only in b for *t*-ACPD, but were significant for all cases with KA.

The effect of the antagonist L-AP3 (L-2-amino-3phosphonoproprionic acid) on *t*-ACPD induced CaBP-IR was tested in four experiments. Slices were incubated in 500 µM or in 1mM L-AP3, for 60 minutes; or in 500 µM *t*-ACPD for 60 minutes; or were first preincubated 60 minutes in L-AP3, then transferred to the same solution with t-ACPD added for another 60 minutes. Preincubation was performed with L-AP3 in order to obtain a maximum effect of the antagonist. The number of CaBP-IR PCs in the sections from slices treated L-AP3 only was not significantly different from the control. When the slices were treated with L-AP3 together with *t*-ACPD, instead of a decreased CaBP-IR there was a clear potentiation (Figure 3).

t-ACPD specifically activates mGluR of the PCs in cerebellar slices (7). The concentration range to obtain minimal to maximal PC depolarization were applied in our work. Nevertheless, the increase in CaBP-IR described above was not always significant. Poor penetration of the drug in the slice is unlikely to be the only explanation since intense immunoreactive PCs were only counted in the superficial sections of the slices. We tentatively conclude that the mGluR contribution to the increased CaBP-IR with glutamate is small. In other neurons the excitotoxicity is induced through NMDA receptor activation (see 15). Since the mature cerebellum is very poor in NMDA glutamate receptors (6), KA/AMPA receptor activation would appear to be the major EAA pathway to enhance CaBP-IR of the PC. However, our present results showed that the *t*-ACPD-induced CaBP-IR is potentiated by L-AP3, the selective antagonist of the mGluRs (14) but in the thalamus (13) and in cerebellar PCs (7) it can also facilitate the responses to AMPA and to mGluR agonists. In

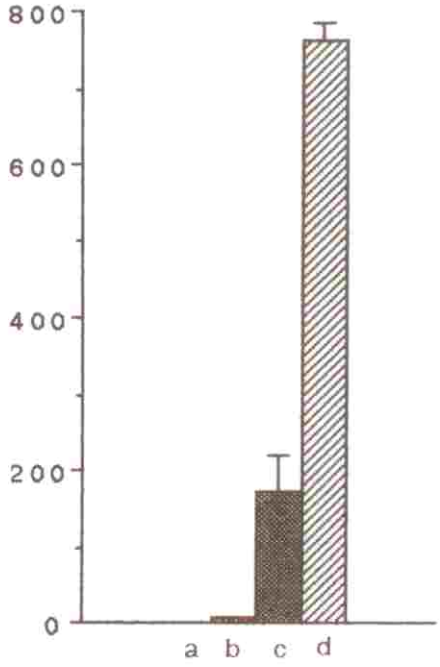

Fig. 3. - *L-AP3 effects on t-ACPD induced CaBP-IR.*

The mGluR antagonist L-AP3 dose dependently potentiated the *t*-ACPD induced CaBP-IR. a) 2 hrs in control medium; b) 2 hrs in medium containing 1 mM L-AP3; c) 2 hrs in 500 µM *t*-ACPD; d) 1 hr in 1 mM L-AP3 then 1 hr in a solution with both 1 mM L-AP3 and 500 µM *t*-ACPD.

our present experiments, L-AP3 acted as an agonist for t-ACPD induced CaBP-IR enhancement. Apparently, L-AP3 could make the mGluRs behave like iGluRs where it concerns CaBP-IR.

The possibility that translational or transcriptional mechanisms may be involved in CaBP-IR enhancement had not been eliminated in the present nor in the previous experiments. We therefore repeated these experiments using slices treated with the translational inhibitors, cycloheximide (n = 5) and emetine (n = 6), and two transcriptional inhibitors, actinomycine D (n = 5), which inhibits RNA synthesis and α-amanitine (n = 4) which blocks mRNA synthesis. KA was used since this agonist gives the best and more reliable increases in CaBP-IR (3). For each animal, one slices was incubated with KA at excitotoxic concentrations for 30 minutes to 3 hours. Two slices were first incubated in medium containing a protein synthesis inhibitor for different periods of time, then transferred to the medium with KA at the same concentration and period of time; finally one slice was kept in normal medium for controls.

KA significantly increased the number of CaBP-IR PCs over controls, whether the slices were treated with protein synthesis inhibitors or not. There was no significant difference between slices with and without synthesis inhibitor treatment (Figure 4). As a further control, a few slices were incubated in media containing only the protein synthesis inhibitors for up to 9 hours (no KA was added). In all of these slices, the immuno reactivity was similar to that of the slices kept only in the control medium for the same period (data not shown).

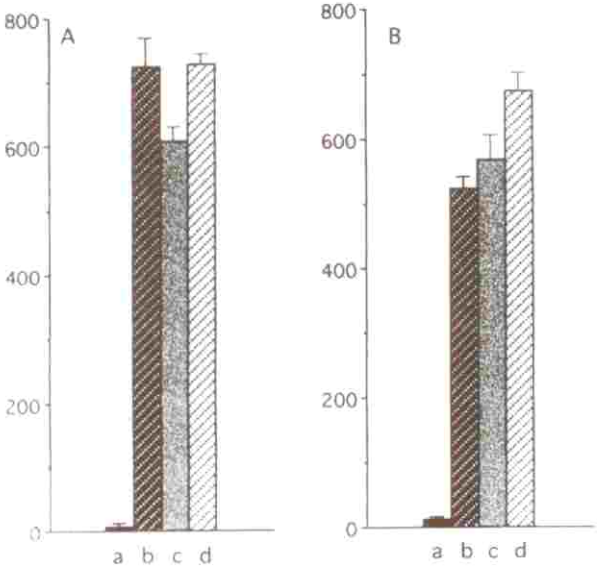

Fig. 4. - *Transcriptional and translational protein synthesis inhibitors do not prevent the upregulation of CaBP-IR by EAA.*

Two experiments are illustrated: A. a) control slices (4 hours in normal medium); b) 2 hours in normal medium then 2 hours in medium with 200 μM KA added; c) 2 hours in medium with 500 μM cycloheximide then 2 hours in medium with KA (200 μM) and cycloheximide added; d) 2 hours in medium with 10 μM α-amanitine then 2 hours in medium with KA and α-amanitine. B. a) control slices, 4 hours in normal medium; b) 1 hour in normal medium then 3 hours in medium containing 200 μM KA; c) 1 hour in medium containing 500 μM emetine then 3 hours in medium with KA and emetine; d) in medium containing 20 μM actinomycin then in medium containing KA and actinomycin for times as in a and b. A small potentiating effect of emetine and actinomycin was seen in B. c and d respectively.

Previous works have shown the efficacy of Cycloheximide, Emetine and Actinomycine D as protein synthesis inhibitors in brain slices at the concentrations and incubation times used in our experiments (17, 8). Thus, our results strongly suggest that increased CaBP-IR in the cerebellar PCs does not depend on *de novo* synthesis of the protein through pathways blocked by the inhibitors we have used. In hippocampal neurons, a decreased CaBP-IR has also been shown to be independent of protein synthesis (16). Taken together, these results suggest that the increased CaBP-IR we have obtained may be due to a conformational change of CaBP increasing its access for antibodies. The mechanism needs to be studied further since it has also been recently reported that the CaBP conformation is calcium and H^+ dependent (4). These and our previous finding that CaBP-IR is reversible (2, 3) support the idea that CaBP is a stable cytoplasmic component present in sufficient quantity without the need for *de novo* synthesis.

SUMMARY

The calcium binding protein Calbindin-D28K (CaBP) is abundantly expressed in cerebellar Purkinje cells and show increased immunoreactivity (CaBP-IR) when challenged with glutamate or an analog agonist for the ionotropic glutamate receptor (iGluR). Here we report that *t*-ACPD, a metabotropic glutamate receptor (mGluR) agonist, produced small increases in CaBP-IR which was potentiated by a mGluR antagonist The increase in CaBPIR was not due to *de novo* protein synthesis because the translational inhibitors (cycloheximide and emetine) or transcrip-

tional inhibitors (actinomycine-D and a-amanitine), did not prevent the EAA enhanced CaBP-IR. The CaBP-IR in the PC appears to be coupled to the ionotropic rather than the metabotropic glutamate receptors, but the latter become effective in the presence of their blocker, L-AP3. The results suggest that CaBP may increase its IR through a conformational change of the protein itself.

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