FEBS Letters 351 (1994) 53-57

FEBS 14434

Induction of calbindin-D 28K gene and protein expression by physiological stimuli but not in calcium-mediated degeneration in rat PC12 pheochromocytoma cells

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Received 27 June 1994; revised version received 19 July 1994

Abstract

To understand the role of calbindin-D 28K in neuronal degeneration, we examined its expression in differentiated PC12 cells in response to calcium intoxication, using the ionophore A23187 treatment, that results in cell degeneration and death. We first established that calbindin-D 28K is expressed in PC12 cells. The amounts of calbindin-D 28K mRNA and protein were increased by the differentiation factors, NGF and retinoic acid, but not by vitamin D_3. Calbindin-D 28K expression was also significantly up-regulated by stimuli (depolarization, low concentrations of Ca^{2+} ionophore A23187) which increase intracellular calcium levels within the physiological range. In contrast, the calbindin-D 28K mRNA and protein concentrations were not modulated by high concentrations of A23187, which resulted in cell degeneration and death. Experiments with the antisense oligonucleotides showed that, although the calbindin-D 28K protein levels were decreased significantly, the progression of degenerative changes induced by calcium via A23187, was not altered.

Key words: Calbindin-D28K; Calcium; Degeneration; Depolarization; PC12 cell; Trophic factor

1. Introduction

The calcium binding protein (CaBP), calbindin-D 28K is widely but heterogeneously distributed in the brain [1,2]. This protein belongs to a family of CaBPs that is characterized by a common structural calcium binding domain designated as the E-F hand [3,4]. In calbindin-D 28K, four such domains are present [5,6], including a unique site to which calcium binds with a very high affinity [7], suggesting that it plays an important role in the maintenance of calcium homeostasis. The evidence that it regulates intracellular calcium by sequestering excess Ca^{2+} is partly indirect and includes: its cytoplasmic localization; its high abundance in specific brain regions, for example, hippocampus, caudate putamen, and cerebellum; its co-localization with inositol triphosphate receptors which are involved in the release of Ca^{2+} from intracellular stores and its increased expression in hippocampal neurons following electrical stimulation [8–10]. Other roles for calbindin-D 28K have also been suggested including modulation of the functions of Ca^{2+}-dependent proteins such as voltage-gated calcium channels [11].

Calbindin-D 28K is believed to protect cells against increases in intracellular Ca^{2+} that in time, could spread and damage the neuron and subsequently threaten its viability. This may also explain the selective vulnerability to pathological processes in neurons lacking this protein compared to those containing it. Several studies have shown a reduction in the expression of calbindin-D 28K specifically in the neurons most affected in neurodegenerative diseases, for example, Alzheimer, Huntington and Parkinsons' disease (PD) [12–14]. In Parkinsonian patients and in the experimental animal model of PD, the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-treated mice, the dopaminergic neurons of substantia nigra pars compacta that have not degenerated are immunopositive for calbindin-D 28K [15–18], reinforcing the idea that this protein has a neuroprotective function.

To understand the role of calbindin-D 28K during cellular degeneration, we studied the expression of its mRNA and protein in differentiated PC12 cells exposed to low and high concentrations of calcium ionophore A23187.

2. Materials and methods

2.1. PC12 cell culture and treatment procedures

PC12 cells were cultivated in RPMI 1640 medium (Gibco, BRL) supplemented with 10% fetal calf serum (FCS), 5% horse serum (HS) (Boehringer) and antibiotics (100 μg/ml streptomycin and 60 μg/ml penicillin). They were differentiated in a modified Leibovitz's L15 medium containing 5% FCS, 10% HS, and 50 ng/ml 2.5S NGF (Boehringer) [19]. The cells were allowed to differentiate for 7 days and the culture medium was changed every 2–3 days. For Northern and Western blot analysis, the cells were grown to about 80% confluency in culture dishes and then treated with the calcium ionophore A23187 (Boehringer), veratridine (Sigma) or maintained in 50 mM KCl medium in which case an equivalent amount of NaCl was omitted from the

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SSDI 0014-5793(94)00821-3

medium. Cells for retinoic acid treatment were grown in RPMI 1640 medium for 5–7 days.

2.2. Immunocytochemistry

Cells were immunolabelled for calbindin-D 28K as described previously [20]. Monoclonal anti-calbindin-D 28K antibody (Sigma) was diluted 1/250 in phosphate-buffered saline (PBS) and the secondary antibody was mouse anti-IgG. Incubation with avidin-biotinylated horseradish peroxidase complex (Vectastain Kit, Vector Lab, Burlingame, USA) was followed by treatment with diaminobenzidine exposure to reveal immunolabelling. The specificity of the antibody had been ascertained by preadsorption assay with commercially (Sigma) obtained calbindin-D 28K protein.

2.3. Northern blot analysis

Total RNA was prepared by the method of Chomczynski and Sacchi [21]. 8 μg total RNA was electrophoresed in 1% agarose-formaldehyde gel and transferred to Hybond N^+ membranes (Amersham). The calbindin-D 28K cRNA probe [22] was prepared by first linearizing the plasmid with *Eco*RI enzyme and then synthetized using an in vitro transcription kit (Promega), radioactively labelling with [^{32}P]UTP. The blots were hybridized with the probe at 50°C for 16–24 h, washed under stringent conditions and autoradiographed.

2.4. Western blot analysis

Total cell protein was extracted in ice-cold RIPA buffer containing PBS, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 5 μg/ml aprotonin. The cells were disrupted by brief sonication, incubated on ice for 30 min and centrifuged in the microfuge for 30 min at 4°C. The amount of protein in the supernatant was measured by the method of Bradford [23]. 15 μg was electrophoresed on 9% PAGE and transferred to Immobilon P (Millipore) membranes. The blots were incubated with anti-calbindin-D 28K antibody (1/250) and processed for immunodetection by treatment with a secondary anti-mouse IgG antibody conjugated to horseradish peroxidase, and the signal detected using the ECL detection kit (Amersham).

2.5. Treatment with antisense oligonucleotides

Three antisense (or sense as controls) phosphorothioate-modified oligonucleotides, (Genset, Paris) each 20–21 bp long, were synthesized. The cells were incubated with a mixture of either 3 antisense or 3 sense oligonucleotides, each at a final concentration of 24 μM, in a serum-free modified L-15 medium but in the presence of NGF. Serum-free conditions were used because short-length oligonucleotides have been reported to degrade rapidly in the presence of serum proteins [24]. The cells were treated with the oligonucleotides every 24 h for 48 h, period during which the differentiation process and cell viability remained unaffected.

The cDNA sequences of oligonucleotides were as follows:

(a) 5′-GCAGAATCCCACCTGCAGTCA-3′
(b) 5′-TCTGGCTTCATTTCGACGGT-3′
(c) 5′-AACAAGACCGTGGATGATACG-3′

2.6. Quantification

mRNA and protein signals were quantified by optical densitometry using a Biocom image analysis system (Les Ulis, France).

3. Results

Almost all the NGF-differentiated PC12 cells contained immunolabelled calbindin-D 28K in the cytoplasm and in the neurite processes (Fig. 1). NGF-induced differentiation of PC12 cells (50 ng/ml, 5 days) resulted in a two-fold increase in both the mRNA and protein levels (Fig. 2A,B). To discern whether calbindin-D 28K synthesis increased as a result of neuronal differentiation induced by NGF, or if other differentiating factors modulate its expression, the cells were treated

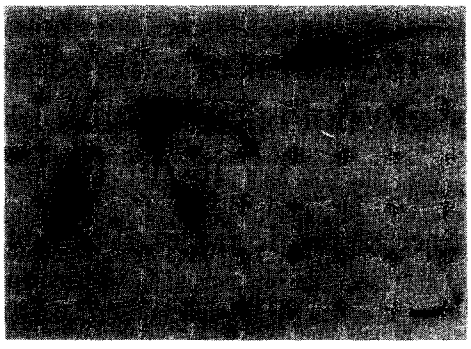

Fig. 1. Differentiated PC12 cells immunolabelled with anti-calbindin-D 28K antibody. Bar = 10 μm.

with retinoic acid or vitamin D_3. While 10 μM retinoic acid treatment did not result in morphological differentiation of PC12 cells, it nevertheless caused a 3-fold increase in the calbindin-D 28K mRNA levels (Fig. 2A). The increase in protein amount was similar to NGF at 10 μM retinoic acid, but a further augmentation was observed at 20 μM of retinoic acid (Fig. 2B). The calbindin-D 28K mRNA remained unchanged after vitamin D_3 (500 nM) treatment (data not shown).

The calbindin-D 28K mRNA levels were measured in NGF-differentiated PC12 cells treated with increasing concentrations of A23187 for 24 h. Most noticeably, at 10 and 50 μM of A23187, there was a 50 and 90–95% loss in cell viability, respectively (Fig. 3A), whereas the lower concentrations did not affect the cell survival significantly. The calbindin-D 28K mRNA levels in cells treated with low concentrations (0.1 and 1 μM) of A23187 increased by 3- and 4-fold, respectively (Fig. 3B), before any morphological changes or cell death occurred. Concentrations of A23187 above 1 μM A23187 caused a dose dependent drop in calbindin-D 28K mRNA; there was significantly smaller increase at 3 μM although the level was still above control. At this concentration, after 24 h of incubation, extensive neurite fragmentation was observed but cell survival was not compromised (data not shown). The calbindin-D 28K mRNA level at 10 μM A23187 was similar to control in the surviving PC12 cells, and had dropped below control at 50 μM (Fig. 3B). We monitored calbindin-D 28K protein levels in cells treated with 10 μM A23187 over a period ranging from 2 to 36 h in order to see whether the calbindin-D 28K levels were augmented prior to cell degeneration and death. There was no change in protein level (Fig. 3C) at all times tested although degeneration was ongoing, and cell death was observed after 12 h.

The above results showing that calbindin-D 28K expression is modulated only at low concentrations of A23187 led us to examine the effect of other stimuli which result in increased intracellular Ca^{2+}. The NGF-differentiated PC12 cells were treated with a depolarizing

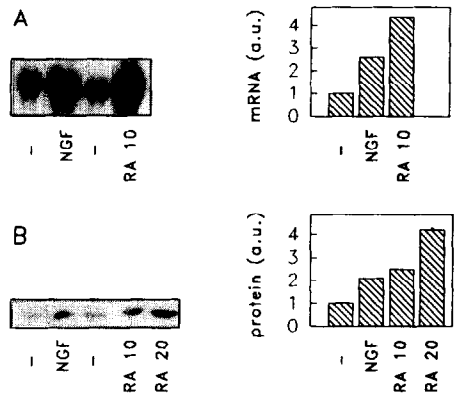

Fig. 2. PC12 cells incubated with NGF (50 ng/ml) and retinoic acid (10 μM) for 5 days. (A) Calbindin-D 28K mRNA levels of control and treated cells, the major transcript size in these cells was 2.8 kb. The mRNA sizes were verified with RNA markers and by rehybridization of the membranes with the β-actin cDNA probe, (mRNA: 1.9 kb). (B) Protein levels were measured by Western blot analysis, the molecular weight (28 kDa) was verified by protein standards. The cells were treated with NGF and with either 10 or 20 μM retinoic acid (RA) for 5 days. The quantified mRNA and protein levels are presented in arbitrary units.

concentration of K^+ (50 mM), or with veratridine (10 μM) for 2 and 24 h. These concentrations, known to increase Ca^{2+} influx, did not induce degenerative changes. Depolarization by K^+ induced a 10-fold increase in calbindin-D 28K mRNA level which was maximal at 2 h; veratridine produced a smaller but noticeable (2-fold) increase on calbindin-D 28K mRNA. For comparison, incubation with 1 μM A23187 resulted in 6-fold increase in calbindin-D 28K mRNA at 2 h which increased to a slightly higher level at 24 h (Fig. 4A). The rise in calbindin-D 28K protein levels paralleled in time the changes in mRNA levels (Fig. 4B), and were all of a similar magnitude.

To determine whether the calbindin-D 28K had a protective function during calcium-induced degeneration induced by calcium ionophore, the differentiated PC12 cells were treated with a mixture of three modified phosphothiorate antisense oligonucleotides in a serum-free medium containing NGF. Untreated cells were more susceptible to A23187 toxicity in the absence of serum proteins; the dose-response curve of ionophore toxicity was shifted toward lower concentrations. At A23187 concentrations of 1–2 μM cell degeneration began after 3–4 h of treatment. Cells incubated with 1.5 μM A23187 alone (control), or with sense or antisense oligonucleotides showed no significant difference in the progression of morphological degenerative changes. However, the calbindin-D 28K protein concentration in antisense-treated cultures was decreased by 50–60%, (Fig. 5), whereas in the sense oligonucleotide-treated cultures, the decrease in the protein level was 10–15% (data not shown).

4. Discussion

We show that both NGF and retinoic acid, well characterized differentiation factors, stimulate the expression of the calbindin-D 28K. These factors have opposing neurochemical and morphological actions in PC12 cells. Whereas NGF causes PC12 cells to differentiate into sympathetic neuron-like cells and promotes catecholaminergic traits [26], retinoic acid has no effect on their morphology but induces a cholinergic neurochemical phenotype [24]. The function of calbindin-D 28K in neuronal development and differentiation has been evoked in ontogenic studies [27] and it is induced during development by target-derived and trophic factors [28–31].

Treatment of NGF-differentiated PC12 cells with low concentrations of A23187 or depolarizing agents which elicit physiologically relevant rises in intracellular Ca^{2+}, and were non-toxic for these cells, resulted in a rapid and prolonged up-regulation of calbindin-D 28K mRNA and

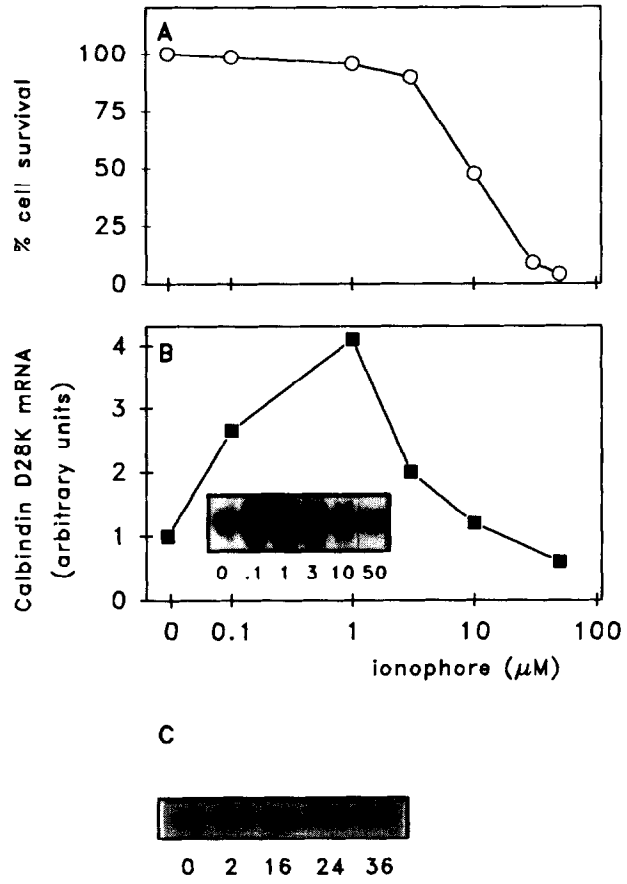

Fig. 3. Treatment of differentiated PC12 cells with increasing concentrations of A23187. (A) Cell viability was measured by Trypan blue exclusion. The results are expressed as % of cells excluding dye after 24 h of A23187 treatment. (B) Northern blot analysis of calbindin-D 28K mRNA after A23187 treatment for 24 h. The quantified results expressed in arbitrary units as the means of three experiments. (C) Differentiated PC12 cells were treated with 10 μM A23187 for varying times and the calbindin-D 28K protein concentrations measured.

protein levels. The low (1 μM) concentration of A23187 used in this study has been shown to elevate basal intracellular Ca^{2+} by 5–10-fold [32], a range comparable to depolarization [11]. The results strongly suggest that Ca^{2+} itself may play a role in maintaining its own homeostasis in physiological situations by regulating the expression of calbindin-D 28K gene. This result is also in keeping with the study of Lowenstein et al. [10] showing the induction of calbindin-D 28K mRNA in the granule cells of the hipppocampus in vivo following neuronal stimulation that increases intracellular Ca^{2+}. In differentiated PC12 cells, this compensatory phenomenon could be an efficient way of safeguarding the cell morphology. In contrast, calbindin-D 28K gene transcripts fell to control levels at A23187 concentrations that resulted in cellular degeneration, and as well, the protein levels remained constant at 10 μM A23187 during both the early non-degenerative period and in the degenerative period. Although there was no up-regulation, the calbindin-D 28K protein levels were not significantly diminished in cells undergoing degeneration suggesting that even though the calcium regulatory processes may be functional they are not sufficient to prevent cell death. As regards to regulation of calbindin-D 28K gene expression, the results suggest that above a threshold concentration of cytosolic Ca^{2+}, the calbindin-D 28K gene expression is not modulated nor is it regulated by any molecular changes occurring during calcium-induced degeneration. In agreement with this finding, Iacopino et al. [33] also reported that the amount of calbindin-D 28K expression remained unchanged in the degenerating and dying neurons of mice treated with MPTP, kainic acid or quinolic acid. When the calbindin-D 28K concentrations were decreased by incubating the cells with antisense oligonucleotides, this had no effect on the progression of cell degeneration induced by A23187, suggesting that calbindin-D 28K makes no important contribution to the paradigm of A23187-induced toxicity.

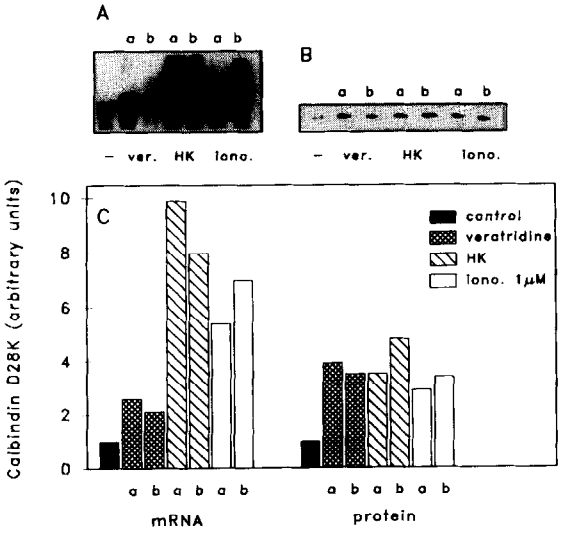

Fig. 4. Treatment of differentiated PC12 cells with veratridine (10 μM), high K^+ (50 mM), and 1 μM A23187 for 2 h (a) and 24 h (b). The mRNA (A) and protein (B) signals were quantified and are expressed in arbitrary units (C).

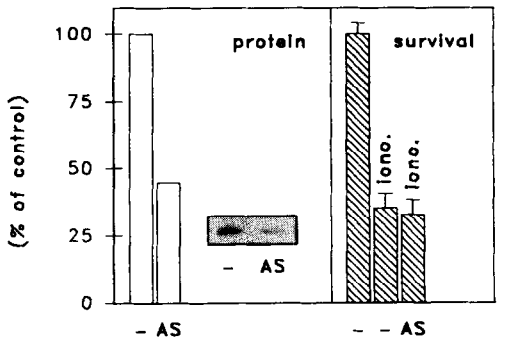

Fig. 5. Treatment of differentiated PC12 cells with antisense oligonucleotides for 48 h in serum-free medium containing NGF. (A) Measurement and quantification of calbindin-D 28K protein in oligonucleotide-treated (AS) and untreated cells. (B) The cells were incubated in the absence of serum and treated with 1.5 μM A23187 (Iono) for 24 h with or without antisense oligonucleotides. Cell survival was measured by Trypan blue exclusion. Results are expressed as % of corresponding controls.

These in vitro results suggest that in response to physiological increase in intracellular Ca^{2+}, the calbindin-D 28K positive neurons in the brain are able to regulate calcium levels aided by up-regulation of calbindin-D 28K, which thus protects these neurons in a continual manner. Above certain Ca^{2+} concentration, the compensatory calbindin-D 28K regulating mechanism becomes nonfunctional contributing to pathway of cell death. Neurons which contain little or no calbindin-D 28K are more susceptible to pathological processes as are the neurons of hippocampus during aging and in hypoxia-ischemia [34,35] or the mesencephalic dopaminergic neurons that degenerate in Parkinson's disease [17,18]. However, calbindin-D 28K is only one of the CaBPs in the brain; parvalbumin and calretinin are also implicated in Ca^{2+} homeostasis [8]. Increased intracellular calcium is suggested to be a final mediator of cell death [15,36] and augmented calbindin-D 28K mRNA and protein expression could contribute in prolonging the survival of degenerating neurons. But this remains hypothetical as the direct causes of cell death in neurodegenerative disorders and aging remain to be elucidated.

Acknowledgements: We thank Dr. O. Parkes for helpful comments. This study was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM). P.P.M. was supported by Fondation France Alzheimer.

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