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# Expression of Seizure-Related PTZ-17 Is Induced by Potassium Deprivation in Cerebellar Granule Cells

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**The aim of this study was to identify changes in gene expression during neuronal apoptosis using the differential display (DD) technique. Potassium deprivation was used to induce neuronal apoptosis in cultured rat cerebellar granule cells. DD analysis of about 1600 transcripts resulted in 8 cDNA clones that confirmed differential expression in a slot blot analysis. One of these clones was homologous to the 3′ end of seizure-related PTZ-17 RNA. Northern blot analysis showed a marked upregulation of a 2.2 kb RNA 24 hours after potassium withdrawal. This upregulation was prevented by the RNA synthesis inhibitor actinomycin D. The increase in PTZ-17 expression was specific for potassium deprivation induced apoptosis, since the other apoptosis inducers, okadaic acid and staurosporine, did not affect PTZ-17 expression. The level of PTZ-17 RNA was not significantly affected by aging in rat cerebellum. Our data suggest that the upregulation of the PTZ-17 RNA is a part of the steps leading to apoptosis during potassium deprivation in cerebellar granule cells.** 

Neuronal apoptosis is considered to play a significant role in several neuropathological conditions, such as stroke, Alzheimer's disease, and Parkinson's disease (1). However, the molecular mechanisms underlying neuronal apoptosis are poorly understood. Several cell culture models have been developed to study the molecular regulation of neuronal degeneration. One widely used model is potassium deprivation induced apoptosis in cerebellar granule cells (2). High level of extracellular $K^+$ promotes survival of several types of neurons (3–5), such as cerebellar granule cells (4), in culture. Furthermore, high potassium concentration has a promoting effect on neuronal differentiation (6) and development (7–9) *in vitro*. On the contrary, lowering of $K^+$ concentration in the culture medium induces apoptosis (2). Neuronal apoptosis requires new RNA and protein synthesis and can be prevented by actinomycin D or cycloheximide (2). Changes in intracellular $Ca^{2+}$ homeostasis are thought to be involved in induced apoptosis (10). In the case of low $K^+$ induced apoptosis, the depletion of intracellular $Ca^{2+}$ stores, which have accumulated during the depolarizing conditions in high $K^+$, could be a trigger for apoptosis during the repolarization after switching to low $K^+$ (11, 12).

We employed the differential display (DD) technique (13) to detect changes in gene expression during neuronal apoptosis induced by potassium deprivation in cerebellar granule cells (2). Differential display of mRNA is a PCR-based method developed by Liang and Pardee (13), which allows the systematic comparison of mRNAs expressed in cells in various biological processes or as a response to experimental treatments. Neurobiological DD studies concerning development (14) and aging (15), for example, have been published previously. The potassium deprivation model is of particular interest because primary cultured cerebellar granule cells are morphologically, biochemically and electrophysiologically differentiated mature neurons (4, 16).

Here we describe the upregulation of seizure-related PTZ-17 RNA during the early phase of apoptosis induced by potassium deprivation in cerebellar granule neurons. According to recent studies (17), PTZ-17 may have a role in the complex regulation of neuronal excitability and calcium homeostasis.

## MATERIALS AND METHODS

*Cell culture.* Primary cultures of cerebellar granule cells were prepared from isolated cerebella of 7-day-old Wistar rats essentially as described by Levi *et al.* (18). The cells (14 million cells per 90 mm dish) were cultured in 37°C for 7 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Life Technologies), 4.5 mg/ml glucose, 2 mM glutamine, 10 $\mu$M Ara-C (added 48 h after plating), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 5 $\mu$g/ml insulin, and 25 mM KCl.

*Induction of apoptosis.* After 7 days in vitro (DIV), the medium was replaced with fresh medium containing a low concentration (5

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mM) of KCl and 10 % dialyzed, heat-inactivated FBS (2, 19). Total RNA was extracted for DD and post-DD analysis using TRIzol reagent (Life Technologies) at several timepoints between 0 h and 48 h after switching to low KCl. To control cultures, a medium containing 25 mM KCl and 10 % dialyzed serum was changed, and samples were taken at 0 h and 24 or 48 h. To analyze the stability of PTZ-17 RNA, actinomycin D (1 $\mu$g/ml) was added to the medium at the time of potassium withdrawal. In separate experiments, okadaic acid (40 nM) or staurosporine (50 nM) were used to induce apoptosis. The drugs were added to the medium at 7 DIV, and RNA was extracted at several timepoints.

*Differential display (DD) and post-DD analysis.* Differential display was performed essentially as described (20). Briefly, RNA was reverse transcribed using $AAGCT_{11}V$ (V = G, A, or C) as the anchored 3′-primer. For the PCR amplification, DyNAzyme II DNA polymerase (Finnzymes), $AAGCT_{11}V$ anchored 3′-primers and 8 different arbitrary 13mers as 5′-primers (GC content ~50%, with a *Hin*d III site at 5′ end), and [[EK]Pa ge: 2 $\alpha$-$^{33}$P]dCTP were used. The following PCR temperature profile was used: initial denaturation 94ºC 5 min; 40 cycles of 94ºC 30 s, 42ºC 1 min, 72ºC 30 s; final extension 72ºC 5 min. After separating the PCR products in a 6 % sequencing gel and autoradiography, differentially expressed bands were excised from the gel, reamplified and cloned into a T-tailed pBluescript SK II+ vector (21). 10 recombinant clones per transformation were analyzed by SSCP (single strand conformational polymorphism) to identify different clones derived from the same DD band. SSCP analysis was performed essentially according to Fujita and Silver (22). All clones that did not produce co-migrating SSCP bands were tested for differential expression using a slot blot hybridization technique essentially as described (23). Briefly, inserts from the selected clones were amplified by vector colony-PCR and slot-blotted to duplicate Hybond N+ membranes (Amersham) in denaturing conditions. The blots were probed using a complex cDNA probe prepared by reverse transcribing 10 $\mu$g of total RNA extracted from either the KCl-deprived or control cultures in the presence of 40 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP. Clones with inserts that showed differential hybridization were sequenced.

*Northern blot analysis.* For Northern blot analysis, total RNA (10–20 $\mu$g/lane) from cerebellar granule cells were separated in a 1% agarose gel containing 1.23 M formaldehyde. The RNAs were transferred to a Hybond N+ membrane, UV crosslinked (70,000 $\mu$J/cm$^2$), and stained with methylene blue as a control of equal loading. A radioactive probe was prepared by PCR amplification of the 287 bp PTZ-17 insert using vector primers in the presence of [$\alpha$-$^{32}$P]dCTP. The PCR labelling reaction consisted of 200 $\mu$M dATP/dTTP/dGTP, 10 $\mu$M dCTP, 1 U DyNAzyme II DNA polymerase (Finnzymes), 1× optimized reaction buffer, 0.2 ng template plasmid, 200 nM T3 and T7 primers, and 30 $\mu$Ci [$\alpha$-$^{32}$P]dCTP. After purification with ProbeQuant G-50 column (Pharmacia), the probe was denatured at 100 °C for 5 min, added to the prehybridization solution (~$10^6$ cpm/ml) and hybridized for 16 h in 42°C. Prehybridization, hybridization and washes were performed as described in (24). The hybridization result was visualized and quantitated using a Storm PhosphorImager and ImageQuaNT software (Molecular Dynamics).

## RESULTS AND DISCUSSION

From a DD analysis of appr. 1600 transcripts, the 27 transcripts that showed a prominent differential amplification in duplicate DD analysis were selected for cloning. After SSCP analysis, slot blot hybridization confirmed the differential expression of 8 clones. Interestingly, one of these rat cDNAs showed a 91 % identity with mouse PTZ-17 (pentylenetetrazol-related clone 17) cDNA (25). This 287 bp fragment spans the bases 1331–1617 of the PTZ-17 sequence (GenBank Accession No. D45203). The rat RNA homolog of the mouse PTZ-17 has not been cloned. However, a search in GenBank with the rat cDNA fragment for 100 % identical rat EST sequences enabled the extension of rat PTZ-17 sequence towards the 5′ end, producing 484 bp of 3′ end sequence of rat PTZ-17 RNA 95 % identical to mouse PTZ-17. Figure 1A shows the DD result where the PTZ-17 transcript shows a marked upregulation at 24 h. In Northern blot analysis, a single transcript of 2.2 ± 0.2 kb was detected, in accordance with the reported mouse PTZ-17 RNA size (25). Furthermore, Northern blot analysis confirmed the result of differential display and showed that the expression of PTZ-17 RNA was upregulated at 6 h after the switch to low-potassium medium and continuously increased until 48 h (Figure 1). Expectedly, potassium-deprived, actinomycin D-treated cerebellar granule cells did not show increase in PTZ-17 expression (Figure 2). The level of the 2.2 kb RNA remains unchanged for at least 24 h after adding the actinomycin D, indicating a relatively high stability of the RNA.

Notably, our results show that the increase in the PTZ-17 expression level is specific for potassium deprivation. Induction of apoptosis by staurosporine (Figure 3A) or okadaic acid (Figure 3B) treatments, known to induce apoptosis in cerebellar granule cells (26, 27), did

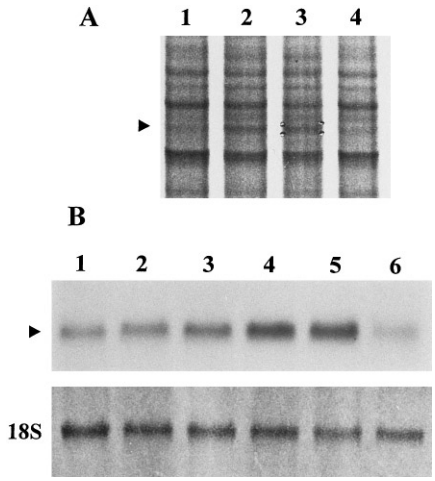


**FIG. 1.** (A) A part of the differential display gel showing the increased intensity of the 287 bp (arrowhead) PTZ-17 fragment 24 h (lane 2), and 48 h (lane 3) after potassium withdrawal in cerebellar granule neurons. Lane 1 shows the cells at 0 h and lane 4 shows the control cells grown in high potassium medium for 48 h. The DD primers used were $AAGCT_{11}C$ (3′-primer) and AAGCTTCGACTGT (5′-primer). (B) Northern blot analysis showing the progressive upregulation of the 2.2 kb PTZ-17 RNA (arrowhead) 6 h (lane 2), 12 h (lane 3), 24 h (lane 4), and 48 h (lane 5) after potassium withdrawal. Lane 1 shows the cells before the experiment and lane 6 shows the control cells grown in high potassium medium for 48 h. Methylene blue staining of 18S rRNA was used as a loading control (lower panel).

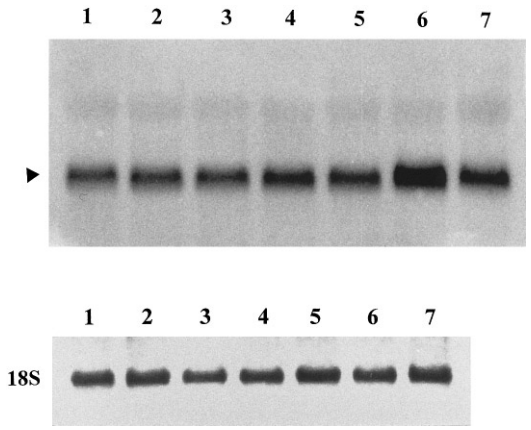


**FIG. 2.** Northern blot analysis showing the effect of actinomycin D (1 $\mu$g/ml) on the PTZ-17 transcript (arrowhead) level in cerebellar granule cells during potassium deprivation. The expression of PTZ-17 RNA remains constant when the cells are treated with actinomycin D during potassium deprivation at 6 h (lane 2), 12 h (lane 3), and 24 h (lane 4) compared to high potassium controls with actinomycin D at 0 h (lanes 1), and 24 h (lane 5). During potassium deprivation at 24 h (lane 6) the expression of PTZ-17 is upregulated compared to control at 24 h, when actinomycin D is not added. Methylene blue staining of 18S rRNA was used as a loading control (lower panel).

not enhance the expression of PTZ-17 in this cell type, but instead reduced the expression slightly after 12 h.

The expression of PTZ-17 RNA was also examined in cerebella of young (5 months), middle-aged (1.5 years)

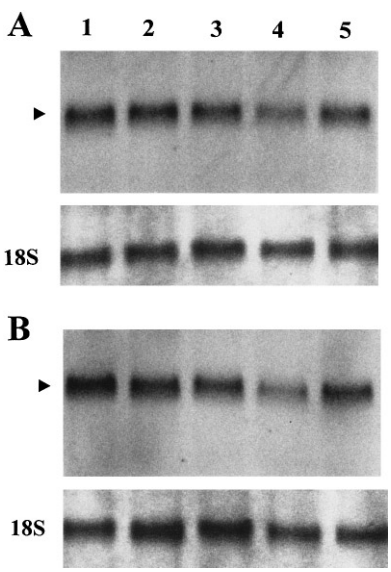


**FIG. 3.** Northern blot analysis showing the effect of staurosporine (A) and okadaic acid (B) on the level of the 2.2 kb PTZ-17 transcript (arrowheads) 4 h (lane 2), 8 h (lane 3), and 12 h (lane 4) in cerebellar granule neurons. Lane 1 shows the cells at 0 h and lane 5 shows the control cells without drugs for 12 h. Staurosporine and okadaic acid concentrations in the culture media were 50 nM and 40 nM, respectively. Methylene blue staining of 18S rRNA was used as a loading control (lower panel).

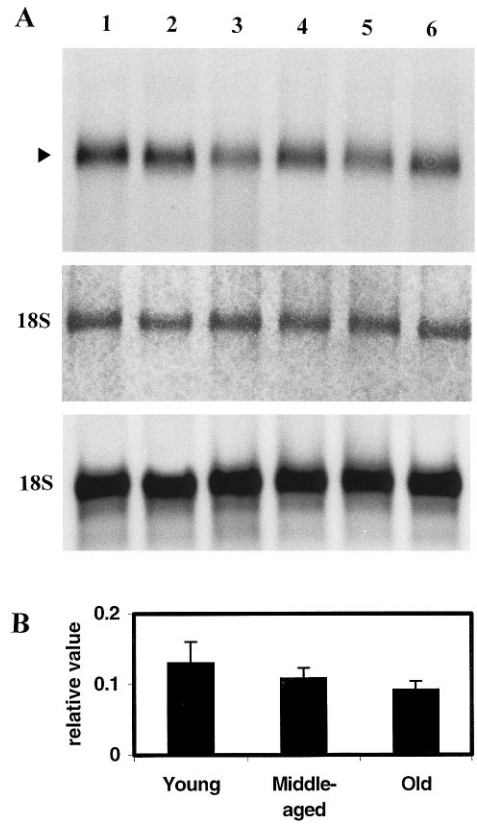


**FIG. 4.** (A) Northern blot analysis showing the expression of PTZ-17 RNA (arrowhead) in young (5 months) (lanes 1 and 4), middle aged (1.5 years) (lanes 2 and 5), and old (3 years) (lanes 3 and 6) female Wistar rats. The expression is slightly reduced during normal aging. Methylene blue staining of 18S rRNA was used as a loading control (middle panel). The RNAs were also hybridized with a probe against 18S rRNA as an internal control (bottom panel). (B) PTZ-17 expression in young, middle-aged, and old Wistar rats presented as relative pixel volumes normalized against 18S rRNA.Values represent mean ± S. D., n = 3.

and old (3 years) female Wistar rats. The expression level of PTZ-17 RNA decreased slightly during aging, but no statistically significant differences were observed (Figure 4). The sustained expression of PTZ-17 in adult rats supports the findings of Studler *et al.* (28) that PTZ-17 could have a physiological role not only in development but also in adults.

Pentylenetetrazol (PTZ) is a potent epileptogenic agent which has been used as a tool to study the mechanism of epileptogenesis (29). Kajiwara *et al.* (25) used a differential hybridization technique to detect PTZ-induced alterations in neuronal gene expression. They cloned and characterized a cDNA designated PTZ-17, the RNA expression of which showed a major down-regulation after PTZ treatment. Interestingly, the injection of PTZ-17 RNA into *Xenopus* oocyte provoked a large calcium inward current following the extracellular application of PTZ. The nucleotide sequence of PTZ-17 (1618 bp) is 99 % identical to the 3′ end of the

3.1 cDNA cloned earlier by Studler *et al.* (28). The expression of 3.1 RNA is high in mouse cerebellum, hippocampus and heart (28).

PTZ-17 RNA shows an interesting expression pattern: depolarization of neurons induces downregulation (25), whereas repolarization by potassium deprivation upregulates the PTZ-17 expression. It is known (5) that the prolonged depolarization of cultured neurons, *e.g.* by high potassium concentration in medium, maintains an elevated intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) concentration and promotes neuronal survival, while low potassium concentration reduces the intracellular $Ca^{2+}$ concentration and triggers an apoptotic cell death (10). Franklin and Johnson (5) have presented a "$Ca^{2+}$ set-point hypothesis" to postulate four steady-state levels of calcium that affect survival. According to this hypothesis, epileptogenic insult by PTZ might present an excitotoxic level of $[Ca^{2+}]_i$ and may induce necrosis, whereas potassium deprivation decreases $[Ca^{2+}]_i$ which triggers an apoptotic cell death. How might PTZ-17 RNA be regulated and related to neuronal death? Recently, Kajiwara *et al.* (17) reported that a particular sequence within the 3′-untranslated region (3′UTR) of PTZ-17 RNA binds to 60 and 47 kDa intracellular proteins. The injection of this 3′UTR sequence into *Xenopus* oocyte induced a calcium current similar to that caused by the injection of whole-length PTZ-17 RNA. Moreover, they showed (17) that the mouse strains with different susceptibilities to epileptic convulsions, DBA/2 and BALB/c, have sequence differences in the 3′UTR of PTZ-17. The injection of PTZ-17 RNA derived from DBA/2 mice, which have a high susceptibility to epileptic convulsions, caused a large calcium current in *Xenopus* oocytes after PTZ treatment, but the RNA from BALB/c mice with a low susceptibility to convulsions showed no PTZ response in oocyte calcium currents.

The above findings suggest that PTZ-17 transcripts and cytoplasmic binding proteins are involved in the regulation of calcium entry into neurons in the case of PTZ-induced epileptic bursts. Interestingly, Studler *et al.* (28) failed to detect any 3.1/PTZ-17 protein in tissues that express the RNA at high level although the antibodies could recognize the protein produced by *in vitro* translation of synthetic 3.1 RNA. The role of PTZ-17 and its interacting RNA-binding proteins in this complex regulation of neuronal excitability and calcium homeostasis will be understood in the future.

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