Direct Alteration of the P/Q-Type Ca^{2+} Channel Property by Polyglutamine Expansion in Spinocerebellar Ataxia 6

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Spinocerebellar ataxia 6 (SCA6) is caused by expansion of a polyglutamine stretch, encoded by a CAG trinucleotide repeat, in the human P/Q-type Ca^{2+} channel α_{1A} subunit. Although SCA6 shares common features with other neurodegenerative glutamine repeat disorders, the polyglutamine repeats in SCA6 are exceptionally small, ranging from 21 to 33. Because this size is too small to form insoluble aggregates that have been blamed for the cause of neurodegeneration, SCA6 is the disorder suitable for exploring the pathogenic mechanisms other than aggregate formation, whose universal role has been questioned. To characterize the pathogenic process of SCA6, we studied the effects of polyglutamine expansion on channel properties by analyzing currents flowing through the P/Q-type Ca^{2+} channels with an expanded stretch of 24, 30, or 40 polyglutamines, recombinantly expressed in baby hamster kidney cells. Whereas the Ca^{2+} channels with $\leq$24 polyglutamines showed normal properties, the Ca^{2+} channels with 30 or 40 polyglutamines exhibited an 8 mV hyperpolarizing shift in the voltage dependence of inactivation, which considerably reduces the available channel population at a resting membrane potential. The results suggest that polyglutamine expansion in SCA6 leads to neuronal death and cerebellar atrophy through reduction in Ca^{2+} influx into Purkinje cells and other neurons. Besides the widely accepted notion that polyglutamine stretches exert toxic effects by forming aggregates, expanded polyglutamines directly alter functions of the affected gene product.

Key words: spinocerebellar ataxia 6 (SCA6); P/Q-type Ca^{2+} channel; CAG repeat expansion; polyglutamine repeat; recombinant expression; neuronal death

Expansion of a polyglutamine stretch, encoded by a CAG trinucleotide repeat, in the human P/Q-type Ca^{2+} channel α_{1A} subunit is associated with spinocerebellar ataxia 6 (SCA6) (Zhuchenko et al., 1997). Expanded polyglutamines cause several diseases, including Huntington's disease (Huntington's Disease Collaborative Research Group, 1993), dentatorubral–pallidoluysian atrophy (Koide et al., 1994; Nagafuchi et al., 1994), spinobulbar muscle atrophy (SBMA) (La Spada et al., 1991), Machado–Joseph disease (also termed SCA3) (Kawaguchi et al., 1994), and other forms of spinocerebellar ataxia (SCA1, 2, and 7) (Orr et al., 1993; Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996; David et al., 1997). SCA6 shares common features with other glutamine repeat disorders: (1) inheritance is autosomal dominant (except for X-linked SBMA); (2) the disorders are progressive; (3) there is an inverse correlation between the age of onset and the CAG repeat number; and (4) the CNS is commonly affected with distinctive distributions of neuronal loss. However, SCA6 exhibits unique features: (1) the CAG repeat is exceptionally small in SCA6, ranging from 21 to 33 (Matsuyama et al., 1997; Yabe et al., 1998), whereas a repeat of >40 units generally leads to disease in other diseases; and (2) clinical features of SCA6 consist predominantly of cerebellar symptoms (Zhuchenko et al., 1997), whereas other diseases involve the brain more extensively.

The mechanisms by which polyglutamine stretches cause neurodegeneration have been the subject of intensive investigation, and it is widely accepted that polyglutamine stretches exert toxic effects by forming aggregates (Ikeda et al., 1996; Christopher, 1997). But there has been no evidence of nuclear inclusions indicative of aggregate formation in neurons of the patients with SCA6. Furthermore, the direct role of intranuclear aggregates in induction of neuronal degeneration has been questioned on the basis of the studies using cellular or animal models of Huntington's disease (Saudou et al., 1998) and SCA1 (Klement et al., 1998). SCA6 is unequaled among glutamine repeat disorders in that the functional properties of the affected gene product, *i.e.* the P/Q-type voltage-gated Ca^{2+} channel, is quantitatively investigated, whereas functional roles of other affected gene products are mostly unknown. To elucidate the pathogenic nexus between expanded polyglutamines and neurodegeneration in polyglu-

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tamine repeat disorders, we studied the direct effects of polyglutamine expansion on channel properties by analyzing currents flowing through the P/Q-type Ca^{2+} channels with an expanded stretch of 24, 30, or 40 polyglutamines, recombinantly expressed in baby hamster kidney (BHK) cells.

MATERIALS AND METHODS

Construction of cDNAs. The 7.9 kb *Hin*dIII (on vector)–*Bam*HI (7739) fragment of pSPCBI-1 carrying the entire protein-coding sequence of the BI-1 Ca^{2+} channel cDNA (Mori et al., 1991; Genbank accession number X57476) was inserted into the *Hin*dIII–*Bam*HI site of pK4K (Niidome et al., 1994) to yield pK4KBI-1. (Nucleotide residues are numbered from the first residue of the ATG-initiating triplet of the unmodified BI-1. Restriction endonuclease sites are identified by numbers indicating the 5′-terminal nucleotide generated by cleavage.) To insert the sequence of GGCAG between nucleotide residues 6819 and 6820, the *Eco*47III (6770)–*Kpn*I (6862) fragment of pK4KBI-1 was replaced by the synthetic oligonucleotides to yield pK4KBI-1-CAG(4); the wild-type sequence contains four CAG trinucleotide repeats. To insert longer CAG repeats, the *Ppu*MI (6963)–*Bal*I (6990) fragment was replaced with synthetic oligonucleotides to yield pK4KBI-1-CAG(n) (n = 24, 30, or 40). In addition to pK4KBI-1-CAG(4), we used pK4KBI-2 (Niidome et al., 1994) as another control. The transiently or stably expressed BI-2 Ca^{2+} channels give the indistinguishable parameters for gating and voltage dependence (Wakamori et al., 1998b).

Expression of the α_{1A} Ca^{2+} channels in BHK cells. The control and mutant P/Q-type Ca^{2+} channels were expressed transiently or stably by introducing α_{1A} subunit cDNAs into the BHK6 cells, which were BHK cells stably expressing the Ca^{2+} channel α_2 and β_{1a} subunits (Wakamori et al., 1998b). The BHK6 cells were grown in DMEM containing 10% fetal bovine serum, penicillin (30 U/ml), and streptomycin (30 μg/ml). BHK6 cells lack endogenous Ca^{2+} channel activity.

For transient expression, BHK6 cells were transfected with pK4KBI-1-CAG(n) (n = 4, 24, 30, or 40) or pK4KBI-2, plus πH3-CD8 containing the cDNA of the T-cell antigen CD8 (Jurman et al., 1994), using SuperFect transfection reagent (Qiagen, Hilden, Germany). Cells were trypsinized and plated onto plastic coverslips (Celldesk; Sumitomo Bakelite, Tokyo, Japan) 18 hr after transfection. Cells were subjected to measurements 36–66 hr after plating on the coverslips. Cells expressing the control or mutant Ca^{2+} channels were selected through detection of CD8 coexpression using polystyrene microspheres precoated with antibody to CD8 (Dynabeads, M-450 CD8; Dynal, Oslo, Norway). For stable expression, BHK6 cells were transfected with pK4KBI-2 using SuperFect transfection reagent and were selected in DMEM containing methotrexate (500 nM) (Sigma). The cells were seeded onto Celldesk and incubated in culture medium for 5–8 d before measurements.

Electrophysiology. Currents were recorded at room temperature (22–25°C) using whole-cell mode of the patch clamp (Hamill et al., 1981) with an Axopatch 200B patch-clamp amplifier (Axon Instruments), as described previously (Wakamori et al., 1998a). Patch pipettes were made from borosilicate glass. Pipette resistance ranged from 1 to 2 MΩ when filled with the pipette solutions described below. The series resistance was electronically compensated to >70%, and both the leakage and the remaining capacitance were subtracted by −P/6 method. Currents were sampled at 10 kHz after low-pass filtering at 2 kHz (−3 dB) using the eight-pole Bessel filter (Frequency Devices), unless otherwise specified. Data were collected and analyzed using the pCLAMP 6.02 software (Axon Instruments). The external solution contained (in mM): 3 $BaCl_2$, 155 tetraethylammonium chloride (TEA-Cl), 10 HEPES, and 10 glucose, pH adjusted to 7.4 with TEA-OH. The pipette solution contained (in mM): 85 Cs-aspartate, 40 CsCl, 2 $MgCl_2$, 5 EGTA, 2 ATP-Mg, 5 HEPES, and 10 creatine phosphate, pH adjusted to 7.4 with CsOH.

Statistics. Statistical comparison between the control BI-1-CAG(4) and the mutant channels was performed by Student's t test (*$p < 0.05$).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay for apoptotic cell death. To detect apoptotic cell death, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was made 48 and 72 hr after transient transfection of pK4KBI-1-CAG(4), pK4KBI-1-CAG(40), or pK4KBI-2 cDNA, plus πH3-CD8 into BHK6 cells using the Apoptosis *in situ* detection kit (Wako, Osaka, Japan) according to the manufacturer's instructions. Expressing cells were selected through detection of CD8 as described above, and occurrence of apoptotic nuclear changes was counted in 100 cells for each measurement. Expression of CD8 itself did not cause apoptotic cell death.

RESULTS

The CAG repeat of the Ca^{2+} channel α_{1A} subunit cDNA is located in the 3′-terminal region, where a considerable variation in alternative splicing has been reported (Mori et al., 1991; Zhuchenko et al., 1997). The insertion and deletion of an exon give rise to two isoforms, BI-1 and BI-2, of the rabbit α_{1A} cDNA (Mori et al., 1991). Among the six alternatively spliced isoforms of the human α_{1A} subunit cDNA, three have GGCAG insertion before the terminal codon; consequently the succeeding ~700 nucleotides containing the CAG repeat being translated (Zhuchenko et al., 1997). Because the BI-1 cDNA, which is highly identical to the human isoforms that contain the CAG repeat, has a $(CAG)_4$ repeat but lacks GGCAG, the pentanucleotide sequence was inserted into the BI-1 cDNA to yield BI-1-CAG(4). The CAG repeat was expanded to yield mutant cDNAs, BI-1-CAG(n) (n = 24, 30, and 40). The control and mutant BI-1 cDNAs, as well as the BI-2 cDNA (Mori et al., 1991) as yet another control, were placed in the pK4K plasmid (Niidome et al., 1994) and were expressed in a BHK cell line, in combination with the Ca^{2+} channel α_2 and β_1 subunit cDNAs (Niidome et al., 1994).

With depolarization from a holding potential of −100 mV, BHK cells expressing the control and mutant Ca^{2+} channels produced significant amplitudes of inward currents in the 3 mM Ba^{2+} external solution (Fig. 1*A*). The currents first appeared at −30 mV and grew with increments of depolarization, reached a peak in the current–voltage relationship at ~0 mV, and then declined with further depolarization (Fig. 1*B*). Figure 1*C* compares peak current densities for the two control and three mutant channels. The current densities of the mutant channels were not statistically different from those of the control channels.

To obtain the voltage dependence of activation, tail currents were recorded at a potential of −50 mV after the termination of 5 msec test pulses to various potentials (Fig. 2*A*). Normalized tail current amplitudes plotted against test potentials were fitted to a single-component Boltzmann equation. The Ca^{2+} channels with a stretch of 30 or 40 polyglutamines showed a slight hyperpolarizing shift with a small, but statistically significant, increase in steepness of the voltage dependence of activation, indicating that polyglutamine expansion exerts only a mild effect on the voltage dependence of activation (Table 1).

The voltage dependence of inactivation was determined by a conventional protocol with 2 sec prepulses followed by a test pulse to 0 mV (Fig. 2*B*). Normalized peak current amplitudes induced by test pulses, plotted against prepulse potentials, were fitted with the Boltzmann equation to yield the half-inactivation potential and the slope factor for the control and mutant channels (Table 1). Whereas the Ca^{2+} channel with a stretch of 24 polyglutamines showed the voltage dependence of inactivation indistinguishable from that of controls, the Ca^{2+} channels with a stretch of 30 or 40 polyglutamines exhibited a significant shift in the voltage dependence of inactivation in the hyperpolarizing direction by 8 mV.

To further characterize the inactivation process, inactivation kinetics were examined by giving test pulses lasting 300 msec to different voltages. The decay phase was well fitted by a two-exponential function with a noninactivating component. The fast and slow time constants and their fractions of the mutant α_{1A} channels were not significantly different from those of the control channels at all test potentials, as exemplified by the values at 10 mV shown in Table 2. And we could not detect the differences in

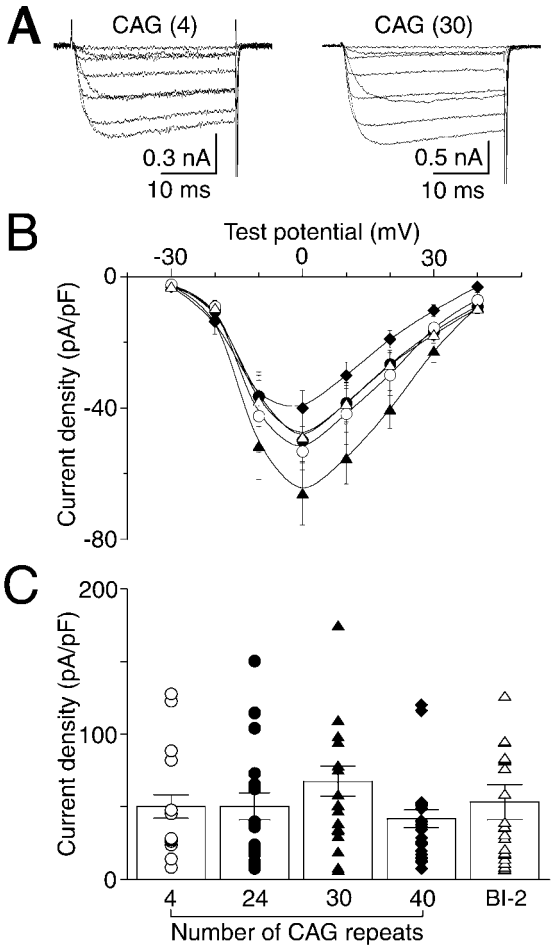

Figure 1. Current–voltage relationships and current density. *A*, Families of Ba^{2+} currents evoked by 30 msec depolarizing pulses from −30 to 40 mV with increments of 10 mV from a holding potential of −100 mV. CAG(4) and CAG(30) channels were transiently expressed in BHK cells. *B*, Current density–voltage relationships. Data are expressed as means ± SEM of 21, 12, 19, 17, and 23 BHK cells transiently expressing CAG(4) (○), CAG(24) (●), CAG(30) (▲), CAG(40) (♦), and BI-2 (△) channels, respectively. *Curves* are drawn by an interpolation process. *C*, Distribution of peak current density. Individual values (*symbols*) and means (*open box*) ± SEM are shown. Symbols and numbers of recorded cells are as in *B*.

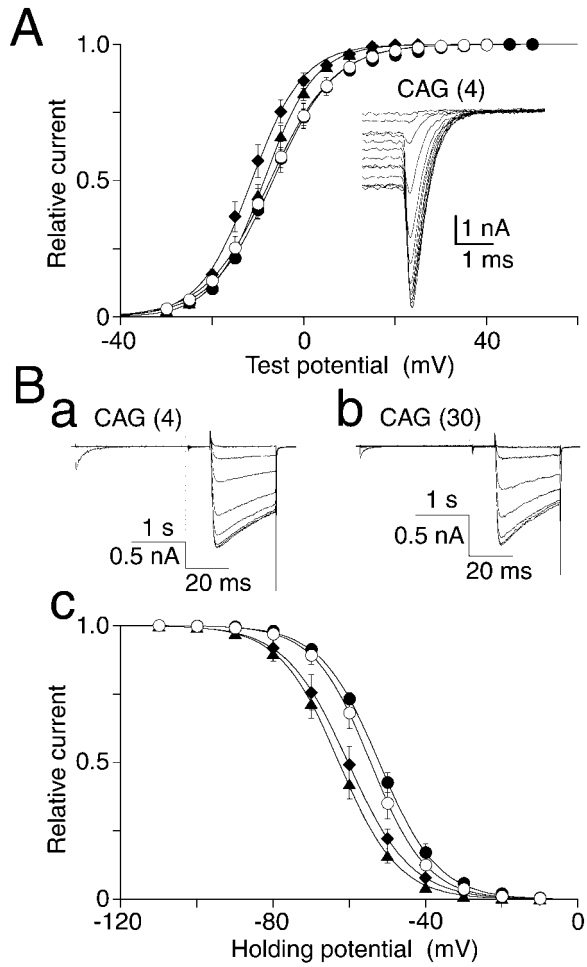

Figure 2. Voltage dependence of activation and inactivation. *A*, Comparison of activation curves. *Inset*, Superimposed tail currents elicited by repolarization to −50 mV after a 5 msec test pulse from −25 to 35 mV with 5 mV increments in CAG(4). Currents were filtered at 10 kHz and digitized at 100 kHz. The amplitude of tail currents was normalized to the tail current amplitude obtained with a test pulse to 50 mV. The mean values from 8–16 cells were plotted against test pulse potentials and fitted to the Boltzmann equation. *Vertical bars* show means ± SEM if they are larger than symbols. ○, CAG(4); ●, CAG(24); ▲, CAG(30); ♦, CAG(40). *B, a, b,* Ba^{2+} currents evoked by 30 msec test pulse to 0 mV after the 10 msec repolarization to −100 mV after 2 sec prepulses from −100 to −20 mV with 10 mV increments in BHK cells expressing CAG(4) or CAG(30). Time scale was changed at the time indicated by the *dotted line*. *B, c*, Comparison of inactivation curves. The amplitude of currents elicited by the test pulses was normalized to the current amplitude induced by the test pulse after a prepulse to −110 mV. The mean values from 5–13 cells were plotted against prepulse potentials and fitted to the Boltzmann equation. *Vertical bars* show means ± SEM if they are larger than symbols. Symbols as in *A*.

the inactivation recovery time course among the channels (data not shown).

To probe the pathogenic process of SCA6 subsequent to the alteration of the P/Q-type Ca^{2+} channel property, we studied whether apoptotic cell death is induced by transiently expressing the BI-1-CAG(n) (n = 4 or 40) or BI-2 using the TUNEL assay. Forty-eight and 72 hr after transient transfection, however, we could not observe apoptotic cell death in cells expressing the Ca^{2+} channels with or without expanded polyglutamines (data not shown).

DISCUSSION

Expansion of CAG repeats encoding polyglutamine tracts has been associated with a group of neurodegenerative diseases. Among the glutamine repeat disorders, SCA6 is unmatched in that functional properties of the affected gene product, the P/Q-type Ca^{2+} channel α_{1A} subunit, have been extensively studied, and that even subtle changes in the properties can be precisely detected, whereas functions of the proteins affected in other glutamine repeat disorders are unknown, with the exception of the androgen receptor in spinobulbar muscle atrophy (La Spada et al., 1991). In this study, we reconstituted the initial triggering step of the SCA6 pathogenic process by recombinantly expressing the α_{1A} Ca^{2+} channel cDNAs with expanded CAG repeats. The results demonstrated that expanded polyglutamines can directly alter the functional property of the affected protein.

The CAG repeat expansion did not affect the expression level of the functional Ca^{2+} channels, based on the unaltered current densities. This result contrasts with that obtained for the Ca^{2+} channels with the *tottering* (*tg*) or *leaner* (tg^{la}) mutations (Waka-

Table 1. Activation and inactivation parameters of α_{1A} channels in BHK cells

	Activation			Inactivation		
Channel	*n*	$V_{0.5}$ (mV)	*k* (mV)	*n*	$V_{0.5}$ (mV)	*k* (mV)
CAG (4)	9	−7.1 ± 1.5	6.47 ± 0.42	5	−54.4 ± 2.0	7.07 ± 0.53
CAG (24)	16	−8.2 ± 0.9	6.20 ± 0.32	11	−51.9 ± 0.9	7.43 ± 0.32
CAG (30)	14	−9.4 ± 1.1	5.21 ± 0.29*	13	−62.9 ± 1.8*	6.82 ± 0.25
CAG (40)	8	−10.8 ± 1.1	5.22 ± 0.32*	11	−61.5 ± 1.9*	7.55 ± 0.35
BI-2	6	−7.6 ± 1.0	5.48 ± 0.38	12	−52.9 ± 1.4	8.72 ± 0.23*

n, Number of cells recorded; $V_{0.5}$, half-maximal voltage of activation and inactivation; *k*, slope factor. Data are expressed as means ± SEM. The BI-2 channel was expressed stably. Statistical comparison between the control BI-1-CAG(4) and the mutant channels was performed by Student's *t* test (*$P < 0.05$).

Table 2. Inactivation kinetics of α_{1A} channels in BHK cells at 10 mV

Channel	*n*	τ_f (msec)	I_f	τ_s (msec)	I_s	I_∞
CAG (4)	4	21.2 ± 1.9	0.29 ± 0.03	91.5 ± 5.8	0.69 ± 0.03	0.02 ± 0.01
CAG (24)	7	20.8 ± 2.2	0.24 ± 0.03	98.2 ± 5.1	0.69 ± 0.03	0.07 ± 0.01
CAG (30)	8	19.4 ± 2.2	0.26 ± 0.04	91.3 ± 10.0	0.73 ± 0.04	0.01 ± 0.01
CAG (40)	5	18.6 ± 0.8	0.26 ± 0.04	107 ± 5.7	0.71 ± 0.03	0.03 ± 0.01
BI-2	20	18.5 ± 1.6	0.27 ± 0.02	97.5 ± 6.0	0.68 ± 0.02	0.05 ± 0.01

n, Number of cells recorded; τ_f, fast inactivation time constant; τ_s, slow inactivation time constant; I_f, the relative fast component of the initial current; I_s, the relative slow component of the initial current; I_∞, the relative noninactivating component. Data are expressed as means ± SEM. The BI-2 channel was expressed stably.

mori et al., 1998b). The *tg* and *tg*^{la} mutations reduced the Ca^{2+} channel current densities in native cerebellar Purkinje neurons, and the reduction was successfully reproduced in the BHK cells expressing mutant recombinant channels. The present result of unaffected current densities in the repeat mutants suggests that the Ca^{2+} channel proteins with a pathologically expanded polyglutamine stretch are transported to the plasma membrane in the normal manner, without forming aggregates.

In contrast to the unaltered expression level, the CAG expansion affected the property of the Ca^{2+} channel. Expansion of 30 or 40 polyglutamines in the distal C terminus causes a significant shift in the voltage dependence of inactivation in the hyperpolarizing direction by 8 mV. Although the proximal portion of the C terminus contributes to determining inactivation kinetics in the L-type Ca^{2+} channel (Soldatov et al., 1998), or to interaction with G-proteins in the N-, P/Q-, and R-type Ca^{2+} channels (Qin et al., 1997; Furukawa et al., 1998), the distal portion of the C terminus is not critically involved in regulating the intrinsic gating properties, because the BI-2 channel, which has a different C terminus, exhibits almost identical gating properties as the control BI-1-CAG(4). The expanded stretches of polyglutamines may impair channel gating by altering interacting with other proteins.

The negative shift in the voltage dependence of inactivation exerts a considerable effect on channel availability. For example, at a resting potential of −55 mV, more than three-fourths of the channels with 30 polyglutamines are inactivated, less than one-fourth being available for activation, whereas more than half of the normal channels are available. A simple estimate predicts that Ca^{2+} influx is almost halved for cells expressing the Ca^{2+} channels with pathogenic polyglutamine expansion. The notion that the voltage dependence of inactivation of the P/Q-type Ca^{2+} channel is a critical factor determining the fate of Purkinje neurons is supported by the recent report that in the seizure-prone, ataxic mutant mice stargazer (*stg*), disrupted expression of the newly identified Ca^{2+} channel γ subunit gene results in a shift in the voltage dependence of inactivation of the P/Q-type Ca^{2+} channel (Letts et al., 1998).

Although it is well established that Ca^{2+} overload triggers excitotoxic neuronal death (Choi, 1995), several lines of evidence suggest that lack of adequate Ca^{2+} influx also causes neuronal death. As mentioned above, the Ca^{2+} influx into cerebellar Purkinje neurons is reduced in the ataxic *tg* mice (Wakamori et al., 1998b) and in the more severely affected *tg*^{la} mice (Lorenzon et al., 1998; Dove et al., 1998; Wakamori et al., 1998b), and apoptotic neuronal cell death is observed in the cerebellum of *tg*^{la} mice (Fletcher et al., 1996). Furthermore, the effect of a low intracellular Ca^{2+} has been demonstrated using neuronal cultures. Decreased intracellular free Ca^{2+} concentrations, brought about by organic Ca^{2+} antagonists or by low extracellular K^+ concentrations, trigger the apoptotic process, which is prevented by the application of Bay K8644, L-type Ca^{2+} channel agonist (Koh and Cotman, 1992; Galli et al., 1995). To look into the subsequent steps of the pathogenic process of SCA6, we studied the possible apoptotic effect in BHK cells of the Ca^{2+} channels with polyglutamine stretches. However, no apoptotic cell death was induced in BHK cells expressing the Ca^{2+} channels with or without expanded polyglutamines. To induce apoptotic cell death in an experimental condition, it seems necessary to use neuronal cell lines and/or a longer duration. Taking these results into consideration, we conclude that the polyglutamine expansion in SCA6 alters the P/Q-type Ca^{2+} channel property to reduce Ca^{2+} influx, which triggers subsequent pathogenic steps in Purkinje cells and other neurons, ultimately leading to neuronal death and cerebellar atrophy.

A number of lines of evidence have suggested that expanded polyglutamines form aggregates in the nucleus and exert a toxic effect (Ikeda et al., 1996; Christopher, 1997). In SCA6, however, the length of glutamine repeats is not long enough to form aggregates, and our data have shown that expanded polyglutamines do not reduce the amount of the functional protein. The Ca^{2+} channel α_{1A} subunit is a membrane protein, whereas proteins affected in other glutamine repeat disorders are cytoplasmic or nuclear proteins. All these facts suggest that aggregate formation is unlikely to be involved in the pathogenesis of SCA6.

Instead, the present study has clearly demonstrated that polyglutamine expansion exerts direct effects on the property of the P/Q-type Ca^{2+} channel. Although we cannot evaluate functional impairments of affected gene products in other glutamine repeat disorders, it is possible that some of their functions are compromised. Because the universal role of aggregate formation in the neurodegenerative process has been questioned (Sisodia, 1998), the direct effect of expanded polyglutamines in other glutamine repeat disorders has to be considered as an additional or alternative mechanism, which may explain the cell specificity that only a selected population of neurons undergo degeneration, whereas the genes carrying the expanded CAG repeat are expressed widely throughout the brain.

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