Abnormal CAG repeat expansion in the α1A voltage-dependent calcium channel gene is associated with spinocerebellar ataxia type 6 (SCA6), an autosomal dominant cerebellar ataxia with a predominant loss of the Purkinje cell. A reverse transcriptase-polymerase chain reaction analysis of mRNA from mouse Purkinje cells revealed a predominant expression of the α1A channel lacking an asparagine-proline (NP) stretch in the domain IV (α1A(NP)). Human α1A channels carrying various polyglutamine length with or without NP were expressed using HEK293 cells, and channel properties were compared using a whole-cell voltage clamp technique. α1A(NP), corresponding to P-type channel, with 24 and 28 polyglutamines found in patients showed the voltage dependence of inactivation shifting negatively by 6 and 11 mV, respectively, from the 13 polyglutamine control. Contrarily, the α1A channel with NP (α1A(+NP)), corresponding to Q-type channel, with 28 polyglutamines exhibited a positive shift of 5 mV. These results suggest that altered function of α1A(NP) may contribute to degeneration of Purkinje cells, which express predominantly α1A(NP), due to the reduced Ca$^{2+}$ influx resulting from the negative shift of voltage-dependent inactivation. On the other hand, other types of neurons, expressing both α1A(NP) and α1A(+NP), may survive because the positive shift of voltage-dependent inactivation of α1A(+NP) compensates Ca$^{2+}$ influx.

Spinocerebellar ataxia type 6 (SCA6) is one of the autosomal dominant neurodegenerative diseases, which is characterized by late-onset slow-progressive cerebellar ataxia and Purkinje cell predominant degeneration in the cerebellum (1, 2). Zuchенко et al. (3, 4) demonstrated abnormal CAG repeat expansion in the coding region of the α1A voltage-dependent calcium channel gene (CACNA1A), which maps to chromosome 19p13.1. All the polyglutamine diseases identified so far are progressive neurodegenerative disorders such as spinal and bulbar muscular atrophy (5), Huntington's disease (6), SCA1 (7), dentatorubral-pallidoluysian atrophy (8, 9), SCA3/Machado-Joseph disease (10), SCA2 (11–13), and SCA7 (14). Although their clinical and pathological features are widely diverse, a common mechanism is presumed to underlie the pathogenesis in which some cytological abnormalities such as nuclear inclusion are claimed to induce cytotoxicity. On the other hand, several unique characteristics have been demonstrated in SCA6 compared with other polyglutamine diseases. The CAG repeat expansion is smaller (4–20 in normal alleles and 21–33 in mutated alleles), the repeat is more stable, and the anticipation is much milder than other polyglutamine diseases (3, 4, 15). Furthermore, other mutations in CACNA1A are known to be associated with both human and mouse diseases such as familial hemiplegic migraine (FHM), episodic ataxia type 2 (EA-2) (16), and dominant cerebellar ataxia (17). These diseases often have a progressive ataxia like SCA6. Considering these together with the fact that CACNA1A encodes an α1A subunit of the P/Q (P and/or Q)-type calcium channel which plays a crucial role in the brain function, especially for the Purkinje cells (18–20), we hypothesized that, unlike the other polyglutamine diseases, functional alterations of the α1A calcium channel are causally related to pathophysiology of SCA6.

An alteration of the P/Q (P and/or Q)-type calcium channel property by the polyglutamine stretches in the rabbit α1A channel expressed in baby hamster kidney (BHK) cells was reported for the channels with 30 or 40 polyglutamines but not with 24 polyglutamines (21). However, it is still obscure whether the observed change reflects functional abnormalities in SCA6 brains, since the authors did not observe the alteration for the channel with 24 polyglutamines, which falls exactly within the range in SCA6 patients. These observations and our hypothesis prompted us to explore the conditions that ensure matching the functional alteration to the disease without such discrepancy. Furthermore, the presence or absence of an NP insertion in the domain IV S3-S4 in the α1A channels is recently postulated as the essential determinant of the P/Q channel-kidney; kb, kilobase pair; RT-PCR, reverse transcriptase-polymerase chain reaction.
nel subtype distinction (22). In SCA6, degeneration occurs dominantly in Purkinje cells, which possess mostly the P-type channel, whereas other neurons such as granule cells, which express Q-type together with P-type, are basically preserved. Therefore, we designed experimental conditions in which the human full-length a1A channels are expressed in human cells to allow an in situ operation of mutated channel functions, and we compare their properties between channels with or without the NP insertion in order to relate the channel subtypes to the altered mechanism. Here, we report that a moderate elongation caused a negative shift of voltage dependence of inactivation in the presumed P-type channel, whereas the same elongation caused a positive shift in the presumed Q-type channel. These may explain a plausible mechanism of the selective Purkinje cell degeneration.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis**

Total RNAs were obtained separately from frozen cerebellar tissues of control and a SCA6 cases. RT-PCR of CACNA1A mRNA was performed as described previously (23). The nucleotide sequences of a coding region of CACNA1A cDNA from a control and a patient with SCA6 were analyzed by directly sequencing the PCR products using ABI 310 Prism Sequencing Analysis (PE Applied Biosystems).

**RT-PCR**

Single Purkinje cells from mouse cerebellum were obtained under microscopic observation using a glass micropipette and were directly subjected to reverse transcription in a tube containing random hexamer (Perkin Elmer, Pomona, CA) and First Strand Buffer (Life Technologies, Inc.). Total RNA was obtained from mouse cerebellum. Young male C57BL/6N (Clea Japan Inc., Japan) mice (20 days old) were used. Primers for amplifying the region spanning the AATCCG insertion site were 5'-GGAAATTGTTGGCTGAGAACCCATGTT-3' for a sense primer and 5'-GACCCCTGGTAACCGAGAAGACCTG-3' for an antisense primer. Quantification was performed using ABI 310 Prism Gene Scan (PE Applied Biosystems) as described (23).

**Expression Vectors**

HS13(+NP), HL24(+NP), and HL28(+NP)—CACNA1A cDNAs, containing 13, 24, or 28 CAG repeats with AATCCG insertion, which correspond to the amino acid NP (asparagine and proline) in the domain IV, were subcloned in the SphI and XbaI sites of pcDNA I/Amp (Invitrogen, Carlsbad, CA), as described previously (23). HS13(-NP), HL24(-NP), and HL28(-NP)—To construct full-length CACNA1A cDNAs containing 13, 24, or 28 CAG repeats and lacking the AATCCG insertion, we first amplified the region spanning the AATCCG insertion site to yield HS13(-NP), HL24(-NP), or HL28(-NP). Thirty-two to 72 h after transfection, cells were used for the electrophysiological analysis.

**RESULTS**

**Expression Vectors**

HS13(+NP), HL24(+NP), and HL28(+NP)—CACNA1A cDNAs, containing 13, 24, or 28 CAG repeats with AATCCG insertion, which correspond to the amino acid NP (asparagine and proline) in the domain IV, were subcloned in the SphI and XbaI sites of pcDNA I/Amp (Invitrogen, Carlsbad, CA), as described previously (23).

**RT-PCR**

**Analysis of NP Insertion in a1A Channel—Two splice variants, with or without insertion of an asparagine-proline (NP) stretch in the domain IV, are known in rat a1A channel (22). In situ hybridization and the pharmacological studies suggested that native P-type channels lack NP and that Q-type channels contain these residues (22).**

**To investigate the difference in the levels of mRNAs spanning the AATCCG insertion site, we conducted RT-PCR analysis of mouse cerebellum and Purkinje cells. In a whole mouse cerebellum, the amount of RT-PCR product of a1A channel without NP (a1A(-NP)) was more than twice as large as that of a1A channel with NP (a1A(+NP)) (Fig. 1A). On the other hand, mRNA for a1A(-NP) was predominant in single mouse Purkinje cells (Fig. 1B). These results suggest that, like the rat a1A channel, a1A(-NP) generally corresponds to the P-type, whereas a1A(+NP) to the Q-type channels, respectively.**

**Electrophysiological Study of Rabbit-Human Chimeric a1A Channel in HEK293 Cells**

To investigate the effect of polyglutamine expansion on the function of a1A voltage-dependent calcium channel, we first made an electrophysiological analysis using rabbit-human chimeric a1A, BI11E-S13 and BI11E-L24. These chimeric constructs carry cDNAs for rabbit a1A subunit, which has the C-terminal tail, replaced with the tail region of human a1A containing 13 or 24 polyglutamines. Since Purkinje cells were suggested to express predominantly a1A channel...
without NP as mentioned above, we used rabbit α1A which lacked the NP insertion.

BI11E-S13 and BI11E-L24 were expressed with α2/δ and β1a subunits in HEK293 cells, and their electrophysiological properties were studied using a whole-cell patch clamp technique with the same protocol as described below. No statistically significant differences were detected between BI11E-S13 and BI11E-L24 in the current density, activation and inactivation kinetics, and voltage dependence of activation and inactivation (data not shown).

Sequencing Analysis of Human Full-length α1A Calcium Channel Subunit—Because we did not observe any noticeable functional alteration for rabbit-human chimeric α1A channels as described above, we speculated that residual regions of the human α1A channel might be needed for alteration of the channel function by polyglutamine expansion. In order to examine this possibility, we directly sequenced the amplified fragments of the full-length CACNA1A cDNA from a control and a SCA6 cerebella. The sequence of human α1A channel was, indeed, different from BI in several regions. Therefore, we constructed full-length CACNA1A with various CAG repeats using the control clone. The amino acid sequence deduced from the cDNA in an SCA6 cerebellum was essentially the same as that in a control cerebellum, although there were several polymorphic alterations between them. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases with the accession numbers AB035727 (control) and AB035726 (patient).

Effect of Polyglutamine Expansion on Human α1A(−NP)—We compared the properties of Ca$^{2+}$ current in HEK293 cells transiently expressing human α1A(−NP) of various numbers of polyglutamine (HS13(−NP), HL24(−NP), and HL28(−NP)), which was suggested to be dominantly expressed in Purkinje cells. Whole-cell Ca$^{2+}$ currents recorded from a HEK293 cell expressing α1A(−NP) coexpressed with α2/δ and β1a subunits are shown in Fig. 2A. Calcium currents were elicited by 400-ms depolarizing test pulses from a holding potential of −80 mV to test potentials (−30 to +50 mV) in 15 mM Ca$^{2+}$ solution. Inward currents of all the three channels were first activated at the threshold potentials of approximately −20 mV and gradually developed to a peak amplitude at approximately 20 mV. To compare the activation and inactivation kinetics of these currents, the time courses of activation and inactivation were quantified. The activating phase of the currents was fitted to a single exponential function, and the obtained time constants for activation at 20 mV are shown in Table I. There is no significant difference in time constants among these channels. Then, the decay phase of the currents was fitted to a double exponential function, and the time constants and their fractions of the components at 20 mV are also shown in Table I. In HL28(−NP), the fraction of the fast inactivating component increased significantly, and oppositely the fraction of the slow inactivating component decreased compared with those of HS13(−NP). However, no difference was observed between HL24(−NP) and HS13(−NP). As for the current density of these channels, there was no noticeable difference among the three types (data not shown).

The voltage dependence of activation for the expressed Ca$^{2+}$ channels was then compared (Fig. 3A and Table II). Conductance was calculated from the peak current (see legend to Fig. 3). The normalized values of conductance were then fitted to a single Boltzmann function. The half-maximal voltage of activation ($V_{1/2}$) and the slope factor (k) revealed no difference among α1A(−NP) of various numbers of polyglutamine stretch.

To examine the voltage dependence of inactivation of the Ca$^{2+}$ channels, a two-pulse protocol was used. A long prepulse (10 s) to different voltages (from −120 to +40 mV with an increment of 20 mV) was applied to allow inactivation to reach a steady state, then a test pulse (20 or 30 mV, the voltage which yielded a peak amplitude) followed. The values of normalized current amplitude were plotted against holding potentials (Fig. 3B). Compared with HS13(−NP), the half-maximal voltage for the voltage-dependent inactivation of HL24(−NP) and HL28(−NP) shifted to a significantly more negative potential.
by 6 and 11 mV, respectively. As for the slope factor, the value was significantly smaller in HL24(−NP) than in HS13(−NP).

**Effect of Polyglutamine Expansion on Human α1A(+NP)**—We analyzed the properties of Ca\(^{2+}\) current in HEK293 cells transiently expressing human α1A(+NP) of different numbers of polyglutamine, HS13(−NP) and HL28(−NP). Whole-cell Ca\(^{2+}\) currents of α1A(+NP) were recorded with the same protocol as that for α1A(−NP) (Fig. 2B). The activation and inactivation kinetics of these currents were not different from those of HS13(−NP) (Table I). The peak current densities of these channels were not significantly different either (data not shown).

Voltage dependence of activation for α1A(+NP) was illustrated...
in Fig. 4A. The slope factors of HS13(+NP) and HL28(+NP) were significantly smaller than that of HS13(−NP) (Table II).

Comparing voltage dependence of inactivation between α1A channels with and without NP, there was no difference in the half-maximal voltage of inactivation between HS13(+NP) and HS13(−NP) (Fig. 4B and Table II). However, this value for HL28(+NP) shifted to significantly more positive potential by 5 mV from that of HS13(+NP), which was in opposite direction to the shift for HL24(−NP) from HS13(−NP).

DISCUSSION

In the present study, we employed a human cell (HEK293) expression system to study the effect of polyglutamine expansion on calcium channel properties of the rabbit-human chimeric α1A channel and the full-length human α1A channel. First, rabbit-human chimeric α1A channel with 13 or 24 polyglutamines was expressed to conduct an electrophysiological study, but no significant differences in channel properties were observed in cells expressing this form of α1A with 24 polyglutamines expressed in BHK cells had a normal channel function. Thus, we speculated that regions other than 1.7-kb 3′ tail were needed to yield a functional difference due to expansion of the CAG repeat. It is well known that two molecular forms with or without an insertion of NP in the domain IV S3-S4 region are expressed in rat α1A channel (22). In situ hybridization to rat brain sections revealed a predominant expression of the variant without NP in the Purkinje cell (22). This gives a good agreement with our data of RT-PCR showing a predominance of the α1A(−NP) in the mouse Purkinje cell. Moreover, a pharmacological study determined by ω-AgaIVA sensitivity in rat suggested that native P-type channels contain α1A subunit lacking NP (22). Therefore, it is most likely that α1A(−NP) is the main component in Purkinje cells and also in human. To study the mechanism of the dominant degeneration of Purkinje cells expressing this form of α1A channel in SCA6, we evaluated the properties of the wild-type and mutated α1A channels with or without NP expressed in HEK293 cells.

In α1A(−NP), the most striking change caused by abnormal expansions of the polyglutamine was hyperpolarizing shift of the half-maximal voltage for the voltage-dependent inactivation in proportion to the increase in the length of polyglutamine. Furthermore, α1A(−NP) with 24 polyglutamines had a steep slope factor, consequently its voltage-dependent inactivation stays at a similar level to that with 13 polyglutamines at hyperpolarized holding potentials, whereas it gets closer to that with 28 polyglutamine at more depolarized holding potentials. Thus α1A(−NP) with 24 polyglutamines is characterized as an intermediate between those with 13 and 28 polyglutamines. As a result of the shift to the hyperpolarizing direction in the voltage-dependent inactivation, available Ca2+ channels near the resting membrane potential are predicted to be reduced in the cells expressing α1A(−NP) harboring extended polyglutamine. The more the polyglutamine expands, the less Ca2+ influx is induced in the cells. Our PCR analysis proved that Purkinje cells predominantly express α1A(−NP). Therefore, we would present a hypothesis that the alteration of inactivation in α1A(−NP) with an abnormal expansion of polyglutamine, inducing the reduction of Ca2+ influx, finally contributes to Purkinje cell death, although there is no molecular linkage suggested between the reduced Ca2+ influx and the degeneration at present. This would also suggest that other cell types expressing both forms of polyglutamine-stretched α1A channels, i.e. α1A(−NP) and α1A(+NP), can be rescued by compensated Ca2+ influx due to the opposite direction of channel dysfunction of α1A(+NP). In other words, Purkinje cells may be selectively damaged because of the lack of this compensation.

Another alteration observed in this study was a slight increase of the fast inactivation component without a change in inactivating time constant for α1A(−NP) with 28 polyglutamines. Although this finding may also contribute to the reduction of Ca2+ influx, we cannot conclude that it is associated with the pathogenesis of SCA6 because α1A(−NP) with 24 polyglutamines did not show a similar tendency.

Among the wide variety of degenerative diseases, SCA6 has valuable features. First, in SCA6, Purkinje cell degeneration is predominant. Therefore, it will be a suitable model for pure cerebellar ataxia from a clinical point of view. Second, SCA6 is one of the calcium channelopathies. In addition to SCA6, the α1A channel is linked to töttering (tg) and leaner (tgα) mice. The tg(α) mice have a severe progressive ataxia, and this mutated channel was reported to exhibit a considerably reduced Ca2+ influx into Purkinje cells (27–29), whereas tg, which expresses mild phenotype, showed a smaller functional alteration (29). Accordingly, the reduced Ca2+ influx by these mutations may account for their ataxic phenotype. On the other hand, although shrinkage and apoptosis of Purkinje cells were revealed in tg and tg(α) mutant mice (30–32), the degeneration
was not strictly specific to the Purkinje cell. Therefore, the selective degeneration of the Purkinje cell, which is seen in SCA6, cannot be explained by the reduced Ca$^{2+}$ influx alone. Cytoplasmic aggregates were observed in more than half of the remaining Purkinje cells (23) and may participate in the cell death, but this mechanism still remains to be confirmed. Furthermore, other mutations in the α1A channel are associated with human diseases of FHM and EA-2 (16). FHM is caused by mutations in the α1A channel functions being considerably refined and means to modulate them being increasingly established, an insight into their dysfunction will potentially lead to the treatment of this disease in the near future. Finally, SCA6 is a member of the polyglutamine disease, which shares several common features. Understanding the pathogenesis of SCA6 as one of polyglutamine disease is very important for elucidation of the common mechanism of these neurodegenerative diseases. In this respect, we would emphasize that both the molecular biological approaches to polyglutamine and the physiological analysis of channel function are essential for clarifying the pathogenic mechanism of this disease.

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Spinocerebellar Ataxia Type 6 Mutation Alters P-type Calcium Channel Function
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