Spinocerebellar ataxia 6 (SCA6) is caused by expansion of a polyglutamine stretch, encoded by a CAG trinucleotide repeat, in the human P/Q-type $\mathrm{Ca}^{2+}$ channel $\alpha_{1A}$ 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 $\mathrm{Ca}^{2+}$ channels with an expanded stretch of 24, 30, or 40 polyglutamines, recombinantly expressed in baby hamster kidney cells. Whereas the $\mathrm{Ca}^{2+}$ channels with $\leq$24 polyglutamines showed normal properties, the $\mathrm{Ca}^{2+}$ 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 $\mathrm{Ca}^{2+}$ 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 $\mathrm{Ca}^{2+}$ channel; CAG repeat expansion; polyglutamine repeat; recombinant expression; neuronal death
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 HindIII (on vector)–BamHI (7739) fragment of pSPCB1-I 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 HindIII–BamHI site of pK4K (Niide 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 first residue of the ATG-initiating triplet of the unmodified BI-1.

**Hin X57476** was inserted into the 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 \(\alpha_{1A}\) 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 \(\alpha_{1A}\) cDNA (Mori et al., 1991). Among the six alternatively spliced isoforms of the human \(\alpha_{1A}\) subunit cDNA, three have GCCGAG insertion before the terminal codon; consequently the succeeding \(\sim700\) 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)\(_5\) repeat but lacks GCCGAG, 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 (Niide et al., 1994) and were expressed in a BHK cell line, in combination with the Ca\(^{2+}\) channel \(\alpha_2\) and \(\beta_1\) subunit cDNAs (Niide et al., 1994).

With depolymerization 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. 1A). The currents first appeared at \(-30\) mV and grew with increments of depolymerization, reached a peak in the current–voltage relationship at \(-50\) mV, and then declined with further depolymerization (Fig. 1B). Figure 1C 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. 2A). 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. 2B). 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 \(\alpha_{1A}\) 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...
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 \(\alpha_{1A}\) 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 \(\alpha_{1A}\) 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 \(\text{tuttinger (tg)}\) or \(\text{leaner (tg}\text{l})\) mutations (Waka-
mori et al., 1998b). The tg and tgla 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 \(\gamma\) subunit gene results in a shift in the voltage dependence of inactivation of the P/Q-type Ca\(^{2+}\) channel (Lets 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 tgla 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 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 \(\alpha_{1A}\) 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.

### Table 1. Activation and inactivation parameters of \(\alpha_{1A}\) channels in BHK cells

<table>
<thead>
<tr>
<th>Channel</th>
<th>Channel Function in SCA6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG (4)</td>
<td>9</td>
</tr>
<tr>
<td>CAG (24)</td>
<td>16</td>
</tr>
<tr>
<td>CAG (30)</td>
<td>14</td>
</tr>
<tr>
<td>CAG (40)</td>
<td>8</td>
</tr>
<tr>
<td>BI-2</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Channel</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel</td>
<td>Function in SCA6</td>
<td></td>
</tr>
<tr>
<td>CAG (4)</td>
<td>9</td>
<td>-7.1 \pm 1.5</td>
</tr>
<tr>
<td>CAG (24)</td>
<td>16</td>
<td>-8.2 \pm 0.9</td>
</tr>
<tr>
<td>CAG (30)</td>
<td>14</td>
<td>-9.4 \pm 1.1</td>
</tr>
<tr>
<td>CAG (40)</td>
<td>8</td>
<td>-10.8 \pm 1.1</td>
</tr>
<tr>
<td>BI-2</td>
<td>6</td>
<td>-7.6 \pm 1.0</td>
</tr>
</tbody>
</table>

\(n\), Number of cells recorded; \(V_{0.5}\), half-maximal voltage of activation and inactivation; \(k\), slope factor. Data are expressed as means \(\pm\) 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 (*\(<\) 0.05).

### Table 2. Inactivation kinetics of \(\alpha_{1A}\) channels in BHK cells at 10 mV

<table>
<thead>
<tr>
<th>Channel</th>
<th>(\tau_f) (msec)</th>
<th>(I_{f})</th>
<th>(\tau_s) (msec)</th>
<th>(I_s)</th>
<th>(I_n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG (4)</td>
<td>4</td>
<td>21.2 \pm 1.9</td>
<td>0.29 \pm 0.03</td>
<td>91.5 \pm 5.8</td>
<td>0.69 \pm 0.03</td>
</tr>
<tr>
<td>CAG (24)</td>
<td>7</td>
<td>20.8 \pm 2.2</td>
<td>0.24 \pm 0.03</td>
<td>98.2 \pm 5.1</td>
<td>0.69 \pm 0.03</td>
</tr>
<tr>
<td>CAG (30)</td>
<td>8</td>
<td>19.4 \pm 2.2</td>
<td>0.26 \pm 0.04</td>
<td>91.3 \pm 10.0</td>
<td>0.73 \pm 0.04</td>
</tr>
<tr>
<td>CAG (40)</td>
<td>5</td>
<td>18.6 \pm 0.8</td>
<td>0.26 \pm 0.04</td>
<td>107 \pm 5.7</td>
<td>0.71 \pm 0.03</td>
</tr>
<tr>
<td>BI-2</td>
<td>20</td>
<td>18.5 \pm 1.6</td>
<td>0.27 \pm 0.02</td>
<td>97.5 \pm 6.0</td>
<td>0.68 \pm 0.02</td>
</tr>
</tbody>
</table>

\(n\), Number of cells recorded; \(\tau_f\), fast inactivation time constant; \(\tau_s\), slow inactivation time constant; \(I_{f}\), the relative fast component of the initial current; \(I_s\), the relative slow component of the initial current; \(I_n\), the relative noninactivating component. Data are expressed as means \(\pm\) SEM. The BI-2 channel was expressed stably.
Instead, the present study has clearly demonstrated that polyglutamine expansion exerts direct effects on the property of the P/Q-type Ca\textsuperscript{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.

REFERENCES


