Developmental Changes in K⁺-Selective Channel Activity During Differentiation of the Purkinje Neuron in Culture

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The cerebellar Purkinje neuron cultured from 20 d rat embryos is electrically inexcitable when immature, and acquires excitable membrane properties according to a programmed developmental sequence, thus providing a useful model for investigating mechanisms of CNS neuronal development. Using conventional patch-clamp techniques, we have characterized the predominant classes of active K⁺-selective channels at a range of ages encompassing the entire developmental process from 5 to 29 d *in vitro* (DIV), and have shown pharmacologically that these channels are important contributors to the patterns of spontaneous activity generated by the Purkinje neurons. The 4 predominant classes of K⁺ channels that are active during steady-state depolarizing voltage commands are identified by unit conductances as the 27, 44, 70, and 100 pS channels, and show differences in several properties, including voltage dependence, sensitivity to tetraethylammonium chloride (TEA), mean open time, and time of appearance during development. Intracellular current-clamp recordings show that physiological maturation of the Purkinje neuron entails increases in the firing rate, the diversity of spike events that comprise spontaneous activity, and the sensitivity of spontaneous activity to disruption by the K⁺ channel blocker TEA. This increase in sensitivity to TEA correlates with the new expression of activity of the larger-conductance TEA-sensitive classes of K⁺ channel (70 and 100 pS types). These data show that developmental regulation of the activity of K⁺-selective channels contributes significantly to the ionic mechanisms that underlie the developmental transitions in spontaneous activity patterns in the Purkinje neuron.

Nervous system development is comprised of significant changes in both the morphology and the excitable membrane properties of the differentiating neuron (for review, see Spitzer and Lomborghini, 1981). Since excitable membrane properties are dictated largely by the types of ion channels present in the membrane (see Hille, 1984, for review), it is likely that transitions in whole cell function may reflect a process of developmental

regulation at the single-channel level. In support of this hypothesis, the results presented here show that the progression in spontaneous activity patterns that are expressed by rat cerebellar Purkinje neurons during development in culture correlate with changes in the types of K⁺ channels that are found to be active. In culture, the Purkinje neuron follows a developmental sequence comparable to that described for these cells *in vivo* (Gruol and Franklin, 1987; Yool and Gruol, 1987) on the basis of comparisons with the morphological and physiological properties documented at different developmental stages by Woodward and colleagues (1969a, b). Purkinje neurons *in vivo* originate on about day 15 (E15) of gestation (Altman and Bayer, 1985), but delay differentiation until after birth (E21). Thus, the establishment of cerebellar cultures from rat embryos at 1 d before birth allows access to these neurons during their entire developmental process.

The aim of this study is to identify the mechanisms underlying the observed changes in spontaneous activity. Using the patch-clamp method for whole cell recording and single-channel analysis (Hamill et al., 1981), we find that immature cells at 5 d *in vitro* (DIV) show only a limited number of the K⁺ channel types (the 27 and 44 pS); by 7 DIV, the 70 pS K⁺ channel is present in some cells, and by 9 DIV, the largest conductance channel (100 pS) is active. Maturation involves differential changes in the frequencies of occurrence, with an apparent decline in the activity of the 27 pS type, increases in the activities of the 70 and 100 pS types, and no discernible change in the occurrence of the 44 pS type. However, the 44 pS channel type does undergo a dramatic 10-fold increase in mean open time during development, changing from 2 msec to more than 20 msec during the second week of development *in vitro*. These changes at the single-channel level are consistent with developmental trends analyzed at the whole cell level. The larger-conductance K⁺ channels are active progressively later in development, and show a higher sensitivity to tetraethylammonium chloride (TEA), a pharmacological blocker for several classes of K⁺ channels (Armstrong and Binstock, 1965; Thompson, 1977). Patterns of spontaneous activity recorded in the whole cell configuration show a comparable increase in sensitivity to TEA. We propose that a developmental sequence in the expression of different K⁺ channels contributes to the changes in spontaneous activity and sensitivity to TEA exhibited by Purkinje neurons during maturation.

Materials and Methods

Cell culture. Modified explant cultures were prepared and maintained in aseptic conditions without antibiotics, as described previously (Gruol, 1983). The immature cerebellar cortices were dissected from sibling

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rat embryos on day 20 of gestation (1 d before birth). The combined cerebella were gently minced and triturated in divalent cation-free saline without enzyme, and plated on polylysine- or collagen-coated culture dishes in modified Eagle's medium (MEM) containing 10% horse and 10% fetal calf serum by volume. Cultures were incubated at 37°C in a humidified 5% CO₂-air atmosphere. After 3 d of incubation, cultures were treated for 3 d with 40–80 μ M 5-fluorodeoxyuridine (FUDR) to inhibit the growth of rapidly dividing cells such as glia, and were subsequently maintained in MEM with 10% horse serum changed twice weekly throughout development to maturity. Cultures were taken at different developmental stages for single-channel and whole cell recording without the use of any enzyme pretreatment.

Electrophysiology. Cell-attached (C/A), intracellular, and outside-out (O/O) recordings were made on the soma and dendrites of identified Purkinje neurons following conventional methods (Hamill et al., 1981). Channel activity was recorded from patches with seal resistances of 1–20 G Ω or more with Sylgard-coated patch electrodes (3–6 M Ω). The bath electrode was connected to the recording dish via an agar bridge. Activity was recorded at potentials set by the List EPC-7 patch-clamp as constant-command voltage steps that were maintained over several minutes. Intracellular records of spontaneously generated cell activity were obtained by whole cell recording with current-clamp. For most O/O and all intracellular recordings, the patch electrodes were filled with K⁺ saline solution, containing (in mM) KCl, 154; NaCl, 6.0; MgCl₂, 2.0; CaCl₂, 0.006; EGTA, 0.008; D-glucose, 10.0; and HEPES-KOH, 10.0, to buffer the saline to pH 7.3. Free Ca²⁺ in this saline was not strongly buffered by EGTA, and was calculated to be approximately 100 nM. Intracellular Ca²⁺ in spontaneously active Purkinje neurons in culture (15–17 DIV) has been estimated by Connor and colleagues (1986) to range from 200 to 300 nM. For C/A recordings, the electrodes were filled either with the K⁺ saline or with bath Na⁺ saline containing (in mM) NaCl, 137; KCl, 3.5; KH₂PO₄, 0.44; Na₂HPO₄, 0.24; MgSO₄, 2.2; CaCl₂, 2.2; D-glucose, 10.0; and HEPES-KOH, 10.0. Experiments were performed at room temperature (21–25°C).

Extracellular recordings of spontaneous activity routinely were made prior to the formation of high-resistance seal in the C/A configuration as a standard for normal activity. The spontaneous activity pattern measured intracellularly at resting membrane potential usually matched the pattern recorded extracellularly. Healthy cells were identified by resting membrane potentials of approximately –60 mV and by stable patterns of spontaneous activity (recorded for up to 45 min). Na⁺ saline was used as bath medium, replacing the MEM culture medium during the recording. Sensitivity to TEA (Eastman Kodak) at 0.5–10 mM was tested by the application of $\geq 5\times$ the bath volume of TEA-containing saline, using a perfusion system (LKB Microplex peristaltic pump). Data were stored on tape (Racal 4-channel FM) and subsequently played back at normal speed for computer-aided digitization and analysis, or at an expanded time scale for high-resolution polygraph records of channel or whole cell activity. Three types of spike events, classified by comparison with those of *in vivo* studies, comprise spontaneous activity. The simple spike is a single-action potential; the doublet spike is paired-action potentials with a short interspike interval; the complex spike is a series of spikes of decreasing magnitude riding on a slow depolarization. Complex spikes are expressed by cultured Purkinje neurons without input from the inferior olivary nucleus, a CNS structure absent from the cerebellar culture preparation.

Data analysis. Data from >100 identified K⁺ channels were recorded in 73 O/O and C/A patches and analyzed by computer (Indec) according to established techniques (Dionne and Leibowitz, 1982; Leibowitz and Dionne, 1984). Records were filtered at 2 kHz (–3 dB; 8-pole Bessel filter; Frequency Devices) and digitized at a sample interval of 200 μ sec. Ionic selectivity of the channels for K⁺ was established from O/O recordings by the calculated reversal potentials. Histograms of current amplitude and open time duration were generated for each applied voltage from 300–1000 channel events, with minimum durations of 400 μ sec. Values for mean open duration (τ) were derived from records with single active channels when possible, or with data selected by amplitude from a multiple-channel record, to represent essentially a single population at the level of one open channel. Analyses from multiple-channel patches are likely to underestimate τ , and are not intended to represent rigorously defined kinetic properties. Duration distributions were fitted with 1 or 2 exponential functions, as determined by the minimum required to give an adequate fit of the distribution. Channel activity was analyzed during steady voltage steps of approximately 1–3 min in duration, and was found to be maintained during prolonged recording sessions (up to 1 hr).

Results

Morphological development of the Purkinje neuron

Morphological criteria for identifying the Purkinje neuron have been established with a selective immunohistochemical marker for cGMP-dependent protein kinase (Lohmann et al., 1981; Franklin and Gruol, 1983). The Purkinje neuron in culture was easily distinguished from glia and interneurons by its large size and, in older cells, by its unique dendritic structure (Fig. 1). At 7 DIV, the immature neuron consisted of a large soma with fine perisomatic processes. By 10–14 DIV, virtually all Purkinje neurons displayed prominent dendritic outgrowth. By 21–24 DIV, the thick dendrites showed several orders of branching, producing an arborization that was characteristic at maturity in culture, although reduced in extent and order with respect to the mature cell *in vivo*.

Development of spontaneous activity

Previous work using extracellular recording demonstrated that Purkinje neurons in culture progress through several stages in the expression of spontaneously generated activity (Gruol and Franklin, 1987; Yool and Gruol, 1987). Further intracellular analyses here were focused on the mechanisms and pharmacological properties of these developmental changes. Spontaneous activity recorded intracellularly from a Purkinje neuron at 7 DIV (Fig. 2A) consisted of a slow, irregular pattern of simple spikes at the resting membrane potential. Average resting potential for cells at 6–7 DIV was -64 ± 1.2 mV (SEM; $n = 33$). The type of activity expressed by the immature cells at 7 DIV (Fig. 2A) was voltage-dependent, ranging from a repetitive firing pattern, generated endogenously at depolarized membrane potentials, to the absence of any spike activity at hyperpolarized levels.

The diversity of components contributing to spontaneous activity increased with developmental age (Fig. 2B). At 13 DIV, activity at the resting potential consisted of a mixture of simple, doublet, and complex spike events. Depolarization evoked a regular firing pattern, which differed from that evoked in immature neurons in having a more pronounced repolarization and a faster maximum firing rate. The average value for the resting potential in cells at 12–14 DIV was -59 ± 1.0 mV (SEM; $n = 31$). At 13 DIV, applied hyperpolarization changed the activity pattern to one of predominantly complex spikes. This voltage-dependent firing pattern contrasted with that seen in the immature (7 DIV) cells, which did not include complex spike activity. Further development of the Purkinje neuron was marked by a shift to a rapid firing pattern of simple spikes at the resting membrane potential, with intermittent periods of bursting comprised of complex spike events. The resting potential of mature Purkinje neurons (21–29 DIV) was -57 ± 1.0 mV (SEM; $n = 20$).

Development of sensitivity to TEA

The K⁺ channel blocker TEA was used to evaluate the role of K⁺ currents in the generation of spontaneous activity at different developmental stages. Immature neurons were found to be less sensitive to TEA than mature neurons (Fig. 2). At 7 DIV (Fig. 2A), TEA applied during whole cell current-clamp recording did not substantially alter the form of spontaneous spike events at a concentration of 1 mM. At 3 mM TEA, the late repolarization phase was prolonged, which appeared to promote the occurrence of a second spike on the falling phase; however,

Figure 1. Immunohistochemical identification of Purkinje neurons at 3 stages of development in culture. Ages of the cultures in days *in vitro* (DIV) are indicated in the upper right corner of each micrograph. Unique characteristic morphologies for the Purkinje neuron (large arrows) at immaturity (6 DIV), at the time of dendritic outgrowth (14 DIV), and at maturity (20 DIV) allow easy identification of these neurons in the unstained cultures used for electrophysiological recording. Identification is verified by staining with antibody to cyclic GMP-dependent protein kinase, a selective marker for the Purkinje neuron, which appears to increase in intensity during neuronal development. Stained cultures were photographed using Hoffman optics (top row, bottom left) and phase-contrast optics (bottom right). Other interneurons in the cerebellar cultures, including the granule cell (small, dark arrow) are not immunopositive; the interneurons are seen more clearly in the phase-contrast micrograph (bottom right).

no overall effect on the pattern of activity was observed. Higher concentrations of TEA, up to 13 mM, further broadened the spike but did not block repetitive firing.

In contrast, neurons at 13 DIV displayed an increased sensitivity to TEA (Fig. 2*B*). TEA at 0.5 mM reduced the magnitude of the spike afterhyperpolarization (AHP), particularly for sin-

glet spikes. At 2 mM, TEA affected complex spike activity, appearing to augment the associated slow depolarization. TEA at 4 mM produced dramatic effects on spike shape and altered spontaneous activity to a slower pattern that typically lacked the regular repetitive component seen in the control. Higher concentrations of TEA blocked spontaneous activity.

Figure 2. Development of spontaneously generated activity, and the sensitivity of spontaneous activity to external TEA. Spontaneous activity patterns of Purkinje neurons at 7 DIV (*A*) and at 13 DIV (*B*). The immature pattern of activity (*A1*) at the resting potential ($E_M = -79$) consisted of simple spikes; depolarization of the holding potential by injection of positive DC current elicited repetitive firing, while hyperpolarization by negative current injection suppressed spike generation. Spontaneous activity from a different cell at 7 DIV (*A2*) at resting membrane potential ($E_M = -64$ mV) similarly showed singlet activity at a slow firing rate; perfusion of bath medium containing progressively higher concentrations of TEA produced alterations in the firing pattern and spike shape at ≥ 3 mM. In contrast, spontaneous activity in the Purkinje neuron at 13 DIV (*B1*) consisted of a mixture of simple (*S*), doublet (*D*), and complex (*C*) spikes; depolarization evoked repetitive firing, while hyperpolarization shifted the activity pattern to predominantly complex spikes. Spontaneous activity from a different cell at 13 DIV (*B2*) at resting membrane potential ($E_M = -65$) similarly showed a mixture of *S*, *D*, and *C* spikes; perfusion of TEA medium produced alterations in the spike shape at 0.5 mM by reducing the afterhyperpolarization phase of singlet spikes (arrows); higher concentrations produced more dramatic effects on firing pattern than were seen with comparable concentrations in the immature cell. Depolarization (5–8 mV) of the resting potential in the presence of TEA was offset with a small negative DC current to maintain the holding potential (E_H) as close to the initial E_M as possible. Scale bars, 20 mV on a time base of 50 msec.

Identification of the classes of K⁺-selective channels

The increased sensitivity of Purkinje neurons to TEA during development was evaluated at the single-channel level by characterizing the K⁺-selective channels present at various stages through maturity. Frequencies of occurrence and average unit conductances for the 4 major classes of voltage-sensitive K⁺ channels are summarized in Table 1. These are referred to by conductance values as the 27, 44, 70, and 100 pS channels. Channel properties were determined under identical experimental conditions, using the O/O patch configuration with Na⁺ saline externally and K⁺ saline internally.

The small-conductance 27 pS K⁺-selective channel was characterized by openings clustered in bursts at depolarized potentials, with usually no more than 1 or 2 channels of this type open simultaneously (Fig. 3*A*). The 27 pS channel was present at all ages, and did not display any apparent changes in mean open time during development. The mean open time in both immature and mature cells ranged from 1.4 msec at 0 mV to 2.4 msec at +60 mV (Table 3). In the O/O patch, with normal ionic conditions (intracellular K⁺, extracellular Na⁺), there was

no measurable activity at hyperpolarized voltages, indicating outward rectification (Fig. 3*B*). The extrapolated reversal potential (E_{rev}) ranged from -68 to -80 mV. This channel was found to be insensitive to the presence of internal or external TEA at concentrations up to 10 mM, and frequently co-occurred in patches with other channel types.

The medium-conductance 44 pS K⁺-selective channel was characterized in immature Purkinje neurons (5–7 DIV) by bursts of openings (Fig. 4*A*), with usually no more than 2 channels open simultaneously. The 44 pS channel in Purkinje neurons at 13–24 DIV (Fig. 4*C*) showed much longer open time durations, with 3–5 or more channels frequently open simultaneously. Maximal activity was most apparent at holding potentials near 0 mV, where one or more channels were open 90–98% of the time. The current–voltage plots (Fig. 4, *B*, *D*) showed a linear relationship for young cells, and apparent limiting conductance for older cells at depolarizing voltages. The extrapolated E_{rev} ranged from -55 to -60 mV. Outward rectification was apparent at both ages in recordings from O/O and C/A patches, with no measurable activity at hyperpolarized potentials. The activity of the 44 pS channel in both immature and mature

Figure 3. Activity of the 27 pS channel recorded in an outside-out (O/O) patch from a Purkinje neuron at 7 DIV. The traces in *A* show a polygraph record of channel activity, filtered by Bessel at 2 kHz, recorded on tape, and played back at an expanded time scale for high resolution. A single type of channel was found to be active (*A*), showing bursts of openings (indicated by square symbols). The linear current-voltage relationship (*B*), generated from computed average current amplitudes at various steady holding potentials, indicated a unit conductance of 27 pS and an extrapolated reversal potential (E_{rev}) of -68 mV, with no measurable channel activity at more hyperpolarized potentials. The high K^+ saline in the pipette contained 10 mM TEA. Scale bars, 5 pA on a time base of 10 msec.

Purkinje neurons was reversibly blocked by 5 mM TEA applied externally (not shown).

The value for mean open time ranged from 2.6 msec at 0 mV to 3.2 msec at depolarized potentials in the immature neuron (Table 3). In cells at 13 DIV (Fig. 4C) and older, all channels classified by conductance values as the 44 pS type showed a substantially longer mean open time, with τ ranging from 17 msec at 0 mV to 24 msec at $+60$ mV. Open duration distributions for the immature and mature 44 pS channel types were fit adequately by one exponential at all voltages. In Figure 5, representative distributions of open times at $+60$ mV from cells at 7 and 14 DIV illustrate the 10-fold increase in mean open time during development.

The larger-conductance 70 pS K^+ channel rarely showed more than one open level in O/O patches (Fig. 6A), with time in the open state representing only 1–2% of the total time; however, high activity with multiple open levels has been seen for this channel type in some C/A patches. The 70 pS channel was not active in cells at 5 DIV, was detected in a few cells at 7 DIV, and showed an increasing frequency of occurrence during maturation (Tables 1 and 2). Open time distributions were best-fitted with 2 exponentials, yielding τ values of 0.6 msec for the fast component, and 1.8–2 msec for the slow component at potentials from 0 to $+60$ mV (Table 3). Mean open times did not show any obvious voltage dependence in this range. We have observed activity of this channel type more often in dendritic patches than in somal patches during later stages of development. The linear current-voltage relationship (Fig. 6B) showed an extrapolated E_{rev} of -60 to -70 mV and outward

rectification. The activity of the 70 pS channel was reduced in amplitude in the presence of 1 mM TEA applied externally, and blocked at 4 mM (Fig. 6C).

The largest-conductance 100 pS K^+ channel (Fig. 7A) was

Table 1. Summary of single-channel data for K^+ -selective channels at different developmental stages of the Purkinje neuron in culture

Parameter measured ^a	Age range (DIV) ^b			
	5	6–7	8–14	15–24
Number of patches	4	20	21	28
Number of observations (by channel type)				
27 pS	3	11	3	6
44 pS	3	10	9	19
70 pS	0	4	7	13
100 pS	0	0	6	14
Average unit conductance ($\pm$ SD)				
27 pS	26 $\pm$ 2	25 $\pm$ 2	25 $\pm$ 2	25 $\pm$ 3
44 pS	44 $\pm$ 2	45 $\pm$ 4	45 $\pm$ 5	43 $\pm$ 5
70 pS	NA	72 $\pm$ 8	66 $\pm$ 9	70 $\pm$ 6
100 pS	NA	NA	106 $\pm$ 8	98 $\pm$ 16
(high K^+)			(158 $\pm$ 78)	(163 $\pm$ 46)

^a Parameters measured are the total number of patches recorded from cells in each age group; the number of observations of activity for each channel type within the respective age group; and the average conductance values $\pm$ SD. An observation is defined as the presence of one or more active channels of a given class in the recorded patch.

^b DIV, days *in vitro*; NA, not active. Increased conductance values for the 100 pS channel in C/A patches with high- K^+ saline are indicated in parentheses.

Figure 4. Activity of the 44 pS channel compared at 2 ages in O/O patches from Purkinje neurons in culture. Representative examples of the activity of the 44 pS channel at 7 DIV (*A, B*) and at 13 DIV (*C, D*) illustrate the developmental change in apparent open time measured for this channel class (suggested to be a single class by comparable properties of conductance and pharmacological sensitivity to 5 mM TEA). Examples of openings of the 44 pS channel are marked by *solid circles*; another K⁺-selective channel of approximately 19 pS (*open circle*) has also been identified in some O/O patches at different ages but has not yet been characterized. The linear current-voltage relationship at 7 DIV (*B*) shows a calculated unit conductance of 41 pS and a E_{rev} of -60 mV. In contrast, the activity of the 44 pS channel at 13 DIV (*4C*) shows long-duration openings with multiple channels open simultaneously, and virtually continuous opening at a potential of 0 mV. The nonlinear current-voltage relationship (*D*) shows a unit conductance in the linear region of 44 pS, and E_{rev} at -55 mV. Apparent rectification at depolarizing potentials is seen for average current amplitude at both the single (one-channel) and double (2 channels open simultaneously) levels. Scale bars, 5 pA (*A*); 4 pA (*B*); time base, 10 msec.

detected only in patches from cells at 9 DIV and older, in both soma and dendrites. Mean open times ranged from 1.9 msec at 0 mV to 5.3 msec at +60 mV in the O/O patch under normal ionic conditions (as defined in Materials and Methods). Manipulations producing an increase in Ca²⁺ at the internal membrane face, such as pulling an inside-out patch in normal bath saline, were found to increase both the opening frequency and number of channels open. In addition to Ca²⁺ sensitivity, the conduc-

tance and opening frequency properties of the 100 pS channel appeared to depend on the ionic environment of the membrane patch. With high concentrations of K⁺ internally and externally (C/A), the 100 pS channel showed an increase in unit conductance to 150–200 pS (Gruol, 1984). The nonlinear current-voltage relationship (Fig. 7*B*) showed reduced conductance levels both near the extrapolated E_{rev} of -78 mV and at depolarized voltages (which may have been due to voltage-dependent Na⁺

Figure 5. Open duration distributions of the 44 pS channel at +60 mV, in O/O patches recorded under identical conditions from Purkinje neurons at 7 and 14 DIV. Mean open times (τ), calculated from open time distributions fitted with a single exponential function, were 1.8 msec at 7 DIV and 24 msec at 14 DIV. Multiple open levels of the 44 pS type in the 14 DIV patch required the selection of events by amplitude to represent single-channel openings, a process likely to underestimate τ since long openings have a higher probability of overlapping, thus disqualifying the events from analysis in the open-duration distribution.

Figure 6. Activity of the 70 and 27 pS channels recorded in an O/O patch from a Purkinje neuron at 7 DIV. The polygraph record (*A*) illustrates representative activities of the 70 pS channel (*triangle*) and the 27 pS channel (*square*). The linear current-voltage relationships (*B*) indicated a calculated unit conductance of 78 pS, with E_{rev} at -60 mV for the larger channel and a unit conductance of 27 pS with E_{rev} at -70 mV for the smaller channel. Sensitivity to TEA (*C*) is shown with another O/O patch displaying activity of only the 70 pS channel from a Purkinje neuron at 8 DIV. Current amplitude at a holding potential of 0 mV was reduced in the presence of 1 mM TEA applied externally; activity was blocked reversibly at 4 mM TEA. Scale bars, 5 pA (*A*); 4 pA (*B*); time base, 10 msec.

blockade; French and Wells, 1977; Singer and Walsh, 1984). The conductance of the channel was determined from the linear central region of the plot. The activity of the 100 pS channel was highly sensitive to external TEA (Fig. 7*C*), showing reduced current amplitude in 0.5 mM TEA and complete blockade at 1.0 mM TEA (determined from other O/O patch recordings). This channel is similar to the large-conductance Ca^{2+} -activated K^{+} channel described in other preparations (Marty, 1981; Pal-lotta et al., 1981; Marty and Neher, 1985; and others).

Development of activity of K^{+} channels

A comparison of data from Purkinje neurons at different stages demonstrated an age-dependent increase in the diversity of K^{+} channels (Fig. 8), and suggested a sequence in their developmental expression. At 5 DIV, only the 27 and 44 pS types of K^{+} -selective channel were found to be present. These channels

persisted as the predominant K^{+} -selective types at 7 DIV. The expression of activity of the larger-conductance channels occurred progressively later in development, with the 70 pS channel first present at 7 DIV and frequent by 9 DIV, and the 100 pS channel not seen at 8 DIV, first present at 9 DIV, and frequent in cells at 13 DIV and older. The measured conductance values of the channels (Table 1) did not change significantly during development (one-way analysis of variance; $p > 0.10$). The 4

Table 2. Frequencies of occurrence of the 4 classes of K^{+} -selective channels at different developmental stages of Purkinje neuron in culture

K^{+} channel type (pS)	Frequency of occurrence (%) ^a			
	5 DIV	6–7 DIV	8–14 DIV	15–24 DIV
27	75	55	14	21
44	75	50	43	68
70	0	20	33	46
100	0	0	29	50

^aFrequency of occurrence represents the number of observations of an active channel type as a percentage of the number of patches recorded within each age group; calculations are based on data presented in Table 1. Summed values exceed 100% owing to the presence of more than one channel type in a patch. DIV, days *in vitro*.

Table 3. Mean open times (τ) determined from open-duration distributions for 4 classes of potassium channels in cultured Purkinje neurons

Channel type (pS)	V range (mV)	τ (mean $\pm$ SEM)	n
27	+50 to +60	2.4 ± 0.8	7
	+20 to +40	1.7 ± 0.3	9
	0	1.4 ± 0.1	8
44 (immature)	+50 to +60	2.6 ± 0.8	4
	+20 to +40	3.2 ± 0.2	5
	0	2.6 ± 0.6	5
44 (mature)	+50 to +60	24 ± 1.6	6
	+20 to +40	20 ± 1.8	7
	0	17 ± 4.4	5
70	+50 to +60	τ_{fast} 0.6 ± 0.3 τ_{slow} 2.0 ± 0.2	4
	+20 to +40	0.6 ± 0.2 1.8 ± 0.4	4
	0	0.6 ± 0.1 2.0 ± 0.2	5
100	+50 to +60	5.3 ± 1.4	2
	+20 to +40	1.9 ± 0.4	2
	0	1.9 ± 0.4	2

Figure 7. Activity of the 100 and 27 pS channels recorded in an O/O patch from a Purkinje neuron at 24 DIV. The polygraph record (*A*) illustrates the voltage-dependent activities of the 100 pS (*diamond*) and 27 pS (*square*) channels. The nonlinear current-voltage relationship for the large channel (*B*) shows a calculated unit conductance of 90 pS in the linear central region and E_{rev} at -78 mV. The linear relationship for the smaller channel indicates a unit conductance of 27 pS and E_{rev} at -78 mV. Sensitivity to TEA (*C*) was tested for the same patch by external application of progressively increasing concentrations of TEA in bath saline at a holding potential of 10 mV. Current amplitude of the 100 pS channel was reduced in 0.5 mM TEA, and activity was blocked at 2.5 mM TEA. Activity of the 27 pS channel was not affected at concentrations up to 8 mM TEA. Only partial recovery of activity of the 100 pS channel was observed after extensive rinsing with bath saline. Traces for 2.5 and 8 mM TEA and recovery were filtered by Bessel at 1 kHz; all other traces were filtered at 2 kHz. Scale bars, 2 pA on a timebase of 10 msec.

major channel types were found to persist through maturity (24 DIV and beyond).

For comparison across age groups, standardized frequencies of occurrence were obtained by representing the number of observations for each channel type as a percentage of the number of patches made within an age group (Table 2). The 27 pS channel was found to occur in 75% of patches from immature neurons at 5 DIV, and in progressively fewer of the patches from older cells, suggesting that this channel type may decrease in abundance during maturation of the Purkinje neuron. The 44 pS channel did not show any strong developmental trend in frequency of occurrence, whereas both the 70 and 100 pS channels showed an increase in frequency during development.

Discussion

Few studies have explored a developmental sequence in the expression of K⁺ channel activity at the single-channel level, although developmental changes in the ionic dependence of action potentials have been documented (for review see Spitzer, 1985). Our results show that the physiological development of the Purkinje neuron involves changes in the currents carried by potassium. The effects of TEA on spontaneous activity can be correlated with the new appearance of activity in TEA-sensitive K⁺ channels. The concentration-dependent selectivity of TEA as a K⁺ channel blocker may prove useful in sorting the individual contributions of each type of K⁺ current into the overall

activity pattern. Immature neurons at 7 DIV exhibited slow patterns of simple spikes during spontaneous activity, and low sensitivity to TEA. Single-channel recordings demonstrated that these cells possessed predominantly the 27 and 44 pS types of K⁺-selective channels, and that the larger-conductance channels, if present, were not active or necessary for the immature functional state. The physiological development of the Purkinje neuron coincides with or precedes the period of morphological differentiation. By 13 DIV, the repertoire of spontaneous activity increased to include complex spike events; this maturation was accompanied by an increased maximal firing rate upon depolarization and an increased sensitivity to TEA. In addition to the 27 and 44 pS channels, these neurons depended on the 70 and 100 pS K⁺ channel types as contributors to spontaneous activity. Low concentrations of TEA (0.5–2 mM), sufficient to block only the 70 and 100 pS channels, produced substantial alterations in both the form and spacing of spike events, evoking a pattern in some ways reminiscent of the immature pattern of activity. These results suggest that the expression or activation of larger-conductance K⁺ channels is essential for the maturation of membrane properties in the Purkinje neuron.

Our results, showing the high sensitivity of the 100 pS channel to TEA, suggest that this channel may be a target of the substantial effects of TEA on the mature pattern of spontaneous activity. For example, the lowest concentration of TEA (0.5 mM) was found to affect only the largest-conductance 100 pS channel

at the single-channel level; thus, the subtle alterations in spike repolarization and the slowing of firing rate seen in intracellular records from 13 DIV cells in 0.5 mM TEA may indicate that this K^+ conductance functions in the maturing neuron to decrease the interspike interval in the rapid repetitive firing mode. This idea is corroborated by the observation that immature Purkinje cells, which lack the active 100 pS channel, show a slower maximal firing rate and a broader AHP that is reminiscent of the AHP in older cells exposed to 0.5 mM TEA. The large 100 pS K^+ channel is similar in several aspects to the Ca^{2+} -activated K^+ channel described in other preparations (Blatz and Magleby, 1984; Marty and Neher, 1985), with a high sensitivity to external TEA, high conductance, increased conductance in symmetrical K^+ saline, and activation by Ca^{2+} at the internal face of the membrane. The later onset of activity for the 100 pS channel may function in regulating Ca^{2+} conductances. Using Mg^{2+} as a Ca^{2+} blocker, we have found that role of Ca^{2+} currents in generating spontaneous activity increases during development, which may explain the relatively greater importance of the large-conductance Ca^{2+} -sensitive K^+ channel in the mature (but not the immature) activity pattern.

The large-conductance 70 pS channel was not active in the youngest cells analyzed, and preceded the 100 pS channel in the appearance of activity. Although the relative abundance of this channel increased with age, intrinsic properties, such as conductance and mean open time of the 70 pS channel, did not appear to change with development. The relatively rare occurrence of the 70 pS channel in neurons at 7 DIV may suggest that expression or activation of this channel is initiated at this age. With the lack of morphological differentiation at young ages, all observed channels were assigned to somatic membrane. In older cells, the activity of the 70 pS channel appeared to show a higher frequency of occurrence in the dendrite. It is possible that the presence of 70 pS channels in nominative somatic membrane of the immature cells may mark membrane destined for dendritic processes. The apparent regionalization of the active 70 pS channel to dendritic membrane remains to be verified, but may suggest that it plays a unique role in regulating dendritic excitability.

In contrast to the large-conductance channels, the 44 pS K^+ channel was present throughout development, and did not show any obvious changes in frequency of occurrence. An alternative mechanism of developmental change that appeared to be more important for this class of K^+ channel was the modification of opening properties. The immature 44 pS channel showed relatively short duration openings. The 44 pS channel in older cells showed more simultaneous open levels, and long-duration openings. These differences may indicate that the Purkinje neuron has 2 different types of K^+ channel at this conductance level, which are preferentially active at different developmental stages, or that a single class of channels is modified selectively to alter properties during development. The similar pharmacological sensitivity of the immature and mature channels to TEA supports their classification as one channel type.

The 27 pS channel was frequently active in immature Purkinje neurons and decreased in relative abundance during maturation. Unlike the 44 pS channel, developmental trends in intrinsic properties were not apparent. The 27 pS K^+ channel described here resembles a channel with properties of a putative delayed rectifier in hippocampal neurons (Rogawski, 1986) in conductance, TEA sensitivity, bursting behavior, and activity at depolarized potentials. The ragged appearance of the open-level

Figure 8. Occurrence of recorded activity in 4 classes of K^+ -selective channels during the development of Purkinje neurons in culture. The occurrence of one or more active channels in a recorded patch is indicated by a *single symbol* for each channel type, as identified by calculated single-unit conductance values and characteristic patterns of activity. The different symbols represent identified channel types: 100 pS (diamond), 70 pS (triangle), 44 pS (circle), and 27 pS (square). Data are summarized as the measured single-unit conductance values plotted against age (DIV) to illustrate the developmental sequence in the occurrence of the different channel types and the range of conductance values found within each category. Patch configurations were predominantly O/O, with high- K^+ saline in the electrode, but included C/A with high- Na^+ or high- K^+ salines. Channel conductance values for the 27, 44, and 70 pS channels were not systematically affected by configuration; however, the 100 pS channel showed increased conductance values, ranging from 150 to 230 pS, in symmetrical K^+ conditions (open diamonds). Actual conductance values for the increased levels are not shown.

current for this channel could be due to incompletely resolved flickering to a closed state, ionic blockade, or the possible existence of substates of conductance, as described for other channels, such as the cardiac inward rectifier (Sakmann and Trube, 1984), the small Ca^{2+} - and voltage-independent K^+ channels of chick ciliary ganglion neurons in culture (Gardner, 1986), and the nicotinic ion channel (Auerbach and Sachs, 1983).

In this study, differential changes in frequency of occurrence suggest that the various classes of K^+ channels are regulated separately during development. Precedent for developmentally regulated changes in K^+ currents has been established in other studies. Maturation of spinal neurons in the frog *Xenopus laevis* involves shortening of the action potential by an increased outward K^+ current, which at the single-channel level correlates with a dramatic increase in Ca^{2+} sensitivity of a large-conductance (155 pS) K^+ -selective channel (Blair and Dionne, 1985) and an increase in the types of K^+ channels present (Harris et al., 1986). The initial expression of 2 small-conductance voltage-dependent K^+ channels (14 and 28 pS) in the spinal neuron membrane is followed by the later occurrence of a voltage-independent large-conductance K^+ channel of 88 pS (Harris et al., 1986). This sequence, involving activation of a larger-conductance channel later in development, is similar to the pattern for the Purkinje neuron shown by our results. The importance of an increase in voltage-dependent K^+ current in the maturation

of the action potential has been shown for *Ambystoma* spinal neurons (Barish, 1986). Developmental differences in the appearance of K⁺ current components have also been described for cultured quail neural crest neurons (Bader et al., 1985).

In summary, the developmental changes in K⁺-selective channel activity involve not only increases in the number of active channel types, but may also include specific changes in the properties within a channel class or segregation of channels according to regional or functional specialization. It is interesting that the 3 channel types that show developmental trends in frequency of occurrence (27, 70, and 100 pS) do not show any obvious changes in intrinsic channel properties; conversely, the channel type that increases in mean open time (44 pS) does not show any obvious trend in frequency of occurrence with developmental age. Further work will be required to establish the functions and interrelationships of these channels. From these results, we suggest that the development of increased diversity in the types of activity patterns generated by the Purkinje neuron appears to depend at least in part on the expression of new classes of K⁺ channels, such as the Ca²⁺-dependent K⁺ channel, during the developmental process.

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