GABAergic activation of an inwardly rectifying K^+ current in mouse cerebellar Purkinje cells

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Cerebellar Purkinje cells integrate motor information conveyed by excitatory synaptic inputs from parallel and climbing fibres. Purkinje cells abundantly express B-type G-protein-coupled γ-aminobutyric acid receptors ($GABA_BR$) that are assumed to mediate major responses, including postsynaptic modulation of the synaptic inputs. However, the identity and function of effectors operated by $GABA_BR$ are not fully elucidated. Here we characterized an inwardly rectifying current activated by baclofen (I_{bacl}), a $GABA_BR$ agonist, in cultured mouse Purkinje cells using a ruptured-patch whole-cell technique. I_{bacl} is operated by $GABA_BR$ via $G_{i/o}$-proteins, as it is not inducible in pertussis-toxin-pretreated cells. I_{bacl} is carried by K^+ because its reversal potential shifts with the equilibrium potential of K^+. I_{bacl} is blocked by 10^{-3} M Ba^{2+} or Cs^+, and 10^{-8} M tertiapin-Q. Upon the onset and offset of a hyperpolarizing step, I_{bacl} is activated and deactivated, respectively, with double-exponential time courses (time constants, <1 ms and 30–80 ms). Based on similarities in the above properties, G-protein-coupled inwardly rectifying K^+ (GIRK) channels are thought to be responsible for I_{bacl}. Perforated-patch recordings from cultured Purkinje cells demonstrate that I_{bacl} hyperpolarizes the resting potential and the peak level achieved by glutamate-evoked potentials initiated in the dendrites. Moreover, cell-attached recordings from Purkinje cells in cerebellar slices demonstrate that I_{bacl} impedes spontaneous firing. Therefore, I_{bacl} may reduce the postsynaptic and intrinsic excitability of Purkinje cells under physiological conditions. These findings give a new insight into the role of $GABA_BR$ signalling in cerebellar information processing.

(Resubmitted 11 December 2004; accepted after revision 4 January 2005; first published online 6 January 2005)
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The cerebellar cortex plays central roles in motor coordination and learning (Thach *et al.* 1992; Llinas & Welsh, 1993; Kawato *et al.* 2003; Ohyama *et al.* 2003). Purkinje cells are the sole output neurones of the cerebellar cortex, and they integrate motor information conveyed by excitatory synaptic inputs from parallel and climbing fibres (Palay & Chan-Palay, 1974; Llinas *et al.* 2003). Purkinje cells express a very high density of B-type $G_{i/o}$-protein-coupled γ-aminobutyric acid receptor ($GABA_BR$) (Jones *et al.* 1998; Kaupmann *et al.* 1998; Kuner *et al.* 1999). $GABA_BR$ in Purkinje cells may be activated by not only GABA synaptically released from the innervating interneurones (Llinas *et al.* 2003), but also by GABA spillover from synapses between neighbouring neurones (Hirono *et al.* 2001). Thus, $GABA_BR$ is assumed to mediate major cellular functions in Purkinje cells. A previous study (Kawaguchi & Hirano, 2000) reported that $GABA_BR$ activation leads to suppression of rebound potentiation of inhibitory synaptic inputs to Purkinje cells (Kano *et al.* 1992). Moreover, $GABA_BR$ is thought to play a role in the postsynaptic modulation of the excitatory synaptic inputs because the peak subcellular density of $GABA_BR$ is found at the excitatory synapses (Fritschy *et al.* 1999; Ige *et al.* 2000; Kulik *et al.* 2002). For example, $GABA_BR$ activation may lead to the enhancement of the postsynaptic responses mediated by type-1 metabotropic glutamate receptors (Hirono *et al.* 2001). However, the identity and physiological actions of effectors operated by $GABA_BR$ are not fully elucidated in Purkinje cells.

In this study, we explored a $GABA_BR$-operated ion current presumably produced by G-protein-coupled inwardly rectifying K^+ (GIRK) channels (Bichet *et al.* 2003) in Purkinje cells. GIRK channels are gated by the $\beta\gamma$ subunit complex released from activated $G_{i/o}$-proteins (Sadja *et al.* 2003), and they form a major class of

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 DOI: 10.1113/jphysiol.2004.081000

$GABA_BR$-operated effectors in various central neurones (Jan & Jan, 1997). GIRK channels can produce a large inward K^+ current at membrane potentials (E_m) more negative than the equilibrium potential of K^+ (E_K, ~ -100 mV), while producing only a small outward K^+ current at more positive membrane potentials (Bichet *et al.* 2003). Thus, K^+ conductance through GIRK channels at physiological membrane potentials is much smaller than the maximum. Nevertheless, this conductance is often capable of hyperpolarizing the resting potential (E_{rest}) and attenuating excitatory postsynaptic potentials (Jan & Jan, 1997; Mark & Herlitze, 2000; Seeger & Alzheimer, 2001). A functional GIRK channel is a tetramer consisting of various combinations of subunits termed GIRK1–4 (Kir3.1–3.4)(Wischmeyer *et al.* 1997; Bichet *et al.* 2003). Some *in situ* hybridization and immunohistochemical studies detect the mRNAs and proteins of GIRK subunits (Karschin *et al.* 1996; Iizuka *et al.* 1997; Lauritzen *et al.* 1997; Murer *et al.* 1997) except GIRK1 (Liao *et al.* 1996; Ponce *et al.* 1996; Miyashita & Kubo, 1997) in Purkinje cells. In certain heterologous expression systems, GIRK2–4 can form functional homo- and heteromeric channels (Wischmeyer *et al.* 1997). However, macroscopic currents produced by these channels are much smaller than that of GIRK1-containing channels (Wischmeyer *et al.* 1997). Therefore, it is important to functionally determine at the whole-cell level whether $GABA_BR$ activation induces a significant inwardly rectifying K^+ current in Purkinje cells.

We used a ruptured-patch whole-cell voltage-clamp technique to extract an inwardly rectifying current activated by baclofen (I_{bacl}), a $GABA_BR$-selective agonist (Bowery, 1993). We characterized the basic properties of I_{bacl}, including the $G_{i/o}$-protein-dependence, carrier ion species, pharmacological profile and kinetics. These results demonstrate that Purkinje cells are equipped with an inwardly rectifying K^+ current most likely produced by GIRK channels. These results also offer a useful tool for studying G-protein signalling in Purkinje cells because the amplitude of GIRK currents directly reflects the activity of G-proteins (Sadja *et al.* 2003). Moreover, we assessed the possible physiological contribution of I_{bacl} to regulation of the postsynaptic and intrinsic excitability of Purkinje cells using a perforated-patch whole-cell technique in cultured cell preparations, and a cell-attached technique in cerebellar slice preparations. These findings give a new insight into the role of $GABA_BR$ signalling in cerebellar information processing.

Methods

Cultured cell preparation

Cerebellar Purkinje cells from C57BL/6 mice were cultured as described elsewhere (Tabata *et al.* 2000). Briefly, perinatal embryos were removed by Caesarian section from pregnant mice, which were deeply anaesthetized, and killed with diethylether. The embryos were deeply anaesthetized by cooling in chilled phosphate-buffered saline, and then killed by decapitation. The cerebella from these embryos were dissociated with trypsin, and plated onto plastic dishes (diameter, 35 mm; Falcon 3001). The neurones were cultured in an ultra-low-serum, hormone/nutrient-supplemented Dulbecco's modified Eagle's medium for 10 days to 3 weeks. Purkinje cells were identified by their large somata (15–30 μm) and thick primary dendrites.

Cerebellar slice preparation

C57BL/6 mice (4–5 weeks old) were deeply anaesthetized with diethylether, and killed by cervical dislocation. Parasagittal cerebellar slices (250 μm thick) were prepared, using a vibrating slicer (VT-1000S, Leica, Wetzlar, Germany), as described elsewhere (Kano *et al.* 1995).

Electrophysiology

We measured whole-cell currents from the somata of cultured Purkinje cells using a ruptured-patch voltage-clamp technique (Marty & Neher, 1995) (25–26°C). The pipette solution consisted of (mM): 130 D-gluconate potassium salt, 10 NaCl, 10 Hepes, 0.5 ethyleneglycol-bis-(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid, 4 Mg-ATP and 0.4 Na_2-GTP; pH was adjusted to 7.3 with KOH or D-gluconic acid; the total concentrations of K^+ and Mg^{2+} were adjusted to 150.6 and 5.2 mM with KCl and $MgCl_2$, respectively. The recording chamber (culture dish) was perfused at a rate of 1–2 ml min^{-1} with a saline whose standard composition was (mM): 116 NaCl, 16 KCl, 1.1 NaH_2PO_4, 23.8 $NaHCO_3$, 2 $CaCl_2$, 0.3 $MgCl_2$, 5.5 D-glucose and 5 Hepes; pH was adjusted to 7.3 with HCl. Voltage-gated Na^+ channels and ionotropic receptors for glutamate and GABA were always blocked by supplementing the saline with (μM): 0.5 tetrodotoxin (TTX), 1000 kynurenic acid (dissolved with 1 eq. NaOH) and 10 (–)-bicuculline methochloride. Current signals were low-pass filtered at 0.5–1 kHz, and sampled at 2–20 kHz using a voltage-clamp amplifier (EPC 8 or 9/2, HEKA, Lambrecht, Germany) driven by PULSE software (version 8.31 or 8.53, HEKA). Command potentials were corrected for a liquid junction potential between the pipette solution and the saline, and given to the examined cell, employing electronic compensation (by 60%) of the series resistance (typically 5–20 MΩ). The capacitance cancellation circuitry was adjusted to erase the slowest component of the capacitive currents (monitored with 5 mV voltage steps; typically 20–70 pF; Tabata *et al.* 2000), which is thought to reflect the capacitance of the soma and proximal dendrites (Llano *et al.* 1991). Upon membrane rupture, we repeatedly monitored the amplitude of current responses at an interval of 30–60 s until it became stable. We then consecutively

obtained basal and test records, applying the control and drug-containing saline to the cell, respectively. When a drug had a detectable effect on a current response, we checked the recovery of the response, washing out the drug with the control saline (30–60 s). Only if the effect of the drug was at least partially reversible, the case was adopted for the following analyses.

We measured the somatic E_m of cultured Purkinje cells using a perforated-patch current-clamp technique (Marty & Neher, 1995) (25–26°C). The pipette solution consisted of (mM): 140 D-gluconate potassium salt, 10 NaOH, 10 Hepes and 8 $MgCl_2$; pH was adjusted to 7.3 with HCl; 0.005 vol. dimethylsulfoxide solution of amphotericin B (0.2 mg μl^{-1}) was added before recordings. The ionic composition of the chamber-perfusing saline was the same as that used for the voltage-clamp measurements, except that the total concentration of K^+ was reduced to 3 mM. Toxins included in the saline are specified in the corresponding figure legends. Current stimulation and E_m signal acquisition were performed using the fast current-clamp circuitry of the EPC-9/2 amplifier driven by the Pulse software. Signals were low-pass filtered at 5–10 kHz, and sampled at 10–20 kHz. We started recording after the series resistance decreased below 90 MΩ. The measured E_m was corrected for a liquid junction potential between the pipette solution and the saline.

We monitored the spontaneous firing of Purkinje cells in cerebellar slices by measuring transient inward currents corresponding to action potentials under voltage clamp (we hereafter term this current a spike current) in a cell-attached mode (Hamill *et al.* 1981) (24–26°C). The pipette solution consisted of (mM): 125 NaCl, 2.5 KCl, 10 Hepes, 2 $CaCl_2$ and 1 $MgSO_4$; pH was adjusted to 7.3 with NaOH. The recording chamber (volume, 1 ml) was perfused at a rate of 0.8 ml min^{-1} with a saline whose standard composition was (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$, and 20 D-glucose (bubbled with 95% O_2 and 5% CO_2). Toxins included in the saline are specified in the corresponding figure legend. The command potential was set to 0 mV. Current signals were low-pass filtered at 2 kHz, and sampled at 20 kHz using a voltage-clamp amplifier (Axopatch-1D, Axon Instruments) driven by PULSE software (version 8.54, HEKA).

Drug preparation and application

Baclofen (0417, Tocris, Avonmouth, Bristol, UK), (–)-N^6-(2-phenylisopropyl)adenosine (R-PIA; P-4532, Sigma-Aldrich), $BaCl_2$, CsCl, L-glutamate sodium salt (G-1626, Sigma), (–)-bicuculline methochloride (0131, Tocris) and CGP55845A (gift from Novartis, Basel, Switzerland) were dissolved into water to concentrations 1000–10 000 higher than the final levels, and kept at ≤4°C until use. Tertiapin-Q (TQ; 1316, Tocris) was dissolved into water to concentrations 300–1000 times higher than the final level, and kept at ≤−20°C until use. (*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 0169, Tocris) was dissolved into the saline to a concentration of 10 mM, and kept at −20°C until use. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; 0190, Tocris) was dissolved into dimethylsulfoxide to a concentration of 100 mM, and kept at 4°C until use. Pertussis toxin (PTX; 516560, Calbiochem, San Diego, CA, USA) was reconstituted into the culture medium to a concentration of 50 $\mu g \cdot ml^{-1}$, and kept at 4°C until use.

In the ruptured-patch recordings, saline containing the drugs or the control agent (0.001 vol. of water) was applied locally to the whole of the examined Purkinje cell through a theta-tube (BT150-10, Sutter, Novato, CA, USA) under the control of gravity. In some experiments, baclofen and TQ were bath-applied with the chamber-perfusing saline. $BaCl_2$ and CsCl were added to both the chamber-perfusing and locally applied saline. For pretreatment with PTX, the drug (0.5 $\mu g\ ml^{-1}$) was added to the culture dishes ≥20 h before recordings.

In the perforated-patch recordings, the glutamate- or AMPA-containing saline was applied locally to the dendrites of the examined Purkinje cell through a pipette (tip diameter, < 1 μm) attached to a pressure ejection system (∼0.35 kg cm^{-2}, PicoSpritzer III, Parker, Fairfield, NJ, USA).

In the cell-attached recordings, CNQX, bicuculline, TQ and CGP55845A were bath-applied with the chamber-perfusing saline.

Data analysis

A baclofen- or R-PIA-induced current was extracted as a difference between the basal and test records (see above) of the whole-cell currents. The amplitude of the extracted component was expressed by the mean current level over a range of test potentials from −129.5 to −131.5 mV (referred to as the amplitude at −130 mV). The reversal potential (E_{rev}) was estimated from the null-current point of a line fitted to the local region (5–10 mV wide) of a *I–V* plot using Igor software (versions 4.00–5.01, WaveMetrics, Lake Oswego, OR, USA). The equilibrium potentials of K^+ and Na^+ (E_K and E_{Na}, respectively) were estimated using Nernst equation, assuming the equality of ion activity coefficients between the intra- and extracellular sides. For analysing the kinetics of a voltage step-activated current response, a double-exponential rise function ($I(t) = a_1[1 - \exp(-t/\tau_1)] + a_2[1 - \exp(-t/\tau_2)] + c$, where $I(t)$, a, τ and c are current level at time t, amplitude, time constant and basal current level, respectively), and a double-exponential decay function [$I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + c$)] were fitted to the activation phase (50–100 ms) and deactivation phase (15–100 ms), respectively, using the Igor software.

The E_{rest} and peak amplitude of action potentials are expressed by the mean values of five records obtained during a 75 s period. The peak level and amplitude of glutamate- or AMPA-evoked potentials (GEPs and AEPs, respectively) are expressed by the mean values of six records obtained during a 100 s period.

Spike currents were analysed, using Mini Analysis Program (version 5.1.1, Synaptosoft, Decatur, GA, USA). Inter-spike current interval (ISCI) was measured as an interval between the maximal inward deflections of consecutive spike currents. The peak amplitude of a spike current is measured as a difference from the mean prespike level (length, 2 ms) to the maximal inward deflection. These measures are expressed by the mean values of all of the events in a 9 s record collected during a 3 min period.

Groups of numerical data are presented as means ± S.E.M. Differences between raw values were tested by Student's paired or unpaired *t* test (with analysis of variance (ANOVA) for more than two groups). Differences between percentage-scored values were tested by rank sum test.

Results

Extraction of a $GABA_BR$-operated inwardly rectifying current

To explore a possible $GABA_BR$-operated inwardly rectifying K^+ current in cerebellar Purkinje cells, we measured the whole-cell currents activated by a voltage ramp (Fig. 1). The E_m was first held at −31 mV for 100 ms to facilitate the inactivation of depolarization-activated currents, and then ramped to −131 mV at a rate of $-100\ mV\ s^{-1}$. In the control saline, the total currents consisted of an apparently inwardly rectifying component at test potentials more negative than −45 mV, and an apparently outwardly rectifying component at more positive test potentials (Fig. 1*A* and *C*, Basal).

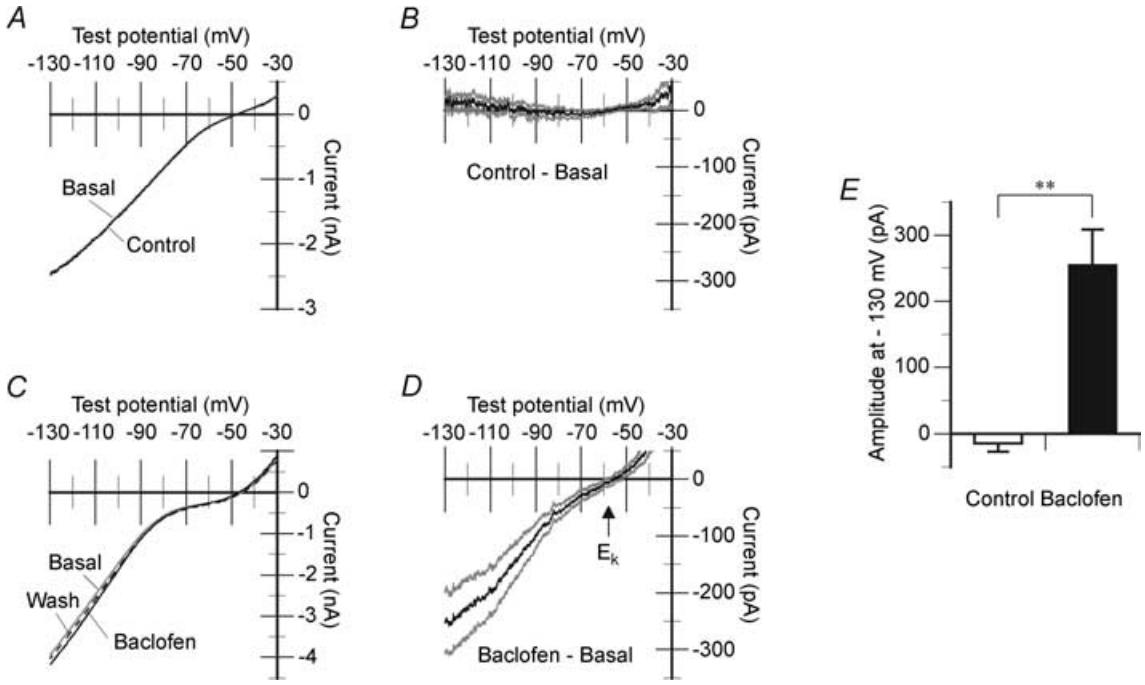

Figure 1. $GABA_BR$-operated inwardly rectifying current in cerebellar Purkinje cells
A and *B*, local application of the control saline does not induce an inwardly rectifying current in cultured Purkinje cells. *A*, sample voltage ramp-activated currents recorded from a cell consecutively before (Basal) and at the end of (Control) a 2 min application of the control saline. The equilibrium potential of K^+ (E_K) = −57.6 mV. In this and the following figures, the test potential was ramped from −31 to −131 mV at a rate of $-100\ mV\ s^{-1}$, and the resultant current was converted to a *I–V* plot. *B*, mean difference of the basal and control currents; grey lines, ±S.E.M.; $n = 10$. *C* and *D*, baclofen, a $GABA_BR$-selective agonist, induces an inwardly rectifying current (I_{bacl}) in cultured Purkinje cells. *C*, sample voltage-ramp-activated currents recorded from a cell consecutively before (Basal), at the end of (Baclofen) and after (Wash) a 2 min application of 3 μM baclofen. $E_K = -57.6$ mV. *D*, mean I_{bacl} (grey lines, ±S.E.M.) extracted by subtracting the basal currents from the baclofen currents. This extraction procedure is used throughout this study. $n = 12$. Note that the reversal potential (E_{rev}) of I_{bacl} is close to the E_K (arrow). *E*, mean amplitudes at −130 mV of the currents induced by the control saline (2 min, $n = 10$) and I_{bacl} (baclofen, 3 μM, 2 min, $n = 12$). Inward deflection is taken as positive. Error bars, ±S.E.M.; $^{**}P < 0.01$, Student's unpaired *t* test.

A major part of the apparently inwardly rectifying component is attributable to the slow activation of the hyperpolarization-activated mixed-cation current (I_h) (Crepel & Penit-Soria, 1986; Li *et al.* 1993) because of its pharmacological profile (analysed in Fig. 4*A* and *B*). The remaining part may include constitutively active inwardly rectifying K^+ current (Liesi *et al.* 2000) presumably produced by IRK channels (Falk *et al.* 1995; Miyashita & Kubo, 1997). The apparently outwardly rectifying component may include the 'tail' currents of the depolarization-activated currents. Continuous application of the control saline (2 min) did not change the overall *I–V* relation of the total currents (Fig. 1*A*, Control; Fig. 1*B*). By contrast, baclofen, a $GABA_BR$-selective agonist (3 μM) augmented the inwardly rectifying component of the total current (Fig. 1*C*). We extracted the I_{bacl} as a difference between the total currents recorded before and at the end of a 2 min application of baclofen (3 μM) (Fig. 1*D*). I_{bacl} displayed a E_{rev} (-60.4 ± 3.6 mV, $n = 11$) close to the E_K (-57.6 mV, Fig. 1*D*, arrow) and inward rectification (more clearly discernible in Fig. 3*B*). Induction of I_{bacl} with 3 μM baclofen was partially or completely reversible (Fig. 1*C*, 'Wash'). When compared at a test potential of -130 mV, the amplitude of I_{bacl} (256.4 ± 54.2 pA, $n = 12$) was significantly larger than a change in the amplitude of the total currents following a 2 min application of the control saline (-14.3 ± 12.9 pA, $n = 10$; Fig. 1*E*). These results exclude that I_{bacl} is an artefact. In experiments described in the following sections, we did not use higher concentrations of baclofen because their effect was often irreversible, and thus not readily distinguished from artefacts. The results in Fig. 1 clearly demonstrate that $GABA_BR$ activation induces an inwardly rectifying K^+ current in cerebellar Purkinje cells.

$G_{i/o}$-protein-dependence of I_{bacl}

Cerebellar Purkinje cell may express GIRK channels (see Introduction) which can be operated by $GABA_BR$ via $G_{i/o}$-proteins (Sadja *et al.* 2003). We examined the $G_{i/o}$-protein-dependence of I_{bacl} (Fig. 2). To this end, we pretreated cultured Purkinje cells for $\geq$20 h prior to the recordings with PTX, which uncouples $G_{i/o}$-proteins from G-protein-coupled receptors. In these cells, baclofen (3 μM) failed to induce I_{bacl} (Fig. 2*A*). A change in the amplitude of the total currents following a 2 min application of baclofen (3 μM) in the PTX-pretreated cells (3.6 ± 20.8 pA, $n = 7$) was significantly smaller than the amplitude of I_{bacl} in the untreated cells (254.6 ± 54.2 pA, $n = 12$; Fig. 2*B*). This result clearly demonstrates that $GABA_BR$ operates I_{bacl} via $G_{i/o}$-proteins.

Ion-selectivity of I_{bacl}

In general, inwardly rectifying currents are classified into K^+-selective currents and I_h which is carried by both K^+ and Na^+ (for review see Tabata & Ishida, 1996). To determine the ion species carrying I_{bacl}, we measured the E_{rev} of I_{bacl} with varying E_K and E_{Na} (Fig. 3). In the standard saline containing 16 mM KCl and 141.9 mM NaCl, the E_{rev} (-60.4 ± 3.6 mV, $n = 11$) was close to the E_K (-57.6 mV), but not the E_{Na} ($+66.2$ mV) (Figs 1*D* and 3*C*). When the E_K was set to -85.5 mV by reducing the concentration of KCl in the saline to 5.4 mM, the E_{rev} shifted to -80.7 ± 3.9 mV ($n = 10$, Fig. 3*A–C*). By contrast, the E_{rev} did not shift (-60.3 ± 4.1 mV, $n = 7$) when the E_{Na} was set to $+22.5$ mV by replacing 120 mM Na^+ in the saline with *N*-methyl-D-glucamine, a channel-impermeant monovalent cation (Fig. 3*D–F*). These results clearly demonstrate that I_{bacl} is carried by K^+ but not Na^+, and is different from I_h.

Pharmacology of I_{bacl}

We analysed the pharmacological profile of I_{bacl} (Fig. 4). Sensitivities to millimolar levels of Ba^{2+} and Cs^+ are widely used criteria to distinguish certain classes of inwardly rectifying K^+ currents from I_h (for review see Tabata & Ishida, 1996). Inwardly rectifying K^+ currents can be blocked by both cations. I_h is blocked by Cs^+ while resistant to Ba^{2+}. When 1 mM $BaCl_2$ ($n = 9$) or 3 mM CsCl ($n = 16$) was included in both the chamber-perfusate and the locally delivered saline, baclofen (3 μM) failed to

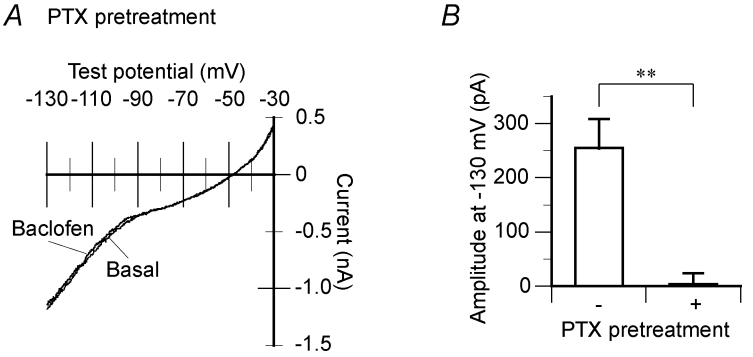

Figure 2. $G_{i/o}$-protein dependence of I_{bacl}
I_{bacl} is not inducible in cultured Purkinje cells pretreated with pertussis toxin (PTX), a $G_{i/o}$-protein inhibitor (0.5 μg ml^{-1}, $\geq$20 h). *A*, sample voltage-ramp-activated currents recorded from a cell consecutively before (Basal) and at the end of (Baclofen) a 2 min application of 3 μM baclofen. $E_K = -57.6$ mV. *B*, mean amplitude at -130 mV of I_{bacl} (baclofen, 3 μM, 2 min) measured in the untreated cells ($n = 12$, reproduced from Fig 1*E*, $-$) and the PTX-pretreated cells ($n = 7$, $+$). Inward deflection is taken as positive. Error bars, $\pm$S.E.M. $^{**}P < 0.01$, Student's unpaired *t* test.

induce I_{bacl} (Fig. 4*A* and *B*). Changes in the amplitude of the total currents induced by a 2 min application of baclofen under these conditions (-12.0 ± 6.6 pA, $n = 9$ and -2.5 ± 6.2 pA, $n = 16$, respectively) were significantly smaller than the amplitude of I_{bacl} in the absence of these cations (254.6 ± 54.2 pA, $n = 12$; Fig. 4*C*). This result indicates that I_{bacl} belongs to inwardly rectifying K^+ currents, but not to I_h. In addition, the apparently inward rectifying component of the total currents (e.g. Fig. 1*A* and *C*, Basal) was persistent in the Ba^{2+}-containing saline (Fig. 4*A*, Basal) while obscured in the Cs^+-containing saline (Fig. 4*B*, Basal). This result suggests that this component largely consists of I_h.

We next examined the sensitivity of I_{bacl} to tertiapin-Q (TQ), a synthetic peptide derived from a bee venom toxin which potently blocks certain classes of inwardly rectifying K^+ channels, including GIRK channels (Jin & Lu, 1999). For the reason of availability, it was difficult to continuously include TQ in the chamber-perfusing saline. Instead, we sequentially applied baclofen alone (3 μM, 2 min; Fig. 4*D*, Baclofen) and baclofen together with TQ (3 μM and 30 nM, respectively; Fig. 4*D*, Baclofen & TQ) locally to the Purkinje cell, and evaluated the reduction of I_{bacl} following the coapplication. The effect of TQ reached the maximum within 30–90 s of the onset of the coapplication. At the maximum, the amplitude at -130 mV of I_{bacl} was significantly reduced by $74.7 \pm 21.4\%$ ($n = 10$, Fig. 4*E*). By contrast, prolonged application of baclofen alone (3 μM, 30–120 s) did not significantly reduce the amplitude (by $2.8 \pm 8.1\%$, $n = 6$; Fig. 4*E*), indicating that the effect of TQ is not an artefact due to the run-down or inactivation of I_{bacl}. This result

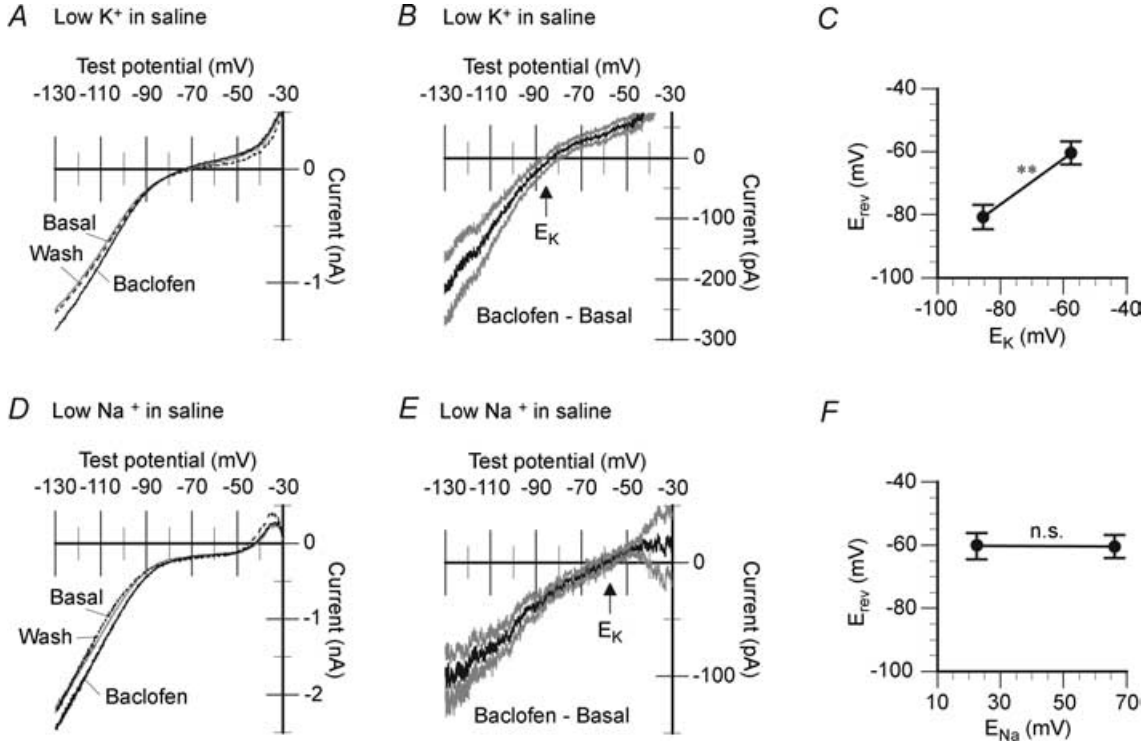

Figure 3. Carrier ion species of I_{bacl}

A–C, the E_{rev} of I_{bacl} shifts with the E_K. *A*, sample voltage-ramp-activated currents recorded from a cultured Purkinje cell consecutively before (grey line, Basal), at the end of (continuous line, Baclofen) and after (dashed line, Wash) a 2 min application of 3 μM baclofen in the saline with reduced KCl (5.4 mM). $E_K = -85.5$ mV. *B*, mean I_{bacl} (baclofen, 3 μM, 2 min) in the 5.4 mM KCl-containing saline. Grey lines, $\pm$S.E.M., $n = 10$. Note that the E_{rev} of I_{bacl} is close to E_K (arrow). *C*, mean E_{rev} of I_{bacl} (baclofen, 3 μM, 2 min) measured in the saline containing 5.4 mM KCl ($n = 10$) and 16 mM KCl ($n = 11$, data taken from Fig. 1*D*) plotted against the corresponding E_K. Error bars, $\pm$S.E.M.; $^{**}P < 0.01$, Student's unpaired *t* test. *D–F*, the E_{rev} of I_{bacl} does not shift with equilibrium potential of Na^+ (E_{Na}). *D*, sample voltage-ramp-activated currents recorded from a cultured Purkinje cell consecutively before (grey line, Basal), at the end of (continuous line, Baclofen) and after (dashed line, Wash) a 2 min application of 3 μM baclofen in the saline with reduced NaCl (25.9 mM; by equimolar replacement with *N*-methyl-D-glucamine). $E_{Na} = +22.5$ mV. *E*, mean I_{bacl} (baclofen, 3 μM, 2 min) in the 25.9 mM NaCl-containing saline. Grey lines, $\pm$ S.E.M.; $n = 7$. Note that the E_{rev} of I_{bacl} is close to the E_K (-57.6 mV, arrow) but not E_{Na}. *F*, mean E_{rev} of I_{bacl} (baclofen, 3 μM, 2 min) measured in the saline containing 25.9 mM NaCl ($n = 7$) and 141.9 mM NaCl ($n = 11$, data taken from Fig. 1*D*) plotted against the corresponding E_{Na}. Error bars, $\pm$ S.E.M.; n.s., $P > 0.05$, Student's unpaired *t* test.

shows that TQ-sensitive classes of inwardly rectifying K^+ channels are responsible for I_{bacl}.

Kinetics of I_{bacl}

We examined the activation and deactivation kinetics of I_{bacl} to abrupt voltage changes (Fig. 5). We recorded the total current responses to a hyperpolarizing voltage step (to −91 mV from a holding potential of −61 mV; duration, 100 ms) before and during baclofen application (3 μM) (Fig. 5*A*). I_{bacl} was extracted as a difference of these currents (Fig. 5*B*, continuous black line). Upon the step onset, a major part of I_{bacl} was activated within a millisecond period, and the remaining part continued to develop onward. These rapid and slow components of the activation phase can be described by a double-exponential rise function (see Methods for definition; Fig. 5*B*, continuous grey line) with two much different time constants (Table 1). I_{bacl} displayed no inactivation during hyperpolarization (Fig. 5*B*). Upon the step offset, a major part of I_{bacl} was deactivated within a millisecond period, and the remaining part more slowly. These rapid and slow components of the deactivation phase can be described by a double-exponential decay

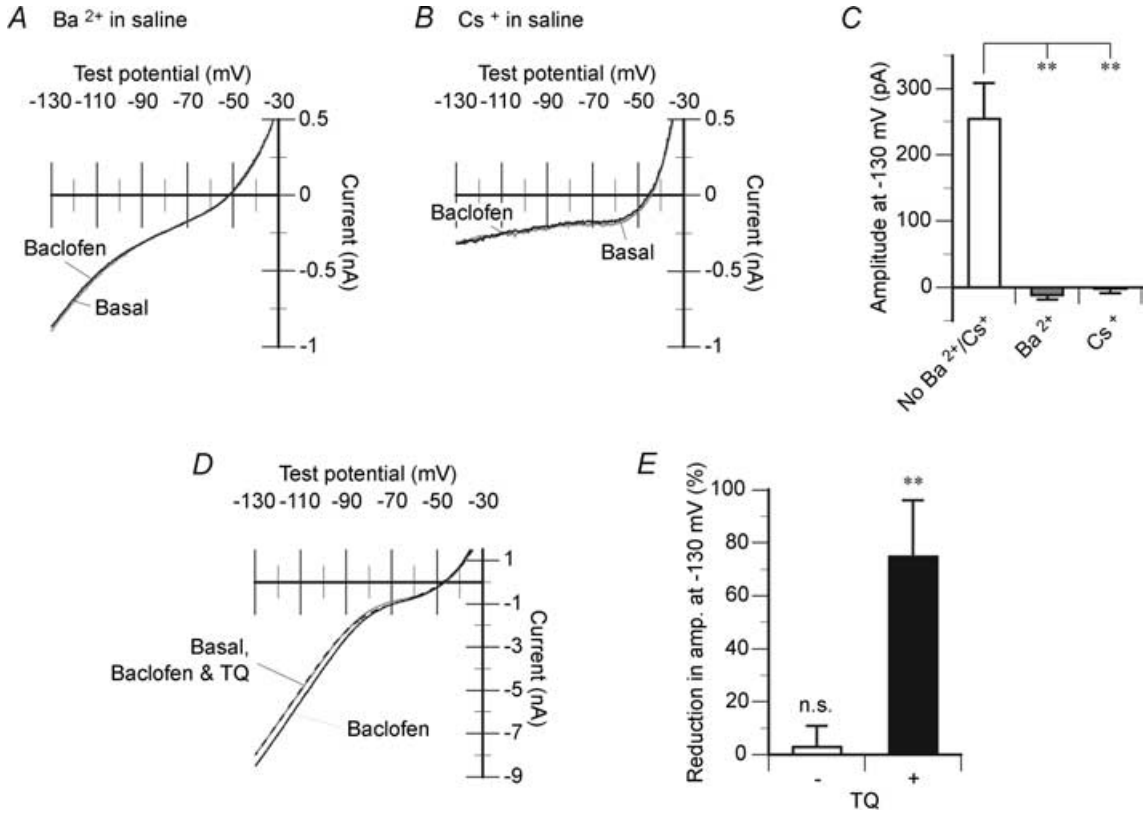

Figure 4. Pharmacology of I_{bacl}

A–C, I_{bacl} is not inducible in the continuous presence of extracellular Ba^{2+} or Cs^+. *A* and *B*, sample voltage-ramp-activated currents recorded from single cultured Purkinje cells consecutively before (Basal) and at the end of (Baclofen) a 2 min application of 3 μM baclofen in the saline containing 1 mM $BaCl_2$ (*A*) or 3 mM CsCl (*B*). In addition to the effect on I_{bacl}, Cs^+ also blocks the inwardly rectifying component of basal current; note that this component is relatively small in the CsCl-containing saline (*B*) compared with that in the CsCl-free saline (cf. Figs 1*A* and C, Basal; see Results for more detailed explanation). *C*, mean amplitudes of I_{bacl} at −130 mV (baclofen, 3 μM, 2 min) measured in the normal ($n = 12$, reproduced from Fig. 1*E*), 1 mM $BaCl_2$-containing ($n = 9$) and 3 mM CsCl-containing ($n = 16$) saline. Error bars, ±S.E.M. $^{**}P < 0.01$, ANOVA and Student's unpaired *t* test. *D–E*, the GIRK channel antagonist TQ reduces I_{bacl}. *D*, sample voltage-ramp-activated currents recorded from a cultured Purkinje cell consecutively before (grey line, Basal) and during application of the labelled drugs. In this experiment, we observed the induction of I_{bacl} at the end of a 2 min application of 3 μM baclofen alone (continuous black line) and then repeatedly monitored at an interval of 30 s the reduction of I_{bacl}, coapplying 3 μM baclofen and 30 nM TQ (dashed black line). The effect of TQ reached a maximum within 30–90 s of the coapplication onset. *E*, mean reduction in the amplitude of I_{bacl} at −130 mV caused by coapplication of baclofen and TQ (3 μM and 30 nM, respectively, $n = 10$, '+'). For each cell, the maximal effect was expressed as a percentage of the amplitude of I_{bacl} induced by baclofen alone (3 μM, 2 min). For control, mean reduction of I_{bacl} was measured during prolonged application of baclofen alone (3 μM, 30–120 s, $n = 6$, −). Error bars, ±S.E.M.; n.s., $P > 0.05$; $^{*}P < 0.01$; Student's paired *t* test.

function (see Methods for definition; Fig. 5*B*, dashed grey line) with two much different time constants (Table 1). These results show that I_{bacl} can rapidly respond to voltage changes, and once activated it continuously functions for a prolonged time.

Possible functional contribution of I_{bacl}

We assessed how I_{bacl} influences the E_{rest} and action potentials of cerebellar Purkinje cells using a perforated-patch current-clamp technique in cultured cell preparations (Fig. 6). To minimize spontaneous firing, we injected a constant background current (typically −15 to +15 pA) as to set the mean E_{rest} to ∼−65 mV at the beginning of a recording session (Fig. 6*A*, Basal), and continued injection at the same intensity for the rest of the recording session. This level of the E_{rest} was naturally observed for Purkinje cells without vigorous spontaneous firing (data not illustrated). When an additional current stimulus was not imposed, the momentary E_m fluctuated by only a few millivolts around the mean E_{rest} (cf. Fig. 6*D*). Baclofen (3 μM, 1–3 min) hyperpolarized the E_{rest} by 3.46 ± 1.15 mV ($n = 14$; Fig. 6*A* and *C*). There was a significant difference between the mean E_{rest} before and during baclofen application (Fig. 6*C*). This effect can be ascribed to I_{bacl} because it was not reproduced with a statistical significance in the continuous presence of 0.1–1 mM $BaCl_2$ in the saline ($n = 12$; Fig. 6*B* and *C*).

Injection of a strong depolarizing pulse (300 pA, 5 ms) evoked an action potential (Fig. 6*A* and *B*). Baclofen (3 μM, 1 min) prolonged the peak latency of the action potential by ∼1 ms in five out of eight Purkinje cells (Fig. 6*A*, inset). Baclofen (3 μM, 1 min) also raised the peak level achieved by the action potential by ∼2 mV in five out of eight cells (Fig. 6*A*, inset). The peak level was higher when the E_m prior to depolarizing pulse was more hyperpolarized in both the absence and presence of baclofen (data pooled from three cells with the most evident effect of baclofen on the peak level, Fig. 6*D*). These effects can be ascribed to I_{bacl} because they were not seen in the continuous presence of 0.1–1 mM $BaCl_2$ in the saline ($n = 7$; Fig. 6*B*, inset). However, the effects of baclofen on the peak latency and peak level of action potentials were not obvious in the remaining cells. Thus, baclofen-induced changes in the mean peak latency (from 3.99 ± 0.56 to 4.77 ± 0.90 ms) and the mean peak level (from +24.8 ± 4.4 to +25.3 ± 4.5 mV) for all of the examined cells ($n = 8$) were not statistically significant ($P > 0.05$, Student's paired t test). Baclofen did not delay the recovery from after-hyperpolarization to the E_{rest} (Fig. 6*A*). The mean time constant of the exponential functions fitted to the main component of the recovery phase before baclofen application was 41.6 ± 13.5 ms, and that during baclofen application was 33.4 ± 5.4 ms ($n = 6$, data not illustrated). There was no significant difference between the time constants ($P > 0.05$, Student's paired t test). These results suggest that I_{bacl} may decrease the readiness of firing by hyperpolarizing the E_{rest}, while it may not much affect the waveform of an action potential itself.

We next assessed how I_{bacl} influences GEPs in Purkinje cells using a perforated-patch current-clamp technique (Fig. 7). We used glutamate puff instead of stimulation of glutamatergic neurones innervating Purkinje cells because baclofen is known to attenuate synaptic release via the presynaptic mechanism (Dittman & Regehr, 1996,

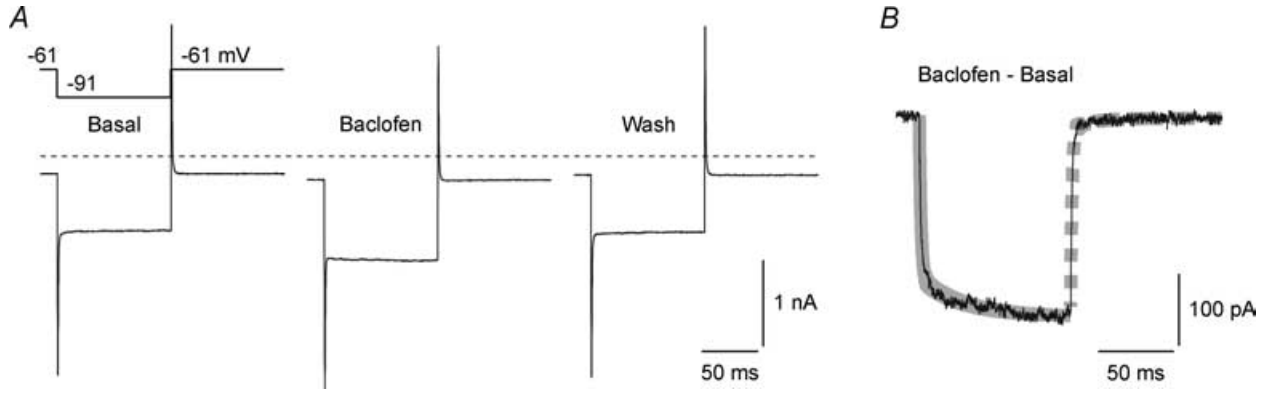

Figure 5. Kinetics of I_{bacl}
I_{bacl} is rapidly activated and deactivated upon membrane potential (E_m) jumps, and sustains without inactivation during hyperpolarization. *A*, sample voltage-step-activated currents recorded from a cultured Purkinje cell consecutively before (Basal), at the end of (Baclofen) and after (Wash) a 1 min application of 3 μM baclofen. The schematic above the left trace indicates the time course of the test potential. Dashed line, null-current level. $E_K = -56.7$ mV. *B*, I_{bacl} extracted as a difference between the basal and baclofen currents in *A*. The activation and deactivation phase of I_{bacl} (continuous black line) can be described by a double-exponential rise function (continuous grey line) and a double-exponential decay function (dashed grey line), respectively (see Methods for the definition of these functions). The parameters of the fitted functions are presented in Table 1.

Table 1. Parameters of double-exponential functions fitted to the activation and deactivation phases of the inwardly rectifying current activated by baclofen

Component	Relative amplitude	Time constant (ms)
Activation, rapid	0.754 ± 0.039	0.81 ± 0.15
Activation, slow	0.246 ± 0.039	82.4 ± 43.1
Deactivation, rapid	0.930 ± 0.028	0.60 ± 0.11
Deactivation, slow	0.070 ± 0.028	30.5 ± 16.4

Data are collected from 8 cells in the experiment shown in Fig. 5, and presented as means ± S.E.M. See Methods for the definition of the functions.

1997). We puffed glutamate (1 mM, 3–6 ms) to the middle of the dendritic arbor of a Purkinje cell repeatedly at an interval of 20 s, and recorded GEPs from the soma (Fig. 7*A*). The peak amplitude of the GEPs ranged from 5 to 20 mV (Fig. 7*B* and *C*, Basal). Baclofen (3 μM, 80–180) had little effect on their waveform and peak amplitude ($n = 6$, Fig. 7*B*). There was no significant difference between the peak amplitudes before (16.8 ± 3.3 mV) and during (16.7 ± 3.7 mV) baclofen application ($n = 6$; $P > 0.05$, Student's paired t test). However, baclofen (3 μM, 80–180 s) significantly lowered the peak level achieved by the GEPs by 6.29 ± 2.19 mV ($n = 6$), along with a hyperpolarizing shift in the E_{rest} (Fig. 7*B* and *D*). This effect of baclofen can be ascribed to I_{bacl} because a significant lowering in the peak level was not observed in the continuous presence of 1 mM $BaCl_2$ in the saline ($n = 5$, Fig. 7*C* and *D*). In Purkinje cells, glutamate may evoke not only ionotropic glutamate receptors but also metabotropic glutamate receptors, and both the types of receptors may contribute to GEPs (see Tabata *et al.* 2004 for review). $GABA_BR$ activation is reported to lead to augmentation of mGluR1-mediated responses (Hirono *et al.* 2001). Thus, baclofen could augment the mGluR1-mediated component of GEPs, and this could mask the attenuation of the ionotropic glutamate receptor-mediated component. However, these possibilities are not the cases because baclofen did not reduce the peak amplitude of postsynaptic potentials evoked by AMPA (AEP), an ionotropic glutamate receptor agonist (0.1–1 mM, 10.1 ± 3.8 mV before baclofen application and 11.3 ± 4.2 mV after baclofen application, $n = 6$; Fig. 7*E*). Ionotropic glutamate receptors might largely mediate GEPs elicited by the glutamate application protocol used in this study. The results shown in Fig. 7 suggest that I_{bacl} may impede excitatory synaptic inputs from triggering action potentials.

Finally, we examined whether I_{bacl} can function *in situ* using Purkinje cells in cerebellar slice preparations. We tried to measure the effects of baclofen on events observed under current clamp in a ruptured-patch whole-cell configuration. However, this attempt was hampered because the effects of baclofen and the intrinsic excitability of the examined cells severely ran down during a prolonged recording in this configuration presumably due to cytoplasmic disturbance ($n = 3$, data not illustrated). Instead, we used a noninvasive recording technique (cell-attached technique), and evaluated the effects of baclofen on spontaneous firing. In a cell-attached configuration, the depolarizing phase of an action potential can be observed as a transient inward current (spike current). In the normal saline, most of the examined cells displayed regular spontaneous firing with an ISCI of 21–93 ms (55.2 ± 8.0 ms, $n = 7$). Inclusion of CNQX and bicuculline, antagonists against ionotropic glutamate and GABA receptors in the saline, did not stop or slow down spontaneous firing (ISCI, 18–37 ms, $n = 4$; data not illustrated), suggesting that the intrinsic mechanisms but not excitatory or inhibitory synaptic inputs initiate spontaneous firing. In the regularly firing cells, baclofen significantly increased the ISCI by 221.8 ± 23.6% ($n = 7$, Fig. 8*A* and *D*). Subsequent application of 2 μM CGP55845A, a $GABA_BR$-selective antagonist, reversed this effect of baclofen ($n = 7$, Fig. 8*A*–*C* and *E*). Baclofen significantly increased the ISCI even in the continuous presence of 0.1 mM $BaCl_2$ (by 73.4 ± 19.7%, $n = 4$) or 150–300 nM TQ (by 74.6 ± 12.3, $n = 7$) in the saline (Fig. 8*B*–*D*). However, the extent of the effect with $BaCl_2$ or TQ was significantly smaller that that without these agents (Fig. 8*D*, # and ##). The baclofen-induced increase in the ISCI was accompanied with augmentation of spike currents (Fig. 8*A* and *F*). This effect was significant only in the absence of 0.1 mM $BaCl_2$ or 150–300 nM TQ (Fig. 8*A*–*C* and *F*), and reversed by 2 μM CGP55845 (Fig. 8*A* and *G*). The results shown in Fig. 8 demonstrate that I_{bacl} can function *in situ* and influence the intrinsic excitability of Purkinje cells.

Discussion

Functional identification of a $GABA_BR$-operated inwardly rectifying current in cerebellar Purkinje cells

In this study, we characterized an inwardly rectifying current activated by the $GABA_BR$-selective agonist baclofen (I_{bacl}) in cultured cerebellar Purkinje cells, using a ruptured-patch whole-cell voltage-clamp technique (Figs 1–5). In central neurones, inwardly rectifying currents may be produced by Na^+-/K^+-permeable channels (HCN channels; Robinson & Siegelbaum, 2003) and/or the supergene family of K^+-selective channels (Kir channels; Bichet *et al.* 2003). Cerebellar Purkinje cells possess I_h (Crepel & Penit-Soria, 1986; Li *et al.* 1993), which is produced by HCN channels (Robinson & Siegelbaum, 2003), and they appear to express GIRK2–4 (Kir3.2–3.4, see below), IRK1,3 (Kir2.1,2.3) (Falk *et al.* 1995; Miyashita & Kubo, 1997), and Kir7.1 (Krapivinsky *et al.* 1998). In this section, we compare the physiological properties of I_{bacl} (Figs 1–5) with these channels.

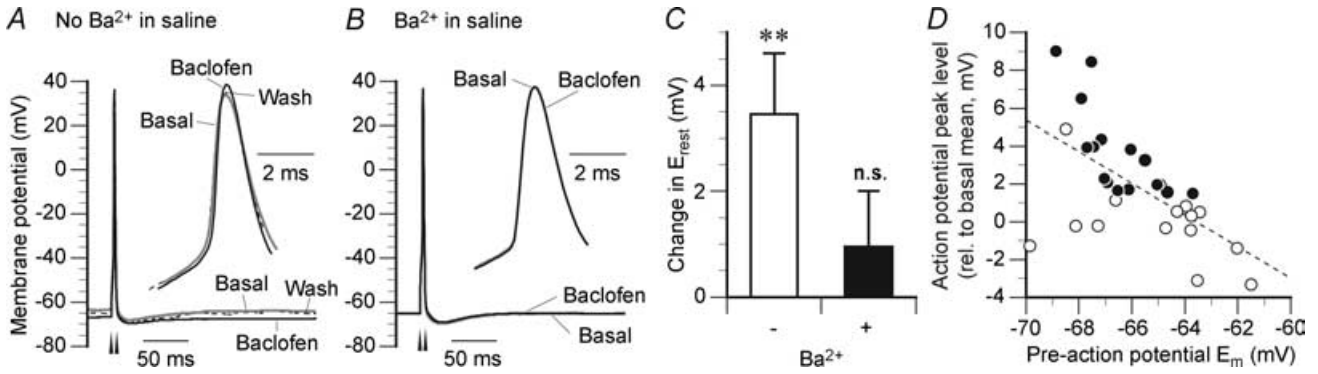

Figure 6. Actions of I_{bacl} on the E_{rest} and action potentials
I_{bacl} hyperpolarizes the resting potential (E_{rest}), while it does not much affect action potentials. *A* and *B*, sample current-clamp traces recorded from single cultured Purkinje cells consecutively before (grey line, Basal), during (1–2 min of baclofen onset, black line, Baclofen) and after (in *A* only; dashed line, Wash) baclofen application (3 μM) in the $BaCl_2$-free (*A*) and 0.1 mM $BaCl_2$-containing (*B*) saline. Note a change in the E_{rest} in *A*. Depolarizing pulses (300 pA, 5 ms, applied between arrow heads) evoke action potentials. Each trace indicates the average of three to five records. Insets, action potentials (single records) expanded in time scale and justified at the peaks. The saline contained 3 mM KCl, 10 μM bicuculline and 1 mM kynurenic acid, but not TTX. The recordings were made in perforated-patch mode. *C*, mean changes in the E_{rest} from the basal to baclofen records in the $BaCl_2$-free ($n = 14$, −) and $BaCl_2$-containing (0.1–1 mM, $n = 12$, +) saline. Hyperpolarizing shift is taken as positive. Error bars, ±S.E.M. $^{**}P < 0.01$ and n.s. ($P > 0.05$) between the basal and baclofen records, Student's paired *t* test. *D*, peak levels of action potentials are plotted against the momentary E_m (mean over 10 ms) prior to the depolarizing pulses. Open and closed symbols indicate the data of the basal and baclofen records, respectively. Each peak level is expressed as a difference from the mean level of 5 basal records. The dashed line indicates a linear function fitted to the data by regression (slope, −0.84). Data were pooled from 3 cells.

The E_{rev} of I_{bacl} was close to the E_K, and shifted depending on the extracellular concentration of K^+ but not that of Na^+ (Figs 1 and 3). These results demonstrate that I_{bacl} is produced by Kir channels but not HCN channels. Uncoupling $G_{i/o}$-proteins from $GABA_BR$ almost completely suppressed I_{bacl} (Fig. 2). This result indicates that I_{bacl} is operated by $GABA_BR$ via $G_{i/o}$-proteins and is produced by GIRK channels which are highly dependent

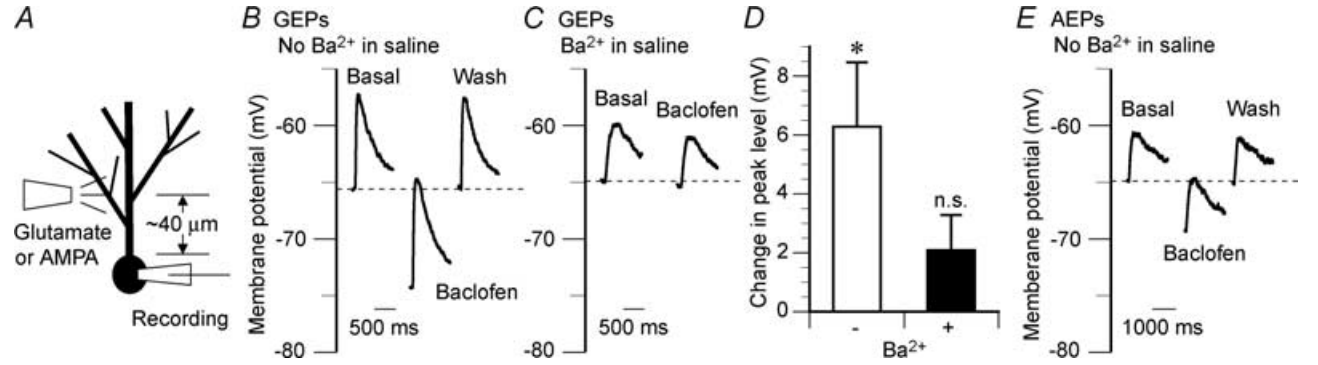

Figure 7. Actions of I_{bacl} on GEPs
I_{bacl} lowers the peak levels achieved by glutamate-evoked potentials (GEPs). *A*, schematic shows the recording configuration. L-Glutamate (1 mM) or AMPA (0.1–1 mM) was repeatedly pressure-applied (duration 3–8 ms; inter-stimulus interval, 20 s) to the middle of the dendritic arbor of a cultured Purkinje cell through a pipette. The agonist-evoked potentials were recorded from the soma using a perforated-patch current-clamp technique. The saline contained 3 mM KCl, 0.3–0.5 μM TTX, and 10 μM bicuculline but not kynureic acid. *B* and *C*, sample GEPs recorded from single cells consecutively before (Basal), during (80–180 s of baclofen onset, Baclofen) and after (Wash) baclofen application (3 μM) in the $BaCl_2$-free (*B*) or 1 mM $BaCl_2$-containing (*C*) saline. Baclofen was bath-applied. Each trace indicates the average of three to six records. *D*, mean change in the peak level achieved by GEPs from the basal to baclofen records in the $BaCl_2$-free ($n = 6$, −) and 1 mM $BaCl_2$-containing ($n = 5$, +) saline. Hyperpolarizing shift is taken as positive. Error bars, ±S.E.M. $^{*}P < 0.05$; n.s., $P > 0.05$; between the basal and baclofen records, respectively, Student's paired *t* test. *E*, sample AMPA-evoked potentials (AEPs) recorded from a cell consecutively before (Basal), during (80–180 s of baclofen onset, Baclofen) and after (Wash) baclofen application (3 μM) in the $BaCl_2$-free saline. Each trace indicates the average of six records.

on $G_{i/o}$-proteins compared with other Kir channels (Isomoto *et al.* 1997; Bichet *et al.* 2003; Sadja *et al.* 2003). Both Ba^{2+} and Cs^{+}, at millimolar levels, completely blocked I_{bacl} (Fig. 4*A*–*C*). I_{bacl} shares these metal ion sensitivities with GIRK (Hommers *et al.* 2003) and IRK (Isomoto *et al.* 1997) channels. By contrast, HCN channels (I_h) are resistant to Ba^{2+} (Crepel & Penit-Soria, 1986; Tabata & Ishida, 1996; Robinson & Siegelbaum, 2003), and Kir7.1 channels are resistant to both Ba^{2+} and Cs^{+} (Krapivinsky *et al.* 1998). TQ at 30 nM blocked a large fraction of I_{bacl} (Fig. 4*D* and *E*). The degree of blockade is consistent with the reported affinity of TQ for GIRK channels (K_i, 13.3 nM) but not that for IRK channels (no significant effect even at 1 μM) (Jin & Lu, 1999). Upon the onset of a hyperpolarizing voltage step, I_{bacl} displayed the activation phase consisting of rapid and slow components

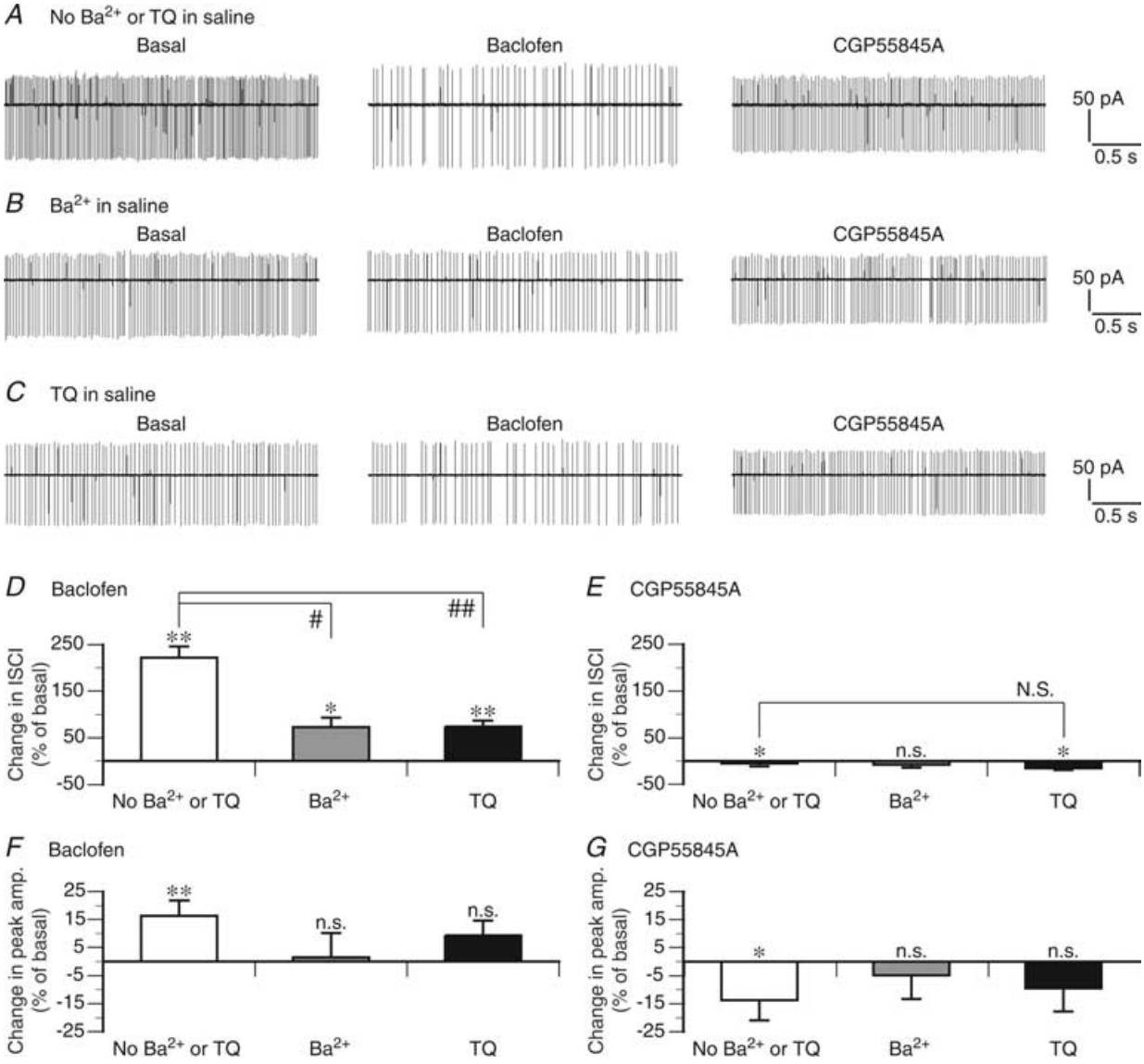

Figure 8. Actions of I_{bacl} on the intrinsic excitability *in situ*

I_{bacl} reduces the rate of spontaneous firing of Purkinje cells in cerebellar slices presumably by hyperpolarizing the E_{rest}. *A*–*C*, sample spike currents (see Methods) recorded from single Purkinje cells consecutively before (Basal) and during baclofen application (10 μM, at 5–8 min of the application onset), and during CGP55845A application (a $GABA_BR$-selective antagonist, 2 μM, at 5–8 min of the application onset) in the $BaCl_2$-/TQ-free (*A*), 0.1 mM $BaCl_2$-containing (*B*) and 300 nM TQ-containing (*C*) saline. The recordings were made in a cell-attached mode. *D*–*G*, mean changes in inter-spike current interval (ISCI; *D* and *E*) and the peak amplitude (*F* and *G*) of spike currents following baclofen and CGP55845A application without ($n = 7$) or with 0.1 mM $BaCl_2$ ($n = 4$) or 150–300 nM TQ ($n = 7$) measured as in *A*–*C*. Error bars, ±S.E.M. For each condition, the effects of baclofen (difference between the basal and baclofen records) and CGP55845A (difference between the basal and CGP55845A records) were tested by rank sum test (n.s., $P > 0.05$; *$P < 0.05$; **$P < 0.01$). When judged significant by the previous test, the effects were further compared between the conditions by rank sum test (N.S., $P > 0.05$; #$P < 0.05$; ##$P < 0.01$).

with much different time constants (Fig. 5, Table 1). The activation phases of GIRK currents in cerebellar granule neurones (Slesinger *et al.* 1997) and GIRK-transfected heterologous cells (Wischmeyer *et al.* 1997) also consist of rapid and slow components. I_{bacl} is distinguished from Kir7.1 current, which is instantaneously activated to the maximal level (Krapivinsky *et al.* 1998), and I_h, which displays single-exponential activation phase with a time constant (50–100 ms; Crepel & Penit-Soria, 1986; Tabata & Ishida, 1996) much longer than that of the main activation component of I_{bacl} (~0.8 ms, Table 1). During a 100 ms hyperpolarization, I_{bacl} sustained without inactivation (Fig. 5). I_{bacl} is distinguished from IRK1 currents, which display apparent inactivation due to open channel block by external Na^+ (Kubo *et al.* 1993). Taken together, our voltage-clamp measurements (Figs 1–5) demonstrate that cerebellar Purkinje cells are equipped with a $GABA_BR$-operated inwardly rectifying current most likely produced by GIRK channels.

The subunit composition of GIRK channels mediating I_{bacl} remains to be elucidated in future studies. Some *in situ* hybridization studies show that Purkinje cells express the mRNAs of GIRK2 (Lauritzen *et al.* 1997), GIRK3 (Karschin *et al.* 1996) and GIRK4 (Karschin *et al.* 1996; Iizuka *et al.* 1997). Some immunohistochemical studies show that GIRK2 protein is present in Purkinje cells (Lauritzen *et al.* 1997) (albeit there are opposing reports; Liao *et al.* 1996; Murer *et al.* 1997), and GIRK4 protein localizes to the proximal dendrites, somata and proximal axonal segments of Purkinje cells (Iizuka *et al.* 1997; Murer *et al.* 1997). Thus, Purkinje cells may express homo- and/or heteromeric channels consisting of GIRK2–4. In certain heterologous expression systems, GIRK4 form functional channels alone or together with GIRK3 (Iizuka *et al.* 1995; Wischmeyer *et al.* 1997). These channels could be candidates for the I_{bacl}-mediating channels. On the other hand, Purkinje cells do not have detectable levels of the mRNA (Karschin *et al.* 1996) and protein of GIRK1 (Ponce *et al.* 1996; Miyashita & Kubo, 1997). Thus, Purkinje cells express virtually no GIRK1/2 heteromeric channel, which is the predominant type in many other central neurones (Jan & Jan, 1997). In the heterologous expression system, GIRK1-free channels produce smaller macroscopic currents than GIRK1-containing channels (Wischmeyer *et al.* 1997). Lack of GIRK1 may explain that the amplitude of I_{bacl} in Purkinje cells is comparable with that in much smaller neurones (e.g. cerebellar granule cells; Slesinger *et al.* 1997).

Possible physiological roles of a $GABA_BR$-operated inwardly rectifying current in cerebellar Purkinje cells

Using a perforated-patch whole-cell current-clamp technique, we assessed how I_{bacl} influence the postsynaptic excitability of cultured cerebellar Purkinje cells (Figs 6 and 7). $GABA_BR$ in Purkinje cells is reported to respond to not only GABA released from the innervating interneurones, but also to GABA spillover from neighbouring inhibitory synapses between other neurones *in situ* (Hirono *et al.* 2001). One study (Dittman & Regehr, 1997) estimates that the spillover elevates the extracellular GABA concentration around Purkinje cells to $\leq 10\ \mu M$. GABA and baclofen have similar binding affinities (~60 nM) and potencies for $GABA_BR$ (Bowery, 1993). Thus, the events observed with $3\ \mu M$ baclofen in Figs 6 and 7 may occur under physiological conditions when cerebellar cortical GABAergic neurones are active.

Baclofen ($3\ \mu M$) did not much affect the waveform of an action potential evoked by a suprathreshold stimulus (Fig. 6*A*). This result indicates that I_{bacl} may not limit the contribution of strong excitatory synaptic inputs (e.g. climbing fibre inputs) to the generation of action potentials, whereas I_{bacl} may affect weak excitatory inputs (e.g. parallel fibre inputs) in a manner mentioned below. In some cells, baclofen raised the peak level achieved by the action potentials (see Results; Fig. 6*A*, inset). In general, such augmentation could result from the increased activity of the voltage-gated Na^+ channels producing the depolarizing phase of an action potential. One possibility is that the $GABA_BR$–$G_{i/o}$-protein signalling cascade suppresses activation of cyclic adenosine monophosphate-dependent kinase, which is reported to reduce the peak current of the voltage-gated Na^+ channels (Catterall, 1999). Another possibility is that a hyperpolarizing shift in the E_{rest} caused by I_{bacl} (Figs 6*A* and *C*) facilitates disinactivation of the Na^+ channels (i.e. recovery from depolarization-induced inactivation; for review see Hayashida & Ishida, 2004). The latter possibility is consistent with the observation that the peak level depended on the E_m prior to the action potential (Fig. 6*D*).

In hippocampal pyramidal neurones, activation of GIRK channels results in attenuation of GEPs initiated in the dendrites (Seeger & Alzheimer, 2001), presumably because GIRK channels effectively reduce the input resistance at the stimulated site (Koch, 1999). In cerebellar Purkinje cells, by contrast, such attenuation was not observed (Fig. 7*B*). Some immunohistochemical studies (Iizuka *et al.* 1997; Murer *et al.* 1997) suggest condensation of GIRK subunits around the soma. Thus, it could be possible that K^+ conductance through the I_{bacl}-producing channels is relatively small in the dendrites, and does not effectively reduce the input resistance for GEPs. However, baclofen significantly hyperpolarized the E_{rest} (Fig. 6*A* and *C*) and the peak level achieved by GEPs in Purkinje cells (Fig. 7*B* and *D*). These effects are due largely to I_{bacl} because they were not observed in the continuous presence of extracellular Ba^{2+} (Figs 6*B* and *C*, and 7*C* and *D*). These results indicate that when activated, I_{bacl} effectively increases K^+ conductance around the soma, and may impede excitatory synaptic inputs from driving

the E_m towards the firing threshold. The coincidence of synaptic inputs from parallel fibres and a climbing fibre in the Purkinje cell is a key step to induce cerebellar long-term depression (LTD), a form of synaptic plasticity crucial for cerebellar motor learning (for review see Ito, 2002). GABAergic activation of I_{bacl} might influence induction of LTD by preferentially limiting the contribution of relatively weak parallel fibre inputs to postsynaptic excitation.

The cell-attached recordings from Purkinje cells in cerebellar slices showed that baclofen decreases the rate of spontaneous firing (Fig. 8*A* and *D*), which is initiated by the intrinsic mechanisms of Purkinje cells but not synaptic inputs (see Results). I_{bacl} activated via the $GABA_BR$ is thought to be the primary mechanism enabling this effect of baclofen because the $GABA_BR$-selective antagonist CGP55845A reversed it (Fig. 8*A* and *E*), and extracellular Ba^{2+} and TQ largely eliminated it (Fig. 8*B–D*). I_{bacl} could exert the effect on the firing rate by hyperpolarizing the E_{rest} (Fig. 6*A* and *C*). This notion is consistent with the observation that baclofen augmented the peak amplitude of spike currents (Fig. 8*A* and *F*); such augmentation is expected to occur with the hyperpolarized E_{rest} (see above; Fig. 6*A* and *D*). These results suggest that I_{bacl} reduces the intrinsic excitability of Purkinje cells, and strongly support the notion I_{bacl} can function in Purkinje cells *in situ*.

In conclusion, we have functionally identified a $GABA_BR$-operated inwardly rectifying K^+ current most likely produced by GIRK channels, and demonstrated its possible inhibitory actions on the postsynaptic and intrinsic excitability of cerebellar Purkinje cells. These findings give a new insight into the physiological roles of $GABA_BR$ signalling in cerebellar cortex.

References

Bichet D, Haass FA & Jan LY (2003). Merging functional studies with structures of inward-rectifier K^+ channels. *Nat Rev Neurosci* **4**, 957–967.

Bowery NG (1993). $GABA_B$ receptor pharmacology. *Annu Rev Pharmacol Toxicol* **33**, 109–147.

Catterall WA (1999). Molecular properties of brain sodium channels: an important target for anticonvulsant drugs. *Adv Neurol* **79**, 441–456.

Crepel F & Penit-Soria J (1986). Inward rectification and low threshold calcium conductance in rat cerebellar Purkinje cells. An *in vitro* study. *J Physiol* **372**, 1–23.

Dittman JS & Regehr WG (1996). Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. *J Neurosci* **16**, 1623–1633.

Dittman JS & Regehr WG (1997). Mechanism and kinetics of heterosynaptic depression at a cerebellar synapse. *J Neurosci* **17**, 9048–9059.

Falk T, Meyerhof W, Corrette BJ, Schafer J, Bauer CK, Schwarz JR & Richiter D (1995). Cloning, functional expression and mRNA distribution of an inwardly rectifying potassium channel protein. *FEBS Lett* **367**, 127–131.

Fritschy J-M, Meskenaite V, Weinmann O, Honer M, Benke D & Mohler H (1999). $GABA_B$-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. *Eur J Neurosci* **11**, 761–768.

Hamill OP, Marty A, Neher E, Sakmann B & Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**, 85–100.

Hayashida Y & Ishida AT (2004). Dopamine receptor activation can reduce voltage-gated Na^+ current by modulating both entry into and recovery from inactivation. *J Neurophysiol* **92**, 3134–3141.

Hirono M, Yoshioka T & Konishi S (2001). $GABA_B$ receptor activation enhances mGluR-mediated responses at cerebellar excitatory synapses. *Nat Neurosci* **4**, 1207–1216.

Hommers LG, Lohse MJ & Bunemann M (2003). Regulation of the inward rectifying properties of G-protein-activated inwardly rectifying K^+ (GIRK) channels by Gbetagamma subunits. *J Biol Chem* **278**, 1037–1043.

Ige AO, Bolam JP, Billinton A, White JH, Marshall FH & Emson PC (2000). Cellular and sub-cellular localisation of $GABA_{B1}$ and $GABA_{B2}$ receptor proteins in the rat cerebellum. *Brain Res Mol Brain Res* **83**, 72–80.

Iizuka M, Kubo Y, Tsunenari I, Pan CX, Akiba I & Kono T (1995). Functional characterization and localization of a cardiac-type inwardly rectifying K^+ channel. *Receptors Channels* **3**, 299–315.

Iizuka M, Tsunenari I, Momota Y, Akiba I & Kono T (1997). Localization of a G-protein-coupled inwardly rectifying K^+ channel, CIR, in the rat brain. *Neuroscience* **77**, 1–13.

Isomoto S, Kondo C & Kurachi Y (1997). Inwardly rectifying potassium channels: their molecular heterogeneity and function. *Jpn J Physiol* **47**, 11–39.

Ito M (2002). The molecular organization of cerebellar long-term depression. *Nat Rev Neurosci* **3**, 896–902.

Jan LY & Jan YN (1997). Voltage-gated and inwardly rectifying potassium channels. *J Physiol* **505**, 267–282.

Jin W & Lu Z (1999). Synthesis of a stable form of tertiapin: a high-affinity inhibitor for inward-rectifier K^+ channels. *Biochemistry* **38**, 14286–14293.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA & Gerald C (1998). $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_BR1$ and $GABA_BR2$. *Nature* **396**, 674–679.

Kano M, Hashimoto K, Chen C, Albeliovich A, Aiba A, Kurihara H, Watanabe M, Inoue Y & Tonegawa S (1995). Impaired synapse elimination during cerebellar development in PKC gamma mutant mice. *Cell* **83**, 1223.

Kano M, Rexhausen U, Dreessen J & Konnerth A (1992). Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. *Nature* **356**, 601–604.

Karschin C, Dissmann E, Stuhmer W & Karschin A (1996). IRK (1–3) and GIRK (1–4) inwardly rectifying K^+ channel mRNAs are differentially expressed in the adult rat brain. *J Neurosci* **16**, 3559–3570.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A & Bettler B (1998). $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. *Nature* **396**, 683–687.

Kawaguchi S & Hirano T (2000). Suppression of inhibitory synaptic potentiation by presynaptic activity through postsynaptic $GABA_B$ receptors in a Purkinje neuron. *Neuron* **27**, 399–347.

Kawato M, Kuroda T, Imamizu H, Nakano E, Miyauchi S & Yoshioka T (2003). Internal forward models in the cerebellum: fMRI study on grip force and load force coupling. *Prog Brain Res* **142**, 171–188.

Koch C (1999). *Biophysics of Computation: Information Processing in Single Neurons*. Oxford University Press, New York.

Krapivinsky G, Medina I, Krapivinsky L, Yang Y & Clapham DE (1998). A novel inward rectifier K^+ channel with unique properties. *Neuron* **20**, 995–1005.

Kubo Y, Baldwin TJ, Jan YN & Jan LY (1993). Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature* **362**, 127–133.

Kulik A, Nakadate K, Nyiri G, Notomi T, Malitschek B, Bettler B & Shigemoto R (2002). Distinct localization of $GABA_B$ receptors relative to synaptic sites in the rat cerebellum and ventrobasal thalamus. *Eur J Neurosci* **15**, 291–307.

Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A & Kornau HC (1999). Role of heteromer formation in $GABA_B$ receptor function. *Science* **283**, 74–77.

Lauritzen I, De Weille J, Adelbrecht C, Lesage F, Murer G, Raisman-Vozari R & Lazdunski M (1997). Comparative expression of the inward rectifier K^+ channel GIRK2 in the cerebellum of normal and *weaver* mutant mice. *Brain Res* **753**, 8–17.

Li S-J, Wang Y, Strahlendorf HK & Strahlendorf JC (1993). Serotonin alters an inwardly rectifying current (I_h) in rat cerebellar Purkinje cells under voltage clamp. *Brain Res* **617**, 87–95.

Liao YJ, Jan YN & Jan LY (1996). Heteromultimerization of G-protein-gated inwardly rectifying K^+ channel proteins GIRK1 and GIRK2 and their altered expression in *weaver* brain. *J Neurosci* **16**, 7137–7150.

Liesi P, Stewart RR & Wright JM (2000). Involvement of GIRK2 in postnatal development of the weaver cerebellum. *J Neurosci Res* **60**, 164–173.

Llano I, Marty A, Armstrong CM & Konnerth A (1991). Synaptic- and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. *J Physiol* **434**, 183–213.

Llinas RR, Walton KD & Lang EJ (2003). Cerebellum. In *The Synaptic Organization of the Brain*, 5th edn, ed. Shepherd GM, pp. 271–309. Oxford University Press, New York.

Llinas R & Welsh JP (1993). On the cerebellum and motor learning. *Curr Opin Neurobiol* **3**, 958–965.

Mark MD & Herlitze S (2000). G-protein mediated gating of inward-rectifier K^+ channels. *Eur J Biochem* **267**, 5830–5836.

Marty A & Neher E (1995). Tight-seal whole-cell recording. In *Single-Channel Recording*, 2nd edn, ed. Sakmann B & Neher E, pp. 31–52. Plenum, New York.

Miyashita T & Kubo Y (1997). Localization and developmental changes of the expression of two inward rectifying K^+-channel proteins in the rat brain. *Brain Res* **750**, 251–263.

Murer G, Adelbrecht C, Lauritzen I, Lesage F, Lazdunski M, Agid Y & Raisman-Vozari R (1997). An immunocytochemical study on the distribution of two G-protein-gated inward rectifier potassium channels (GIRK2 and GIRK4) in the adult rat brain. *Neuroscience* **80**, 345–357.

Ohyama T, Nores WL, Murphy M & Mauk MD (2003). What the cerebellum computes. *Trends Neurosci* **26**, 222–227.

Palay SL & Chan-Palay V (1974). *Cerebellar Cortex: Cytology and Organization*. Springer-Verlag, Berlin.

Ponce A, Bueno E, Kentros C, Vega-Saenz de Miera E, Chow A, Hillman D, Chen S, Zhu L, Wu MB, Wu X, Rudy B & Thornhill WB (1996). G-protein-gated inward rectifier K^+ channel proteins (GIRK1) are present in the soma and dendrites as well as in nerve terminals of specific neurons in the brain. *J Neurosci* **16**, 1990–2001.

Robinson RB & Siegelbaum SA (2003). Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu Rev Physiol* **65**, 453–480.

Sadja R, Alagem N & Reuveny E (2003). Gating of GIRK channels: details of an intricate, membrane-delimited signaling complex. *Neuron* **39**, 9–12.

Seeger T & Alzheimer C (2001). Muscarinic activation of inwardly rectifying K^+ conductance reduces EPSPs in rat hippocampal CA1 pyramidal cells. *J Physiol* **535**, 383–396.

Slesinger PA, Stoffel M, Jan YN & Jan LY (1997). Defective gamma-aminobutyric acid type B receptor-activated inwardly rectifying K^+ currents in cerebellar granule cells isolated from *weaver* and *Girk2* null mutant mice. *Proc Natl Acad Sci U S A* **94**, 12210–12217.

Tabata T, Araishi K, Hashimoto K, Hashimotodani Y, Van der Putten H, Bettler B & Kano M (2004). Ca^{2+} activity at $GABA_B$ receptor constitutively promotes metabotropic glutamate signaling in the absence of GABA. *Proc Natl Acad Sci U S A* **101**, 16952–16957.

Tabata T & Ishida AT (1996). Transient and sustained depolarization of retinal ganglion cells by I_h. *J Neurophysiol* **75**, 1932–1943.

Tabata T, Sawada S, Araki K, Bono Y, Furuya S & Kano M (2000). A reliable method for culture of dissociated mouse cerebellar cells enriched for Purkinje neurons. *J Neurosci Methods* **104**, 45–53.

Thach WT, Goodkin HP & Keating JG (1992). The cerebellum and the adaptive coordination of movement. *Annu Rev Neurosci* **15**, 403–442.

Wischmeyer E, Doring F, Spauschus A, Thomzig A, Veh R & Karschin A (1997). Subunit interactions in the assembly of neuronal Kir3.0 inwardly rectifying K^+ channels. *Mol Cell Neurosci* **9**, 194–206.

Acknowledgements

We thank Dr K. Hashimoto for his advice, Ms A. Imai for cell preparation, and Professor K. Yamada, Department of Pharmacy and Health Science, Graduate School of Natural Science and Technology, Kanazawa University, for the opportunity to pursue this project. This work was supported by the Grants-in-Aid for Scientific Research, and the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Science, Sports, Culture and Technology of Japan, and a grant from the Cell Science Research Foundation.

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