Hypoxic Depolarization of Cerebellar Granule Neurons by Specific Inhibition of TASK-1
Leigh D. Plant, BSc; Paul J. Kemp, DPhil; Chris Peers, PhD; Zaineb Henderson, PhD; Hugh A. Pearson, PhD

**Background and Purpose**—The mechanisms underlying neuronal excitotoxicity during hypoxic/ischemic episodes are not fully understood. One feature of such insults is a rapid and transient depolarization of central neurons. TASK-1, an open rectifying K⁺ leak channel, is significant in setting the resting membrane potential of central neurons by mediating a standing outward K⁺ current. In this study we investigate the theory that the transient neuronal depolarization seen during hypoxia is due to the inhibition of TASK-1.

**Methods**—Activity of TASK-1 in primary cultures of rat cerebellar granule neurons was investigated by the whole-cell patch-clamp technique. Discriminating pharmacological and electrophysiological maneuvers were used to isolate the specific channel types underlying acute hypoxic depolarizations.

**Results**—Exposure of cells to acute hypoxia resulted in a reversible and highly reproducible mean membrane depolarization of 14.2±2.6 mV (n=5; P<0.01). Two recognized means of inhibiting TASK-1 (decreasing extracellular pH to 6.4 or exposure to the TASK-1–selective inhibitor anandamide) abolished both the hypoxic depolarization and the hypoxic depression of a standing outward current, identifying TASK-1 as the channel mediating this effect.

**Conclusions**—Our data provide compelling evidence that hypoxia depolarizes central neurons by specific inhibition of TASK-1. Since this hypoxic depolarization may be an early, contributory factor in the response of central neurons to hypoxic/ischemic episodes, TASK-1 may provide a potential therapeutic target in the treatment of stroke. (Stroke. 2002;33:2324-2328.)

**Key Words:** brain ■ ion channels ■ ischemia ■ potassium channels
Materials and Methods

Culturing of Rat Central Neurons

All experiments were performed with the use of primary cultures of rat cerebellar granule neurons. Cells were obtained by enzymatic and mechanical dissociation, as previously described. Briefly, tissue was removed from 6- to 8-day-old rat pups and triturated after a 15-minute trypsin digestion (EC 4.4.21.4, 2.5 mg · mL$^{-1}$ in PBS), which was halted by the addition of PBS containing soybean trypsin inhibitor (0.1 mg · mL$^{-1}$). Cells were pelleted by centrifugation (1 minute at 100g) and resuspended in minimum essential medium supplemented with 10% fetal calf serum, 2.5% chick embryo extract, 26 mmol/L glucose, 19 mmol/L KCl, 2 mmol/L L-glutamine, and penicillin/streptomycin (50 IU · mL$^{-1}$/50 μg · mL$^{-1}$). The cells were seeded at a density of $0.25 \times 10^6$ cells per well on circular 13-mm-diameter poly-l-lysine-coated coverslips. Multiwell plates were incubated in a humidified atmosphere containing 5% CO$_2$/95% air at 37°C. After 48 hours, the culture medium was exchanged for one consisting of minimum essential medium supplemented with 10% horse serum, 2.5% chick embryo extract, 26 mmol/L glucose, 19 mmol/L KCl, 2 mmol/L L-glutamine, penicillin/streptomycin (50 IU · mL$^{-1}$/50 μg · mL$^{-1}$), and 80 μmol/L fluorodeoxyuridine to prevent proliferation of nonneuronal cells. Culture medium was exchanged every 3 days, and all recordings were made from cells between days 5 to 12 in culture.

Electrophysiology

K$^+$ currents were recorded from cells at room temperature (measured in all experiments as 22°C) with the use of either the amphotericin B perforated patch-clamp technique for TASK or the conventional whole-cell patch-clamp technique for measurement of voltage-gated currents. Glass micropipettes (2 to 4 MΩ) were fabricated from borosilicate glass and filled with solution containing (in mmol/L): KCl 140, CaCl$_2$ 0.5, EGTA 5, HEPES 10, K$_2$ATP 2, MgCl$_2$ 1; pH was adjusted to 7.2 with KOH. Patch perforation was achieved by including amphotericin B (240 μg · mL$^{-1}$) in the pipette solution. The external solution with which cells were continually perfused comprised the following (in mmol/L): NaCl 120, KCl 2.5, MgCl$_2$ 2, CaCl$_2$ 0.5, glucose 10, HEPES 10, pH was adjusted to 7.4 or 6.4 with NaOH, as appropriate. Cells were made hypoxic by perfusion with an external solution that had been bubbled with nitrogen for at least 30 minutes before perfusion. Oxygen tension was measured at the cell with the use of a polarized carbon fiber electrode and was $\sim 30$ mm Hg in all experiments reported herein. This degree of hypoxia was reached within 1 minute of switching perfusion. For all electrophysiological measurements, series resistance and capacitance transients were electronically compensated. For measurement of voltage-gated currents, linear leak and residual transients were electronically compensated. For measurement of voltage-gated currents, a voltage protocol modified from Millar et al$^{23}$ was used. Cells were held at $-20$ mV, and the membrane potential was ramped to $-100$ mV over a period of 800 ms before reduction to $-20$ mV (Figure 2, inset). Ramp hyperpolarizations were repeated every 20 seconds. Voltage-gated K$^+$ currents were evoked as described by Ramsden et al$^{17}$ by depolarizing from a prepulse potential of $-140$ mV to test potentials ranging from $-70$ mV to +90 mV. Membrane potential was measured in current clamp ($I=0$ pA), with the use of the same solutions as those used in voltage-clamp experiments.

Currents were recorded and analyzed with the use of the Patch v6.0 program by Cambridge Electronic Design. Further analyses were performed with the use of Microsoft Excel 97 and Microcal Origin version 6.1. Student's $t$ tests (paired and unpaired, as appropriate) were used to determine the significance of differences between the means, with probability values of $<0.05$ considered significant.

Materials

Standard reagents were obtained from Sigma-Aldrich or BDH. All culture reagents were obtained from Gibco BRL. Anandamide was purchased from Tocris Cookson Ltd.

Results

Acute Hypoxia Depolarizes Cerebellar Granule Neurons

Exposure of cells to acute hypoxia resulted in a reversible and highly reproducible depolarization (mean value, 14.2±2.6 mV; n=5; P<0.01), as exemplified in Figure 1A (see also the Table). Such a depolarization is consistent with an inhibitory effect of hypoxia on channels that set the resting membrane potential.

Figure 1. Modulation of hypoxic depolarization and excitability. A, Sample recording of membrane potential under current-clamp conditions. For the period represented by the horizontal bar, perfusate PO$_2$ was reduced from 150 to $\sim 30$ mm Hg. B and C, Similar to A, except that hypoxia was applied in the continued presence of either pH 6.4 (B) or 1 μmol/L anandamide (C). Scale bar shown in A applies also to B and C. Each experiment was repeated at least 5 times with similar results.

Figure 2. Effect of acidosis or hypoxia on standing outward current. A and B, Sample tracings recorded during the voltage-clamp protocol shown in the inset before, during, and after application of perfusate at pH 6.4 (A) or hypoxic perfusate (B). C and D, Current amplitudes measured at $-20$ mV before, during, and after application of perfusate at pH 6.4 (C) or hypoxic perfusate (D). Mean values (n=4) are indicated by the horizontal bars.
Similarly, subjecting cells to a discriminating concentration (1 μmol/L) of the selective TASK-1 blocker anandamide also resulted in marked depolarization (16.7 ± 4 mV; n=5; P<0.001) and prevented further significant depolarization by acute hypoxia (Figure IC and Table). These data show clearly that an acid- and anandamide-sensitive current is inhibited by hypoxia and strongly suggest that TASK-1 is the channel underlying this conductance. To confirm this hypothesis and identify definitively the nature of this hypoxia-sensitive current, we studied hypoxic modulation of the standing outward current using voltage clamp.

**Standing Outward Current Is Hypoxia Sensitive**

In confirmation of previous suggestions that the standing outward current is the acid-sensitive TASK-1, figures 2 and 4 demonstrate that either reducing extracellular pH to 6.4 or application of anandamide causes significant current depression. Thus, at physiological pH of 7.4, cerebellar granule neurons, held at −20 mV, had mean outward current and current density of 218 ± 13 pA and 97.7 ± 0.1 pA/pF, respectively (n=34). The effect of extracellular acidification on this standing outward current is shown in the sample tracing of Figure 2A. Reducing pH from 7.4 to 6.4 resulted in a substantial and reversible decrease in the current (Figure 2A and 2C) but was without significant effect on the voltage-gated currents that were activated on return of the membrane potential to −20 mV after the hyperpolarizing ramp (Figure 2A). This current was also sensitive to inhibition by the muscarinic receptor agonist carbachol (100 μmol/L), which gave rise to a 55 ± 1% decrease in the standing outward current (n=4; P<0.001; data not shown). When cells were exposed to hypoxia, a similar inhibition of the standing outward current was seen (Figure 2B and 2D). This suggests that the TASK-1 currents are O2 sensitive in these neurons. To ensure that noninactivating voltage-gated K+ channel currents (eg, delayed rectifier) did not contribute to the O2-sensitive component of the standing outward current,
acid-sensitive current-voltage relationships appear similar (ie, inhibition of voltage-gated channels. Cells appears to be specific for TASK and does not involve presence of pH 6.4 (A) or 1/1000 mol/L acids and B, Typical time courses of current amplitudes measured at -20 mV, showing hypoxic inhibition and its modulation in the presence of pH 6.4 (A) or 1 μmol/L anandamide (B). Periods of application are shown by horizontal bars. C, Mean subtracted current-voltage relationships showing the hypoxic and acid-sensitive currents (n = 4). Also shown is the minimal residual O₂-sensitive current in the presence of pH 6.4. D, Typical time course of current amplitude measured at -20 mV, showing anandamide inhibition and its modulation in the presence of pH 6.4. A, B, and D are representative of 5 repeated experiments in each case.

Voltage-gated currents were activated by repetitive depolarizations to +50 mV from a prepulse potential of -140 mV with the use of conventional ruptured patch (as opposed to perforated patch) whole cell. Under these conditions, TASK currents are minimal because of "run-down" of the channel. No inhibition of voltage-gated currents was observed on application of hypoxia (Figure 3A and 3C). Voltage-current relationships were constructed before and during perfusion with hypoxic solutions (Figure 3B). No effect of hypoxia was seen on voltage-gated currents at any of the potentials tested. Thus, the inhibitory effect of hypoxia on K⁺ currents are minimal because of "run-down" of the channel. 19

To substantiate the claim that the O₂- and acid-sensitive currents were one and the same, we investigated the effect of hypoxia in the presence of extracellular acidosis. Plotted in Figure 4A is a sample time course of the amplitude of the standing outward current during these maneuvers. Thus, exposing cells to hypoxia resulted in a rapid and reversible decrease in the current amplitude by 47±3% (n = 6; P<0.001). Subtraction of the ramp current recorded in hypoxia from the ramp current recorded in normoxia resulted in the difference (ie, O₂-sensitive) current exemplified in Figure 4C. When the perfusing solution was switched from a pH of 7.4 to 6.4, a more substantial decrease in the standing current at -20 mV was observed (75±2%; Figure 4A); the subtracted acid-sensitive current is shown in Figure 4C. It is noteworthy that, apart from absolute magnitude, the O₂- and acid-sensitive current-voltage relationships appear similar (ie, follow Goldman-Hodgkin-Katz rectification), which suggests that these currents are flowing through open rectifying K⁺ channels. Importantly, exposure of cells to hypoxia at pH 6.4 produced no further significant inhibition (Figure 4A); this is seen clearly in the third tracing of Figure 4C, which plots the negligible O₂-sensitive current at pH of 6.4.

Further evidence for the involvement of TASK-1 in the hypoxic inhibition of the standing outward current is provided by the data shown in Figure 4B. Anandamide (1 μmol/L) inhibited the standing outward current by 46±3% (n = 4). This effect was maximal because a higher concentration of anandamide (3 μmol/L) produced no further blocking effect (n = 4; data not shown). In the presence of this discriminating concentration of anandamide (1 μmol/L), hypoxia was no longer effective (Figure 4B). The ability of anandamide to inhibit the current was absent in hypoxia. Under this condition, application of anandamide resulted in a further inhibition of only 2±1% of the original current (n = 4). Consistent with these findings for anandamide and for low pH and hypoxia were changes in cell input resistance (Table). These were calculated by approximating currents to straight lines for 10 mV positive to and 10 mV negative to the cell resting potential (Table). Thus, significant increases in input resistance were found for hypoxia (P<0.05), pH 6.4 (P<0.001), and anandamide (P<0.01). Furthermore, at pH 6.4 or in the presence of anandamide, no further significant increase in cell input resistance to hypoxia was observed (Table).

Even though the degree of current inhibition by low pH was much greater than the inhibition seen with anandamide, both completely occluded the effect of hypoxia. This suggests that the standing outward current in these cells is composed of more than one channel type, with only one (the anandamide-sensitive TASK-1) being inhibited by hypoxia. To address this possibility we applied anandamide and low pH to the same cell. An example of the time course for this effect is shown in Figure 4D. Extracellular acidification blocked an additional component of the standing outward current when applied in the presence of anandamide (inhibition by anandamide, 42±1%; inhibition by anandamide and pH 6.4, 77±4%; n = 4). Furthermore, when anandamide was removed from the acidified extracellular medium, no recovery could be observed (inhibition by pH 6.4 alone, 78±3%; eg, Figure 4D), clearly indicating that low pH blocked the anandamide-sensitive component of current. Thus, there appear to be 2 components of the acid-sensitive standing outward current in these cells: one that is inhibited by anandamide and one that is anandamide insensitive. A recent study by Talley and coworkers20 indicates that both TASK-1 and TASK-3 are highly expressed in the granule cell layer of the cerebellum, suggesting that the anandamide-insensitive current that we observe is carried by TASK-3. If such a suggestion is true, this would indicate that TASK-3 is also hypoxia insensitive in these cells.

**Discussion**

The standing outward current observed in cerebellar granule neurons held at a potential of -20 mV is thought to be a consequence of TASK expression. The current is sensitive to muscarinic inhibition and modulation by pH,10 and previous
studies have claimed that TASK-1 is the specific channel involved. However, these previous claims were based on positive immunoreactivity with the use of a commercially available antibody raised against TASK-1 and sensitivity to pH, a characteristic common to all expressed TASK-like channels. In this study we confirm that TASK-1 underlies a proportion of this current by showing that it can be inhibited by the endocannabinoid anandamide, a selective inhibitor of TASK-1 when used at 1 μmol/L. However, we also found evidence for a pH-sensitive, anandamide-insensitive current component and, on the basis of expression studies in rat brain, suggest that this is carried by TASK-3. In addition to these findings, we show that hypoxia can selectively inhibit native TASK-1. Such an inhibition of twin pore domain K⁺ channels has previously been shown for a TASK-like current in carotid body glomus cells and TASK-3 in a human neuroepithelial cell line. The findings that both anandamide and pH 6.4 occluded the inhibition by hypoxia (a characteristic of recombinant TASK-1) provide compelling evidence that inhibition of TASK-1 accounts for the reduction of the standing outward current and cell depolarization of cerebellar granule neurons. This depolarization, which is accompanied by an increase of cell input resistance (Table), is consistent with the idea that acute hypoxia, by inhibiting a hyperpolarizing conductance active at resting membrane potential, would increase neuronal excitability. However, it should also be noted that more prolonged episodes of hypoxia are likely to lead to an accumulation of K⁺ extracellularly, an effect that would in itself depolarize neurons. In addition to increasing excitatory output from these neurons, this depolarization would also facilitate the glutamatergic excitatory input into these cells by relieving Mg²⁺-dependent blockage of N-methyl-D-aspartate receptors. However, and in common with other groups, we found it difficult to activate repetitive, all-or-none action potentials in these cultured neurons. Such poor excitability has been shown to be a consequence of the damping effect of the relatively large voltage-gated K⁺ channel current that these cells exhibit. This does not diminish the importance of our suggestion that the increased excitability observed in hypoxia/ischemia is likely due to inhibition of TASK-1.

There are potential pathological consequences arising from inhibition of TASK-1 in cerebellar granule neurons. The depolarization caused may lead to increased firing patterns, which in turn could cause excitotoxicity via excess glutamate release (this is especially so since the cell input resistance increases), a major determinant of ischemic cell death in central neurons. Indeed, transient depolarizations during hypoxia have been demonstrated in more intact central neuronal preparations. However, hyperpolarizations have also been reported, arising because of activation of ATP-dependent and Ca²⁺-sensitive K⁺ channels, although it should be noted that these studies examined the effects of anoxia, not hypoxia. Clearly, neuronal responses to acute hypoxic/ischemic episodes are complex. However, since TASK-1 appears to be a major determinant of cell input resistance and membrane potential, its specific inhibition by acute hypoxia is likely to be a major contributory factor in the overall response of neurons during infarction; as such, it represents a potentially important therapeutic target for treatment of conditions characterized by ischemia/hypoxia, such as stroke.

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References

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