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Review

Protective role of neuronal K_{ATP} channels in brain hypoxia

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Summary

During severe arterial hypoxia leading to brain anoxia, most mammalian neurons undergo a massive depolarisation terminating in cell death. However, some neurons of the adult brain and most immature nervous structures tolerate extended periods of hypoxia–anoxia. An understanding of the mechanisms underlying this tolerance to oxygen depletion is pivotal for developing strategies to protect the brain from consequences of hypoxic-ischemic insults. ATP-sensitive K^+ (K_{ATP}) channels are good subjects for this study as they are activated by processes associated with energy deprivation and can counteract the terminal anoxic-ischemic neuronal depolarisation. This review summarises *in vitro* analyses on the role of K_{ATP} channels in hypoxia–anoxia in three distinct neuronal systems of rodents. In dorsal vagal neurons, blockade of K_{ATP} channels with sulfonylureas abolishes the hypoxic-anoxic hyperpolarisation. However, this does not affect the extreme tolerance of these neurons to oxygen depletion as evidenced by a moderate and sustained increase of intracellular Ca^{2+} (Ca_i). By contrast, a sulfonylurea-induced block of K_{ATP} channels shortens the delay of occurrence of a major Ca_i rise in cerebellar Purkinje neurons. In neurons of the neonatal medullary respiratory network, K_{ATP} channel blockers reverse the anoxic hyperpolarisation associated with slowing of respiratory frequency. This may constitute an adaptive mechanism for energy preservation. These studies demonstrate that K_{ATP} channels are an ubiquituous feature of mammalian neurons and may, indeed, play a protective role in brain hypoxia.

Key words: anoxia, ATP-sensitive K^+ channels, brainstem, calcium, fura-2, mitochondria.

Introduction

Most mammalian neurons have a low tolerance to severe arterial hypoxia resulting in brain anoxia (Hansen, 1985; Haddad and Jiang, 1993). In many brain regions, this is due to the fact that anoxia-evoked extracellular accumulation of glutamate is excitotoxic (Choi, 1994; Lipton, 1999). The anoxia-induced vulnerability appears to be related to the profound rise of the free intracellular concentration of Ca^{2+} (Ca_i) associated with the depolarisation caused by increased interstitial levels of glutamate. Such neuronal Ca_i elevation results primarily from influx *via* Ca^{2+}-permeable glutamate receptors and voltage-activated Ca^{2+} channels during the anoxic depolarisation (Haddad and Jiang, 1993; Kristian and Siesjö, 1996; Bickler and Buck, 1998). Also, impairment of Ca^{2+} extrusion and sequestration due to inhibition of mitochondria and ATP-dependent ion pumps such as the Na^+/K^+-ATPase contribute to the excessive anoxic rise of Ca_i (Hansen, 1985; Kristian and Siesjö, 1996). The Ca^{2+} overload in combination with cellular changes such as ATP consumption by depolarised mitochondria activate a cascade of events leading to cell death (Lipton, 1999; Nicholls and Budd, 2000; Müller and Ballanyi, 2003).

In a variety of central mammalian neurons, the terminal anoxic depolarisation is preceded by a K^+ channel-mediated hyperpolarisation (Misgeld and Frotscher, 1982; Hansen, 1985; Haddad and Jiang, 1993). This hyperpolarisation reduces neuronal activity, and thus transmembrane ion fluxes, and consequently attenuates the activity of ion pumps that consume about 50% of the energy supplied to the brain (Hansen, 1985; Hochachka, 1986). Accordingly, if the anoxic hyperpolarisation persists for a reasonable length of time, it may well have a protective effect. The cellular mechanisms leading to activation of the anoxic K^+ conductance are still under discussion. Several reports on hippocampal neurons indicated that the anoxic Ca_i rise promotes activation of Ca^{2+}-dependent K^+ channels (Leblond and Krnjevic, 1989; Nowicky and Duchen, 1998; for further references, see Kulik et al., 2002). But, an increasing number of studies provides evidence that ATP-sensitive K^+ (K_{ATP}) channels mediate the anoxic hyperpolarisation (Mourre et al., 1989; Luhmann and Heinemann, 1992; for further references, see Kulik et al., 2002). The pharmacological and biophysical properties as well

as the structure of K_{ATP} channels have been investigated thoroughly in muscle tissues and pancreatic β-cells (Ashcroft and Gribble, 1998; Aguilar-Bryan and Bryan, 1999). By contrast, the structure–function relationship of the neuronal isoforms of these metabolism-regulated K^+ channels has only recently been explored using molecular techniques such as *in situ* hybridisation or polymerase chain reaction (PCR) combined with patch-clamp recording (Karschin et al., 1998; Liss et al., 1999; Zawar and Neumcke, 2000; Haller et al., 2001). Although agents such as tolbutamide or glibenclamide block the anoxic activation of neuronal K_{ATP} channels, it has only been shown in a few cases that blockade of K_{ATP} channels by such sulfonylureas increases the vulnerability of neuronal structures to anoxia (Pek-Scott and Lutz, 1998; Garcia de Arriba et al., 1999).

In this review, the latter aspect is addressed for three neuronal systems (Fig. 1). Patch-clamp recording was combined with fluorometric measurements of Ca_i to determine whether K_{ATP} channels are involved in the response to oxygen depletion of dorsal vagal neurons and Purkinje cells in brain slices from mature rodents. The potential for K_{ATP} channels to contribute to the anoxic slowing of respiratory frequency in neonatal rats was also investigated. For this purpose, the response to anoxia of the respiratory network in isolated brainstems of newborn rats was studied using nerve recordings of respiratory activity combined with 'blind' patch-clamp recordings.

K_{ATP} channels in anoxia-tolerant dorsal vagal neurons

Subpopulations of mammalian neurons (Ballanyi et al., 1996a; Richter and Ballanyi, 1996) are highly tolerant to anoxia in a fashion similar to brain structures of some cold-blooded vertebrates (Lutz and Nilsson, 1994; Hochachka and Lutz, 2001). By elucidating the mechanisms of the anoxia tolerance of these mammalian neurons, it may be possible to develop pharmacological strategies to protect the brain from the consequences of hypoxic-anoxic insults. Tonically active dorsal vagal neurons in brainstem slices from juvenile rats (Fig. 1) represent a population of mammalian neurons in which both the structure and properties of K_{ATP} channels have been studied quite extensively. Interestingly, these cells are particularly resistant to oxygen depletion, at least in rats. They respond to hypoxic solutions resulting in anoxia of the dorsal vagal nucleus (Ballanyi et al., 1996a) with a sustained K^+ channel-mediated hyperpolarisation (Fig. 2A; Cowan and Martin, 1992; Trapp and Ballanyi, 1995). Neither hypoxic anoxia nor chemical block of aerobic metabolism with

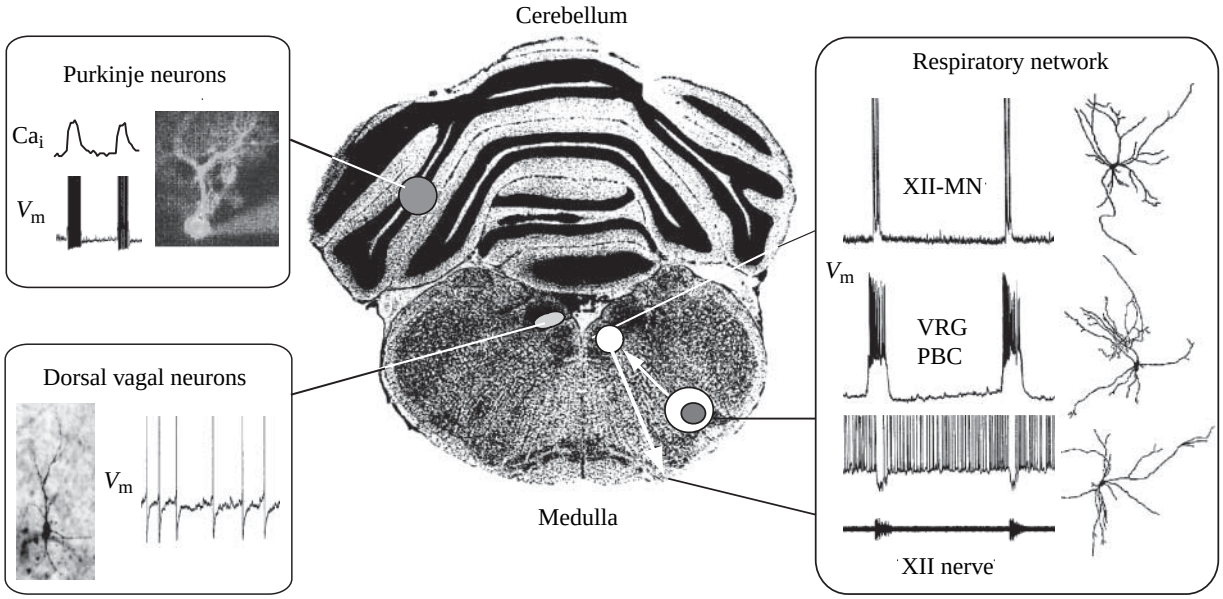

Fig. 1. Model systems for analysis of the involvement of neuronal ATP-sensitive K^+ (K_{ATP}) channels in brain hypoxia–anoxia. Coronal brain slices from rodents are used to study the electrophysiological response to oxygen depletion in three types of central neurons. Anoxia-vulnerable cerebellar Purkinje neurons from 16–20-day-old mice with a characteristic flat dendritic tree show pronounced rises of intracellular Ca^{2+} (Ca_i) during rhythmic or tonic activity of membrane potential (V_m). Ca_i is monitored in superficial cells filled *via* the recording patch-electrode with 50–200 μmol l^{-1} of the Ca^{2+}-sensitive dye fura-2. The same techniques are applied to tonically active dorsal vagal neurons in medullary slices from juvenile rats or mice. These neurons innervate organs of the gastrointestinal tract, such as pancreatic β-cells, in which K_{ATP} channel properties and functions are being thoroughly explored. Whole-cell patch-clamp recording is done in neurons of the ventral respiratory group (VRG) including the rhythmogenic pre-Bötzinger complex (PBC) or inspiratory active hypoglossal motoneurons (XII-MN) in coronal medullary slices or brainstem–spinal cord preparations from neonatal rodents. The cells can be labelled with dyes such as lucifer-yellow or biocytin for subsequent (immuno)histochemical analysis of their structure and neurotransmitter receptors. Rhythmic inspiratory activity is recorded with glass suction electrodes from hypoglossal (XII) nerve rootlets in the slices or from cervical nerve rootlets in an *en bloc* preparation. Reconstructed respiratory neuron data from K. Ballanyi and S. Schwarzacher. Brain section taken from Paxinos (1982).

cyanide evokes a secondary, terminal depolarisation, at least not within time periods of up to 30 min following the insult (Figs 2A, 3A; Trapp and Ballanyi, 1995; Ballanyi and Kulik, 1998). By contrast, a progressive depolarisation due to extracellular accumulation of glutamate is revealed in slices of dorsal vagal neurons preincubated in glucose-free superfusate and subsequently exposed to hypoxic or chemical anoxia in the absence of glucose, mimicking ischemia *in vitro* (Ballanyi et al., 1996a; Kulik et al., 2000). The anoxic hyperpolarisation and the underlying outward current and increase in membrane conductance of the dorsal vagal neurons are blocked by the sulfonylurea K_{ATP} channel blockers tolbutamide, glibenclamide and gliquidone (Figs 2A, 3A). Furthermore, the sulfonamide K_{ATP} channel opener diazoxide causes a hyperpolarisation and conductance increase

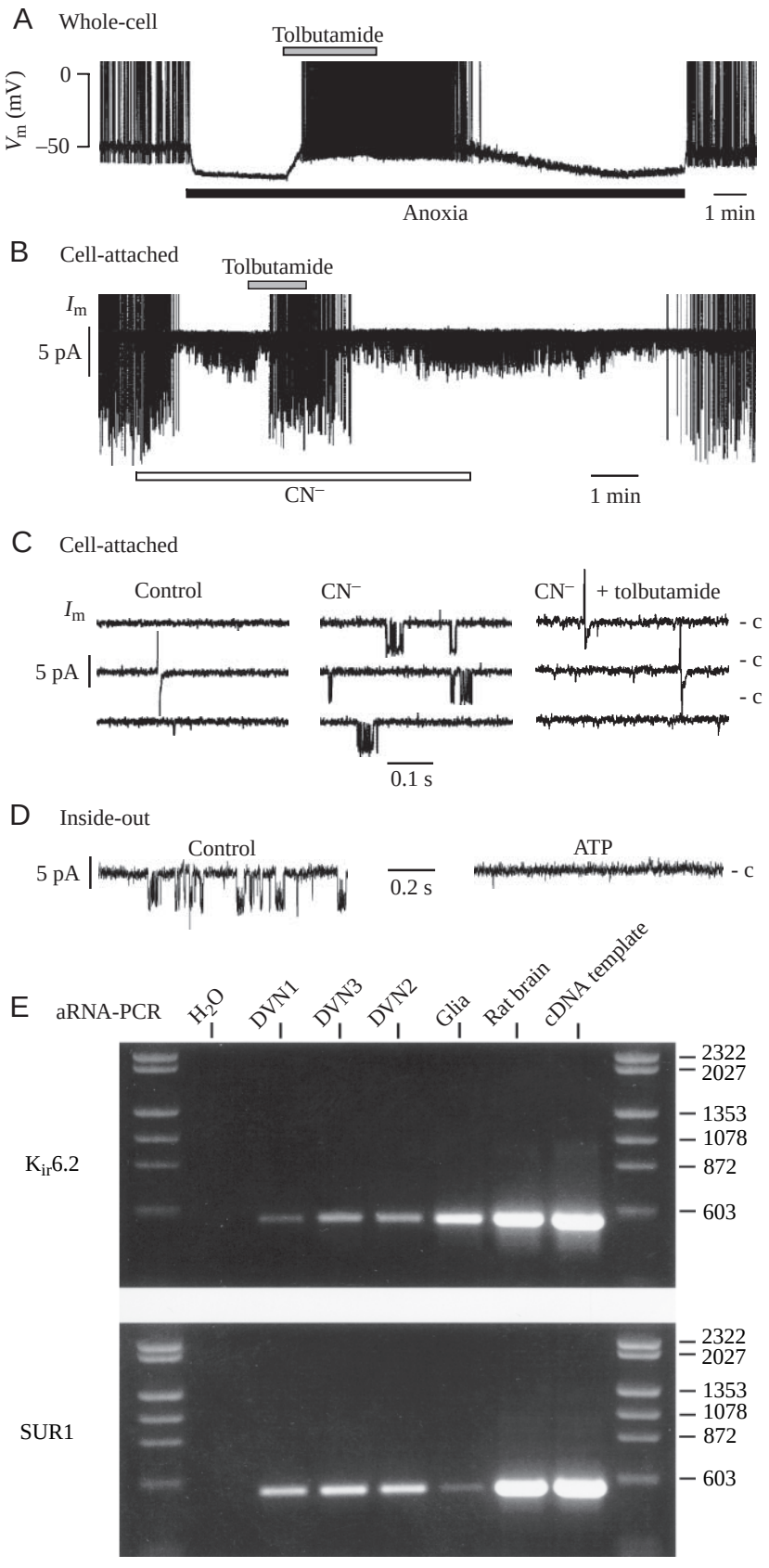

Fig. 2. K_{ATP} channels in dorsal vagal neurons of juvenile rodents. (A) Superfusion of nitrogen-gassed hypoxic saline causes tissue anoxia in the dorsal vagal nucleus of medullary slices kept at 30°C. In dorsal vagal neurons of rats, such anoxia results in a sustained hyperpolarisation and concomitant suppression of tonic action potential discharge that are reversed by the sulfonylurea K_{ATP} channel blocker tolbutamide (200 μmol l^{-1}). Whole-cell recordings were done using patch-electrodes containing (in mmol l^{-1}) 140 K-gluconate, 1 $MgCl_2$, 0.5 $CaCl_2$, 1 NaCl, 10 Hepes, pH 7.4. The electrodes also contained Na_2ATP at different concentrations, in most cases 1 mmol l^{-1}. However, varying the ATP concentration between 0 and 20 mmol l^{-1} did not affect the membrane response to anoxia (Müller et al., 2002). (B) The anoxic hyperpolarisation is due to opening of single K_{ATP} channels, as revealed in this example for chemical anoxia due to bath application of 1 mmol l^{-1} cyanide (CN^-). The sharp deflections on the cell-attached current (I_m) trace during control, CN^- plus tolbutamide (200 μmol l^{-1}) and wash are caused by tonic spiking. Holding potential: 0 mV. (C) Current traces of the recording in B at higher time resolution. (D) In an inside-out patch from a mouse dorsal vagal neuron, K_{ATP} channel activity is abolished by addition of 20 μmol l^{-1} ATP to the superfusate mimicking the intracellular solution. Holding potential: –50 mV. (E) Antisense RNA-polymerase chain reaction (aRNA-PCR) analysis of cytoplasm obtained during whole-cell recording reveals that three dorsal vagal neurons (DVN1–3) of rats coexpress mRNA for the inward-rectifying K^+ (K_{ir}) channel isoform, $K_{ir}6.2$, and the sulfonylurea receptor (SUR) isoform, SUR1. Obviously, dorsal vagal neurons express the same type of K_{ATP} channels as pancreatic β-cells innervated by a subpopulation of these neurons. A, reproduced from Ballanyi and Kulik (1998); B, C and E, reproduced from Karschin et al. (1998); D, data from K. Ballanyi and J. Brockhaus.

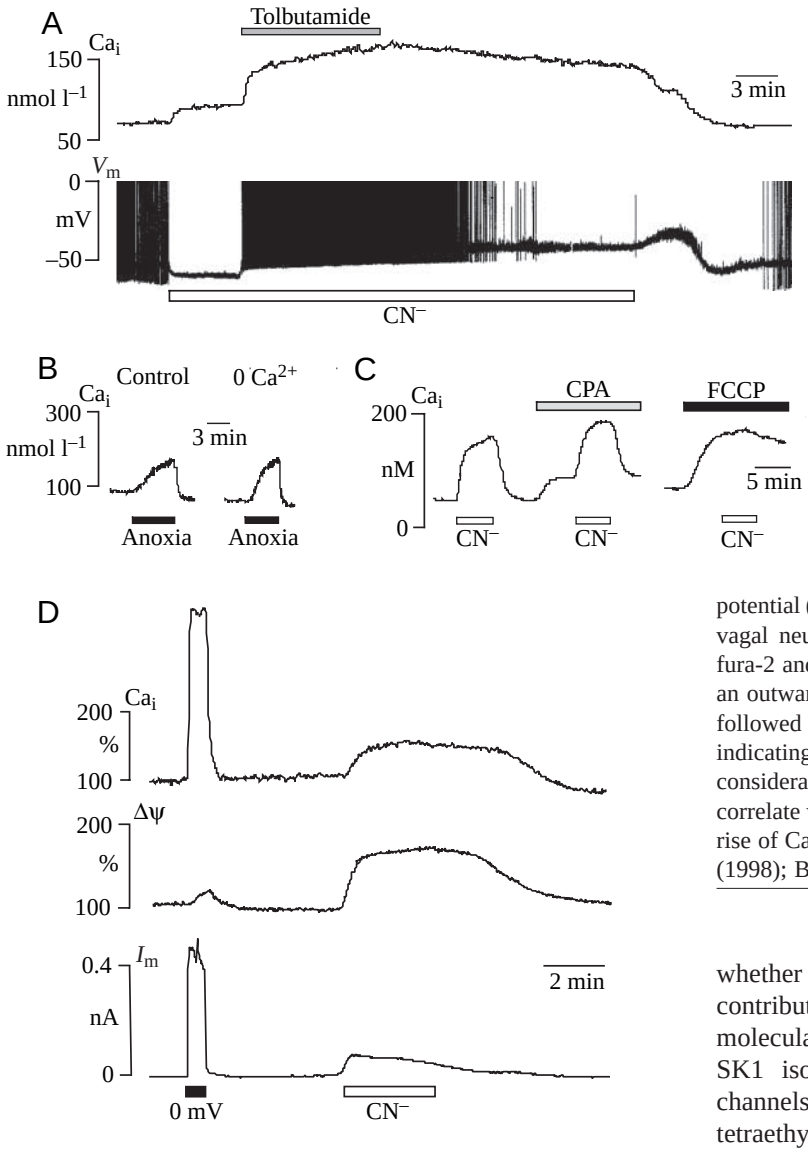

Fig. 3. Relationship between intracellular Ca^{2+} concentration (Ca_i) and anoxic K_{ATP} channel activation in dorsal vagal neurons from juvenile rats. (A) Chemical anoxia due to 1 mmol l^{-1} CN^- produces a persistent hyperpolarisation and block of tonic spiking while Ca_i increases by <50 nmol l^{-1}. Tolbutamide (200 μmol l^{-1}) reverses the anoxic hyperpolarisation. The concomitant reappearance of spiking induces a further stable increase of Ca_i that is, nevertheless, not much larger than Ca_i levels during physiological activity of these cells. (B) The persistence of the anoxic Ca_i rise in Ca^{2+}-free superfusate suggests that this Ca^{2+} signal is due to release from intracellular stores. (C) The Ca_i rise associated with chemical anoxia is not substantially attenuated following depletion of endoplasmic reticulum Ca^{2+} stores with the Ca^{2+} pump blocker cyclopiazonic acid (CPA; 30 μmol l^{-1}), while the anoxia response is mimicked and occluded by the mitochondrial blocker FCCP (1 μmol l^{-1}). (D) Simultaneous recording of Ca_i, mitochondrial potential ($\Delta\psi$) and membrane current (I_m) in a voltage-clamped dorsal vagal neuron filled *via* the patch-electrode with both 100 μmol l^{-1} fura-2 and 5 mg ml^{-1} rhodamine-123. A rapid increase of Ca_i during an outward current due to depolarisation from –50 to 0 mV (20 s) is followed by a modest increase in rhodamine-123 fluorescence, indicating a depolarisation of $\Delta\psi$. In response to CN^- (1 mmol l^{-1}), a considerably larger mitochondrial depolarisation whose onset kinetics correlate with that of the K_{ATP} outward current is observed, while the rise of Ca_i is notably slower. A, reproduced from Ballanyi and Kulik (1998); B–D, data from K. Ballanyi and A. Kulik.

very similar to the effects of anoxia (Trapp et al., 1994; Trapp and Ballanyi, 1995; Ballanyi and Kulik, 1998).

The view that K_{ATP} channels are responsible for the anoxic hyperpolarisation is supported by results from single-channel studies. The single-channel conductance is 70 pS in both cell-attached (Fig. 2B,C; Karschin et al., 1998) and excised inside-out patches, the latter obtained from dorsal vagal neurons of mice (Fig. 2D; Müller et al., 2002). This value, and also the half-maximal inhibitory concentration (IC_{50}) for blocking channel activity by intracellular ATP, 5 μmol l^{-1} (Müller et al., 2002), resembles closely that described for K_{ATP} channels in pancreatic β-cells (Ashcroft and Gribble, 1998; Aguilar-Bryan and Bryan, 1999). Consequently, single-cell aRNA-PCR analysis revealed that dorsal vagal neurons coexpress mRNA for the β-cell type sulfonylurea receptor (SUR) isoform, SUR1, and for the inwardly rectifying K^+ (K_{ir}) channel subunit, $K_{ir}6.2$ (Fig. 2E; Karschin et al., 1998). These findings established that K_{ATP} channels mediate the persistent anoxic hyperpolarisation of dorsal vagal neurons. Despite this, it is necessary to test whether activation of Ca^{2+}-dependent K^+ channels may contribute, at least in part, to the anoxic hyperpolarisation. A molecular analysis demonstrated that the apamin-sensitive SK1 isoform of 'small conductance' Ca^{2+}-dependent K^+ channels and the 'big conductance', iberiotoxin- and tetraethylammonium-sensitive BKα-subunit are expressed in dorsal vagal neurons (Pedarzani et al., 2000). However, the SK1-mediated current is blocked by anoxia while the BK current is not changed (Kulik et al., 2002). For some neuronal systems, it appears that accumulation of interstitial adenosine due to anoxic degradation of ATP acts *via* A_1 receptors on K^+ channels, possibly including K_{ATP} channels, to exert a protective role by suppressing electrical activity (Pek-Scott and Lutz, 1998; Mironov et al., 1999; Mironov and Richter, 2000). However, in dorsal vagal neurons, adenosine does not mimic the hyperpolarising effect of anoxia while the anoxic hyperpolarisation is not blocked by the A_1 receptor antagonist DPCPX (Ballanyi and Kulik, 1998).

The extent to which anoxia elevates Ca_i was studied in dorsal vagal neurons filled *via* the patch-electrode with the Ca^{2+}-sensitive dye fura-2. ⩾20-min periods of (chemical) anoxia only evoke a very moderate (<100 nmol l^{-1}) and stable rise of Ca_i (Fig. 3A; Ballanyi and Kulik, 1998; Kulik et al., 2000). As the anoxic rise of Ca_i is not affected by removal of extracellular Ca^{2+}, it must be due to release from intracellular stores (Fig. 3B). The Ca_i rise related to (chemical) anoxia is

also not affected by depleting endoplasmic reticulum Ca^{2+} stores with cyclopiazonic acid (Fig. 3C). On the contrary, the Ca_i response to anoxia or cyanide is both mimicked and occluded by the protonophore FCCP (Fig. 3C), which dissipates the mitochondrial transmembrane H^+ gradient (Schuchmann et al., 2000). These results suggest that the moderate anoxic rise of Ca_i is caused by mitochondrial Ca^{2+} release (Kulik and Ballanyi, 1998).

This view is supported by observations in dorsal vagal neurons filled *via* the patch-electrode with fura-2 and the mitochondrial potential-sensitive dye rhodamine-123 together (Fig. 3D). These results showed that the kinetics of the cyanide-induced K_{ATP} current closely correlate with those of a rapid and pronounced increase in rhodamine-123 fluorescence, indicating a major depolarisation of mitochondrial potential, while the accompanying Ca_i rise has considerably slower kinetics (Fig. 3D). By contrast, a major rise of Ca_i in response to membrane depolarisation in voltage-clamp is only reflected by a minor mitochondrial depolarisation (Fig. 3D; Kulik and Ballanyi, 1998).

The very similar time courses of the anoxic outward current and the mitochondrial depolarisation suggest that metabolic activation of K_{ATP} channels is due to a rapid cellular process associated with loss of the mitochondrial membrane potential. The nature of this process is currently under investigation. A change in the redox state of the K_{ATP} channels is unlikely to be involved as neither reduced/oxidised glutathione nor the oxidase blocker diphenyliodonium has an effect on the cyanide-induced outward current (Müller et al., 2002). Also, an anoxia-related change in the actin cytoskeleton or the composition of the plasma membrane does not seem to have a major contribution as cytochalasin-D does not affect the cyanide-induced hyperpolarisation and phosphatidyl-inositol 4,5-bisphosphate fails to decrease the ATP sensitivity of single K_{ATP} channels (Müller et al., 2002). The same study also suggests no major role for ATP in anoxic activation of dorsal vagal neuronal K_{ATP} channels, as dialysing the cells *via* the patch-electrode with either 0, 1 or 20 mmol l^{-1} ATP does not affect the amplitude or delay of onset of the cyanide-induced outward current. Furthermore, the current peaks within 1 min, independent of whether or not the cells are exposed to ouabain and vanadate that block the ion pumps that constitute the major source of ATP consumption (Müller et al., 2002). Finally, the fall of intracellular pH of up to 0.5 units that accompanies the modest rise of Ca_i does not appear to contribute to anoxic activation of K_{ATP} channels. In most cells, the intracellular acidosis starts to develop after the outward current reaches its maximum amplitude (Trapp et al., 1996; Raupach and Ballanyi, 2004).

The K_{ATP} channels of dorsal vagal neurons do not seem to play a protective role during anoxia. Upon sustained exposure to hypoxic or chemical anoxia, the rise of Ca_i by ~100 nmol l^{-1} is less than doubled upon sulfonylurea-induced block of the K_{ATP} channel-mediated hyperpolarisation or outward current (Fig. 3A; Ballanyi and Kulik, 1998). The additional moderate Ca_i increase is related to reappearance of tonic spiking that is typical for these cells and promotes a notable influx of Ca^{2+} (Ballanyi and Kulik, 1998). A progressive depolarisation or rise of Ca_i is not observed as in anoxia-vulnerable cells (see below) (Hansen, 1985; Haddad and Jiang, 1993; Kristian and Siesjö, 1996; Lipton, 1999). This is certainly not due to the fact that the cells were whole-cell recorded with patch-electrodes containing 1–2 mmol l^{-1} ATP. A similar lack of occurrence of a terminal depolarisation or massive Ca_i increase is observed when the dorsal vagal neurons are recorded with sharp microelectrodes (Cowan and Martin, 1992; Ballanyi et al., 1996a) or optically in a non-invasive manner using a membrane-permeable form of fura-2 (Ballanyi and Kulik, 1998). According to these results, it is proposed that the tolerance to anoxia of these mammalian neurons is rather due to a low resting metabolic rate in conjunction with effective utilisation of anaerobic metabolism (Ballanyi et al., 1996a; Trapp et al., 1996; Ballanyi and Kulik, 1998).

Which alternative function might these K_{ATP} channels have? Since the dorsal vagal neurons innervate several organs of the gastrointestinal tract, their metabolism-gated K^+ channels may be involved in nutritive functions. Accordingly, stimulation of the dorsal vagal nucleus *in vivo* results in a substantial release of insulin as a subpopulation of dorsal vagal neurons innervates pancreatic β-cells (Laughton and Powley, 1987). In line with a putative role in glucose homeostasis, the K_{ATP} channels of the dorsal vagal neurons are not only activated by anoxia but also by a fall of interstitial glucose levels (Ballanyi et al., 1996a). Thus, these neuronal K_{ATP} channels may represent a central nervous glucose sensor such as in hypothalamic neurons (Miki et al., 2001). However, the sensor mechanism may be different as the K_{ATP} channels of hypothalamic neurons respond within much shorter time periods to a fall of interstitial glucose levels (Miki et al., 2001).

K_{ATP} channel-mediated delay of anoxic Ca_i rise in Purkinje neurons

Cerebellar Purkinje neurons are exceptionally vulnerable to hypoxic and ischemic damage (Pulsinelli et al., 1982; Horn and Schlote, 1992; Balchen and Diemer, 1992; Brasko et al., 1995; Barenberg et al., 2001). Similar to dorsal vagal neurons, these cells are tonically or rhythmically active when kept in brain slices (Fig. 1) and respond to hypoxic or chemical anoxia as well as to diazoxide with a tolbutamide- and gliquidone-sensitive pronounced hyperpolarisation and increase in membrane conductance (Fig. 4). This suggests that K_{ATP} channels mediate the anoxic hyperpolarisation of Purkinje neurons although their molecular identity has not been explored yet. In contrast to the dorsal vagal neurons, anoxia induces a major perturbation of Ca_i in Purkinje cells dialysed *via* the patch-electrode with fura-2. The Ca_i baseline increases by <50 nmol l^{-1} during the initial phase of the cyanide-induced hyperpolarisation and remains at that level for several minutes while membrane potential starts to recover to resting level with no change in membrane conductance. After that period, a

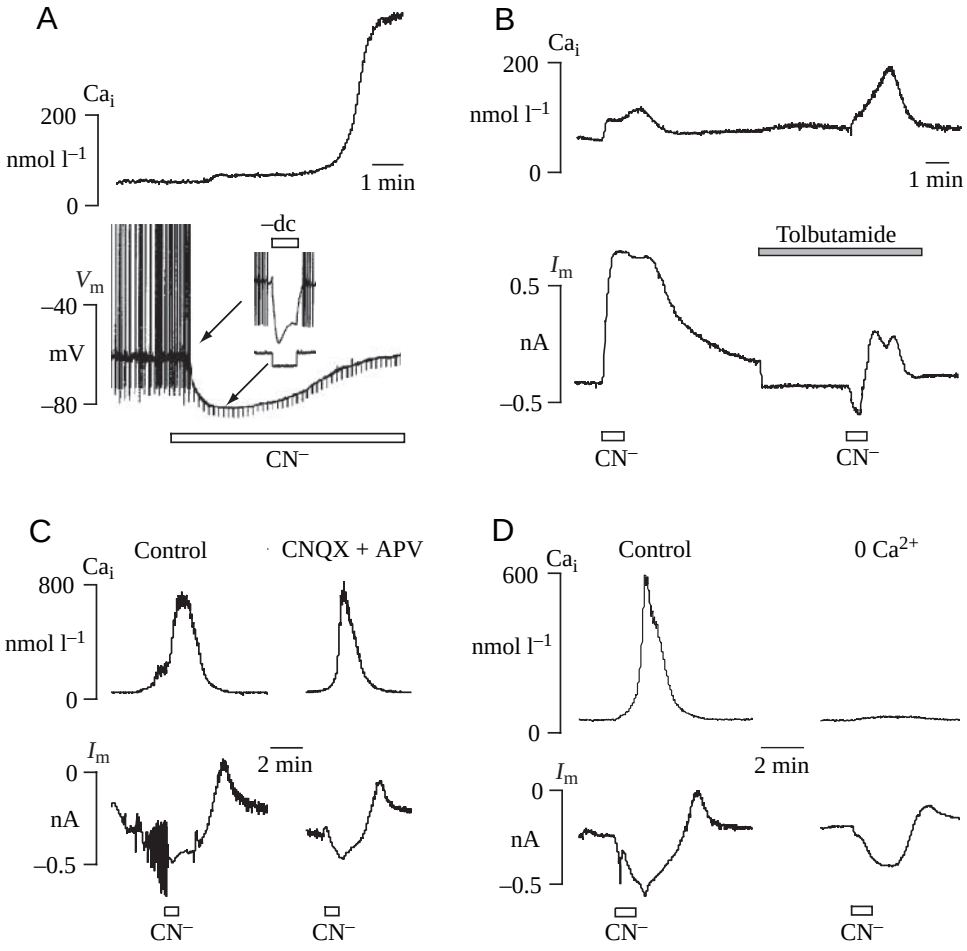

Fig. 4. K_{ATP} channels delay a progressive anoxic Ca_i rise due to Ca^{2+} influx in whole-cell-recorded Purkinje neurons of cerebellar slices from juvenile mice. (A) CN^- (1 mmol l^{-1}) blocks spontaneous spiking due to a hyperpolarisation and concomitant increase in membrane conductance as measured in response to regular injection of hyperpolarising dc current pulses (see insets). During this phase of chemical anoxia, Ca_i shows a stable increase by <50 nmol l^{-1}. Several minutes after the onset of a spontaneous repolarisation of membrane potential in the presence of CN^-, a secondary progressive rise of Ca_i starts to develop. (B) The anoxic hyperpolarisation is caused by a prominent tolbutamide-sensitive outward current. In the presence of tolbutamide, the onset of the secondary progressive phase of the anoxic Ca_i rise develops almost immediately at the beginning of a CN^--induced inward current that is usually masked by the K_{ATP} outward current. Holding potential: –60 mV. (C) In a Purkinje neuron, filled *via* the patch-electrode with 100 mmol l^{-1} Cs^+ and 30 mmol l^{-1} TEA^+ to block K^+ currents, CN^- evokes an inward current accompanied by a major rise of Ca_i. These responses are not notably affected by bath application of 20 μmol l^{-1} CNQX and 100 μmol l^{-1} APV to block ionotropic glutamate receptors. (D) In a Cs^+/TEA^+ filled cell, the CN^--induced Ca_i rise is abolished by Ca^{2+}-free superfusate that also contains 5 mmol l^{-1} Mg^{2+} to block Ca^{2+} channels and 1 mmol l^{-1} EGTA to buffer extracellular Ca^{2+}. All recordings from K. Ballanyi, M. Lückermann and D. W. Richter.

secondary progressive rise of Ca_i develops that can exceed 1 μmol l^{-1} but is not reflected by a major change in membrane potential or conductance (Fig. 4A). As exemplified in Fig. 4B, cyanide induces a K_{ATP} outward current that can exceed 1 nA. Since the agent was applied for only 1 min in that experiment, the rise of Ca_i was rather moderate without occurrence of the steep secondary phase. Subsequent application of tolbutamide reduced the outward current that had not yet fully recovered from the first cyanide application. In the presence of tolbutamide, cyanide evokes an inward rather than an outward current while a minor outward current develops during washout of this toxin. In contrast to the control response to cyanide, Ca_i starts to progressively increase right from the beginning of the application of cyanide in the presence of tolbutamide. However, micromolar levels of Ca_i are not reached in the example of Fig. 4B due to the short application time period of cyanide.

Analysis of the spatiotemporal pattern of the anoxic rise of Ca_i using digital CCD camera imaging revealed that levels of Ca_i are very similar in somatic and dendritic regions of the Purkinje cells at rest. As shown in Fig. 5, application of cyanide for 5 min in the presence of tolbutamide induces first a comparable moderate Ca_i rise in both the soma and dendrites. After about 1 min, a major rise of Ca_i develops in the distal dendrites and proceeds within the following 90 s towards the soma until a similar elevation of Ca_i is seen in both. Upon washout of cyanide, Ca_i recovers first in the soma and then in the dendrites.

These findings suggest that anoxia promotes initially a moderate rise of Ca_i, possibly due to release from intracellular (mitochondrial) stores as suggested above for the dorsal vagal neurons. In contrast to the anoxia-tolerant dorsal vagal cells, the vulnerable Purkinje neurons are subjected to a secondary progressive Ca_i rise that is irreversible in a major population of cells when cyanide or hypoxic anoxia are applied for several minutes, particularly in the presence of tolbutamide. The secondary cyanide-induced rise of Ca_i appears to be related to membrane depolarisation, as it develops more rapidly when the anoxic hyperpolarisation is suppressed with sulfonylurea K_{ATP} channel blockers. Similarly, dialysing the cells with a mixture of Cs^+ and TEA^+ reveals an immediate onset of the progressive anoxic Ca_i rise with no occurrence of a cyanide-induced

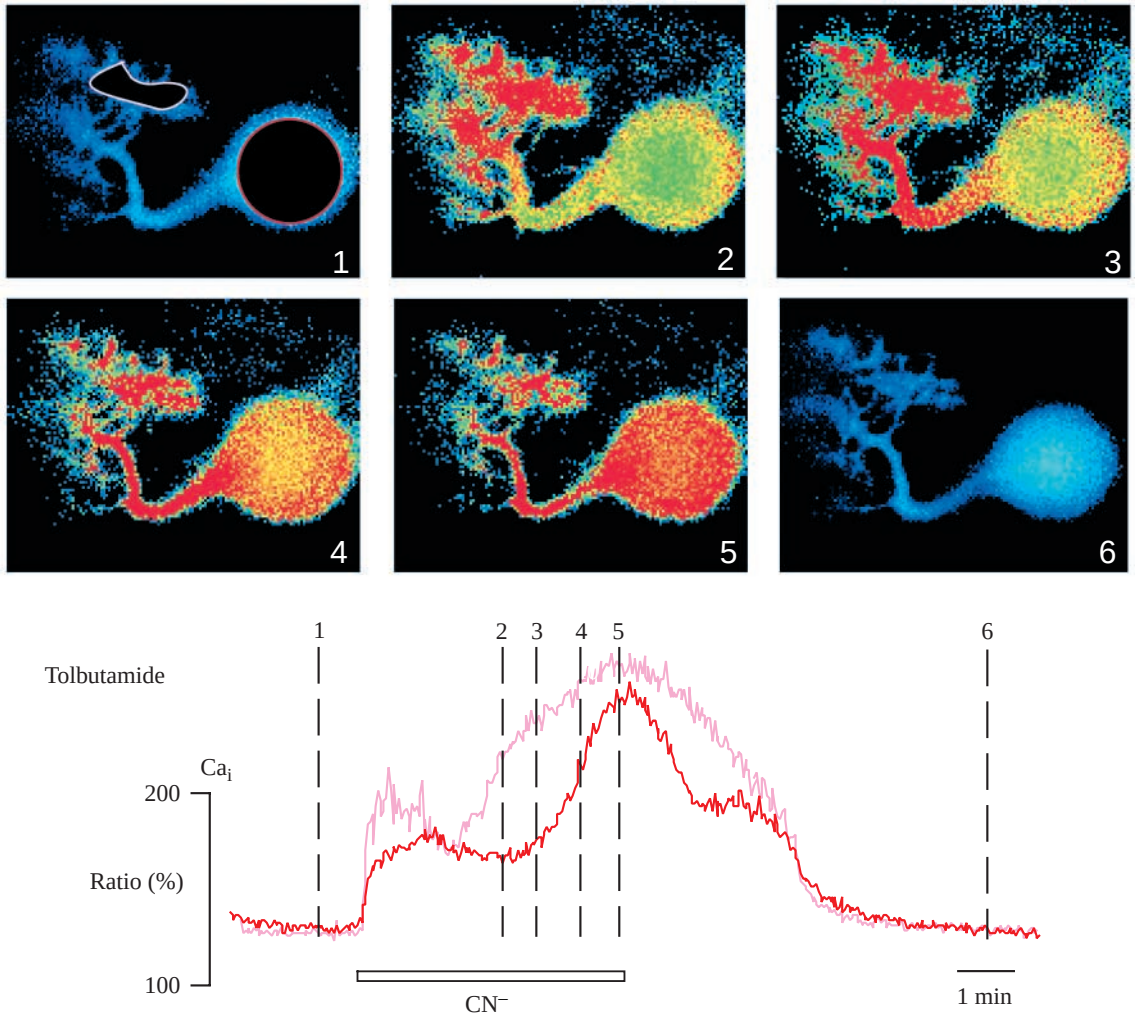

Fig. 5. Spatiotemporal relation of anoxic Ca_i rises in Purkinje neurons. Digital imaging was used to measure Ca_i in two regions of interest (pink, dendritic tree; red, soma). The continuous Ca_i traces illustrate that administration of CN^- (1 mmol l^{-1}) in the presence of tolbutamide (200 μmol l^{-1}) induces instantly a similar moderate Ca_i rise in both the soma and dendrites. During the following 60 s, a secondary progressive Ca_i increase occurs first in the dendritic tree. Subsequently, a Ca^{2+} wave progresses from the dendrites to the soma as shown by the sequence of individual images whose numbers correspond to those on the continous Ca_i trace. Data and images from K. Ballanyi et al.

outward current (Fig. 4C,D). The latter prominent rises of Ca_i are not affected by a mixture of 6-cyano-7-nitroquino-xaline-2,3-dione and 2-amino-5-phosphonovalerate to block ionotropic glutamate receptors (Fig. 4C; for references, see Kulik et al., 2000) while they are abolished by Ca^{2+}-free superfusate or extracellular Cd^{2+} (Fig. 4D). The lack of effects of glutamate receptor blockers on the progressive Ca_i rise and the potency of Cd^{2+} and Ca^{2+}-free superfusate to suppress this response indicates, on the one hand, that anoxia affects the activation–inactivation characteristics of voltage-gated Ca^{2+} channels in these cells as shown for other types of mammalian neurons (Sun and Reis, 1994; Brown et al., 2001; Lukyanetz et al., 2003). On the other hand, it is obvious that K_{ATP} channels counteract, at least during the time course of several minutes, the secondary massive rise of Ca_i and thus have a protective role during short periods of anoxia in these vulnerable mammalian neurons.

K_{ATP} channel-mediated anoxic slowing of breathing in newborn rats

Breathing movements are mediated by a neuronal network in the lower brainstem (Feldman, 1986; Richter et al., 1992). Neurons of the ventral respiratory group (VRG), including the respiratory centre of the pre-Bötzinger complex (PBC), can be isolated *in vitro* in inspiratory active brainstem–spinal cord preparations or 200–800 μm-thick coronal medullary slices from neonatal rodents (Fig. 1; Smith et al., 1991; Ballanyi et al., 1999). Membrane potentials or currents from PBC or other VRG cells can be monitored with whole-cell recording techniques, and the recorded cells can be labelled with dyes for subsequent (immuno)histochemical analysis (Fig. 1; Ballanyi et al., 1999; Ballanyi, 2004). Studies on these *in vitro* preparations have indicated that rhythmogenesis within the respiratory network depends on PBC conditional burster neurons (Smith et al., 1991) whose burst frequency is

determined by intrinsic regenerative membrane properties such as a persistent Na^+ conductance that operates in concert with leak, e.g. TASK-1 or K_{ir}-type K^+ conductances coupled to receptors for diverse neuromodulators (Fig. 6A; for references, see Ballanyi et al., 1999; Ballanyi, 2004). In neonates, the respiratory network is highly tolerant to hypoxia–anoxia (Ballanyi et al., 1994; Richter and Ballanyi, 1996; Richter et al., 1999). Respiratory movements persist in intact, unanesthetised neonatal rats for more than 30 min during anoxia evoked by nitrogen breathing whereas terminal apnea occurs within less than 5 min of anoxia in rats older than one week (Fazekas et al., 1941; Adolph, 1969; for further references, see Ballanyi, 2004). In perinatal rats *in vivo*, the frequency of breathing is profoundly reduced during anoxia from ~1 breath s^{-1} to <1 breath min^{-1} (Ballanyi, 2004). A similar persistence of respiratory activity at greatly reduced frequency for anoxia periods of up to 1 h is observed in brainstem–spinal cord preparations from newborn rats (Fig. 7A; Ballanyi et al., 1994, 1999; Ballanyi, 2004).

A series of reports on the latter preparation (Ballanyi et al., 1992, 1994, 1999; Brockhaus et al., 1993; Voipio and Ballanyi, 1997; Ballanyi, 2004) has led to the view that the high tolerance of the respiratory network in newborn mammals to oxygen depletion includes two cooperative processes. On the one hand, anoxia appears to effectively stimulate anaerobic glycolysis ('Pasteur effect'; Lutz and Nilsson, 1994; Hochachka and Lutz, 2001). In agreement with the efficacy of the Pasteur effect, up to 50% of the ATP production under normoxic conditions in neonates appears to be due to anaerobic metabolism, in contrast to <20% in adults (Hansen, 1985). Accordingly, a high lactate dehydrogenase activity supports the notion of an important role of anaerobic glycolysis during the perinatal period (Booth et al., 1980). By contrast, the activity of cytochrome *c* oxidase (an indicator of oxidative glucose utilisation) increases only after P12–17 (Wong-Riley, 1989). Due to the low efficacy of anaerobic ATP production, survival of neurons during extended periods of anoxia is only possible as the metabolic rate of neonatal brain tissue is very low, i.e. in postnatal day-1 rats <5% of that in adults (Duffy et al., 1975; Hansen, 1985). Thus, in the perinatal period, cerebral glucose consumption appears to be <10% of that in adult rats (Vannucci and Vannucci, 1978). Despite a reduced *in vitro* temperature (25–28°C), the metabolic rate is not extremely low in the brainstem–spinal cord preparation of newborn rats as the glycolytic blocker iodoacetate or removal of glucose from the superfusate profoundly perturbs both the respiratory rhythm

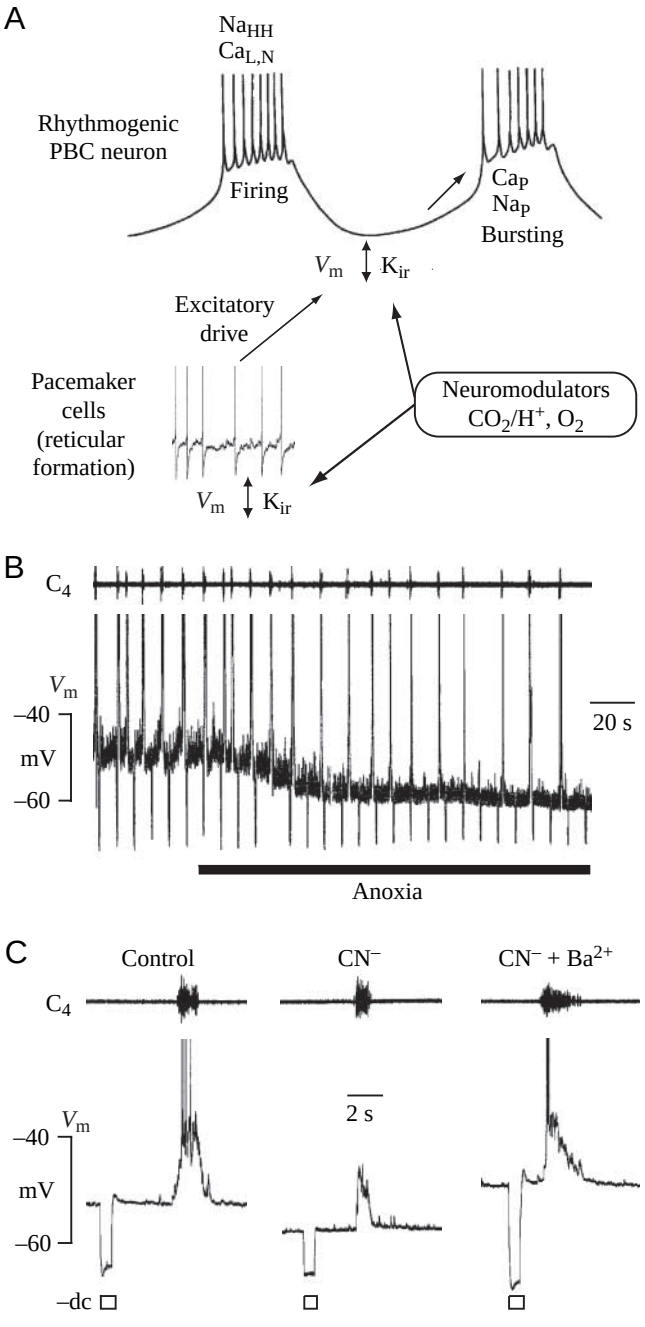

Fig. 6. Effects of anoxia on rhythmic bursting of respiratory neurons in isolated medulla preparations from neonatal rats. (A) Bursting of rhythmogenic pre-Bötzinger complex (PBC) neurons is possibly caused by the cooperative interaction between regenerative intrinsic ion conductances such as persistent Na^+ channels (Na_P) or intermediate-voltage-activated (P/Q-type) Ca^{2+} channels (Ca_P) with K_{ir} channels (including K_{ATP} channels) or leak (e.g. TASK-1 channels) that contribute to resting membrane potential (V_m) and are affected by various neuromodulators, CO_2/H^+ and/or O_2. These neuromodulators may also indirectly affect PBC neurons *via* an action on K_{ir} (or TASK-1) channels of pacemaker cells within the reticular formation proposed to provide excitatory drive to rhythmogenic PBC cells. Spike firing during individual bursts is mediated by Hodgkin-Huxley-type Na^+ channels (Na_{HH}) plus L- and N-type Ca^{2+} channels ($Ca_{L,N}$). (B) In a minor subpopulation of inspiratory (PBC) neurons in a brainstem–spinal cord preparation, a hyperpolarisation induced by anoxia does not block the rhythmic drive potential, as also evident from persistence of inspiratory-related cervical (C_4) nerve rootlet activity. (C) In other respiratory neurons, such as this inspiratory cell in a brainstem–spinal cord preparation, anoxia depresses the drive potential and abolishes spiking. This effect is antagonised by the K_{ir} and K_{ATP} channel antagonist Ba^{2+}. The downward deflections on the membrane potential traces in B and C are responses to injection of dc current for measurement of membrane conductance. Data from K. Ballanyi.

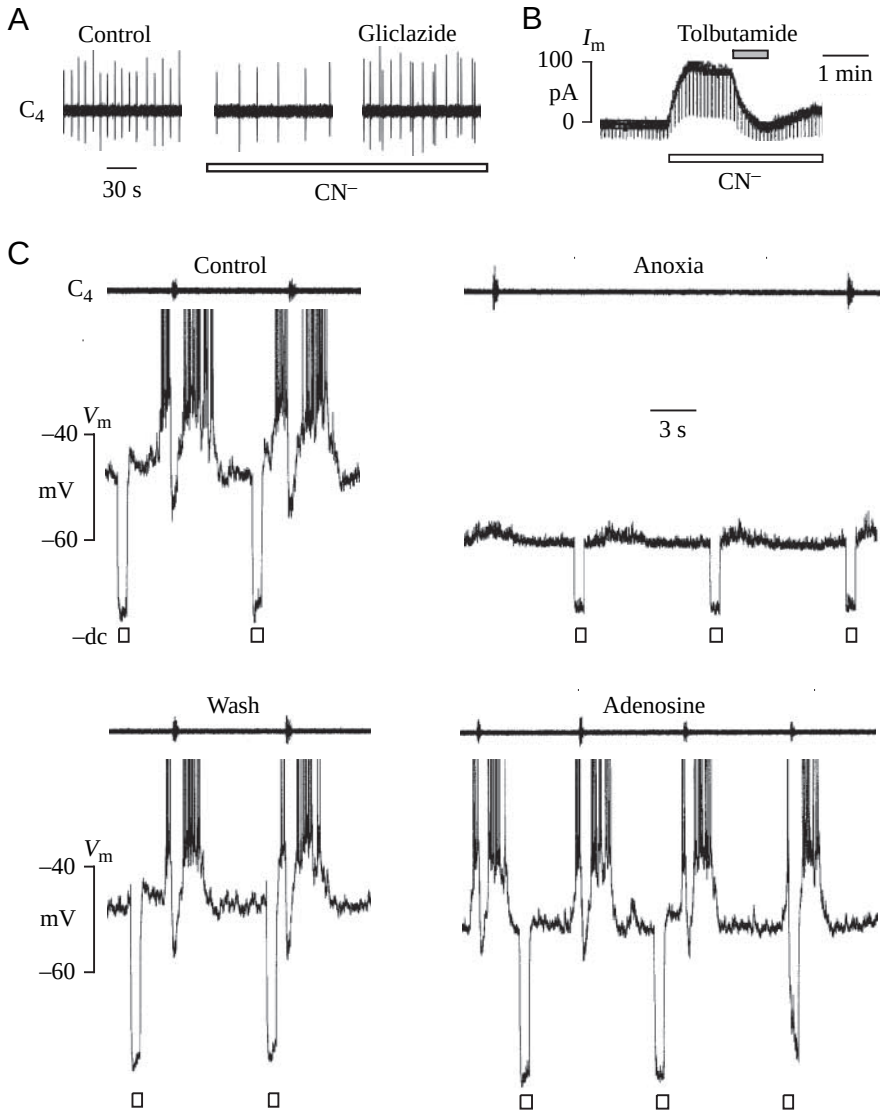

Fig. 7. Involvement of K_{ATP} channels, but not adenosine receptors, in anoxic hyperpolarisation of neonatal respiratory neurons and frequency depression of respiratory rhythm. (A) Chemical anoxia due to 1 mmol l^{-1} CN^- notably depresses respiratory frequency in the brainstem–spinal cord preparation from newborn rats. In the presence of CN^-, this effect is reversed by the K_{ATP} channel blocker gliclazide (200 μmol l^{-1}). (B) In a neuron in the region of the pre-Bötzinger complex (PBC) of a non-rhythmic medullary slice, tolbutamide blocks the outward current and conductance increase underlying the anoxic hyperpolarisation. Membrane conductance is measured by injection of hyperpolarising dc current pulses. (C) In a pre-inspiratory neuron (also classified as 'biphasic-expiratory'; Ballanyi et al., 1999), the anoxia-induced hyperpolarisation and conductance increase abolishes rhythmic fluctuations of membrane potential. After recovery from anoxia (wash), administration of adenosine (500 μmol l^{-1}) fails to mimic the anoxic hyperpolarisation, conductance increase or block of respiratory-related membrane potential fluctuations. A, data from L. Secchia and K. Ballanyi; B,C, data from K. Ballanyi.

and ion homeostasis within the respiratory network within 30 min (Ballanyi et al., 1996b, 1999). These effects are accompanied by a progressive depolarisation of neonatal respiratory neurons often preceded by a hyperpolarisation lasting between 2 and 4 min (Ballanyi et al., 1999).

The second process contributing to the anoxia tolerance of the neonatal respiratory network is constituted by downregulation of metabolic rate and demand by 'functional inactivation' of ion conductances as described for cold-blooded vertebrates (Hochachka and Lutz, 2001). Most (>60%) of the respiratory neurons in neonatal rats *in vitro* respond with a sustained K^+ channel-mediated hyperpolarisation and conductance increase in response to blockade of aerobic metabolism by either anoxia or cyanide (Figs 6B,C, 7C; Ballanyi et al., 1994, 1999). In about 50% of these cells, the anoxic hyperpolarisation coincides with suppression of respiratory-related membrane potential fluctuations (Fig. 6C). This is particularly obvious in pre-inspiratory neurons that appear to constitute a second respiratory centre in addition to the PBC (Ballanyi et al., 1999). More than 90% of these cells are hyperpolarized and inactivated during anoxia, while a subpopulation of these VRG cells receives burst-type subthreshold synaptic inputs that are not in phase with the slowed inspiratory motor output (Fig. 7C). Such anoxic functional inactivation of a major portion of respiratory neurons does not reflect a pathophysiological impairment of synaptic transmission or membrane excitability. Action potentials can still be evoked in these cells while drive potentials of a different subpopulation of inspiratory VRG neurons remain vitually unaltered by anoxia (Fig. 6B; Ballanyi et al., 1994, 1999; Ballanyi, 2004). This tolerance to anoxia of excitatory synaptic transmission in a subclass of neonatal respiratory neurons coincides with the ability of inspiratory premotoneurons and motoneurons to generate (respiratory-related) spiking as obvious from the persistence of inspiratory motor output during anoxia.

For some neuronal tissues, evidence has been presented that adenosine formed during anoxia may act *via* A_1 receptors on

K_{ATP} or other K^+ channels to exert a protective function (Pek-Scott and Lutz, 1998; Mironov and Richter, 2000). However, the effects of adenosine on the respiratory network are not yet clear. The agent strongly depresses breathing *in vivo* and this is antagonised by aminophylline, i.e. theophylline (Hedner et al., 1982; Eldridge et al., 1985). On the other hand, adenosine stimulates breathing in conscious, unanesthetised humans (Fuller et al., 1987; Griffith et al., 1997). The depressant effect of adenosine on breathing *in vivo* is more potent during the time period around birth and/or is also more severe in anesthetised animals (Herlenius et al., 2002). Hypoxia–anoxia elevates adenosine levels in the VRG of adult cats *in vivo* (Richter et al., 1999) and in the rostral brainstem of fetal sheep (Koos et al., 1994). The rostral brainstem has a high density of A_1 adenosine receptors (Bissonnette and Reddington, 1991) that are possibly involved in central respiratory inhibition *in vitro* and *in vivo* (Herlenius and Lagercrantz, 1999; Mironov et al., 1999). Furthermore, the adenosine receptor antagonist theophylline blocks, at least partly, the depressant response to oxygen deprivation *in vivo* (Darnall, 1985). A similar antagonistic effect of theophylline (165 μmol l^{-1}) was revealed in the brainstem–spinal cord preparation of newborn rats (Kawai et al., 1995). By contrast, 500 μmol l^{-1} theophylline or 2.5 μmol l^{-1} of the A_1 adenosine receptor blocker 8-cyclopentyl-1,3-dipropyl-xanthine fails to reverse both the anoxic frequency decrease and the hyperpolarisation of respiratory neurons in the same *in vitro* preparation (Ballanyi et al., 1999). 8-cyclopentyl-1,3-dipropyl-xanthine is effective *in vitro* as it blocks adenosine-mediated suppression of non-respiratory excitatory postsynaptic potentials in VRG neurons and abolishes the anticonvulsant effect of adenosine on spinal motor circuits (Brockhaus and Ballanyi, 2000). Finally, adenosine neither mimics the anoxic slowing of the neonatal respiratory rhythm nor the concomitant hyperpolarisation of VRG neurons (Fig. 7C).

According to the proposed pivotal role of PBC conditional burster neurons in respiratory rhythm generation, an anoxic hyperpolarisation of these cells and/or of cells that provide a tonic excitatory drive to these neurons would slow the respiratory rhythm. Thus, it is possible that the K^+ channel-mediated anoxic hyperpolarisation, observed in the majority of neonatal respiratory neurons (Ballanyi et al., 1994, 1999; Ballanyi, 2004), is causally related to the secondary respiratory depression. In line with this view, Ba^{2+}, which blocks inwardly rectifying K^+ channels including K_{ATP} channels (Ashcroft and Gribble, 1998; Töpert et al., 1998; Aguilar-Bryan and Bryan, 1999), antagonises not only the anoxic hyperpolarisation of neonatal VRG cells (Fig. 6C) but also the accompanying frequency depression of respiratory rhythm (Ballanyi et al., 1999; Ballanyi, 2004). The same reports provide more direct evidence for an involvement of K_{ATP} channels, as both tolbutamide and the more potent sulfonylurea gliclazide reverse both the depressing effect of anoxia on respiratory frequency (Fig. 7A) and the anoxic hyperpolarisation or outward current of VRG neurons (Fig. 7B) (Ballanyi et al., 1999; Ballanyi, 2004; see also Haller et al., 2001; Mironov and Richter, 2000).

This raises the question of whether the cellular ATP concentration decreases considerably in neonatal respiratory neurons during anoxia. The above findings in medullary dorsal vagal neurons suggest that anoxic activation of neuronal K_{ATP} channels occurs in the absence of a major fall in ATP concentrations (Müller et al., 2002). Furthermore, cellular ATP levels do not appear to decrease profoundly in the neonatal respiratory network during anoxia periods of up to 30 min (Duffy et al., 1975; Wilken et al., 1998). In addition to Ba^{2+} and tolbutamide, muscarine and thyrotropin-releasing hormone reverse the respiratory response to anoxia in the brainstem–spinal cord preparation (Ballanyi et al., 1999; Ballanyi, 2004). This suggests that a yet undetermined mediator acting on (G protein-regulated) K_{ATP} channels is released (or suppressed) during anoxia. Alternatively, cellular processes such as changes in phosphorylation or redox state may be responsible for K_{ATP} channel activation and, thus, for anoxic slowing of rhythm, although these processes do not appear to contribute to activation of the K_{ATP} channels in dorsal vagal neurons (Müller et al., 2002). The observation that the anoxic slowing of the neonatal respiratory rhythm is mediated by K_{ATP} channels shows that this is not a pathological consequence of anoxic perturbation of cellular function. Rather, it may represent an adaptive mechanism serving for conservation of energy during severe hypoxia such as that occurring during birth (Ballanyi, 2004). Interestingly, K_{ATP} channels appear to be active during normoxia, as sulfonylureas depolarise dorsal vagal neurons of medullary slices (Trapp et al., 1994) as well as respiratory neurons *in vivo* (Pierrefiche et al., 1998) and *in vitro* (Haller et al., 2001). That the physiological activity of K_{ATP} channels is relevant for respiratory functions is indicated by the observation that sulfonylureas increase the frequency of the respiratory rhythm in medullary slices from newborn rats (K. Ballanyi and L. Secchia-Ballanyi, unpublished observations).

Conclusions

This article has presented three examples for the involvement of K_{ATP} channels in the response of neuronal structures to oxygen depletion. In mature Purkinje neurons and neonatal respiratory neurons, these metabolism-gated K^+ channels appear to play a protective role during brain hypoxia–anoxia. In the Purkinje neurons, these channels delay the onset of the profound secondary rise of the cytosolic Ca^{2+} concentration as a major factor of hypoxic-ischemic cell death. And, in the neonatal respiratory network, K_{ATP} channels functionally inactivate a major population of respiratory neurons not required for rhythm generation, while the K_{ATP} channel-mediated hyperpolarisation of rhythmogenic burster neurons, or of tonic pacemaker cells providing excitatory drive to these neurons, does not lead to inactivation of the cells. Rather, this anoxic hyperpolarisation appears to reduce the frequency of their bursting and thus likely suppresses breathing movements. In contrast to these examples, K_{ATP} channels of dorsal vagal neurons do not seem to play a protective role in

hypoxia–anoxia but rather may be involved in other metabolic functions such as glucose sensing. There is increasing evidence that K_{ATP} channels are not only active during energy depletion but also during physiological activity of brain cells. This suggests that they serve multiple functions to couple cellular energy metabolism with neuronal excitability.

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