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K_{ATP} channel mediation of anoxia-induced outward current in rat dorsal vagal neurons *in vitro*

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1. Thin brainstem slices (150 μm thickness) were taken from mature rats, and membrane potentials (E_m) and currents (I_m) in the dorsal vagal neurons (DVN) were analysed with whole-cell patch clamp techniques during anoxia.
2. At a holding potential (V_h) of −50 mV, a sustained anoxia-induced outward current (AOC) of 92 ± 44 pA (reversal potential (E_{rev}), −78 ± 12 mV) and a concomitant increase of membrane conductance (g_m) from 2·2 ± 0·45 to 5·9 ± 2·4 nS were revealed in 40% of 142 DVN analysed. The AOC led to a hyperpolarization of the cells by 14·4 ± 6·1 mV from a mean resting E_m of −51 ± 6 mV, and to blockade of spontaneous action potential discharges. In the remaining DVN, anoxia had almost no effect on E_m, I_m or g_m and did not block spontaneous action potential discharges.
3. The AOC was not affected by 0·5 μM tetrodotoxin (TTX), 2 mM Mn^{2+}, 50 μM cyanonitroquinoxaline dione (CNQX) or 100 μM bicuculline.
4. Elevation of the extracellular [K^+] from 3 to 10 mM resulted in a positive shift of E_{rev} of the AOC by 23 mV, whereas an increase in the [Cl^-] of the patch pipette solution from 5 to 144 mM had no effect on E_{rev}.
5. In DVN responding with an AOC, addition of 200 μM diazoxide, an activator of ATP-sensitive K^+ (K_{ATP}) channels, to oxygenated solutions elicited a similar outward current (E_{rev} = −79 ± 5·5 mV, n = 12) and increase in g_m. Diazoxide did not affect E_m, I_m or g_m in cells which did not show an AOC.
6. In a subpopulation of DVN (n = 26), spontaneous activation of a K_{ATP} current with an E_{rev} of −80 ± 6 mV was observed. As analysed in four of these cells, an AOC was revealed during the initial phase of development of the spontaneous outward current but not under steady-state conditions.
7. The AOC, the diazoxide-induced current, and the spontaneous outward current were completely blocked upon bath application of the K_{ATP} channel blocker tolbutamide (100–200 μM).
8. The results indicate that the sustained anoxia-induced outward current of dorsal vagal neurons is due to activation of K_{ATP} channels. A possible physiological role of functional inactivation of these cells during metabolic disturbances is discussed.

The function of the mammalian brain is extremely sensitive to energy depletion as induced by hypoxia or ischaemia (Hansen, 1985; Ben-Ari, 1992; Haddad & Jiang, 1993). Suppression of neuronal excitability occurring in the early phase of oxygen depletion has been attributed to a hyperpolarization which is due to an increase in K^+ conductance (Hansen, Hounsgaard & Jahnsen, 1982). Such hyperpolarization is thought to have a protective function since the accompanying reduction of activity-related transmembraneous ion fluxes lowers the activity of ion pumps, which consume about 50% of the energy supply of the brain (Hansen, 1985; Hochachka, 1986).

The cellular mechanisms leading to activation of this K^+ conductance are still under discussion. *In vitro* studies on

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hippocampal neurons indicated that an anoxia-evoked rise in intracellular free Ca^{2+} leads to activation of Ca^{2+}-dependent K^+ (BK_{Ca}) channels (Leblond & Krnjevic, 1989; Krnjevic & Xu, 1991). Similar results were obtained from whole-cell patch clamp recordings in isolated dorsal root ganglion cells (Duchen, 1990). In these cells, the K^+ outward current, evoked by either oxygen depletion or metabolic inhibition, was not affected by intracellular ATP, which was adjusted via diffusion from the patch electrodes.

Other studies, however, presented evidence that an anoxia-induced fall in neuronal ATP (and/or a concomitant rise in ADP) leads to opening of K_{ATP} channels, as has been demonstrated in mammalian heart and pancreatic β-cells (for review see Ashcroft & Ashcroft, 1990, 1992; Nichols & Gross, 1994). In different types of central neuron sulphonylurea blockers of K_{ATP} channels suppress the initial anoxia-induced hyperpolarization and activators of these channels mimic such a response to metabolic blockade (Mourre, Ben-Ari, Bernardi, Fosset & Lazdunski, 1989; Grigg & Anderson, 1989; Ben-Ari, 1990; Röper, Hainsworth & Ashcroft, 1990; Luhmann & Heinemann, 1992; Murphy & Greenfield, 1992).

A detailed analysis of the ionic mechanism of the anoxia-induced hyperpolarization of neurons is often hampered by development of a secondary progressive depolarization (e.g. Hansen *et al.* 1982; Grigg & Anderson, 1989; Luhmann & Heinemann, 1992). Dorsal vagal neurons (DVN) are appropriate for such an analysis since anoxia evokes stable hyperpolarizations in these cells (Cowan & Martin, 1992), which are caused by an anoxia-induced outward current (AOC; Ballanyi, Doutheil & Richter, 1992*a*). In the present study, we have used the thin slice patch clamp technique (Edwards, Konnerth, Sakmann & Takahashi, 1989) to investigate the mechanism of the AOC. The voltage clamp analysis of the reversal potential (E_{rev}) of the AOC, its ionic dependence and the effects of modulators of metabolism-regulated K^+ channels suggests that this anoxia-induced current is due to activation of K_{ATP} channels.

Parts of the results have been published in abstract form (Doutheil, Trapp, Ballanyi & Richter, 1993; Trapp, Ballanyi & Richter, 1994*a*).

METHODS

Preparation

Wistar rats (16–22 days old) of either sex were anaesthetized with ether and decapitated. The brain was removed, the brainstem with the cerebellum was isolated and kept in ice-cold artificial cerebrospinal fluid (standard solution, for composition see below; Ca^{2+} concentration reduced to 0·5 mM) for 5 min. Afterwards, the cerebellum was removed, the brainstem glued to the stage of a vibratome (FTB; Vibracut, Weinheim, Germany) and eight to ten transverse slices (150 μm thick) were cut around the obex level in low-Ca^{2+} solution. Prior to transfer to the recording chamber, the slices were stored at 30 °C in standard solution.

Solutions and superfusion system

After transfer, and fixation of individual slices with a net, the recording chamber (volume, 3 ml) was superfused with oxygenated standard solution (temperature, 30 °C; flow rate, 5 ml min^{-1}) of the following composition (mM): 118 NaCl, 3 KCl, 1 $MgCl_2$, 1·5 $CaCl_2$, 25 $NaHCO_3$, 1·2 NaH_2PO_4 and 10 D-glucose. The pH was adjusted to 7·4 by gassing with 95% O_2–5% CO_2. In some experiments, the temperature in the experimental chamber was raised to 37 °C. Stainless steel tubing was used for gas supply to closed buffer flasks, as well as for the superfusion system, which was driven by gravity and gas pressure. Partial pressure of oxygen (P_{O_2}), measured with O_2-sensitive microelectrodes (for details, see Brockhaus, Ballanyi, Smith & Richter, 1993), was between 580 and 620 mmHg in the experimental chamber during superfusion of the standard solution. Superfusion with hypoxic standard solution, gassed with 95% N_2 instead of O_2, led to a fall in P_{O_2} in the experimental chamber to below 30 mmHg, and to a fall of P_{O_2} in the slices to zero levels indicating tissue anoxia (K. Ballanyi, unpublished data). Drugs, purchased from Sigma or Tocris Cookson were added from stock solutions to the superfusion fluid.

Intracellular recordings

Patch pipettes were obtained from borosilicate glass capillaries (GC 150TF; Clark Electromedical Instruments, Pangbourne, UK) using a horizontal electrode puller (Zeitz, Munich, Germany). The standard low-Cl^- (5 mM) patch pipette solution contained (mM): 120 potassium gluconate, 1 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 11 K-BAPTA, 10 Hepes, 0·5 Na-ATP. High-Cl^- (144 mM) solution consisted of (mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 Hepes, 2 Na-ATP. These solutions, which had an osmolarity of between 270 and 290 mosmol l^{-1}, were adjusted to a pH of 7·3–7·4 with 1 M KOH. DC resistances of patch electrodes ranged from 3 to 8 MΩ depending on the filling solutions.

Whole-cell recordings were performed on superficial DVN as described by Edwards *et al.* (1989) using an EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany). Seal resistances were between 1·5 and 6 GΩ. Series resistance (8–20 MΩ) and cell capacitance (39·43 $\pm$ 9·44 pF) were compensated by more than 70%. Experimental protocol was dependent on the behaviour of membrane current (I_m) after establishing the whole-cell configuration. Responses to anoxia were analysed in detail in cells ($n = 168$) in which I_m (at a holding potential, V_h, of −50 mV) was stable for at least 15 min, whereas the relation between AOC and K_{ATP} current was studied in a subpopulation of DVN ($n = 30$) in which a spontaneous outward current developed 1–8 min after establishing the whole-cell configuration (Trapp, Ballanyi & Richter, 1994*b*). Current–voltage (I–V) relations were obtained by application of depolarizing or hyperpolarizing voltage (in voltage clamp mode) or DC (in current clamp mode) steps (duration 300 ms) and measuring steady-state I_m or membrane potential (E_m) responses. Averages of the responses to three pulses were analysed. Resting membrane conductance (g_m) was measured by injection of hyperpolarizing DC or voltage pulses (duration 500 ms, amplitude 10–100 pA or 20 mV) at a V_h of −50 mV, which was close to resting E_m (see Results).

During the experiments, signals were also displayed on a chart recorder (low-pass filtered at 20 Hz; Easygraph, Gould). Current and voltage signals were digitized (VR-100A; Instrutech, Elmont, NY, USA) and recorded on magnetic tape. 'Analysis' software by HEKA was used for data analysis. Values are given as means $\pm$ S.D. Significance values were obtained from Student's unpaired t tests.

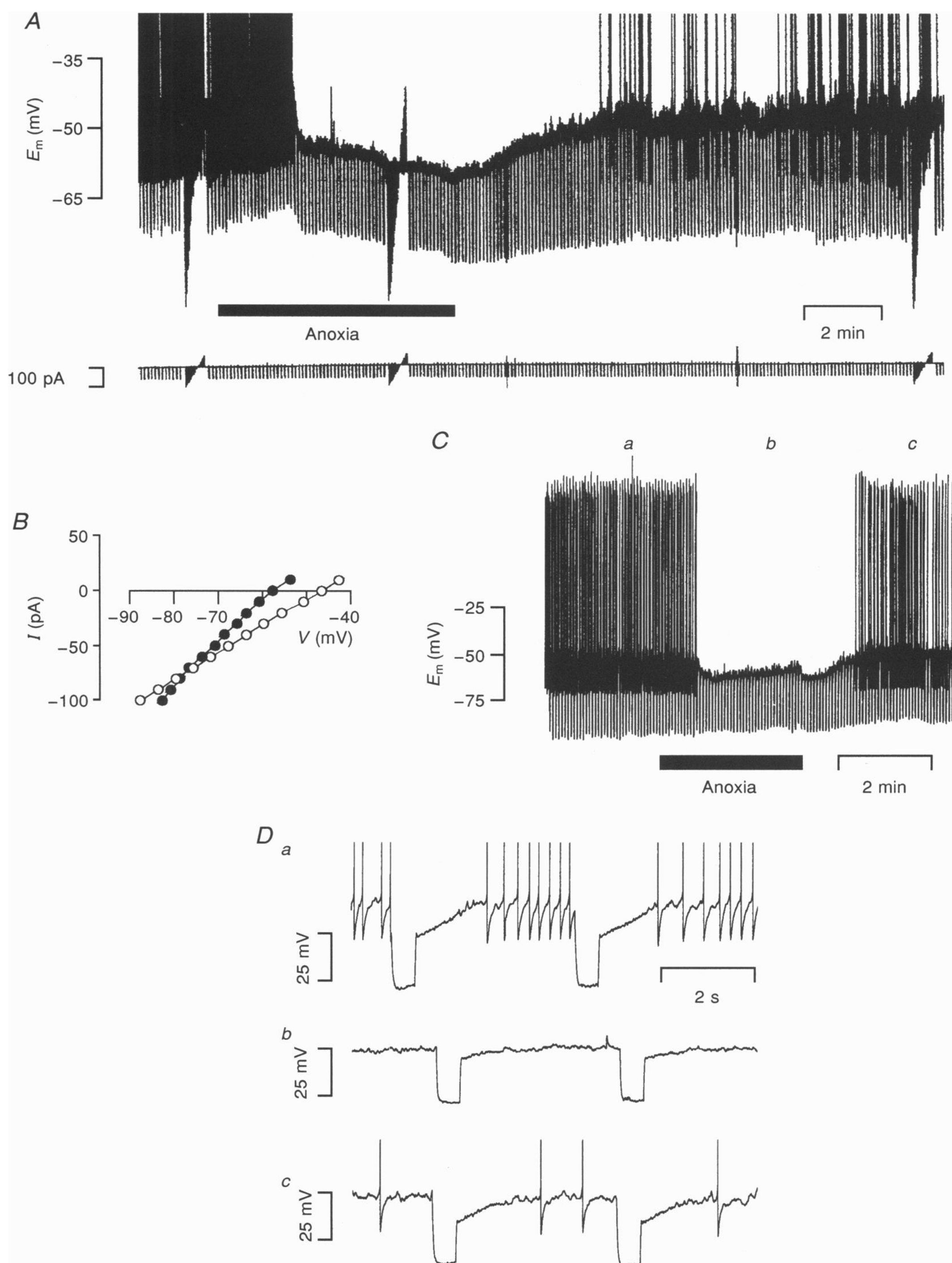

Figure 1. Anoxia-induced hyperpolarization and inactivation

A and *C*, anoxia led to inhibition of spontaneous action potential discharges caused by a hyperpolarization and an increase in membrane conductance (g_m, measured by regular injection of hyperpolarizing DC pulses; for protocol see lower trace in *A*). *I–V* relations in *B* show that the anoxia-induced hyperpolarization of the cell shown in *A* reversed at −78 mV. *D*, extended time scale recordings, corresponding to *a–c* in *C*, illustrating that the delayed recovery of membrane potential (E_m) after termination of hyperpolarization was blocked during anoxia.

RESULTS

Response to anoxia

Immediately after establishing the whole-cell configuration using patch pipettes filled with 5 mM Cl^- and 0·5 mM ATP, a steady-state I_m of −7 ± 21 pA and a g_m of 2·2 ± 0·45 nS were observed in 168 DVN at a V_h of −50 mV. After switching to the current clamp mode in twenty-six DVN, a mean E_m of −51 ± 6 mV was revealed (Fig. 1). More than 80% of these cells showed spontaneous firing of action potentials at a frequency of 2–6 Hz. A further characteristic feature of DVN was a delayed recovery of E_m and action potential discharges after termination of hyperpolarizing DC pulses, injected via the patch electrode (Fig. 1*D*). These responses were due to activation of an A-type K^+ current (Yarom, Sugimori & Llinas, 1985).

In ten of these DVN, anoxia led to a persistent hyperpolarization by a maximal 23 mV (−14·4 ± 6·1 mV), accompanied by an increase in g_m of 42 ± 27%, and to suppression of action potential discharges after a delay of 103 ± 46 s. As tested in three DVN, these hyperpolarizations were stable for more than 15 min. Upon reoxygenation, E_m and g_m recovered to control levels within 1–3 min. In some cells, however, recovery of spontaneous action potential discharges to control values took several minutes. *I–V* relations obtained from four cells during control and anoxic conditions intersected at −79 ± 4 mV; this corresponds to the E_{rev} of the anoxia-induced hyperpolarization (Fig. 1*B*).

In eleven of the remaining sixteen DVN, E_m and g_m were not affected by anoxic exposure for more than 15 min, whereas a depolarization by a maximal 4 mV with no apparent change in g_m was detected in five cells. The frequency of spontaneous action potential discharges decreased by up to 65% in nine of these DVN, but anoxia had no effect on the frequency of spontaneous activity in the remaining seven cells, which did not hyperpolarize

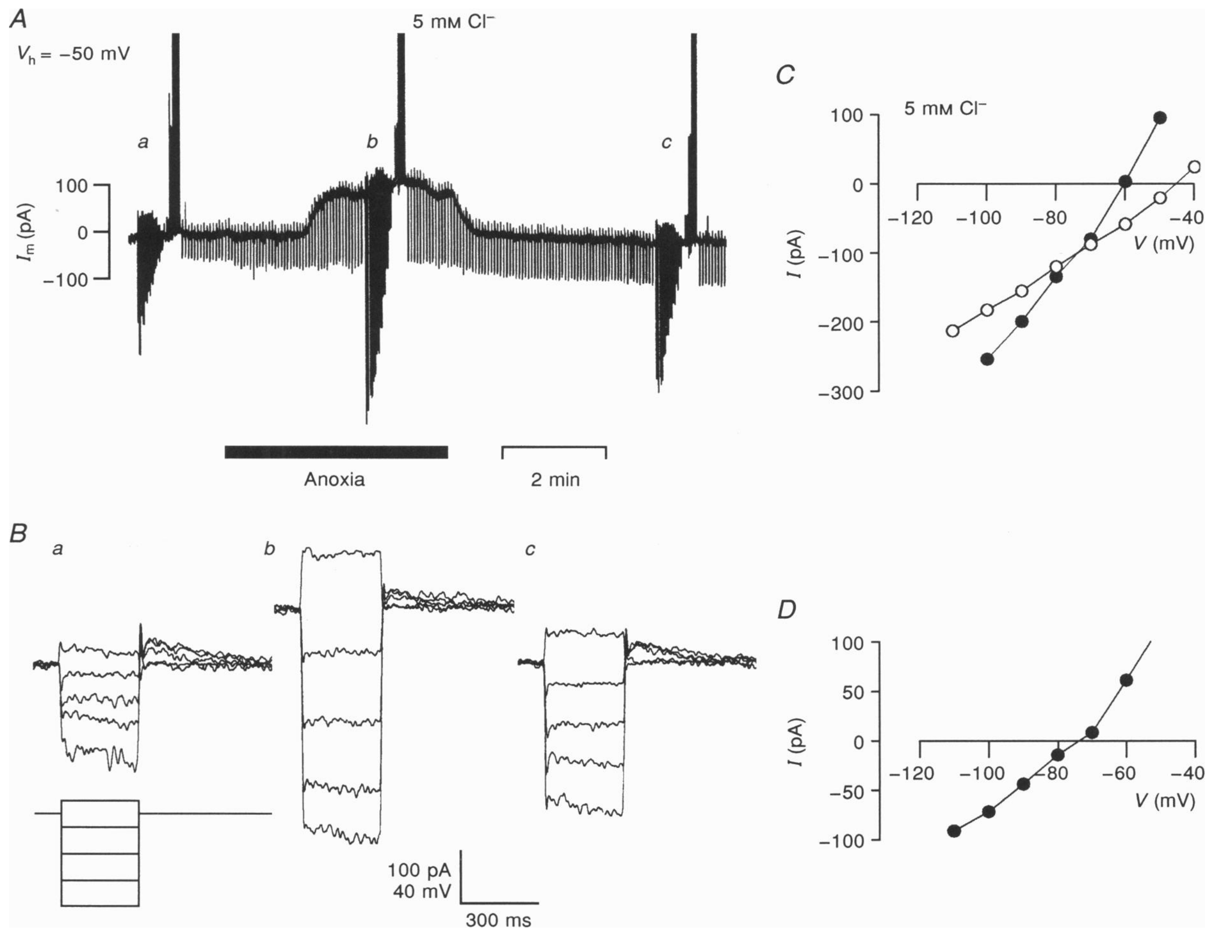

Figure 2. Anoxia-induced outward current

Anoxia resulted in an outward current (AOC) and a concomitant increase in g_m (measured by regular injection of hyperpolarizing voltage pulses). *B* illustrates, on an extended time scale, currents in *A* before (*a*), during (*b*) and after (*c*) anoxia in response to depolarizing and hyperpolarizing voltage steps (lower traces in *a*). *C* shows the resulting *I–V* relation before (○) and during (●) anoxia. *D* shows that the AOC (obtained by subtraction of the control *I–V* relation from that during anoxia) reversed polarity at −78 mV.

during anoxia. Blockade of such activity occurred only during the early phase of reoxygenation in five of these cells. Independent of the type of E_m response to anoxia, the A-current appeared to be suppressed in about 50% of DVN (Fig. 1*D*, see below). Under voltage clamp conditions, no significant changes in I_m or g_m were observed in those cells which did not hyperpolarize upon anoxic exposure.

Ionic mechanism of AOC

The ionic mechanism of anoxia-induced hyperpolarization was analysed under voltage clamp conditions. In 40% of 142 DVN tested, an AOC with an amplitude of 30–300 pA (92 ± 44 pA) and a concomitant, significant ($P < 0{\cdot}001$) increase of g_m from $2{\cdot}2 \pm 0{\cdot}45$ to $5{\cdot}9 \pm 2{\cdot}4$ nS developed after a delay of 99 ± 38 s (Fig. 2). The equilibration time from the onset of this response to steady-state conditions in individual cells varied between 0·5 and 2 min (compare Figs 6*A* and 7*A*), whereas the recovery upon reoxygenation was typically complete after 1–2 min. The AOC remained stable as tested during periods of anoxia of up to 15 min and had an E_{rev} of -78 ± 12 mV ($n = 63$; e.g. Fig. 2*C* and *D*). As observed in five DVN during anoxia periods of 4–15 min, these characteristics of the AOC were not affected by increasing the temperature of the superfusion fluid in the recording chamber from 30 to 37 °C. (In four different DVN which did not show an AOC, I_m and g_m remained stable during anoxic exposure under these conditions.) Furthermore, the AOC was not influenced by suppression of synaptic transmission with 0·5 μM tetrodotoxin (TTX) and 2 mM Mn^{2+} ($n = 8$; Fig. 3*A*) or blockade of postsynaptic neurotransmitter receptors (Ben-Ari, 1990) with a 'cocktail' consisting of 0·5 μM TTX, 25 μM cyanonitroquinoxaline (CNQX) and 100 μM bicuculline ($n = 4$; Fig. 3*C*).

In a minority of fifteen cells, a transient outward current with a maximal amplitude of 50 pA and a duration of less than 1 min was detected during the early phase of reoxygenation (Fig. 3*A*). In the initial phase of the whole-cell recordings, an A-current with an amplitude of 30–150 pA was revealed upon termination of hyperpolarizing pulses with an amplitude of 20 mV (Fig. 4*A* and *C*). However, in most cases, this current was reduced in amplitude with time ('washout'; see Figs 5 and 6). In about 60% of cells, in which the A-current was stable, the A-current was reduced by up to 80% during the AOC (Fig. 4*A*), whereas in the remaining DVN it remained almost unaffected (Fig. 4*C*). Similar observations were made under voltage clamp conditions in cells in which anoxia did not affect I_m and g_m (not illustrated).

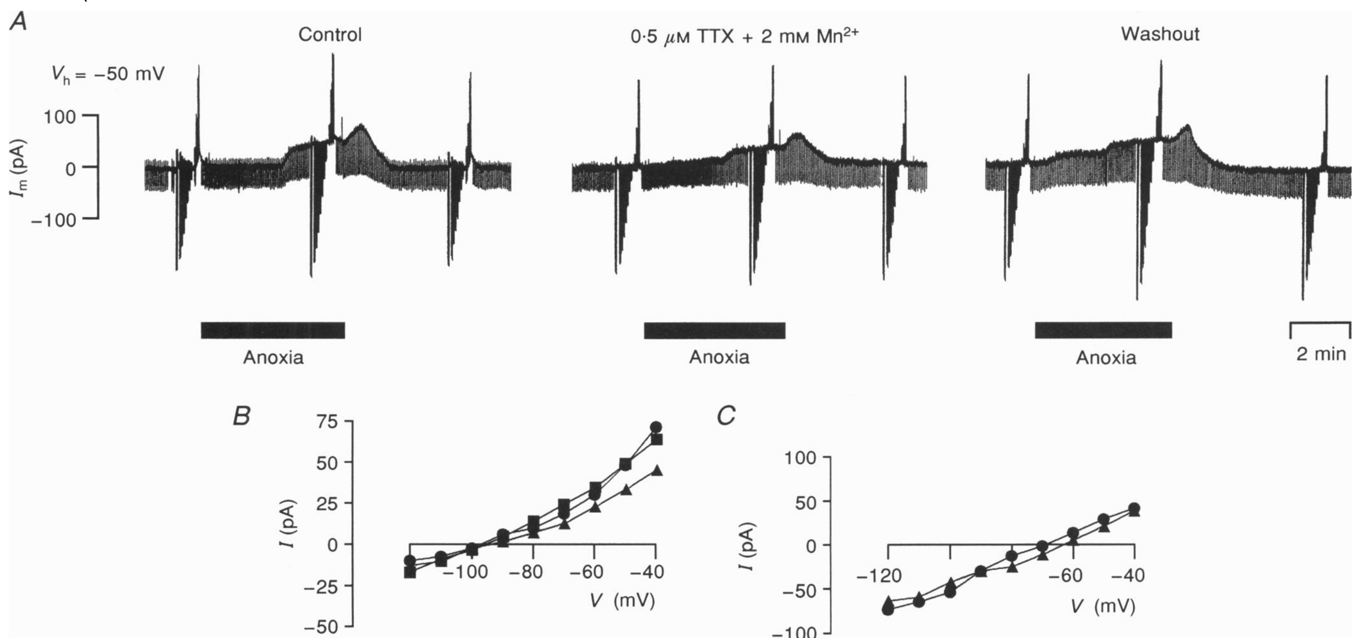

Figure 3. Anoxia-induced outward current during blockade of synaptic transmission or of neurotransmitter receptors

Blockade of synaptic transmission by 0·5 μM tetrodotoxin (TTX) and 2 mM Mn^{2+} did not affect the AOC and g_m increase (*A*) and its reversal potential (*I–V* relations of this cell (*B*) were obtained by subtraction of the control *I–V* curve from that during anoxia). AOC was measured under control conditions (●), during blockade of synaptic transmission (■), and after washout of the drugs (▲). Chart recordings in *A* were interrupted for 5 and 8 min, respectively. *C*, in a different cell, the *I–V* curve of the AOC (●) was unaffected by 0·5 μM TTX and blockade of glutamate and $GABA_A$ receptors with 25 μM cyanonitroquinoxaline (CNQX) and 100 μM bicuculline (▲).

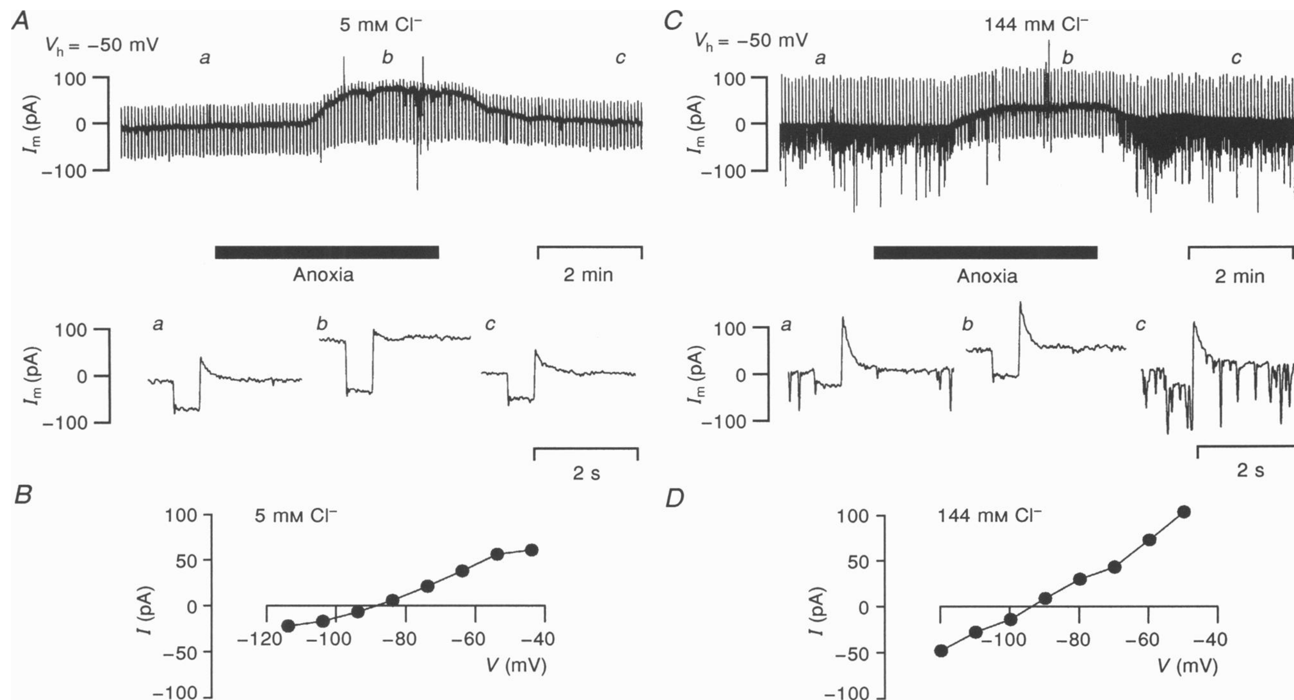

Figure 4. Effects of intracellular Cl^- on anoxia-induced outward current

The AOC (upper traces in *A* and *C*) and its reversal potential (see *I–V* relations of AOC in *B* and *D* as obtained by subtraction of the control *I–V* relation from that during anoxia) were almost identical in cells recorded with patch pipettes containing 5 or 144 mM Cl^-. The A-type K^+ current, revealed after termination of hyperpolarizing voltage steps, was almost blocked in the cell shown in *A*, whereas it was only slightly depressed in the cell shown in *C* (see lower traces with individual pulses on extended time scale corresponding to *a–c* in upper traces). Note that spontaneous synaptic noise which was observed using high-Cl^- patch pipette solution (*C*) was reduced during anoxia.

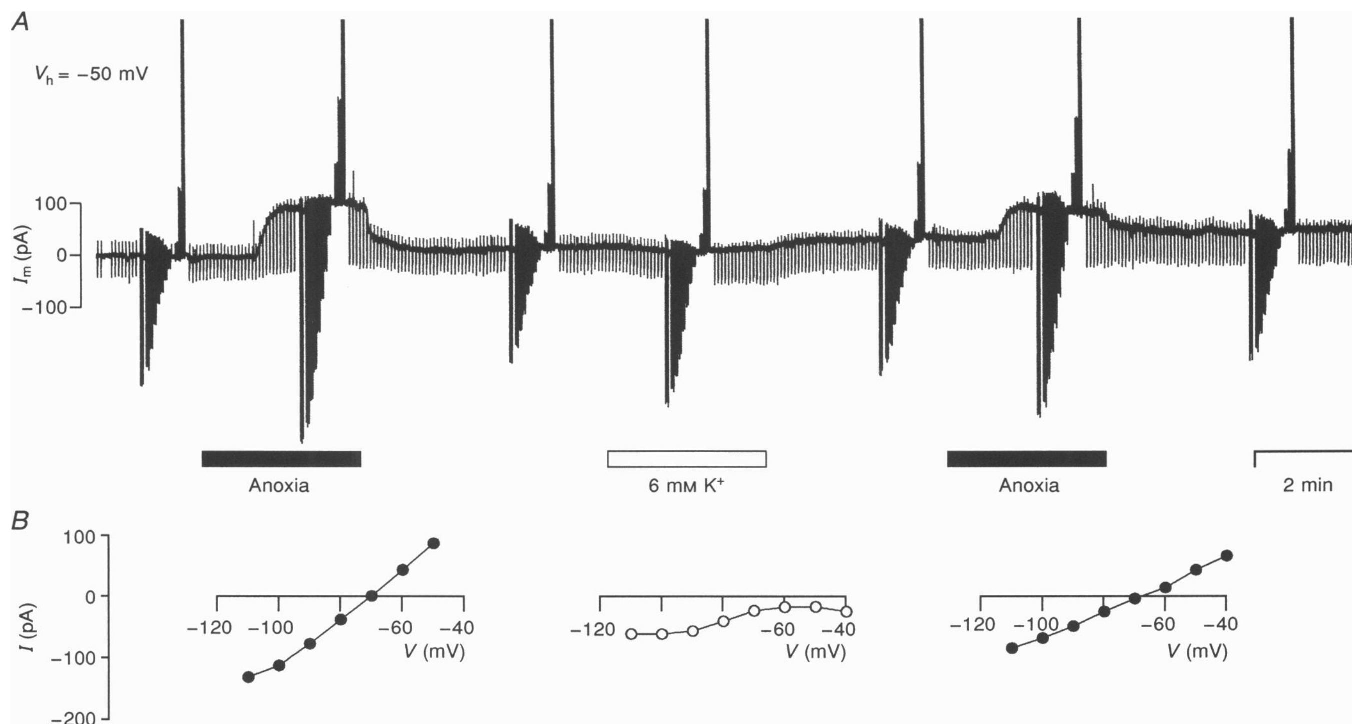

Figure 5. Membrane currents during anoxia and elevation of extracellular K^+

A, anoxia evoked the AOC and increase in g_m, whereas elevation of $[K^+]$ to 6 mM in the superfusion fluid elicited a small inward current and rise in g_m. *B* illustrates the corresponding *I–V* relations obtained by subtraction of the control curves from those upon anoxia (●) and elevated K^+ (○).

In a series of experiments, the effects of Cl^- on the AOC were tested using patch pipettes containing 144 mM instead of 5 mM Cl^-. The calculated equilibrum potentials for Cl^- (E_{Cl}) were +3·5 and −85·5 mV, respectively. In twelve of twenty-six cells analysed, an AOC with a kinetics of development, amplitude, increase in g_m and an E_{rev} (−79 ± 10 mV) was revealed which did not differ significantly ($P > 0·05$) from those of cells recorded with the low-Cl^- solution (Fig. 4). In some of these measurements with high-Cl^- patch electrodes, spontaneous inhibitory postsynaptic currents (IPSCs) were observed which reversed polarity at 0 to +5 mV ($n = 4$) and were blocked by 50 μM bicuculline. Such IPSC activity was typically reduced during anoxia as illustrated in Fig. 4*C*.

In nine experiments, the effects of anoxia on I_m were compared with those of elevation of the extracellular K^+ concentration to 6 mM, which corresponds to the maximal increase of extracellular K^+ observed in the dorsal vagal nucleus of conventional brainstem slices during anoxia (Ballanyi, Kuipers, Doutheil & Richter, 1992). As illustrated in Fig. 5, this led to an inward current of 33 ± 14 pA which was accompanied by an increase in g_m of between 15 and 23% (Fig. 5*A*).

The effects of elevation of the K^+ content of the superfusate to 10 mM on E_{rev} of the AOC were analysed in four further DVN. Superfusion of this solution led to an inward current with an amplitude of 91 ± 25 pA and an increase in g_m of between 20 and 33%. As illustrated in Fig. 6*A*, the AOC was apparently blocked under these conditions, although the typical anoxia-induced increase in g_m was still detectable. The *I–V* relations shown in Fig. 6*B* revealed that the apparent blockade was caused by a positive shift in E_{rev} of the AOC from −78 ± 8 to −55 ± 6 mV.

In the experiment illustrated in Fig. 6, the reduced amplitude of the AOC after return to normal K^+ concentrations indicates a 'washout' effect which was observed in 65% of the cells upon repetitive anoxic exposure. Such a reduction in the amplitude did, however, not affect E_{rev} of the AOC (cf. Fig. 5).

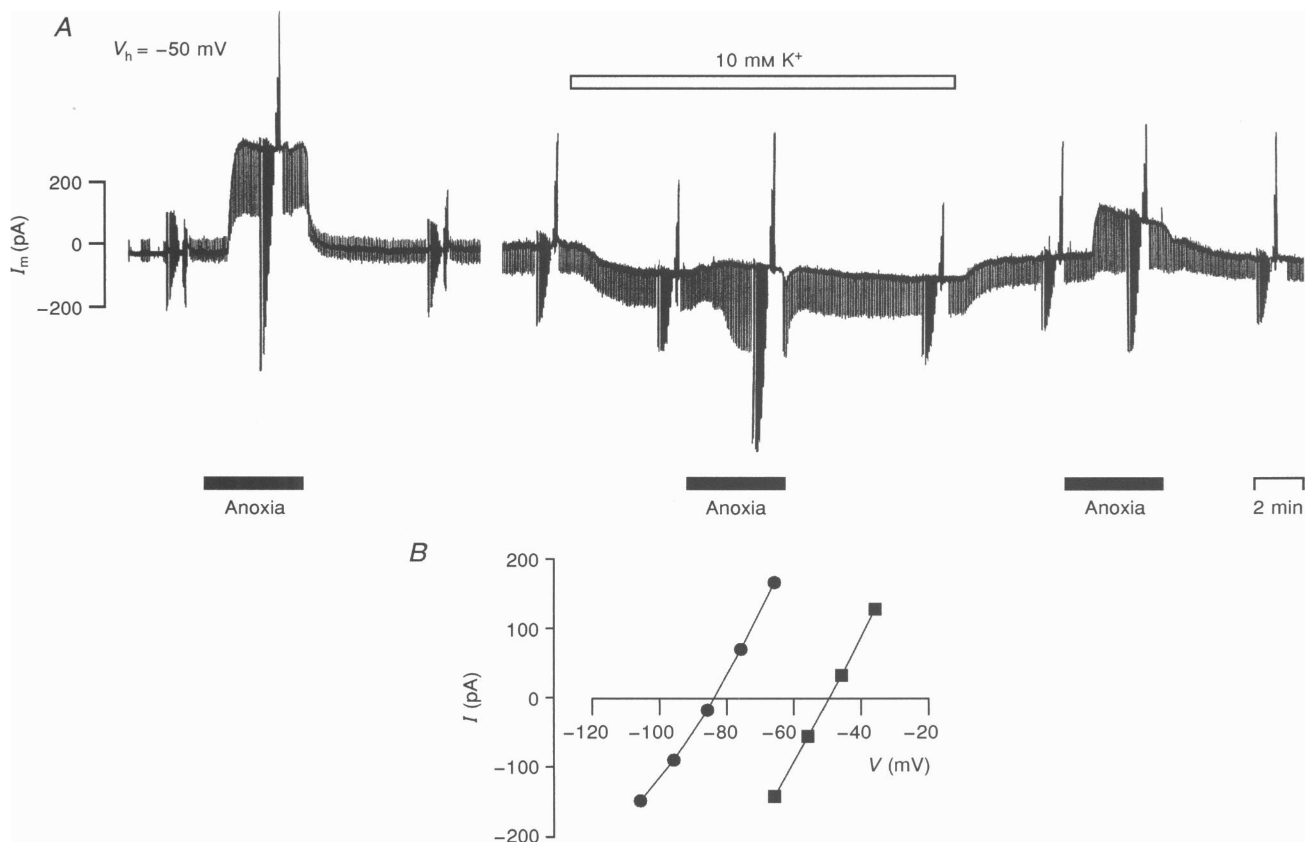

Figure 6. K^+ dependence of anoxia-induced outward current

A, both the AOC and the inward current upon elevation of $[K^+]$ of the superfusion fluid were accompanied by an increase in g_m. In the high-K^+ solution, AOC was apparently blocked whereas the concomitant increase in g_m was still detectable. Note that the AOC and rise in g_m after return to 3 mM K^+ were reduced in amplitude indicating a 'washout effect'. The gap in the trace indicates an interruption in recording of 8 min. *B*, *I–V* relations revealed a positive shift in the AOC along the voltage axis. ●, 3 mM K^+; ■, 10 mM K^+.

Effects of modulators of K_{ATP} channels on AOC

The experiments described so far suggest that the AOC occurs due to activation of a K^+ current. To test, whether this K^+ current might be caused by activation of K_{ATP} channels (see Introduction), the effects of modulators of these metabolism-regulated K^+ channels were analysed. Application of 100–200 μM of the K_{ATP} channel blocker tolbutamide (Ashcroft & Ashcroft, 1990, 1992) led, after 1–2 min, to a complete suppression of the AOC, and g_m returned to $2{\cdot}2 \pm 0{\cdot}83$ nS, which did not significantly ($P > 0{\cdot}05$) differ from control values. After such blockade of AOC, I_m and g_m remained stable during periods of anoxia of up to 15 min duration. The E_{rev} values of the AOC (see above) and of the tolbutamide-sensitive current (-80 ± 6 mV, $n = 11$) were almost identical (Figs 7 and 9). In twelve DVN, in which an AOC was revealed, bath application of 200 μM diazoxide, a K_{ATP} channel activator (Ashcroft & Ashcroft, 1990), led to a similar persistent outward current (E_{rev}, $-79 \pm 5{\cdot}5$ mV) and an increase in g_m. The kinetics of development of the diazoxide-induced current, however, was about 3-fold slower than that of the AOC and recovery time after washout of the drug was between 8 and 20 min (Fig. 8A). Similar to the AOC, the diazoxide-evoked outward current was blocked by 100 μM tolbutamide in four cells tested (Fig. 8A). In contrast, diazoxide did not affect I_m or g_m in seven cells, which did not respond with an AOC to oxygen depletion.

Relation between AOC and a spontaneous outward current

The effects of tolbutamide and diazoxide on the AOC resembled those of a spontaneously developing outward current mediated by activation of K_{ATP} channels in these cells (Trapp et al. 1994b). In the present study, such spontaneous outward currents with an E_{rev} of -80 ± 6 mV ($n = 14$) were observed in thirty DVN in addition to the 168 neurons in which I_m and g_m were stable (see above and Methods). Anoxia, when applied during the initial phase of the whole-cell recording in four of these cells, evoked an AOC which was almost identical to the steady-state spontaneous K_{ATP} current (Fig. 9). Similar to the AOC, the spontaneous outward current was completely blocked by 100–200 μM tolbutamide ($n = 13$) and not affected by

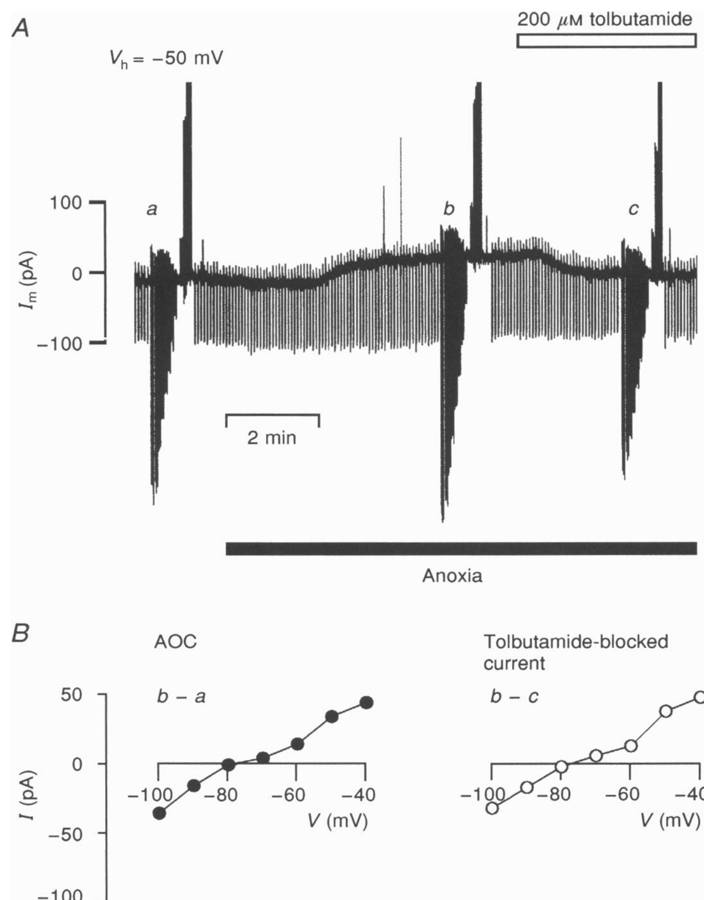

Figure 7. Sulphonyl urea-induced blockade of anoxia-induced outward current

A, AOC was completely blocked by bath application of 200 μM tolbutamide. *B*, left panel, *I–V* relation illustrating the AOC obtained by subtraction of currents ($b - a$) in *A*. Right panel, *I–V* relation illustrating the tolbutamide-sensitive current obtained by subtraction of currents ($b - c$) in *A*.

0·5 μM TTX, 25 μM CNQX and 100 μM bicuculline ($n = 4$; Fig. 9). After the spontaneous K_{ATP} current reached a steady state, oxygen depletion did not evoke an additional AOC or increase in g_m (not illustrated).

DISCUSSION

In the present study we report that a major proportion of dorsal vagal neurons (DVN) in thin brainstem slices of rats responds to superfusion of hypoxic solutions with a sustained outward current. This AOC causes hyperpolarization of the cells leading to suppression of spontaneous action potential discharges.

Suitability of thin slice patch clamp technique for analysis of AOC

The amplitudes and kinetics of the AOC (and the anoxia-induced hyperpolarization) were very similar to those recorded with fine-tipped microelectrodes from DVN in conventional 400 μm 'thick' brainstem slices (Ballanyi *et al.* 1992*a*; Doutheil *et al.* 1993; see also Cowan & Martin, 1992). In these previous studies, it was demonstrated with oxygen-sensitive microelectrodes that tissue P_{O_2} during exposure to hypoxic solutions falls to zero levels thus indicating tissue anoxia. The similarity of these responses to those in the present study using 'thin' slices (150 μm) suggests that superficial DVN are also under anoxic conditions.

One major difference from observations in 'thick' brainstem slices is the lack of occurrence of anoxic depolarizations (or underlying inward currents) in a proportion of those DVN in which no sustained AOC (or anoxia-related hyperpolarization) was revealed (Dean, Gallman, Zhu & Millhorn, 1991; Cowan & Martin, 1992; Donnelly, Jiang & Haddad, 1992; Doutheil *et al.* 1993). This was certainly not due to temperature difference, since in the present study responses to anoxia were not altered after increasing the *in vitro* temperature from 30 to 37 °C. It is possible that the anoxia-induced increase in extracellular K^+ could be larger in conventional slices (Ballanyi *et al.* 1992*b*; Donnelly *et al.* 1992) than in superficial layers of the thin brainstem slices. Accordingly, the depolarizations of DVN in 'thick' slices might be secondary to anoxic K^+ release. We could indeed show that DVN in the thin slices respond to elevation of the K^+ concentration of the superfusion fluid, mimicking the anoxic K^+ release (Ballanyi *et al.* 1992*b*) with inward currents which could cause a depolarization of several millivolts.

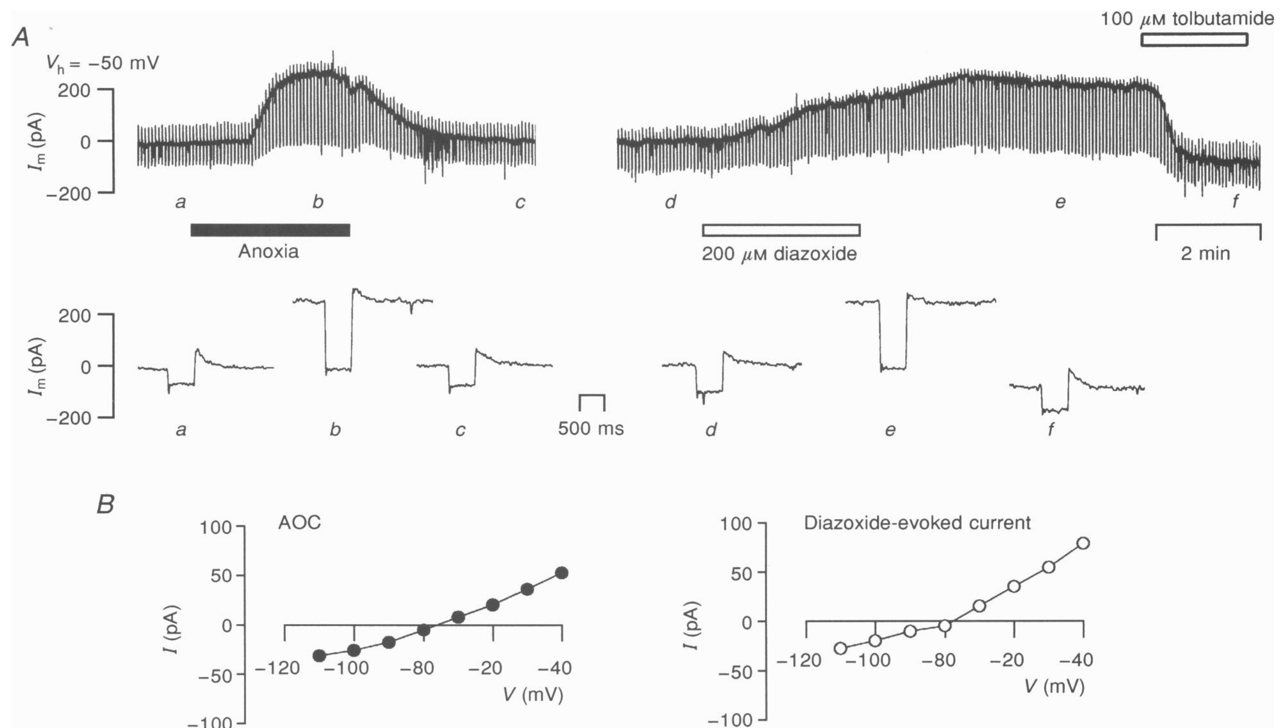

Figure 8. Anoxia- and diazoxide-induced outward currents

A, in this DVN the AOC and g_m increase were similar to those elicited by bath application of 200 μM diazoxide. The diazoxide-induced outward current which persisted after washout of the drug was effectively blocked upon addition of 100 μM tolbutamide. Lower traces show individual currents elicited by hyperpolarizing voltage steps (20 mV, 500 ms) corresponding to *a–f* in the upper traces, on an expanded time scale. Interruption in the recording was 15 min. *B*, in a different cell, almost identical *I–V* curves were measured during anoxia and diazoxide application.

At present we cannot exclude the possibility that diffusional exchange between the patch electrode and the cell interior (Pusch & Neher, 1988) might either provide intracellular ATP levels which are too high for activation of such inward currents (see below) or induce 'run down' or inactivation of ion channels responsible for these responses. Two observations, however, provide an argument against such a view: (i) preliminary results indicate that, under the experimental conditions of the present study, a progressive inward current develops in DVN during anoxic exposure after preincubation in glucose-free solutions (authors' unpublished observations) and (ii) an almost identical relative number of depolarizing, hyperpolarizing and non-responding DVN was found in conventional slices using both microelectrodes and patch electrodes (Ballanyi *et al.* 1992*a*; see also Cowan & Martin, 1992). These results are in contrast to recent findings in CA1 hippocampal neurons, where minor depolarizations were revealed with whole-cell recordings instead of hyperpolarizations found using fine-tipped microelectrodes (Zhang & Krnjevic, 1993). The observations that the probability of occurrence of the AOC in the DVN was not changed and that these responses were stable for periods of more than 15 min under the experimental conditions of the present study suggest that whole-cell patch clamp techniques are well suited for an analysis of the anoxia-induced hyperpolarization and the underlying outward current in these neurons.

Ionic mechanism and pharmacology of the AOC

Using the standard low-Cl^- patch pipette solution, the mean E_{rev} of the AOC was −78 mV, which is close to the value expected for the equilibrium potential for Cl^- (−82 mV). However, E_{rev} of the AOC was almost identical using high-Cl^- patch pipettes, which provides an E_{Cl} of between 0 and 5 mV as judged by the observed reversal of spontaneous Cl^--mediated IPSPs. This excludes the possibility that the AOC (and the anoxia-induced hyperpolarizations) might be due to activation of Cl^- channels. Accordingly, the AOC is most likely to be due to an increase in K^+ conductance, as was previously suggested for the initial hyperpolarization during oxygen depletion in hippocampal neurons (e.g. Hansen *et al.* 1982; Fujiwara, Higashi, Shimoji & Yoshimura, 1987; Leblond & Krnjevic, 1989). This view is supported by the positive shift of E_{rev} along the voltage axis upon elevation of $[K^+]_o$ from 3 to 10 mM. However, the mean values of E_{rev} of the AOC in

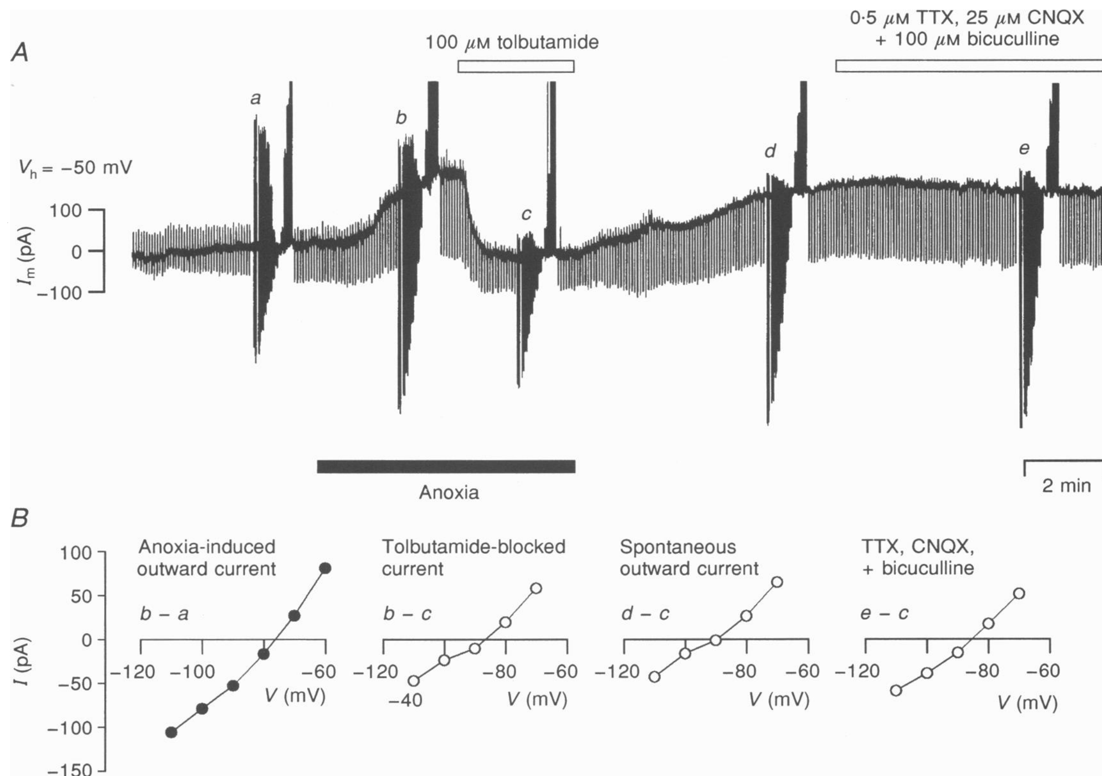

Figure 9. Spontaneous and anoxia-induced outward currents

A, at the beginning of recording, a spontaneous progressive outward current (I_m) and g_m increase developed. During this period, the typical AOC was revealed and effectively blocked by 100 μM tolbutamide. After washout of tolbutamide, I_m and g_m recovered to values similar to those observed during anoxia. Blockade of synaptic transmission and postsynaptic glutamate and $GABA_A$ receptors with 0·5 μM TTX, 25 μM cyanonitroquinoxaline (CNQX) and 100 μM bicuculline did not affect the steady-state spontaneous outward current. Curves in *B* were obtained by subtraction of *I–V* relations as indicated by letters corresponding to points on the trace in *A*.

both normal and high-K^+ solutions (−78 and −55 mV) are more positive than the values for the calculated equilibrium potential for K^+ (E_K) (−100 and −66 mV) although E_{rev} was close to −100 mV in individual cells (Fig. 3*B*). Such a variation could, for example, be due to local differences in the anoxia-induced release of K^+ from neighbouring neurons or glial cells, which would cause a positive shift of E_K. Furthermore, in some cells a small inward current, possibly carried by Ca^{2+}, is possibly activated in addition to the K^+ outward current as hypothesized by Cowan & Martin (1992). Whole-cell measurements in isolated DVN or measurements of single AOC K^+ channels are necessary to discriminate between these or other possible mechanisms.

Several arguments indicate that activation of K_{ATP} channels is responsible for the AOC in DVN. The AOC was effectively blocked by the hypoglycaemic sulphonylurea tolbutamide (Ashcroft & Ashcroft, 1990, 1992). Although recent evidence suggests that sulphonylureas are not exclusively selective for K_{ATP} channels (Ashcroft & Ashcroft, 1992; Crépel, Krnjevic & Ben-Ari, 1993; see below), the outward current induced by the K_{ATP} channel opener diazoxide (Ashcroft & Ashcroft, 1990), which was almost identical to the AOC, supports the assumption that K_{ATP} channels mediate the AOC. Similar antagonistic effects of modulators of K_{ATP} channels are found in an increasing number of neurons from different regions of the brain responding to energy depletion with a hyperpolarization (Mourre *et al.* 1989; Grigg & Anderson, 1989; Ashford, Boden & Treherne, 1990*a*,*b*; Röper *et al.* 1990; Luhmann & Heinemann, 1992; Murphy & Greenfield, 1992; Schwanstecher & Panten, 1994; see also Pierrefiche & Richter, 1994). These results confirm previous suggestions that the tolbutamide- and glibenclamide-sensitive spontaneous outward current of DVN, which was also revealed in a subpopulation of DVN of the present study after establishing the whole-cell configuration, is mediated by activation of K_{ATP} channels (Trapp *et al.* 1994*b*; see below).

Recent studies showed that tolbutamide-sensitive ATP inhibitable channels in substantia nigra neurons can be activated by elevation of intracellular Ca^{2+} (Jiang & Haddad, 1994; Jiang, Sigworth & Haddad, 1994). However, it is not likely that the AOC of DVN is due to activation of Ca^{2+}-activated K^+ (BK_{Ca}) channels, since these channels, but not K_{ATP} channels, are blocked by charybdotoxin and activated by ATP (for references, see Jiang & Haddad, 1994; Jiang *et al.* 1994). Our results do not confirm previous assumptions that the hyperpolarization (and the AOC) is due to enhancement of an outward current through K^+ channels of the delayed rectifier type (Cowan & Martin, 1992). However, it was recently suggested that the K_{ATP} channel, at least in some tissues, might be a dephosphorylated form of the delayed rectifier K^+ channel (Edwards & Weston, 1994) although it is widely accepted that these channels represent different entities (Ashcroft *et al.* 1994).

Mechanism of K_{ATP} channel activation

Our results show that in DVN spontaneous as well as anoxia-evoked activation of K_{ATP} channels can occur despite dialysing the cells with 2 mM ATP via the patch pipette ('high-Cl^- solution'). This finding is surprising, since single channel studies, mainly on pancreatic β-cells or muscle tissue, revealed that the activity of ('type 1') K_{ATP} channels is blocked by micromolar intracellular ATP concentrations ($[ATP]_i$; cf. Ashcroft & Ashcroft, 1990). However, activation of such K_{ATP} channels during ischaemia appears to occur prior to a fall in cellular ATP (cf. Ashcroft & Ashcroft, 1990; Terzic, Tung & Kurachi, 1994). This led to the assumption that the local physiological $[ATP]_i$ in the vicinity of the channels, which is relevant for regulation of these channels, might be two orders of magnitude lower than that of the bulk cytosolic solution (for references, see Benndorf, Bollmann, Friedrich & Hirche, 1992).

In this context, it was demonstrated for isolated myocytes that development of a spontaneous K_{ATP} outward current was delayed, but not blocked, during whole-cell recordings using patch pipettes containing 20 mM ATP (Belles, Hescheler & Trube, 1987). The authors hypothesized that ATP catabolism, providing such putative low submembraneous ATP levels, might not be sufficiently compensated by the slow diffusion of ATP from the patch pipette. Such a mechanism could possibly also explain development of the spontaneous K_{ATP} outward current, observed in a subpopulation of DVN of the present study. However, spontaneous activation of K_{ATP} channels was not observed during intracellular recordings using conventional fine-tipped microelectrodes which did not contain ATP and in which normoxic metabolism should provide submembraneous $[ATP]_i$ levels sufficient for blockade of the K_{ATP} channels (Ballanyi *et al.* 1992*a*; Doutheil *et al.* 1993). Since $[ATP]_i$ can be as high as 10 mM (cf. Ashcroft & Ashcroft, 1990), ATP diffusion into the patch pipette solution might occur, lowering the cytosolic (and also the submembraneous) levels thus leading to activation of the K_{ATP} channels. If this were true, the value for half-maximum inhibition (K_i) of K_{ATP} channels in DVN should be in the millimolar range, as was demonstrated for 'type 2' K_{ATP} channels in cortical and hypothalamic neurons (Ashford, Sturgess, Trout, Gardner & Hales, 1988; Ashford *et al.* 1990*b*).

However, a spontaneous K_{ATP} outward current did not principally develop in DVN which were recorded with 0·5 mM ATP-containing patch pipettes ('standard solution') and which responded to anoxia with opening of such metabolism-regulated K^+ channels. This could mean that in different types of DVN that serve distinct physiological functions (see below) 'type 1' or 'type 2' K_{ATP} channels

might be expressed. To answer this question, single channel studies are necessary to analyse the distribution and properties of (different types of) K_{ATP} channels in DVN. Recent findings that tolbutamide and glibenclamide can reduce the steady-state whole-cell current indicate that a proportion of K_{ATP} channels in DVN might be functional even during normoxia (Trapp *et al.* 1994*b*; see also Pierrefiche & Richter, 1994).

Whereas the opening of the K_{ATP} channels by diazoxide can be explained by a direct pharmacological modulation of the channel opening (Ashcroft & Ashcroft, 1990, 1992; Nichols & Gross, 1994) it is surprising that modification of the intracellular milieu by diffusional exchange via the patch pipette and by disturbance of metabolism (which should have distinct effects on the interior of the cell) have a similar activating effect on these K^+ channels. Accordingly, a large body of single channel data from excised patches suggests that cell constituents, in addition to $[ATP]_i$, are primarily involved in the regulation of K_{ATP} channel activity under physiological and pathophysiological conditions (cf. Ashcroft & Ashcroft, 1990; Nichols & Gross, 1994; Terzic *et al.* 1994). It was recently proposed for substantia nigra neurons that activation of K_{ATP} channels during metabolic disturbances requires the cooperative effects of a decrease in $[ATP]_i$ (and a concomitant increase of $[ADP]_i$), a rise in intracellular Ca^{2+} and depolarization of the cell membrane (Jiang *et al.* 1994; see also Haddad & Jiang, 1993). Indeed, it is well established that the ratio of $[ATP]_i/[ADP]_i$ rather than the absolute $[ATP]_i$ determines regulation of channel activity. In addition, other nucleoside diphosphates like UDP, GDP or CDP have also been demonstrated to activate K_{ATP} channels (cf. Terzic *et al.* 1994). This strongly suggests that in intact cells the degree of ATP blockade of K_{ATP} channels is determined by a complex set of cellular constituents. In line with that suggestion it has been demonstrated that high intracellular Cl^- levels lead to inhibition of K_{ATP} channels (McKillen, Davies, Stanfield & Standen, 1994). In the present study, we found that neither the kinetics, amplitude, E_{rev} or the accompanying g_m increase during the AOC or the spontaneous K_{ATP} current were significantly different using high- or low-Cl^- patch pipette solutions. This is in agreement with recent observations that the Cl^--induced blockade can be reversed by addition of Mg-GDP (Takano & Ashcroft, 1994). There is also evidence from studies on muscle tissues that lactate, produced during stimulation of anaerobic metabolism during anoxia or ischaemia, as well as the concomitant fall in intracellular pH can activate K_{ATP} channels by decreasing their sensitivity to inhibition by $[ATP]_i$ (for references, see Ashcroft & Ashcroft, 1990; Terzic *et al.* 1994). Although the persistence of the AOC during blockade of synaptic transmission and postsynaptic neurotransmitter receptors suggests a direct effect of anoxia on the DVN, it should be noted that non-synaptic release of neuroactive substances like adenosine or serotonin could, by activation of G protein-coupled receptors, indirectly activate the DVN K_{ATP} channels (cf. Terzic *et al.* 1994). Finally, the decrease of cellular O_2 could directly affect K_{ATP} channels, for example, by changing the redox state of amino acid residues (Jiang & Haddad, 1994). Further studies analysing the properties of K_{ATP} channels in solutions imitating the intracellular milieu and energy state will hopefully point out, in more detail, the links responsible for activation of these channels during metabolic disorders.

Functional role of K_{ATP} channels in DVN

After tolbutamide blockade of the AOC, I_m and g_m remained stable and an anoxia-related inward current did not develop subsequently as analysed for periods of anoxia of 15 min duration. This indicates that activation of K_{ATP} channels in these brainstem cells does not primarily have a protective function by delaying development of a progressive anoxia-induced depolarization as has been suggested for medullary hypoglossal motoneurons (Jiang, Ying & Haddad, 1992; see also above). It was found that DVN in conventional brainstem slices respond to removal of glucose with a stable hyperpolarization and blockade of spontaneous action potential discharges (Ballanyi *et al.* 1992*a*). This response is almost identical to that found during anoxia, suggesting that opening of K_{ATP} channels is also responsible for the hyperpolarization upon glucose depletion. Activation of K_{ATP} channels in glucose-sensing neurons of the ventromedial hypothalamus has been demonstrated to play an important role in appetite control (Ashford *et al.* 1990*a*, *b*). We speculate whether DVN might represent a further type of central glucose sensor (see also Röper *et al.* 1990). Since early observations that puncture lesions of the medulla oblongata caused diabetes mellitus (Bernard, 1855) it has been assumed that this brain region is involved in the control of carbohydrate metabolism. Accordingly, it was found that electrical stimulation of the lateral regions of the dorsal vagal motonucleus resulted in insulin secretion (Laughton & Powley, 1987). It remains to be determined whether particular neurons in this subregion of the dorsal vagal motonucleus possess functional K_{ATP} channels and whether a hyperpolarization-induced functional inactivation of these cells, innervating β-cells of the pancreas, affects glucose levels in the brain.

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