Intracellular Sodium and Calcium Homeostasis During Hypoxia in Dopamine Neurons of Rat Substantia Nigra Pars Compacta

EZIA GUATTEO,1 NICOLA B. MERCURI,1,2 GIORGIO BERNARDI,1,2 AND THOMAS KNÖPFEL1,3
1Department of Pharmacology, Istituto di Ricoovero e Cura a Carattere Scientifico, Ospedale S. Lucia, 00179 Rome; 2Clinica Neurologica, Università di Tor Vergata, 00133 Rome, Italy; and 3Laboratory for Neuronal Circuit Dynamics, Brain Science Institute, The Institute of Physical and Chemical Research, Saitama 351-0198, Japan

Guatteo, Ezia, Nicola B. Mercuri, Giorgio Bernardi, and Thomas Knöpfel. Intracellular sodium and calcium homeostasis during hypoxia in dopamine neurons of rat substantia nigra pars compacta. J. Neurophysiol. 80: 2237–2243, 1998. We investigated the hypoxia-induced disturbance of cytosolic sodium concentration ([Na+]i) and of cytosolic calcium concentration ([Ca2+]i) in dopamine neurons of the substantia nigra pars compacta in rat midbrain slices, by combining whole cell patch-clamp recordings and microfluorimetry. Transient hypoxia (3–5 min) induced an outward current (118.7 ± 15.1 pA, mean ± SE; Vm = −60 mV). The development of this outward current was associated with an elevation in [Na+]i, and in [Ca2+]i. The hypoxia-induced outward current as well as the elevations in [Na+]i, and [Ca2+]i, were not affected by the ionotropic and metabotropic glutamate receptor antagonists d-amino-phosphonovalerate (50 μM), 6-nitro-7-sulfamoyl-benzo[ f ]quinoxaline-2,3-dione (10 μM) and S-(α)-methyl-4-carboxyphenylglycine (500 μM). Tolbutamide, a blocker of ATP-dependent K+ channels, depressed the hypoxia-induced outward current but did not affect the increases in [Na+]i or [Ca2+]i. Increasing the concentration of ATP in the internal solution from 2 to 10 mM strongly reduced the hypoxia-induced outward current but did not reduce the rise in [Na+]i. Decreasing the concentration of extracellular Na+ to 19.2 mM depressed the hypoxia-induced outward current and resulted in a decrease in resting [Na+]i. Under this condition hypoxia still increased [Na+]i, albeit to levels not exceeding those of resting [Na+]i observed under control conditions. We conclude that 1) a major component of the hypoxia-induced outward current of these cells is caused by a depletion of intracellular ATP in combination with an increase in [Na+]i, 2) that the [Na+]i, and [Ca2+]i, responses are not mediated by glutamate receptors, 3) that the [Na+]i, and [Ca2+]i, responses are not depressed by activation of sulfonylurea receptors, and 4) that the rise in [Na+]i, induced by short-lasting hypoxia is not due to a ATP depletion-induced failure of Na+ extrusion.

INTRODUCTION

Mammalian neurons react rapidly to a lack of oxygen supply. This reaction is a consequence of their extraordinary high energy consumption together with a limited reservoir of energy-rich metabolites. It is well documented that different neuron types exhibit distinct initial responses to hypoxia. Thus, in the majority of neurons studied, hypoxia induces an initial membrane hyperpolarization that might be followed, after a characteristic period, by a depolarization.

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current and changes in $[Na^+]$, and $[Ca^{2+}]$, and to address the above issues.

METHODS

Electrophysiology

Preparation of rat midbrain slices was performed as described previously (Lacey et al. 1989; Mercuri et al. 1995). In brief, horizontal slices including the substantia nigra and the ventral tegmental area were cut from the ventral mesencephalon of Wistar rats (150–250 g) anesthetized with halothane and killed. The brain was removed, and horizontal slices (300 μm thick) were cut by a vibratome starting from the ventral surface of the midbrain. A single slice was then transferred into a recording chamber and completely submerged in an artificial cerebrospinal fluid with continuously flowing (2.5 ml/min) solution at 35–36°C (pH 7.4). This solution contained (in mM) 126 NaCl, 2.5 KCl, 1.2 MgCl$_2$, 1.2 NaHPO$_4$, 2.4 CaCl$_2$, 10 glucose, and 18 NaHCO$_3$, gassed with 95% O$_2$-5% CO$_2$. In a series of experiments, an extracellular solution containing 19.2 mM Na$^+$ was used. This solution was prepared by equimolar substitution of NaCl by choline chloride with the addition of 3 μM scopolamine to prevent activation of muscarinic receptors.

The recording chamber was mounted on the stage of an upright microscope (AxioScope, Carl Zeiss) equipped for infrared video microscopy (Hamamatsu, Japan) and video microfluorometry (Im- proVision). Whole cell patch-clamp recordings were obtained from visually identified dopamine neurons (Bonci and Williams 1996) using pipettes made from borosilicate glass (WPI, 1.5 mm) and pulled with a PP 88 Narishige puller. The resistance of the pipette was ~4 MΩ when filled with the standard pipette solution containing (in mM) 145 K$^+$ gluconate or K$^+$ methysulfate, 0.1 CaCl$_2$, 2 MgCl$_2$, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 0.75 ethylene glycol-bis-(β-aminoethyl)ether-N,N,N’,N’-tetraacetic acid (EGTA), 2 Mg-ATP, and 0.3 Na,GTP, pH 7.3. For microfluorometry (see section entitled Microfluorometry), 250 μM sodium-binding benzofuran (SBFI) or 250 μM fura-2 (both Moleular Probes) were added to the pipette solution.

The membrane voltage and current were acquired using pClamp and Axoscope software (Axon Instruments) or with the MacLab analog/digital interface and Chart software (AD Instruments).

Microfluorometry

Fluorescence of neurons loaded either with SBFI or fura-2 was excited via a x40 water immersion objective (Olympus) by epi-illumination with light provided by a 75-W Xenon lamp band-pass filtered alternatively at 340 or 380. Emissiion light passed a barrier filter (510 nm) and was detected by a charge-coupled device (CCD) camera (Photonic Science). Pairs of 340- and 380-nm images were acquired at 12-s intervals and analyzed off-line with a software (IonVision, ImproVision) running on a PowerMac 8100. Ratio images were calculated from pairs of 340- and 380-nm images corrected for background fluorescence (measured from image regions free of dye fluorescence). Time courses of fluorescence ratio values were calculated from regions that included the cell bodies (“regions of interest,” defined as those pixels that exhibit at least 20–30% of maximal specific fluorescence) and were transformed into ion concentration using the method of Grynkiewicz (Grynkiewicz et al. 1985). The calibration parameters $R_{max}$ and $R_m$ (Grynkiewicz et al. 1985) for the transformation of SBFI and fura-2 signals were obtained in situ by bathing perforated cells (1 μM nystatin/gramicidin D or 1 μM ionomycin, respectively) in Na$^+$-free or Ca$^{2+}$-free (1 mM EGTA) and in 140 mM Na$^+$ and 1 mM Ca$^{2+}$, respectively, containing solution. In three perforated neurons, calibration parameters for the transformation of SBFI were obtained in situ by bathing perforated cells in solutions containing varying extracellular Na$^+$ concentrations (Na$^+$ substituted by K$^+$). The apparent $K_d$ value (Grynkiewicz et al. 1985) for fura-2 was estimated by measuring fluorescence ratios obtained from solution containing fura-2 and known concentrations of free calcium (Fura-2 Calcium Imaging Calibration Kit, Molecular Probes) trapped between two coverslips spaced by pieces of coverslips and imaged by the water immersion objective. A corresponding approach was also employed to estimate the $K_d$ of SBFI with calibration solution containing 1 mM EGTA, 0.1 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM HEPES, 0–145 mM NaCl and 145–0 mM potassium gluconate ([K$^+]$ + [Na$^+]$ = 145 mM, pH 7.4). This in vitro apparent $K_d$ was consistent with the $K_d$ estimated from perforated cells.

Values in the text are expressed as means ± SE. Student’s t-test was used for statistical analysis.

Drug application

The following drugs were used: dopamine hydrochloride, tolbutamide (Sigma), N,N,N,N’-tetraethylamide (n-APV), 6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), and S-(α)-methyl-4-carboxyphenylglycine (S-MCPG; all Tocris Cookson, Bristol, UK). Stock solutions of tolbutamide were made in dimethyl sulfoxide and diluted to the appropriate concentrations. The vehicle had no effect when applied alone. Drugs were bath-applied by bath-supplying the solution to one containing known concentrations of drugs. In all figures horizontal bars labeled “hypoxia” indicate time during which the superfusion was switched to a solution equilibrated with 95% N$_2$-5% CO$_2$. An exchange of the solution in the recording chamber occurred in ~1 min.

RESULTS

The database was derived from 75 visually and electrophysiologically identified dopamine neurons of the substantia nigra pars compacta. Forty-three of these cells were patch clamped with pipettes containing SBFI to measure $[Na^+]$, and the remaining portion of the population were neurons loaded with fura-2 to determine $[Ca^{2+}]$. The criteria for electrophysiological identification were, as described previously, a hyperpolarizing response to dopamine (30 μM) and a pronounced hyperpolarization-induced slowly relaxing inward current, $I_h$ (Grace and Onn 1989; Lacey et al. 1989; Mercuri et al. 1993, 1995; Yung et al. 1991). Cells were visually identified by their fusiform cell body and long unbranched proximal dendrites extending in the plane of the slice (Grace and Onn 1989).

Transient hypoxia induces an outward current and elevations in $[Na^+]$, and $[Ca^{2+}]$.

Cells were exposed to hypoxia by switching the control solution to one depleted of O$_2$ for 3–5 min. After ~1 min of hypoxia, the cells invariably developed an outward current amounting 118.7 ± 15.1 pA (mean ± SE, $n = 39$). Figure 1, A and B, shows the responses to hypoxia of two cells, one loaded with SBFI and the other loaded with fura-2. With the onset of the hypoxic outward current, the level of $[Na^+]$, started to increase and rose in parallel with the outward current until return to control solution (Fig. 1, A and C). Hypoxia induced also a rise in $[Ca^{2+}]$, which onset typically was more abrupt than that of the outward current and elevation of $[Na^+]$ (Figs. 1B, 2B, and 4). During 4 min of
Hypoxic \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\) Changes in Dopamine Neurons

Glutamate antagonists and compared with control responses obtained before application of the cocktail and after wash out of the cocktail. The cocktail contained 10 \(\mu\text{M}\) NBQX and 50 \(\mu\text{M}\) D-APV, blockers of ionotropic glutamate receptors (Collingridge and Lester 1989), and 500 \(\mu\text{M}\) S-MCPG, a broad-spectrum antagonist of metabotropic glutamate receptors (Watkins and Collingridge 1994). Neither the hypoxic outward current nor the \([\text{Na}^+]\), or \([\text{Ca}^{2+}]\), signals were affected by these glutamate antagonists.

Thus the hypoxic current in the presence of the cocktail was 96.2 \(\pm\) 17.2\% of the current measured under control conditions. In the presence of the cocktail, resting \([\text{Na}^+]\), hypoxia, \([\text{Na}^+]\), and \([\text{Ca}^{2+}]\), increased from basal values of 13.5 \(\pm\) 0.9 mM and 32.7 \(\pm\) 5 nM to 19.7 \(\pm\) 1.3 mM \((n = 15)\) and 121.4 \(\pm\) 14.6 nM \((n = 9)\), respectively. After termination of the transient \(O_2\) deprivation, the outward current and the changes in \([\text{Na}^+]\), and \([\text{Ca}^{2+}]\), reversed to resting levels. In some cells, there was a clear posthypoxic outward current (Figs. 2A, 3B, 4A, and 5A), whereas in other cells such current was not obvious (Figs. 1, A and B, 2B, and 3, A and C).

**Hypoxia-induced elevations in \([\text{Na}^+]\), and \([\text{Ca}^{2+}]\), are not due to activation of glutamate receptors**

It is conceivable that the elevations of \([\text{Na}^+]\), or \([\text{Ca}^{2+}]\), during transient hypoxia are mediated by ionotropic glutamate receptors or caused by the activation of metabotropic glutamate receptors. To test for this possibility, hypoxic responses were determined in the presence of a cocktail of glutamate antagonists and compared with control responses obtained before application of the cocktail and after wash out of the cocktail. The cocktail contained 10 \(\mu\text{M}\) NBQX and 50 \(\mu\text{M}\) D-APV, blockers of ionotropic glutamate receptors (Collingridge and Lester 1989), and 500 \(\mu\text{M}\) S-MCPG, a broad-spectrum antagonist of metabotropic glutamate receptors (Watkins and Collingridge 1994). Neither the hypoxic outward current nor the \([\text{Na}^+]\), or \([\text{Ca}^{2+}]\), signals were affected by these glutamate antagonists.

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and the hypoxia-induced [Na\(^+\)] increase were 102.4 ± 4.8% and 115.4 ± 11.2% of control values (n = 4), respectively. The corresponding values for resting and hypoxia-induced [Ca\(^{2+}\)] were 102.2 ± 13.7% and 115.3 ± 7.7% of control values (n = 5), respectively.

**Tolbutamide inhibits the hypoxia-induced outward current but not the associated disturbances in ion homeostasis**

The sulfonylurea tolbutamide efficiently depresses the hypoxia-induced outward current (Guatteo et al. 1998; Jiang et al. 1994). Sulfonylureas are well known to block ATP-inhibitable K\(^+\) channels (Ashcroft and Ashcroft 1992; Edward and Weston 1993), but their receptor is an independent protein, which might associate also with other membrane proteins (Ammala et al. 1996). The depression of the hypoxic outward current by sulfonylureas does, therefore, not unequivocally prove that ATP-inhibitable K\(^+\) channels mediate this current. This possibility prompted us to test whether the [Na\(^+\)], or [Ca\(^{2+}\)], signals were affected by tolbutamide. Tolbutamide (100 \(\mu M\)) reversibly reduced the hypoxic outward current to 10.2 ± 6.9% of control (n = 17, Fig. 3, A and D). Tolbutamide did, however, affect neither the hypoxia-induced elevation in [Na\(^+\)], nor that in [Ca\(^{2+}\)] (Fig. 3, B, C, and E, and Fig. 4). In the presence of tolbutamide, resting [Na\(^+\)], and the hypoxia-induced [Na\(^+\)], increase were 99.2 ± 2.3% and 106 ± 8.1% of control values (n = 7), respectively. The corresponding values for resting and hypoxia-induced [Ca\(^{2+}\)], were 107.9 ± 16.3% and 102.2 ± 12.6% (n = 3), respectively.

It might be noted that in many cells depression of the hypoxic outward current by the sulfonylurea uncovered an hypoxic inward current (Figs. 3C and 4) and isolated a posthypoxic outward current (Figs. 3B, 4, and 5) (Mercuri et al. 1994a).

**Addition of 10 mM ATP to the intracellular solution prevents the induction of the hypoxia-induced outward current but does not prevent the rise in [Na\(^+\)].**

Addition of 10 mM ATP to the intracellular solution efficiently prevents the development of the hypoxic outward current, supporting the conclusion that this current is generated by ATP-inhibitable K\(^+\) channels (Guatteo et al. 1998). Similarly, the accompanying elevation in [Na\(^+\)], could result from a failure of ATP-dependent extrusion mechanisms caused by a depletion of ATP. We found, however, that an increased concentration of ATP in the patch pipette did not prevent the hypoxia-induced rise in [Na\(^+\)], (Fig. 5). Thus, with 10 mM ATP in the pipette, the outward current mea-
HYPOXIC \([\text{Na}^+]\) AND \([\text{Ca}^{2+}]\) CHANGES IN DOPAMINE NEURONS

Previous work has revealed that brief hypoxia induces an outward current in dopamine-sensitive neurons of the rat substantia nigra pars compacta. The hypoxic outward current recorded under whole cell patch-clamp conditions is essentially generated by an ATP-dependent and sulfonylurea-sensitive \(K^+\) conductance (Guateo et al. 1998). Recordings obtained with sharp microelectrodes revealed an additional, sulfonylurea-insensitive (and charybotoxin-sensitive) component of the hypoxia-induced outward current (Guateo et al. 1998).

In the present work we report that brief hypoxia causes reversible elevations in \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\) in these cells. Elevations in \([\text{Ca}^{2+}]\), and/or \([\text{Na}^+]\), have been implicated as triggers for hypoxia-induced neuronal death (Choi 1995; Friedman and Haddad 1994b; Goldberg et al. 1986). Identification of the mechanisms underlying the hypoxic \([\text{Na}^+]\) and/
or Ca\(^{2+}\) influx might provide a basis for the design of a therapeutic intervention.

We observed that hypoxia increase [Ca\(^{2+}\)], and [Na\(^{+}\)] at the level of the cell body under conditions of voltage clamp in the whole cell patch-clamp configuration. We can therefore exclude the possibility that voltage-activated Ca\(^{2+}\) and Na\(^{+}\) channels mediate the hypoxic changes in [Ca\(^{2+}\)], and [Na\(^{+}\)], respectively.

Both ionotropic and group I metabotropic glutamate receptors mediate Na\(^{+}\)-dependent inward currents in these neurons (Mercuri et al. 1993, 1996). We found, however, that neither the rise in [Na\(^{+}\)], nor the outward current was affected by blocking these glutamate receptors. We also reasoned that the increase in [Na\(^{+}\)], was caused by a failure of ATP-dependent Na\(^{+}\) extrusion mechanisms following a hypoxia-induced fall in intracellular ATP concentration. Addition of 10 mM ATP into the recording pipette prevented the generation of the hypoxic outward current and hence, at least, the fall in ATP concentration during brief hypoxia. However, the supply of ATP via the pipette did not prevent the hypoxic rise in [Na\(^{+}\)]. We therefore conclude that the hypoxia-induced elevation in [Na\(^{+}\)], is not due to an ATP depletion-induced failure of Na\(^{+}\) extrusion. On the other hand, we cannot exclude an impairment of Na\(^{+}\) extrusion due to factors other than ATP and that are associated with the response to hypoxia. In contrast to the hypoxic outward current, the hypoxia-induced increase in [Na\(^{+}\)], and [Ca\(^{2+}\)], were both not affected by tolbutamide, demonstrating that these disturbances in ion homeostasis are not regulated by sulfonylurea-sensitive receptors. We also wondered whether the hypoxic [Ca\(^{2+}\)], elevation was mediated by glutamate receptors and found that this was not the case.

The striking similarity in time course of the hypoxic sodium and calcium elevations is consistent with the hypothesis that the K\(^{+}\) channels mediating this current are regulated by [Na\(^{+}\)]. (Egan et al. 1992). This hypothesis is supported by two additional observations. In agreement with a previous study employing sharp microelectrode techniques (Mercuri et al. 1994b), we found that the generation of the hypoxia-induced outward current was almost completely prevented when the cells were bathed in an extracellular solution containing a reduced Na\(^{+}\) concentration. With reducing the concentration of extracellular Na\(^{+}\) from 145.2 to 19.2 mM, hypoxia still increased [Na\(^{+}\)], with respect to a reduced resting [Na\(^{+}\)]. However, under this condition the hypoxia-induced elevation in [Na\(^{+}\)], did not exceed the level of resting [Na\(^{+}\)], measured under control conditions and would, therefore, not reach the threshold for activation of K\(^{+}\) channels activated [Na\(^{+}\)], levels above the normal resting [Na\(^{+}\)].

As discussed above, the hypoxic outward current is blocked by sulfonylureas and prevented if a high ATP concentration is supplied via the patch pipette, suggesting that the hypoxic outward current is both ATP and Na\(^{+}\) dependent as well as regulated by sulfonylurea receptors. Indeed, a potassium conductance, which is sensitive to sulfonylureas and induced by a large elevation in [Na\(^{+}\)], has been recently reported in substantia nigra neurons (Seutin et al. 1996).

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Address for reprint requests: T. Kaupfél, Laboratory for Neuronal Circuit Dynamics, Brain Science Institute, The Institute of Physical and Chemical Research, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan.

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