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Anoxia-Induced Dopamine Release from Rat Striatal Slices: Involvement of Reverse Transport Mechanism

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Abstract: Incubation of rat striatal slices in the absence of oxygen (anoxia), glucose (aglycemia), or oxygen plus glucose (ischemia) caused significant increases in dopamine (DA) release. Whereas anoxia decreased extracellular 3,4-dihydroxyphenylacetic acid levels by 50%, aglycemia doubled it, and ischemia returned this aglycemia-induced enhancement to its control level. Although nomifensine, a DA uptake blocker, completely protected the slices against anoxia-induced DA depletion, aglycemia- and ischemia-induced increases were not altered. Moreover, hypothermia differentially affected DA release stimulated by anoxia, aglycemia, and ischemia. Involvement of glutamate in DA release induced by each experimental condition was tested by using MK-801 and also by comparing the glutamate-induced DA release with that during anoxia, aglycemia, or ischemia. MK-801 decreased the anoxia-induced DA depletion in a dose-dependent manner. This treatment, however, showed a partial protection in aglycemic conditions but failed to improve ischemia-induced DA depletion. Like anoxia, DA release induced by exogenous glutamate was also sensitive to nomifensine and hypothermia. These results indicate that anoxia enhances DA release by a mechanism involving both the reversed DA transporter and endogenous glutamate. Partial or complete lack of effect of nomifensine, hypothermia, or MK-801 in the absence of glucose or oxygen plus glucose also suggests that experimental conditions, such as the degree of anoxia/ischemia, may alter the mechanism(s) involved in DA depletion. **Key Words:** Dopamine release—Anoxia—Reverse transport—Aglycemia—Ischemia—Glutamate.
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As brain critically depends on its blood flow for a continuous supply of oxygen and glucose, a reduction in one or both of them causes a cascade of biochemical events and eventually leads to neuronal death. Results from in vivo and in vitro studies indicate that ischemia- and/or anoxia-induced enhancements in neurotransmitter release and subsequent activation of postsynaptic receptors are important processes in developing neuronal damage (for reviews, see Choi, 1990; Martin et al., 1994; Szatkowski and Attwell, 1994). Although accumulating evidence suggests that increases in extracellular glutamate concentrations and overstimulation of NMDA receptors by glutamate appear to play a major role in ischemia-induced neuronal damage (Martin et al., 1994; Szatkowski and Attwell, 1994), other endogenous neurotransmitters, especially dopamine (DA) and serotonin, also seem to be involved. In support of this conclusion, a massive DA release can be seen repeatedly during ischemic conditions (Globus et al., 1988; Akiyama et al., 1991; Richards et al., 1993; Santos et al., 1996; Toner and Stamford, 1997*a–c*), and strategies that attenuate ischemia-induced DA release (Weinberger and Cohen, 1983; Weinberger et al., 1985; Globus et al., 1987; Clemens and Phebus, 1988; Buisson et al., 1992) or block dopaminergic receptors (Globus et al., 1989) have been shown to be potentially neuroprotective.

One of the most obvious effects of anoxia and/or ischemia is rapid depletion of intracellular ATP levels (Whittingham et al., 1984; Santos et al., 1996; see also reviews by Schurr and Rigor, 1989; Martin et al., 1994). Because this endogenous substrate is required by the sodium pump as well as for vesicular uptake, a reduction of intracellular ATP levels leads to a decrease of the Na^+ gradient by inactivating Na^+,K^+-ATPase and may also enhance cytoplasmic free DA levels by inhibiting vesicular DA uptake. As a decrease or reversal of the electrochemical Na^+ gradient and an increase in the cytoplasmic concentration of DA facilitate the reverse transport mechanism (Adam-Vizi, 1992; Levi and Raiteri, 1993), it is likely that reversed transport of DA might also contribute to ischemia/anoxia-induced DA depletion as a secretory mechanism, and its blockade might be also neuroprotective. In support of this conclusion, in PNS (Schomig et al., 1987; Russ et al., 1991; Kurz et al., 1995, 1996) and CNS (Kim et al., 1998) ischemia has been shown to enhance carrier-mediated norepinephrine release. Although in CNS blockade of DA uptake by nomifensine is able to abolish any hypoxia-induced in-

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Abbreviations used: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid.

crease in extracellular DA levels measured by in vivo microdialysis, this effect has not been attributed to blockade of the reverse transport mechanism (Akiyama et al., 1991). Results derived from in vitro striatal slices, on the other hand, indicated that the hypoxia/hypoglycemia-induced DA release does not result from reversal of the uptake carrier (Toner and Stamford, 1997*a*). In contrast to these results, it has been reported that nomifensine decreases ischemia-induced [^{3}H]DA depletion from rat striatal slices (Kim et al., 1995), suggesting a possible involvement of the reverse transport mechanism in ischemic conditions.

Although results derived from CNS and PNS seem to be contradictory, it is possible that differences among the experimental conditions, such as the degree of hypoxia, hypoglycemia, or ischemia used in these studies, may also alter the mechanism involved in DA depletion. In the present study, we reinvestigated the mechanism of anoxia-induced DA release from rat striatal slices and compared it with those of aglycemia- or aglycemia plus anoxia-induced DA depletion. Results presented in this study suggested that reversal of the uptake carrier is a significant contributor to anoxia-induced DA release. Involvement of this mechanism in DA release induced by omission of glucose or oxygen plus glucose seems unlikely.

MATERIALS AND METHODS

Materials

Ouabain was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Glutamate, MK-801, and nomifensine were purchased from Research Biochemicals International (Natick, MA, U.S.A.). [^{3}H]DA was obtained from Amersham, U.K. Other chemicals were pure analytical grade and obtained from Merck KGaA (Darmstadt, Germany).

Preparation and incubation of striatal slices

Male and female Sprague–Dawley rats (weighing 250–300 g and obtained from Experimental Animals Breeding and Research Center, Bursa, Turkey) were used. All experimental protocols were approved by the Uludağ University Medical Center Institutional Review Board for animal research.

Rats were killed by decapitation, and brains were removed quickly and placed in cold oxygenated physiological medium with 95% O_2 and 5% CO_2 (in mmol/L: 120 NaCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 3.5 KCl, 25 $NaHCO_3$, and 10 glucose). This medium also contained ascorbic acid (0.3 mmol/L) and Na_2EDTA (0.029 mmol/L) as antioxidant. When glucose-free medium was used, the constant osmolarity was maintained by increasing the NaCl concentration. After dissection of the corpus striatum, slices 0.3 mm thick were prepared with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY, U.S.A.). Slices were washed with physiological medium to remove the membrane debris and then transferred to 2-ml incubation tubes. Each tube was divided into four separate chambers and contained three slices that did not touch each other. The slices were incubated in a water bath at 37°C, and the medium was changed every 10 min with fresh oxygenated medium. Following a 1-h equilibration period, striatal slices were incubated in each condition—control (gassed with 95% O_2 and 5% CO_2), anoxic (first degassed under vacuum and then gassed with 95% N_2 and 5% CO_2), aglycemic (no added glucose but gassed with 95% O_2 and 5% CO_2), or ischemic (glucose and oxygen-free medium)—for another 1 h at 37°C. To test the sensitivity of DA depletion to hypothermia, the temperature of the incubation medium was decreased during the last 10 min of the equilibration period to the degrees as indicated in the text and kept constant until the end of the experiments. During this period, incubation medium was changed at 10-min intervals with fresh medium, and collected samples were acidified with $HClO_4$ (final concentration 0.4 *M*) and then assayed for DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels. When nomifensine or MK-801 was used, they were added to the medium during the last 10-min equilibration period and were present in the medium until the end of the experiments. At the end of the incubation period, slices were removed from the tubes and homogenized in $HClO_4$ (2 ml; final concentration, 0.4 *M*), and homogenates were used for determination of tissue DA, DOPAC, and protein levels.

Quantification of DA and DOPAC released into medium

DA and DOPAC levels in the medium were determined by a small modification of previously reported HPLC methods (Keller et al., 1976; Mefford et al., 1980). Acidified samples were transferred into the glass vials. Activated alumina (50 mg) and 5 ml of Tris buffer [pH 8.6, contained 10% (wt/vol) Na_2EDTA and 10% (wt/vol) sodium metabisulfite] were added, and the pH was adjusted to 8.6 with 5 *M* NaOH. Vials were capped and shaken for 10 min. After aspiration of the supernatant, alumina was washed with 10 ml of distilled water, and then DA and DOPAC were eluted from the alumina with 150 μl of 0.4 *M* $HClO_4$. Fifty microliters of the eluate was injected onto an HPLC system (model PU-980 liquid chromatography pump; Jasco, Japan). DA and DOPAC were separated on a C_{18} reversed-phase column (Macherey-Nagel GmbH, Düren, Germany) with a flow rate of 1 ml/min. An amperometric detector (HP1049 EC; Hewlett-Packard, U.S.A.) with the glass carbon electrode set at 0.7 V versus an Ag–AgCl reference electrode was used to detect the DA and DOPAC. The chromatograms were integrated using a Hewlett-Packard (HP3396) integrator. The mobile phase (pH 4.5) consisted of 0.15 *M* NaH_2PO_4, 1 m*M* sodium octyl sulfate, 0.1 m*M* Na_2EDTA, and 10% (vol/vol) methanol. DA and DOPAC standards were prepared in acidified incubation medium with $HClO_4$ (final concentration, 0.4 *M*) and processed together with the samples.

Measurement of tissue DA, DOPAC, and protein levels

After homogenization of the slices in 2 ml of 0.4 *M* $HClO_4$, 1 ml of the homogenate was centrifuged for 5 min in a Beckman microfuge, and a portion of the supernatant (20 μl) was injected onto the HPLC system without further purification. Tissue DA and DOPAC levels were calculated by comparing peak heights of the samples with DA and DOPAC standards prepared in 0.4 *M* $HClO_4$. Because tissue DOPAC levels were ~5% or less than the tissue DA levels, they will not be discussed in the article. Tissue protein levels were measured in 50 μl of homogenate according to the procedure of Lowry et al. (1951). Protein standards were also prepared in 0.4 *M* $HClO_4$ and processed together with the tissue samples.

[^{3}H]DA uptake inhibition in rat striatal synaptosomes

The uptake of [^{3}H]DA by striatal synaptosomes was studied by a small modification of the procedure used by Tateyama et

al. (1993). Striatal tissue obtained from Sprague–Dawley rats were homogenized in 40 volumes of cold 0.32 *M* sucrose in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 1,000 *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 15,000 *g* for 30 min. The mitochondrial-rich pellet was resuspended in sucrose to get a final concentration of 2 mg of protein in 1 ml and then used immediately for [^{3}H]DA uptake assay. The incubation medium contained 100 mmol/L NaCl, 11 mmol/L glucose, 4 mmol/L KCl, 0.11 mmol/L ascorbic acid, and 40 mmol/L sodium phosphate buffer (pH 7.4). Fifty microliters of the synaptosomal suspension was added to 400 μl of cold incubation medium containing various concentrations of nomifensine (0–10 μM), and the mixture was preincubated for 5 min at 37°C. Fifty microliters of [^{3}H]DA (final concentration, 0.15 n*M*) was added into the mixture, and tubes were incubated for 3 min at 37 or 0°C. Incubation was terminated by rapid filtration of the mixture through Whatman GF/B glass fiber filters. Filters were washed three times with 4 ml of cold incubation medium. Radioactivity on the filter was determined in 5 ml of scintillation cocktail using a liquid scintillation counter (model 1600-TR; Packard). Net uptake was calculated by subtracting the 0°C value from the 37°C value.

Data analysis

All results are presented as mean ± SEM values. Differences between results were tested by Tukey–Kramer multiple comparisons test or Student's *t* test. A probability of $p < 0.05$ was considered significant.

RESULTS

Anoxia-, aglycemia-, and ischemia-induced alterations in DA release and extracellular DOPAC levels

Under control conditions (in the presence of both glucose and oxygen), striatal slices released 3.2 ± 0.5 pmol of DA/mg of protein (n = 31) and 19 ± 1 pmol of DOPAC/mg of protein (n = 31) during the first 10 min of the incubation period. As indicated in Fig. 1A, incubation of striatal slices in oxygen-free medium (anoxic conditions) caused a significant increase in DA release; this anoxia-induced enhancement peaked during the third 10 min of the incubation period in anoxic conditions (83 ± 10 pmol/mg of protein/10 min; n = 12) and then declined gradually (Fig. 1A). In contrast, extracellular DOPAC levels slightly decreased in the absence of oxygen (Fig. 1B). When incubated in glucose-free medium, DA release was less than anoxia-induced DA depletion (Fig. 1A), but extracellular DOPAC levels were also enhanced under this experimental condition (Fig. 1B). When glucose and oxygen were omitted together (ischemia), DA depletion was more rapid and peaked in 20 min (162 ± 17 pmol/mg of protein/10 min). As indicated in Fig. 1B, the aglycemia-induced enhancement in extracellular DOPAC levels was returned to control levels by omission of oxygen from glucose-free medium. Table 1 summarizes the total amounts of DA and DOPAC measured in the medium during 60-min incubation periods and the DA levels remaining in the slices at the end of experiments. It is clearly seen from Table 1 that the sum of the DA and DOPAC released from and remaining in the slices was significantly lower in striatal slices that were incubated in the absence of oxygen, glucose, or oxygen plus glucose compared with the control groups.

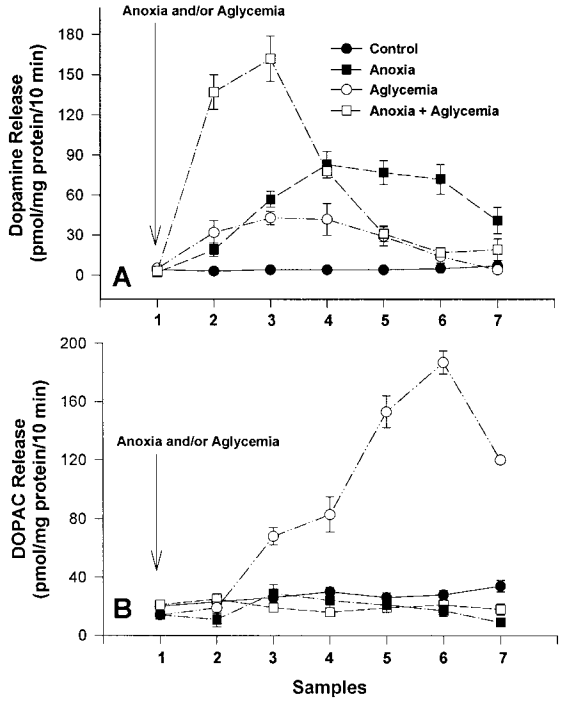

FIG. 1. Effect of anoxia, aglycemia, and anoxia plus aglycemia on (**A**) DA and (**B**) DOPAC release from rat striatal slices. After the preincubation period, striatal slices were incubated for another 10 min in normoxic conditions for determination of basal DA and DOPAC release (sample 1). Incubation of the slices was then continued for 60 min in control, anoxic, aglycemic, or anoxic plus aglycemic conditions. During this period, the incubation medium (2 ml) was changed at 10-min intervals to fresh medium. One milliliter of the collected samples was acidified with $HClO_4$ and assayed for DA and DOPAC levels. Another half of the samples was pooled in $HClO_4$ (final concentration, 0.4 *M*) and then assayed for total DA and DOPAC release during the 60-min incubation period, which is shown in Table 1. At the end of the experiments, slices were removed from the incubation tubes and homogenized in 2 ml of 0.4 *M* $HClO_4$, and homogenates were used for determination of both tissue DA and protein levels. Data are mean ± SEM (bars) values, in pmol/mg of protein/10 min. The number of determinations is 11 for control, 12 for anoxia, and four for both aglycemia and aglycemia plus anoxia.

Involvement of reverse transport mechanism in anoxia-, aglycemia-, and ischemia-induced DA release

A possible involvement of the reverse DA transporter in anoxia-, aglycemia-, and ischemia-induced DA release was tested with nomifensine, a DA uptake blocker. When added to the incubation medium, nomifensine (20 μM) almost completely prevented anoxia-induced DA release (Fig. 2A) and reversed tissue DA depletion (Fig. 2B). Neither aglycemia- nor ischemia-induced changes in DA release, however, were altered significantly by nomifensine (Table 2). To test whether the potencies of nomifensine in blocking [^{3}H]DA uptake and protecting

TABLE 1. *Effects of anoxia, aglycemia, and anoxia plus aglycemia on DA and DOPAC release and tissue DA levels in rat striatal slices*

	Release		Content	
Experimental condition	DA	DOPAC	Tissue DA	Total[a]
Control	17 ± 3	164 ± 17	1,016 ± 34	1,197 ± 50
Anoxia	349 ± 21[b]	108 ± 16[c]	447 ± 55[b]	904 ± 70[d]
Aglycemia	175 ± 11[b]	462 ± 48[b]	63 ± 20[b]	723 ± 67[b]
Aglycemia plus anoxia	479 ± 47[b]	123 ± 14	34 ± 5[b]	712 ± 33[b]

After the preincubation period, striatal slices were incubated in normoxic (control), anoxic, aglycemic, or anoxic plus aglycemic conditions for 60 min. During this period, the incubation medium (2 ml) was changed at 10-min intervals with fresh medium. One milliliter of the collected samples was acidified with $HClO_4$ and assayed for DA and DOPAC levels to obtain the release kinetics, which are shown in Fig. 1. Another 1 ml of the collected samples was pooled in $HClO_4$ and then assayed for DA and DOPAC to get total release values during the 60-min incubation period. In some experiments, samples were collected for determination of total DA and DOPAC release only. At the end of the experiments, striatal slices were transferred to 2 ml of 0.4 *M* $HClO_4$ and homogenized for determination of tissue DA, DOPAC, and protein levels. Release data are mean ± SEM values, in pmol/mg of protein/60 min, from 13 and 15 determinations for control and anoxia, respectively, and seven determinations for the other two points. Content data are mean ± SEM values, in pmol/mg of protein.

[a] Represents the sum of DA and DOPAC that were released from and remained in the slices.

[b] $p < 0.001$, [c] $p < 0.05$, [d] $p < 0.01$, significantly different from the corresponding control value.

the slices against anoxia-induced DA depletion were similar, we studied its [^{3}H]DA uptake blocking potency in striatal synaptosomes and its protecting potency in striatal slices. Studies performed on striatal synaptosomes and on striatal slices indicated that nomifensine has a similar potency in blocking both [^{3}H]DA uptake (IC_{50} = 59 ± 20 n*M*, n = 5) and anoxia-induced DA depletion (IC_{50} = 39 ± 3 n*M*, n = 3; Fig. 3).

Sensitivity of anoxia-, aglycemia-, and ischemia-induced DA depletion to hypothermia

Decreasing of the incubation temperature by 1°C (to 36 from 37°C) diminished anoxia-induced DA release to 49 ± 14 (n = 26) from 353 ± 25 pmol/mg of protein/60 min (n = 12; Fig. 4A) and protected the slices against anoxia-induced DA depletion (684 ± 45 vs. 237 ± 17 pmol/mg of protein; Fig. 4B). As shown in Fig. 4, additional decrements in incubation temperature further protected the slices against anoxia. In contrast to anoxia, aglycemia-induced DA release was less sensitive to hypothermia: Decreasing the incubation temperature by 2°C (to 35°C) caused only 45% inhibition of aglycemia-induced DA depletion (Table 2). DA release induced by ischemia, on the other hand, was not altered by decreasing the incubation temperature to 35°C (Table 2).

Differences between DA depletion induced by anoxia and Na^+,K^+-ATPase inhibition

Inhibition of Na^+,K^+-ATPase with 100 μM ouabain caused an enhancement in DA release similar to that observed under anoxic conditions (Table 3). The extracellular DOPAC level, moreover, was also enhanced by ouabain (data not shown). Neither hypothermia nor nomifensine, on the other hand, affected ouabain-induced DA release and tissue DA depletion (Table 3) or the enhancement of extracellular DOPAC level (data not shown).

Involvement of endogenous glutamate in anoxia-, aglycemia-, and ischemia-induced DA depletion

Involvement of endogenous glutamate in DA release induced by anoxia, aglycemia, or ischemia was tested with MK-801, an ionotropic glutamate receptor antagonist, and by comparing the glutamate-induced DA release with that during anoxia. The presence of MK-801 in the medium protected the slices against anoxia-induced DA release in a dose-dependent manner (Fig. 5). Whereas DA release induced by aglycemia was inhibited partially, ischemia-induced DA release was not affected by MK-801 (Table 2). As shown in Table 4, L-glutamate added into the medium increased DA release by a mechanism that was sensitive to nomifensine and hypothermia, indicating similarities between the DA release induced by glutamate and anoxia.

DISCUSSION

Results presented here clearly indicate that DA release, as observed repeatedly, is highly sensitive to anoxic conditions. Because DA, in addition to excitatory amino acids, is widely recognized to be a neurotoxic neurotransmitter in anoxic/ischemic conditions, protecting dopaminergic neurons against anoxia/ischemia-induced DA depletion might have therapeutic potential. Indeed, several pharmacological or surgical approaches that prevent DA depletion have been shown to improve striatal histologic alterations following ischemia (Weinberger and Cohen, 1983; Weinberger et al., 1985; Globus et al., 1987, 1989; Clemens and Phebus, 1988; Buisson et al., 1992). Thus, clarifying the mechanism(s) responsible in DA depletion and demonstrating the differences among the DA release induced by different experimental conditions, such as anoxia, aglycemia, or anoxia plus aglycemia, seem to be important for evaluating new

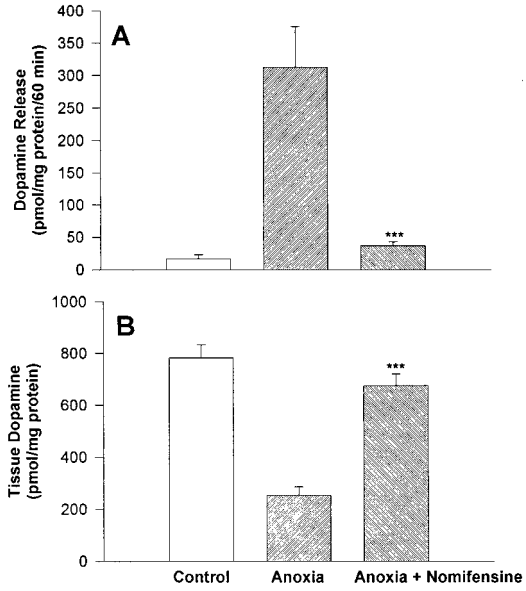

FIG. 2. Effect of nomifensine on anoxia-induced DA release and tissue DA depletion in rat striatal slices. After the preincubation period, striatal slices were incubated in the presence (control) or absence (anoxia) of oxygen for 60 min. Nomifensine (20 μM) was added to the incubation medium 10 min before anoxia and was present in the medium during the 60-min incubation period. Samples collected at 10-min intervals were pooled in $HClO_4$ (0.4 M) and assayed for their DA (**A**) levels. At the end of the experiment, slices were homogenized in 2 ml of 0.4 M $HClO_4$, and homogenates were used for determination of both tissue DA (**B**) and protein levels. Data are mean ± SEM (bars) values (n = 5 determinations for each point). ***$p < 0.001$, significantly different from the value obtained in anoxic conditions without nomifensine.

pharmacological strategies against anoxic/ischemic damage.

In addition to DA release, omission of glucose from normoxic medium also caused a significant increase in extracellular DOPAC levels (Fig. 1 and Table 1). Nomifensine added to the medium, on the other hand, decreased this aglycemia-induced enhancement in extracellular DOPAC levels only 17% (Table 2), indicating that most of the DA might be exposed to monoamine oxidase before being released to the extracellular medium in aglycemic conditions. When oxygen and glucose were omitted together from the medium, aglycemia-induced DOPAC output was returned to control levels, but a greater release of DA was obtained. It is noteworthy that the total amount of DA and DOPAC released in aglycemic conditions was almost equal to the value found in aglycemic plus anoxic conditions (Table 1), probably as a result of the inhibition of monoamine oxidase in the absence of oxygen in the medium. It is also clear from Table 1 that the sum of the DA and DOPAC released from and remaining in the slices was significantly less in striatal slices that were incubated in anoxic, aglycemic, or ischemic conditions, suggesting that omission of oxygen and/or glucose from the medium also inhibits DA synthesis during the 60-min incubation period.

TABLE 2. *Effects of nomifensine, hypothermia, and MK-801 on DA release induced by aglycemia or aglycemia combined with anoxia*

	Aglycemia-induced release		Aglycemia plus anoxia-induced release	
Treatment	DA	DOPAC	DA	DOPAC
None	217 ± 6	471 ± 22	601 ± 12	105 ± 6
Nomifensine (20 μM)	202 ± 11	390 ± 15[a]	557 ± 16	102 ± 4
None	285 ± 41	535 ± 68	615 ± 50	146 ± 12
Hypothermia (35°C)	156 ± 16[a]	528 ± 50	552 ± 42	113 ± 11
MK-801 (100 μM)	181 ± 17[b]	442 ± 46	511 ± 29	140 ± 24

After a 60-min preincubation period in normoxic conditions, striatal slices were incubated in aglycemic or aglycemic plus anoxic conditions for another 60 min. When tested, the temperature of the incubation medium was decreased to 35°C 10 min before aglycemia or aglycemia plus anoxia and kept constant at this temperature until the end of the experiment. Similarly, nomifensine or MK-801 was added into the medium 10 min before aglycemia or aglycemia plus anoxia and was present in the medium during the entire experimental period. As indicated in the text, the incubation medium was changed at 10-min intervals to fresh medium, and collected samples were pooled in $HClO_4$ (final concentration, 0.4 M) and then assayed for their DA, DOPAC, and protein levels. Data are mean ± SEM values, in pmol/mg of protein/60 min, for five to seven determinations for each point except MK-801 in the aglycemic plus anoxic condition (n = 3 for this point).

[a] $p < 0.01$, [b] $p < 0.05$, significantly different from the corresponding control value.

One of the most obvious effects of anoxia or ischemia is a rapid depletion of intracellular ATP levels (Whittingham et al., 1984; Santos et al., 1996; see also reviews by Schurr and Rigor, 1989; Martin et al., 1994). ATP is

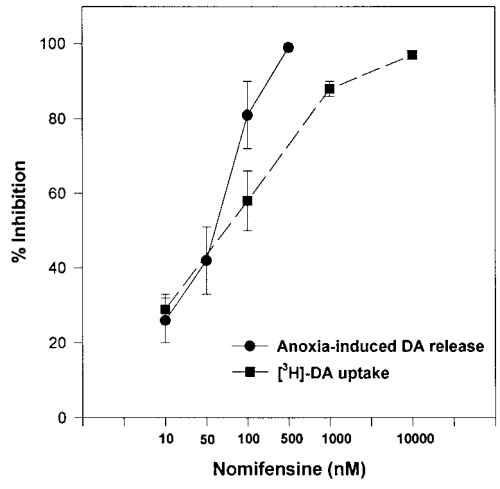

FIG. 3. Dose–response relationship of nomifensine in blocking [^{3}H]DA uptake in striatal synaptosomes and in protecting the slices against anoxia-induced DA release. Data are mean ± SEM (bars) percent inhibition of both [^{3}H]DA uptake or anoxia-induced DA release by nomifensine (n = 5 determinations for [^{3}H]DA uptake values and 3 for DA release studies).

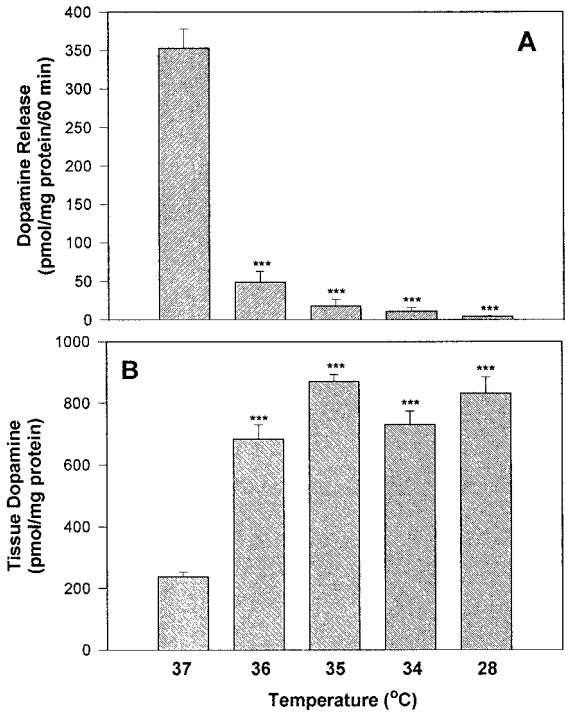

FIG. 4. Effect of hypothermia on anoxia-induced DA release from rat striatal slices. After the preincubation period in normoxic conditions at 37°C, striatal slices were incubated in the absence of oxygen (anoxia) at different temperatures for 60 min. Striatal slices were transferred to hypothermic conditions during the last 10 min of the preincubation period, and hypothermia was kept constant during the entire experimental period. Samples collected at 10-min intervals were pooled in $HClO_4$ and assayed for DA (**A**) and DOPAC levels. At the end of the experiments, slices were homogenized in 2 ml of 0.4 *M* $HClO_4$, and homogenates were used for determination of both tissue DA (**B**) and protein levels. Data are mean ± SEM (bars) values (n = 26 determinations for 37°C, 12 for 36°C, 10 for 35°C, and 4 for the other two points). ***$p < 0.001$, significantly different from the value obtained at 37°C.

known to be required for vesicular uptake of DA as well as by the sodium pump. Thus, a reduction in intracellular ATP levels not only leads to a decrease of the Na^+ gradient, but also may enhance cytoplasmic free DA levels. Because both reversal of the electrochemical Na^+ gradient and an increase in cytoplasmic DA concentration facilitate reverse DA transport (Adam-Vizi, 1992; Levi and Raiteri, 1993), it is likely that this mechanism might play a secretory role in anoxia/ischemia-induced DA release. The reverse transport mechanism has been shown to contribute to ischemia-induced norepinephrine release in the PNS and the CNS (Schomig et al., 1987; Russ et al., 1991; Kurz et al., 1995, 1996; Kim et al., 1998). Although a few published reports also indicate a possible involvement of this mechanism (Akiyama et al., 1991; Kim et al., 1995), the importance of the reverse DA transporter in anoxia/ischemia-induced DA release seems to be questionable. In the present study we observed that nomifensine, a DA uptake-blocking drug, almost completely prevented anoxia-induced DA release (Fig. 2A), indicating a significant contribution of the reverse transport system in anoxia-induced DA depletion. In support of this conclusion, we also determined that IC_{50} values of nomifensine in blocking [^{3}H]DA uptake in striatal synaptosomes and inhibiting anoxia-induced DA release in striatal slices were similar (59 ± 20 and 39 ± 3 n*M*, $p > 0.05$; Fig. 3). In contrast to DA release induced by anoxia, neither aglycemia- nor ischemia-induced enhancements were lessened significantly by nomifensine (Table 2), suggesting that experimental conditions such as the degree of anoxia/ischemia may alter the mechanisms involved in DA release. Indeed, in preliminary studies we observed that the glucose concentration of the medium was critical for the role of the reverse transport mechanism in anoxia-induced DA release; whereas nomifensine caused only 10% inhibition in anoxia-induced DA release at a concentration of 2 m*M*, this inhibition was enhanced to 65% when the glucose concentration of the anoxic medium was raised to 5 m*M* (data not shown).

Because Na^+,K^+-ATPase (sodium pump) uses as fuel most of the neuronal ATP, it is very reasonable to conclude that experimental conditions that cause a rapid depletion of intracellular ATP levels inhibit this enzyme and then stimulate DA release. To test this possibility, we blocked Na^+,K^+-ATPase in normoxic slices with ouabain (100 μM) and compared ouabain-induced DA release with that of anoxia. As shown in Table 3, incubation of striatal slices with ouabain or in anoxic medium caused comparable increases in DA release. Nomifensine, as mentioned above, almost completely prevented anoxia-induced DA depletion. Ouabain-induced DA release, however, like aglycemia- or ischemia-induced DA release (see Table 2), was not altered by

TABLE 3. *Differences between DA release induced by anoxia and ouabain*

Experimental condition	DA release (pmol/mg of protein/60 min)	Tissue DA content (pmol/mg of protein)	n
Anoxia	337 ± 13	266 ± 21	10
Anoxia + nomifensine	42 ± 6[a]	705 ± 36[a]	8
Anoxia + hypothermia	9 ± 6[a]	854 ± 3[a]	5
Ouabain	476 ± 46	30 ± 6	10
Ouabain + nomifensine	408 ± 27	31 ± 5	7
Ouabain + hypothermia	406 ± 20	21 ± 3	5

After the preincubation period, striatal slices were incubated in anoxic conditions or in normoxic conditions plus 100 μM ouabain for 60 min. When tested, addition of nomifensine (20 μM) to the medium or cooling of the medium to 35°C was performed 10 min before anoxia or ouabain, and these conditions were present during the entire experimental period. Samples were collected at 10-min intervals, pooled in $HClO_4$, and then assayed for DA levels. At the end of the experiments, slices were homogenized in 2 ml of 0.4 *M* $HClO_4$, and homogenates were used for determination of both tissue DA and protein levels. Data are mean ± SEM values of the given number of determinations (n).

[a] $p < 0.001$, significantly different from the corresponding control value obtained in the absence of nomifensine or at 37°C.

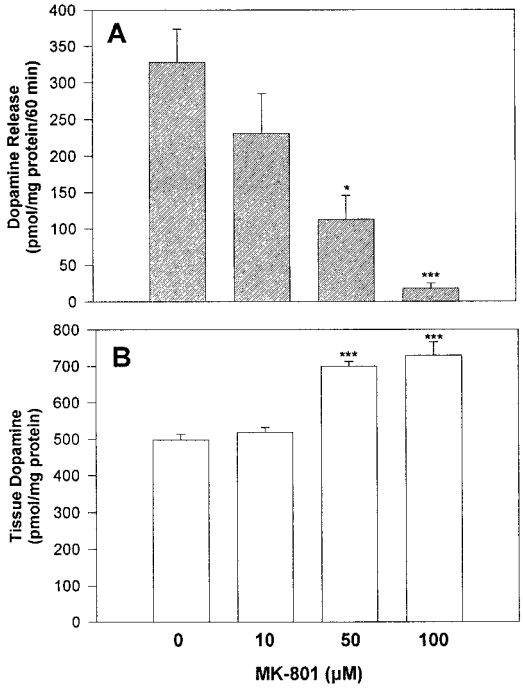

FIG. 5. Protecting effect of MK-801 against anoxia-induced DA release and tissue DA depletion in rat striatal slices. After the preincubation period, striatal slices were incubated in the absence or presence of MK-801 in anoxic conditions. When tested, MK-801 was added to the medium 10 min before anoxia and was present in the medium until the end of the experiment. Samples were collected at 10-min intervals, pooled in $HClO_4$, and assayed for DA level (**A**). At the end of the experiments, slices were homogenized in 2 ml of 0.4 *M* $HClO_4$, and homogenates were used for determination of both tissue DA (**B**) and protein levels. Data are mean ± SEM (bars) values (n = 6 determinations for control and 10 and 100 μM MK-801 and 3 for 50 μM MK-801). $^*p < 0.05$, $^{***}p < 0.001$, significantly different from the control value found in the absence of MK-801.

nomifensine or hypothermia (Table 3). These results indicate that the mechanism(s) involved in anoxia-induced DA depletion seems clearly different from that of ouabain, and additional mechanisms might also contribute to anoxia-induced DA release.

Cooling of the brain is the most effective means known for protecting the brain against ischemic injury. Although results mentioned above indicate distinct differences among the DA release induced by anoxia, aglycemia, and ischemia, we also made a further comparison by testing their sensitivities to hypothermia. Results presented in Fig. 4 clearly show that DA release induced by anoxia is highly sensitive to cooling of incubation medium by as little as 1°C. Neither aglycemia- nor ischemia-induced DA depletion, on the other hand, was altered as much as anoxia by decreasing the incubation temperature to 35°C (Table 2). Although we did not test the efficacy of lower temperatures in aglycemic or ischemic conditions, the present results indicate that the degree of anoxia/ischemia may alter the sensitivity of DA depletion to hypothermia. What remains unclear is the mechanism by which small temperature changes protect the slices against anoxia-induced DA release. Hypothermia, in addition to decreasing cerebral metabolic demand (Hagerdal et al., 1975; Yager and Asselin, 1996), can also inhibit neurotransmitter release (Nakashima and Todd, 1996; Thoresen et al., 1997). Thus, its protective effect against anoxia-induced DA release could be attributed to its inhibiting ability on release of neurotransmitters, such as excitatory amino acids. Because hypothermia, as mentioned in the present study, also protects the slices against glutamate-induced DA release, it seems very reasonable to conclude that cooling of the medium might have some additional effects beyond the inhibitory effect on glutamate release, such as a decline in responsiveness of NMDA receptors to glutamate or a direct and/or indirect inhibition of the reverse DA transporter.

Because anoxic/ischemic conditions cause a significant increase in extracellular glutamate levels (Globus et al., 1988; Rubio et al., 1991; Milusheva et al., 1992; Nakashima and Todd, 1996; Santos et al., 1996) and because glutamate can also stimulate DA release by a mechanism that involves the reverse DA transporter

TABLE 4. *Similarities between the DA release induced by anoxia and exogenous glutamate (Glu)*

Experimental condition	DA release	(n)
Effect of nomifensine		
Anoxia	336 ± 21	(5)
Anoxia + nomifensine	50 ± 20	(5)[a]
Glu (1 m*M*)	44 ± 4	(8)
Glu (1 m*M*) + nomifensine	31 ± 3	(5)[b]
Glu (2 m*M*)	168 ± 2	(8)
Glu (2 m*M*) + nomifensine	36 ± 2	(5)[a]
Glu (5 m*M*)	404 ± 31	(7)
Glu (5 m*M*) + nomifensine	179 ± 40	(5)[a]
Effect of hypothermia		
Anoxia	324 ± 32	(5)
Anoxia + hypothermia	26 ± 3	(5)[a]
Glu (1 m*M*)	53 ± 3	(6)
Glu (1 m*M*) + hypothermia	2 ± 1	(5)[a]
Glu (2 m*M*)	203 ± 11	(7)
Glu (2 m*M*) + hypothermia	34 ± 7	(7)[a]
Glu (5 m*M*)	408 ± 21	(10)
Glu (5 m*M*) + hypothermia	236 ± 15	(5)[a]

After the preincubation period, striatal slices were incubated in anoxic conditions or in normoxic conditions combined with different concentrations of Glu. When tested, addition of nomifensine (20 μM) to the medium or cooling of the medium to 35°C (hypothermia) was performed 10 min before anoxia or Glu, and these conditions were present during the entire experimental period. Samples were collected at 10-min intervals, pooled in $HClO_4$, and then assayed for their DA and DOPAC levels. At the end of the experiments, slices were homogenized in 2 ml of 0.4 *M* $HClO_4$ and homogenates were used for determination of both tissue DA (data not shown) and protein levels. Data are mean ± SEM values (no. of determinations), in pmol/mg of protein/60 min.

[a] $p < 0.001$, [b] $p < 0.05$, significantly different from the corresponding control value obtained in the absence of nomifensine or at 37°C.

(Lonart and Zigmond, 1991), it is likely that anoxia-induced DA release depends, at least partly (if not completely), on enhancements of endogenous glutamate levels and/or on overstimulation of NMDA receptors. Although a good correlation has been reported between glutamate content and release of monoamines (including DA) from the crude synaptosomes during hypoglycemia, anoxia, or ischemia, MK-801 and 6-cyano-7-nitroquinoxaline-2,3-dione, which are antagonists of NMDA and non-NMDA receptors, respectively, were unable to alter ischemia-induced monoamine release (Santos et al., 1996). In contrast, involvement of an NMDA receptor/ion complex in hypoxia/hypoglycemia-induced DA release has been well documented in rat striatal slices (Toner and Stamford, 1997*c*). In the present study, a possible contribution of endogenous glutamate to DA release induced by anoxia, aglycemia, or ischemia was tested with MK-801. As shown in Fig. 5, the presence of MK-801 in incubation medium protected the slices against anoxia-induced DA depletion, indicating a role of the glutamatergic system in DA release under anoxic conditions.

To support further the role of endogenous glutamate in anoxia-induced DA release, we also attempted to show the similarities between the anoxia- and L-glutamate-induced DA release. In parallel studies performed in normoxic and anoxic conditions, L-glutamate added to normoxic medium increased both DA release (Table 4) and extracellular DOPAC levels (data not shown). The magnitude of the L-glutamate-induced DA release was less than that induced by anoxia when 2 m*M* and, especially, 1 m*M* L-glutamate were tested. However, the sum of the DA and DOPAC released by 2 m*M* glutamate was calculated to be 454 ± 28 pmol/mg of protein/60 min (n = 6), and this value was not different from anoxia-induced DA plus DOPAC release (405 ± 32 pmol/mg of protein/60 min; $p > 0.05$). When the glutamate concentration was raised to 5 m*M*, on the other hand, DA depletion was found to be almost equal to anoxia-induced DA release, but the sum of the DA and DOPAC was higher than the release value obtained in anoxic conditions (652 ± 36 vs. 405 ± 32 pmol/mg of protein/60 min; $p < 0.001$). As observed in anoxic conditions, nomifensine (20 μM) significantly decreased both 1 and 2 m*M* glutamate-induced DA release to control levels measured in the presence of nomifensine only (23 ± 5 pmol/mg of protein/60 min, n = 6). Extracellular DOPAC levels, which were stimulated by these concentrations of glutamate, on the other hand, were also decreased to control levels by nomifensine (data not shown). A similar inhibition in glutamate-induced DA release was also observed in hypothermic conditions (Table 3); cooling of the incubation temperature to 35°C prevented the DA release stimulated by both 1 and 2 m*M* glutamate and decreased the enhancements of extracellular DOPAC levels to their control levels (data not shown). These findings support the possibility that both endogenous glutamate and anoxia share a similar mechanism in stimulating DA release from rat striatal slices. When DA release was stimulated with 5 m*M* glutamate, however, nomifensine and hypothermia showed only a partial protection (Table 3). This finding indicates that not only the reverse transport mechanism, but also additional mechanisms seem to be responsible in DA release if the glutamate concentration is raised to 5 m*M* in incubation medium.

In contrast to the significant inhibitory effect of MK-801 in anoxic conditions, this antagonist exerted only a partial protection in aglycemic conditions (Table 2), suggesting a partial involvement of endogenous glutamate in aglycemia-induced DA release. Ischemia-induced DA release, however, as observed by Santos et al. (1996), did not respond to MK-801 in the present study. When differences observed with nomifensine or in hypothermic conditions were considered together with the differences observed with MK-801, it is very reasonable to conclude that experimental conditions used in anoxia/ischemia studies may alter the mechanism(s) involved in DA release and probably explain the differences among the findings observed by different laboratories. In support of this conclusion, it has been shown that oxidative stress, hypoxia, and ischemia-like conditions increase the release of endogenous amino acids from cultured retinal cells by distinct mechanisms (Rego et al., 1996).

In summary, results presented in this study show that omission of oxygen from incubation medium causes a rapid and significant DA release from rat striatal slices by a mechanism involving both reversed DA transport and endogenous glutamate. The partial or complete lack of effect of nomifensine, hypothermia, or MK-801 in preventing aglycemia- or aglycemia plus anoxia-induced DA release also suggests that experimental conditions, such as the degree of anoxia/ischemia, may alter the mechanism(s) involved in DA depletion. Therefore, considering the experimental conditions seems to be important when evaluating new therapeutic approaches against anoxia/ischemia-induced DA release.

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