Hypoglycemia Enhances Ionotropic But Reduces Metabotropic Glutamate Responses in Substantia Nigra Dopaminergic Neurons

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Marinelli, Silvia, Mauro Federici, Patrizia Giacomini, Giorgio Bernardi, and Nicola B. Mercuri. Hypoglycemia enhances ionotropic but reduces metabotropic glutamate responses in substantia nigra dopaminergic neurons. *J Neurophysiol* 85: 1159–1166, 2001. It is widely accepted that energy deprivation causes a neuronal death that is mainly determined by an increase in the extracellular level of glutamate. Consequently an excessive membrane depolarization and a rise in the intracellular concentration of sodium and calcium are produced. In spite of this scenario, the function of excitatory and inhibitory amino acids during an episode of energy failure has not been studied yet at a cellular level. In a model of cerebral hypoglycemia in the rat substantia nigra pars compacta, we measured neuronal responses to excitatory amino acid agonists. Under single-electrode voltage-clamp mode at −60 mV, the application of the ionotropic glutamate receptor agonists *N*-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, kainate, and the metabotropic group I agonist (S)-3,5-dihydroxyphenilglycine (DHPG) produced reversible inward currents in the dopaminergic cells. In addition, an outward current was caused by the superfusion of the metabotropic $GABA_B$ agonist baclofen. Glucose deprivation enhanced the inward responses caused by each ionotropic glutamate agonist. In contrast, hypoglycemia depressed the DHPG-induced inward current and the baclofen-induced outward current. These effects of hypoglycemia were reversible. To test whether a failure of the Na^+/K^+ ATPase pump could account for the modification of the agonist-induced currents during hypoglycemia, we treated the midbrain slices with strophanthidin (1–3 μM). Strophanthidin enhanced the inward currents caused by glutamate agonists. However, it did not modify the $GABA_B$-induced outward current. Our data suggest that glucose deprivation enhances the inward current caused by the stimulation of ionotropic glutamate receptors while it dampens the responses caused by the activation of metabotropic receptors. Thus a substantial component of the augmented neuronal response to glutamate, during energy deprivation, is very likely due to the failure of Na^+ and Ca^{2+} extrusion and might ultimately favor excitotoxic processes in the dopaminergic cells.

INTRODUCTION

The progress of neuronal dysfunction and damage during energy deprivation is a complex process that includes presynaptic and postsynaptic mechanisms (Auer and Siesjo 1988; Martin et al. 1994). Two main events have been described when energy levels are reduced: an increased release of excitatory amino acids (EAA) and a reduced concentration of intracellular ATP, which leads to diminished Na^+/K^+-ATPase activity (Benveniste et al. 1984; Erecinska and Silver 1989; Hansen 1985; Lees 1991; Roettger and Lipton 1996). It is well accepted that the excessive stimulation of EAA receptors associated with metabolic inhibition hampers the recovery of $[Na^+]_i$ and $[Ca^{2+}]_i$ loads and facilitates cell death (Cebers et al. 1998; Lees 1991; Monyer et al. 1989; Novelli et al. 1988; Rose et al. 1998; Rothman et al. 1987). However, the neuronal vulnerability caused by energy deprivation might not only result from an excess of extracellular glutamate and aspartate that stimulates NMDA and/or AMPA/kainate receptors (Choi 1988) but also from an impaired function of metabotropic responses. For instance, a deficient hyperpolarization mediated by GABA metabotropic receptors might not be able to counteract the harmful ischemia-induced membrane depolarization produced by glutamate receptors superactivation. In addition, the neuronal damage resulting from energy failure might not be simply dependent on the increased extracellular levels of EAAs. In fact, the reduced metabolic state might aggravate neuronal depolarizations to glutamate and aspartate, and this can influence outcome.

In spite of the importance of the excitatory and inhibitory processes in the pathogenic sequences caused by energy deprivation, there are only few studies that have examined the changes in responses to excitatory and inhibitory amino acids under a reduced metabolic condition.

Considering that the early electrophysiological events caused by hypoxia and hypoglycemia have been extensively studied in the dopaminergic neurons of substantia nigra pars compacta (Guatteo et al. 1998a,b; Hauser et al. 1991; Jiang et al. 1994; Marinelli et al. 2000; Mercuri et al. 1994a,b; Stanford and Lacey 1995; Watts et al. 1995), that these cells possess well-characterized postsynaptic responses to ionotropic and metabotropic glutamate receptor agonists, and to the $GABA_B$ agonists (Lacey et al. 1988; Mercuri et al. 1992a,b, 1993), and that a selective toxicity of these cells occurs during impairment of energetic metabolism and activation of NMDA receptors (Marey-Semper et al. 1995), we used single-electrode voltage-clamp recordings from dopaminergic neurons of the rat mesencephalon maintained in vitro to examine changes in gluta-

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matergic and GABAergic responses during glucose removal. We report that hypoglycemia enhances the cellular responses caused by the activation of ionotropic glutamate receptors and depresses the responses mediated by second messengers.

METHODS

Tissue preparation

Wistar rats (150–300 g) were killed under deep halothane anesthesia. All efforts were made to minimize animal suffering. The brain was rapidly removed from the skull, and horizontal slices (300 μm thick) of the ventral midbrain were cut by a vibratome (Mercuri et al. 1995). A single slice containing the substantia nigra and the ventral tegmental area was transferred to a recording chamber, immobilized with titanium grids, and perfused at a rate of 2.5 ml/min, with a solution maintained at 35°C and oxygenated with a mixture of 95% O_2-CO_2 5% O_2. The standard solution contained (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 10 glucose, and 19 $NaHCO_3$, pH of 7.4.

Intracellular recording techniques

The recording electrodes (Clark 1–1.5 mm, thick wall), pulled by a Flaming-Brown horizontal puller (Sutter Instruments, Novato, CA), were filled with 2 M KCl and had a tip resistance of 30–80 MΩ. Membrane voltage and current signals were recorded using an amplifier (Axoclamp-2A, Axon Instruments, Foster City, CA). During the single-electrode voltage-clamp procedures, the amplifier headstage was monitored on a separate oscilloscope to ensure correct operation of the switch clamp: switching frequency was 3–4 kHz, 30% duty cycle. The signals were displayed on a pen recorder and on a digital oscilloscope or digitized by use of an A/D converter (Digidata 1200, Axon Instruments) and saved in a computer with the Axotape software (Axon Instruments) for off-line analysis. To obtain *I-V* plots, voltage commands (40–100 ms, between −110 and −40 mV) were delivered before and during the application of the agonists in the presence of TTX (1 μM) and barium (300 μM). The *I-V* curves in the presence of *N*-methyl-D-aspartate (NMDA) were also done in magnesium-free solutions. The substantia nigra pars compacta was visually identified as the region caudal to the medial terminal nucleus of the accessory optic tract using a dissecting microscope.

Induction of hypoglycemia and application of drugs

To induce hypoglycemia, the control solution was substituted with aglycemic artificial cerebrospinal fluid (ACSF, 0 mM glucose) saturated with 95% O_2-5% CO_2. In some experiments, equimolar mannitol (10 mM) was replaced with glucose. The drugs were bath-applied at a known concentration. Drug solutions entered the recording chamber no later than 20 s after turning a three-way tap. Complete replacement of the medium in the chamber took 90 s. The following drugs

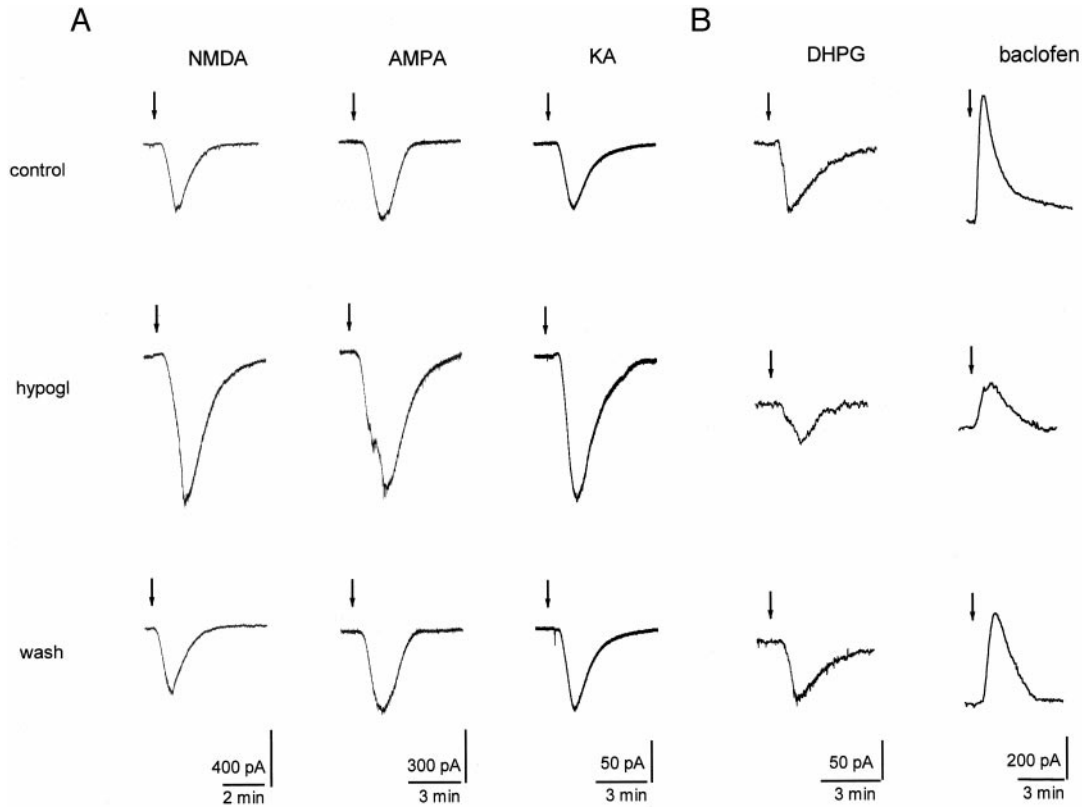

FIG. 1. Hypoglycemia potentiates ionotropic glutamate responses and depresses metabotropic responses. *A*, *top*: the inward currents produced by *N*-methyl-D-aspartate (NMDA, 50 μM for 12 s), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA, 10 μM for 11 s), and kainate (KA, 50 μM for 18 s) when applied to substantia nigra pars compacta dopaminergic cells (held at −60 mV) in control conditions (control–barium and TTX present). In this and the following figures, ↓, the point of drug application. *Middle*: after 20 min of glucose deprivation (hypogl), the whole cell currents produced by the ionotropic glutamate agonists were enhanced. *Bottom*: the changes of the glutamate currents were reversible (wash). *B*, *top*: the inward currents caused by (S)-3,5-dihydroxyphenilglycine (DHPG, 50 μM for 12 s, in TTX and barium) and outward current caused by baclofen (10 μM for 17 s) when applied to substantia nigra pars compacta dopaminergic cells (held at −60 mV) in control conditions (control). *Middle*: after 20 min of glucose deprivation (hypogl), the inward current to DHPG and the outward response to baclofen was depressed. *Bottom*: the modifications caused by hypoglycemia on the metabotropic currents were reversible (wash).

were used: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA, 10 μM) and (S)-3,5-dihydroxyphenilglycine (DHPG, 50 μM) from Tocris Cookson, NMDA (50 μM), kainic acid (KA, 50 μM), strophanthidin (1–3 μM), tetrodotoxin (TTX, 1 μM), and dopamine hydrochloride (10–30 μM) from Sigma. Baclofen (30 μM) was obtained from Roche.

Data analysis

Numerical data were expressed as means ± SD. Student's *t*-test for paired observations was used to compare the data. $P < 0.05$ was considered significant. The areas of the currents induced by ionotropic and metabotropic agonists have been calculated with Microcal Origin-Analysis/Calculus/Integrate-program running on an IBM computer.

RESULTS

Properties of the dopaminergic cells

The present study is based on intracellular recordings from 86 "principal" dopaminergic neurons of the rat substantia nigra pars compacta. These cells were identified by their location in the slice and their well-defined electrophysiological and pharmacological characteristics (Grace and Ohn 1989; Lacey et al. 1988, 1989; Mercuri et al. 1995).

Effects of ionotropic glutamate agonists

Under single-electrode voltage-clamp condition (−60 mV, holding potential), transient application of NMDA (50 μM for 15–30 s) induced a rapidly developing inward current (Fig. 1*A*). This inward current could be repeatedly produced on the same neuron. The average inward current caused by NMDA was 261 ± 63 pA ($n = 8$). The total charge of the NMDA current was 37 ± 8 pC ($n = 8$, Fig. 2*A*). The rapid application AMPA (10 μM for 10–20 s) also produced a rapidly developing inward current that washed quickly (Fig. 1). This inward response was consistently caused on each application of AMPA. The average inward current caused by 10 μM AMPA was 678 ± 121 pA ($n = 8$). The total charge caused by AMPA was 69 ± 8 pC ($n = 8$, Fig. 2*A*).

The rapid superfusion of kainate (50 μM for 15–30 s) caused an inward current of 191 ± 48 pA ($n = 6$). This type of response could be repeatedly evoked on the same neuron. The total charge induced by kainate was 31.5 ± 11 pC ($n = 6$, Fig. 2*A*).

Responses to metabotropic agonists

The rapid superfusion of DHPG (50 μM for 20–30 s), a Group I metabotropic agonist, produced an inward current of 107 ± 20 pA ($n = 6$) that could be consistently reproduced on the same cell (Fig. 1*B*). The total current area caused by DHPG was 15.5 ± 3 pC ($n = 6$, Fig. 2*B*).

The rapid superfusion of baclofen (30 μM for 15–20 s) determined an outward current of 395 ± 52 pA ($n = 5$, Fig. 1*B*). The outward response to this $GABA_B$ agonist could be consistently reproduced on the same cell. The total charge caused by baclofen was 46.3 ± 16 pC ($n = 5$, Fig. 2*B*).

Effects of hypoglycemia on the agonist-mediated responses

The perfusion of a glucose-free ACSF for 15–20 min produced a slowly developing outward current (Marinelli et al. 2000) that was 96 ± 8 pA ($n = 19$) at 15 min and 304 ± 33 pA ($n = 19$) at 20 min. We routinely tested the effects of the agonists after 20 min of glucose depletion. The inward current caused by NMDA was greatly augmented by hypoglycemia (Fig. 1*A*). The peak current was 329 ± 59% ($n = 8$, $P < 0.05$) of control. In addition we observed an increase of the duration of the current. Thus the inward area was 932 ± 242% ($n = 8$, $P < 0.05$) of control (from 37 ± 8 to 345 ± 68 pC). Similar results were obtained with AMPA and kainate (Fig. 1*A*). In fact, the peak AMPA and kainate currents were 157 ± 30% $P < 0.05$ ($n = 8$) and 344 ± 96% $P < 0.05$ ($n = 6$) of control, respectively. In addition, the total AMPA current was 453 ± 105% of control ($n = 8$; from 69 ± 8 to 313 ± 89 pC; $P < 0.05$) and the total kainate current was 251 ± 44% of control ($n = 6$; from 31.5 ± 11 to 78 ± 28 pC; $P < 0.05$; Fig. 2*A*). After having examined

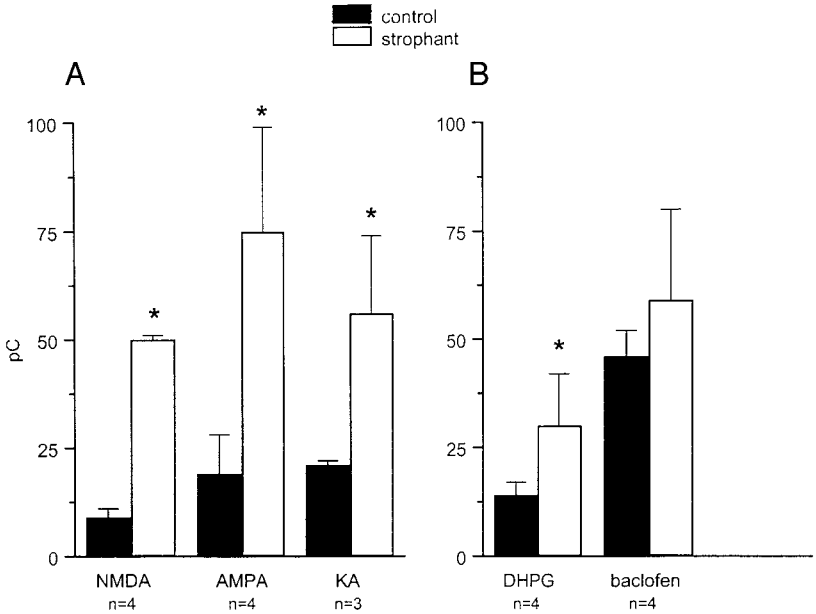

FIG. 2. Changes in ionotropic and metabotropic responses produced by hypoglycemia. *A*: the plot shows the total charge by NMDA, AMPA, and KA in control condition (■) and during the hypoglycemic phase (□). *B*: the plot shows the total charge induced by DHPG and baclofen,in control condition (■) and during the hypoglycemic phase (□). Note that hypoglycemia increases the ionotropic responses and depresses the metabotropic ones. The number of experiments for each column is indicated. Error bars represent SE. *, significant differences.

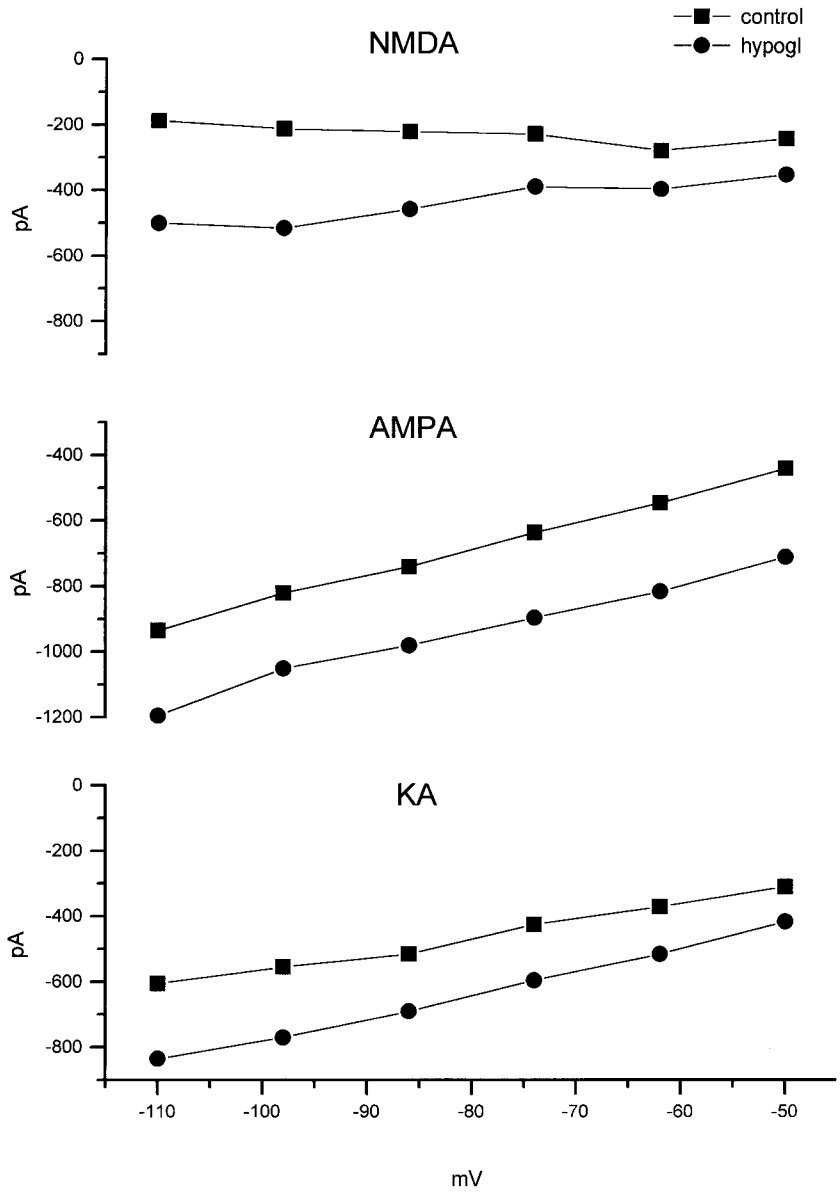

FIG. 3. Enhancement of the ionotropic currents along the voltage axes. The NMDA-, AMPA-, and KA-induced currents were increased by hypoglycemia at all the explored potentials (between −50 and −110 mV). Note that the currents evoked by the glutamate agonists were generated in the presence of TTX and barium.

the effects of hypoglycemia on the glutamate-evoked ionotropic currents, we also tested the effects on the metabotropic current caused by the group I agonist DHPG. After 20 min of glucose depletion, the inward charge caused by DHPG was depressed by 64 ± 8% of control ($n = 6$; from 15.5 ± 3 to 5.6 ± 1.5 pC; $P < 0.05$; Figs. 1*A* and 2*B*). A depressant effect of hypoglycemia on the agonist-induced current was also observed when we tested the effect of the metabotropic $GABA_B$ agonist baclofen (Fig. 1*B*). In fact, the outward charge caused by baclofen was reduced by 70.4 ± 4%, of control ($n = 5$; from 46.3 ± 16 to 13.7 ± 3.9 pC; $P < 0.001$, Fig. 2*B*). All the enhancing or depressing effects of hypoglycemia on the agonist-induced currents (3 cells for each agonist) were reversible and were also observed in the presence of TTX (1 μM, which was used to block fast sodium channel and synaptic transmission). In a series of experiments using the glutamate agonists, we also applied barium (300 μM) to reduce either the potassium conductance increase caused by energy deprivation (Guatteo et al. 1998a; Marinelli et al. 2000; Mercuri et al. 1994b) or the outward current that follows the application of the glutamate agonists (Mercuri et al. 1996); under the treatment with this divalent cation, hypoglycemia enhanced NMDA, AMPA, and kainate responses and reduced the DHPG-induced inward current (2 neurons for each agonist). In addition, hypoglycemia did not cause changes of the *I-V* relationship of the currents caused by ionotropic (Fig. 3) and metabotropic agonists (3 neurons for each compound; data not shown).

Effects of strophanthidin on the agonist-induced currents

To investigate the role of the Na^+/K^+-ATPase pump in determining changes in the agonist-induced currents in the dopaminergic neurons (voltage-clamped at −60 mV), the agonists were tested before, during, and after the superfusion of the pump inhibitor, strophanthidin (1–3 μM). The superfusion of this compound initially induced an inward current (87 ± 35

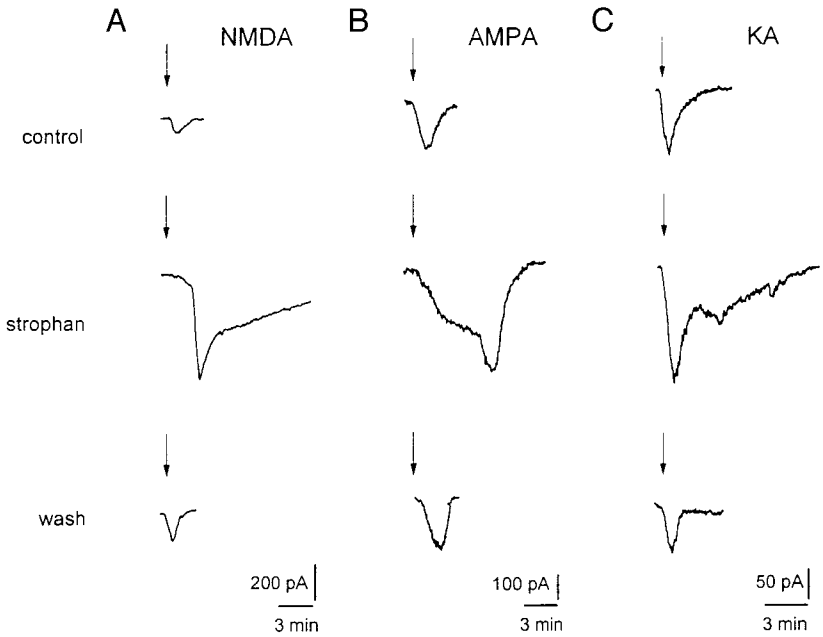

FIG. 4. Effects of strophanthidin on the currents induced by ionotropic agonists. *Top*: the inward responses induced by NMDA (50 μM for 18 s, *A*), AMPA (50 μM for 19 s, *B*), and KA (50 μM for 22 s, *C*), when applied to substantia nigra pars compacta dopaminergic cells (held at −60 mV) in control condition (control). *Middle*: after 15 min of strophanthidin superfusion (strophan) the inward responses to the ionotropic glutamate agonists were enhanced. *Bottom*: the modifications caused by strophanthidin on the ionotropic currents were reversible (wash).

pA, $n = 17$), but after 13–15 min, perfusion caused an outward shift of the holding current (109 ± 15 pA, $n = 17$). In the presence of strophanthidin, the responses induced by the glutamate agonists were increased (Fig. 4). In fact, the NMDA-, AMPA-, KA-, and DHPG-induced inward currents were increased to 555 ± 29% ($n = 4$, $P < 0.05$), 394 ± 99% ($n = 4$, $P < 0.05$), 266 ± 52% ($n = 3$, $P < 0.05$), and 214 ± 109% ($n = 4$, $P < 0.05$) of control, respectively (Fig. 6). Interestingly, while the metabotropic (DHPG-induced) current was increased, the baclofen-induced current was not significantly modified (128 ± 58% of control, $n = 4$, $P = 0.32$) in the presence of strophanthidin (Figs. 5 and 6). In addition, strophanthidin did not cause a clear-cut change of the *I-V* relationship during the effects of the excitatory and inhibitory agonists (2 cells for each compound; not shown).

DISCUSSION

The major observation of the present study is that a lack of glucose differentially changes the ionotropic and metabotropic responses caused by glutamate agonists in midbrain dopaminergic cells. In fact, the ionotropic-induced inward currents were increased, while the metabotropic inward current were decreased. In addition the metabotropic $GABA_B$-induced outward current was depressed. Altogether, these phenomena could be key elements in the constitution of brain damage during energy deprivation.

The observation that the inward responses caused by the ionotropic glutamate agonists were potentiated by glucose removal is, at least in part, consistent with the hypoglycemia induced prolongation of NMDA-induced inward current previously observed in dissociated dopaminergic cells of the substantia nigra (Nakashima et al. 1996). In addition, here we show that hypoglycemia not only prolongs but also increases the peak NMDA inward currents.

Previous studies have already demonstrated that anoxia either suppresses the NMDA-induced current (Krnjevic et al. 1989) or does not affect the postsynaptic responses to AMPA and NMDA (Khazipov et al. 1995) in hippocampal neurons. The differences between the present results and those obtained

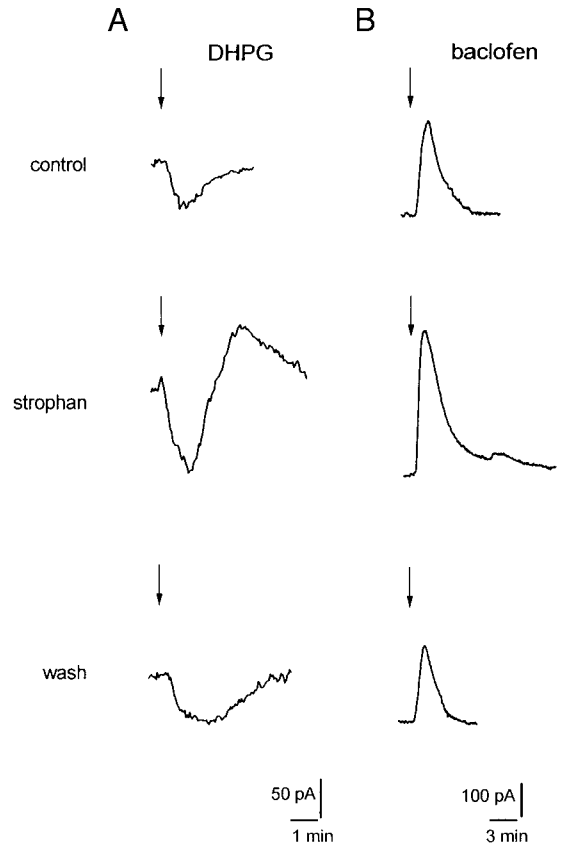

FIG. 5. Effects of strophanthidin on the metabotropic currents. *Top*: the typical current responses of 2 dopaminergic cells (held at −59 mV) to DHPG (50 μM for 25 s, *A*) and baclofen (10 μM for 24 s, *B*). *Middle*: during a treatment with the Na^+/K^+ pump inhibitor strophanthidin (3 μM for 15 min) both responses were slightly augmented. *Bottom*: the effects of strophanthidin were reversible (wash).

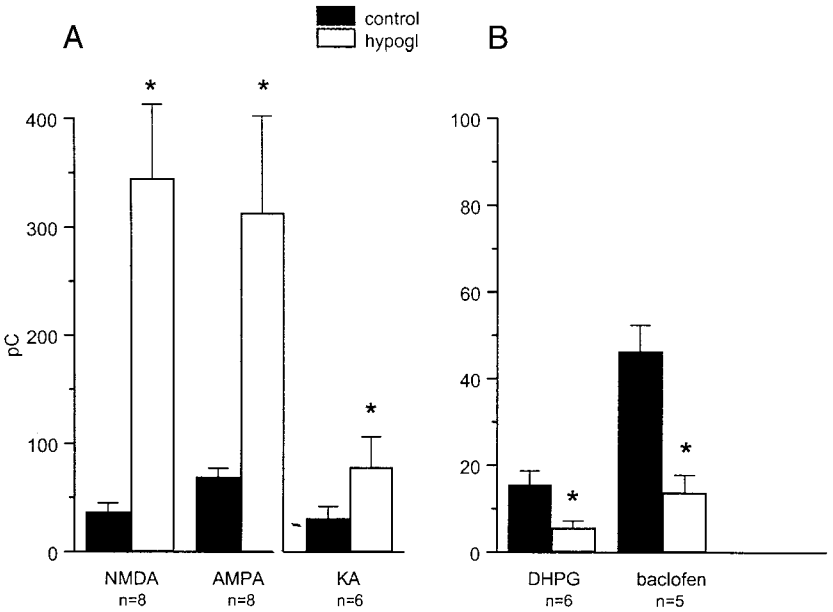

FIG. 6. $Na^{+/}K^{+}$ pump inhibition and ionotropic and metabotropic currents. *A*: the application of the $Na^{+/}K^{+}$ pump inhibitor (3 μM for 15 min), strophanthidin augmented the mean total current caused NMDA, AMPA, and KA on the dopaminergic cells. *B*: strophanthidin significantly increased the whole cell inward current caused by DHPG but did not significantly increase the outward current caused by baclofen. ■, controls (expressed in pC); □, taken during the effects of strophanthidin (strophant). Error bars represent SE. The number of experiments for each column is indicated. *, significant differences.

in the hippocampus might be due to the use of different cell types or different methods of obtaining energy deprivation (hypoglycemia vs. anoxia).

The basis for the hypoglycemia enhancement of the glutamate currents is an unbalanced sodium and calcium clearance. In fact, during the activation of NMDA, AMPA, and KA channels there is an increased permeability to sodium and calcium. Under normal conditions (normoglycemic), the Na^{+}/K^{+}-ATPase extrudes Na^{+} and energizes other secondary ion transporters (e.g., Na^{+}/Ca^{2+} exchange). If a profound drop in the level of extracellular glucose occurs, the consequent ATP depletion causes a reduced extrusion of Na^{+} and Ca^{2+} ions from the intracellular compartment (Haddad and Jiang 1993). It is important to note that during metabolic inhibition, a relevant component of the $[Na^{+}]_i$ load derives from glutamate-gated Na^{+} influx (Auer and Siesjo 1988; Choi and Rothman 1990). In addition, an increase in the content of $[Ca^{2+}]_i$ might derive not only from the activation of calcium permeable glutamate receptors but also from the reversal of Na^{+}/Ca^{2+} exchanger (Amoroso et al. 1993; Stys et al. 1991). All these events certainly cause a harmful disturbance of ion regulation (Kiedrowski et al. 1994), leading to a reduction of membrane potential, cell swelling, and irreversible damage.

In agreement with the hypothesis that an impaired function of the Na^{+}/K^{+}-ATPase augments the cellular responses to excitatory amino acids during hypoglycemia, we observed that the strophanthidin-induced block of this pump amplifies the inward currents caused by glutamate agonists. Consistent with the present results, it has been previously reported that the activity of the Na^{+}/K^{+}-ATPase pump regulates the glutamate-induced depolarization of striatal and hippocampal neurons (Calabresi et al. 1995; Fukuda and Prince 1992). However, changes of postsynaptic glutamate receptor conformation may also be involved during hypoglycemia.

The reduced glycolysis caused by the lack of energy substrates would be likely to produce a drop in intracellular ATP and GTP levels, which would limit the activation of G-protein-mediated events. Consequently, during hypoglycemia, the activation of metabotropic responses such as those caused by DHPG and baclofen (Tanabe et al. 1998) is reduced. In accordance, the baclofen-induced currents were not significantly modified by a treatment with strophanthidin. The fact that the agonist-induced responses along the explored voltage range was not modified by hypoglycemia or strophanthidin treatment further suggests that alteration of ion homeostasis and not of ion reversal potential be involved.

Physiopathological implications

It is widely accepted that the neuronal damage caused by energy deprivation is highly dependent on the release of excitatory amino acids (Auer 1986; Choi 1988; Siesjo et al. 1988; Szatkowski and Attwell 1994; Vornov 1995), which has negative consequences, including a sustained membrane depolarization, an increase in intracellular Na^{+} and Ca^{2+}, increase of extracellular potassium, and activation of reactive oxygen species (Coyle and Puttfarchen 1993; Dugan et al. 1995; Guatteo et al. 1998b; Peng and Greenamyre 1998; Rose et al. 1998; Szatkowski and Attwell 1994; Tymianski et al. 1993; Waxman et al. 1994).

In particular the dopaminergic cells are highly sensitive to excitotoxicity and oxidative stress when the energetic metabolism is impaired (Chan et al. 1994; Jenner et al. 1992; Marey-Semper et al. 1995; Shapira 1994).

The present electrophysiological data suggest that a reduced activity of Na^{+}/K^{+}-ATPase pump, induced by a reduction of energy levels due to glucose deprivation, enhances glutamate ionotropic responses in the dopaminergic cells of the substantia nigra pars compacta. Thus not only an increased release of excitatory amino acids but also amplified postsynaptic responses to glutamate associated to depressed $GABA_B$-mediated hyperpolarizations might contribute to the vulnerability of these neurons during an episode of energy failure.

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