Na
+<sup>+</sup>/Ca
2+<sup>+</sup> Exchanger Maintains Ionic Homeostasis in the Peri-Infarct Area

Anna Tortiglione, PhD; Barbara Picconi, PhD; Ilaria Barone, PhD; Diego Centonze, MD; Silvia Rossi, MD; Cinzia Costa, MD; Massimiliano Di Filippo, MD; Alessandro Tozzi, PhD; Michela Tantucci, PhD; Giorgio Bernardi, MD; Lucio Annunziato, MD; Paolo Calabresi, MD

**Background and Purpose**—A prominent feature of cerebral ischemia is the excessive intracellular accumulation of both Na
+<sup>+</sup> and Ca
2+<sup>+</sup> ions, which results in subsequent cell death. The plasma membrane Na
+<sup>+</sup>/Ca
2+<sup>+</sup> exchanger (NCX), regulates the distribution of these ions acting either in the forward mode or in its reverse mode and it can play a critical role in brain ischemia. However, it is unclear whether the activity of NCX leads to detrimental or beneficial effects.

**Methods**—Extracellular field potentials and whole-cell patch clamp recordings were obtained from rat corticostriatal brain-slice preparations in the peri-infarct area 24 hours after the permanent middle cerebral artery occlusion. Ischemia was induced in rats by permanent middle cerebral artery occlusion.

**Results**—Bepridil, an inhibitor of NCX, reduced in a concentration-dependent manner (IC
<sub>50</sub>
= 68 µmol/L) the field potential amplitude recorded from the peri-infarct area of corticostriatal slices. Conversely, no change was observed in sham-operated animals. The effect of bepridil was mimicked by 5-(N-4-chlorobenzyI)-2',4'-dimethylbenzamil (CB-DMB) (IC
<sub>50</sub>
= 6 µmol/L), a more selective inhibitor of NCX. In whole-cell patch clamp experiments, bepridil and CB-DMB caused an inward current in spiny neurons recorded from the peri-infarct area but not in the same cells recorded from controls. Interestingly, cholinergic interneurons recorded from the striatal peri-infarct area did not develop an inward current after the application of NCX inhibitors, suggesting that the electrophysiological alterations induced by NCX inhibition are cell-type specific. Bepridil and CB-DMB also induced a suppression of excitatory synaptic currents in most of spiny neurons recorded from the peri-infarct area. This effect was not coupled to a significant change of paired-pulse facilitation suggesting a postsynaptic site of action.

**Conclusions**—Our data indicate that NCX plays a critical role in the maintenance of ionic homeostasis in the peri-infarct area. (Stroke. 2007;38:1614-1620.)

Key Words: electrophysiology ■ field potential ■ ischemia ■ Na
+<sup>+</sup>/Ca
2+<sup>+</sup> exchanger ■ permanent middle cerebral artery occlusion ■ striatum

In neuronal cells, the Na
+<sup>+</sup>/Ca
2+<sup>+</sup> exchanger (NCX) plays a fundamental role in controlling Na
+<sup>+</sup> and Ca
2+<sup>+</sup> homeostasis. Depending on the intracellular concentrations of Ca
2+<sup>+</sup> and Na
+<sup>+</sup>, NCX can operate either in the forward mode, coupling the extrusion of Ca
2+<sup>+</sup> to the influx of Na
+<sup>+</sup> ions, or in the reverse mode, mediating the extrusion of Na
+<sup>+</sup> and the influx of the Ca
2+<sup>+</sup> ions. This feature of NCX is of critical importance considering that changes in the intracellular concentrations of Na
+<sup>+</sup> and Ca
2+<sup>+</sup> ions are known to occur in pathophysiologic states, such as brain ischemia.

Moreover, a prominent feature of cerebral ischemia is the excessive extracellular accumulation of glutamate and subsequent impairment of intracellular Na
+<sup>+</sup> and Ca
2+<sup>+</sup> homeostasis, which results in subsequent cell death. Thus, by controlling intracellular homeostasis of these 2 ions, NCX may play a pivotal role in the events leading to ischemic damage. Reduction of the energy supply leads to a cascade of events including depolarization, influx of Na
+<sup>+</sup>, and the subsequent reverse operation of the membrane protein that ultimately terminates in intracellular Ca
2+<sup>+</sup> overload and irreversible neuronal injury.

Conflicting reports have been published on the role played by NCX during brain ischemia. The inhibition of NCX prevents the activation of phospholipases that occurs after an ischemic insult of the brain. Similarly, activation of NCX in its reverse mode plays an important role in cellular Ca
2+<sup>+</sup> homeostasis.
overload and irreversible damage after energy depriva-
tion.13–16 Conversely, by using in vitro and in vivo models of
anoxia and ischemia, it has been demonstrated that the
stimulation of NCX activity may help neurons to survive,
whereas its pharmacological blockade can compromise their
survival.6,7,17,18 Accordingly, we found that the activation of
the NCX exerts a protective role during the early phase of
oxygen and glucose deprivation in striatal neurons.19

Considering this controversial issue, we have analyzed the
effect of the pharmacological inhibition of NCX on the
physiological properties of neurons recorded from the stria-
tum, a structure that is highly vulnerable to ischemic in-
sults.4,5,20,21 The mRNA and proteins of the 3 isoforms NCX1,
NCX2, and NCX3 are widely expressed in the striatum.9,22
However, their exact location on specific striatal neuronal
subpopulations as well as their role in cell-type–specific postis-
chemic striatal damage has been not yet analyzed. Thus, we
have recorded the physiological activity of striatal neurons in the
peri-infarct area after permanent middle cerebral artery occlu-
sion and their electrical response to the pharmacological inhibi-
tion of NCX.

In the peri-infarct area, we have also studied the possible
differential sensitivity to NCX inhibition of striatal spiny
neurons, a neuronal subtype that is highly sensitive to
ischemia and energy deprivation, versus striatal cholinergic
interneurons, that have been reported to be resistant to these
pathological conditions.4,5,20,21

Materials and Methods

Animals

Sprague-Dawley male rats (250 to 270 grams; Charles River, Lecco,
Italy) were used. All the experiments were conducted in conformity
with the European Communities Council Directive of November

Permanent Middle Cerebral Artery Occlusion,
Monitoring of Regional Cerebral Blood Flow, and
Infarct Size Analysis

Methods for permanent middle cerebral artery occlusion have been
previously described.23,24 Regional cerebral blood flow was moni-
tored in the ipsilateral parietal cortex to the occluded middle cerebral
artery using laser Doppler flowmeter (Periflux System 5000), pro-
vided with PC software (PeriSoft PSW 2.0) used to record the blood
flow.25

For the staining of infarct size analysis, 2,3,5-triphenyltetrazolium
chloride was used as previously described.24 The results are given as
percentage of the infarcted volume, adjusted for edema index, as the percentage of the
whole ipsilateral cerebral hemisphere.26

Electrophysiology and Identification of the
Peri-Infarct Area

Preparation and maintenance of rat corticostriatal slices (270 to
300 μm) have been previously described.19,20,27 Slices were kept in
artificial cerebrospinal fluid (34°C, O2/CO2) whose composition was
(in mmol/L): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4
CaCl2, 11 glucose, and 25 NaHCO3. For synaptic stimulation, bipolar
electrodes were used, located in the white matter to activate
corticostriatal fibers.

Electrophysiological electrodes for extracellular recording (15 to
20 mol/1L) were filled with 2 mol/L NaCl.

The peri-infarct area was defined through electrophysiological
criteria.28 Recording electrodes were initially positioned within the
striatum. The stimulating electrode, constantly held 0.3 mm apart,
artery occlusion was dose-dependent (n=6 for each dose; IC₅₀=68 μmol/L).

The inhibitory action of bepridil was also reproduced with CB-DMB as shown in the dose-response curve of Figure 2 (IC₅₀ of 6 μmol/L). This compound, synthesized by the amiloride discoverer Cracoe, is the most potent and selective pyrazine derivative, which inhibits forward and reverse mode of NCX operation. Interestingly, this NCX inhibitor, at the incubation time intervals used in the present study, does not interfere with L-type Ca²⁺ channels. However, more recent putative NCX inhibitors including KB-R7943

Figure 1. The inhibition of the NCX induced by bepridil reduces the field potential amplitude recorded from the peri-infarct area in rat corticostrital slices. A. Electrophysiological and morphological characterization of the peri-infarct area (P) and of the ischemic core (C). B. The plot represents the effect of bath application of 100 μmol/L bepridil in corticostrital slices obtained at 24 hours after permanent middle cerebral artery occlusion in multiple experiments and in slices obtained from sham-operated animals. C. Traces represent examples of corticostrital field potentials recorded before, during, and after bath application of 100 μmol/L bepridil in slices obtained from sham-operated animals (upper row) and from animals exposed to focal ischemia (lower rows).

Figure 2. Bepridil decreases the field potential amplitude in ischemic slices in a dose-dependent manner (A). The selective NCX inhibitor CB-DMB mimics the inhibitory effects of bepridil of field potential amplitude in a dose-dependent manner (B). The graph shows the dose-response curve obtained from ischemic tissue and from sham-operated striata. Each data point represents at least 3 single experiments. Traces were obtained from single experiments in ischemic and sham-operated animals (C).

Figure 2. Bepridil decreases the field potential amplitude in ischemic slices in a dose-dependent manner (A). The selective NCX inhibitor CB-DMB mimics the inhibitory effects of bepridil field potential amplitude in a dose-dependent manner (B). The graph shows the dose-response curve obtained from ischemic tissue and from sham-operated striata. Each data point represents at least 3 single experiments. Traces were obtained from single experiments in ischemic and sham-operated animals (C).
and SN-6 can also interfere with several ion transporters and channels.\textsuperscript{38} To rule out that the reduction of the field potential observed in the peri-infarct area after the administration of NCX inhibitors could be caused by tissue deterioration during electrophysiological recordings rather than by the specific drug effect, we performed field potential recordings in the peri-infarct area also in control medium. Under this experimental condition, we have observed no change of the field potential amplitude throughout the recording time (n=9, data not shown).

**Effect of NCX Inhibitors on Membrane Currents of Medium Spiny Neurons and Large Aspiny Interneurons Recorded in the Peri-Infarct Area**

To assess the possible mechanism underlying the irreversible loss of the field potential amplitude caused by bepridil in spiny neurons after ischemia, we measured, by using whole-cell patch clamp recordings, the effects of NCX inhibitors on somatic currents recorded either in the striatal peri-infarct area or in the striatum of sham-operated rats. As shown in Figure 3A through 3C, 100 μmol/L bepridil induced an inward current in most of the medium spiny neurons (8/12) recorded from the peri-infarct area of striatal slices obtained either from sham-operated animals (upper rows) or from animals exposed to focal ischemia (lower rows). The holding potential was kept constant during all the experiment and it was −80 mV for spiny neurons and −60 mV for cholinergic interneurons. The histogram shows the percentage of spiny neurons showing irreversible inward currents in sham-operated rats and in animals exposed to ischemia 24 hours before. The plot shows the time-course of the bepridil-induced inward currents in spiny neurons recorded from the peri-infarct area. The plot shows an example of a current–voltage relationship measured from spiny neurons recorded in the peri-infarct area and obtained by delivering both positive and negative voltage steps from the holding potential.

Figure 3. The inhibition of the NCX produces an inward current in striatal medium spiny neurons (MS), but not in large cholinergic interneurons (LA) recorded from the peri-infarct area. A, Changes of the membrane currents detected by whole-cell patch clamp recordings in spiny neurons versus cholinergic interneurons in slices obtained either from sham-operated animals (upper rows) or from animals exposed to focal ischemia (lower rows). The holding potential was kept constant during all the experiment and it was −80 mV for spiny neurons and −60 mV for cholinergic interneurons. B, The histogram shows the percentage of spiny neurons showing irreversible inward currents in sham-operated rats and in animals exposed to ischemia 24 hours before. C, The plot shows the time-course of the bepridil-induced inward currents in spiny neurons recorded from the peri-infarct area. D, The plot shows an example of a current–voltage relationship measured from spiny neurons recorded in the peri-infarct area and obtained by delivering both positive and negative voltage steps from the holding potential.

**Effect of NCX Inhibitors on Evoked Excitatory Synaptic Current Recorded From Medium Spiny Neurons in the Peri-Infarct Area**

Figure 4 shows that application of bepridil causes a progressive reduction of the amplitude of excitatory postsynaptic currents (EPSCs) recorded from medium spiny neurons in the peri-infarct area (n=7, P<0.05). Also in this case, as well as for the bepridil-induced inward current, the EPSCs did not recover after the interruption of the drug application. In medium spiny neurons obtained from the striatum of sham-operated rats, no change of EPSC amplitude was observed (n=7, P>0.05).

To investigate whether the depression of EPSCs induced by bepridil in spiny neurons from the peri-infarct area was dependent on presynaptic or postsynaptic sites of action, we measured synaptic responses to a pair of stimuli before and during the application of the NCX inhibitor. In these experiments, interstimulus interval was 60 ms. Paired-pulse modification of neurotransmission has been studied extensively and is attributed to a presynaptic change in release probabil-
ity. An increase in the ratio of the second pulse response to the first pulse response (EPSC2/EPSC1) indicates a decrease in the release probability. The depression of the EPSC amplitude induced by 100 μmol/L bepridil was not associated with a significant increase in this ratio (n=5, P>0.05; Figure 4B), suggesting that postsynaptic mechanisms play a role in the observed effect.

The synaptic effects of bepridil were mimicked by 10 μmol/L CB-DMB (n=7, data not shown).

Discussion

The present study represents the first electrophysiological demonstration that the activation of NCX maintains ionic homeostasis in the peri-infarct area after focal ischemia. We found, in fact, that the pharmacological inhibition of NCX by bepridil and CB-DMB causes an irreversible loss of field potential amplitude recorded from the striatal area surrounding the ischemic core. These effects were dose-related and irreversible suggesting that inhibition of NCX can trigger a cascade of detrimental events leading to neuronal damage. The hypothesis that the electrophysiological action of bepridil and CB-DMB are closely linked to the ischemia-triggered events is also confirmed by the evidence that these drugs failed to alter the field potential amplitude in slices obtained from sham-operated rats.

Bepridil is not considered a selective NCX inhibitor. In fact, this drug inhibits sodium and calcium channels and modulates potassium conductances. Nevertheless, the use of a more specific NCX inhibitor, such as CB-DMB, mimicking the electrophysiological effects of bepridil strongly support the hypothesis that the effects we observed are dependent on the selective inhibition of NCX. The change of the expression in NCX1, NCX2, and NCX3 gene products after permanent middle cerebral artery occlusion seems to suggest that these isoforms can be involved in the process leading to cell death or survival in those areas that are interested by the ischemic insult.5,7,9

According to our electrophysiological and pharmacological data, it has been shown that after knocking down 2 of these proteins, NCX1 and NCX3, a remarkable enlargement of the infarct volume occurred. Such a phenomenon was associated with a worsening of neurological deficits.7 Accordingly, the pharmacological inhibition of NCX leads to an enlargement of the infarct size.6

By using single-cell patch clamp recordings, we have demonstrated that the pharmacological blockade of NCX produced an inward current in spiny neurons recorded in the peri-infarct area, whereas no current was detected in cells obtained from sham-operated animals. The estimated reversal potential for this inward current was around -45 mV, indicating that multiple events rather than a single conductance seem to be involved.27 The single cell experiments gave us also the possibility to demonstrate that the pharmacological action of bepridil and CB-DMB were cell-type–specific. In fact, no inward current was detected from striatal large cholinergic interneurons recorded from the peri-infarct area. Noticeably, striatal spiny neurons are vulnerable to energy deprivation, whereas cholinergic interneurons have been reported to be resistant to ischemia.4,5,20,21 Thus, we can argue that the different expression of NCX among various striatal neuronal subtypes might take part in the mechanisms controlling cell-type–specific vulnerability to ischemia within the striatum and, possibly, in other brain areas. However, as an alternative hypothesis, we can consider the possibility that although the expression of NCX is similar in spiny neurons and cholinergic interneurons, these 2 neuronal subtypes respond in a distinct manner to NCX inhibition because they have a differential capability to compensate alterations of ion concentrations.

The inward current recorded from spiny neurons during application of NCX inhibitors was paralleled by a progressive...
decrease of the amplitude of the evoked EPSCs. This latter effect may also represent the synaptic event underlying loss of the field potential amplitude detected by extracellular recordings. In fact, the field potential amplitude reflects the synchronous discharge of several neurons adjacent to the recording electrode and activated by excitatory fibers. Interestingly, the reduction of the EPSC amplitude was not coupled to changes in EPSC2/EPSC1 ratio, suggesting that postsynaptic rather than presynaptic mechanisms seem to underlie this event.

An interesting issue that should be considered in future studies is related to the potential recovery of the peri-infarct area and its relationship with the functional activity of NCX at different time windows after the ischemic insult.

The 3 isoforms of NCX that are present in the striatum are constitutively expressed not only in neurons but also in glia.39 Interestingly, the expression of this antiporter can be altered under particular conditions, such as hypoxia.9

Cerebral edema is known to exacerbate neuronal damage induced by ischemia. Accordingly, it has been recently reported that a nonselective cation channel is expressed after brain ischemia and is crucially involved in development of cerebral edema.40 However, no evidence has never been provided on an involvement of NCX on water distribution either in normal tissue or in focal ischemia.

NCX is also expressed in peripheral cells such as macrophages and leukocytes5 migrating in the brain after the ischemic insult; therefore, a hypothetical role played by the modification of NCX in these cells cannot be excluded. Nevertheless, the changes observed in the electrophysiological behavior of spiny and cholinergic interneurons seem to favor the idea that the action of the NCX inhibitors is mainly directed to neuronal cells rather than in peripheral cells.

In conclusion, our data suggest that in the peri-infarct area, NCX plays a role in maintaining ionic homostasis because its pharmacological block leads to detrimental effects after focal cerebral ischemia. Moreover, this effect is selectively expressed in neurons particularly vulnerable to energy deprivation.

Acknowledgments
We thank Mr Cristiano Spaccatini and Mr Massimo Tolu for their excellent technical support.

Sources of Funding
This work was supported by grants from Ministero della Salute (Ricerca Finalizzata 2004, 2005 IRCCS to P.C. and B.P.), FIRB 2001, Fondazione Cassa Risparmio Perugia (to P.C.).

Disclosures
None.

References


Na⁺/Ca²⁺ Exchanger Maintains Ionic Homeostasis in the Peri-Infarct Area
Anna Tortiglione, Barbara Picconi, Ilaria Barone, Diego Centonze, Silvia Rossi, Cinzia Costa, Massimiliano Di Filippo, Alessandro Tozzi, Michela Tantucci, Giorgio Bernardi, Lucio Annunziato and Paolo Calabresi

Stroke. 2007;38:1614-1620; originally published online March 29, 2007; doi: 10.1161/STROKEAHA.106.478644
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/5/1614

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/