Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger Maintains Ionic Homeostasis in the Peri-Infarct Area

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Background and Purpose—A prominent feature of cerebral ischemia is the excessive intracellular accumulation of both Na\textsuperscript{+} and Ca\textsuperscript{2+} ions, which results in subsequent cell death. The plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} 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\textsubscript{50}=68 \(\mu\)mol/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-chlorobenzyl)-2',4'-dimethylbenzamil (CB-DMB) (IC\textsubscript{50}=6 \(\mu\)mol/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 ion homeostasis in the peri-infarct area. (Stroke. 2007;38:1614-1620.)

Key Words: electrophysiology ■ field potential ■ ischemia ■ Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger ■ permanent middle cerebral artery occlusion ■ striatum

In neuronal cells, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) plays a fundamental role in controlling Na\textsuperscript{+} and Ca\textsuperscript{2+} homeostasis.\textsuperscript{1} Depending on the intracellular concentrations of Ca\textsuperscript{2+} and Na\textsuperscript{+}, NCX can operate either in the forward mode, coupling the extrusion of Ca\textsuperscript{2+} to the influx of Na\textsuperscript{+} ions, or in the reverse mode, mediating the extrusion of Na\textsuperscript{+} and the influx of the Ca\textsuperscript{2+} ions.\textsuperscript{1} This feature of NCX is of critical importance considering that changes in the intracellular concentrations of Na\textsuperscript{+} and Ca\textsuperscript{2+} ions are known to occur in pathophysiologic states, such as brain ischemia.\textsuperscript{2,9}

Moreover, a prominent feature of cerebral ischemia is the excessive extracellular accumulation of glutamate and subsequent impairment of intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} homeostasis, which results in subsequent cell death.\textsuperscript{3,10,11} 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\textsuperscript{+}, and the subsequent reverse operation of the membrane protein that ultimately terminates in intracellular Ca\textsuperscript{2+} overload and irreversible neuronal injury.\textsuperscript{2,12}

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.\textsuperscript{13} Similarly, activation of NCX in its reverse mode plays an important role in cellular Ca\textsuperscript{2+} overload and irreversible neuronal injury.
overload and irreversible damage after energy depreviation.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 striatum, a structure that is highly vulnerable to ischemic insults.\textsuperscript{4,5,20,21} The mRNA and proteins of the 3 isoforms NCX1, NCX2, and NCX3 are widely expressed in the striatum.\textsuperscript{9,22} However, their exact location on specific striatal neuronal subpopulations as well as their role in cell-type–specific postischemic 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 arterial occlusion and their electrical response to the pharmacological inhibition 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.\textsuperscript{4,5,20,21}

\section*{Materials and Methods}

\subsection*{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 1986 (86/609/EEC).

\subsection*{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.\textsuperscript{23,24} Regional cerebral blood flow was monitored in the ipsilateral parietal cortex to the occluded middle cerebral artery using laser Doppler flowmeter (Periflux System 5000), provided with PC software (PeriSoft PSW 2.0) used to record the blood flow.\textsuperscript{25}

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

\subsection*{Electrophysiology and Identification of the Peri-Infarct Area}
Preparation and maintenance of rat corticostriatal slices (270 to 300 \textmu m) have been previously described.\textsuperscript{19,20,27} Slices were kept in artificial cerebrospinal fluid (34° C, O\textsubscript{2}/CO\textsubscript{2}) whose composition was (in mmol/L): 126 NaCl, 2.5 KCl, 1.2 MgCl\textsubscript{2}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 2.4 CaCl\textsubscript{2}, 11 glucose, and 25 NaHCO\textsubscript{3}. 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/L) were filled with 2 mol/L NaCl.

The peri-infarct area was defined through electrophysiological criteria.\textsuperscript{28} Recording electrodes were initially positioned within the striatum. The stimulating electrode, constantly held 0.3 mm apart, was progressively placed in up to 8 distinct positions around the recording electrode (360°) to try to elicit extracellular field potentials. We assumed we were in the ischemic core when no reliable field potential (negative deflection from the baseline of at least 0.2 mV) was evocable despite the progressive increase of the stimulation intensity. From the “core” position, the electrodes were progressively (0.5 mm) moved along a single line toward the periphery of the striatum, with the stimulating electrode preceding the recording one. When the field potential appeared, we considered we were in the peri-infarct area. In the “ischemic core” it was not possible to obtain single cell recordings by using whole-cell patch clamp. Conversely, in the peri-infarct area this technique allowed to obtain reliable electrophysiological recordings. This was confirmed analyzing the extension of the ischemic core after 2,3,5-triphenyltetrazolium chloride staining of the slices obtained immediately before and after the one used for the electrophysiological experiments.

\subsection*{Whole-Cell Patch Clamp Recordings}
Whole-cell patch clamp recordings and identification of individual neurons were performed in situ using a differential interference contrast (Nomarski) optical system as previously described.\textsuperscript{29} Striatal spiny neurons were clamped at −80 mV, whereas striatal aspiny interneurons were clamped at −60 mV, close to their respective resting membrane potentials. All the experiments were performed in the presence of 30 \mu mol/L bicuculline.\textsuperscript{29}

Brain slices were prepared 24 hours after the ischemic insult. Whole patch clamp recordings were obtained within 0.5 to 3 hours after slice preparation. Within this period, the viability of the neurons within the slice was perfect. In fact, although in the ischemic core no recording was possible, in the peri-infarct area as well as in the surrounding normal tissue both spiny neurons and cholinergic interneurons showed intrinsic and synaptic membrane properties similar to those previously reported for “healthy” neurons.\textsuperscript{27-29}

Although from each single slice we could obtain several whole-cell recordings, we used only one cell to perform the pharmacological experiments with NCX inhibitors to avoid multiple applications of these drugs on the same brain slice preparation.

\subsection*{Statistical Analysis and Drug Application}
Values represent mean±SEM of changes in the respective cell populations. Student t test was used to compare the means. Bepridil (Sigma, Milan, Italy) was applied by dissolving it to the desired final concentration in the saline solution. S-(N-4-chlorobenzylidene)-2,4'-dimethylbenzamil (CB-DMB) was from Division of Pharmacology, Department of Neuroscience, School of Medicine, “Federico II” University of Naples, Italy.

\section*{Results}

\subsection*{Pharmacological Blockade of NCX and Field Potentials Recorded in the Peri-Infarct Area}
As shown in Figure 1A, the peri-infarct area was detected by morphological and electrophysiological analysis by field potential detections\textsuperscript{28} at 24 hours after the ischemic insult. From sham-operated animals, slices were obtained at the same time window. Application of bepridil (100 \mu mol/L) in the extracellular medium for 15 minutes significantly reduced the amplitude of the field potential obtained 24 hours \textit{(n}=8, ***P<0.0001 field potential amplitude \textit{t}=−15 versus \textit{t}=40) after the permanent middle cerebral artery occlusion (Figure 1B and 1C). No significant inhibition was detected in slices prepared from sham-operated animals at the same time window (100 \mu mol/L, \textit{n}=8, \textit{P}>0.05 field potential amplitude \textit{t}=−15 versus \textit{t}=40; Figure 1B and 1C).

As shown in Figure 2, the inhibitory action of bepridil on the amplitude of field potentials recorded from the peri-infarct area of rats exposed to permanent middle cerebral
artery occlusion was dose-dependent (n=6 for each dose; IC50 = 68 μmol/L).

Figure 1. The inhibition of the NCX induced by bepridil reduces the field potential amplitude recorded from the peri-infarct area in rat corticostriatal 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 corticostriatal 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 corticostriatal field potentials recorded before, during, and after bath application of 100 μmol/L bepridil in slices obtained from sham-operated animals and from animals exposed to focal ischemia.

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 Cragoe, 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...
and SN-6 can also interfere with several ion transporters and channels. 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 24 hours after the ischemic insult (P < 0.01 15 minutes after the application of bepridil). Conversely, spiny neurons recorded from sham-operated striata did not show significant changes of resting membrane currents (11/11) (P > 0.05, 15 minutes after the application of bepridil). Interestingly, cholinergic interneurons did not show bepridil-induced current when recorded either from the peri-infarct area (7/7) (P > 0.05, 15 minutes after the application of bepridil), or from control tissue (6/6) (P > 0.05, 15 minutes after the application of bepridil). The bepridil-induced inward current detected in the spiny neurons from the peri-infarct area developed within 5 minutes from the onset of the application of bepridil and reached a peak at ≈15 minutes. This inward current did not tend to reverse after bepridil removal.

Figure 3D shows a voltage clamp experiment performed by applying increasing voltage steps in both directions to detect a reversal potential for the bepridil-induced inward current. The reversal potential for this current was estimated close to −45 mV (n = 4). CB-DMB 10 μmol/L mimicked the effects of bepridil, inducing an inward current (at least 50 pA) in 5 of 6 spiny neurons recorded in the peri-infarct area (data not shown). No current was detected in 5 spiny neurons obtained from sham-operated rats. Moreover, no current was induced in cholinergic interneurons recorded either from ischemic tissue (n = 4) or from sham-operated animals (n = 5) (data not shown).

### 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 in the expression of 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 ≈ −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,4,20,21 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 post synaptic 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. Interestingly, the expression of this antiporter can be altered under particular conditions, such as hypoxia.

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. 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 leukocytes 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.

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Disclosures

None.

References


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