Dynamic Recording of Cell Death in the In Vitro Dorsal Vagal Nucleus of Rats in Response to Metabolic Arrest

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Müller, Michael and Klaus Ballanyi. Dynamic recording of cell death in the in vitro dorsal vagal nucleus of rats in response to metabolic arrest. J Neurophysiol 89: 551–561, 2003. 10.1152/jn.00559.2002. Anoxic/ischemic neuronal death is usually assessed in cell cultures or in vivo within a time window of 24 h to several days using the nucleic acid stain propidium iodide or histological techniques. Accordingly, there is limited information on the time course of such neuronal death. We loaded acute rat brain stem slices with propidium iodide for dynamic fluorometric recording of metabolic arrest-related cell death in the dorsal vagal nucleus. This model was chosen because dorsal vagal neurons show a graded response to metabolic inhibition: anoxia and aglycemia cause a sustained hyperpolarization, whereas ischemia induces a glutamate-mediated, irreversible depolarization. We found that the number of propidium iodide–labeled cells increased from 27% to 43% of total cell count within 1–7 h after preparation of slices. Compared with these untreated control slices, cyanide-induced anoxia (30 min) or aglycemia (1 h) did not cause further cell death, whereas 3-h aglycemia destroyed an additional 13% of cells. Ischemia (1 h) due to cyanide plus iodoacetate immediately labeled an additional 20% of cells, and an additional 48% of cells were destroyed within the following 3 h of posts ischemia. Continuous recording of propidium iodide fluorescence showed that loss of membrane integrity started within 25 min after onset of the ischemic depolarization and the concomitant intracellular Ca2+ rise. The results show that propidium iodide can be used to monitor cell death in acute brain slices. Our findings suggest that pronounced cell death occurs within a period of 1–4 h after onset of metabolic arrest and is apparently due to necrotic/oncotic mechanisms.

INTRODUCTION

In most mammalian nervous structures, anoxia/ischemia-induced impairment of cellular function culminates in neuronal death (Lipton 1999; Luhmann and Heinemann 1992; Somjen et al. 1993). For example, neurons of the mature mammalian forebrain were demonstrated to respond within minutes of metabolic arrest with a nearly complete depolarization and a massive disturbance in ion homeostasis (Bureš and Burešová 1957; Haddad and Jiang 1993; Hansen 1985; Müller and Somjen 2000a,b). The occurrence of this “terminal depolarization” is one of the early events in the cascade terminating in cell death. The very event resembling cell death cannot be identified exactly, but an unambiguous and commonly used sign is the loss of integrity of the plasma membrane that can be detected by dye-exclusion techniques (Beversee et al. 1995; Laake et al. 1999; Lipton 1999; Loo and Rillema 1998). So far, little is known about the temporal correlation of metabolic insults, terminal depolarization, and the loss of membrane integrity. In the present study we therefore used the nucleic acid stain propidium iodide (PI) to assess the extent and time course of cell death resulting from chemical anoxia, aglycemia, and in vitro ischemia, and we elucidated the time span between the occurrence of the terminal depolarization and the loss of membrane integrity.

The red fluorescing dye PI is excluded from vital cells, but readily stains necrotic and/or late apoptotic cells. PI is a standard tool to assess cell viability in cultured cells (Beversee et al. 1995; Coco-Martin et al. 1992; Juurlink and Hertz 1993; Loo and Rillema 1998). It has also been used as a cell death marker in some recent studies on cultured brain slices (Laake et al. 1999; Lahtinen et al. 2001; Sakaguchi et al. 1997; Zimmer et al. 2000), but rather rarely in situ (Ingefield and Schwartz-Bloom 1998; Scarabelli et al. 1999; Tekkók and Goldberg 2001; Wolff et al. 2000). In most of the latter in vitro studies, cell death was routinely tested 24 h after an insult. Also in vivo, excitotoxic or anoxic/ischemic neuronal death is typically analyzed using histological techniques within a time window of days to weeks (Fukuda et al. 1999; Leite et al. 1996; Lipton 1999; Sugawara et al. 2002). This may reliably yield the full extent of cell death, but no information on its onset and the time course of neuronal death is obtained.

In this study, we aimed to determine the time span between the occurrence of the terminal depolarization and the diagnosis of cell death. For that purpose, we modified the PI-staining protocol, making PI fluorescence measurements feasible for the dynamic quantification of cell death in acute tissue slices. Our main focus was on viability changes in the metabolically challenged dorsal vagal nucleus. Dorsal vagal neurons (DVN), the principal cell type of that nucleus (Loewy and Spyer 1990), were shown in previous studies to tolerate anoxia or aglycemia periods of more than 30 min (Cowan and Martin 1992; Müller et al. 2002; Trapp and Ballanyi 1995), but to undergo a glutamate-mediated terminal depolarization during “in vitro ischemia” (Ballanyi and Kulik 1998; Ballanyi et al. 1996; Kulik et al. 2000; Martin 1999). With that clearly graded response to metabolic insults of differing severity, DVN seem...
well suited to study the correlation of terminal depolarization, cell death, and the time span in between these two events.

In detail, we compared cell death in untreated control slices and slices previously exposed to anoxia, aglycemia, or ischemia. To allow for the correlation of cell death and electrophysiological responses of single cells, we also analyzed the membrane potential responses and the associated changes in the free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) of single whole cell–recorded DVN. Furthermore, increased light transmittance of tissue slices was used as a marker for severe cell swelling during ischemia.

Parts of this study have been published as an abstract (Müller and Ballanyi 2001).

**METHODS**

**Preparation**

Medullary tissue slices were prepared from ether-anesthetized juvenile Wistar rats (16–22 days old). Following decapitation, the brain was rapidly removed from the skull and placed in ice-cold artificial cerebrospinal fluid (ACSF; for composition, see **Solutions**) with a reduced Ca$^{2+}$ concentration (0.5 mM) for 2–4 min. The brain stem was isolated, glued to the stage of a vibrorslicer (FTB Vibracut; Weinheim, Germany), and submerged in ice-cold, 0.5 mM Ca$^{2+}$-containing ACSF. Six to eight transverse 200-μm slices were cut around the obex level and stored in oxygenated saline at 30°C. To ensure for recovery from surgical trauma, slices were allowed to rest for ≥1 h before experiments were started. For this purpose, they were kept in storage chambers at 30°C, where they also underwent the various metabolic disturbances. Slices were then transferred to a submersion style recording chamber (1 ml), immobilized with a net, and superfused at a rate of 4–5 ml/min. Experiments were performed at a temperature of 30°C to allow for comparison with earlier studies of our laboratory and to assure long-term stability of whole cell recordings, which is difficult to obtain at higher temperatures.

**Solutions**

The ACSF had the following composition (in mM): 118 NaCl, 3 KCl, 1 MgCl$_2$, 1.5 CaCl$_2$, 25 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, and 10 d-glucose. Osmolarity was approximately 290 mOsm/l; pH was adjusted to 7.4 by aeration with 95% O$_2$–5% CO$_2$. Sodium cyanide and iodoacetate (Sigma-Aldrich, Taufkirchen, Germany) were kept frozen as aqueous 1 M stock solutions; dilutions were prepared freshly before each application. PI (Molecular Probes Europe, Leiden, The Netherlands) was prepared as an aqueous 1 mg/ml stock solution. Fura-2 (Molecular Probes) was dissolved as 5 mM aqueous stock solution and kept frozen. Triton X-100 (polyethylene glycol tert-octylphenyl ether; Fluka, Taufkirchen, Germany) was dissolved as a 20% stock solution in ACSF.

**Electrical recordings**

Patch pipettes for whole cell recording were pulled from thin-walled borosilicate glass (Clark GC150TF-10; Harvard Apparatus, Edenbridge, UK) using a horizontal puller (DMZ Universal Puller, Augsburg, Germany). The pipette solution contained the following (in mM): 140 K-glucuron, 1 Na$_2$ATP, 1 MgCl$_2$, 0.5 CaCl$_2$, 1 NaCl, and 10 HEPES. Osmolarity was approximately 285 mOsm/l, and pH 7.4 was adjusted with 1 M KOH, increasing the K$^+$ concentration by approximately 3 mM. Since whole cell recording was combined with microfluorometric recordings of [Ca$^{2+}$]$_i$, 100 μM Fura-2 were added to the pipette solution. Pipette resistance was 5–7 MΩ.

DVN were identified according to their location in the vagal nucleus (Fig. 1), spontaneous spike discharges and the occurrence of an A-type K$^+$ current in response to current-induced membrane hyperpolarization (Cowan and Martin 1992; Loewy and Spyer 1990; Trapp and Ballanyi 1995). Whole cell current-clamp recordings were performed using an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Data were sampled at an acquisition rate of 2.5 kHz, transferred to a PC (Labmaster TL-1 Interface) and analyzed with the pClamp6 suite of programs (Axon Instruments; Foster City, CA). Input resistance of DVN was measured every 10 s by a hyperpolarizing current pulse of 500-ms duration and 50-pA amplitude.
Changes in membrane potential and input resistance were referred to the pretreatment baseline and the input resistance changes were expressed in percent.

Microfluorometric recordings of $[\text{Ca}^{2+}]_i$

Changes in $[\text{Ca}^{2+}]_i$ were monitored using a photomultiplier-equipped upright microscope (Standard 16, Zeiss, Göttingen, Germany) and a monochromator/illumination unit (Polychrome I, TILL-Photonics, Martinsried, Germany). DVN were dye-loaded in the whole cell configuration via the patch pipette, and Fura-2 was excited by 20-ms light pulses of alternating wavelengths (360 nm/385 nm). Fluorescence emission was measured through a 510-nm dichroic mirror and a 515- to 656-nm band-pass filter and a pinhole diaphragm (20 μm). For all experiments a 63× water immersion objective lens (Zeiss Achromplan) was used. Fura-2 fluorescence was calibrated according to the in vitro method described by Neher (1989). The maximum ratio ($R_{\text{max}} = 2.94$), the minimum ratio ($R_{\text{min}} = 0.37$), and the dissociation constant of Fura-2 ($K_{\text{eff}} = 523 \text{nM Ca}^{2+}$) were determined from 10 mM Ca$^{2+}$, 0 mM Ca$^{2+}$, and 300 nM Ca$^{2+}$ pipette solutions, respectively, which also contained 100 μM Fura-2. The measured fluorescence ratios ($R$) were converted into Ca$^{2+}$ concentrations using the following equation (Gryniewicz et al. 1985)

$$[\text{Ca}^{2+}]_i = K_{\text{eff}} (R - R_{\text{min}}) / (R_{\text{max}} - R)$$

Evaluation of cell death

Our major aim was to quantify cell death resulting from metabolic disturbances in the dorsal vagal nucleus (Fig. 1A). Therefore analysis was restricted to that region. Nevertheless, all images also show parts of the hypoglossal nucleus to facilitate identification of the dorsal vagal nucleus and some images also contain parts of the central canal. Cell death was evaluated using the “dead cell” marker propidium iodide (PI) (Bevensee et al. 1995; Laake et al. 1999; Loo and Rillema 1998), which on loss of membrane integrity binds to nucleic acids and responds with increased fluorescence emission (Arndt-Jovin and Jovin 1989).

PI was excited at 535 nm and fluorescence emission was measured beyond 590 nm, using the Omega Opticals XF34 filter set (exciter: 535/35 nm band-pass, dichroic mirror: 570 nm, emitter: 590 nm longpass). PI fluorescence was recorded using an imaging system equipped with a 12-bit CCD camera (TILL-Photonics) that was mounted to an upright microscope (Axioskop I, Zeiss). Images for off-line analysis and documentation were taken using a 20× water-immersion objective lens (Zeiss Achromplan). Snapshots of single DVN were taken with a 63× water-immersion objective lens (Zeiss Achromplan).

Superfusion or incubation of slices with PI (2 μg/ml) resulted within approximately 5 min in bright red staining of the nuclei of some individual cells (Fig. 1, B and C), and the build-up of PI fluorescence then started to level off (Fig. 2). Obvious unspecific PI staining was observed in the cell layers lining the central canal and the periphery of the slice (see Fig. 2A), but not in the dorsal vagal nucleus. For statistical comparison of different slices, cell death was normalized to the total number of cells present in the dorsal vagal nucleus of a given slice. For that purpose, a slice was first stained by incubation in PI-containing ACSF for 5–8 min. It was then transferred to the recording chamber to take images and to assess the amount of dead cells resulting from a certain treatment. The total number of cells in the dorsal vagal nucleus of that slice was then determined by subsequent Triton-induced cell permeabilization in the presence of PI, superfusing the slice with ACSF containing 1% Triton X-100 and PI (2 μg/ml) for ≤25 min. Under these conditions, intense staining of

FIG. 2. Feasibility of PI labeling for assessing cell viability in tissue slices. A: PI staining of an untreated control slice (2.5-h-old) results in brightly red stained individual nuclei. Slices are oriented with their dorsal side up and the central canal in the lower right corner; the dorsal vagal nucleus is marked. As can be seen from the plotted time course of the fluorescence intensity recorded in the dorsal vagal nucleus, PI fluorescence starts to develop within 3–5 min of PI application (arrow). The differentiated time course ($dF/dt$) reveals the presence of 2 or more different time constants. The most intense increase in fluorescence intensity occurred during the first few minutes of PI application and the fluorescence changes then started to level off. Prolonged PI treatment intensified the staining, but the number of labeled cells did not increase further. B: following membrane permeabilization by absolute ethanol, virtually every cell was labeled by PI. Accordingly, PI fluorescence was much more intense than in control slices (note different ordinate scaling). In addition, the time course of PI fluorescence was steeper, and PI fluorescence still noticeably increased even after 80 min of PI treatment, as is also indicated by the differentiated curve.
virtually every cell in the slice usually occurred within 15 min; prolonged permeabilization just increased the intensity of PI labeling, but not the cell count (Fig. 3). Since cell permeabilization was final, each slice could be used for a single treatment only.

Statistics

The data were obtained from 35 rats, using ≤6 slices from each brain. Each experimental series was performed on at least three different animals. All numerical values are represented as mean ± SD. Since quantification of cell death required cell permeabilization, control and drug effects could only be investigated in different slices (unpaired observations). Significance of the observed changes was tested using two-tailed, unpaired Student’s t-test and a significance level of 5%. In the figures, significant changes are marked by asterisks (*P < 0.05, **P < 0.01). Statistical calculations were done with the Excel 7.0 or QuattroPro 3.0 software.

RESULTS

Using PI in acute tissue slices

In initial trials we assured the feasibility of PI as a “dead cell” marker in brain stem slices, DVN show a characteristic bipolar spindle-like shape that is best seen on dye-loading (Ballanyi 1999; Yarom et al. 1985). Due to their shape, superficially located cells can easily be identified and cells of obvious vital cell shape and appearance—our criteria for selecting DVN for patch-clamp recordings—were never found to be labeled by PI (Fig. 1). Also, in untreated slices, PI clearly stained only some of the present cells, while in slices that were permeabilized by absolute ethanol, virtually every single cell was stained (Fig. 2). Studying the kinetics of PI labeling revealed that dead cells can be detected within <5 min of PI staining (Fig. 2). Cell permeabilization by Triton X-100 in the presence of PI (Fig. 3) gave us a tool to quantify cell death, to normalize cell death in a given slice to the total number of cells present, and to compare its extent in different slices (for details, see METHODS).

Cell death in untreated slices

Before testing the impact of metabolic disturbance, we first evaluated the cell death occurring in untreated slices that rested after the slicing procedure for 1–7 h. These data were then used as the control group to judge the impact of metabolic disturbance. After the respective resting periods, slices were first PI stained (2 μg/ml; 5–8 min) in a storage chamber. They were then transferred to the experimental chamber where images were taken to determine the number of dead cells. The same slice then underwent cell permeabilization in the presence of PI to determine the total number of cells and finally the relative amount of dead cells was calculated. In 1-, 3-, 5-, and 7-h-old slices the amount of PI-labeled cells averaged 26.7 ± 10.3%, 34.6 ± 8.8%, 36.0 ± 10.4%, and 43.0 ± 14.8%, respectively (n = 7 each; Fig. 4). In 1-h-old slices, PI-labeled cells were mostly superficially located, indicating that they were obviously damaged by the slicing procedure itself (Fig. 4A). Their nuclei were mostly compact, had sharp contour, and showed intense red staining. Only a few nuclei were swollen, showing irregular staining, which may indicate nuclear blebbing. Cells from deeper tissue layers became increasingly stained more than 3 h after slicing (Fig. 4A). They could easily be identified by their blurred appearance, which disappeared when the focal plane was moved from the slice surface into the tissue. Even following 7 h in vitro, more than 50% of cells were still viable, as judged by exclusion of PI (Fig. 4B).

Impact of metabolic insults on cell viability

After quantifying cell viability in untreated control slices, we analyzed the impact of metabolic disturbance on the dorsal vagal nucleus, exposing 1-h-old slices to either anoxia, aglycemia, or ischemia-like conditions (in vitro ischemia). If not otherwise mentioned, the amount of dead cells was determined right after the respective treatment. Chemical anoxia was induced by application of 1 mM cyanide (Ballanyi and Kulik 1998; Müller et al. 2002; Way 1984). Slices exposed to cyanide-containing solutions showed less intense PI staining. This could indicate that cyanide slows the kinetics or extent of PI binding to nucleic acids, or that it might induce DNA fragmentation (Bhattacharya and Lakshmana 2001). After 30 min of chemical anoxia, the amount of PI-labeled cells (33.6 ± 11.9%, n = 6) did not significantly differ from untreated, 1- or 3-h-old control slices (Fig. 5A). Also, following 1 h of aglycemia, which was induced by superfusion of glucose-free ACSF, the amount of PI-labeled cells (28.1 ± 6.5%, n = 5) did not significantly differ from 1- or 3-h-old untreated control slices either (Fig. 5A). Yet after 3 h of aglycemia, the fraction of PI-labeled cells was significantly higher than in 3-h-old control slices (34.6 ± 8.8%, n = 7), averaging 47.2 ± 8.8% (n = 5).

In vitro ischemia was first induced by combined glucose withdrawal and cyanide application (1 mM cyanide, 30 min), and it was preceded by 30-min pretreatment with glucose-free ACSF (Ballanyi et al. 1996; Kulik et al. 2000). Cell death during such ischemia was not significantly higher than in 1-h-old control slices; PI labeled 34.3 ± 10.9% of cells (Fig. 5A). Neither did the amount of PI-labeled cells markedly increase during the next 3 h following the ischemic treatment (Fig. 5B). This may indicate that glucose was only incompletely removed from the tissue and therefore for at least some time, may have upheld neuronal function. We therefore thought out a more severe treatment and induced in vitro ischemia by combined application of 1 mM cyanide and 5 mM iodoacetate to pharmacologically block mitochondrial respiration and glycolysis, respectively (Reiner et al. 1990). In addition, the duration of the ischemic insult was extended to 1 h. After that treatment, the amount of PI-labeled cells was significantly higher compared with 1-h-old control slices; PI labeled 34.3 ± 10.9% of cells (Fig. 5A). Cell death gradually increased further during the following postischemic episode (Fig. 5B), and 3 h after the ischemic insult a total of 83.6 ± 10.3% (n = 4) of cells—compared with 36% in 5-h-old control slices—was labeled by PI.

In translucent light, opacity of the tissue exposed to ischemia was markedly increased, and cell structures could not be identified anymore (Fig. 5C). Dynamic recordings of the intensity of light transmitted through the dorsal vagal nucleus confirmed an irreversible increase in light transmittance by 17.5 ± 1.5% (n = 3), which is an indication of severe cell swelling (Andrew et al. 1999; Fayuk et al. 2002; Müller and Somjen 1999; Ørskov 1935). Judged by its time to onset (3.4 ± 0.7 min, n =
3) it seemed to coincide with the terminal depolarization that was observed in single DVN after about 3 min of ischemia (Fig. 6C). By contrast, 30 min of chemical anoxia caused a reversible and more moderate increase in light transmittance, averaging 6.8 ± 4.7% (n = 3) at the end of the treatment.

Electrophysiological responses of metabolically impaired DVN

To elucidate the correlation of the immediate membrane responses of single neurons and the loss of membrane integrity, we performed current-clamp recordings on single DVN and simultaneously monitored changes in [Ca\(^{2+}\)]. DVN had an average membrane potential of −48.5 ± 4.2 mV, an input resistance of 642 ± 146 MΩ (n = 27), and tonic spontaneous spike discharges occurred at a rate of 0.5–5 Hz.

Chemical anoxia (1 mM cyanide) induced within <1 min a hyperpolarization of about 12 mV, decreased the input resistance and abolished spontaneous spike discharges (Fig. 6A). In parallel, a moderate rise in [Ca\(^{2+}\)] occurred (for statistical details see: Table 1). The initial hyperpolarization ceased within 20 min of anoxia, turning into a slow repolarization, and the initial rise in [Ca\(^{2+}\)] increased further, averaging at the end of anoxia 203 ± 279 nM (n = 7). A massive and sudden terminal depolarization or a dramatic increase in [Ca\(^{2+}\)] did, however, not occur during 30 min of anoxia. Membrane parameters and [Ca\(^{2+}\)] recovered following withdrawal of cyanide and spontaneous activity returned (Fig. 6A).

Since the impact of ischemia was much more severe when cyanide was combined with iodoacetate instead of glucose withdrawal, we elucidated whether iodoacetate alone would be sufficient to induce a terminal depolarization. Metabolic inhibition by 5 mM iodoacetate resulted within 2–13 min in a delayed hyperpolarization averaging 10 mV, a decrease in input resistance and the block of spontaneous activity (Fig. 6B; Table 1). In parallel, [Ca\(^{2+}\)] increased slightly. Note that by contrast to cyanide treatment, these changes did not occur immediately after the start of IAc administration, but were delayed by several minutes. The hyperpolarization then turned into a slow depolarization, and after 19 min of iodoacetate treatment, it culminated in a sudden, nearly complete, terminal depolarization. A massive rise in [Ca\(^{2+}\)], coincided with the sudden depolarization, shifting [Ca\(^{2+}\)] to levels beyond 1 μM (Fig. 6B). Membrane potential and input resistance did not recover on wash-out of iodoacetate. The apparent decrease in fluorescence ratio that was observed in some cells reflects leakage of fura-2 from cells; this was probably due to mechanical disruption of tight seal recording as a result of cell volume changes.

In vitro ischemia, induced by combined application of cyanide (1 mM) and iodoacetate (5 mM), caused within 1 min the characteristic initial hyperpolarization of 11 mV, the decrease in input resistance, block of spontaneous activity, and a concomitant moderate rise in [Ca\(^{2+}\)]. The terminal depolarization occurred within 3 min of ischemia, and it was paralleled by a

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**FIG. 3.** Quantification of cell death by Triton-induced cell permeabilization. The translumination image shows the location of the dorsal vagal nucleus. The relative amount of dead cells in the dorsal vagal nucleus of that slice was determined by PI staining followed by combined Triton X-100/PI treatment. While the initial PI staining labels necrotic and/or late apoptotic cells only, Triton-induced cell permeabilization in the presence PI results in the labeling of every single cell, thereby yielding the relative amount of dead cells. Maximum cell count was usually obtained within 15 min of permeabilization; continued treatment only increased the intensity of PI labeling.
massive increase in [Ca$^{2+}$]$_i$ by more than 1 μM (Fig. 6C; Table 1). These membrane changes did not recover on wash-out of the drugs, and as already observed with iodoacetate alone, the fluorescence ratio decreased in most cells due to the cytoplasmic loss of fura-2.

**Dynamic changes in PI fluorescence mark the onset of ischemic cell death**

Ischemia, induced by cyanide plus iodoacetate, triggered the terminal depolarization of DVN within 3 min, caused a massive Ca$^{2+}$ load, and—among the metabolic insults tested—resulted in the most pronounced cell death. In a final experimental approach, we therefore attempted to estimate the time span in between loss of membrane potential and the loss of membrane integrity. A slice was pretreated for 1 h with PI to ensure that PI fluorescence reached a stable baseline, and it then underwent cyanide plus iodoacetate treatment in the permanent presence of PI. The intensity of PI fluorescence in the dorsal vagal nucleus was continuously measured. Approximately 4 min following addition of the drugs, a transient decrease in PI fluorescence was observed, probably a result of the cell swelling associated with the terminal depolarization known to occur after such a delay (Table 1). Within 29 ± 17 min of in vitro ischemia, a gradual increase in PI fluorescence came about ($n = 3$) and continued for the entire duration of the experiment (>2 h; Fig. 7A). Comparing the images taken from the same slice before and after 2 h of in vitro ischemia clearly demonstrates the amount of cells that lost their membrane integrity during the ischemic insult (Fig. 7B). It also confirms that the dynamically recorded increase in PI fluorescence is indeed related to the additional loss of cells.

**DISCUSSION**

The dead cell marker PI was used to assess the extent of cell death in intact tissue, and valuable additional information on the electrophysiological responses of single neurons was obtained from the combined electrophysiological/microfluorometric recordings.
Reliability of PI as a dead cell marker in intact tissue

Unspecific PI labeling was observed in the cell layer lining the central canal and in the periphery of the slice. This might suggest that these ependymal and meningeal cells actively take up PI by endocytosis. In the dorsal vagal nucleus, however, the following observations verified that PI labeling reliably reflects the loss of membrane integrity: unspecific PI labeling was not observed, and cells of vital appearance were never found to be stained by PI. In fresh control slices only a few, mostly superficially located cells were labeled, while following membrane permeabilization by ethanol or Triton X-100, it appeared that virtually every cell was stained.

FIG. 5. Impact of metabolic impairment on cell viability. A: cyanide was applied for 30 min, while aglycemia and in vitro ischemia lasted 1 h. Except for cyanide plus iodoacetate treatment and 3-h aglycemia, short-time metabolic arrest did not significantly increase the number of PI-labeled cells. Number of trials is reported above bars; asterisks indicate significant changes (*P < 0.05; **P < 0.01) compared with untreated time-matched control slices (see bars on right). B: cell death during the postischemic episode. Acute ischemia did not immediately result in pronounced cell death, but cell death gradually increased during postischemic “recovery,” especially in those slices that were previously exposed to cyanide plus iodoacetate. C: comparing the images taken from the same slice before and after in vitro ischemia (40 min) clearly shows that cyanide plus iodoacetate increased the translucency of the tissue and that cell boundaries could not be identified anymore.

The extent of cell death is often judged by measuring the intensity of PI fluorescence (Sakaguchi et al. 1997) or score-rating the degree of staining (Simantov et al. 1999). As revealed in our study, cyanide affected the intensity of PI fluorescence. Other agents inducing cell death may have similar effects as well, and would thereby severely disturb a quantitative analysis of cell death that is based on the intensity of PI fluorescence. Furthermore, due to the slow binding and satu-
The slow binding kinetics of PI may have somewhat dampened the time resolution, testing alternative nucleic acid stains with faster binding kinetics may be fruitful in view of optimized time resolution and more distinct fluorescence/time profiles.

PI labels only cells that, at the time of staining, already lost their membrane integrity, i.e., necrotic and/or late apoptotic cells. Those cells being in the state of early apoptosis and whose fate is already predetermined, are likely still able to exclude PI and thus remain undetected. Also, PI staining does not discriminate between cell types. DVN do constitute the principal cell type of the dorsal vagal nucleus that does not appear to contain a major number of interneurons (Huang et al.

### Table 1. Electrophysiological responses of metabolically impaired single DVN

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<th>Initial Hyperpolarization</th>
<th>Terminal Depolarization</th>
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<td></td>
<td>( \Delta V_m ) (mV)</td>
<td>( \Delta R_i ) (%)</td>
</tr>
<tr>
<td>CN(^-)</td>
<td>(-11.9 \pm 8.7)</td>
<td>(-59.0 \pm 22.5)</td>
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<tr>
<td>IAc</td>
<td>(-10.2 \pm 8.7)</td>
<td>(-71.1 \pm 14.5)</td>
</tr>
<tr>
<td>CN(^-) + IAc</td>
<td>(-11.0 \pm 9.0)</td>
<td>(-57.1 \pm 20.2)</td>
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<td></td>
<td>( \Delta V_m ) (mV)</td>
<td>( \Delta R_i ) (%)</td>
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<tr>
<td>CN(^-)</td>
<td>(44.2 \pm 3.0)</td>
<td>(-91.9 \pm 3.3)</td>
</tr>
<tr>
<td>IAc</td>
<td></td>
<td>(43.6 \pm 5.8)</td>
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<td>CN(^-) + IAc</td>
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Statistical summary of the changes in membrane potential (\( \Delta V_m \)), input resistance (\( \Delta R_i \)), and intracellular Ca\(^{2+}\) concentration (\( \Delta [Ca^{2+}]_i \)) that were induced by cyanide-induced anoxia (CN\(^-\)), metabolic poisoning by iodoacetate (IAc), and in vitro ischemia (CN\(^-\) + IAc). All changes were referred to the respective pretreatment baseline. The times to onset (\( \Delta t \)) were corrected for the time it took the experimental solutions to reach be experimental chamber (30 s). Data are represented as mean ± SD.
to successfully exclude PI. Metabolic arrest related cell death clearly depended on the severity of the insult. Aglycemia only caused major acute damage when its duration was extended to 3 h. Due to the low metabolic rate of DVN (Ballanyi et al. 1996; Kulik et al. 2000), the glucose remaining in the tissue as well as the utilization of the phosphocreatine pool (Wilken et al. 2000) are apparently sufficient to uphold cellular metabolism for ≃1 h. Our previous analysis of the electrophysiological effects of aglycemia (Ballanyi et al. 1996; Kulik et al. 2000) confirms this assumption. A hyperpolarization and concomitant loss of spontaneous activity occur in the DVN with a marked delay of ≃12 min after glucose withdrawal. This hyperpolarization due to activation of $K_{ATP}$ channels was found to be stable for about 1 h, and a terminal depolarization was not observed within that time period (Ballanyi et al. 1996; Kulik et al. 2000).

Similarly, chemical anoxia did not cause additional cell death. This is consistent with our present (Fig. 6) and previous findings that cyanide induces a moderate and stable $[Ca^{2+}]_i$ rise (Ballanyi and Kulik 1998; Kulik et al. 2000) and a concomitant $K_{ATP}$-mediated hyperpolarization (Cowan and Martin 1992; Müller et al. 2002; Trapp and Ballanyi 1995). In contrast, anoxia is known to induce a terminal depolarization in neurons from various parts of the mammalian brain (Bureš and Burešová 1957; Haddad and Jiang 1993; Hansen 1985; Lipton 1999; Müller and Somjen 2000a, b). Long time effects of cyanide, as were reported by others, can of course not be excluded on the basis of our data. For example, repeated systemic cyanide intoxication of mice caused necrotic lesions within the substantia nigra and apoptotic cell death in motor cortex (Mills et al. 1999); however, these changes did not occur before 3 days of continuous treatment. Similar cyanide-induced delayed necrotic/apoptotic cell death was also observed in cultured neurons (Jensen et al. 2002; Shou et al. 2000).

One might argue that the moderate effects of anoxia and aglycemia on cell viability are due to the “hypothermic” bath-temperature of 30°C (Wang et al. 2000). For anoxia lasting 4–15 min, the electrophysiological responses of DVN were not affected by raising the bath temperature to 37°C (Trapp and Ballanyi 1995); however, no data are available for prolonged anoxia/aglycemia. As revealed by the present study, pronounced neuronal damage is linked to the occurrence of a terminal depolarization and the concomitant massive $Ca^{2+}$ load. So the question to be answered is whether anoxia and aglycemia induced at higher temperatures would be sufficient to trigger these events. In view of the obvious low metabolic rate of DVN (Ballanyi et al. 1996; Kulik et al. 2000) and the fact that $K_{ATP}$ channels are activated in response to metabolic disturbances well before intracellular ATP is depleted (Müller et al. 2002), this may, however, be difficult to achieve within the investigated time window of 0.5–3 h and by blocking either mitochondrial respiration or glycolysis.

A “neuroprotective effect” arising from whole cell recording with 1 mM ATP-containing pipettes can be excluded as well. because the time course and magnitude of anoxia-induced changes in $[Ca^{2+}]_i$ was identical in intact, fura-2 AM-loaded and whole cell recorded DVN (Ballanyi and Kulik 1998). Also, the anoxic activation of $K_{ATP}$ channels was found to be independent of intracellular ATP levels, showing identical time courses and latencies with 0, 1, and 20 mM ATP-containing pipettes (Müller et al. 2002).

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FIG. 7. Dynamic recording of PI fluorescence in the dorsal vagal nucleus during ischemia. A: in vitro ischemia caused a moderate, initial decrease in PI fluorescence, probably due to cell swelling. After 30 min, it turned into a clear increase of PI fluorescence that continued for the entire duration of the experiment (≻2 h). The bottom graph shows the differentiated time course of the changes in PI fluorescence ($dF/dt$). B: images show the amount of PI-labeled cells before and after the slice underwent in vitro ischemia, confirming that additional cell death generated the dynamic increase in PI fluorescence plotted in A.

1993; Loewi and Spyer 1990). Nevertheless, interneurons and also glial cells may have contributed to the cell counts to a certain degree.

Extent of cell death

In untreated control slices, cell death increased with time. But even following 7 h, more than 50% of cells were still able
In contrast to anoxia and aglycemia, in vitro ischemia caused pronounced cell death, especially when it was induced by combined application of cyanide plus iodoacetate. Under the latter conditions, loss of membrane potential, massive intracellular Ca^{2+} load, and severe cell swelling occurred within 3 min (Fig. 5B). These findings are in line with our previous results showing that these effects are due to ischemia-induced interstitial accumulation of glutamate (Kulik et al. 2000). The augmentation of cellular damage during the posts ischemic episode suggests that a “point of no return” may already have been reached during the 1-h ischemic insult, and that elimination of the cell, i.e., loss of membrane integrity, just took some more time to be completed (Fig. 5B). The different impact of the two modes of in vitro ischemia tested obviously reflects their different modes of action. While combined glucose withdrawal and cyanide application allows for the consumption of glucose remaining in the tissue and the utilization of alternative metabolites, pharmacological inhibition of glycolysis and oxidative phosphorylation does act immediately and inevitably.

**Morphology and classification of metabolically induced cell death in the dorsal vagal nucleus**

In the present study, PI labeling revealed that in fresh control slices, most labeled cells were superficially located. This suggests that they were obviously damaged during the slicing procedure (Frotscher et al. 1981; Richerson and Messer 1995). More than 3 h following dissection, cells from deeper layers became increasingly stained. In slices previously exposed to aglycemia or ischemia, cells from all layers were equally affected. In general, PI staining was found to be restricted to the nucleus, i.e., the highest density of nucleic acids. The nuclei either were compact of sharp contour and regularly stained or they appeared swollen and irregularly stained. This irregular staining may indicate the onset of chromatin breakdown.

Typical morphological features of necrotic cell death are the early loss of membrane integrity, cell and organelle swelling, and the rapid energy loss, whereas apoptosis is characterized by cell shrinkage, cytoplasmic condensation, intranucleosomal DNA fragmentation and the appearance of half-moon shaped chromatin particles (Earnshaw 1995; Loo and Rillema 1998; Majno and Joris 1995). Most importantly, apoptosis spans over several hours or even days and membrane integrity is maintained until the late stages of apoptosis. A clear separation of these two processes is, however, difficult, especially since apoptotic cells do undergo necrosis (termed “secondary necrosis”) during their late stages.

Most intense cell death resulted from in vitro ischemia, and it was preceded by a terminal depolarization, excessive intracellular Ca^{2+} load, and severe cell swelling. The occurrence of cell swelling and loss of structure rather than cell shrinkage and increased cytoplasmic density argues against apoptotic mechanisms to be responsible for the observed cell death, especially since the breaking up of nuclei (karyorrhexis), which is assumed the best cytological marker of apoptosis, was not observed. Also, the short time in which cell death occurred excludes the involvement of lengthy, days spanning apoptotic programs and rather favors necrosis as the primary cause for the observed cell death.

**Concluding remarks**

We extended the use of PI, making it feasible for the quantitative analysis of cell death in acute brain slices. With the dynamic recordings of PI fluorescence, a tool is now available for time-resolved analysis of cell death in intact tissue. Of the metabolic disturbances tested, in vitro ischemia induced by cyanide plus iodoacetate had the most devastating impact on the neuronal survival rate. Only 3 h after an 1-h ischemic insult, 85% of cells within the dorsal vagal nucleus had lost their membrane integrity. This severe impact of ischemia is obviously based on the rapid occurrence of the terminal depolarization, the associated massive intracellular Ca^{2+} load and the pronounced, irreversible cell swelling. As indicated by the dynamic recordings of PI fluorescence, loss of membrane integrity, an unambiguous sign of neuronal death, started within approximately 25 min of the terminal depolarization.

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