Intracellular and Extracellular Changes of [Ca^{2+}] in Hypoxia and Ischemia in Rat Brain In Vivo

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ABSTRACT Changes in intra- and extracellular free calcium concentration were evaluated with ion-selective microelectrodes during periods of anoxia and ischemia in three different regions of intact rat brain. Recordings stable for at least 2 min and in most cases for 4–6 min were chosen for analysis. Under normoxic conditions neuronal [Ca^{2+}]$_i$ varied between $<10^{-8}$ and 10^{-7} M from cell to cell but no systematic regional differences were observed. Elimination of O$_2$ or interruption in blood flow caused, within 30–60 s, slight intracellular alkalinization followed by a small rise in [Ca^{2+}]$_i$, a mild degree of hyperpolarization, and disappearance of electrical activity in the cortex, in that order. It is postulated that a decline in cellular energy levels, as manifested by H$^+$ uptake associated with creatine phosphate hydrolysis, leads to an increase in [Ca^{2+}]$_i$, which activates Ca^{2+}-dependent K$^+$ channels and consequently enhances g_K. 2–4 min later there was a sudden, large rise in [K$^+$]$_e$, a fall in [Ca^{2+}]$_e$ and a rapid elevation of [Ca^{2+}]$_i$. The magnitude of the latter was greatest in a high proportion of hippocampal neurons in area CA1 and some cortical cells, while it was smallest and relatively delayed in thalamic neurons. In the hippocampus area CA1 increases in [Ca^{2+}]$_i$ to as much as 6–8 × 10^{-4} were observed; some of these could be reversed when O$_2$ or blood flow were restored to normal. Pretreatment of animals with ketamine and MK-801, antagonists of excitatory amino acid transmitters, markedly slowed and decreased the rises in [Ca^{2+}]$_i$. The effects of the two agents were most pronounced in the hippocampus. It is concluded that the receptor-operated channels are largely responsible for Ca^{2+} entry into certain cells during hypoxia/ischemia. This pathway may be of primary importance in parts of the hippocampus and cortex, regions of the brain that are particularly vulnerable to O$_2$ deprivation and which receive high glutamatergic input and have an abundance of excitatory amino acid receptors.

INTRODUCTION

It is well established that cerebral anoxia and ischemia lead to a decrease in cellular energy production (Lowry et al., 1964; Siesjö, 1978), a resultant reduction in the

efficacy of the membrane ion pumps and a consequent shift in intra-/extracellular ionic balance (Hansen, 1985). Numerous studies have shown that a small initial rise in $[K^+]_e$ is followed, 2–4 min later, by a massive increase in $[K^+]_e$ and a concomitant decrease in $[Na^+]_e$ and $[Ca^{2+}]_e$ (Astrup et al., 1977; Hansen and Zeuthen, 1981; Harris et al., 1981; Siemkowicz and Hansen, 1981). Based on the large decline in $[Ca^{2+}]_e$ it has been proposed (Siesjö, 1981) that neurons serve as a sink for calcium and that an increase in the intracellular concentration of this cation during oxygen deprivation is one of the major causes of irreversible brain damage. However plausible, this hypothesis has not been tested directly because there is as yet no information available on $[Ca^{2+}]_i$ in intact brain during anoxia/ischemia.

Ample evidence exists that in many tissues influx of calcium occurs through two pathways: via the voltage-operated channels and via the receptor-controlled channels (Hagiwara and Byerly, 1981; Meldolesi and Pozzan, 1987; Miller, 1987; Tsien et al., 1988). The latter are especially prominent in neurons of the mammalian central nervous system (CNS) in which excitatory amino acid transmitters markedly increase g_{Ca} (Dingledine, 1983; Mayer and Westbrook, 1985). Since anoxia and ischemia are accompanied by depolarization of the neuronal membrane (Hansen, 1985) as well as by massive leakage of glutamate and aspartate into the extracellular space (Benveniste et al., 1984; Drejer et al., 1985; Hagberg et al., 1985) both mechanisms would be expected to contribute to a rise in $[Ca^{2+}]_i$.

The purpose of this study was to evaluate with ion-selective microelectrodes the changes in intra- and extracellular calcium during periods of anoxia and ischemia in intact rat brain. Measurements were made in three different regions of the CNS to establish whether or not the known vulnerability of cells in certain areas, such as the hippocampus (Ito et al., 1975; Diemer and Siemkowicz, 1981; Jorgensen and Diemer, 1982; Brierley and Graham, 1984; Petito and Pulsinelli, 1984), correlated with larger rises in $[Ca^{2+}]_i$. The effects of two excitatory amino acid receptor antagonists, ketamine (Anis et al., 1983; Thompson et al., 1985) and MK-801 {(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]-cyclohepten-5,10-imine maleate} (Wong et al., 1986) were investigated to determine the contribution from the agonist-operated channels, while a dihydropyridine (nifedipine) was used to block the L-type voltage-controlled channels (Fox et al., 1987). Our results show that ischemia/anoxia lead to a rapid and massive increase in $[Ca^{2+}]_i$, especially in some hippocampal and cortical neurons. This rise in calcium was markedly delayed and attenuated by the administration of ketamine and MK-801 but not of nifedipine. Hence the data suggest that when oxygen supply is limited in regions receiving a rich glutamatergic input the receptor-operated channels are of the utmost importance in controlling the influx of calcium into neurons.

METHODS

Preparation of the Rats

Anesthesia. Measurements were made on 483 white Sprague-Dawley–derived rats of both sexes; body weight 250–300 g anesthetized by intraperitoneal injection of pentobarbitone sodium (60 mg · kg^{-1} as a 6% aqueous solution; Nembutal, Abbott Laboratories Inc., Irving, TX) together with atropine sulfate (0.3 mg · kg^{-1}; Roche Products Ltd., Welwyn Garden City, Herts, UK). Supplementary pentobarbitone was administered as required. In exper-

iments in which a dissociative anesthetic, ketamine, was used, the latter was administered at a rate of 5 mg · kg⁻¹ while the dose of pentobarbitone was reduced slightly to provide a constant level of anesthesia.

Surgery. The trachea was cannulated and connected to a small-animal ventilator. Local analgesic (lignocaine 2% with epinephrin; Xylocaine, Astra Pharmaceuticals, Ltd., St. Albans, Herts, UK) was infiltrated subcutaneously over the scalp and at the prospective pressure points of the head holder. The animal was fixed in a modified "Baltimore" stereotaxic head holder and positioned for use of stereotaxic coordinates as described by de Groot (1959). The caudal artery was cannulated and connected to a Gould-Statham P50 miniature blood pressure transducer. EKG was recorded from left and right forelimbs via subcutaneous needle electrodes after which the rat was given a muscle relaxant (pancuronium bromide, 70 μg · kg⁻¹; Pavulon, Organon-Teknika, Cambridge, UK) to minimize respiratory movements. An artificial pneumothorax was created and ventilation with 30% O_2 in N_2 and minimum tidal volume, adjusted to give an end-tidal PCO_2 of 4.6–5.26 kPa measured by an infrared CO_2 analyzer. Periodic blood samples (0.1 ml) were taken from the caudal artery and blood gas analyses were performed to check the end-tidal measurements. A median longitudinal skin incision was made over the skull, the periosteum reflected on each side of the central suture and two stainless steel miniature bone screws inserted into the frontal bones to serve as electrocorticogram (ECoG) electrodes. The level of anesthesia was assessed constantly by reference to the ECoG, EKG, and blood pressure traces, and supplementary doses of anesthetic were given when required. A 3.0 mm diameter hole was drilled 1.5–3 mm lateral and 0–2 mm caudal to the bregma point (depending on the region to be studied) and enclosed in a polycarbonate ring (i.d., 3 mm; o.d., 5 mm; depth, 2.5 mm), which was fixed to the skull with dental acrylic to form a well which was flushed with warm (38°C) artificial CSF during recording. The de Groot stereotaxic coordinates used were for cingulate cortex A6.0-4.5 L1–2 and D+6-+4, for dorsal thalamus A6-4.5, L0.5–2.3, and D+1.2-0 and for hippocampus A4.4-2.4, L0.5–4.5, and D+3.2-0.0. The underlying dura was removed and the hole was sealed with warm 4% agar in artificial CSF. Before recordings were made from cells the agar was removed and replaced by a small C-shaped, teflon-coated pressure foot (o.d., 3 mm; i.d., 2 mm) derived from the design of Phillips (1956) that minimized brain movement due to cardiovascular and respiratory pulsation. For superficial recording it was necessary to align the "opening" of the horseshoe with the route of the local blood vessels in order to ensure that capillary flow was not unduly compromised by the stabilization system. The pia-arachnoid was perforated and electrodes were inserted through the open center of the pressure foot with a micromanipulator.

In most experiments a concentric microneedle O_2 electrode was inserted into the brain through a second (2 mm diameter) hole in the left side of the skull, together with an extracellular ion-selective probe, and left in this position throughout the experiment. These electrodes were placed at a predetermined site that corresponded to the expected region of intracellular recording on the right side. The left craniotomy opening was sealed with molten wax (m.p., 38°C) which solidified immediately. A ground-reference electrode of sintered Ag/AgCl was placed subcutaneously on the rat's head.

In studies to test the effect of L-type channel blockers, nifedipine was injected at a dose of 0.2–0.3 mg · kg⁻¹ i.p. In experiments with MK-801, the drug was injected at the rate of 1.0–10 mg · kg⁻¹ i.p.

Hypoxic and Ischemic Models

Hypoxemia. Cerebral hypoxemia was induced by ventilation of the animal with 5% O_2 in N_2. This level of hypoxia was chosen because: (*a*) it is possible for rats to survive for at least 30 min when ventilated with 5% O_2 provided they are in good condition; (*b*) ventilation with 3%

oxygen while producing a more severe hypoxic stress is rapidly fatal to most rats because of the onset of heart failure. Thus there is not only hypoxemia but also rapidly developing ischemia. (c) Although brain blood flow increases by at least 50% in moderate to severe hypoxemia, brain tissue PO_2 falls dramatically during ventilation with 5% O_2 to levels that are in many places, at or close to zero, as measured with platinum needle microelectrodes.

Ischemia. In the great majority of experiments brain ischemia was produced by direct arterial compression. A midsagittal incision was made on the ventral aspect of the base of the neck and a polyethylene cannula was inserted into the trachea. The vertebral arteries were exposed by blunt dissection at the point where they enter the vertebraterial canals ventral to Ce7 and thrombosed by thermocautery. The carotid arteries were separated from the other components of the carotid sheath and snared with a loop of #1 linen thread, the ends of which were passed through a 3 mm o.d. polyethylene tube, 3 cm long. The tubes and linen threads were brought out through the skin incision and arranged so that the internal ends of the tubes lay against the walls of the carotids. The skin wound was closed with 3/0 nylon monofilament sutures. Ischemia was induced by traction on the threads that compressed the carotids against the ends of the tubes.

In a few experiments a modification of the technique of Siemkowicz and Hansen (1981) was used which involves both arterial and venous compression. The trachea was cannulated, the vertebral arteries were thrombosed, and the skin was closed as described above. The rat was secured in the head holder and a hydraulic cuff was placed around its neck. Inflation of the cuff by means of a water-filled 10-ml hypodermic syringe with a Luerlok fitting caused readily adjustable changes in flow through the neck vessels. Reduction in blood volume by withdrawal of blood through the cannula in the caudal artery permitted fine control of blood flow to the brain.

Reversal of Hypoxia/Ischemia

Hypoxia. After animals had been ventilated with low O_2 mixtures they were returned to the control gas mixture (30% O_2/70% N_2). Reoxygenation of the brain was monitored with PO_2 microelectrodes.

Ischemia. The brains of ischemic rats were reperfused by releasing the carotid snares or deflating the hydraulic cuff. Recovery of flow was monitored with hydrogen clearance microelectrodes to ensure that perfusion was reestablished satisfactorily, although the very simple observation of return of color to the eyes was a good general indicator of effective reperfusion in albino rats.

Electrodes

Double-barreled microelectrodes (tip diameter, 0.08–0.15 μm; mean, 0.10 μm) were fabricated on a standard electrode puller from theta configuration borosilicate glass capillaries (o.d., 1.5 mm; i.d., 1.0 mm). In some cases, triple-barreled electrodes were made from three glass capillaries (containing solid fibers) annealed together. Electrode barrels that were to be filled with ion sensors were rendered hydrophobic by siliconization with the vapor of dimethyldichloro- or tri-n-butylchlorosilane and subsequently baked at 150°C for 1 h. The tips of most electrodes used for intracellular recording were ground to a short chisel point on a dry, rapidly rotating, optical flat glass disc incorporating very fine diamond dust. The tips of electrodes destined for extracellular recording were broken back to give an appropriate diameter of ~3 μm. The reference barrels for recording membrane potentials were back-filled with filtered 3 M KCl. Ion-sensitive barrels were filled at the tip only by back-injection with a short (150–350 μm) column of liquid ion sensor and then with the appropriate electrolyte. In cases where there was difficulty in filling the electrodes these were placed in a specially designed teflon jig that fitted the sockets on the rotor of a Beckman Microfuge (Beckman Instruments

Inc., Palo Alto, CA), and centrifuged for 10–15 s at 10,000 g, which usually removed any air bubbles and forced ion sensor or electrolyte into the tips of the electrodes unless they were blocked by debris or silicone deposit.

Ion Sensors and Calibration of Electrodes

For extracellular probes the sensors were: for potassium, valinomycin as Fluka "cocktail A" (Wuhrmann et al., 1979) (No. 60031; Fluka AG, Buchs, Switzerland); for calcium and ligand ETH 1001 (N,N'-di[11-ethoxy-carbonyl]undecyl-N,N',4,5-tetramethyl 3,6 dioxaoctane-1,8 dioamide (Lanter et al., 1982) in the Fluka "cocktail" 21192. For intracellular probes with greater sensitivity to calcium in the presence of high concentrations of interfering ions ETH 1001 was used either as a polyvinylchloride (PVC)-gelled neutral ligand or with 1% tetraphenyl borate in 2-nitrophenyl ether to improve selectivity for calcium (Lopez et al., 1983). However, in most experiments, intracellular probes were filled with ETH 129 (N,N,N',N'-tetracyclohexyl-3-oxapentanediamide) in the form of the "cocktail" described by Schefer et al. (1986) (Fluka 21193). The proton-sensitive neutral ligand tri-n-dodecylamine was used in the formulation described by Ammann et al. (1981) (Fluka 82500) for both intra- and extracellular measurements of pH.

The ion-sensitive barrels were back-filled with the appropriate electrolyte i.e., 0.5 M KCl, 0.1 M $CaCl_2$ or 0.1 M HCl, respectively. An electrolyte mixture of 0.1 M Mg acetate, 100 mM $CaCl_2$ and 80 mM KCl, was found to be more reliable for use with the ETH 129 ligand for intracellular, calcium-sensitive probes.

Ion-sensitive electrodes were calibrated at 37.5°C in terms of ionic concentration, in electrolyte mixtures that closely simulated their expected intra- and extracellular counterparts. For intracellular Ca^{2+} probes the calibration mixture was 80 or 90 mM KCl, 10, 20, and 25 mM NaCl, 1 and 1.8 mM $MgCl_2$ (to check the effects of changes in "interfering" ions) and a range of $CaCl_2$ from 0.1×10^{-8} to 10^{-4} M. The calcium buffers were made according to the rate constants quoted by Martell and Smith (1984), which include a correction for changes in proton ionization at different ionic strengths. The stock solutions were made up in deionized (Millipore/Continental Water Systems, Bedford, MA) water kept in plastic containers. The calibration buffers were prepared in plastic tubes that had been rinsed in deionized water. Two types of calcium calibration buffers were used initially: (a) those at an ionic strength of 0.1 M described by Tsien and Rink (1980) in which the calcium ligands, pH buffers, and pH of the solution vary with the pCa; and (b) buffers with an ionic strength of 0.17 M containing EGTA (20 mM) and HEPES (10 mM) at pH 7.1 (Table I). Electrodes cross calibrated in the two types of buffer gave similar responses. Subsequently most calibrations were performed in the second type of buffer, details of which are given in Table I. All chemicals were of Analar grade or better. The calcium source was $CaCl_2$, AVS (analytical volumetric standard) grade (BDH Chemicals Ltd., Bristol, UK). Considerable care is needed in preparing the 1 and 3 μM solutions since the EGTA is almost saturated. For calibrations at 37.5°C and at different [K^+], [Na^+], and [Mg^{2+}], appropriate adjustments were made to compensate for the changes in rate constants for these ions and for H^+ and Ca^{2+}. For extracellular calibration the mixture was 120 mM NaCl, 3.35 mM KCl, and 0.1–10 mM $CaCl_2$ in 10 mM MOPS buffer at pH 7.3, with adjustments of NaCl concentration to maintain isoosmolarity. Proton-sensitive electrodes were calibrated in the same mixtures with the pH adjusted by addition of NaOH or HCl to provide a range from pH 5.5 to 8.5. The valinomycin-based K^+ sensor showed virtually no interference from other ions in the physiological range. The calcium sensors based on ETH 1001 were highly selective provided that only minimal amounts of tetraphenyl borate were included in the diluent. The PVC-gelled formulation had a high impedance and Nernstian behavior at calcium concentrations above 10^{-8} M encountered in the cells in this study, in accordance with Tsien and Rink (1980).

All electrodes were calibrated individually and only those showing a near-Nernstian response to changes in appropriate ion concentration and a lack of sensitivity to other ions, were used for experimental measurements. For calcium-sensitive electrodes a log/linear response of between 25 and 29 mV/decade and for pH and K⁺ electrodes 51–59 mV/decade were considered acceptable. Probes giving voltage changes outside these ranges, or which were nonlinear, were discarded. Electrodes giving responses above the theoretical maximum had a tendency to become unstable and were used for short-term measurements.

The impedances of all electrodes were measured before use: reference barrels were routinely 20–40×10^6 Ω and ion-sensitive barrels were always $\geq 10^9$ Ω. The typical 87% response time of ion-selective electrodes in the recording system ranged from 500 ms (Ca^{2+}) to 5 s (K^+) for an increase and 700 ms (Ca^{2+}) to 8 s (K^+) for a decrease, to an order of magnitude step change of concentration for K^+.

Calculations

All results were calculated from standard calibration curves constructed for the various cations as described above and are given as (free) ion concentrations. This assumes that ionic strength and hence activity coefficients in extra- and intracellular compartments are of comparable magnitudes.

TABLE I

Composition of Buffers Used for the Calibration of Electrodes Used in Measurements of $[Ca^{2+}]_i$

Free Ca^{2+}	Free Mg^{2+}	Added Ca^{2+}	Added Mg^{2+}
nM	mM	mM	mM
0	1	0.00	1.64
10	1	0.72	1.62
50	1	3.16	1.54
100	1	5.46	1.47
200	1	8.57	1.37
500	1	13.05	1.22
1,000	1	15.79	1.13
3,000	1	18.37	1.05

Base solution: 20 mM EGTA, 90 mM KCl, 10 mM NaCl, 20 mM HEPES adjusted to pH 7.10. To this solution is added sufficient 1 M $CaCl_2$ and 1 M $MgCl_2$ to give the apparent concentrations of Ca^{2+} and Mg^{2+} shown in columns 3 and 4 but which at 20°C give the actual concentrations in columns 1 and 2 by virtue of the action of EGTA.

Recording

The electrodes were connected via chlorided silver wires to a capacitance-compensated, high-impedance ($>10^{14}$ Ω) DC amplifier, the output of which drove a storage oscilloscope, chart recorder, loud speaker and BBC microcomputer via an A to D converter. Intracellular records always included the membrane potential (the voltage from the reference barrel), measured against ground potential, which was subtracted electronically from the output of the ion-sensitive electrode.

Immediate recording of membrane potential and ion concentrations were made after cell penetration. Since this investigation was concerned with the characteristics of neurons, if no spontaneous action potentials were observed after cell penetration a 5-ms square pulse of 1.5

μA was applied through the reference barrel and the behavior of the cell was noted. If there was no spike response the stimulus was repeated twice at 15-s intervals before the cell was classified as "nonneuronal" and disregarded. It was very occasionally possible to retain a cell for 15–20 min but most showed signs of membrane damage, probably due to vascular pulsation, after 3–8 min. For spontaneously responsive cells a minimum recording period of 30 s was required before they were regarded as a reliable source of data, but most cells were recorded for at least 2–4 min. In addition, membrane resistance was measured routinely by current injection through the reference barrel of the electrode.

ECoG and EKG was recorded using appropriate standard amplifiers (Gould Electronics VB, Bilthoven, the Netherlands).

Identification of Cell Position

Reference barrels of electrodes were filled with electrolyte containing a 10% solution of an anionic fluorescent marker dye, Procion yellow (M-4RS; Imperial Chemicals Industries, Organics Public Liability Co., Macclesfield, Cheshire, UK) and at the end of a successful recording, dye was injected iontophoretically into the site using 100-ms current pulses of 2 × 10^{-8} A at 5 s^{-1} for 30–60 s, to assist in identification of the cell and to provide an estimate of any brain shrinkage or distortion during subsequent processing and reconstruction.

Identification of Cell Type

At the end of each experiment an electrode was left at the final recording site, the animal was killed with an anesthetic overdose and the brain was perfused through the carotid arteries with ice-cold 4% glutaraldehyde. The animal in the headholder was placed in a cold room at 4°C. After 12 h the electrode was removed and the brain was extracted from the skull and placed in cold 4% glutaraldehyde for a further 48 h. It was then cut serially in a cryostat to give 40-μm sections which were stained with Cresyl violet or Toluidine blue. Recording positions were identified from stereotaxic coordinates, electrode tracks, and microscopic examination of fluorescent markers under UV excitation (Fig. 1 right). More precise identification of individual cells was sometimes possible by the removal of small blocks of tissue, which included a recording site, embedding in Epon resin, and sectioning at 1–3 μm. These sections were stained with Toluidine blue.

Blood Flow and Tissue Oxygen Measurements

Changes in blood flow were measured by a modification of the local hydrogen generation and clearance method of Stosseck et al. (1974) using a concentric palladinized needle detector polarized at +150 mV, surrounded by a platinized H_2 generator surface (diffusion gap, 300 μm). The method gave a reasonably good indication of the effectiveness of measures used to induce ischemia or reperfusion. It also allowed a distinction to be made between changes in flow, or lack of them, in different but closely contiguous parts of the brain, such as cortex and hippocampus.

Changes in tissue oxygen tension were measured with platinum-iridium-in-glass needle microelectrodes (Silver, 1965) or with the palladinized probe described above, polarized at -700 mV. PO_2 monitoring served as a useful indicator of the efficacy of the hypoxic and ischemic insults in the regions where ion measurements were being made. It should be noted that (a) values of tissue PO_2 are indicators of free O_2 availability to the electrode in relation to the balance of O_2 supply and demand and (b) at very low oxygen tensions the consumption of oxygen by the electrode itself may alter local PO_2. Thus the electrode readings may become unreliable below ~0.13 kPa (Silver, 1965) and fail to distinguish consistently between true anoxia and severe hypoxia.
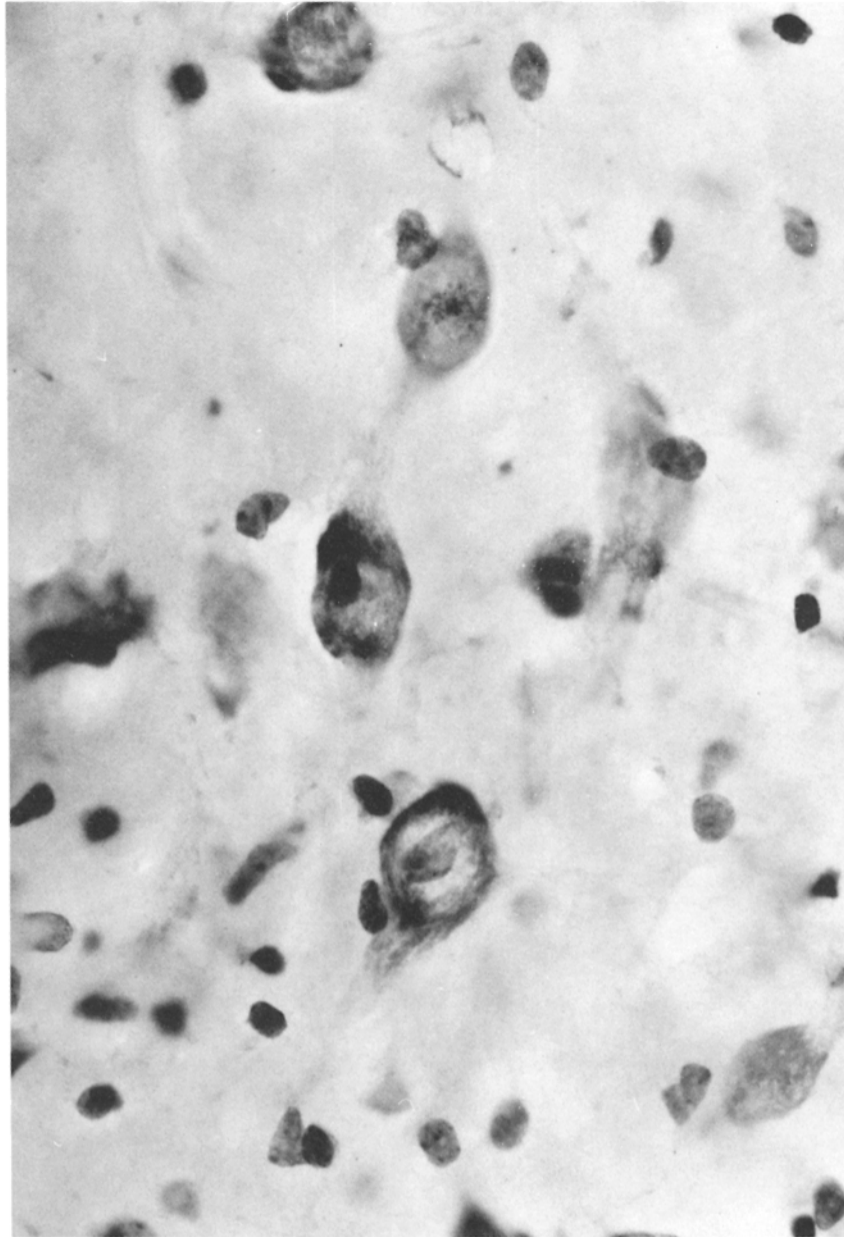
FIGURE 1.   Photomicrographs of a thick section (40 μM) in hippocampal area CA1 showing large neurons and small glial nuclei. (*Left*) Bright field illumination. A recording was made from the large cell at the bottom of the photograph which shows Nissl granules peripherally and an axon emerging from the soma. Centrally the nucleus can be seen outlined with a pale zone of injected Procion yellow.
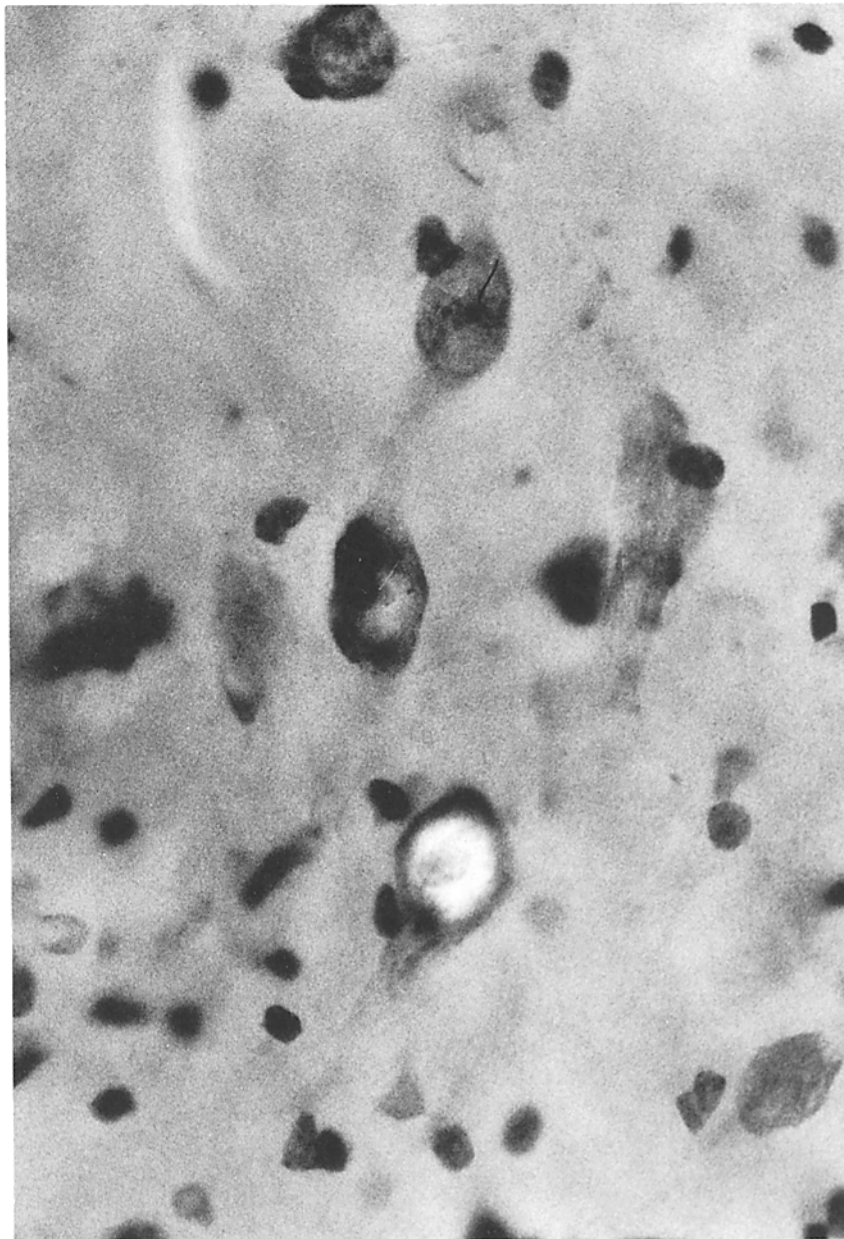
FIGURE 1, continued. (*Right*) The same field with mixed illumination including incident UV, showing fluorescent emission from the Procion yellow marker. This cell had been injected at the end of an experiment with a very small amount of dye which has remained localized and has not completely obscured the internal cell structure. Magnification, 868×. Cresyl violet.

Experimental Design

The ideal recordings are those in which changes in ionic concentrations and membrane potentials are followed in a series of individual cells during a control period and through the development of and recovery from the insult. However, both hypoxemia and ischemia are accompanied by changes in brain volume which frequently cause damage to cells impaled in the control period. For this reason it was often necessary to compare measurements of ionic concentration in one set of cells in the control period with those in other nearby cells that were penetrated after the onset of hypoxia or ischemia. The collection of data under such conditions was time consuming but when sufficient cells were penetrated, it provided a faithful reflection of the events taking place during hypoxic/ischemic insults. In this series of investigations recordings were made from more than 1,200 responsive cells in 483 anesthetized rats in most of which local brain blood flow and/or tissue PO_2 were measured simultaneously.

90% of ischemic measurements were made on the arterial occlusion preparation and the rest on the hydraulic cuff model that involved some venous congestion. No obvious differences were detected in the findings from the two models and the results from them have therefore been combined.

RESULTS

Identity of Cells from which Recordings Were Made

The great majority of cells from which recordings were made had the following characteristics:

(*a*) Generation of spontaneous or induced action potentials.

(*b*) A resting membrane potential in the range 45–80 mV (most 65–75 mV). The relatively few glial cells penetrated had higher potentials in the range 80–98 mV.

(*c*) A high membrane resistance typical of neural cells (>30 MΩ; range 34–58 MΩ). No statistically significant variation of membrane resistance was identified among neurons at different anatomical sites although cells in hippocampal area CA1 had a slightly higher proportion of resistances at the lower end of the range. Among cells subsequently identified morphologically as glia resistance was generally lower (10–20 MΩ).

(*d*) Morphologically recognizable Nissl substance. All brains were fixed by perfusion, sectioned serially, and stained routinely with Toluidine blue or Cresyl violet. Nissl substance, which is characteristic of neurons, was identified microscopically under white light in intact cells from which records had been obtained (Fig. 1, *left*). Since these were routinely injected with Procion yellow at the end of a recording period the individual cells were located first under UV illumination by activation of Procion yellow fluorescence (Fig. 1, *right*). Attempts to confirm with FITC-labeled, neuron-specific antibodies the types of cells from which recordings were made, were unsuccessful because the FITC fluorescence was not visible in the presence of the highly fluorescent Procion yellow injected into the cell. More than 90% of identified cells from which records were obtained had both morphological and functional characteristics of neurons. In the case of cells that were penetrated during ischemia/hypoxia and were therefore functionally inactive, only those that could be positively classified as neurons by morphological criteria have been included in the data. Tables II and III and Figs. 2–4 show continuous data from cells that generated

action potentials during normoxia. In Tables IV and V all records showing responses up to 3 min, 70% of those recorded for 4–6 min and 50% for 8 min were also identified in this way. These latter cells had been penetrated during normoxia and the recording continued through the developing insult. The remaining cells (penetrated while "silent" in the 4–6-min period) were recognized subsequently on classical morphological grounds as neurons and initially retained high membrane resistance. In the later stages of hypoxia/ischemia membrane resistance varied markedly among individual cells (5–20 MΩ). Recordings from cells that could not subsequently be identified as neurons, at least through their staining characteristics, were disregarded.
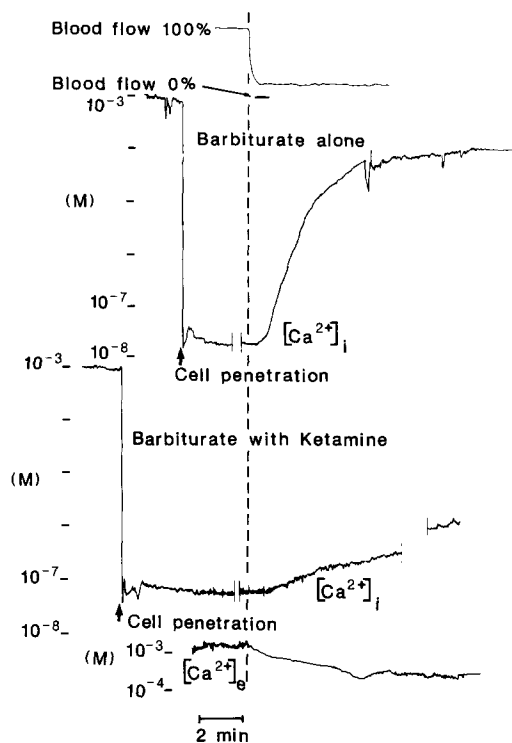

FIGURE 2. Composite traces showing the response of intra- and extracellular $[Ca^{2+}]$ to 80% reduction in local cerebral blood flow (right hippocampal area CA1). From top to bottom: Trace 1: Change in local blood flow measured with micro hydrogen-clearance electrode (see text). Trace 2: Recording of $[Ca^{2+}]_i$ from a neuron in the left CA1 area of hippocampus of a rat anesthetized with pentobarbitone sodium. Trace 3: As for Trace 2 except that the rat was anesthetized with pentobarbitone and ketamine (see text). Trace 4: Extracellular $[Ca^{2+}]$ in right hippocampal area CA1 in the absence of ketamine. Records 1 and 3, and 2 and 4, respectively, were made simultaneously in the same rats. The recording system included a low-pass filter with a cut-off at 30 Hz which has supressed high-frequency transients, including the injury-induced action potentials.

Intra- and Extracellular Calcium in Brain in Normoxia

Recordings were made from cells impaled at random in cingulate cortex, dorsal thalamus, and hippocampus. The only localized anatomical site in which records of neuronal behavior were specifically sought and obtained was hippocampal area CA1 and these are identified in table and figure legends. A typical record of cell penetrations is shown in Fig. 2 which demonstrates the abrupt change of $[Ca^{2+}]$ when the electrode moves from the interstitial to the intracellular environment.

Table II presents the values of $[Ca^{2+}]_i$ in cortex, hippocampus, and thalamus of the brains of anesthetized rats together with simultaneously recorded $[Ca^{2+}]_e$ in the same regions on the opposite side of the brain. It can be seen that the levels of

extracellular calcium were constant at a value of ~1.5 × 10^{-3} M. The concentrations of internal calcium were also similar in all three regions and had a mean of ~7 × 10^{-8} M. However, relatively large variations were seen from cell to cell that were independent of the area investigated. In some cells levels as low as 10^{-9} M were observed and in others [Ca^{2+}]$_i$ reached 10^{-7} M.

Table II also lists for reference purposes simultaneously recorded membrane potentials. All values fall between −50 and −80 mV and indicate that the cells penetrated were neurons because glia show higher voltages (Erecińska and Silver, 1990 and references therein). Neurons from the dorsal thalamic areas exhibited somewhat smaller membrane potentials than those in cortex and hippocampus, which were nearly identical.

Brain PO$_2$ in Normoxia and during Limitation in Oxygen Supply

Under normoxic conditions tissue oxygen availability in the three regions investigated in the anesthetized rat brain showed considerable variation. In area CA1 of

TABLE II

[Ca^{2+}]$_i$, [Ca^{2+}]$_e$, and Membrane Potential in Rat Brain Cortex, Hippocampus, and Thalamus

Brain region	[Ca^{2+}]$_i$	[Ca^{2+}]$_e$	E$_m$
	10^{-8} M	10^{-3} M	mV
Cortex	7.44 ± 2.7	1.56 ± 0.32	−68 ± 8.7
	(n = 81)	(n = 72)	(n = 108)
Hippocampus	6.93 ± 2.2	1.49 ± 0.4	−67 ± 9.8
	(n = 104)	(n = 80)	(n = 124)
Thalamus	6.89 ± 1.9	1.51 ± 0.3	−58 ± 7.3
	(n = 89)	(n = 87)	(n = 97)

[Ca^{2+}]$_i$, [Ca^{2+}]$_e$, and membrane potential were measured as described in the Methods. Values represent means ± SD for the number of cells indicated in parentheses.

hippocampus the levels were 1.06–1.48 kPa, in dorsal thalamic nuclei 1.48–2.23 kPa, and in cingulate cortical grey matter 1.98–3.30 kPa, which is in excellent agreement with the earlier detailed study of Cater et al. (1961). To test differential sensitivity of neurons to lack of oxygen, three insults of varying severity were used: ventilation with 5% O$_2$ in N$_2$ and reduction in blood flow by 20 and 80%. Ventilation with 5% O$_2$ produced hypoxemia with enhanced brain bloodflow rates but the PO$_2$ in area CA1 of hippocampus was almost indistinguishable from zero. The same degree of hypoxemia left some sites in cortex and thalamus with a low but measurable PO$_2$ of 0.26–0.66 kPa. Reduction in flow of 20% increased heterogeneity of tissue PO$_2$. In area CA1, oxygen tension was virtually zero but in CA3, in dorsolateral thalamic nuclei and in parts of the cingulate cortex, it did not fall initially below 0.26–1.32 kPa. Hippocampal area CA3 had an average PO$_2$ similar to that of cortex (2.64–3.30 kPa), which fell to 0.53–1.32 kPa in response to 20% reduction of blood flow. By contrast, 80% reduction of flow gave PO$_2$ readings of nearly or exactly zero in all regions and invariably we got zero in hippocampus and cortex.

Early Changes in Calcium in Hypoxia and Ischemia

When tissue PO_2 was reduced to near zero either by hypoxemia or limitation in blood flow, within 60 s there was a small rise in $[Ca^{2+}]_i$ that was noted in all regions studied and was of approximately the same magnitude in each. To gain an insight into the possible mechanisms involved in this phenomenon, $[Ca^{2+}]_i$, $[K^+]_e$, $[H^+]_i$, and membrane potentials were recorded simultaneously during arterial occlusion. All parameters are displayed graphically in Fig. 3 and summarized numerically in Table III. The results show that 15–20 s after O_2 fell to zero, there was a small alkaline shift of 0.1–0.2 units in the intracellular pH. Immediately thereafter $[Ca^{2+}]_i$ began to rise in all neurons examined; the change amounted to between 50 and
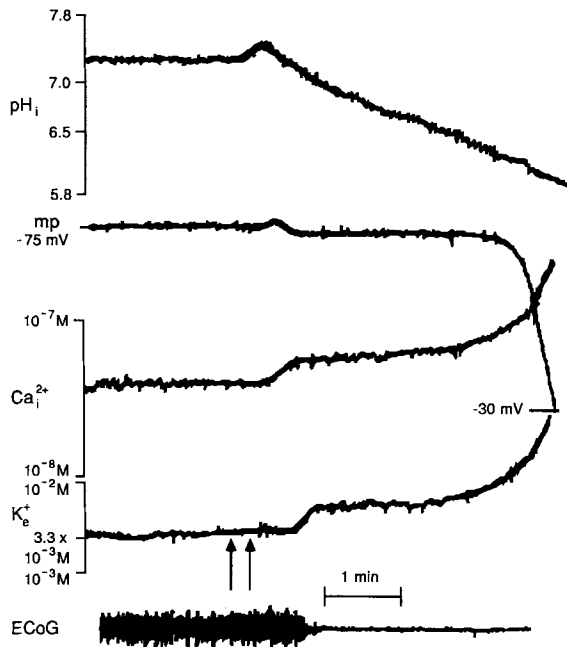

FIGURE 3. Simultaneous records using a triple-barrelled microelectrode, of intracellular pH, $[Ca^{2+}]$, and membrane potential (*mp*) from a cell in area CA1 of the left hippocampus, together with extracellular $[K^+]$ in the right hippocampus and frontal ECoG during the onset of acute, low-flow ischemia induced by arterial occlusion (*bold arrow*). Tissue PO_2 fell to zero at the second arrow as measured by a tissue oxygen microelectrode (trace not shown). The concentrations of K^+ and Ca^{2+} are shown on a log scale and $[H^+]$ as pH. Note that the first change is a small rise in pH_i and that ECoG becomes isoelectric shortly after the rise in $[Ca^{2+}]_i$ and concurrently with the increase in $[K^+]_e$.

200%. In the majority of cells there was a small hyperpolarization. When the $[Ca^{2+}]_i$ started to plateau, there was a rise in $[K^+]_e$ from 3.35 to 4–5 mM that was accompanied by a rapid decline in ECoG activity, which became isoelectric (Fig. 3). At this stage the membrane potential began to fall and the initial alkalosis was succeeded by increasing intracellular acidosis.

During the next 2–4 min, the time depending on the individual rat, there was a progressive fall in pH_i, the slope of which was somewhat steeper in anoxemic than in ischemic rats, but there were only minor changes in $[Ca^{2+}]_i$ or $[K^+]_e$ both of which remained on a nearly constant level. The "quiet" phase, which characterized the second stage of the early response to hypoxia/ischemia, ended with the development of marked differences in behavior not only between individual cells in the

same area but also among the cell populations in the different regions of the brain studied. Towards the end of this phase $[Ca^{2+}]_e$ began to fall at the same time as the $[K^+]_e$ started to rise significantly (Fig. 3).

Late Changes in Intracellular Calcium Concentration in Ischemia and Hypoxia

Low-flow ischemia. In low-flow ischemia ~40% of the cells showed changes in $[Ca^{2+}]_i$ (responsive cells) while the remaining 60% showed values not significantly different from control. The "responsive" group could be divided according to their behavior into two general categories which were designated for convenience "fast responders" and "slow responders." The fast responders were characterized by a very abrupt acceleration of the slow increase in $[Ca^{2+}]_i$ in the second stage of the initial phase of the response to ischemia which became converted to a sudden and dramatic rise by as much as two or three orders of magnitude in 3–5 min (Fig. 4 *A*). Slow-responding cells showed a later, smoother transition from the initial rise and

TABLE III

Early Changes in Intra- and Extracellular Ion Concentrations and Membrane Potentials in Rat Brain Hippocampus during Acute Ischemia

Time after tissue PO_2 reached zero	Ion concentrations					Membrane potential
	$[Ca^{2+}]_i$	$[Ca^{2+}]_e$	$[K^+]_e$	$[H^+]_i$	$[H^+]_e$	
s	$10^{-8} M$	$10^{-3} M$	$10^{-3} M$	*pH*		*mV*
0	6.93 ± 2.2	1.64 ± 0.20	3.32 ± 0.12	7.28 ± 0.11	7.30 ± 0.1	−72.3 ± 6.2
	(*n* = 104)	(*n* = 63)	(*n* = 70)	(*n* = 31)	(*n* = 31)	(*n* = 85)
30	6.95 ± 1.8	1.63 ± 0.24	3.38 ± 0.50	7.41 ± 0.15*	7.28 ± 0.1	−75.1 ± 5.4*
	(*n* = 93)	(*n* = 63)	(*n* = 32)	(*n* = 23)	(*n* = 23)	(*n* = 79)
60	12.5 ± 6.2‡	1.65 ± 0.18	4.30 ± 0.82‡	7.32 ± 0.1	7.19 ± 0.13	−73.2 ± 6.8
	(*n* = 91)	(*n* = 63)	(*n* = 39)	(*n* = 19)	(*n* = 19)	(*n* = 42)

Concentrations of ions and membrane potentials were measured as described in the Methods. Values represent means ± SD for the number of cells in parentheses. *p* values are given with respect to zero time.
*$p < 0.01$.
‡$p < 0.001$ (*t* statistics for two means).

stabilization of $[Ca^{2+}]_i$ to a steadily but relatively slowly rising concentration which eventually reached a level not very different from that of the fast responders (Fig. 4 *B*). Thus the slow responders exhibited a rise in $[Ca^{2+}]_i$ that was not dissimilar to that of the fast-responding cells but which was relatively delayed and slower, being complete in 8–10 min, i.e., twice as long as for the fast responders.

The values of $[Ca^{2+}]_i$ from a number of responsive cells in dorsal thalamus, hippocampus (largely area CA1), and cingulate cortex are summarized in Table IV. To facilitate comparison, recordings are shown at 3 and 8 min after the onset of ischemia. The first time point corresponds to the early phase of changes and allows distinction between the slow and fast responders. The second reflects the relative stabilization of the level of intracellular calcium. Table IV shows that the distribution of the two types of cells was markedly dependent on the region of the brain. Whereas in the hippocampal area Ca1 over 90% of cells from which recordings were obtained fell into the fast-responder category, in the dorsal thalamic nuclei the

ischemia-sensitive cells were almost exclusively slow responders. In the cingulate cortex ~20% of responsive cells belonged to the former category. Analysis of the numerical values for $[Ca^{2+}]_i$ provides several interesting points. First, under normoxic conditions (time 0), the fast- and slow-responder cells had essentially the same concentration of intracellular calcium. Second, in the slow responders, $[Ca^{2+}]_i$ rose by about twofold in 3 min and, moreover, by the same amount in all three regions studied. Third, in fast-responding cells the increases in intracellular Ca^{2+} by 3 min were larger by an order of magnitude and more pronounced in hippocampus than
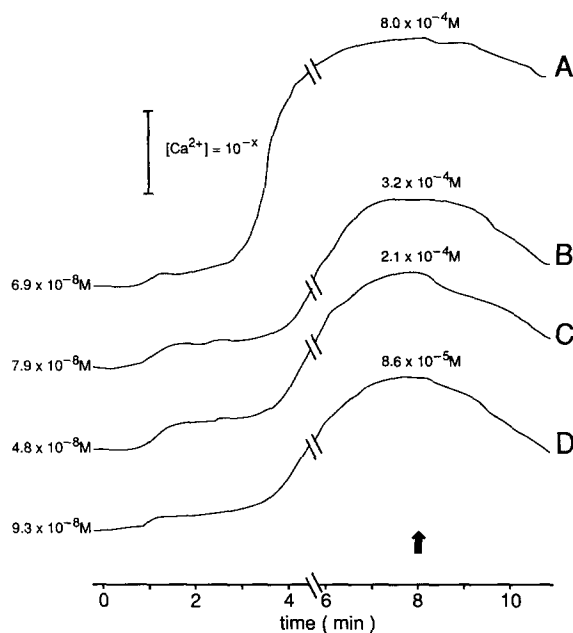

FIGURE 4. Four superimposed continuous records of intracellular calcium concentrations measured with ion-specific microelectrodes from neurons in four rats subjected to low-flow cerebral ischemia. The figures on the left side of the traces show the control, normoxic $[Ca^{2+}]_i$ for each cell and the vertical scale bar indicates one decade (log scale) for calcium concentration. Arterial occlusion was performed at time zero in the *x*-axis. The double bars indicate a break in the traces between 4.5 and 5.5 min. The arrow indicates the time of release of arterial compression and restoration of cerebral blood flow. The figures at the top of each trace are the maxima of the intracellular $[Ca^{2+}]$ for each cell after 8 min ischemia. (*A*) Fast-responder cell from hippocampal area CA1 in an untreated rat. (*B*) Slow-responder cell from dorsolateral thalamus in untreated rat. (*C*) Record from cell in area CA1 (where over 90% were fast responders) in a rat treated with ketamine. (*D*) Record from cell in area CA1 in a rat treated with MK-801. Note that these four cells all showed evidence of ability to recover from high $[Ca^{2+}]_i$ when blood flow was restored.

in cortex. Fourth, at 8 min of low flow ischemia, the differences in $[Ca^{2+}]_i$ between the two categories of cells were much smaller, especially in the hippocampus. Fifth, there were large variations in the level of intracellular calcium at each time point. In some cells $[Ca^{2+}]_i$ rose only to 1×10^{-5} M whereas in others it reached 8×10^{-4} M.

High-flow ischemia and hypoxia. Similar measurements were carried out in high-flow ischemia and hypoxia and are presented in Table V. With these milder insults only scattered cells in dorsal thalamus, 5–10% of cells in the cingulate cortex, and 20–30% in the hippocampus, primarily in area CA1, responded with rises in $[Ca^{2+}]_i$.

Moreover, even in hippocampus the predominant group of cells belonged to the slow-responding category. The differences between the number of cells responding and their behavior in high-flow ischemia and hypoxia were rather small.

Cell Membrane Potentials in Late Hypoxia and Ischemia

Membrane potentials (measured against ground potential) were routinely recorded simultaneously with $[Ca^{2+}]_i$ (Table IV). Membrane potential declined in parallel to the rise in $[Ca^{2+}]_i$. Depolarization was larger and faster in fast-responder than in slow-responder cells but reached about the same level after 8 min.

TABLE IV

$[Ca^{2+}]_i$ $(10^{-8}$ M) and Membrane Potentials (in millivolts) in Thalamic, Hippocampal, and Cortical Neurons after 3 and 8 min of Low-Flow Ischemia

Time		Thalamus	Hippocampus		Cortex	
		Slow response	Fast response	Slow response	Fast response	Slow response
min						
0	$[Ca^{2+}]_i$	6.9 ± 1.8	6.93 ± 2.2	7.3 ± 3.1	7.28 ± 3.5	7.44 ± 2.7
		(n = 76)	(n = 104)	(n = 8)	(n = 18)	(n = 77)
	mp	−67.2 ± 7.4	−71.4 ± 6.7	−73.1 ± 5.4	−69.0 ± 9.1	−63.1 ± 9.0
		(n = 107)	(n = 96)	(n = 28)	(n = 29)	(n = 91)
3	$[Ca^{2+}]_i$	14.7 ± 5.2*	86.3 ± 54*	17.6 ± 5.8*‡	52.0 ± 23*	16.7 ± 7.1*‡
		(n = 53)	(n = 61)	(n = 8)	(n = 18)	(n = 58)
	mp	−52.0 ± 10.7*	−27.4 ± 12.8*	−56.6 ± 9.1*‡	−42.0 ± 11.3*	−58.0 ± 7.9*‡
		(n = 62)	(n = 73)	(n = 22)	(n = 23)	(n = 71)
8	$[Ca^{2+}]_i$	2400 ± 800*	3480 ± 760*	3320 ± 870*	3270 ± 910*	2760 ± 890*
		(n = 15)	(n = 22)	(n = 3)	(n = 7)	(n = 18)
	mp	−19.0 ± 12.9*	−12.7 ± 8.6*	−17.8 ± 14.2*	−14.1 ± 7*	−20.1 ± 9*
		(n = 17)	(n = 28)	(n = 7)	(n = 12)	(n = 20)

$[Ca^{2+}]_i$ and membrane potential (mp) were measured as described in the Methods. Values represent means ± SD for the number of cells indicated in parentheses.
*$p < 0.0001$ vs. time zero (t statistics for two means).
‡$p < 0.001$ vs. fast responders (t statistics for two means).

Effect of Treatment with Ketamine, MK-801, and Nifedipine on Intracellular Calcium Concentration

Administration of ketamine or MK-801 had no effect on neuronal $[Ca^{2+}]_i$ under normoxic conditions: the values recorded were indistiguishable from those in control cells in untreated animals. On the other hand, both drugs markedly attenuated the behavior of $[Ca^{2+}]_i$ in response to a limitation of the oxygen supply, especially that caused by ischemia. Although there was no apparent influence on the early rise in intracellular calcium (Figs. 2, 4, C and D) the later increase was clearly delayed and slowed. However, differences were seen with respect to the response from various brain regions to both drugs and of individual cells to either of the two. In

general the most susceptible region was the hippocampus in which almost all the cells tested in area CA1 responded to ketamine and MK-801. Table V shows that after 5 min of low-flow ischemia $[Ca^{2+}]_i$ rose in control cells by over two orders of magnitude whereas in the presence of either drug the increase was only fourfold. MK-801 might have been more effective than ketamine although it is difficult to prove on the basis of the data available due to the large standard deviation.

Analysis of the figures also shows that both drugs produced on a relative basis

TABLE V

Changes in $[Ca^{2+}]_i$ in Fast-Responder Hippocampal Cells in Area CA1 during Hypoxia and Low- and High-Flow Ischemia, with and without Pretreatment with Ketamine or MK-801

Time (min)	0	3	5	8
			10^{-8} M	
Low-flow ischemia				
Control	6.93 ± 2.2	86.3 ± 54	2280 ± 830	3480 ± 760
	(n = 104)	(n = 62)	(n = 28)	(n = 26)
Ketamine	6.91 ± 2.3	19.79 ± 6.2*	28.6 ± 14*	2650 ± 830*
	(n = 58)	(n = 39)	(n = 32)	(n = 21)
MK-801	6.89 ± 1.9	14.93 ± 5.7*	22.4 ± 12.6*	2590 ± 630*
	(n = 43)	(n = 28)	(n = 18)	(n = 11)
High-flow ischemia				
Control	6.93 ± 2.2	38.9 ± 8.5	1570 ± 390	3170 ± 940
	(n = 104)	(n = 57)	(n = 40)	(n = 14)
Ketamine	6.91 ± 2.3	15.8 ± 7.3*	26.6 ± 8.9*	3010 ± 710
	(n = 58)	(n = 23)	(n = 21)	(n = 13)
MK-801	6.89 ± 1.9	14.2 ± 4.9*	24.9 ± 7.3*	2890 ± 830
	(n = 37)	(n = 23)	(n = 21)	(n = 11)
Hypoxia				
Control	6.93 ± 2.2	18.6 ± 6.3	78.8 ± 21	3380 ± 560
	(n = 104)	(n = 71)	(n = 45)	(n = 18)
Ketamine	6.91 ± 2.3	13.8 ± 5.9*	38 ± 12.6*	3280 ± 620
	(n = 58)	(n = 26)	(n = 20)	(n = 11)

$[Ca^{2+}]_i$ was measured as described in the Methods. Values represent means ± SD for the number of cells indicated in parentheses.

*All significantly different at a $p < 0.001$ from controls at the respective time points (t statistics for two means).

(i.e., with respect to nontreated controls) smaller attenuation in high-flow than in low-flow ischemia, whereas in hypoxia ketamine reduced the rise in $[Ca^{2+}]_i$ at 5 min by only 50%. A similar pattern of response was seen in the cortex: fewer cells showed fast-responder behavior and the number of those affected was larger in animals to which ketamine had been administered than in those injected with MK-801. In thalamus, MK-801 was without any apparent effect on the ischemic rises in $[Ca^{2+}]_i$, whereas ketamine produced a small delay and attenuation in some cells,

which did not decrease the mean values for $[Ca^{2+}]_i$. At 8 min of ischemia or hypoxia the differences in $[Ca^{2+}]_i$ between responsive cells in treated and untreated animals were small in all three regions of the brain including the hippocampus (Table V), although in low-flow ischemia ketamine and MK-801 still afforded significant protection ($P < 0.001$).

Simultaneous recordings of membrane potentials showed that the changes occurred in parallel with those in $[Ca^{2+}]_i$, i.e., there was a smaller depolarization in the presence of ketamine and MK-801 (data not given).

17 rats were pretreated with nifedipine and the same measurements were performed. Normoxic values of $[Ca^{2+}]_i$ were in the same range as for control cells in the same areas in untreated rats. During ischemic and hypoxic insults and data obtained from nifedipine-treated animals were in the same range as those from untreated animals. The same parallelism was observed in high-flow ischemia and hypoxemia (data not shown).

Extracellular Calcium Ion Concentrations

While $[Ca^{2+}]_e$ was almost uniform in different regions of the brain during normoxia it changed rapidly at the interface between the early and late phases of the hypoxic/ischemic response. Between 3 and 6 min after the onset of oxygen deprivation, $[Ca^{2+}]_e$ fell to 0.5×10^{-3} M or less. The exact timing of the change varied in the different regions and correlated with the rise in $[Ca^{2+}]_i$. Thus it occurred earlier in the hippocampus than thalamus. These measurements were made for control purposes since alterations in $[Ca^{2+}]_e$ during hypoxia/ischemia are well documented in previous publications (Hansen and Zeuthen, 1981; Harris et al., 1981; Siemkowicz and Hansen, 1981).

Effect of O_2 Reintroduction or Restoration of Blood Flow on $[Ca^{2+}]_i$

Recovery from hypoxia and ischemia was not the subject of this investigation, however, in several cases changes in $[Ca^{2+}]_i$ were followed to ascertain whether or not the large increases ($1-8 \times 10^{-4}$ M) recorded in many cells were synonymous with irreversible damage to the neuronal plasma membrane. Records such as those presented in Fig. 4 A demonstrate that this is not necessarily the case. Although $[Ca^{2+}]_i$ rose in this fast-responder cell to 8×10^{-4} M, resuscitation of the animal with reinstatement of brain blood flow was followed by progressive elimination of calcium from the cytosol.

DISCUSSION

The results summarized in this work provide a clear demonstration that limiting the O_2 supply to the brain causes an increase in the concentration of intracellular calcium in some cell populations, which is especially rapid and pronounced in parts of the hippocampus. The changes that occur in these sensitive neurons upon transition from normoxia to severe ischemia are large, 10^3–10^4-fold. This situation is unlike that in many other cells or organs, including hepatocytes (Lemasters et al., 1987), myocytes (Allen and Orchard, 1984; Allshire et al., 1987), cultured kidney cells (Snowdowne et al., 1985), and perfused heart (Steenbergen et al., 1987) in which

lack of oxygen or addition of respiratory chain inhibitors induces only slow and marginal increases. This unique response of large numbers of cells in the CNS and its underlying causes require detailed discussion.

$[Ca^{2+}]_i$ in Brain in Normoxia

Our data show that the concentration of intracellular calcium in brains of pentobarbitone-anesthetized rats is 10^{-7}–10^{-8} M, i.e., comparable to or slightly lower than that in isolated or cultured neurons (Alvarez-Leefmans et al., 1981; Murphy et al., 1987) and other cells (Carafoli, 1987), but by one to two orders of magnitude smaller than the values reported for cat spinal cord motoneurons in situ (Morris et al., 1985). This large discrepancy between our study and that of Morris et al. (1985) may be due to differences in microelectrode technology, including the use of more sensitive, calcium-selective ligands. The levels of Ca^{2+} do not seem to differ significantly among the individual regions of brain but vary by over 10-fold from cell to cell. By contrast, the $[Na^+]_i$, $[K^+]_i$ and $[H^+]_i$ are maintained within ±15% of their mean values (Erecińska and Silver, 1990). The differing concentrations of calcium in cerebral neurons may reflect either variable levels of activity of individual cells, or of regions within the cell which microelectrode tips penetrate at random. This is not an unexpected conclusion owing to the abundance of processes in brain that are directly or indirectly dependent on calcium. These could be controlled locally by modulating calcium levels through nonuniform distribution of its entry and exit pathways. In support of our suggestion are observations with fluorescent Ca^{2+} indicators which have also shown inter- and intracellular variation, in the concentration of this cation (Connor, 1986; Tank et al., 1988; Tsien, 1988).

The maintenance of very low $[Ca^{2+}]_i$ requires the existence of efficient mechanisms to remove the cation from neural cells. It is generally accepted that this task is accomplished by two processes: the operation of the ATP-dependent calcium pump and Na^+/Ca^{2+} exchange (Carafoli, 1987; Blaustein, 1988; DiPolo and Beaugé, 1988). The former is considered to have high affinity but low capacity for calcium, while the opposite is true for the latter. Several studies have indicated that nervous tissue in general and synaptosomes in particular (Javors et al., 1981; Michaelis et al., 1983; Sorensen and Mahler, 1981; Lin and Way, 1982; Gill et al., 1984) are equipped with an active pump which may contribute substantially to calcium removal. Neurons as well as synaptosomes also contain a powerful Na^+/Ca^{2+} exchange (Blaustein and Ector, 1976; Blaustein, 1977; Gill et al., 1984; Nachschen et al., 1986; Sanchez-Armass and Blaustein, 1987) with a reported stoichiometry of $3Na^+$ that moved inwards for each calcium transported outwards (Blaustein, 1977). If the exchanger is the predominant mechanism in maintaining the cytosolic free Ca^{2+} (see e.g., Blaustein, 1988) it should be able to reduce its level to 10^{-7}–10^{-8} M. Since the electrochemical Na^+ gradient is the sole source of energy for the Na^+-coupled Ca^{2+} movement, the concentration of cytosolic free calcium at equilibrium would be given by:

$$[Ca^{2+}]_i = [Ca^{2+}]_o([Na^+]_i/[Na^+]_o)^3/\exp[(2 - n)\,EF/RT] \qquad (1)$$

where E is the transmembrane electrical potential and F, R, and T have their usual meanings. Substituting into Eq. 1 the appropriate values for $[Na^+]_i$, $[Na^+]_o$, $[Ca^{2+}]_o$,

and E from this and our earlier work (Erecińska and Silver, 1989) one obtains:

$$[Ca^{2+}]_i = 1.5 \text{ mM } (29 \text{ mM}/133 \text{ mM})^3 \exp$$

$$[(2 - 3) 23{,}060 \text{ cal}/V \cdot (-0.068V)/1.98 \text{ cal/degree} \cdot 310 \text{ degree}] = 1.21 \ \mu M \quad (2)$$

The calculated value of 1.21 μM is 10–100-fold larger than that measured directly. This is different from the situation observed in, for example, cardiac myocytes (Carafoli, 1987) or squid axon (Blaustein, 1977) where the calculated $[Ca^{2+}]_i$ is very close to the measured value. Hence, our results mean that in resting cerebral neurons under normoxic conditions the cytosolic $[Ca^{2+}]$ is either maintained predominantly by the operation of the ATP-fueled pump or that the stoichiometry of the Na^+/Ca^{2+} exchange is larger, e.g., 4:1.

$[Ca^{2+}]_e$ and pH in Brain in Normoxia

Concentrations of external calcium in the three regions of the brain investigated in this work were 1.49–1.56 mM (Table II), which is somewhat higher than the 1.2–1.3-mM value found by earlier investigators (Hansen and Zeuthen, 1981; Harris et al., 1981; Siemkowicz and Hansen, 1981; see Hansen, 1985 for review). We have no explanation for this difference although our figure is in good agreement with the value of 1.39 mM obtained by Krnjević et al. (1982) for the rat hippocampus.

External pH was 7.3 (Table III), essentially the same as that reported in numerous previous studies (e.g., Kraig et al., 1983; von Hanwehr et al., 1986; see Hansen, 1985 for review). pH_i was determined to be ~7.28, which is ~0.2 pH units higher than that calculated either from values of $PtCO_2$ (tissue PCO_2), TCO_2 (total CO_2 content), $[HCO_3^-]_e$, and CSF volume (Siesjö et al., 1985; von Hanwehr et al., 1986), or from near equilibrium in the creatine phosphokinase reaction (Mabe et al., 1983). The umbelliferone technique, which measures predominantly pH_i, yielded a value of 7.2 for tissue pH (Csiba et al., 1985) while nuclear magnetic resonance measurements give figures from 7.08 (e.g., Behar et al., 1985) to 7.2–7.3 (e.g., Thulborn et al., 1982; Naruse et al., 1983). It is interesting that both snail neurons (Thomas, 1976) and central neurons of the lamprey (Chesler and Nicholson, 1985) bathed in HCO_3^-/CO_2 buffer at pH 7.4–7.5 exhibit pH 7.4–7.5, i.e. no substantial pH gradient is maintained across their plasma membrane at physiological pH.

Changes in $[Ca^{2+}]_i$ during Ischemia/Hypoxia

The early phase of the rise in $[Ca^{2+}]_i$. The earliest events after the fall of O_2 tension to zero are intracellular alkalinization and a small rise in calcium. These are followed by a slight hyperpolarization and a disappearance of the cortical electrical activity. A similar early increase in $[Ca^{2+}]_i$ in cortical cells loaded with quin-2 has been observed by Uematsu et al. (1988) during middle cerebral artery occlusion, and was shown to precede the loss of ECoG. This interesting finding obtained by two independent techniques in two different models is not easy to explain because calcium influx mechanisms in general require depolarization for their activation. However, some speculation seems appropriate because elucidation of this puzzling phenomenon may prove crucial for our understanding of ischemic/hypoxic events.

Lack of O_2 and consequent inhibition of mitochondrial energy production reduces cerebral ATP synthesis (Lowry et al., 1964; Siesjö, 1978). However, in brain, as in heart and skeletal muscle, the decline in ATP level is "buffered" and delayed by the operation of the creatine phosphate/creatine (CrP/Cr) system (Meyer et al., 1984). Because the concentrations of CrP and Cr are greater than those of the adenine nucleotides and the equilibrium in the CrP reaction is shifted towards ATP synthesis (Lawson and Veech, 1979), the initial hydrolysis of CrP that occurs in anoxia and ischemia is accompanied by almost no change in [ATP] (Siesjö, 1978, 1981). Because ATP concentration remains constant, the hydrolysis of this nucleotide, even though it precedes that of CrP, cannot be the source of net proton production (Busa and Nuccitelli, 1984). On the other hand, operation of CrP in the direction of ATP formation, a reaction that consumes H^+, may lead to alkalinization because a net fall in [CrP] will be accompanied by a net decrease in proton concentration. At physiological pH the stoichiometry of this reaction is 1 H^+ per 1 CrP (Lawson and Veech, 1979). The brain contains 5–6 μmol of CrP/g of tissue (Siesjö, 1978) and complete hydrolysis of CrP would take up an equivalent amount of H^+ which, at a buffering capacity in nervous tissue of 15–20 μmol $\cdot$ g^{-1} $\cdot$ pH unit^{-1} (Moody, 1984), should cause an alkalinization of ~0.3 pH unit. The smaller than predicted intracellular alkaline shift observed as the first event in anoxia and ischemia (0.1–0.15 pH unit, Table III) means either that only a portion of CrP is initially hydrolyzed or that the lack of O_2 simultaneously stimulates net proton-producing pathways which oppose the rise in pH.

It should be noted that the intracellular alkalinization discussed above is transient in nature and is followed by a steadily increasing and marked acidification. The latter is one of the most consistent features of brain ischemia and hypoxia (Siesjö, 1978). A decrease in CrP and ATP and a rise in ADP, AMP, and inorganic phosphate, which occur during the early stages of O_2 deprivation, lead to both deinhibition and stimulation of phosphofructokinase, the rate-limiting enzyme in the glycolytic pathway. Enhanced glycolysis in combination with impaired mitochondrial energy synthesis and decreasing ATP levels leads to accumulation of a large excess of H^+ and acidosis. The early activation of proton production may also explain our observation that the initial alkalinization was smaller than that predicted from the calculation of the total CrP content of whole brain.

The increase in $[Ca^{2+}]_i$ that follows could be caused by an inability of neurons to maintain proper operation of the Ca^{2+} buffering systems (McBurney and Neering, 1987; Blaustein, 1988) because a decrease in cellular energy levels curtails calcium sequestration mechanisms in endoplasmic reticulum (Tanford, 1981), and mitochondria (Fiskum and Lehninger, 1982) and also limits the activity of the plasma membrane Ca^{2+} pump (Carafoli, 1987). Failure of the first of these two mechanisms should result in a release of calcium from the intracellular stores whereas inhibition of the membrane pump could raise $[Ca^{2+}]_i$ without activation of specific routes of cation entry. The possibility that the early increase in $[Ca^{2+}]_i$ is of intracellular origin is supported by three lines of evidence. (a) No measurable decrease of $[Ca^{2+}]_e$ was found (Table III). (b) There was no detectable membrane depolarization to activate voltage-dependent Ca^{2+} channels. (c) Ketamine and MK 801 were without effect, which argues against a possible opening of certain classes of receptor-linked chan-

nels. It is worth noting that the early anoxic/ischemic events occur primarily at the synapses (Grossman and Williams, 1971) and it is not unreasonable to suppose that increases in $[Ca^{2+}]_i$ at these sites may be larger than those recorded in the cell soma. The early rise in $[Ca^{2+}]_i$ could have at least two important consequences. Firstly, it may open the calcium-activated K^+ channels known to be present in nervous tissue (Meech, 1978; Blatz and Magleby, 1987) and the transient membrane hyperpolarization that we observed together with the concomitant increase in $[K^+]_e$ are consistent with their activation. Secondly, it could stimulate transmitter release including the acidic amino acids (Katz and Miledi, 1967; Llinas and Nicholson, 1975). Although we do not know whether or to what extent such a release occurs at this early stage of anoxia, if it does take place it could contribute to the subsequent ion movements through the activation of postsynaptic receptors.

It has long been known that these disappearance of the ECoG during anoxia and ischemia precedes generalized ionic shifts (Hansen, 1985). It can be argued that loss of integrated electrical activity is initiated by large ion movements that occur only at very localized sites, such as certain synapses, and thus escape detection. However, our results, which show that ECoG activity becomes isoelectric very shortly after the initial increases in $[Ca^{2+}]_i$ and $[K^+]_e$, may indicate that this change occurs as a result of alteration in membrane permeability to K^+. This is because not only does an increase in $[Ca^{2+}]_i$ open Ca^{2+}-sensitive K^+ channels as mentioned above, but the resulting rise in $[K^+]_e$ would also activate the inward K^+ rectifier channels, the existence of which has been shown in nerve cells (Kandel and Tauc, 1966). The increase in g_k thus produced could be responsible for depressing spontaneous neural activity and could lead to the observed loss in ECoG. A similar relationship between K^+ permeability and cell activity has been suggested by Godfraind et al. (1971).

Late changes in $[Ca^{2+}]_i$. Our results show, in agreement with earlier studies (Hansen and Zeuthen, 1981; Harris et al., 1981; Siemkowicz and Hansen, 1981) that 3–6 min after deprivation of O_2 or a decrease in blood flow, there is a rapid decline in $[Ca^{2+}]_e$ and an increase in $[K^+]_e$. This is accompanied by a rise in $[Ca^{2+}]_i$, the magnitude of which is dependent on the type and severity of insult (Table V). In general the smallest changes and lowest proportion of responsive cells were seen in hypoxia and the most pronounced and generalized in severe ischemia. While in the former the proportion of cells responding to the result was only ~5–15%, in the latter it was at least 40% and in hippocampal region CA1 at least 90%.

CNS cells have in abundance two pathways that control calcium entry: the voltage-activated channels and the receptor-operated channels (Hagiwara and Byerly, 1981; Meldolesi and Pozzan, 1987; Miller, 1987; Tsien et al., 1988). The former are opened upon depolarization, an event which occurs in hypoxia and ischemia (Hansen, 1985). Although in our studies nifedipine was without a measurable effect on the rise in $[Ca^{2+}]_i$, it is known that dihydropyridines do not block all types of neuronal voltage-dependent channels (Fox et al., 1987). Thus, in spite of this apparent lack of influence of the Ca^{2+} channel blocker, but based on the widely occurring depolarization (Table III), we would like to suggest that during limitation in O_2 supply calcium may enter neurons through this pathway.

The receptor-operated channels in neurons are activated predominantly by the acidic amino acid transmitters. It has been shown (Pumain and Heinemann, 1985)

that iontophoretic application of aspartate and glutamate to brain slices causes large decreases in $[Na^+]_e$ and $[Ca^{2+}]_e$ whereas addition of these amino acids to isolated or cultured neurons induces increases in g_{Na} and g_{Ca} (Mayer and Westbrook, 1985) as well as a rise in $[Ca^{2+}]_i$ (Bührle and Sonnhof, 1983; MacDermott et al., 1986). These responses are mediated to a large extent by a subclass of acidic amino acid receptors specifically activated by N-methyl-D-aspartate (NMDA) (MacDermott et al., 1986). Brain hypoxia and ischemia lead to a release of amino acid transmitters into the external environment (Benveniste et al., 1984; Drejer et al., 1985; Hagberg et al., 1985) at concentrations that are high enough to activate postsynaptic receptors (Foster and Fagg, 1984). Certain regions of the brain, such as the hippocampus, which are known to be very vulnerable to lack of oxygen (Brierley and Graham, 1984), receive a rich glutamatergic input (Cotman et al., 1987) and contain a high density of acidic amino acid receptors, especially those sensitive to NMDA (Greena-myre et al., 1985; Monaghan and Cotman, 1985). One would predict, therefore, that in such areas an important pathway for calcium entry would be via the recep-tor-operated channels. The following results obtained in this study provide direct experimental evidence for this prediction. (a) During low-flow ischemia, i.e., under conditions that would favor release and accumulation of acidic amino acid neuro-transmitters, cells exhibiting rapid-responder behavior were found to comprise the majority of the population in hippocampal area CA1. (b) Ketamine and MK-801, two noncompetitive antagonists of the NMDA receptors (Kemp et al., 1987), mark-edly inhibited rises in $[Ca^{2+}]_i$ especially in the hippocampus. (c) The increases in $[Ca^{2+}]_i$ in the early stages of high-flow ischemia and even more in hypoxemia, i.e., those insults during which blood flow was maintained, were much smaller than in low-flow ischemia and were not significantly affected by ketamine and MK-801. Although this may be due to better preservation of the high-energy phosphate com-pounds in these less severe insults, it is also possible that removal of acidic amino acids via sustained circulation (Rafalowska et al., 1975) and consequent lowering of their concentrations in the external environment of neurons can ameliorate large increases in $[Ca^{2+}]_i$ in these cells by reducing influx via the receptor-mediated path-way.

In addition to the two discussed above, there are other mechanisms that could contribute to the rise in cytosolic $[Ca^{2+}]_i$ in hypoxia and ischemia. Lack of ATP either directly or indirectly curtails reactions that under physiological conditions eliminate calcium from the cytosol: the Ca^{2+} pump, Na^+/Ca^{2+} exchange, and uptake by endoplasmic reticulum and mitochondria. Failure of any or all of these would result in an increase in free $[Ca^{2+}]_i$. Finally, nonspecific leaks through damaged plasma membrane may also be of importance although their contribution is difficult to assess.

Within each category of cells sensitive to the lack of oxygen described in this study, relatively large variations in $[Ca^{2+}]_i$ were seen in hypoxia/ischemia which can-not be explained on the basis of the time factor because in many instances the mea-surements were done concurrently. At least two explanations can be offered to account for this variability. The first is that small differences in the distribution of the capillaries and/or capillary blood flow that favor some cells and disadvantage others could make them appear more resistant or more vulnerable to hypoxia/

ischemia. Consistent with this is the recent report by Feng et al. (1988) that oxygenation in hippocampus is significantly less than expected from measurement of hippocampal blood flow, which could be due to wide intercapillary spacing and hence large diffusion distances for oxygen (and other substrates) or to a particularly high local metabolic rate. An alternative explanation for selective hippocampal vulnerability to hypoxia/ischemia is that the number and/or distribution of the calcium channels as well as the efficacy of mechanisms that lead to their activation may vary from cell to cell. Consequently, cells with more Ca^{2+} entry pathways or those susceptible to their rapid activation would be expected to be especially sensitive to reduction in O_2 and/or blood flow.

In summary, our results show that hypoxia and ischemia induce large increases in $[Ca^{2+}]_i$ in some cerebral neurons via activation of voltage-operated and receptor-operated channels. The latter may be the predominant pathway for calcium entry in regions that receive rich glutamatergic input and are highly sensitive to the lack of O_2 or interruption of blood flow. It is suggested that the abundance of the acidic amino acid receptors, and consequently receptor-operated channels, is responsible, to a large extent, for the uniquely high increases in cerebral $[Ca^{2+}]_i$ during anoxia/ischemia. Our data support the hypothesis advanced on the basis of experiments in vitro (Rothman, 1983; Garthwaite et al., 1986; Golberg et al., 1986; Weiss et al., 1986; Choi et al., 1987; Rothman et al., 1987) that increased permeability to calcium induced by acidic amino acid transmitters contributes to their neurotoxic action (Olney, 1984; Rothman and Olney, 1986; Choi, 1988). The observation here that ketamine and MK-801 delay the rise in $[Ca^{2+}]_i$ in the rapid responder cells is consistent with studies in vivo that show that blockade of the NMDA-receptor ameliorates hypoxic/ischemic brain damage (Simon et al., 1984; Foster et al., 1987; Gill et al., 1987; Ozyurt et al., 1988). Our results suggest that this beneficial effect is exerted by reducing the time over which the cells are exposed to very high internal levels of calcium during the late stages of oxygen deprivation. However, to what extent the rise in calcium during short periods of O_2 or flow deprivation is correlated with irreversible brain damage cannot be answered by this study. It may be speculated that the final outcome will depend on how quickly and to what extent cells can reduce their $[Ca^{2+}]_i$ after normal conditions are reinstated and/or how reversible are the changes incurred during the hypoxic/ischemic episode.

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