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Inhibition of Bioenergetics Alters Intracellular Calcium, Membrane Composition, and Fluidity in a Neuronal Cell Line

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The effect of inhibited bioenergetics and ATP depletion on membrane composition and fluidity was examined in cultured neuroblastoma-glioma hybrid NG108-15 cells. Sodium cyanide (CN) and 2-deoxyglucose (2-DG) were used to block oxidative phosphorylation and anaerobic glycolysis, respectively. Endoplasmic reticulum (ER) Ca^{2+}-pump activity measured by $^{45}Ca^{2+}$ uptake was >92% inhibited in intact cells incubated with CN (1 mM) and 2-DG (20 mM) for 30 min. In addition, exposure of cells to CN and 2-DG caused a 134% increased release of isotopically labeled arachidonic acid (^{3}H-AA) or arachidonate-derived metabolites from membranes. Removal of Ca^{2+} from the incubation medium ablated the CN/2-DG induced release of ^{3}H-AA or its metabolites. Membrane fluidity of intact cells was measured by electron spin resonance spectroscopy using the spin label 12-doxyl stearic acid. The mean rotational correlation time (τ_c) of the spin label increased 49% in CN/2-DG exposed cells compared to controls, indicating a decrease in membrane fluidity. These results show that depletion of cellular ATP results in inhibition of the ER Ca^{2+}-pump, loss of AA from membranes, and decreased membrane fluidity. We propose that impaired bioenergetics can increase intracellular Ca^{2+} as a result of Ca^{2+}-pump inhibition and thereby activate Ca^{2+}-dependent phospholipases causing membrane effects. Since neurons derive energy predominantly from oxidative metabolism, ATP depletion during brain hypoxia may initiate a similar cytotoxic mechanism.

KEY WORDS: NG108-15 cells; ATP; calcium; arachidonic acid; spin label; membrane fluidity.

INTRODUCTION

The cell membrane consists of a fluid phospholipid bilayer with embedded macromolecules (1). These macromolecules include receptors for hormones and neurotransmitters, enzymes, and the effectors of ion and electrolyte transport. These specialized functions are particularly important for excitable membranes in the nervous system where energy-dependent and stimulus-dependent ion transport regulates nerve conduction. Macromolecular function is strongly influenced by the composition and consequently the physical state fluidity of the membrane lipids (2–7). Lipid composition of the cell membrane is maintained by the normal incorporation of fatty acids into the membrane and their degradation due to phospholipases. Phospholipases such as phospholipase A_2 (PLA_2) are modulated by the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (8) that is buffered by Ca^{2+}-

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ATPases (Ca^{2+}-pumps) in the mitochondria and endoplasmic reticulum (ER). The mitochondrial and ER Ca^{2+}-pumps sequester Ca^{2+} when $[Ca^{2+}]_i$ rises above the resting level. However, the ER Ca^{2+}-pump is a high affinity system compared to the mitochondrial Ca^{2+}-pump and plays the predominant role in buffering $[Ca^{2+}]_i$ (9).

We hypothesized that inhibited bioenergetics would alter membrane function by the following pathway: decreased ATP synthesis → decreased ER Ca^{2+}-pump activity → increased cytoplasmic Ca^{2+} → increased PLA_2 activity → a loss of arachidonic acid from cellular membranes → a decrease in membrane fluidity. We tested our hypothesis in the clonal neuroblastoma-glioma hybrid NG108-15 cell line. This cell line has been demonstrated to be a model for neurons in that these cells synthesize and secrete acetylcholine, have receptors for several neurotransmitters, and have excitable membranes (10). The results presented in this report support the proposed hypothesis.

EXPERIMENTAL PROCEDURE

Materials. Reagents and materials were obtained from the following sources: Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/L) from Grand Island Biological Co. (Grand Island, NY); Fetal calf serum from Hyclone Laboratory (Logan, UT); [5,6,8,9,11,12,14,15-^{3}H (N) Arachidonic acid (76.0 Ci/mmol) from New England Nuclear (Boston, MA); manoalide from Biomole Laboratories, Inc. (Plymouth Meeting, PA). The spin probe 12-doxyl stearic acid was obtained from Syva, Ltd, Palo Alto, CA. Fura-2 acetoxymethyl ester (Fura-2 AM) was obtained from Molecular Probes (Eugene, OR). All other reagents including quinacrine dihydrochloride and 4-bromophenacyl bromide (4-BPB) were from Sigma Chemical Co. (St. Louis, MO); The NG108-15 cell line (a mouse neuroblastoma × rat glioma hybrid) was generously donated by Dr. M. Nirenberg (NIH, Bethesda, MD). NaCN solution was prepared in distilled water just before experimentation and was stored in a tightly closed vial. The addition of NaCN up to 1 mM concentration to the final experimental medium did not change pH significantly.

Cell Culture. The clonal NG108-15 cell line was routinely maintained as a monolayer in plastic culture vessels in a humidified aerobic environment (10% CO_2/90% air) at 37°C (11).

Inhibition of Bioenergetics. NG108-15 cell monolayers (grown in 24-well plates to 90–100% confluency) were incubated in isosmotic (340 mOsmol/L) HEPES buffered salt solution (HBSS, 116 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, 25 mM D-glucose, 25 mM HEPES, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, adjusted to pH 7.4 with NaOH) with 1.0 mM CN or 20 mM 2-DG or both at 37°C for 0.5, 1.0, 5.0, 15, and 30 min. The incubation medium was removed by aspiration, and cells were precipitated with 1 ml of 10% trichloroacetic acid in cold and centrifuged for 1 min at 20,000 *g* in a microfuge. Supernatants were adjusted to pH 7.0 by addition of 0.6N NaOH in 20 mM Tris. Aliquots of this preparation were assayed for ATP content by a luminometric method using a purified luciferin-luciferase system from the Analytical Luminescence Laboratory, San Diego, CA. The contents of one vial of luciferin-luciferase mixture were dissolved in 2 ml of 50 mM HEPES-KOH buffer pH 7.5 containing 5 mM $MgCl_2$. The reaction mixture consisted of 20 μl of reconstituted luciferin/luciferase solution and 80 μl of HEPES buffer to which 10 μl of the cell extracts or ATP standard were added to initiate the reaction. Light was measured with a custom-made light detector (12). In a separate set of experiments, cells were solubilized by keeping in 1 ml of 0.14 N HNO_3 at 70°C for 20 min. The extracts were processed as TCA treated cells for ATP assay. Assay results were identical in both TCA precipitated and HNO_3 solubilized cells.

Endoplasmic Reticulum Calcium Pump Activity. Fully confluent NG108-15 cells grown in 75 cm^2 flasks were washed twice by suspension and centrifugation using 10 ml each time of isotonic (340 mOsmol/L) sucrose in 10 mM Tris-HCl buffer, pH 6.8. Cells were detached by gentle shaking and centrifuged at 200 *g* for 5 min. Washed cells were suspended in Ca^{2+}-free isosmotic medium HBSS and adjusted to 10^6 cells/ml. In experiment a, one ml of suspended cells was incubated without additives or with 1 mM CN plus 20 mM 2-DG for various times at 37°C. The cells were then microfuged and the cell pellet was suspended in ice cold 20 mM Tris-HCl buffer without additives or with 1 mM CN plus 20 mM 2-DG at pH 7.6 and homogenized using a Dounce homogenizer with 50 strokes of a type B pestle until >90% of cells were disrupted. In experiment b, one ml of suspended cells was first homogenized in Ca^{2+}-free 10 mM Tris-HCl buffer at ice cold temperature and then incubated with the indicated agents for different times at 37°C. Endoplasmic reticulum Ca^{2+}-pump activity in experiments a and b was measured by the method of Moore et al. (13). In this assay, homogenates were incubated with $^{45}CaCl_2$ in a medium containing 100 mM KCl, 30 mM imidazole-histidine buffer (pH 6.8), 5 mM ammonium oxalate, 5 mM sodium azide, 5 mM $MgCl_2$, 5 mM ATP, 20 μM $CaCl_2$ with 0.1 μCi of $^{45}CaCl_2$/ml, CN and 2-DG as indicated, and approximately 1 mg/ml of homogenate protein. The reaction was initiated by the addition of homogenate. Following incubation, 0.2 ml aliquots of samples were removed and filtered through cellulose nitrate filters (0.45 micron pores). The nonspecific $^{45}Ca^{2+}$ uptake was determined using the same $^{45}Ca^{2+}$ medium without 5 mM ATP. The filters were thoroughly washed in cold using 100 mM KCl in 30 mM imidazole-histidine buffer, pH 6.8. The filters were then air dried, and $^{45}Ca^{2+}$ retained by the filters was determined by liquid scintillation counting. Uptake of $^{45}Ca^{2+}$ was calculated as the difference between the nanomoles of $^{45}Ca^{2+}$ per mg of protein found in the homogenate in the presence and absence of ATP. Protein concentration was determined by the method described by Lowry et al. (14).

Measurement of Intracellular Free Calcium Level. Intracellular free calcium concentrations were determined using the calcium specific fluorescent probe fura-2 by a modification of the methods described (15,16). Confluent monolayer NG108-15 cells grown in 75 cm^2 tissue culture flasks (10 to 15 × 10^6 cells per flask) were first rinsed twice using 10 ml HBSS at room temperature. These cells were then detached by gentle shaking in 10 ml HBSS and centrifuged at 200 *g* for 5 min to obtain a pellet. The pellet was suspended in 1 ml HBSS containing 4 μM fura-2 AM to load cells with fura-2 AM for 30 min at 37°C. Cells were washed twice to remove extracellular fura-2 AM by suspending in 10 ml HBSS and centrifugation at 200 *g* for 5 min. Washed cells were suspended in HBSS to a concentration of 10^6 cells/ml. A two-ml aliquot of cell suspension was then placed in a polystyrene cuvette and was continuously stirred with a small magnetic stirring bar to maintain an uniform cell suspension and to ensure adequate mixture of agents. A SPEX spectrofluorometer (CM system, Edison, NJ) equipped with a 37°C water-jacketed cuvette compartment

was used for fluorescence determinations. Fura-2 fluorescence was measured at an excitation of 340/380 nm and emission of 510 nm.

Determination of Arachidonic Acid Release. Arachidonic acid release assay was performed by a modification of the methods of Kanterman *et al.* (17) and Fink et al. (18) as described. NG108-15 cells were plated in 24-well plates. Confluent cells were incubated with ^{3}H-AA (0.25 μCi/ml/well) for 20 hour to isotopic equilibrium. The cells were washed with 0.5 ml of serum-free "incubation medium" [138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 0.5 mM $MgCl_2$, 1 mg/ml fatty acid-free bovine serum albumin to trap free ^{3}H-AA in the extracellular medium] containing Ca^{2+} (0.9 mM $CaCl_2$). The cells were then incubated in "incubation medium" without or with Ca^{2+} (0.9 mM $CaCl_2$) and without or with additives [1 mM CN, 20 mM 2-DG, 20 μM Quin II, or 20 mM ammonium oxalate] in a final volume of 0.5 ml at 37°C for 30 min. The reaction was stopped by removing the incubation medium which was then centrifuged at 12,000 *g* for 1 min to remove nonadherent cells. After 400 μl of the supernatant was removed, released ^{3}H-AA or its derivatives was measured by scintillation counting.

Membrane Fluidity Assay. A quantity (50 μg) of the spin-label 12-doxyl stearic acid in ethanol was dried under a stream of dry nitrogen for 2 min at the bottom of a 10 × 75 mm glass test tube which was wrapped in aluminum foil to exclude light. Untreated (control) and experimental cell suspensions (250 μl containing 5 mg protein) were added to tubes containing spin-label, gently stirred and incubated at room temperature for 30 min to incorporate spin label into membrane lipid. To determine the effect of freezing and thawing of cells on membrane fluidity, a suspension of cells (10^7 cells in 0.25 ml of isotonic HBSS) with mean viability of 88% (trypan blue dye exclusion assay) was frozen at −20°C for 4 hr. Frozen cells were thawed at room temperature (5 min), viability was determined (<1%), and they were immediately used for membrane fluidity assay. All ESR spectral measurements were carried out in an aqueous solution sample cell with a type E238 cavity in a Varian model E109 Century line spectrometer. A 250 μl aliquot of spin-labeled cell suspension was placed in the ESR sample cell and the spectrum was recorded at ambient temperature. All spectra were stored in the memory of a Varian E935 data-acquisition system and recorded later with the Varian digital drive recorder.

Rotational correlation time (τ_c) of the spin label was calculated from the ESR spectrum after free spin subtraction using the relationship (19) $\tau_c = 6.5 \times 10^{-10} H_o [(h_o/h_{-1})^{1/2} - 1]$, where H_o is the linewidth of the medium field line, and h_o and h_{-1} are the heights of the medium and high field lines respectively as shown in Figure 1. An increase in τ_c means a decrease in membrane fluidity and vice versa.

Cell Viability Assay. Cell viability was determined by two different assays (a) trypan blue dye exclusion assay, and (b) cell growth assay. In the trypan blue assay, cells were incubated in HBSS with 0.2% trypan blue for 5 min at room temperature. Viability was determined by the proportion (%) of unstained cells as observed by light microscopy. In the cell growth assay, separate samples (10^6 cells/sample) of untreated cells and cells treated under sterile condition with 1 mM CN plus 20 mM 2-DG in HBSS at 37°C for 30 min were centrifuged at 200 *g* to obtain cell pellets. These pellets were washed once by resuspending in 10 ml growth medium followed by centrifugation. Washed individual pellets were suspended separately in 25 ml growth medium and placed in a 75 cm^2 tissue culture flask and incubated inside a CO_2 incubator for cell growth. Cell growth was determined by counting total cells per flask after incubation for 5–6 days.

Data Analysis. Statistical differences were determined among group means by using a Student's *t*-test.

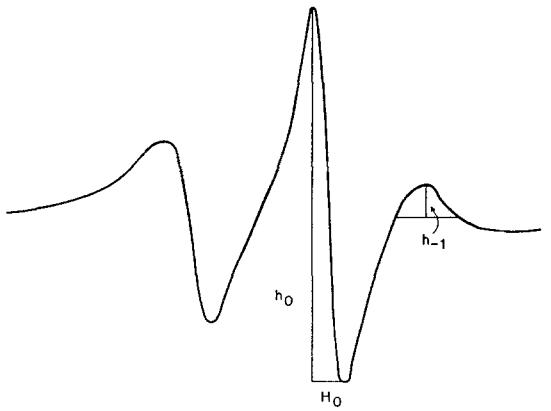

Fig. 1. A typical ESR spectrum of the spin label 12-doxyl stearic acid incorporated in NG108-15 cell membrane. The calculation of the mean rotational correlation time of the spin label was carried out using the relationship: $\tau_c = 6.5 \times 10^{-10} H_o [(h_o/h_{-1})^{1/2} - 1]$, where H_o is the linewidth of the medium field line, and h_o and h_{-1} are the heights of the medium and high field lines respectively.

RESULTS

Inhibition of Bioenergetics. We determined the time-course of ATP depletion in NG108-15 cells exposed to inhibitors of bioenergetics. In an aerobic environment, NG108-15 cells derive energy from oxidative phosphorylation. As for other clonal neuronal cell lines, these cells can switch to anaerobic glycolysis when the oxidative pathway is blocked (20). To mimic a state of complete energy deprivation, we used CN (an inhibitor of oxidative metabolism) in combination with 2-DG (an inhibitor of anaerobic glycolysis) to completely prevent the production of ATP from both aerobic and anaerobic metabolism. ATP content of control cells was 10 ± 0.2 nmol per 10^6 cells. Exposure of cells to CN plus 2-DG resulted in an almost total depletion of ATP within 1 min (<0.3 nmol per 10^6 cells). CN (1 mM) alone did not reduce ATP synthesis, whereas 2-DG (20 mM) lowered ATP level to 80 ± 3%, 62 ± 2% and 30 ± 2% of control in 5, 15 and 30 min respectively. Because the sequelae of inhibition of ATP synthesis would require some minutes to occur, the effect of inhibited bioenergetics on ATP-dependant processes was determined 30 min after addition of CN plus 2-DG.

The Effect of CN + 2-DG on Cell Viability. The effect of ATP depletion, if any, on cell viability was examined by two different methods: (a) the trypan blue dye exclusion assay, and (b) comparing growth (cell number and viability) of untreated and CN + 2-DG treated cells as described under "Experimental Proce-

dure". After 30 min incubation, CN + 2-DG had no effect on cell viability as determined by both methods.

In the trypan blue assay, viability of CN + 2-DG treated cells was 80–90%, whereas that of untreated control cells was more than 90%, indicating no gross adverse effect of ATP depletion up to 30 min.

In the cell growth assay, total number (mean ± SEM, N = 4) of cells per flask was 10.1 ± 0.43 × 10^6 for control and 10.4 ± 0.34 × 10^6 for CN + 2-DG treated cells after 5–6 days of growth. Both control and CN + 2-DG treated cells looked similar, firmly attached and healthy. Trypan blue assays of these cells showed % viabilities (mean ± SEM, N = 4) of 98 ± 0.11 for control and 96 ± 0.96 for CN ± 2-DG cells.

Endoplasmic Reticulum Calcium Pump Activity. Since the ATP-dependant ER Ca^{2+}-pump is the most important intracellular mechanism for buffering Ca^{2+}, we examined the effects of inhibited ATP synthesis on the ER Ca^{2+}-pump. When NG108-15 cells were pretreated with 1 mM CN + 20 mM 2-DG for 30 min at 37°C, Ca^{2+}-pump activity was reduced to approximately 10% of controls (Table 1, section a). Inhibition of Ca^{2+}-pump activity was not due to a lack of ATP during the assay period because the assay medium contained 5 mM ATP. To further demonstrate that the Ca^{2+}-pump inhibition was due to a lack of ATP during incubation with CN and 2-DG, in one experiment the cells were homogenized and then preincubated with 5 mM ATP along with CN + 2-DG for 30 min at 37°C prior to assay of Ca^{2+}-pump activity (Table I, section b). When ATP was present during the incubation with CN plus 2-DG, Ca^{2+}-pump activity was equal to control values. These results indicate that diminution of intracellular ATP irreversibly damages ER Ca^{2+}-pump activity. Following CN + 2-DG treatment, calcium pump activity of either intact cells or homogenate did not alter in 5 min (data not shown). At 15 min, intact cells had Ca^{2+} uptake (nmol/mg protein) of 4.1 ± 0.3 and 3.0 ± 0.2 (73% of control) in control and due to CN + 2-DG treatment respectively, whereas cell homogenate had Ca^{2+} uptake of 4.2 ± 0.2 and 3.1 ± 0.2 (74% percent of control) under identical conditions. No significant decrease in uptake of Ca^{2+} resulted when cells were treated with either CN or 2-DG alone in these experiments.

Table I. Effect of Inhibited Bioenergetics on Endoplasmic Reticulum ATP-Dependent Ca^{2+}-Pump Activity in Cultured NG108-15 Cells

Tissue	Incubation condition	Ca^{2+} uptake nmol/mg protein	% control
a. Intact cells	(no aditives)	3.54 ± 0.09	100.0
	CN + 2-DG	0.27 ± 0.06*	7.6
b. Homogenate	(no additives)	4.23 ± 0.30	100.0
	CN + 2-DG	0.47 ± 0.13**	11.0
	5 mM ATP	5.00 ± 0.15	118.0
	CN + 2-DG + 5 mM ATP	4.96 ± 0.14	117.0

NG108-15 cells were suspended in an isosmotic medium and adjusted to 10^6 cells/ml. Experiment a: one ml of suspended cells was incubated without additives or with 1 mM CN + 20 mM 2-DG for 30 min at 37°C. The cell pellet was homogenized until >90% of cells were disrupted. Experiment b: one ml of suspended cells was first homogenized and then incubated with the indicated agents for 30 min at 37°C. Endoplasmic reticulum Ca^{2+}-pump activity in experiments a and b was measured by the method of Moore et al. [13].

* Significantly different from intact cell control ($P < 0.001$)

** Significantly different from all other homogenate groups ($P < 0.05$).

Intracellular Free Calcium Level. Fluorometric determination of intracellular free calcium ($[Ca^{2+}]_i$) levels as described under "Experimental Procedure" showed a significant ($p < 0.05$) increase in $[Ca^{2+}]_i$ due to treatment of cells with 1 mM CN + 20 mM 2-DG. In cells treated with CN + 2-DG, $[Ca^{2+}]_i$ increased two-fold compared to untreated control cells as early as 5 min following treatment. Control cells had $[Ca^{2+}]_i$ of 169 ± 10 nM (mean ± SEM., N = 3). The increased level of $[Ca^{2+}]_i$ observed at 5 min was sustained at 15 and 30 min post CN + 2-DG treatment. Treatment of cells with 1 mM CN or 20 mM 2-DG alone did not cause any significant change in $[Ca^{2+}]_i$ compared to control cells.

Effect of Inhibited Bioenergetics on Arachidonic Acid Release. Incubation of NG108-15 cells with CN + 2-DG for 30 min resulted in a significant generation of AA or its metabolites (Table II: line 2 vs line 1). The magnitude of increased AA release was 134% over the control. Incubation of cells with CN + 2-DG for 5 min did not increase the release of AA or its metabolites, whereas at 15 min, there was an enhanced release of AA (18% over control) in response to CN + 2-DG (59 ± 2 and 70 ± 2 fmol/mg protein in control and CN + 2DG treated samples respectively). To investigate if lowering of the cytosolic calcium level would prevent AA release due to CN + 2-DG, AA release was assessed in the absence of extracellular Ca^{2+} (Table II: line 3). When Ca^{2+} was omitted from the incubation medium, there was a statistically significant decrease in basal release of AA or its metabolites. To make sure that no Ca^{2+} remained in the incubation system, Ca^{2+} chelators were added (Quin II and ammonium oxalate). Addition of these chelators to incubation medium without Ca^{2+} did not change the result of Ca^{2+} elimination: release of AA or its metabolites was two-thirds of that obtained with Ca^{2+} (Table 2: lines 3–7). Since the data in lines 3–7 are from experimental groups treated with CN and 2-DG for 30 min as well as with other additives,

Table II. Effect of Inhibited Bioenergetics on AA Release

Incubation Condition	AA release fmol/mg protein (mean ± SEM)	% control
Medium with Ca^{2+}	66.6 ± 1.8*	100
Medium with Ca^{2+}, with CN/2-DG	156.3 ± 4.6*	234
Medium without Ca^{2+}	46.3 ± 1.2	70
Medium without Ca^{2+}, with CN/2-DG	44.0 ± 2.1	66
Medium without Ca^{2+}, with Quin II	42.3 ± 0.9	64
Medium without Ca^{2+}, with Quin II, and CN/2-DG	42.6 ± 1.8	64
Medium without Ca^{2+}, Quin II, oxalate	41.3 ± 0.9	62
Medium without Ca^{2+}, Quin II, oxalate and CN/2-DG	43.0 ± 1.7	65

NG108-15 cells were exposed to ^{3}H-AA acid for 20 hr to isotopic equilibrium. The cells were then incubated with "incubation medium" with or without Ca^{2+} (0.9 mM $CaCl_2$) and with or without additives [1 mM CN, 20 mM 2-DG, 20 μM Quin II, or 20 mM ammonium oxalate] in a final volume of 0.5 ml at 37°C for 30 min. The reaction was stopped by removing the incubation medium which was then centrifuged at 12,000 *g* for 1 min to remove nonadherent cells. ^{3}H-AA released into 400 μl of the supernatant was measured by a liquid scintillation counter. Each value represents the mean of triplicate determinations performed in three independent experiments.
* Significantly different from all other groups.

these data also indicate that AA release is not a non-specific event putatively due to cell deterioration after 2-DG and CN treatment. When cells were incubated with either CN (1 mM) or 2-DG (20 mM), no increase in the release of AA was detected up to 30 min.

Effect of PLA_2 Inhibitors on ^{3}H-AA Release Due to CN + 2-DG. To examine whether the increase in ^{3}H-AA release following CN + 2-DG treatment was due to PLA_2 activation, cells were incubated with 1 mM CN + 20 mM 2-DG for 30 min at 37°C in the absence and presence of 20 μM 4-BPB (PLA_2 inhibitor). The observed increase in ^{3}H-AA release due to CN + 2-DG compared to untreated control was completely prevented by 4-BPB (^{3}H-AA release (fmol/mg protein): control, 68 ± 2; CN + 2-DG, 162 ± 4, 4-BPB and CN + 2-DG, 73 ± 3) indicating that the increased ^{3}H-AA release following CN + 2-DG treatment was due to PLA_2 activation. Similar results were obtained when other known PLA_2 inhibitors (20 μM quinacrine or 1μM manoalide) were used (data not shown).

Effect of Inhibited Bioenergetics on Membrane Fluidity. The state of membrane fluidity is expressed in terms of the mean rotational correlation time (τ_c, nsec) of a spin probe calculated from the parameters of the ESR spectrum as described in "Experimental Procedure". A typical spectrum of the spin probe 12-doxyl stearic acid incorporated into NG108-15 cell membrane is shown in Fig. 1. We determined whether there was a change in τ_c in NG108-15 cells in which bioenergetics was inhibited. τ_c of cells treated for 30 min at 37°C with 1.0 mM CN plus 20 mM 2-DG, which ablates ATP synthesis, increased 49% compared to controls (Table III). Treatment of cells with either CN (1.0 mM) or 2-DG (20 mM) alone under similar conditions, which does not decrease cellular ATP, did not alter membrane fluidity (Table III). If cells were treated with a lower (0.5 mM) CN concentration plus 20 mM 2-DG, the increase in τ_c was not reproducible. There was no cell death due to CN + 2-DG treatment as discussed above. However, to verify that the increase of the τ_c was not a non-specific event possible due to the inability of cells to exclude trypan blue during experimental procedures in determining τ_c, cells were deliberately killed by freezing and thawing (1% cell viability by trypan blue exclusion assay) as described under "Experimental Procedure" and then subjected to ESR spectroscopic measurements. Killed cells had a τ_c of 5.75 and 5.16 (two measurements); viable (88%) control cells in the same experiment had a τ_c of 5.70 and 4.88.

DISCUSSION

Neurones are extremely sensitive to inhibition of oxidative metabolism resulting in ATP depletion. The objective of this study was to elucidate the mechanism of neuronal damage following energy deprivation. Models such as brain tissue slices have the disadvantage of a

Table III. Effect of Inhibited Bioenergetics on Membrane Fluidity

Cell Treatment	Mean Rotational Correlation Time τ_c (nsec)
none (control)	3.55 ± 0.03
CN (1.0 mM)	3.39 ± 0.13
2-DG (20 mM)	3.57 ± 0.16
CN (1.0 mM) + 2-DG (20 mM)	5.30 ± 0.60*

Cell suspensions (approximately 5 mg protein) without additives (controls) or treated with experimental agents as indicated in the Table were added to a tube containing spin-label (12-doxyl stearic acid) and incubated at room temperature for 30 min. A 200 μl aliquot of spin-labeled cell suspension was placed in the ESR sample cell and the spectrum was recorded at ambient temperature. Calculations of the mean rotational correlation time of the spin label were carried out using the relationship: $\tau = 6.5 \times 10^{-10} H_0 [(h_o/h_{-1})^{1/2}-1]$, where H_0 is the linewidth of the medium field line, and h_o and h_{-1} are heights of the medium and high field lines respectively as shown in Fig. 1. Each value represents the mean of triplicate determinations performed in three independent experiments.
* Significantly different from all other groups ($p < 0.05$).

large number of cell types which makes it difficult to interpret biochemical changes. We, therefore, investigated the effect of decreased ATP synthesis on membrane composition and fluidity in a clonal cell line (neuroblastoma-glioma hybrid NG108-15 cells). Because this and other neuronal cell lines respond to decreased oxygen by synthesis of ATP via anaerobic mechanisms, we totally inhibited ATP synthesis by exposing the cells to a combination of CN (1 mM) and 2-DG (20 mM). Inhibition of ATP synthesis required only 1 min under these conditions. Intracellular free calcium level increased two-fold in cells treated with 1 mM CN + 20 mM 2-DG as early as 5 min after exposure of cells to these agents and remained elevated at this level until 30 min. Johnson et al. (21) have reported an three- and six-fold increase in $[Ca^{2+}]_i$ in PC12 cells treated with 10^{-3} and 10^{-2} M KCN respectively for 15 min. Neither ER Ca^{2+}-pump activity nor the release of AA or its metabolites was modified at 5 min following CN and 2-DG treatment, but both of these parameters were altered at 15 min following treatment (data not shown). However, a major parameter in which we were interested, namely, membrane fluidity consistently required 30 min to demonstrate modification following cyanide plus 2-DG treatment. This is presumably due to the fact that a change in membrane fluidity can only be demonstrated in the ESR assay after a critical mass of AA is released from the membrane. Therefore, we were most interested in alteration of ER Ca^{2+}-pump activity, membrane composition, and membrane fluidity 30 min after the cells were exposed to CN + 2-DG. Controls showed that AA release and membrane fluidity were not non-specifically modified under these conditions. Kurzinger et al. reported the effect of a reduced ATP level on ATP-dependent Ca^{2+}-pumps (Ca^{2+}-ATPases) and on intracellular Ca^{2+} homeostasis in NG108-15 cells (20). The consequence of a disturbed Ca^{2+} homeostasis as a result of ATP depletion in NG108-15 cells is described in this report. Since alteration in membrane fluidity may have a profound effect on membrane function (2–6, 22), these results explain the significance of a disturbance in Ca^{2+} homeostasis in NG108-15 cells.

Ablated ATP synthesis in NG108-15 cells resulted in 90% inhibition of the ER Ca^{2+}-pump activity that was irreversible in the sense that the pump could not be activated by addition of ATP to the reaction mixture. A 134% increased liberation of AA or its metabolites from cell membranes was associated with the inhibition of ATP synthesis. This increase in AA release could be prevented by treatment of cells with a PLA_2 inhibitor 4-BPB, suggesting the involvement of PLA_2 in AA release. We hypothesize that increased cytoplasmic Ca^{2+} resulting from the inhibited ER Ca^{2+}-pump can activate membrane-bound phospholipases such as PLA_2 which in turn release AA from membranes (8). The finding that incubation of cells with CN + 2-DG in Ca^{2+}-free or Ca^{2+}-complexed medium did not stimulate the release of ^3H-labeled products suggests the specificity and Ca^{2+}-dependence of AA release possibly due to PLA_2 activation. This dependence on extracellular Ca^{2+} also suggests that under these experimental conditions the $[Ca^{2+}]_i$ elevation following ATP depletion is due to Ca^{2+} influx. In the absence of extracellular Ca^{2+}, the release of Ca^{2+} from intracellular stores, if any, is insufficient to stimulate PLA_2 and AA release. Although the sequence of ATP depletion $\rightarrow$ Ca^{2+}-pump inhibition $\rightarrow$ $[Ca^{2+}]_i$ increase $\rightarrow$ PLA_2 stimulation $\rightarrow$ AA release $\rightarrow$ membrane fluidity decrease is logical, it is important to document that this sequence in fact pertains to a particular cell type. In another report, oxygen deprivation resulted in liberation of radiolabeled AA from cultured myoblasts but not from cultured fibroblast-like cells (23).

The most important observation in the present study is the decrease in membrane fluidity following energy deprivation possibly as a result of AA release from cellular membranes. The release of AA, an unsaturated fatty acid, from the membrane would be expected to decrease membrane fluidity (24). We studied the change in membrane fluidity by the electron spin resonance (ESR) technique using the spin-labeled fatty acid probe 12-doxyl stearic acid. Such probes incorporate themselves into biological membranes so that the carboxyl group lies at the membrane surface while the hydrophobic tail extends into the lipid region (25,26). The parameters of the ESR spectrum of these probes reflect the state of fluidity of the surrounding membrane.

For 12-doxyl stearic acid, the doxyl group places itself about one-third of the way inward across the membrane. Changes in τ_c values in these experiments therefore implies alteration in membrane fluidity at this membrane region. Inhibition of ATP synthesis led to a 49% increase in τ_c, i.e., to a marked decrease in membrane fluidity as hypothesized by the loss of arachidonic acid. This change in τ_c is statistically significant ($p < 0.05$) compared to the control(s) as shown in Table III. However, the physiological significance of this change can only be a subject of speculation due to the lack of information in this field at present. Altered membrane composition (loss of AA) and fluidity may affect the normal distribution of the membrane spanning peptide segments of functional macromolecules such as transport ATPases, and as such their tertiary structure and function. Published reports have demonstrated alteration in the activity of several membrane bound enzymes (2–4),

ion transport molecules including voltage-sensitive sodium channels (5,6), and diffusion controlled membrane processes (22) as a result of membrane fluidity change.

In conclusion, our results suggest that one mechanism by which energy deprivation may lead to neuronal injury is likely to involve irreversible damage to the ER Ca^{2+}-pump, the attendant release of AA from membranes, and a decrease in membrane fluidity. The alteration in membrane fluidity would modify the panoply of cellular functions that require cellular membranes to be in their normal configuration.

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