Cytochrome *c* release from brain mitochondria is independent of the mitochondrial permeability transition

Alexander Y. Andreyev, Brenda Fahy, Gary Fiskum*

Department of Anesthesiology, University of Maryland, School of Medicine, MSTF Building, Room 534, 685 W. Baltimore Street, Baltimore, MD 21201, USA

Received 12 October 1998

Abstract Ca^{2+} uptake by brain mitochondria induces the release of up to 40% of total cytochrome *c* in a cyclosporin A-insensitive manner. In the presence of ATP and Mg^{2+}, this process is not accompanied by mitochondrial swelling. There is a moderate decrease in membrane potential under these conditions, but it is completely reversible upon removal of accumulated Ca^{2+} by addition of EGTA+A23187 but not by EGTA alone. These observations provide evidence that cytochrome *c* release from brain mitochondria does not require the membrane permeability transition. However, brain mitochondria can undergo the permeability transition in the absence of ATP and Mg^{2+}, which results in cyclosporin A-sensitive large amplitude swelling, loss of Ca^{2+} uptake capacity and release of matrix solutes.

Key words: Cytochrome *c*; Permeability transition; Apoptosis; Brain mitochondrion; Calcium

1. Introduction

In various apoptotic cell death paradigms, release of mitochondrial cytochrome *c* into the cytosol is a key step linking the triggering phase of apoptosis to the executioning phase [1–3]. This process is widely regarded as mediated by the onset of the mitochondrial permeability transition [4,5], a complex phenomenon in which mitochondria uniformly respond to different challenges by opening a Ca^{2+}-dependent, cyclosporin A-sensitive inner membrane pore non-selectively permeable to solutes of MW up to 1500 [6]. In experiments with isolated mitochondria, pore opening leads to mitochondrial membrane depolarization, equilibration of ions and other low MW solutes across membranes, and large amplitude swelling due to the osmotic pressure of large MW mitochondrial matrix polymers.

The pathway by which cytochrome *c* leaves its normal location in the intermembrane space proposed independently by Skulachev [7] and Kroemer and co-authors [8] is based upon the above mechanism. It is known that large amplitude swelling in saline media causes reversible respiratory inhibition due to cytochrome *c* loss as a result of outer membrane rupture [9]. Several different laboratories have demonstrated that the permeability transition induced by various triggers leads to swelling and cytochrome *c* release from liver mitochondria [10–13].

An alternative mechanism was proposed to explain cell death experiments in which cytochrome *c* release occurs before or independently of mitochondrial depolarization [3,14,15]. This mechanism involves release of cytochrome *c* through an outer membrane pore or channel that does not require the mitochondrial permeability transition or mitochondrial swelling.

Unlike mitochondria isolated from many other tissues, e.g. liver, brain can accumulate very little Ca^{2+} in the absence of adenine nucleotides [16,17]. On the other hand, brain mitochondria are less prone to undergo the permeability transition-mediated swelling than liver mitochondria [18,19]. Considering the increasing evidence that brain mitochondrial Ca^{2+} uptake is involved in both buffering normal elevations in intraneuronal Ca^{2+} [20,21] and mediating both necrotic and apoptotic death caused by excitotoxic neurotransmitters [22–25], the objective of this study was to determine the relationship between Ca^{2+} uptake and cytochrome *c* release in brain mitochondria and to determine what role, if any, the permeability transition plays in this process.

2. Materials and methods

Rat brain mitochondria were isolated according to the procedure of Rosenthal et al. [26] yielding non-synaptosomal plus synaptosomal mitochondria. Mitochondrial protein was determined by a modified biuret reaction [27]. Mitochondria were incubated at 37°C in media containing 125 mM KCl, 2 mM K_2HPO_4, 5 mM HEPES-KOH (pH 7.0) and supplemented with respiratory substrates, 3 mM ATP and 4 mM $MgCl_2$ as indicated. Ca^{2+} fluxes were monitored fluorometrically with calcium green 5N (0.1 μM). Light scattering of the mitochondrial suspension was measured at a right angle with a Perkin-Elmer LS-3 fluorescence spectrometer at 520 nm or alternatively as absorbance at 660 nm. Mitochondrial membrane potential was monitored using a TPP^+-selective electrode and 2 μM TPP-Cl.

For measurements of mitochondrial cytochrome *c*, glutathione or Quin-2 release, an aliquot of the experimental suspension was centrifuged at $13\,000 \times g$ for 2 min. An 0.75 ml aliquot of supernatant was carefully removed and used for the assays or supplemented with 15 μl of Protease Inhibitor Cocktail (Sigma), frozen on dry ice and stored at −70°C. The pellet was resuspended in 1 ml of the medium and stored likewise. For cytochrome *c* immunoblots, 16 μl aliquots of the samples (diluted to 0.12 mg mitochondrial protein/ml for the pellets) were run on to 4–20% Tris-glycine gradient gels. Proteins were electrotransferred to PVDF membranes, membranes were rinsed with TBS-T buffer and blocked overnight in TBS-T supplemented with 10% dry milk. Cytochrome *c* was immunostained with primary 7H8 mouse anti-cytochrome *c* antibody (PharMingen) plus secondary anti-mouse Ig bound to horseradish peroxidase (Amersham) (1:2000 dilution each). Peroxidase activity was detected using the Enhanced Chemiluminescence detection kit (Amersham) and X-ray film. Band intensities were analyzed densitometrically using GelExpert system (NucleoTech). Glutathione in the supernatant samples was measured in enzymatic recycling assay [28]. For measurements of permeability to Quin-

*Correspondence author. Fax: (1) (410) 706-2550.
E-mail: gfiskum@anesthlab.ab.umd.edu

Abbreviations: EGTA, ethyleneglycol bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCCP, *p*-trifluoromethoxyphenylhydrazone carbonyl cyanide; DNP, 2,4-dinitrophenol; TPP, tetraphenylphosphonium

PII: S0014-5793(98)01394-5

2, mitochondria were preloaded by incubating with 100 μM Quin-2AM for 1 h at 37°C in the dark. Following the experimental incubation, Quin-2 was measured in the supernatant sample fluorometrically.

3. Results and discussion

3.1. Rat brain mitochondria do not undergo Ca^{2+}-induced permeability transition in the presence of ATP and Mg^{2+}

Rat brain mitochondria incubated in physiologically relevant KCl-based medium in the presence of ATP and Mg^{2+} rapidly accumulate added Ca^{2+} up to approximately 4 μmol/mg of protein (data not shown). Ca^{2+} uptake is associated with the normal transient drop in mitochondrial membrane potential (Fig. 1A) which, however, is not followed by spontaneous complete depolarization typical of the permeability transition. Moreover, instead of this process being accompanied by the classical large amplitude swelling resulting in decrease in light scattering, Ca^{2+} uptake leads to a light scattering increase (Fig. 1B). Neither the membrane potential nor the light scattering changes are sensitive to cyclosporin A (data not shown).

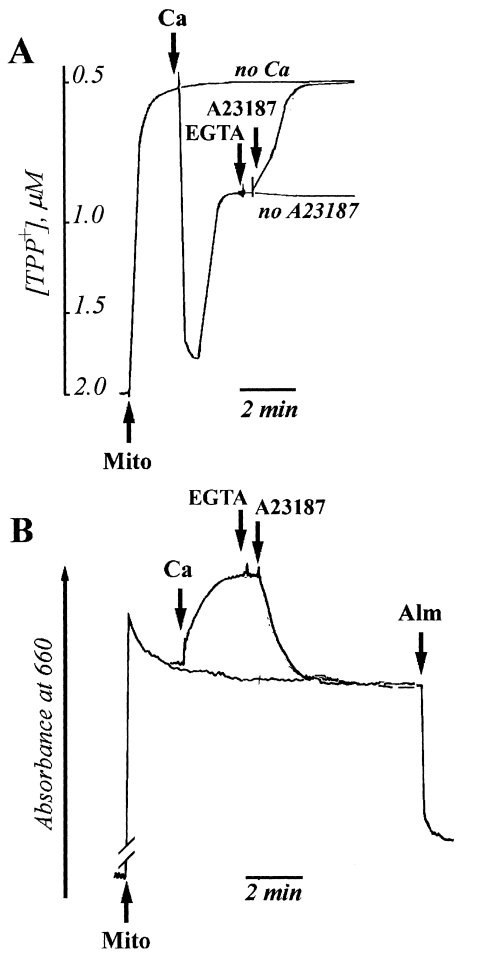

Fig. 1. Brain mitochondria do not undergo the permeability transition in the presence of ATP and Mg^{2+}. Membrane potential (A) and absorbance at 660 nm (B) are shown. See Section 2 for conditions; 5 mM succinate in the presence of 2 μM rotenone was used as respiratory substrate. Additions: Mito: 0.5 mg mitochondrial protein/ml; Ca: 2.1 μmol $CaCl_2$/mg protein; EGTA: 2 mM; A23187: 0.4 μg/ml; Alm: 40 μg alamethicin/mg protein.

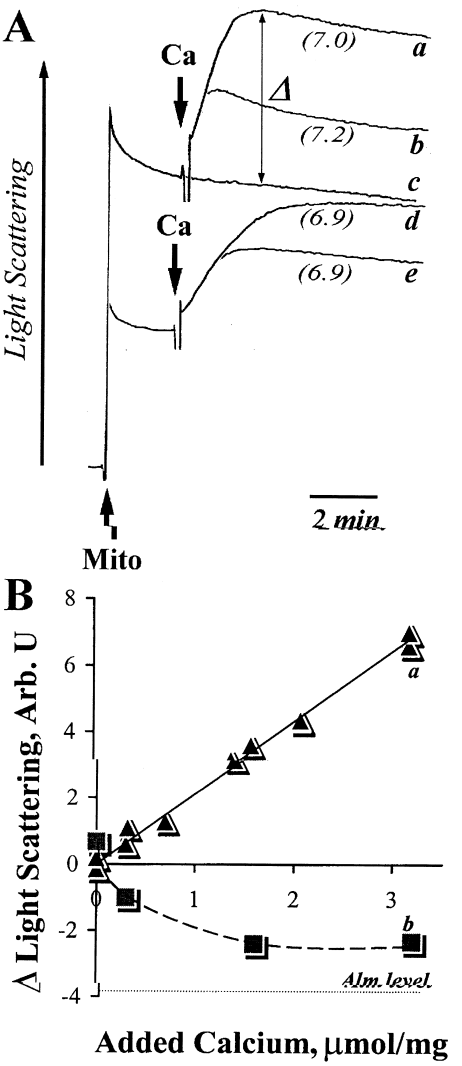

Fig. 2. Light scattering changes in suspensions of brain mitochondria in the presence or absence of ATP and Mg^{2+}. See Section 2 for conditions; 5 mM glutamate plus 5 mM malate were used as respiratory substrates. A: Ca^{2+}-induced light scattering increase in the presence of ATP and Mg^{2+} at different concentrations of mitochondria. Additions: Mito: 0.25 mg mitochondrial protein/ml (a–c) or 0.12 mg/ml (d, e); Ca: 0 (c), 260 (b, e), 400 (d) or 525 (a) μM $CaCl_2$. Numbers in parentheses show ratios of Δ light scattering/$[Ca^{2+}]$. B: Ca^{2+} dose dependence of Δ light scattering changes (Δ) in the presence (a) or absence (b) of ATP and Mg^{2+}. Dotted line shows the level of light scattering changes upon permeabilization with alamethicin.

The decrease in membrane potential observed upon the completion of Ca^{2+} uptake is completely reversible when Ca^{2+} is sequestered with the Ca^{2+} chelator EGTA plus the Ca^{2+} ionophore A23187 (Fig. 1A). This effect is not observed upon addition of EGTA alone, indicating that the mitochondria remain impermeable to Ca^{2+} and EGTA. We hypothesize that Ca^{2+}-induced decrease in membrane potential is due to the moderate and reversible respiratory inhibition observed under these conditions (data not shown).

Further experiments indicated that the Ca^{2+}-induced increase in brain mitochondrial light scattering results from

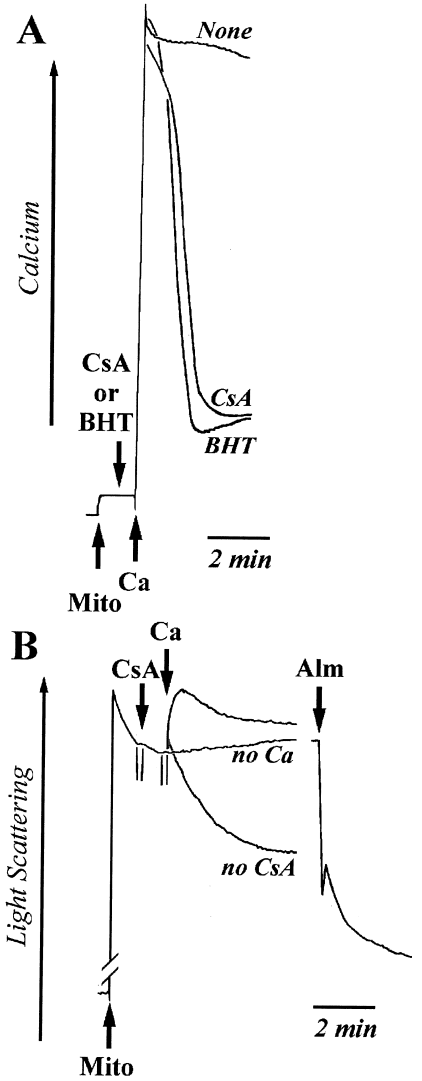

Fig. 3. Brain mitochondria are capable of the permeability transition in the absence of ATP and Mg^{2+}. Calcium uptake (A) and light scattering (B) are shown. See Section 2 for conditions; 5 mM succinate in the presence of 2 μM rotenone was used as respiratory substrate. Additions: Mito: 0.25 mg mitochondrial protein/ml; Ca: 1.6 μmol $CaCl_2$/mg protein (A) or 0.8 μmol/mg (B); CsA: 0.5 μM cyclosporin A; BHT: 50 μM butylated hydroxytoluene; Alm: 40 μg alamethicin/mg protein. Mitochondrial samples collected at the end of the experimental period (A) were used for immunoblots shown in Fig. 4D.

Ca^{2+} precipitation in the matrix rather than mitochondrial shrinkage. The increase in light scattering that can be observed in highly hypertonic medium (550 mosM) is much smaller than what is observed following Ca^{2+} accumulation (data not shown). Ca^{2+} accumulation also leads to an increase in light scattering proportional to the amount of Ca^{2+} added over a wide concentration range (Fig. 2B, curve a) that is approximately the same at both 0.25 and 0.12 mg mitochondrial protein/ml (Fig. 2A). As the change in light scattering is dependent on the total Ca^{2+} accumulated rather than on the amount taken up per mg mitochondrial protein, the increase in scattering seen in these experiments is likely due to the formation of calcium phosphate-based precipitates, as has been previously described [29,30].

The increase in light scattering observed in the presence of ATP and Mg^{2+} could, but does not obscure a significant decrease in light scattering due to swelling. As shown in Fig. 1B, the light scattering changes are completely reversible by releasing accumulated Ca^{2+} with EGTA and A23187. The same complete reversibility is observed by eliminating the membrane potential with the addition of a protonophoric uncoupler (0.5 μM FCCP) which normally causes a net decrease in light scattering in liver mitochondria by promoting the permeability transition (data not shown) [31,32].

3.2. *Brain mitochondria are capable of Ca^{2+}-induced permeability transition in the absence of ATP and Mg^{2+}*

The ability of brain mitochondria to undergo the permeability transition under any circumstances is questionable [17–19]. We therefore performed experiments in the absence of ATP and Mg^{2+}, which are known to inhibit the transition in liver mitochondria [33,34]. In the absence of ATP and Mg^{2+} only a small fraction of an addition of 1.6 μmol Ca^{2+}/mg is accumulated by isolated brain mitochondria (Fig. 3A). However, in the presence of inhibitors of the permeability transition (i.e. cyclosporin A or butylated hydroxytoluene (BHT)) (Fig. 3A), all of the added Ca^{2+} was accumulated. A similar protective effect on Ca^{2+} accumulation is observed in the presence of 15–30 μM ADP in the presence of oligomycin (data not shown), which is also known to inhibit the permeability transition [33].

The severe reduction in Ca^{2+} uptake capacity observed with brain mitochondria in the absence of ATP and Mg^{2+} is accompanied by large amplitude swelling (i.e. a decrease in light scattering) upon addition of Ca^{2+} (Fig. 3B). This effect is also sensitive to cyclosporin A (Fig. 3B). The extent of swelling is

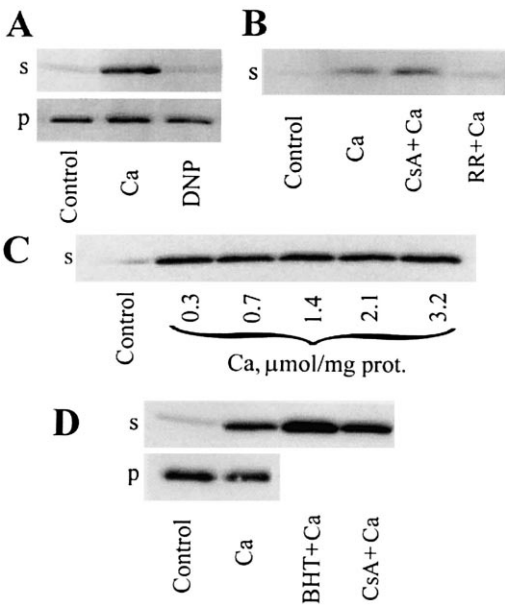

Fig. 4. Cytochrome *c* release from brain mitochondria in the presence (A–C) or absence (D) of ATP and Mg^{2+}. Representative results are presented for supernatants (s) and pellets (p). Each panel represents results from an individual immunoblot. See Section 2 for conditions of incubations; 5 mM glutamate plus 5 mM malate (A–C) or 5 mM succinate in the presence of 2 μM rotenone (D) were used as respiratory substrates. Control: incubation without additions; Ca: 3.2 μmol $CaCl_2$/mg protein (A, B) or 1.6 μmol/mg (D); DNP: 90 μM dinitrophenol; CsA: 0.5 μM cyclosporin A; RR: 0.2 μM ruthenium red; BHT: 50 μM butylated hydroxytoluene.

substantially lower than the maximal swelling obtained upon permeabilization of the mitochondria with the artificial pore-forming compound alamethicin (Fig. 3B; Fig. 2B, curve b). These results suggest that only a fraction of the mitochondrial population undergoes permeabilization under these conditions. This observation may relate to the fact that the heterogeneous mitochondrial preparation used in these studies contains both synaptosomal and non-synaptosomal mitochondria.

Additional evidence that brain mitochondria can undergo a permeability transition was obtained by measuring Ca^{2+}-induced release of matrix solutes into the incubation medium. Ca^{2+}-induced release of matrix glutathione reaches 1.0 nmol/mg protein compared to 1.3 nmol/mg protein elicited by alamethicin. The Ca^{2+}-induced release is inhibited by cyclosporin A (to the level of 0.1 nmol/mg protein). Ca^{2+} also induces the release of up to 70% of Quin-2 from preloaded mitochondria. Therefore, in the absence of ATP and Mg^{2+}, brain mitochondria can undergo the permeability transition, but the extent of the transition and particularly the extent of swelling is reduced compared to what has been reported for liver mitochondria. Most importantly, in the presence of ATP and Mg^{2+}, brain mitochondria do not undergo the transition even at extremely high levels of Ca^{2+}.

3.3. *Cytochrome c release from brain mitochondria occurs independently of the permeability transition*

When brain mitochondria are incubated for 10 min in the presence or absence of ATP and Mg^{2+} but in the absence of added Ca^{2+} and then centrifuged, cytochrome *c* is associated mainly with the mitochondrial pellet. Background cytochrome *c* release into the medium supernatant is only 3–5% of the total (Fig. 4A,D). Addition of Ca^{2+} causes release of cytochrome *c* both in the presence and in the absence of ATP and Mg^{2+} (Fig. 4A,B,D). In the presence of ATP and Mg^{2+}, this release reaches a maximum of approximately 40% of the total at 0.3–0.7 μmol/mg protein and does not increase at higher levels of Ca^{2+} (Fig. 4C), despite the fact that the maximal Ca^{2+} uptake capacity exceeds 4 μmol/mg.

The release of cytochrome *c* is blocked by ruthenium red, an inhibitor of the Ca^{2+} uptake uniporter (Fig. 4B). The mechanism of Ca^{2+} uptake-stimulated cytochrome *c* release is not simply the consequence of the membrane depolarization that accompanies Ca^{2+} uptake since depolarization of the mitochondrial membrane with the uncoupler DNP does not evoke release (Fig. 4A).

Cyclosporin A does not inhibit Ca^{2+}-stimulated release of cytochrome *c* from brain mitochondria in the presence of ATP and Mg^{2+} (Fig. 4B). Moreover, neither cyclosporin A nor BHT inhibits cytochrome *c* release in the absence of ATP and Mg^{2+} (Fig. 4D), even though they do inhibit the membrane permeability transition under these conditions (Fig. 3). The findings that cyclosporin A had no effect on release under conditions where it does inhibit the permeability transition, and that the amount of cytochrome *c* release under these conditions is not greater than when the permeability is suppressed by ATP and Mg^{2+} are surprising. These findings could be interpreted as due to the presence of a fraction of brain mitochondria that do not undergo the permeability transition. Alternatively, unlike liver mitochondria, Ca^{2+}-evoked swelling of brain mitochondria may be insufficient to disrupt the mitochondrial outer membrane. As membrane disruption is not necessarily responsible for Ca^{2+}-induced cytochrome *c* release by brain mitochondria, studies are in progress to assess the possible roles of outer membrane pores in this process.

Acknowledgements: The authors are grateful to Drs. V.P. Skulachev, A.A. Starkov and Y.E. Kushnareva for helpful discussions and to Mr. S. Russell for expert technical assistance. This work was supported by NIH Grant NS34152.

References

[1] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) Science 275, 1132–1136.
[2] Zhivotovsky, B. and Orrenius, S. (1998) Nature 391, 449–450.
[3] Green, D.R. and Reed, J.C. (1998) Science 281, 1309–1312.
[4] Lehninger, A.L. (1962) Physiol. Rev. 42, 467–517.
[5] Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, C755–C786.
[6] Hunter, D.R. and Haworth, R.A. (1979) Arch. Biochem. Biophys. 195, 460–467.
[7] Skulachev, V.P. (1997) Biosci. Rep. 17, 347–357.
[8] Kroemer, G., Zamzami, N. and Susin, S.A. (1997) Immunol. Today 18, 44–51.
[9] Jacobs, E.E. and Sanadi, D.R. (1960) Biochim. Biophys. Acta 38, 12–33.
[10] Kantrow, S.P. and Piantadosi, C.A. (1997) Biochem. Biophys. Res. Commun. 232, 669–671.
[11] Scarlett, J.L. and Murphy, M.P. (1997) FEBS Lett. 418, 282–286.
[12] Yang, J.C. and Cortopassi, G.A. (1998) Free Radical Biol. Med. 24, 624–631.
[13] Petit, P.X., Goubern, M., Diolez, P., Susin, S.A., Zamzami, N. and Kroemer, G. (1998) FEBS Lett. 426, 111–116.
[14] Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T. and Thompson, C.B. (1997) Cell 91, 627–637.
[15] Bossy-Wetzel, E., Newmeyer, D.D. and Green, D.R. (1998) EMBO J. 17, 37–49.
[16] Tjioe, S., Bianchi, C.P. and Haugaard, N. (1970) Biochim. Biophys. Acta 216, 270–273.
[17] Rottenberg, H. and Marbach, M. (1990) Biochim. Biophys. Acta 1016, 87–98.
[18] Kristal, B.S. and Dubinsky, J.M. (1997) J. Neurochem. 69, 524–538.
[19] Berman, S.B., Watkins, S.W. and Hastings, T.G. (1998) Soc. Neurosci. Abstr. 24, 553.
[20] Nicholls, D.G. (1985) Prog. Brain Res. 63, 97–106.
[21] Budd, S.L. and Nicholls, D.G. (1996) J. Neurosci. 66, 403–411.
[22] Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A. and Nicotera, P. (1995) Neuron 15, 961–973.
[23] Dugan, L.L., Sensi, S.L., Canzoniero, L.M.T., Handran, S.D., Rothman, S.M., Lin, T.-S., Goldberg, M.P. and Choi, D.W. (1995) J. Neurosci. 15, 6377–6388.
[24] Isaev, N.K., Zorov, D.B., Stelmashook, E.V., Uzbekov, R.E., Kozhemyakin, M.B. and Victorov, I.V. (1996) FEBS Lett. 392, 143–147.
[25] White, R.J. and Reynolds, I.J. (1997) J. Physiol. 498, 31–47.
[26] Rosenthal, R.E., Hamud, F., Fiskum, G., Vardhese, P.J. and Sharpe, S. (1987) J. Cereb. Blood Flow Metab. 7, 752–758.
[27] Skarkowska, L. and Klingenberg, M. (1963) Biochem. Z. 338, 674–697.
[28] Griffith, O.W. (1980) Anal. Biochem. 106, 207–212.
[29] Greenawalt, J.W., Rossi, C.S. and Lehninger, A.L. (1964) J. Cell Biol. 23, 21–38.
[30] Weinbach, E.C. and von Brand, T. (1965) Biochem. Biophys. Res. Commun. 19, 133–137.
[31] Igbavboa, U. and Pfeiffer, D.R. (1988) J. Biol. Chem. 263, 1405–1412.
[32] Novgorodov, S.A., Gudz, T.I., Kushnareva, Y.E., Eriksson, O. and Leikin, Y.N. (1991) FEBS Lett. 295, 77–80.
[33] Hunter, D.R. and Haworth, R.A. (1979) Arch. Biochem. Biophys. 195, 453–459.
[34] Hunter, D.R., Haworth, R.A. and Southard, J.H. (1976) J. Biol. Chem. 251, 5069–5077.