Brain Research Protocols 3 (1998) 76–82

BRAIN RESEARCH PROTOCOLS

Protocol

Cryopreservation of rat cortical synaptosomes and analysis of glucose and glutamate transporter activities, and mitochondrial function

James G. Begley [a], D. Allan Butterfield [b,c], Jeffrey N. Keller [a,d], Tanuja Koppal [b], Jennifer Drake [b], Mark P. Mattson [a,c,e,*]

[a] *Sanders-Brown Research Center on Aging, University of Kentucky, Lexington, KY 40536, USA*
[b] *Department of Chemistry, University of Kentucky, Lexington, KY 40536, USA*
[c] *Center of Membrane Sciences, University of Kentucky, Lexington, KY 40536, USA*
[d] *Department of Biological Sciences, University of Kentucky, Lexington, KY 40536, USA*
[e] *Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40536, USA*

Accepted 29 April 1998

Abstract

Direct comparisons of synaptic functional parameters in brain tissues from different groups of experimental animals and different samples from post mortem human brain are often hindered by the inability to perform assays at the same time. To circumvent these difficulties we developed methods for cryopreservation and long-term storage of neocortical synaptosomes. The synaptosomes are suspended in a cryopreservation medium containingwhereas mitochondrial function and cellular esterase activity were largely maintained. Electr conjunction with a protein-specific spin label indicated that cryopreservation did not alter the physical state of synaptosomal membrane proteins. These methods provide the opportunity to generate stocks of functional synaptosomes from different experiments or post mortem samples collected over large time intervals.

Themes: Excitable membranes and synaptic transmission

Topics: Presynaptic mechanisms

Keywords: Cerebral cortex; Electron paramagnetic resonance spectroscopy; Esterase; Excitatory amino acid; Mitochondrial respiration

1. Type of research

- Establishment of ‘synaptosome banks’ for studies of synaptic physiology and pathophysiology.
- Simultaneous analyses of synaptic functional parameters in post mortem brain tissues from adult rats and human patients collected at different times.
- Quantification of glucose and glutamate transport in synaptosomes.
- Quantification of mitochondrial function in synaptosomes.

2. Time required

- Synaptosome preparation. The time period from removal of brain tissue until pure synaptosomes are obtained is 5–6 h.
-

* Corresponding author. 211 Sanders-Brown Building, University of Kentucky, Lexington, KY 40536-0230, USA. Fax: +1-606-323-2866; E-mail: mmattson@aging.coa.uky.edu

magnetic resonance spectroscopy (EPRS) analysis of protein conformation using the MAL-6 spin label requires 3–4 h.
- Assay of membrane integrity. The protocol for assessing the ability of synaptosomes to accumulate the fluorescent probe calcein-AM takes approximately 2 h.

3. Materials

3.1. Animals

Adult male Sprague–Dawley rats (250–300 g) were purchased from Harlan (Indianapolis, IN).

3.2. Chemicals, reagents and kits

- Homogenization buffer. 0.32 M sucrose, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 4 μg/ml leupeptin, 0.2 mM PMSF, 2 mM EDTA, 2 mM EGTA, and 20 mM HEPES (all from Sigma, St. Louis, MO).
- Locke's buffer. NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.3 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 3.6 mM; glucose, 5 mM; HEPES, 5 mM (reagents from Sigma).
- Cryovials (1.0 ml capacity) were purchased from Fisher Scientific (Pittsburgh, PA).
- [^{3}H]-2-deoxy-glucose and [^{3}H]glutamate were purchased from Amersham (Chicago, IL).
- Whatman GF/C glass microfibre filters with 1.2-μm retention (Whatman #1822-024) were purchased from Fisher Scientific.
- Scintillation vials and Scintiverse solution were purchased from Fisher Scientific.
- Calcein-AM was purchased from Molecular Probes (Eugene, OR).
- 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) was purchased from Sigma.
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma.
- 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was purchased from Molecular Probes.
- Other reagents. Sucrose, dimethylsulfoxide (cell culture grade), and all other chemicals were purchased from Sigma.

3.3. Special equipment

- A Millipore Cytofluor 2350 fluorescence plate reader was employed to quantify calcein-AM fluorescence in synaptosomes.
- A Bruker 300 EPR spectrometer (Bruker, Billerica, MA) was used to acquire EPR spectra.
- A CERES 900 visible light plate reader was used to quantify levels of MTT reduction.
- A Millipore Cytofluor 2350 fluorescence plate reader was used to quantify JC-1 fluorescence.

4. Detailed procedure

4.1. Synaptosome preparation and cryopreservation

Adult male Sprague–Dawley rats (250–300 g) were anesthetized with halothane and decapitated. The brain was removed and the cerebral cortex cut into small fragments, and homogenized in a solution (cortex from 2 brains/10 ml buffer) containing 0.32 M sucrose, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 4 μg/ml leupeptin, 0.2 mM PMSF, 2 mM EDTA, 2 mM EGTA, and 20 mM HEPES (pH 7.4). The homogenate was centrifuged for 10 min at $300 \times g$ at 4°C in a Sorvall RT6000B centrifuge with a swinging bucket rotor. The supernatant was then centrifuged for 10 min at $12{,}400 \times g$ at 4°C in a Beckman Model J2-21 centrifuge using a JA-20 fixed angle rotor; the pellet was resuspended in homogenization buffer and re-centrifuged at $20{,}250 \times g$. The pellet was collected and resuspended in 1.5 ml of 0.32 M sucrose and placed atop a sucrose gradient (7 ml 1.18 M sucrose, pH 8.5; 7 ml 1 M sucrose, pH 8.0; 7 ml 0.85 M sucrose, pH 8)

4 8 Cpt Beckman=g for

tion lacking glucose, and the assay was started by the addition of either [^{3}H]-2-deoxy-glucose (1.5 μCi) or [^{3}H]glutamate (0.1 μCi/ml; specific activity 0.2 μCi/ml). Seven minutes later the assay was stopped by placing the synaptosomes on Whatman filters in a vacuum filtration apparatus and rapidly washing three times with Locke's solution (3 ml/wash). The filters were then placed in scintillation vials containing Scintiverse and radioactivity determined by scintillation spectroscopy.

4.3. Assays for membrane integrity / esterase activity and protein conformation

In order to evaluate the integrity of synaptic membranes and, at the same time, determine whether the synaptosomes retain functional cytoplasmic enzymes, we employed the acetoxymethyl ester form of the fluorescent dye calcein (calcein-AM; Molecular Probes, Eugene, OR). Synaptosomes were incubated for 30 min in the presence of 5 μM calcein-AM. The synaptosomes were then washed twice with Locke's buffer and fluorescence was quantified

5(e(plos3)8171(the).86[(protei-(specific)8009spein)8323labeld)]TJ -669(Mol-21.121tified)]TJ1.196 -1.1-317(Lo8(-21.121t10o7(65[(2e,)9(2e,)9(6e,)

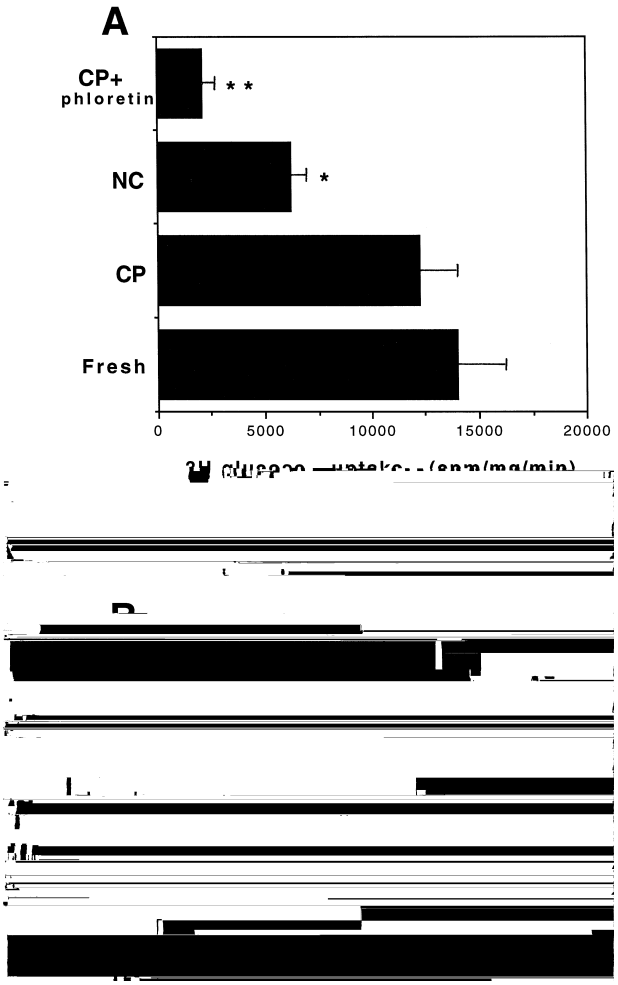

Fig. 1. Glucose and glutamate transport activities are retained in cryopreserved synaptosomes. [^{3}H]glucose uptake (A) and [^{3}H]glutamate uptake (B) were quantified in freshly prepared (unfrozen) synaptosomes, cyropreserved synaptosomes, and non-cryopreserved (frozen) synaptosomes (see Section 4). Phloretin (50 μM; a specific inhibitor of high-affinity glucose transport) or unlabeled glutamate (100 μM) were included in some samples of cryopreserved synaptosomes. Values are the mean and S.E.M. of determinations made in 4–8 synaptosome preparations. $*\ p < 0.01$, $**\ p < 0.001$ compared to values for fresh and cryopreserved synaptosomes (ANOVA with Scheffe's post-hoc tests).

The resulting composite EPR spectrum reflects both environments (Fig. 2A). The ratio of the EPR spectral amplitudes of the weakly-immobilized line (W) to that of the strongly-immobilized line (S) in the low-field region of the EPR spectrum of MAL-6-labeled synaptosomal membranes, the W/S ratio, is highly sensitive to protein conformational changes and protein–protein interactions [1,4,13]. The W/S ratio of MAL-6 was not significantly different in CP synaptosomes compared to fresh control synaptosomes (Fig. 2B) indicating no change in the physical state of membrane proteins. In contrast, NC synaptosomes exhibited a significantly decreased W/S ratio ($p < 0.005$), suggesting an altered conformation of membrane proteins (Fig. 2B).

5.3. Membrane integrity and esterase activity in cryopreserved synaptosomes

We next incubated fresh, CP and NC synaptosomes in the presence of the acetyoxymethyl ester form of the fluorescent dye calcein (calcein-AM). This lipophilic dye passes into cells 3(dye)-413(calcein)-74152l53(a98 1 T-41ome))-381T

sucrose/1.18 M sucrose interface are present, then it is likely that the sucrose solutions were improperly prepared. Finally, there are alternative methods for preparing synaptosomes which are less time-consuming including the use of a Percoll gradient; the effectiveness of such methods in preserving synaptosomal functions following cryopreservation remains to be determined.

7. Discussion

Synaptosomes, which are prepared by centrifugation of nervous tissue homogenates through a density gradient, consist of pre- and post-synaptic elements of neurons, and associated astrocytic end feet [5,11,19]. Studies of synaptosomal preparations have provided insight into regulation of a variety of synaptic functions including neurotransmitter release and reuptake [21,27], energy metabolism [6] and ion transport systems [7]. In addition, mechanisms of synaptic dysfunction and degeneration that may occur in neurological and neurodegenerative disorders such as Parkinson's disease [18] schizophrenia [24] and Alzheimer's disease [14,15,25] may be studied in synaptosomal preparations. Unfortunately, analyses of many functional parameters in synaptosomes require that the synaptosomes be freshly prepared because, as with intact cells, a freeze–thaw cycle will disrupt membranes and damage the synaptosomes and render them dysfunctional. In many circumstances (e.g., studies of synaptosomes from patients with neurodegenerative disorders) the requirement for fresh synaptosomes precludes direct comparisons of synaptosomes among patients.

Adapting procedures previously employed for cryopreservation of primary rat hippocampal neurons [16], we have developed a protocol for cryopreservation of synaptosomes prepared from neocortex of adult rats. The present findings demonstrate that plasma membrane glutamate and glucose transport systems, and mitochondrial transmembrane potential and electron transport activities, are maintained in CP synaptosomes at levels essentially identical to freshly prepared synaptosomes. Additionally, magnetic resonance studies showed no alteration in the physical state of synaptosomal membrane proteins in cryopreserved samples. The slight reduction in the ability of CP synaptosomes to accumulate calcein-AM may be due to reduced esterase activity, because the data from glucose and glutamate transport studies make it unlikely that the membranes were disrupted in the CP synaptosomes. The altered membrane protein conformation detected by EPR analysis of MAL-6 in NC synaptosomes, and the reduced ability of NC synaptosomes to retain calcein-AM strongly suggest that freeze–thaw causes severe damage to the plasma membranes. Increased protein–protein interactions, decreased segmental motion, and/or conformational changes in spin-labeled proteins leads to decreased molecular motion, resulting in a lowering of the W/S ratio. Such changes in membrane protein conformation can be caused by a variety of perturbations including exposure of cells to oxidative stress [3,4,10,13,25]. The membrane damage was correlated with severe compromise of glucose and glutamate transport systems in NC synaptosomes. These results suggest that failed membrane transport systems were probably not secondary to energy failure.

Previous studies have shown that neurotransmitter release and uptake can be quantified in cyropreserved synaptosomes prepared from post mortem human and rodent brains [8,9,12,24]. We have found that the same methods for cryopreservation of rat cortical synaptosomes described in the present study can be successfully applied to synaptosomes prepared from post mortem human brain tissues (M.P.M. and J.N.K., unpublished data). The ability to cryopreserve synaptosomes therefore provides the opportunity to establish 'banks' of synaptosomes from patients with different neurological disorders (and controls), and then to simultaneously perform various functional assays on synaptosomes from different cases. Synaptosomal cryopreservation can also be employed as a time-saving approach that allows the maintenanc1430gTJ /F1 1 Tf 17.95(j /F7