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Protein Half-Lives of Calmodulin and the Plasma Membrane Ca-ATPase in Rat Brain

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We report the half-lives for two proteins involved in the regulation of intracellular calcium in the brain: the plasma membrane Ca-ATPase and its regulatory protein, calmodulin. [^{14}C]-labeled leucine was injected into seven month old adult Fischer 344 rats and the time-dependent appearance and loss of radioactivity was monitored in both the serum and proteins from the brains of rats sacrificed from 4 hours to 13 days after injection. Experimental data obtained for calmodulin and the plasma membrane Ca-ATPase are best described by theoretical curves accounting for leucine reutilization that assume apparent half-lives of 18 (±2) hours and 12 (±1) days, respectively.

The plasma membrane Ca-ATPase (PM-Ca-ATPase)(E.C.3.6.1.38), present in all eukaryotic tissues, is responsible for the extrusion and fine regulation of intracellular calcium (1). In response to rising intracellular calcium levels, calmodulin (CaM) binds and activates the PM-Ca-ATPase. Both CaM and the PM-Ca-ATPase have been shown to exhibit functional defects in the aged brain that may be responsible for the observed age-related increases in intracellular calcium levels (2-5). The diminished ability of CaM from aged brain to activate the PM-Ca-ATPase is accompanied by the oxidation of multiple methionines within CaM to methionine sulfoxide (5). Similarly, the age-related decreased enzymatic activity of the PM-Ca-ATPase may also result from post-translational modifications. Since such modifications are likely to accumulate to a greater extent for proteins with long cellular lifetimes, knowledge of the in vivo half-life of these proteins would facilitate our understanding of possible cellular mechanisms leading to these defects. Therefore we have measured the protein half-life of CaM and the PM-Ca-ATPase from whole brain homogenates of seven month adult Fischer 344 rats after injection of radiolabeled [^{14}C]-leucine, correcting for the leucine reutilization.

MATERIALS AND METHODS

Reagents. The following chemicals were obtained from the indicated sources: uniformly labeled L-[^{14}C]-leucine (292 mCi/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO); primary antibody to CaM (polyclonal) from Signal Transduction (San Diego, CA); primary antibody to the PM-Ca-ATPase (monoclonal, clone 5F10), from Affinity Bioreagents (Golden, CO); alkaline phosphatase-conjugated secondary antibodies: goat anti-rabbit IgG (A+L) and anti-mouse IgM from Zymed Laboratory (So. San Francisco, CA); 5-bromo-4-chloro-3-indolyl phosphate, from Sigma (St. Louis, MO); Immobilon-P PVDF membrane (0.45 μm) for Western immunoblots, from Millipore (Bedford, MA); all reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA); benzamidine and benzamidine/HCl from Fisher Scientific (Pittsburgh, PA); 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) from Calbiochem (San Diego, CA); and bicinchoninic acid (BCA) protein assay reagents from Pierce Chemical Co. (Rockford, IL).

Animals. The animals used in this study were young adult (7 months, 359 ± 12 g) Fischer 344 male rats obtained from the National Institute of Aging colonies maintained at Harlan Sprague-Dawley, Inc (Indianapolis, IN). Rats were housed separately during the study and maintained under strictly controlled environmental conditions for temperature (25° C) and light/dark cycles (12 hr intervals). Food (NIH-31, Harlan Teklad Laboratory diet, Madison, WI) and tap water were provided *ad libitum*. Pulse injections were not scheduled until at least three weeks after shipping of rats. The research described in this report was conducted in compliance with all applicable federal statutes and regulations relating to animals and adheres to the principles in *The Guide for Care and Use of Laboratory Animals*, NIH publication 86-23, 1985 edition.

Injection of radioisotopes. [^{14}C]-leucine (specific activity 292 μCi/mmol) was diluted with 0.9% normal sterile saline and administered

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Abbreviations: AEBSF, 4-(2-Aminoethyl)-benzenesulfonyl fluoride; BCA, bicinchoninic acid; CaM, calmodulin; PM-Ca-ATPase, plasma membrane Ca-ATPase; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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by intraperitoneal injection at a (non-flooding) dose rate of 75 μCi/100 g body weight. In order to control for diurnal fluctuations in amino acid absorption, all isotope injections were administered at the same time (10:00–10:30 a.m.) each day. Handling and disposal of radioactive material was done in compliance with instructions from the University of Kansas Office of Radiation Safety.

Determining serum free-leucine specific activity. Following sacrifice the chest cavity was opened and blood was withdrawn from the heart. The blood was centrifuged (3000 $\times$ *g* for 10 minutes) and the serum frozen at −70°C for later amino acid analysis and scintillation counting. Free leucine content in serum was determined as described previously following phenylisothiocyanate derivatization (6) using a Beckman (System Gold) amino acid analyzer (Biochemical Research Services Laboratories, University of Kansas). Parallel samples were assayed (in triplicate) for radioactivity by scintillation counting.

Isolation and radioactive counting of proteins from brain homogenate. Immediately following sacrifice, brains were removed from the animal and transferred to an ice-cold medium containing 0.32 M sucrose (pH 7.4) and the protease inhibitors AEBSF (0.1mM), benzamidine (0.1 mM) and benzamidine/HCl (0.1 mM). Brains were homogenized in the above medium by mechanical disruption using a glass homogenizer with a Teflon pestle (7) and subsequently centrifuged (3500 $\times$ *g* for 5 min) at 4°C. The resulting supernatant was centrifuged (44,000 $\times$ *g* for 15 min); the resulting synaptic membrane pellet was resuspended in the same medium as above, and frozen at −70°C until later use. Protein concentrations were determined using the BCA assay (8). Constituent proteins were separated by SDS-PAGE using an 8.75% separating gel according to the method of Laemmli (9). For each animal, 2.5 mg of brain homogenate was loaded onto preparative gels (16 cm $\times$ 20 cm) containing a large, single lane. Immediately following electrophoresis, proteins were electroblotted to a PVDF membrane, transferring at 4°C for 5 hours as previously described (10). The protein-blotted PVDF membrane was divided in half, and the portions corresponding to lower and higher molecular weight proteins were incubated with primary antibodies directed against CaM (using a 1:800 dilution) and against the PM-Ca-ATPase (using a 1:1000 dilution), respectively. Alkaline-phosphatase conjugated secondary goat anti-mouse IgM and anti-rabbit IgG (1:800) were used in concert with the primary antibodies to CaM and the PM-Ca-ATPase, respectively. The substrate 5-bromo-O-4-chloro-3-indolyl phosphate was used for color development. Individual bands containing immunoreactive product were excised from PVDF membranes for scintillation counting.

Computer simulation. Theoretical curvers were developed using Origin 4.1-32 bit software from Microcal (Northampton, MA).

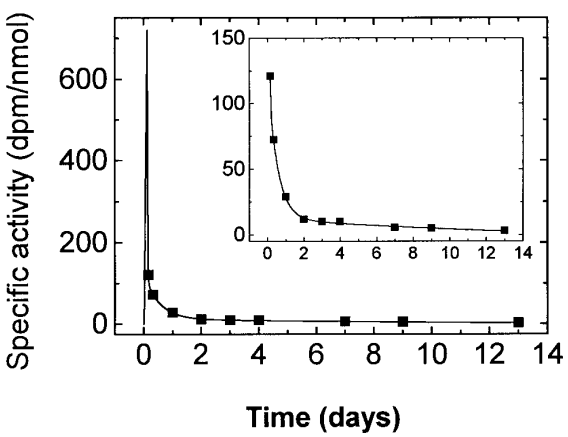

FIG. 1. The time-dependent change in the radioactivity of [^{14}C]-leucine (in dpm/nmol leucine) associated with serum. The data (■) is best described by a three exponential decay (solid line; inset). For the generation of curves for proteins of different half-lives (Fig. 2), this fit was projected to a maximum specific activity value of 720 (±60) dpm/nmol at 3.0 (±0.6) hours, with a linear increase from 0 to 3 hrs (solid line).

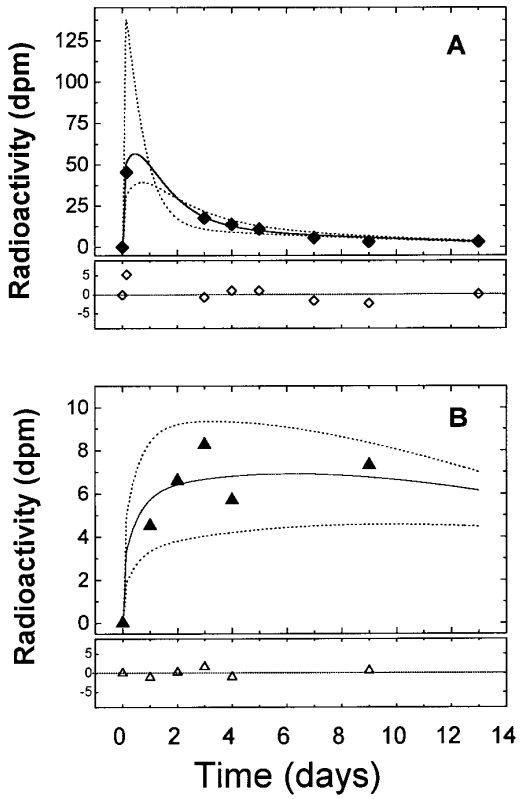

FIG. 2. The time-dependent change in radioactivity (dpm/mg total protein) associated with CaM (A) and the PM-Ca-ATPase (B). Theoretical curves were generated based on the changes in the serum radioactivity (defined in Fig. 1) and assuming different protein half-lives as described in Methods. Panel A. Radioactivity of ^{14}C-Leu associated with CaM (♦) fits best to a theoretical curve assuming a half-life of 18 (±2) hours (solid line) as judged by a reduced chi-squared (χ^2_R) of 1.7. The associated deviations between data and curves are shown below (◇). Dotted lines describe curves assuming half-lives corresponding to the 95% confidence limits, i.e., 6 and 30 hours. Panel B. Radioactivity of ^{14}C-Leu associated with the PM-Ca-ATPase (▲) fits best to a theoretical curve assuming a half-life of 12 (±1) days (solid line) as judged by a reduced chi-squared (χ^2_R) of 1.1. The associated deviations between data and curves (△) are shown below. Dotted lines describe curves for proteins with half-lives which correspond to the 95% confidence limits, i.e., 8 and 21 days.

RESULTS AND DISCUSSION

The time-dependence of the incorporation and loss of radioactivity associated with serum leucine and with both CaM and the PM-Ca-ATPase in brain was measured following a single injection of [^{14}C]-leucine in order to estimate the apparent half-lives of these proteins. The use of whole brain homogenate in this study does not permit possible differentiation of turnover

rates of proteins from different brain regions or for the different neuronal isoforms of the PM-Ca-ATPase. After electrophoretic separation of brain proteins by SDS-PAGE, bands corresponding to CaM and the PM-Ca-ATPase were identified by immunoblotting. These bands were excised for scintillation counting. [^{14}C]-leucine from parallel serum samples was also quantitated. As illustrated in Figures 1 and 2, the radiolabeled leucine disappears rapidly from the serum concomitant with its incorporation into protein. The measurable radioactivity present in the blood up to 13 days after isotopic injection indicates the occurrence of a small but significant degree of leucine reutilization. Therefore, we accounted for this reutilization in our analysis modeling the experimental data for protein-associated radioactivity to theoretical curves generated from the decay of serum leucine and assuming different half-lives for proteins according to the model of Zilversmit (11) as described by Martin and co-workers (6).

Theoretical curves for the incorporation and loss of [^{14}C]-leucine radioactivity into proteins were generated by numerical calculations according to:

$$\frac{dP}{dt} = k\,(F(t) - P(t))$$

where $P(t)$ is the time-dependent specific radioactivity of [^{14}C]-leucine associated with either CaM or the PM-Ca-ATPase, $F(t)$ is the time-dependent specific radioactivity of [^{14}C]-leucine in the precursor pool, i.e., the serum (Fig. 1), and k is the first order rate constant of protein degradation which is equal to 0.693 divided by the protein half-life. Theoretical curves were generated based on the incorporation and loss of radioactivity from serum (F(t)) and assuming different values of protein degradation rates (k). These curves were compared with the experimental data obtained from either CaM (Fig. 2A) or the PM-Ca-ATPase (Fig. 2B) and best fits were determined by chi-squared minimization. Curves assuming a protein half-life of 18 ($\pm$2) hours and 12 ($\pm$1) days best fit the experimental data from CaM and the PM-Ca-ATPase, respectively. For data from both proteins, the residuals were small and random (Fig. 2), demonstrating the absence of systematic errors in the fits.

The very short half-life of brain CaM measured in this study is consistent with the 10 hour half-life reported for the δ- subunit (identified as CaM) of phosphorylase kinase from skeletal muscle which has been described as one of the shortest half-lives of any protein in rabbit skeletal muscle (13). Despite this rapid turnover of CaM in the brain, a remarkable degree of protein modification in the form of methionine oxidation has been observed for CaM from aged brain suggesting defects in pathways responsible for repair or degradation of modified CaM proteins (5).

In contrast, the 12 day half-life of the PM-Ca-ATPase in brain indicates a most stable protein. For example, based on turnover rates for a large number of liver enzymes, proteins classified with the slowest turnover consists of those with a half-life of 24 hours or more. Even taking into account the slower turnover of proteins in skeletal muscle (2-5 fold) compared to liver, the PM-Ca-ATPase can be classified as a protein with one of the slowest turnover rates (reviewed in (14)). Two other ion transporters, the sarcoplasmic reticulum Ca-ATPase from rat skeletal muscle and the Na^+-K^+-ATPase from rat kidney, have half-lives of a similar magnitude, i.e., 10-14 days and 4 days, respectively, suggesting that active transport proteins may be extremely stable, and thus more liable to the accumulation of post-translational modifications (12,15).

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