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Protein Half-Lives of Two Subunits of an NMDA Receptor-like Complex, the 71-kDa Glutamate-Binding and the 80-kDa CPP-Binding Protein

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We determined the half-lives for two subunits of a complex that functions as a glutamate and N-methyl-D-aspartate (NMDA) receptor-ion channel in synaptic membranes. These two proteins are a 71 kDa glutamate-binding protein (GBP) and an 80 kDa CPP-binding protein (CBP). Seven month-old Fischer 344 rats were injected with L-[^{14}C] leucine. The radioactivity in the two proteins was determined in a crude synaptosomal membrane fraction obtained from the brains of rats sacrificed from 4 hours to 13 days after the injection. The previously reported data on time-dependent appearance and loss of L-[^{14}C] leucine radioactivity in the serum (Ferrington et al., 1997, *Biochem. Biophys. Res. Commun.* 237, 163-165) was used in the present study to estimate the half-lives of GBP and CBP. Theoretical curves best fit the experimental data obtained for the two proteins assuming apparent half-lives of 14 (± 2.4) and 18 (± 1.2) hours for CBP and GBP, respectively.

L-Glutamate is the most widespread excitatory transmitter in the mammalian brain. The N-methyl-D-aspartate (NMDA) receptors are a class of ion-channel forming receptors activated by L-glutamic acid (1-4). The NMDA class of receptors are important in the expression of both physiological processes, such as synaptic plasticity, memory formation, and learning, and pathological conditions, such as neuronal damage in focal ischemia, trauma, and Alzheimer's disease. Neuronal NMDA receptors are tetrameric or pentameric protein complexes composed of the NMDAR1 (NR1) and NMDAR2 (NR2A, 2B, 2C, and 2D) subunits (1-4). In addition to these NMDA receptor complexes, there is another complex of proteins in neuronal membranes that forms NMDA and glutamate-activated ion channels but does not contain either the NR1 or NR2 proteins (5-7). This complex may also play an important role in neuronal development (8), neuronal adaptation to chronic ethanol exposure (9-11), and neuronal toxicity induced by exposure to high concentrations of L-glutamate or NMDA (12, 13). Two subunits of this protein complex, the 71 kDa glutamate-binding protein (GBP) and the 80 kDa (3-((±)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP)-binding protein (CBP), have been purified and characterized biochemically and immunochemically (14-16).

The cDNA for the GBP has been cloned (17) and anti-sense oligonucleotides designed on the basis of this cDNA sequence cause down-regulation of GBP expression in hippocampal and cerebellar granule cell neurons (10, 13). Treatment of hippocampal neurons in primary cultures with antisense oligonucleotides protects these neurons from L-glutamate and NMDA-induced neurotoxicity (13) and causes a diminution in Ca^{2+} influx in response to NMDA receptor activation (10, 13). Maximum suppression of GBP expression following oligonucleotide treatment occurs within 24 hours and recovery to control levels within 40-48 hours after treatment (13). These results are indicative of a fairly rapid turnover rate for GBP with a half-life that may be less than 24 hours. In another series of studies, it was observed that chronic ethanol treatment of experimental animals for 15 days leads to a 50-60% increase in the expression of both the GBP and CBP subunits of this NMDA receptor-like complex and withdrawal from ethanol for as short a period as 36 h is sufficient to bring the levels of both proteins back to normal (18). These observations also indicate that both

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Abbreviations used: CBP, (3-((±)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid binding protein; CPP, (3-((±)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid; GBP, glutamate-binding protein; NMDA, N-methyl-D-aspartate; NMDARn (NRn), N-methyl-D-aspartate receptor protein subunit; P2 fraction, crude synaptosomal membrane fraction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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GBP and CBP have relatively short half-lives. However, the turnover rate of either of these two proteins in neurons is not known. The present study was designed to obtain a direct measure of the half-lives of GBP and CBP in synaptosomal membranes isolated from whole brain homogenates of seven month-old rats injected with L-[^{14}C]leucine (19).

MATERIALS AND METHODS

Reagents. 5-Bromo-4-chloro-3-indolylphosphate was obtained from Sigma, benzamidine and benzamidine/HCl from Fisher Scientific, [4-(2-aminoethyl)benzenesulfonylfluoride from Calbiochem, bicinchoninic acid protein assay kits from Pierce Chemical, and L-[^{14}C]leucine (292 mCi/mmol) from American Radiolabeled Chemicals. The polyclonal anti-GBP and anti-CBP antibodies were those raised in our laboratory (20, 21). The secondary antibody, goat anti-rabbit IgG -alkaline phosphatase conjugate, was obtained from Zymed Laboratory. Immobilon-PVDF membranes (0.45 μm) used in immunoblotting were from Millipore.

Animals and radioisotope injections. Animals used, animal care, radioisotope injection procedures, and radioactive material disposal were as previously described (19) and were in compliance with all applicable statutes and regulations of the federal and state governments and the University of Kansas. The seven month-old Fischer 344 male rats (body weight 359 $\pm$ 12 g) were obtained from the National Institute of Aging colonies located at Harlan Sprague-Dawley Inc. The rats were maintained in separate cages at conditions of 25°C and in alternating 12 h dark-light cycles. L-[^{14}C]Leucine (specific activity 292 μCi/mmol) was injected intraperitoneally into rats (75 μCi/100g body weight) three weeks after the animals were shipped to us. To minimize the diurnal fluctuations in amino acid absorption, all [^{14}C]leucine injections were performed at the same time (10:00-10:30 am) of the day.

Determination of the specific activity of free L-[^{14}C]leucine in serum. The specific activity of free leucine in serum was determined as in our previous report (19) and was used in calculations described in this paper. All measurements reported in the present study were conducted using brain tissue obtained from the same animals as those reported previously (19).

Protein isolation and measurement of radioactivity associated with the GBP and CBP. A crude synaptosomal membrane fraction (P2 fraction) was isolated according to the procedures described in detail previously (19). The resulting membrane fraction was stored at -70 °C and prepared for sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the methods described previously (19, 22). PAGE and immuno-blotting were performed on protein samples (2.5 mg of protein from each brain membrane preparation) dissolved in sample buffer containing 2% (w/v) SDS, 250 mM dithiothreitol, and 500 mM iodoacetamide. Each sample was loaded onto a preparative 8.75% polyacrylamide gel (16 cm $\times$ 20cm) containing a large, single lane. After separation by electrophoresis, the proteins were transferred to a PVDF membrane, and then incubated in solutions containing either polyclonal anti-GBP or anti-CBP sera (1:800 dilution). Following incubation of the membranes with alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:1000) and development of the color reaction products, the bands corresponding to GBP and CBP were excised and the radioactivity associated with each band was measured (19). The radioactivity of an area of PVDF membrane of equal size that contained no proteins was used as a measure of a blank value.

Computer simulation. Computer simulation was conducted as described previously (19) and was performed using Origin 4.1-32 bit software (Microcal).

RESULTS AND DISSCUSSION

The incorporation of L-[^{14}C]leucine into GBP and CBP following a pulse injection of L-[^{14}C]leucine, and the loss of such radioactivity from the two proteins over a period of 3 h to 13 days were estimated by the methods described above. We previously found that L-[^{14}C]-leucine disappears rapidly from serum and is incorporated into proteins (19). We also showed that there is a small but significant amount of residual [^{14}C]leucine in serum which represents the amount of [^{14}C]leucine that is released from proteins and may be incorporated into newly synthesized proteins (19). This [^{14}C]leucine reutilization is accounted for in the estimation of the half-lives of GBP and CBP as described in our previous study (19).

The radioactivity associated with the immunostained bands for GBP and CBP at various periods following the pulse injection of L-[^{14}C]leucine was measured and used to estimate the apparent half-lives of these two proteins. The band of GBP labeled by the anti-GBP antibodies had an estimated molecular size equal to 66-70 kDa and that for CBP was 80-83 kDa (6). The estimates of GBP and CBP half-lives represented global measures of the turnover of these proteins in the brain since the membranes used were isolated from whole brain homogenates. A series of theoretical curves were generated on the basis of the following formula for the incorporation and decay of [^{14}C]leucine radioactivity associated with GBP or CBP:

$$dP/dt = k[F(t) - P(t)]$$

F(t) is the specific radioactivity of L-[^{14}C]leucine in the precursor pool, i.e., the serum, at time t, and this was reported previously (19). P(t) is the specific radioactivity of L-[^{14}C]leucine associated with either GBP or CBP at time t, and k is the first order constant of protein degradation described by the equation:

$$k = 0.693/t_{1/2}$$

where $t_{1/2}$ is the protein half-life.

Theoretical curves were generated using the previously estimated value for F(t) (19) and a different k value was calculated on the basis of different $t_{1/2}$ values. These curves (P(t) vs t) were then fit with the experimental data for either GBP or CBP. The best fit to the experimental data was determined by chi-squared analysis. Curves assuming a protein half life ($t_{1/2}$) of 14.4 ($\pm$ 2.4) hours for CBP and 18 ($\pm$ 1.2) hours for GBP best fit the experimental data obtained for these two proteins (Fig. 1). Little, if any, systematic errors were found because the residuals for the

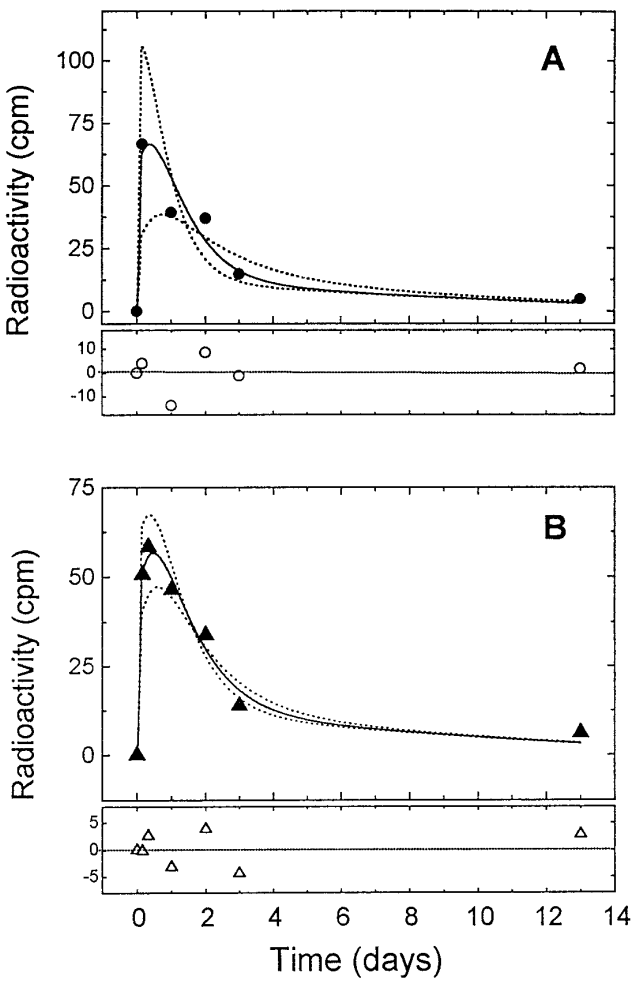

FIG. 1. Time-dependent change in radioactivity (cpm/mg total protein) associated with CBP (A) and GBP (B). Theoretical curves were generated based on the change in the serum radioactivity (reported in Ref. 1) and assuming different protein half-lives as described in Methods. **A.** Radioactivity of L-[^{14}C] leucine associated with CBP (●) fits best to a theoretical curve assuming a half-life of 14.4 (± 2.4) hours (solid line). 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., 8 hours and 1.3 days. **B:** Radioactivity of L-[^{14}C] leucine associate with GBP (▲) fits best to a theoretical curve assuming a half-life of 18 (± 1.2) hours (solid line). 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., 14 and 23 hours.

experimental data of both GBP and CBP are small and random (Fig. 1).

The half lives of both GBP and CBP that were measured in the present study were less than 20 hours, which is consistent with previous observations with regard to these two proteins from studies with primary neuronal cultures and intact experimental animals. Mattson and colleagues (13) reported that the expression of GBP in hippocampal cells in primary cultures reached its lowest level after 24 hours of treatment with antisense oligonucleotides to this protein's cDNA, an indication of a relatively short half-life of the GBP. Chen and colleagues (18) indicated that recovery to baseline levels of expression of both GBP and CBP after chronic exposure of rats to ethanol occurred within 36 h of withdrawal of the animals from ethanol treatment. This observation is consistent with the measurements reported in the present studies which indicate a half-life of 18 h for the GBP and 14 h for the CBP. The results of the present study provide a direct measure of the half-life of these two subunits of an NMDA receptor-like complex in vivo which can be used not only to account for the previous observations of Mattson et al. (13) and Chen et al. (18), but also to compare them with other neuronal membrane proteins. For example, in our previous studies we determined the half-life of synaptic plasma membrane Ca^{2+}-ATPases to be 12 days (19). Unlike the relatively long half-life of ATP-dependent transport proteins such as the neuronal plasma membrane Ca^{2+}-ATPases, muscle sarcoplasmic reticulum Ca^{2+}-ATPases (23) and kidney plasma membrane (Na^+ + K^+)-ATPases (24), receptor proteins may have a relatively short half-life. The half-lives for the GBP and CBP were similar to those observed for the extrasynaptic population of nicotinic acetylcholine receptors in muscle cells in culture ($t_{1/2}$ = 17 hours) (25) and folate receptors expressed in chinese hamster ovary cells ($t_{1/2} \cong$ 24hours) (26). The short half-lives of receptor proteins may be related to their function and the need for rapid adjustments in receptor expression in neurons and other types of cells. The possible generality of such a phenomenon of short half-lives of receptor proteins needs to be evaluated further by estimating the turnover of other neurotransmitter receptor proteins, and especially the NMDA receptor proteins NR1 and NR2.

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