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Increased Fragility of Neuronal Membranes with Aging

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Changes in membrane integrity have been implicated as a contributing factor in the aging process. We have examined the relative stability of synaptosomal membranes from young and aged mice, in response to chemically induced damage. The basal characteristics of the resting synaptosomes prepared from mice of either age were indistinguishable, as judged by intracellular levels of ionic calcium and by efflux of the fluorescent probe employed, fura-2. However, the response of isolated nerve endings to the presence of a neurotoxic agent, the insecticide chlordecone, was markedly different. Exposure to this chemical invariably elevated free calcium and increased the rate of leakage of fura-2 into the extracellular space. These effects were much more pronounced in preparations derived from aged animals. The implication of these findings is that neural membranes of aged animals are more susceptible to malfunction induced by xenobiotic agents and are less able to maintain homeostasis under such circumstances. © 1989 Academic Press, Inc.

INTRODUCTION

The status of membrane integrity during the aging process has been a subject of interest in recent years. Deterioration of membrane properties has been invoked as a mechanism underlying senescence. Within the nervous system of aged animals, altered functioning of various components of the plasma membrane has been described. These include changes in membrane rigidity (10, 11), neurotransmitter-related receptor sites (1, 13), and ion transport systems (5). We have compared levels of ionic calcium in a crude synaptosomal fraction from 3- and 26-month-old mice. The response of this subcellular fraction to a neurotoxic organochlorine, chlordecone, was also studied in order to evaluate resistance to a chemical insult. Chlordecone (decachlorooctahydro-1,3,4-methano-2H-cyclobuta-pentylene-2-one), originally developed as a persistent insecticide, is known to be destructive to neuronal membranes and effects a time- and dose-dependent increase in synaptosomal $[Ca^{2+}]_i$ (7). While resting levels of $[Ca^{2+}]_i$ were very similar in crude synaptosomes from mice of both ages, chlor-

decone treatment caused a significantly greater elevation of $[Ca^{2+}]_i$ in aged mice. In addition, membrane permeability was also disproportionately increased in the chlordecone-exposed preparation from older mice, as judged by dye leakage into the surrounding medium.

METHODS

Whole brains (except cerebellum) were excised from mice, homogenized in 10 vol 0.32 M sucrose, and centrifuged (1500g, 10 min). The supernatant from this was recentrifuged (20,000g, 10 min) in order to obtain the P2 fraction comprised of synaptosomes, myelin fragments, and mitochondria (3). This fraction was resuspended in a buffer containing (mM) NaCl, 125; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$, 0.1; $NaHCO_3$, 5; glucose, 6; and Hepes, 25, at a pH of 7.4. After loading this fraction with the acetoxymethyl ester of the fluorescent indicator dye fura-2, $[Ca^{2+}]_i$ was determined. The diffusion of this ester into cells or synaptosomes is followed by hydrolysis by nonspecific esterases and the anionic fura-2 is then trapped within cellular material. Fura-2 can reversibly interact with calcium ions to form a complex that emits a characteristic fluorescent signal, differing from that of the free anion (4). In the case of crude synaptosomal preparations, the mitochondrial contribution to the signal is minimal probably due to both the absence of major esteratic activity within mitochondria and to the poor metabolic condition of free mitochondria in the low potassium Hepes buffer. We have found that mitochondria isolated under these conditions hydrolyzed fura-2 at less than 5% of the corresponding synaptosomal rate (8).

Samples contained 170–250 μ g protein and were allowed to equilibrate for 10 min before addition of chemicals. A correction was made for any fura-2 leaking out of the particulate fraction by quenching extracellular fluorescence of fura-2 calcium complex with $MnCl_2$ (6). In order to calculate $[Ca^{2+}]_i$ before addition of chlordecone, a separate correction was made for each batch of synaptosomes. This correction was between 15–21% of the fluorescence at 340 nM. For calibration of the synaptosomal fura-2- Ca^{2+} signal (R), R_{min} (the ratio of fluorescence at 340 nm/380 nm in the absence of Ca^{2+}) and R_{max} (the ratio when all fura-2 of the sample was saturated

FIG. 1. Effect of chlordecone upon free calcium within crude synaptosomes from young and old mice. Synaptosomal fractions prepared from whole brains of either 3- or 26-month-old male CBA/Ca mice (Charles River Breeding Laboratory, National Institute of Aging contract colony) were preincubated in Hepes buffer prior to a 15-min incubation with various concentrations of chlordecone. This agent was in a dimethyl sulfoxide (DMSO) vehicle, and control samples received an equivalent volume of DMSO (final concentration 1% (v/v)). The number of mice in each group was 6. Means $\pm$ SE are presented. *Value of preparation from aged animals differs significantly from corresponding value for young mice ($P < 0.05$). Bartlett's test for homogeneity of variance validated nonparametric Kruskal-Wallis analysis followed, when appropriate, by Mann-Whitney U test between paired groups (12).

with Ca^{2+}) were determined for each batch of fura-2-loaded synaptosomes. $[\text{Ca}^{2+}]_i$ was then calculated using the formula of Grynkiewicz *et al.* (4). $R_{\min}$ was determined following solubilization of synaptosomes with sodium dodecyl sulfate and chelation of calcium with EGTA, while $R_{\max}$ was measured after the addition of sufficient CaCl_2 to give a $[\text{Ca}^{2+}]$ of 1 mM (8).

RESULTS

The resting value of $[\text{Ca}^{2+}]_i$ in the P2 fraction from brains of young adult mice was 366 ± 15 nM ($n = 12$). The response of $[\text{Ca}^{2+}]_i$ to a 15-min exposure to chlordecone was measured. A significant elevation of $[\text{Ca}^{2+}]_i$ occurred in all preparations tested at both concentrations of chlordecone used (Fig. 1). The increase in $[\text{Ca}^{2+}]_i$ was greater in the P2 fraction from aged mice. This difference between young and old fractions was significant at the high concentration of chlordecone (50 μM), this difference between young and old fractions was significant. The higher standard error of $[\text{Ca}^{2+}]_i$ values for 50 μM chlordecone-treated synaptosomes from aged animals was due to a relatively uniform greater population variance.

The extent to which fura-2 had leaked back into the surrounding buffer after hydrolysis was determined by quenching the extracellular fura-2- Ca^{2+} signal with MnCl_2 (6). This allowed quantitation of the degree of permeability of the plasma membrane of cytoplasmic inclusions within the P2 fraction. This leakage was enhanced in a dose-dependent manner by chlordecone but

was more prominent in the preparations from aged mice. This age-related difference was also significant in the presence of 50 μM chlordecone (Fig. 2).

Both $[\text{Ca}^{2+}]_i$ and leakage of fura-2 reflect the integrity of the plasma membrane (7) and neither of these parameters was demonstrably different under basal incubation conditions in the absence of a chemical stressor. In order to unmask the especial vulnerability of synaptosomes from aged animals, exposure to a toxic, xenobiotic agent was necessary.

DISCUSSION

Alterations in neuronal calcium translocation mechanisms have been intensively studied, but conflicting data exist concerning the nature of age-related deficits. Data can be divided into results suggesting abnormally low levels of free ionic intracellular calcium, $[\text{Ca}^{2+}]_i$, and the opposite, namely, excessive concentrations of $[\text{Ca}^{2+}]_i$ in neurons of aged animals. These views are well summarized by Gibson and Peterson (2). There is evidence that the aging process may reduce the efficiency of appropriate calcium translocations under normal metabolic conditions (2, 9). However, our results imply that an excessive entry of calcium across neuronal membranes in response to a xenobiotic challenge can be effected as a result of aging. The ability of limiting membranes from a crude synaptosomal fraction from aged mouse brain to withstand a neurotoxic insult was clearly impaired using either $[\text{Ca}^{2+}]_i$ or leakage of fura-2 as a criterion.

Subsensitivity of calcium movements in regard to physiological cellular requirements, concurrent with a heightened susceptibility to deleterious extraneous factors, may account for some of the contradictory results described earlier. Loss of functionality of specific receptor and transport systems within the membrane can coexist with a general loss of coherent structure of the membrane as a whole.

An impaired replacement rate of α -adrenergic receptors in cerebral membranes of aged rats following their

FIG. 2. Effect of chlordecone on integrity of crude synaptosomes from young and old rats as judged by leakage of fura-2 into the extracellular medium. Preparation and incubation conditions as described in Fig. 1. Data represent mean values $\pm$ SE derived from six animals per group. *Value of preparation from aged animals differs significantly from corresponding value for young mice ($P < 0.05$).

irreversible inhibition has been reported (13). This raises the possibility of a reduced metabolic adaptive response to pharmacologically induced damage. The combination of increased membrane fragility and reduction of repair-related anabolic responses may act in concert in retarding neuronal responsivity in the aging nervous system.

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