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Deficits in the Sensitivity of Striatal Muscarinic Receptors Induced by ^{56}Fe Heavy-Particle Irradiation: Further "Age–Radiation" Parallels

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We had previously shown that there was a loss of sensitivity of muscarinic receptors (mAChR) to stimulation by cholinergic agonists (as assessed by examining oxotremorine enhancement of K^+-evoked release of dopamine from neostriatal slices) in animals that had been exposed to energetic particles (^{56}Fe, 600 MeV/n), an important component of cosmic rays. This loss of mAChR sensitivity was postulated to be the result of radiation-induced alterations in phosphoinositide-mediated signal transduction. The present experiments were undertaken as a first step toward determining the locus of these radiation-induced deficits in signal transduction by examining K^+ enhancement of release of dopamine in ^{56}Fe-exposed animals (0, 0.1, and 1.0 Gy) with agents [A23187, a potent Ca^{2+} ionophore, or 1,4,5-inositol trisphosphate (IP_3)] that "bypass" the mAChR–G protein interface and by comparing the response to oxotremorine-enhanced K^+-evoked release of dopamine. Results showed that although oxotremorine-enhanced K^+-evoked release of dopamine was reduced significantly in the radiation groups, no radiation effects were seen when A23187 or IP_3 was used to enhance K^+-evoked release of dopamine. Since similar findings have been observed in aging, the results are discussed in terms of the parallels between aging and radiation effects in signal transduction that might exist in the neostriatum.

INTRODUCTION

Among the myriad of problems that must be solved if astronauts are to travel to distant planets in our solar system, such as Mars, are those dealing with the effects of long-term exposure to space radiation, such as cosmic rays [for reviews, see Refs. (*1–5*)]. Such considerations will become especially important because shielding against heavy particles such as ^{56}Fe, an important component of cosmic rays, is presently very difficult. In attempts to elucidate these effects, our research has focused on the consequences of whole-body exposure to ^{56}Fe particles (600 MeV/n) on motor behavioral performance and related neurochemical parameters of the corpus striatum in rats. This structure has been shown to mediate a variety of motor behavioral functions such as coordination and muscle strength [for reviews, see Refs. (*6–8*)].

In our initial experiment (*9*), motor behavior was assessed in various groups of rats at 12 h to 14 days after ^{56}Fe-particle irradiation by examining the length of time that a rat could remain suspended by its forepaws from a wire. Biochemical assessments of striatal function were carried out immediately after the behavioral tests and up to 180 days after irradiation by determining the sensitivity of muscarinic acetylcholine receptors (mAChR)[4] to stimulation by muscarinic agonists. This latter assessment was made by examining the oxotremorine (OXO)-enhanced K^+-evoked release of dopamine (DA) from perifused striatal slices obtained from these animals. The results indicated that profound decrements occurred in both indices. The effects on K^+-evoked release of DA were evident as early as 12 h after irradiation and were seen as long as 180 days after irradiation.

Thus, there were deficits in the sensitivity of mAChR to agonist stimulation. However, the locus of these deficits within putative mAChR-initiating phosphoinositide (PI)-mediated signal transduction pathways remained unresolved by these studies. The mAChR are linked to G proteins, which are heterotrimers composed of $\alpha\beta\Gamma$ subunits. After stimulation of mAChR (specifically the PI-linked M_1 and M_3 subtypes) in the presence of Mg^{2+}, there is an exchange of GTP for GDP on the α subunit (*10*). The activated α_{GTP} subunit dissociates from the $\beta\Gamma$ subunits and interacts with effector molecules of phospholipase C, which catalyzes the cleavage of phosphatidylinositol 4,5-bisphos-

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[4] Abbreviations used: DA, dopamine; HPLC, high-performance liquid chromatography; IP_3, 1,4,5,-inositol trisphosphate; mAChR, muscarinic acetylcholine receptors; OXO, oxotremorine; PI, phosphoinositide.

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phosphate (PIP_2). One product resulting from this reaction is 1,4,5-inositol trisphosphate (IP_3), one of the second messengers of this system (*11, 12*). An intrinsic low-K_M GTPase activity of the a subunit hydrolyzes GTP to GDP, releasing inorganic phosphate (P_i) and α_{GDP}, and reassociates with $\beta\Gamma$, ending the activation cycle (*13, 14*). IP_3 acts to evoke the quantal (*15*) release of Ca^{2+} from internal stores to carry the impulse further or impinge upon an effector. It is clear that alterations induced by exposure to ^{56}Fe particles at any point in this signal transduction pathway could affect the responsiveness of mAChR to agonist stimulation, resulting in reduced Ca^{2+} mobilization, alterations in neuronal processing, and ultimately, reduced behavioral performance.

As a first step toward making these analyses, the present experiment was carried out to determine if the locus of the deficit in mAChR-initiating PI-mediated signal transduction after exposure to ^{56}Fe particles occurred after the receptor–G protein complex in the biochemical pathway of signal transduction. For that reason we chose to examine K^+-evoked release of DA in striatal tissue from rats exposed to ^{56}Fe particles by "bypassing" this complex and stimulating signal transduction at later points in the pathway. In this regard, K^+-evoked release of DA was assessed after application of IP_3 or A23187 [a potent Ca^{2+} ionophore (*16*)] to perifused striatal slices from irradiated and control animals.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley Crl:CD(SD)BR rats (Charles River Laboratories, Kingston, NY) weighing 200–300 g were used in these experiments. The rats were housed at an AAALAC-accredited vivarium at the Lawrence Berkeley Laboratory (LBL), Berkeley, CA. The rats were maintained in polycarbonate cages that contained autoclaved hardwood contact bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY). They were given food and water *ad libitum*. The animal holding rooms were kept at 21 ± 1°C with 50 ± 10% humidity.

Radiation and Dosimetry

Rats were exposed to whole-body irradiation with high-energy ^{56}Fe particles (600 MeV/n) produced by the BEVALAC at LBL. In each experiment one rat was irradiated at a time. This energy provided a Bragg curve with the plateau region extending 8 cm in water. Since the diameter of the rat (including the plastic restrainer) was 7 cm, the animals were within this plateau region of the curve. Entrance dose measurements were made by the staff of the BEVALAC facility using parallel-plate ionization chambers with Mylar windows and N_2 gas flow positioned in the beam line (*17, 18*). The rats were irradiated in well-ventilated plastic holders. Rats were given one of three doses (0.0, 0.1, or 1.0 Gy) at a dose rate that averaged 1 Gy/min. The specified doses were received by all organs.

Perifusion Procedure

At 2–3 days after irradiation the animals were killed by decapitation; their brains were removed quickly, and the striata were dissected rapidly on ice. The tissue was then treated as described previously (*9, 16, 19, 20*). Briefly, cross-cut striatal slices (300 μm) were prepared from each animal using a McIlwain tissue chopper (Westbury, NY); slices from each animal in the individual experimental and control groups were pooled and were placed into small glass vials that contained a modified Krebs-Ringer basal release medium containing 21 m*M* $NaHCO_3$, 3.4 m*M* glucose, 1.3 m*M* NaH_2PO_4, 1 m*M* EGTA, 0.93 m*M* $MgCl_2$, 127 m*M* NaCl, and 2.5 m*M* KCl (pH 7.4). The medium had been bubbled for 30 min with 95% O_2/5% CO_2. Slices from each vial were washed twice in this medium and aliquots were placed into the chambers of a perifusion apparatus. After being placed in the chambers of the perifusion apparatus, the tissue was allowed to equilibrate for 30 min while being perifused continuously with basal release medium at a rate of 124 μl/min. Gillson peristaltic pumps (Middleton, WI) controlled the flow rate of the medium. After the equilibration period, a 5-min baseline fraction was collected on ice. The tissue was then exposed to a HiKCl (release) medium that contained 30 m*M* KCl, 1.26 m*M* $CaCl_2$, and 57 m*M* NaCl, as well as the other components described above (pH 7.4). The tissue from each control or radiation group, depending upon the particular experiment, was treated with one of the agents (delivered in the release medium) under study. These included: (a) 100 μ*M* of A23187 (Behring Diagnostics, La Jolla, CA), (b) 20 μ*M* IP_3 (Sigma, St. Louis, MO) (c) 500 μ*M* OXO, or (d) HiKCl alone. Five-minute fractions continued to be collected on ice for 30 min. The fractions were collected into tubes containing 0.3 ml of cold 0.4 *M* perchloric acid, 0.05% sodium metabisulfite, and 0.10% EDTA. These samples were then stored at −80°C for later analysis of DA using high-performance liquid chromatography (HPLC) coupled to electrochemical detection.

The HPLC system consisted of a Varian Model 5000 ternary chromatograph, a Varian 401 data system, a Varian Model 8055 autosampler, and a Valco air-actuated injector with a 50-ml loop (all from Varian Associates, Sunnyvale, CA). The effluent was monitored with a Bioanalytical Systems LC-4B amperometric detector using a glassy carbon electrode. The detector potential was set at 0.72 V as an $Ag/AgCl_2$ reference electrode with a sensitivity of 10 nA/V. The mobile phase consisted of a filtered, degassed 100 m*M* KH_2PO_4 buffer containing 3 m*M* 1-heptanesulfonic acid, 100 μ*M* EDTA, and 8% (V/V) acetonitrile (pH 3.6.) The components were eluted off a Waters 10 μm particle, μBondapak C_{18} reverse-phase column (30 × 0.39 cm; flow rate, 1 ml/min) maintained at 30°C. Results were calculated relative to known previous standards that were analyzed on the HPLC under similar conditions. Data were expressed as pmol/mg protein as analyzed using the procedure of Lowry *et al.* (*21*).

Data Analysis

Data from the perifusion experiments were analyzed by first computing difference scores by subtracting the peak amount (pmol/mg protein) of DA released to the release medium alone from that released under each of the other conditions (i.e., OXO, A23187, IP_3; see Results). These difference scores were then analyzed by analyses of variance and post-hoc *t* tests.

RESULTS

Release of DA to HiKCl Alone

An examination of the release of DA to HiKCl indicated that there were significant differences among the groups [$F(2,52) = 6.25$, $P < 0.005$]. Subsequent post-hoc *t* tests revealed that the 1.0-Gy group had significantly higher release of DA than either the 0.1-Gy or control groups [$t(53)$ 1.0 Gy vs 0.1 Gy = 3.62, $P < 0.001$; 1.0 Gy vs control = 2.59, $P < 0.02$]. The release of DA in the 0.1-Gy group did not differ significantly from that in the control group (0.1 Gy vs control $t < 1$, $P > 0.05$). For this reason subsequent analyses of the degree of enhancement by OXO, IP_3, or A23187 were carried out by analyzing the differences be-

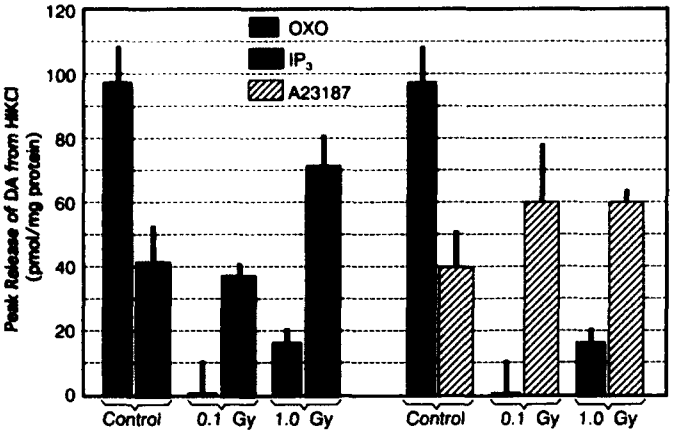

FIG. 1. Effects of exposure to 0.1 or 1.0 Gy of ^{56}Fe particles on the release of dopamine (DA) stimulated by oxotremorine (OXO), 1,4,5-inositol triphosphate (IP_3), or A23187.

tween HiKCl and the respective pharmacological treatment.

Enhancement of Release of DA by Oxotremorine, IP_3, or A23187

Comparisons of A23187 and oxotremorine. As shown in Fig. 1, there were differences among the groups in the ability of A23187 or OXO to enhance the release of DA from striatal tissue. Analysis of variance revealed these differences to be significant [F test for radiation condition × OXO-A23187 (2,32) = 15.85, $P < 0.001$]. Subsequent Duncan's post tests ($df = 32$) showed that OXO-enhanced K^+-evoked release of DA was significantly greater in the nonirradiated group than that by either the 0.1-Gy ($P < 0.001$) or 1.0-Gy ($P < 0.001$) irradiated groups, and that the irradiated groups did not differ from each other ($P > 0.05$). There were no differences among the various groups in the A23187 enhancement of K^+-evoked release of DA (all P values > 0.05, Duncan's tests). However, as can be seen from Fig. 1, A23187 produced significantly less enhancement of K^+-evoked release of DA than OXO in the control group (Duncan's test, $P < 0.05$). Because it was possible that the failure to find any group differences in the enhancement of K^+-evoked release of DA by A23187 was the result of reductions in the responses of the tissue from the controls, we also examined the differences between the effect of OXO and A23187 on K^+-evoked release of DA within each irradiated group. Duncan's tests revealed significant differences in both groups (0.1 Gy A23187 vs 0.1 Gy OXO, $P < 0.01$; 1.0 Gy A23187 vs 1.0 Gy OXO, $P < 0.05$), indicating that A23187 was significantly more efficacious than OXO in enhancing K^+-evoked release of DA in these irradiated groups.

Comparisons of IP_3 and oxotremorine. Similar results were seen with respect to IP_3 and OXO. As shown in Fig. 1, IP_3 was more effective in enhancing K^+-evoked release of DA than OXO in the irradiated groups [F test for radiation condition × OXO-IP_3 (2,27) = 9.21, $P < 0.001$]. There were no differences among the various groups in IP_3-enhanced K^+-evoked release of DA (Duncan's tests, all Ps > 0.05), while as shown above, enhancement of K^+-evoked release of DA was seen only in the control (nonirradiated) groups. However, as with A23187, enhancement of K^+-evoked release of DA by IP_3 was actually less than that seen with OXO in the control group (Duncan's test, $P < 0.5$; $df = 27$). Thus we also examined the differences between IP_3 and OXO in the irradiated groups. Comparisons between IP_3 and OXO within each irradiated group indicated that there was a significant difference between enhancement of K^+-evoked release of DA by OXO and IP_3 in the 1.0-Gy group, indicating that IP_3 was effective in enhancing the release of DA in this group (see Fig. 1), but this effect was not seen in the 0.1-Gy group. Thus these findings indicated that both IP_3 and A23187 were more effective than OXO in enhancing K^+-evoked release of DA in the 1.0-Gy group, while the reverse was true for the control group.

DISCUSSION

The present results showed that once the initial "steps" in the signal transduction sequence were "bypassed," and Ca^{2+} was mobilized directly by the addition of A23187, enhancement of K^+-evoked release of DA was significantly greater in both irradiated groups (0.1 and 1.0 Gy) than it was in OXO groups. Similar results were seen with respect to the enhancement of K^+-evoked release of DA by IP_3. However, in this case statistically significant effects of IP_3 were seen only in the 1.0-Gy group. The 0.1-Gy group showed a trend toward an increase, but it was not significant.

While both A23187 and IP_3 appeared to have greater effects than OXO in the irradiated groups, they were less effective than OXO in the controls. While we cannot explain the loss of effectiveness of these compounds in the controls, these data suggest that the signal transduction initiated by mAChR in the irradiated rats is primarily intact below the ligand–mAChR–G protein interface. Preliminary results from ongoing experiments undertaken to determine the locus of the radiation-induced deficits in mAChR signal transduction support this hypothesis. They have revealed that this decrement occurs early in the process and probably involves alterations in the signal transduction process, possibly in the mAChR or its respective G protein. There are three critical areas that may be important. First, there may be radiation-induced neuronal loss which includes reductions in mAChR concentrations. It is known that exposure to ^{56}Fe at these doses can produce profound neuronal loss in the substantia nigras (Joseph *et al.*, in preparation). There is also some evidence in aged animals which suggests

that the degree of OXO-enhanced K^+-evoked release of DA is dependent on the number of striatal mAChR (*22*). If this is also true after irradiation, then this would also contribute to the reductions in OXO-enhanced K^+-evoked release of DA.

A second area that may contribute to these reductions probably involves radiation-induced deficits mAChR–G protein coupling (the first step in signal transduction). At least two indices of receptor–G protein coupling appear to be altered in the striata of irradiated rats: (a) the ability of the receptor to uncouple from its respective G protein (Joseph *et al.*, in preparation) and enter a low-affinity state upon stimulation, and (b) the ability of muscarinic agonists to stimulate low-K_M GTPase activity.[5]

Third, it is also possible that the free radicals produced during heavy-particle irradiation may induce neuronal membrane structural and functional alterations that may involve changes in lipid content, increases in membrane rigidity, or protein crosslinking (*23*). These membrane changes probably occur through free radical-induced lipid peroxidation.

We are presently carrying out experiments on putative effects of free radicals on membranes. However, until they are completed some evidence can be extrapolated from the findings in animals where damage from free radicals has taken its toll on central nervous system function. Extensive research has indicated that senescent organisms represent one such group (*24–26*). It has been postulated for a number of years that the accumulated effects of free radicals produced during normal metabolism are responsible for the alterations in the structure and function of membranes seen in senescence (*23*).

Thus, when these same parameters [i.e., motor behavior (*6, 7*) and enhancement of K^+-evoked release of DA by OXO (*20*)] were examined in middle-aged (12 months) and aged (24 months) rats, similar deficits were observed. These experiments indicated that there were declines in motor behavior and OXO-enhanced K^+-evoked release of DA that were similar to those found in irradiated animals. Subsequent experiments have shown that: (a) A23187 or IP_3 applied to striatal slices obtained from senescent rats yielded results similar to those seen here, (b) carbachol-stimulated low-K_M GTPase activity is reduced in striatal tissue obtained from aged animals (*27*), and (c) the ability of mAChR to uncouple from or remain coupled to its respective G protein is altered during aging (*28*).

Taken together, these results suggest that the deficits induced by exposure to ^{56}Fe particles or those that occur during aging may involve alterations in membrane integrity through lipid peroxidation. These changes, in turn, can alter the responsiveness, integrity, and transduction in a variety of systems. In the present case it could mean that radiation-induced alterations in the membrane may alter mAChR–G protein coupling/uncoupling, resulting in decreased receptor sensitivity.

These experiments provide further evidence for the "age–radiation parallel" hypothesis (*29–32*). However, there are at least two other important implications of these findings: (a) Since many of the astronauts are middle-aged or approaching middle age, these particles will be impinging upon nervous systems that are already showing the deleterious effects of age. Thus there may be immediate or delayed (as the individual continues to age) effects upon motor or cognitive performance. (b) Since oxidative damage may also be responsible for the deficits seen in aging, as well as in diseases such as tardive dyskinesia (*33*), Parkinson's disease (*34*), and Alzheimer's disease,[6] continued efforts to use radiation and aging models in concert should provide useful information on the role of lipid peroxidation induced by free radicals in central neuronal function.

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