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Decreased Efficacy of Inositol 1,4,5-Trisphosphate to Elicit Calcium Mobilization from Cerebrocortical Microsomes of Aged Rats

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SUMMARY

The effects of aging on the ability of brain microsomes to sequester calcium in response to ATP stimulation and to release calcium in response to inositol 1,4,5-trisphosphate (IP_3) stimulation were examined. Calcium uptake and release were compared in microsomal preparations from the cerebral cortex, hippocampus, thalamus, and cerebellum of 3-, 16-, and 28-month-old male Fischer 344 rats. No age-related differences were found in the ability of brain microsomes to sequester calcium in response to ATP stimulation. A maximally effective concentration of IP_3 (1 μM) released approximately 30% of the calcium sequestered by microsomes. This was observed in all brain regions and age groups studied except in the cerebral cortex, where the amount of calcium released by IP_3 was reduced by 50% in the oldest age group. Concentration-response curves for IP_3 in this brain region from 3- and 28-month-old rats confirmed that the maximally effective concentrations, the EC_{50} values, and the Hill coefficients did not differ with aging. These data indicate that the efficacy of IP_3 is selectively diminished in the cerebral cortex of aged rats and that this age-related change may contribute to the attenuated responsiveness of neurons in this brain region to activation by phosphoinositide-coupled receptors.

It is now well established that many hormones, neurotransmitters, and therapeutic agents exert their effects on target cells via the phosphoinositide second messenger system. Through this mechanism, receptor stimulation causes the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate into two products, 1,2-diacylglycerol, which activates protein kinase C (1), and IP_3, which serves to increase the concentration of cytosolic free calcium (2). Changes in the concentration of intracellular calcium are known to regulate many cellular processes (3).

As the proposed second messenger in the phosphoinositide transduction system, IP_3 links receptor activation with the release of internally stored calcium. IP_3 has been shown to trigger calcium mobilization from a wide variety of peripheral cells and tissues (4), as well as from the brain (5–8). Its mechanism of action is thought to involve an IP_3-specific receptor that is located intracellularly (9). Binding sites specific for IP_3 have been shown to exist in many peripheral and central tissues (10–14). The IP_3 receptor protein has been purified and functionally reconstituted into lipid vesicles (15), and its gene has been cloned, sequenced, and expressed in transfected mammalian cells (16). Through its receptor, IP_3 is thought to trigger the mobilization of calcium from intracellular stores that are distinct from the mitochondrial pools (17). A recent immunohistochemical labeling study has localized the IP_3 receptor protein to the endoplasmic reticulum in cerebellar Purkinje neurons (18). However, other possible sites of action for IP_3 have been suggested, such as a specialized vesicular system in close association with the plasma membrane (11) and the calciosome (19).

Many processes associated with calcium homeostasis are known to change with the aging process. For example, sodium-dependent calcium transport across the plasma membrane is diminished with aging in brain synaptic membrane preparations (20), as well as in renal cells (21), from the rat. Voltage-dependent calcium uptake into rat brain synaptosomes is also diminished in aged animals (22–24). In addition, the rate of ATP-stimulated calcium uptake by mitochondria from both rat heart (25) and brain (23) is attenuated with aging. Collectively, these studies indicate that the aging process can decrease the movement of calcium across membrane systems.

In the present study, we have examined the effects of aging

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ABBREVIATIONS: IP_3, D-*myo*-inositol 1,4,5-trisphosphate; 2,4,5-IP_3, D-*myo*-inositol 2,4,5-trisphosphate; VO_4^{3-}, sodium orthovanadate; EGTA, ethylene glycol bis(betaaminoethylether)-*N*,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

on the ability of brain microsomes to sequester calcium in response to ATP stimulation and to release calcium in response to the agonist IP_3. Microsomal calcium uptake and release were examined and compared in the cerebral cortex, hippocampus, thalamus, and cerebellum of 3-, 16-, and 28-month-old male Fischer 344 rats. Our results indicate that the aging process does not affect the capacity of brain microsomes to sequester calcium but selectively diminishes the efficacy of IP_3 to release calcium in the rat cerebral cortex.

Experimental Procedures

Materials. Indo-1, pentapotassium salt, was purchased from Molecular Probes, Inc. IP_3 and 2,4,5-IP_3 were obtained from Calbiochem and Boehringer Mannheim, respectively. ATP, VO_4^{3-}, and sodium azide were purchased from Sigma. All other chemicals were reagent grade.

Animals and tissue preparation. Male Fischer 344 rats of three age groups were used, 3, 16, and 28 months. All animals were obtained from the National Institute of Aging colony (Harlan Laboratories, Indianapolis, IN) and were housed in cages in laminar air-flow hoods with a 12-hr light/dark cycle. Water and food were available *ad libitum*. The animals were anesthetized with halothane before decapitation. The brains were removed quickly and chilled on ice. All subsequent procedures were conducted at 4°. Microsomes were prepared by a modification of the procedure described by Shah *et al.* (26). Whole brain or isolated brain areas were homogenized at approximately 500 rpm in 8 volumes of sucrose-HEPES buffer (320 mM sucrose, 3 mM HEPES, 0.1 mM EDTA, pH 7.4 adjusted with Tris base) using a Teflon/glass homogenizer. The homogenate was centrifuged at 1,800 × *g* for 6 min and the resulting supernatant (S_1) was saved. The pellet was resuspended in half of the original volume and centrifuged at 1,000 × *g* for 6 min. The resulting supernatant (S_1) was combined with the first S_1 supernatant and centrifuged at 17,000 × *g* for 20 min. The S_2 supernatant was then centrifuged at 100,000 × *g* for 35 min, and the resulting microsomal pellet (P_3) was resuspended gently in assay buffer [150 mM KCl, 20 mM HEPES, 3 mM $MgCl_2$, 0.5 mM sodium azide (a mitochondrial poison), pH 7.0 adjusted with Tris base].

Microsomal calcium flux assay. The procedure used for measuring microsomal calcium uptake and release has been fully described elsewhere (5). In brief, aliquots of microsomal suspension (approximately 0.2–0.3 mg of protein) were combined in a quartz cuvette with warm assay buffer (35°). The extramicrosomal calcium concentration was measured using the fluorescent indicator Indo-1 (final concentration, 0.45 μM). Emission of Indo-1 was monitored at 410 and 500 nm (10 nm bandpass filters; Microcoatings, Inc.) at an excitation wavelength of 345 nm, using a T-format H&L Instruments HH-3 spectrofluorometer equipped for continuous data acquisition. To determine the extramicrosomal calcium concentration, the method of Grynkiewicz *et al.* (27) was used. R_{max} was determined in the presence of 2.7 mM free calcium and R_{min} in the presence of 2 mM EGTA. Sample temperature was maintained thermostatically at 32°. ATP was added at 32 sec (final concentration, 1 mM) following the initiation of data acquisition and VO_4^{3-} at 90 sec (final concentration, 50 μM). Water or varying concentrations of 1,4,5- or 2,4,5-IP_3 were added at 152 sec. The amount of calcium sequestered or released by the microsomes was calculated from the difference between the free calcium concentration of the incubation buffer before and after stimulation and was expressed as nmol/mg of microsomal protein. Protein concentrations were determined by the method of Bradford (28), using bovine serum albumin as a standard.

Marker enzyme assays. Na^+/K^+-ATPase (EC. 3.6.1.3) activity in initial homogenates and microsomal preparations was measured after freeze-thawing by using an enzyme-coupled kinetic assay with pyruvate kinase and lactate dehydrogenase (29). Cytochrome *c* reductase (EC 1.6.2.1) and succinic dehydrogenase (EC 1.3.99.1) activities were determined by the methods of Baron and Tephly (30) and Seubert (31), respectively. Protein concentrations were determined, using bovine serum albumin as a standard, by the method of Lowry *et al.* (32).

Data analyses. Concentration-response curves were analyzed by computer-assisted curve fitting based on the logistic function. For the cortical experiments, data for calcium uptake and calcium release at each age group studied were compared using one-way analysis of variance with age as a between-groups factor. When a significant interaction was indicated, follow-up analyses were performed using the Neuman-Keuls test. Data from the marker enzyme assays, as well as from the IP_3 concentration-response analyses, were compared using the unpaired Student's *t* test. Differences were considered statistically significant when $p < 0.05$.

Results

Characteristics and specificity of the calcium flux assay. Data from a typical microsomal calcium influx assay are shown in Fig. 1. Extramicrosomal calcium was measured spectrofluorometrically as a function of time. The addition of 1 mM ATP (at 32 sec following the initiation of data acquisition) resulted in a rapid uptake of extravesicular calcium. In the absence of ATP, microsomes slowly leaked calcium. By 90 sec, approximately 3 nmol of calcium/mg of protein were sequestered as a result of ATP stimulation. In this system, ATP-stimulated calcium uptake routinely achieved equilibrium by 90 sec. At 90 sec, the addition of 50 μM VO_4^{3-} effectively inhibited Ca^{2+}-ATPase activity and prevented further calcium uptake (5). IP_3 was then added at 152 sec; this immediately and rapidly induced the mobilization of vesicularly stored calcium. A maximal response was achieved within 10 sec. A maximal concentration of IP_3 (1 μM) released approximately 1 nmol of calcium/mg of protein, or 33% of the amount taken up due to ATP stimulation. The presence of VO_4^{3-} prevented the reuptake of calcium following IP_3 stimulation. In this assay system, the calcium-mobilizing effects of IP_3 were totally dependent upon prior stimulation of calcium uptake with ATP (data not shown).

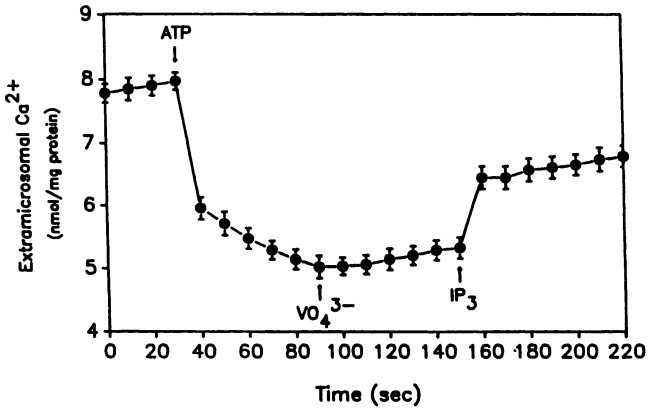

Fig. 1. Time course of changes in extramicrosomal calcium measured spectrofluorometrically. These data are representative of a typical experiment and were generated using microsomes prepared from the whole brain of a 3-month-old rat. An aliquot of microsomes (approximately 0.3 mg of protein) was combined with assay buffer and Indo-1 in a quartz cuvette. Under basal conditions, microsomes slowly leaked calcium. At 32 sec following initiation of data acquisition, ATP (final concentration, 1 mM) was added to stimulate calcium uptake into the microsomes and, at 90 sec, VO_4^{3-} (final concentration, 50 μM) was added to inhibit Ca^{2+}-ATPase activity. At 152 sec, IP_3 (final concentration, 1 μM) was added to elicit calcium release from the microsomes. At this concentration, IP_3 stimulated the release of 1 nmol of calcium/mg of protein or 33% of the amount sequestered following ATP addition. Mean values ± standard deviations are shown for a single experiment performed in triplicate.

In order to characterize the pharmacological selectivity of the microsomal calcium-flux response, concentration-response curves were generated using two isomeric forms of IP_3 and cortical microsomes. The biologically active isomer 1,4,5-IP_3 was approximately 8-fold more potent. The EC_{50} value for 1,4,5-IP_3 was 150 nM, whereas that for 2,4,5-IP_3 was 1.2 μM. Although 2,4,5-IP_3 was less potent, it displayed full agonist activity relative to that of the 1,4,5-isomer; approximately 30% of the calcium taken up following ATP addition was released by a maximally effective concentration of either compound. The Hill coefficients for the two concentration-response curves were 1.1 for 1,4,5-IP_3 and 1.2 for 2,4,5-IP_3, suggesting agonist interaction with a single class of receptor. Similar results were obtained when concentration-response curves were generated using microsomes from other brain regions (data not shown).

Effects of aging on microsomal calcium uptake and mobilization. The effects of aging on the ability of brain microsomes to sequester calcium in response to ATP stimulation and to release calcium in response to IP_3 stimulation were compared in four separate brain regions, cerebral cortex, hippocampus, thalamus, and cerebellum. As a preliminary study, the same brain regions from six animals of each age were pooled and a single microsomal pellet representing a particular brain region from each age group was prepared and assayed. There were no apparent differences between age groups in the capacity of microsomes to sequester calcium in response to ATP stimulation in any of the brain areas examined (Fig. 2A). The average amount of calcium sequestered by the cerebellum was 3.4 nmol/mg of protein, followed by the cerebral cortex at 2.6, the hippocampus at 2.1, and the thalamus at 1.4 nmol/mg of protein.

The ability of IP_3 to elicit calcium mobilization from microsomes loaded with calcium in the presence of ATP was tested using a maximally effective concentration of IP_3 (1 μM) (Fig. 2B). Like the calcium uptake data, the rank order of responsiveness to the effects of IP_3 was cerebellum > cerebral cortex > hippocampus > thalamus. In addition, the results suggested that there were no differences between age groups in the extent to which 1 μM IP_3 could elicit calcium mobilization in the cerebellum, hippocampus, or thalamus. The average amounts of calcium mobilized from these brain areas were approximately 1.1, 0.59, and 0.42 nmol/mg of protein, respectively. In the cerebral cortex, however, the data suggested that there was a diminished responsiveness to the effects of this concentration of IP_3 at 28 months of age, as compared with 3 or 16 months (Fig. 2B). The amount of calcium released in response to IP_3 at the two younger ages was approximately 0.82 nmol/mg of protein, whereas that released from the microsomes at 28 months of age was 0.47 nmol/mg of protein. This apparent difference was also evident when the amount of calcium released in response to IP_3 was expressed as a percentage of that sequestered in response to ATP stimulation. In all four brain regions at all ages, except in the cerebral cortex at 28 months, 1 μM IP_3 stimulated the release of approximately 30% of the calcium sequestered due to ATP stimulation. In the cerebral cortex at 28 months of age, however, the response was approximately half of that found for all other brain areas and ages.

To test for the significance of the apparent age-related decrease in IP_3 responsiveness observed in the pooled cerebral cortical tissue (Fig. 2B), microsomes were prepared from cerebral cortex from three animals of each age and tested individually for their responsiveness to ATP and IP_3 stimulation. No differences were found between age groups in the capacity of the cortical microsomes to sequester calcium in response to 1 mM ATP (Fig. 3). The amount of calcium sequestered was approximately 2.5 nmol/mg of protein at all ages. However, the magnitude of the calcium-mobilizing effect of 1 μM IP_3 was significantly attenuated by 50% at 28 months of age, as compared with 3 or 16 months (Fig. 3).

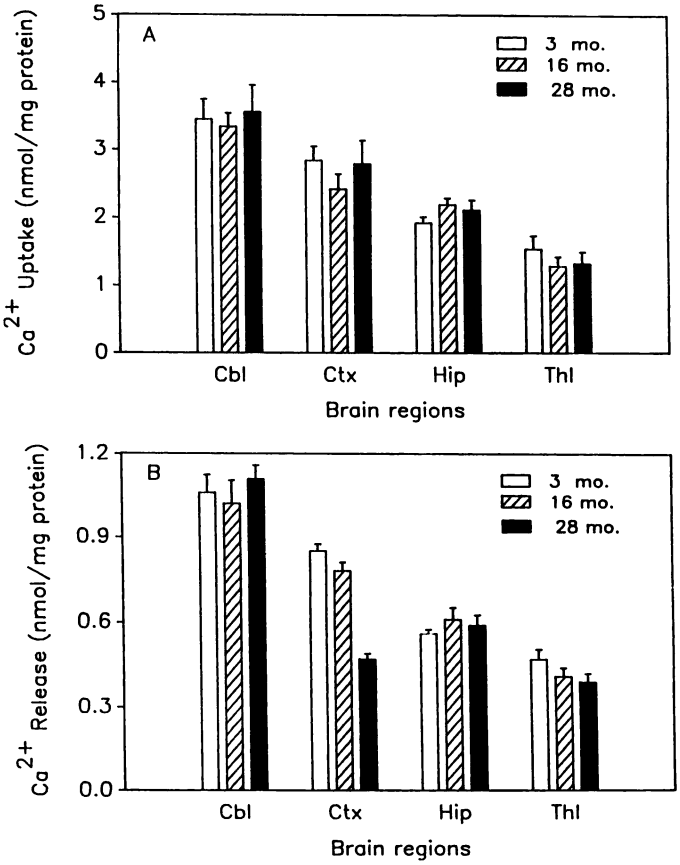

Fig. 2. Uptake and release of microsomal calcium from four different brain regions as a function of age. Microsomes were prepared from the cerebellum (*Cbl*), cerebral cortex (*Ctx*), hippocampus (*Hip*), and thalamus (*Thl*) by pooling the same brain regions from six rats at each age. Thus, data are representative of a population mean and were not analyzed for statistical differences. Assay conditions were identical to those in Fig. 1. Mean values ± standard errors are shown for a single experiment performed in triplicate. A, The capacity of different microsomal preparations to sequester calcium upon addition of 1 mM ATP. B, The ability of a maximally effective concentration of IP_3 (1 μM) to elicit calcium release from microsomes loaded with calcium in the presence of ATP.

To determine whether the aging process alters the efficacy or the potency of IP_3 in the cerebral cortex, full concentration-response curves were generated using microsomal preparations from young and aged rats. IP_3 was a more powerful agonist at 3 months of age than at 28 months of age (Fig. 4). A maximally effective concentration of IP_3 (1 μM) released a statistically larger amount of the ATP-dependent calcium pool from cortical microsomes of young animals than from the aged animals; mean values ± SE were 32 ± 2.0% and 19 ± 1.8%, respectively. Neither the EC_{50} values nor the Hill coefficients were significantly different. The EC_{50} values were 150 ± 4.2 nM (mean ± SE) at 3 months and 160 ± 3.7 nM at 28 months, and the Hill coefficients were 0.96 ± 0.05 and 1.1 ± 0.08, respectively.

Marker enzyme composition of cortical microsomes.

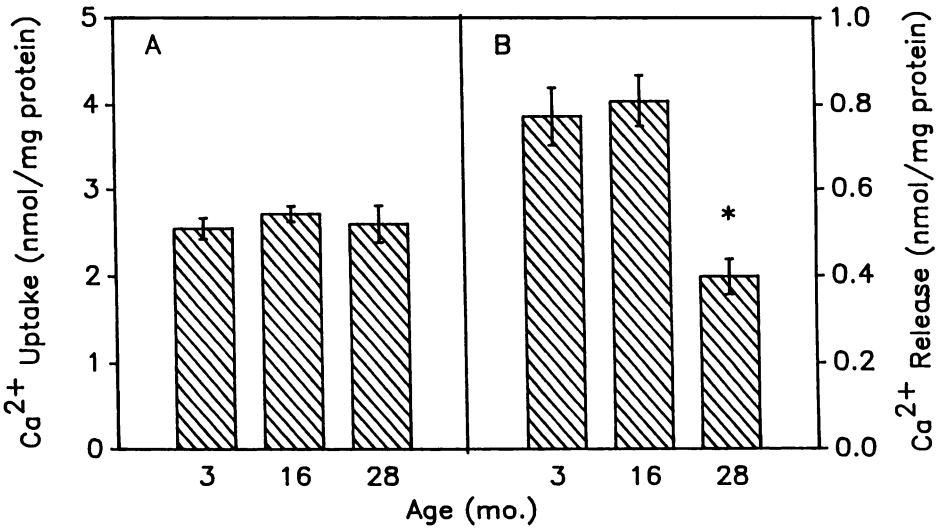

Fig. 3. Comparison of the effects of ATP on calcium uptake (A) and of IP_3 on calcium release (B) in cortical microsomes from three different age groups of rats. Assay conditions were identical to those in Fig. 1. Data are the mean values ± standard errors for three separate experiments and three rats in each age group. One-way analysis of variance revealed a significant interaction between age and responsiveness to IP_3 ($F(2,6) = 114.61$). *$p < 0.05$.

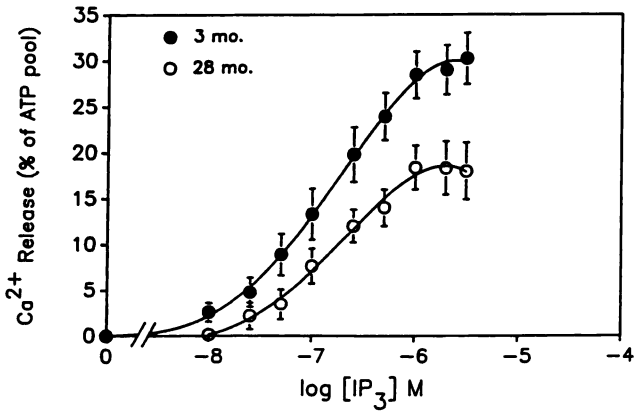

Fig. 4. The efficacy of IP_3 is reduced in cortical microsomes from 28-month-old rats. Assay conditions were identical to those in Fig. 1 except that various concentrations of IP_3 were used to elicit calcium release. The amount of calcium released at each concentration is expressed as a percentage of that which was sequestered due to ATP stimulation. Mean values ± standard errors are shown for four separate experiments and four rats in each age group.

To determine whether the composition of the microsomes was uniform between the youngest and the oldest age groups, the specific activities of three marker enzymes were measured in both the initial tissue homogenates and the final microsomal pellets from young and old animals. As seen in Table 1, no differences were found between young and aged animals in the specific activities of cytochrome *c* reductase, succinic dehydrogenase, or Na^+/K^+-ATPase in either the initial tissue homogenates or the final microsomal pellets. The specific activities of cytochrome *c* reductase, an endoplasmic reticulum marker, and of Na^+/K^+-ATPase, a plasma membrane marker, were enriched 4- and 2-fold, respectively, in the final microsomal pellets. Succinic dehydrogenase activity, a mitochondrial marker, was approximately 25-fold lower in the final microsomal pellets than in the initial homogenates from both young and aged animals.

Discussion

In the present study, we have examined the effects of aging on the agonist properties of IP_3 in the rat brain. Our results revealed that the efficacy of this second messenger is selectively diminished in the cerebral cortex of aged rats. At 28 months of age, the maximal effectiveness of IP_3 in eliciting calcium mobilization was reduced by 50%, as compared with the responsiveness of this tissue from young adult animals. Agonist potency was unaltered. In the hippocampus, thalamus, and cerebellum, however, no age-related changes in the agonist properties of IP_3 were observed.

The general characteristics of the microsomal calcium flux assay used in the present study and the responses elicited by IP_3 are very similar to those reported by other investigators. Under the conditions used in this study, the ability of IP_3 to induce microsomal calcium efflux was totally dependent upon prior stimulation of Ca^{2+}-ATPase activity by the addition of ATP. This dependence of the IP_3 response on preloading of microsomes with calcium has been documented in several studies (5, 7, 8, 33, 34). The amount of calcium sequestered by microsomes and, consequently, the absolute amount released

TABLE 1

Specific activities of marker enzymes in homogenates and microsomes from cerebral cortex of young and aged rats

Enzyme	Specific activity					
	Initial homogenate		Microsomal pellet		Enrichment[a]	
	Young[b]	Aged[c]	Young	Aged	Young	Aged
	μmol/mg of protein/hr					
Cytochrome *c* reductase	0.24 ± 0.01	0.25 ± 0.03	0.89 ± 0.02	0.95 ± 0.08	3.8 ± 0.18	4.0 ± 0.46
Succinic dehydrogenase	9.7 ± 0.35	11 ± 1.1	0.39 ± 0.07	0.47 ± 0.05	0.04 ± 0.01	0.05 ± 0.01
Na^+/K^+-ATPase	17 ± 0.87	17 ± 1.2	31 ± 0.77	36 ± 3.5	1.9 ± 0.05	2.2 ± 0.24

[a] Enrichment is the ratio of the enzymatic activity in the microsomal pellet to that in the initial homogenate.
[b] Young animals were 3 months of age; mean ± standard error ($n = 4$).
[c] Aged animals were 28 months of age; mean ± standard error ($n = 4$).

by the actions of IP_3 varied with brain region. The cerebellum possessed the largest capacity to accumulate calcium and, likewise, exhibited the greatest response to a maximally effective concentration of IP_3 (see Fig. 3). All four of the brain areas, however, produced the same 30% response to a maximally effective concentration of IP_3. Other investigators have reported similar results (6, 33). The significance of the fact that IP_3 was able to mobilize only a small fixed percentage of the calcium accumulated by the microsomes is unclear. One explanation is that calcium is sequestered into many different pools, only a small percentage of which are sensitive to IP_3 stimulation (35).

The responses elicited by IP_3 in this study were rapid and concentration dependent and displayed appropriate pharmacological selectivity. The calcium-mobilizing effects of IP_3 were complete within 10 sec (Fig. 1). This observation is again consistent with the results of other studies (6, 7). 1,4,5-IP_3 is the naturally occurring agonist and possesses greater potency for mobilizing calcium than its purely synthetic 2,4,5-isomer, due to the phosphate group in the 1-position (2). In agreement with our results, Stauderman *et al.* (7) reported EC_{50} values for these two agonists of 60 and 800 nM, respectively, and Supattapone *et al.* (36) found an EC_{50} value of 100 nM for IP_3. These results agree well with those found in this study. Other studies, however, have reported larger EC_{50} values for IP_3 (8, 33, 37). The range in the reported potency of IP_3 may be the result of several factors. It has been suggested that metabolism is the principle mechanism responsible for terminating the actions of IP_3 (7, 37). Varying amounts of 5-phosphatase or 3-kinase activities in different preparations could alter the observed agonist potency. Another explanation may be differences in the phosphorylation state of the IP_3 receptors present in the various preparations. Recently, Supattapone *et al.* (36) found that phosphorylation of purified IP_3 receptors from rat cerebellum by cyclic AMP-dependent protein kinases reduces agonist potency by 90%. A third possibility would be the existence of heterogeneous IP_3 receptor subtypes (38) with a range of affinities for IP_3.

A general finding in the central nervous system has been that aging alters many processes associated with calcium homeostasis (see Ref. 39 for review). A majority of the studies using rats suggest that aging attenuates calcium regulation. Some of the processes affected include calcium channel affinity for dihydropyridines (40), sodium-dependent transport (20), ATP-dependent mitochondrial uptake (25), and calmodulin content (41). Ishikawa *et al.* (42), using rat parotid cells, were the first to show that aging reduces by approximately 40% the ability of IP_3 to stimulate calcium efflux. Our results show that this age-related change also occurs in the central nervous system. Interestingly, however, this change was only observed in one of the brain regions investigated.

The results of the concentration-response analysis shown in Fig. 4 indicate that aging diminishes the efficacy, but not the affinity, of IP_3 to mobilize calcium in cortical microsomes. Many factors could govern the tissue responsiveness to IP_3. These factors include differences in 1) the rate of IP_3 inactivation; 2) IP_3 receptor density, affinity, or coupling; 3) the availability of calcium within IP_3-sensitive pools; and 4) the composition of the microsomal preparations. Our results cannot be explained by an age-related change in the metabolism of IP_3, inasmuch as a shift in the potency of IP_3 would be expected if its inactivation rate were altered. On the other hand, our results are consistent with the suggestion that aging reduces the number or functional coupling of IP_3 receptors in the cerebral cortex of rats. Although specific binding sites for IP_3 have been identified and localized throughout rat brain (14) and have been functionally linked to calcium release (15, 16), the effect of aging on these receptors has not been reported. Our findings indicate that such studies would be of interest. The fact that aging did not reduce the capacity of brain microsomes to sequester calcium in response to ATP stimulation in any of the brain regions studied suggests that the observed loss of IP_3 efficacy with aging is not due to a reduction in the availability of releasable calcium. However, a limitation is that we might not have detected a specific age-related reduction in calcium sequestration into IP_3-sensitive pools (35). Microsomes are a preparation enriched in endoplasmic reticulum, but other membrane components are also present that might affect the relative distribution of calcium during ATP-stimulated uptake. However, this explanation is unlikely because, based on the specific activities of marker enzymes for endoplasmic reticulum, plasma membrane, and mitochondria (Table 1), no age-related differences in the composition of the microsomal preparations were detected.

Several electrophysiological studies in rat brain have shown that neuronal responsiveness to certain neurotransmitters is diminished with aging. In the cerebral cortex, the response of neurons to both norepinephrine and acetylcholine is attenuated (43) and, in the cerebellum, the effect of norepinephrine on neuronal firing rate is reduced in aged rats (44). Likewise, in the hippocampus, neuronal responsiveness to norepinephrine, acetylcholine, and serotonin is diminished (45, 46). Of potential significance is the fact that each of these neurotransmitter systems possesses a receptor subtype (i.e., α_1-adrenergic, M_1 muscarinic cholinergic and 5-HT_2 serotonergic receptors) that is coupled to the phosphoinositide transduction system (47). In accordance, we originally hypothesized that a common mechanism might exist that would account for the diminished responsiveness to these neurotransmitters, an age-related loss in responsiveness to the second messenger IP_3. Although our data in the cerebellum and hippocampus do not support this hypothesis, our results in the cerebral cortex do. The diminished efficacy of IP_3 in this latter brain region may contribute to the reported decrease in neuronal responsiveness to the effects of both norepinephrine and acetylcholine with aging. Indeed, our findings suggest that the effects of any neurotransmitter or hormone that is dependent on the phosphoinositide transduction system should be diminished in the cerebral cortex of aged rats.

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