Age-related changes in muscarinic receptor and post-receptor mechanisms in brain and ileum strip of rats

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Abstract. Age-related differences in the response of the cerebral cortex and ileum strip to a repeated treatment with an anticholinesterase compound, diisopropyl fluorophosphate (DFP) were evaluated in 3- and 24-month Sprague-Dawley rats. The response was measured in terms of acetylcholinesterase (AChE) inhibition and total muscarinic receptor density (MACHRs, measured as $^3$H-QNB binding). At the end of DFP treatment there was a 75% inhibition of brain AChE and 30% inhibition of ileal AChE, independently of age. The adaptive down-regulation of brain MACHRs was more pronounced in aged than in young rats (50 and 25%, respectively), while that of ileal MACHRs was greater in young than in aged rats (50 and 35%). The normalization of cortical MACHRs was delayed in aged rats that of ileal MACHRs was delayed in young rats. As regards age-related changes of AChE and MACHRs in untreated rats, there was a 30% decrease of cortical and ileal AChE, no changes in Bmax of cortical MACHRs and a 45% deficit of ileal MACHRs. This was accompanied by only a little age-related decrease in sensitivity of the isolated ileum to cholinergic agonists. Additional experiments on the responsiveness of phosphatidyl inositol system stimulated with carbachol showed that accumulation of inositol phosphate both in cortical and ileum strip slices was higher in aged than in young rats. The overall data indicate that treatment- and age-related changes of AChR mechanisms in the ileum strip differ considerably from those in the brain. However, the increased efficiency of post-receptor mechanisms in old age is their common feature.

Key words: muscarinic acetylcholine receptors, rat aging, inositol phosphate accumulation, cerebral cortex, ileum smooth muscle
INTRODUCTION

The anticholinesterase (antiChE) compounds are widely used as pesticides. Their utilization in agriculture and household, in spite of neurotoxicity, increased considerably over the past decades, mainly in relation to their relatively short persistence in the environment. An additional interest for antiChE agents depends on their recent use in pharmacotherapy of Alzheimer disease, the most frequent dementia of old age (Becker and Giacobini 1988, Kumar and Calache 1991).

It is well known that repeated administration of such compounds induces the development of tolerance, and down-regulation of brain muscarinic acetylcholine receptor sites (MAChRs) has been recognized as the main mechanism responsible for this phenomenon (Russell and Overstreet 1987).

It has been shown in this laboratory that such adaptive mechanisms are present in the brain during the whole life-span, from late foetal life up to senescence (Michalek et al. 1986, Pintor et al. 1988, Michalek and Pintor 1990). However, the recovery of cortical, hippocampal and striatal MAChRs in aged rats was considerably delayed with respect to young and adult animals (Pintor et al. 1990).

As regards the autonomic nervous system, it is well known that in the ileum strip there is cholinergic innervation. About 90% of the specifically bound 3H-quinuclidinyl benzilate (3H-QNB) in the whole rat ileum is distributed in the longitudinal layer muscle (Ehlert et al. 1980). Moreover, the utilization of the ileum allows the evaluation of functional parameters such as contractile responsiveness to cholinergic agonists. It has been shown that persisting ChE inhibition causes a down-regulation of MAChRs in the ileum longitudinal muscle of young rats (Ehlert et al. 1980).

As a part of our study (Fortuna et al. 1991) there are no data in the literature regarding such adaptive changes of MAChRs in the ileum longitudinal muscle of aged rats. In the first part of the present study potential age-related changes in the response to an antiChE compound of cholinergic innervation of the ileum were evaluated in terms of acetyl-ChE (AChE) and MAChRs. As antiChE compound diisopropyl fluorophosphate (DFP), a liposoluble, irreversible ChE inhibitor, frequently used in biochemical investigations, was used as a model compound. Moreover, the recovery of AChE and MAChRs after termination of DFP treatment was also studied in young and aged rats. The data are compared with those obtained for the cerebral cortex of the same rats.

Since independently of DFP treatment considerable age-related changes in the ileal MAChRs have been found, the investigation was extended to age-related changes in post-receptor mechanisms, namely responsiveness of phosphatidyl inositol system stimulated by a muscarinic agonist, both in the cerebral cortex and ileum. Moreover, in the case of the latter tissue a functional parameter, i.e., in vitro contractility of the isolated ileum upon stimulation by cholinergic agonists, was measured in young and aged rats.

A part of the investigation has been already published (Fortuna et al. 1991).

METHODS

Animals and treatment

The experiments on AChE, MAChRs and isolated ileum contractility were performed on male Sprague Dawley rats, 3-4 (young) and 23-25 (aged) months purchased from Sprague Dawley (USA). The respective mean SE body weights (in g) were 280±6.1 and 592±20. The animals were maintained in an air conditioned room (21±1°C and 50±10% relative humidity, with light from 7 a.m. to 7 p.m.) and allowed free access to standard chow and tap water. Repeated treatment with DFP was performed according to a schedule previously standardized, which caused about a 75% inhibition of AChE and down-regulation of MAChRs in the cerebral cortex (Pintor et al. 1990). The rats were s.c. injected with DFP in arachis oil (doses in mg/kg: first 1.1, two of 0.7 and four of 0.35) on alternate days for 2 weeks. Young and aged control rats were treated with the vehicle. The rats were killed by decapitation 2, 7,
14, 21, 28 and 35 days after the last treatment (4 rats for each interval).

The experiments on age-related responsiveness of cholinergic muscarinic receptors in terms of accumulation of inositol phosphate were performed on male Sprague-Dawley rats of similar ages and weights purchased from Charles River Italia, (Calco, Como).

**Acetylcholinesterase activity**

Acetylcholinesterase activity was measured according to Ellman et al. (1961), as previously described (Michalek et al. 1990). The homogenates of the cerebral cortex were prepared at the ratio 1:10, those of ileum strip (longitudinal and circular muscle layers previously minced) at the ratio 1:25, with 0.32 M sucrose solution using Polytron. The samples of fresh homogenates (0.6-2.5 µl for brain and 5-7.5 µl for the ileum strip) were incubated in 39 mM phosphate buffer, pH 7.2, containing 0.21 mM 5,5'-dithiobis-2-nitrobenzoic acid in a final volume of 1.4 ml, at 25°C for 30 min (in duplicate). The determinations were performed in the presence of a selective inhibitor of pseudoChE, 0.01 mM iso-OMPA, added to the incubation mixture 15 min before the substrate acetylthiocholine (AcThCh) 0.56 mM. The absorbance at 412 nm was measured in a Gilford 2400 spectrophotometer. The enzymatic activity was expressed as nanomol of AcThCh hydrolyzed per min per mg protein.

**Muscarinic receptor binding sites**

Muscarinic receptor binding sites in the brain and ileum strip homogenates (frozen and stored at -80°C up to 2 weeks) were determined using the specific ligand L-^3^H-QNB, by the rapid filtration method as previously described (Michalek et al. 1990). The samples (50 µl, corresponding to 10-20 µg protein in the case of brain, and to 80-200 µg protein in the case of ileum strip) were incubated in 50 mM phosphate buffer, pH 7.4, containing six different concentrations of ^3^H-QNB (from 0.005 to 1.8 nM) in a final volume of 0.5 ml (in triplicate) in order to obtain linearity and saturation conditions. Parallel tubes which also contained 1 µM atropine sulfate were prepared to determine non-specific binding. After incubation at 37°C for 45 min the reaction was terminated by the addition of 4 ml of ice-cold phosphate buffer and immediate filtration through Whatman GF/B filters. The tubes were washed twice with an additional 4 ml of buffer, the filters placed in vials with 10 ml of Filter count (Packard) and radioactivity measured in a Packard Tricarb 4640 at a counting efficiency of 50%. The specific binding was calculated as total minus non-specific binding. The Scatchard analyses of specific ^3^H-QNB binding were carried out on six points with computer fitted regression lines (correlation coefficients r>0.86) and results expressed as Bmax in femtomol per mg protein, and Kd (pM).

**Accumulation of inositol phosphate (IP) induced by a cholinergic agonist carbachol**

The assay of IP was performed according to Brown et al. (1984) as previously described (Nalepa et al. 1989). The experiments were performed in balanced replications. Every day one young and one aged rat were processed. The cerebral cortex and the ileum strip were cut into 300x300 µm and 500x500 µm slices, respectively, using a McIlwain tissue chopper (Ek and Nahorski 1988). The slices of a single rat were immediately suspended in 50 ml of Krebs-Ringer medium (mM: NaCl 118, KCl 5, CaCl2 1.3, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, glucose 11.7; pH 7.4) and gassed with O2:CO2 (95:5) for 1 h at 37°C. Fifty µl of density-packed slices were pipetted into vials containing the above mentioned buffer, LiCl 5 mM and ^3^H-myoinositol 0.3 µM, (all final concentrations) in a final volume of 290 µl. After preincubation at 37°C for 30 min carbachol at 5 concentrations (from 10 to 1,000 µM) was added. The incubation continued for 45 min and was stopped by addition of 1 ml of a chloroform-methanol (1:2) mixture. The separation of phases and elution of IP was performed as previously described (Nalepa et al. 1989). The radioactivity of the eluate was counted in a Packard Tricarb 4640 scintillation
counter at approx. 50% efficiency. All assays were
carried out in triplicate. The basal IP accumulation
was expressed in dpm per tube (containing about
0.27 and 0.40 mg protein for the brain and ileum,
respectively). The IP accumulation elicited by car-
bachol was expressed as percentage of basal IP ac-
ccumulation.

Proteins were determined in the two preparations
according to the method of Lowry et al. (1951)
using bovine serum albumine as standard.

**Isolated ileum experiments**

The experiments were performed as previously
described (Fortuna et al. 1991). Two cm segments
of whole ileum taken about 1 cm above the ileo-
caecum junction were suspended in a 10 ml organ
bath containing Tyrode solution (mM: NaCl 137;
NaHCO₃ 12; KCl 2.7; MgSO₄ 1.0; NaH₂PO₄ 0.4;
CaCl₂ 1.8; glucose 5, pH 7.4) maintained at 37°C
and continuously gassed with O₂:CO₂ (95:5).

Isometric contractions were measured with a force
displacement transducer and recorded with a poly-
graph (Grass). Three muscarinic agonists: ace-
tylycholine, oxotremorine and carbachol were tested
consecutively on each organ. Acetylcholine and ox-
otremorine were added at final concentrations from
0.05 to 1 µM, while carbachol from 0.1 to 2 µM,
and washed immediately after full contraction. Maxi-
mal contractions of the ileum segments were tested
before and after the dose-response curve for each
agonist. Computer regression lines (correlation
coefficients r>0.75) based on at least five points
(each in duplicate) were obtained. The data were ex-
pressed as EC50 (µM).

**Statistics**

The data were processed by factorial analysis of
variance for repeated measures (2-way ANOVA).
Degrees of freedom were 1,42 for age, and 6,42 for
recovery rate and age x recovery rate interaction
calculations. For the data on IP formation degrees
of freedom were 1,40 (brain) and 1,36 (ileum strip)
for age, and 4,40 (brain) and 5,36 (ileum strip) for
agonist concentration and age x concentration inter-
action calculations. The accepted level of signific-
ance was P<0.05. Subsequently post-hoc analysis
was performed. To assess differences between
values of young and aged animals at each interval
(or concentration) Student’s t-test was used. To as-
sess differences between values at each interval and
controls in each age-group the calculations were
made by Student’s t-test with Bonferroni correction
for multiple comparisons (Godfrey et al. 1985).

**Materials**

³H-QNB (46 Ci/mmol) and ³H-myoinositol 15.4
Ci/mmol were purchased from New England Nu-
clear Corp., Boston MA (USA). DFP was obtained
from Fluka AG, Buchs (Switzerland).

**RESULTS**

**Age- and DFP-treatment related changes in
muscarinic receptor mechanisms**

During the first week of the treatment, i.e., from
the first to the third DFP administration, both young
and aged rats showed a typical syndrome of cho-
linergic stimulation (tremors, sweating, salivation
and diarrhoea). Severity and duration of the toxic
syndrome was markedly more pronounced in aged
rats, with a higher mortality rate (35%, P<0.05)
than in young rats (10%). During the second week of
treatment the cholinergic syndrome disappeared in
both age-groups. The overall body weight changes
(final vs. initial) consisted of 14% increase in young
and 9% loss in aged DFP-treated rats; there was a
15% increase in body weight of control young rats
and there were no changes in aged rats.

The data on brain and ileal AChE activity in the
two age-groups and the effects of DFP treatment are
presented in Fig.1. In untreated rats there was an
age-related decrease of enzymatic activity both in
the cerebral cortex and in the ileum strip (by about
30%). ANOVA showed significant age-related dif-
ferences for the cerebral cortex (F1,42=69.1,
P<0.001) and the ileum strip (F1,42=97.8,
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Fig. 1. Age-related differences in inhibition and recovery rate of AChE activity in the cerebral cortex and ileum strip following repeated administration of DFP to rats. First pair of columns on the left indicates untreated rats. Means ±SE (n=4). Significant differences between two ages at each interval (+++ P<0.001, ++ P<0.01, + P<0.05) according to Student’s t-test. Significant differences from pretreatment levels in the same age-group (** P<0.01, * P<0.05) according to Student’s t-test with Bonferroni correction for multiple comparisons. From Fortuna et al. 1991.

P<0.001). With regard to the effects of DFP, the AChE inhibition at the end of treatment was considerably higher in the brain (about 75%) than in the ileum strip (about 30%), independently of age. As regards the recovery rate, ANOVA showed significant differences both for the cerebral cortex (F6,42=57.9, P<0.001) and the ileum strip (F6,42=7.1, P<0.001). There was a significant interaction between age and recovery rate for the cerebral cortex (F6,42=5.9, P<0.01) but not for the ileum strip (F6,42=3.0, NS). Post-hoc analysis confirmed that the recovery rate was rapid in the cerebral cortex of young rats (normalization within 2 weeks) and considerably slower in aged rats (5 weeks); there were significant age-related differences in the enzymatic activity at almost all intervals, except at the termination of DFP treatment. With regard to the ileum strip, post-hoc analysis confirmed the lack of significant differences in the recovery rate between young and aged rats. In fact, the recovery of ileal AChE was rapid in both age-groups, with full normalization on the 7th day. There was a slight elevation of AChE levels above control values, present in young rats 2-3 weeks after the end of DFP treatment.

The data on brain and ileal MACHRs in the two age-groups and the effects of DFP treatment are presented in Fig. 2. In untreated rats there were no age-related differences in density of cortical MACHR sites but there was a considerable deficit of ileal MACHRs in aged rats (by 45%). ANOVA showed, however, for all rats significant age-related differences in the cerebral cortex (F1,42=87.5, P<0.001) and ileum strip (F1,42=226.4, P<0.001). At the termination of DFP treatment the adaptive down-regulation was present both in the brain and ileum strip of young and aged rats. The percentage decrease of brain MACHRs was greater in aged than in young rats (by about 50 and 25%, respectively). On the contrary, the decrease of ileal MACHRs was greater in young than in aged rats (by about 50 and 35%, respectively). ANOVA showed significant differences for recovery rate both for the cerebral cortex (F6,42=24.4, P<0.001) and the ileum strip (F6,42=26.5, P<0.001). There were also significant interactions between age and recovery rate both for the brain (F6,42=9.1, P<0.001) and ileum strip (F6,42=10.9, P<0.001). Post-hoc analysis confirmed that in the case of the brain there were age-related differences at all intervals, except when MACHRs attained full recovery. In fact, the recovery rate was rapid in young rats (normalization within 2 weeks), and considerably slower in aged rats.
Fig. 2. Age-related differences in down-regulation and recovery rate of MACHR binding sites in the cerebral cortex and ileum strip following repeated administration of DFP to rats. Bmax of H-QNB binding was calculated from Scatchard analyses performed on six points with computer fitted regression lines (correlation coefficients r>0.86). Means ±SE. For significance levels see Fig 1. From Fortuna et al. 1991.

rats (5 weeks). With regard to the ileum strip, post-hoc analysis showed age-related decrease of Bmax at all intervals after DFP treatment. Unexpectedly, however, the recovery rate was more rapid in aged than in young rats (normalization within 3 and 5 weeks, respectively). The affinity of MACHR binding sites was not substantially influenced by age, treatment or during recovery process. Kd (pM) varied from 136±18 to 179±5 for the brain and from 82±14 to 146±41 for the ileum strip.

**Age-related changes in post-receptor mechanisms**

The data on brain and ileal responsiveness of phosphatidyl inositol system after stimulation with a cholinergic agonist, carbachol, are presented in Fig. 3. ANOVA showed significant age-related differences in IP accumulation for the cerebral cortex (F1,40=40.8, P<0.001) and the ileum
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Fig. 4. Age-related differences in sensitivity to cholinergic agonists of the isolated ileum of rats. Means ±SE (n=4). For significance levels see Fig. 1.

strip ($F_{1,36}=80.7, P<0.001$); the concentration related differences were also significant, with $F_{4,40}=43.2, P<0.001$ for the brain and $F_{5,36}=17.7, P<0.001$ for the ileum strip. There was an interaction between age and agonist concentration in the case of the ileum strip ($F_{5,36}=3.14, P<0.05$) but not in the case of brain ($F_{4,40}=2.05, \text{NS}$). In fact, the carbachol-induced IP accumulation was higher in cortical slices from aged than from young rats at all agonist concentrations, and statistically significant at the maximum agonist concentration. The carbachol-induced IP accumulation in slices of ileum strip was significantly higher in aged rats at most agonist concentrations. Therefore the data indicate a greater responsiveness of MACHRs in aged rats.

The data on age-related changes in contractility of the isolated ileum are presented in Fig. 4. There was a slight, although significant, age-related decline in sensitivity, as shown by the increase of EC50 values for acetylcholine and carbachol, but not to oxotremorine.

DISCUSSION

The data presented here indicate age-related differences in the response of muscarinic receptors to the antiChE compound, DFP both in the central and autonomic nervous system. Moreover, they indicate age-related changes in muscarinic receptor density in the latter but not in the former. On the other hand the efficiency of post-receptor mechanisms (measured as accumulation of IP elicited by a cholinergic agonist) is increased in old age both in the cerebral cortex and ileum strip.

With regard to the response of cortical MACHR system to DFP, the data confirm its marked plasticity resulting in down-regulation, previously shown also for the hippocampus and striatum, and for another strain of aged rats (Pintor et al. 1988, 1990). It is of interest that the down-regulation of cortical MACHR is even more pronounced in aged than in young rats. The adaptive down-regulation of ileal MACHR system after DFP treatment is also present both in young (in agreement with the literature data: Ehlert et al. 1980) and aged rats, although to a lesser extent in the latter age-group. The age-related differences in the response of MACHR system are not accompanied by differences in the effects of DFP on its primary target, AChE. In fact, the inhibition of both cortical (about 75%) and ileal AChE (about 30%) 2 days after the end of treatment is identical in the two age-groups. A considerably lower inhibition of AChE in the ileum than in the brain has been shown to depend on a faster initial recovery - within 2 days - in the former (Fortuna et al. 1991).

After termination of DFP treatment the subsequent recovery rate of cortical AChE and MACHRs in aged rats is impaired; this results in a considerable delay in normalization with respect to young rats (5 and 2 weeks, respectively). It is well known that the recovery depends mainly on biosynthesis of enzymatic and receptor molecules. Although possible age-related changes in degradation rate cannot be excluded, the delay in recovery probably depends on impaired biosynthesis. Besides, the greater adaptive reduction of MACHRs observed in aged rats may also contribute to its slow normalization (see below). As already mentioned, the recovery rate of ileal AChE is fast, with full normalization within 7 days. The recovery rate of ileal MACHRs, in contrast to those in brain, appears more rapid in aged (3 weeks) than in young rats (5 weeks). The smaller extent of reduction may be responsible, at least partially, for the early normal-
ization of MACHRs in the former. It is of interest that in experiments on down-regulation and recovery of MACHRs in various brain areas of aged rats, a similar phenomenon was observed. In fact, more delay in normalization has been observed in the cerebral cortex, where a higher degree of adaptive reduction (40%) occurred, than in the hippocampus and striatum (20%) (Pintor et al. 1990).

With regard to MACHRs system in untreated rats it must be noted that density of MACHRs in the brain is about 13 times higher than in the ileum of young rats. No age-related changes in MACHRs density are observed in the cerebral cortex, in agreement with literature data for Sprague-Dawley strain (for references see Michalek et al. 1989); on the other hand, a considerable decrease (by about 50%) occurs in the ileum. This deficit is very similar to that of ileal choline acetyltransferase, another marker of cholinergic innervation (Fortuna et al. 1991). This indicates a considerable loss of cholinergic neurotransmission in the ileum of aged rats.

The marked age-related loss of ileal MACHRs sites, detected in binding studies, only slightly influence physiological responses to cholinergic agonists, measured as contractions of isolated ileum. The EC50 values of the three agonists for young rats are very similar to those obtained by Ehler et al. (1980). The age related decline of sensitivity to agonists, although significant for acetylcholine and carbachol, is rather small in terms of EC50 values. The data support the view that an agonist may elicit maximal contraction of the ileum while occupying only a small percentage of the total available receptor sites, with the large receptors reserve in this tissue (Ringdahl 1986).

The extension of the study to post-receptor mechanisms allows also to assess functional response of the receptors. In the present study a larger maximal stimulation of carbachol-induced IP formation in the cerebral cortex than in the ileum smooth muscle was observed in young rats. This is consistent with the data of Ek and Nahorski (1988) obtained for the same tissues of the guinea pig. An increase of IP formation in aged rats in the absence of changes in total muscarinic receptor density, measured as QNB binding, in the cerebral cortex was described also by Mundy et al. (1991).

It has been demonstrated that in the brain M1-MACHRs, showing high affinity for pirenzepine, are linked to phosphoinositide hydrolysis (Ek and Nahorski 1988). Therefore some preliminary experiments have been performed on age-related changes in this receptor subtype. They showed, using the method of Watson et al. (1983) Bmax of 975±96 and 434±40 femtomol/mg protein (n=6, P<0.01) for young and aged Sprague-Dawley rats, respectively. It is not clear, however, why the decrease of M1 receptors is not accompanied by a decrease of $^3$H-QNB binding.

With regard to the ileum strip of aged rats, an increase in IP formation observed in the present study is accompanied by a considerable decrease of $^3$H-QNB binding. In the rat ileum most binding sites are M2 and remaining sites M3 subtypes (Entzeroth and Mayer, 1991). In fact, in the experiments done in this laboratory, M1 receptor sites in the ileum were not detected. The data obtained on guinea pig ileum suggest that the responses are mediated by activation of M2 receptors (Konno and Takayanagi 1989). Thus $^3$H-QNB binding reflects mainly these receptors. Therefore it would appear that both in the cerebral cortex and the ileum smooth muscle a decrease of receptors coupled to phosphatidyl inositol system unexpectedly leads to an increase in their hydrolysis. This may depend on an increase in receptor coupling to the guanine nucleotide binding protein or an enhanced activation of phospholipase C. The phenomenon may be probably also regarded as a compensatory response to changes in Ca$^{++}$ homeostasis (Barritt 1987).

In conclusion, the overall data indicate that treatment- and age-related changes in MACHR mechanisms in the ileum smooth muscle differ from those in the brain. However, the increased efficiency of IP accumulation in old age is their common feature.

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