Aging-Induced Decrease of Cholinergic Response and Calcium Sensitivity on Rat Jejunum Contractions

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The role of aging on contraction or relaxation through muscarinic or α-adrenergic receptors, respectively, was studied in isolated rat jejunum. Furthermore, the influence of extracellular calcium was analyzed, through functional and radioligand binding assays. The rank order of potency for selective muscarinic antagonists for M₁, M₂, and M₃ receptor subtypes, measured from affinity (pA₂) values, was p-fluorohexahydrosiladifenidol (pFHHSiD) (M₁) > pirenzepine (M₁) > methoctramine (M₃), indicating a predominance of M₃ subtype. This order was unchanged with age. Contractions by muscarinic agonist methacholine (MCh) were diminished in aged rats, resulting in lower apparent affinity (pD₂) values, compared with adult controls. A larger decrease of MCh contractions occurred in aged rats after Ca²⁺ withdrawal or after the calcium channel blocker isradipine. Changes were not detected for relaxation by adrenergic agonists. In conclusion, aging caused a decrease of MCh potency, which is probably related to the reduction of calcium sensitivity in jejunum.

THERE is evidence for the existence of five muscarinic receptor subtypes (M₁–M₅), revealed by molecular cloning and functional studies (1,2). Gastrointestinal smooth muscle contraction involves predominantly the M₃ subtype (3,4), although the M₂ subtype may also participate (1,5,6). In addition, M₁, M₄, and M₅ subtypes were shown to be expressed in human esophagus (2), although their function is still unknown. In rat jejunum, the main role of the M₃ subtype has been recently indicated by functional studies in intestine (7), but its characterization based on the hierarchy of agonist affinities is still lacking, to our knowledge.

Aging may decrease the responsiveness of cholinergic receptors on isolated ileum of mice and rats, as well as responses involving cholinergic neurotransmission (8,9). It was also observed that ileal smooth muscles of aged rats show a decreased relaxation (10). Bitar (11) suggested that a reduced contractility of the colon from aged rats might be due to alterations on signaling transduction pathways. Furthermore, we have recently found that aging affects intracellular calcium stores of sarcoendoplasmic reticulum and mitochondria of smooth muscle of rat large intestine (12,13). Calcium plays a key role in activation of muscarinic receptors in intestine (14), being released from intracellular stores, which are responsible for an initial phasic peak of tension (15–17), whereas a second, tonic contraction, is sustained basically by the influx through receptor-operated Ca²⁺ channels (ROCs) or voltage-operated Ca²⁺ channels (VOCs) (18,19). As a consequence, contractions are greatly influenced by the withdrawal of external calcium (15,17,20). However, information is still lacking about the role of external calcium in rat jejunum.

Concerning intestinal relaxation, the role of the sympathetic system has been generally neglected, as attention has been mostly directed to calcium translocation (21,22), and to other neurotransmitters, such as adenosine triphosphate (ATP), nitric oxide, and vasoactive intestinal peptide (VIP) (6). Therefore, information about the role of adrenergic agonists seems to be necessary.

Our purpose was to study the role of aging on the contraction or relaxation through respectively muscarinic or α-adrenergic receptors, and the influence of extracellular calcium, by functional and radioligand binding studies. Our results showed that in aged rats the contraction induced by methacholine (MCh) were diminished, resulting in lower pD₂ values. In addition, the decrease of MCh effects after Ca²⁺ withdrawal or after adding the calcium channel blocker isradipine (PN) was more pronounced in aged animals.

MATERIALS AND METHODS

Animals and Jejunum Preparation

Male Wistar rats, were maintained in polypropylene cages (usually 5 per cage) in our animal facilities, under central air conditioning at controlled temperature (22 ± 2°C) and humidity (about 55%). All the air entering the room was filtered for 95% of particles larger than 5 microns. Air flow rate was maintained at about 15 renewals/hr. Usually, the animals were transferred to clean autoclave sterilized cages 3 times/wk. Sterilized commercial food and water were given ad libitum. Under these conditions, at least 50% of the animals showed a survival time of 30 months or more. At the age of 5 months (controls), 12 months, or 24 months, the animals were killed with an overdose of ether. The jejunum was removed and cleared from adjacent tissues and intestinal contents. Pieces (about 15 mm) were suspended...
vertically and immersed in 10 mL organ baths containing Tyrode nutrient solution (mM: NaCl 138; KCl 5.7; CaCl$_2$ 1.8; MgCl$_2$ 1.8; NaH$_2$PO$_4$ 0.36; NaHCO$_3$ 5.5; glucose 5.5, pH 7.4, bubbled with 97% O$_2$/3% CO$_2$, at 37°C). The resting tension was adjusted for isotonic recording, under a load of 1 g.

Measurements of Contractions and Relaxation

After an equilibration period of 30 minutes, the preparation was initially exposed to barium chloride (BaCl$_2$, usually 1 × 10$^{-3}$ M to 1 × 10$^{-2}$ M). Barium was used as a control to check the organ responsiveness, because it was shown to be among the agents that cause the highest contractions in a large number of smooth muscle preparations, even when cholinergic or adrenergic responses are blocked or absent (20). After washout for at least 15 minutes, the organ tonus was allowed to return to the baseline, and cumulative concentrations (1 × 10$^{-9}$ M to 1 × 10$^{-4}$ M) of the muscarinic agonist MCh were added to construct a dose-response curve. The values of median concentration–relaxation curves.

In separate experiments to evaluate the relaxation by adrenoceptor agonists, the tissue was contracted by a maximum dose of MCh (10$^{-4}$ M) followed by the addition of cumulative concentrations (1 × 10$^{-8}$ M to 1 × 10$^{-5}$ M) of adrenaline or noradrenaline to obtain cumulative concentration–relaxation curves.

Effect of Competitive Muscarinic Antagonists

The subtypes of muscarinic receptors were characterized based on the effects of selective antagonists. The following antagonists were used: pirenzepine (M$_1$ selective, 3 × 10$^{-7}$ to 10$^{-5}$ M), methoctramine (M$_2$ selective, 10$^{-6}$ to 3 × 10$^{-5}$ M) and p-fluorohexahydrosiladifenidol (pFHHSiD) (M$_3$ selective, 3 × 10$^{-9}$ to 10$^{-6}$ M). The nonselective antagonist atropine was also used in some experiments (not shown).

After an equilibration period of 30 minutes, concentration-response curves for MCh were obtained in the absence and presence of at least three different concentrations of one of the muscarinic receptor antagonists. Each antagonist was incubated for 45 minutes before the curve of MCh and during the performance of the curve for the agonist. The antagonist affinities or dissociation constants (K$_B$) of antagonists for muscarinic receptors were estimated by the parameter pA$_2$, as described below.

Effect of MCh After Calcium Withdrawal From Nutrient Solution

To test whether the influence of aging on contractile responses was dependent on changes of the influx of extracellular Ca$^{2+}$, the ion was removed from the nutrient solution, as previously described (20). After adding the Ca-free solution, a single dose of MCh (10$^{-5}$ M, for 2 minutes) was added after a given time interval and its effect recorded. This was followed by preparation washout with the same solution and repetition of the procedure at different intervals after up to 45 minutes.

Effect of MCh in the Presence of the L-Type Ca$^{2+}$ Channel Blocker PN

Experiments were also performed in the presence of PN (10$^{-8}$ M). After 10-minute incubation with PN, a single dose (10$^{-5}$ M) of MCh was added, and its effect was recorded for 2 minutes. After recording, the preparation was washed out and the procedure repeated at different time intervals after the addition of PN, as described above for the calcium-free solution.

Radioligand Binding Assay

Membrane preparation.—The mucosa of jejunum was removed, and smooth muscle (longitudinal and circular layers) was pooled from 10 male rats of each group for binding assays to characterize the L-type Ca$^{2+}$ voltage-dependent channel. The tissue was suspended in 50 volumes of Tris-buffer (50 mM, pH 7.4) and homogenized using the Ultra-Turrax T25 homogenizer (IKA, Germany) at a speed setting of 20,500 rpm for 30 minutes. The supernatant was filtered through four layers of gauze, and centrifuged at 45,000 g for 20 minutes, followed by a centrifugation of the supernatant at 45,000 g for 20 minutes, and resuspension of the final pellet. The samples contained a range of 100–150 g/mL proteins. The protein in the samples was measured by the method of Lowry and colleagues, as previously described (23,24).

Binding studies.—The incubation mixture contained 50 µL of membrane suspension, 50 µL of eight increasing concentrations of $^3$H-PN, and 150 µL of Tris buffer solution. Incubation was initiated by adding the membrane suspension to the assay mixture, and carried out at 37°C for 90 minutes. The reaction was ended by adding 5 mL of ice-cold buffer solution followed by rapid vacuum filtration over Tris buffer-soaked Whatman GF/C filter (Florham Park, NJ), as previously described by Castillo and colleagues (24). After rapid filtration, the filter was rinsed three times with 5 mL of ice-cold buffer, dried for 60 minutes at 80°C, and placed in vials with a scintillation mixture of PPO/5 g/L with POPOP 0.1 g/L. The radioactivity bound to the membranes was counted by a scintillation counter (Tri-Carb 1500 TR; Packard, Downers Grove, IL).

The nonspecific binding was defined as that not displaced by 1 µM unlabelled PN. Specific binding of $^3$H-PN was defined as total binding radioactivity minus nonspecific binding. In every experiment, filter blanks were also included, and the apparent specific binding observed in the filters was subtracted from each data point to obtain the actual value of specific binding to the membranes.

Data Analysis

In general, the effect of agonists was measured as g or mm changes on tension or contraction. The EC$_{50}$ values and the slopes of concentration-response curves were calculated by using regression analysis. Agonist potency was defined as the $-\log EC_{50}$ and was expressed as pD$_2$ values.

The other parameter used was pA$_2$, which is defined as a negative logarithm of the molar concentration of the
antagonist that reduces 50% of the effect of an agonist. The pA2 was calculated according to the equation:

\[
pA_2 = - \log[B] + \log([A_2]/[A_1] - 1)
\]

where B is the concentration of the antagonist, and [A2]/[A1] is a concentration ratio, in which A1 is the concentration of the agonist in the absence of the antagonist, and A2 is the concentration of the agonist in the presence of the antagonist.

Using Schild plots (25,26), the pA2 values were obtained from the intercept on the x-axis, in a plot relating the log ([A2]/[A1] - 1) vs log [B]. The antagonism was considered to be competitive if the slope of the Schild plot was not significantly different from unity. Data were expressed as percentage of the maximal response obtained in each preparation.

**Statistical Analysis**

Statistical analysis was carried out using mean values ± standard error of the mean. Student’s t test was used to compare the results, and the .05 level of probability was accepted as significant.

**RESULTS**

**Effects of MCh and BaCl2 in Jejunum of Aged Rats**

Concentration-response curves for MCh and BaCl2 are shown in Figure 1. The maximal response (E_max) to MCh was not significantly changed in aged groups, but the pD2 values decreased significantly for 12- and 24-month-old groups, as compared with 5-month-old controls. BaCl2-induced contractions were not affected by aging, as pD2 values were similar in all groups (Figure 1).

**Muscarinic Receptor Characterization**

The experiments with pirenzepine (M1 antagonist) and methoctramine (M2 antagonist) were performed in jejunum of 5- and 12-month-old rats, and pFHHSiD (M3 antagonist) was also tested in 24-month-old rats. Atropine was used as a nonselective antagonist (data not shown).

All curves were shifted to the right, and the Schild lines were not significantly different from unity, which is consistent with competitive inhibitions (Figures 2 and 3). The rank order of potency of the antagonists, measured from the corresponding pA2 values (Figures 2 and 3), was pFHHSiD > pirenzepine > methoctramine, showing that the M3 subtype is predominant in this preparation. The corresponding pA2 values and rank orders of potency showed no significant differences between aged and adult rats (Figures 2 and 3).

**Effects of Adrenergic Agonists on Precontracted Jejunum**

The relaxant response to noradrenaline and adrenaline to a contraction induced by MCh did not show significant differences between aged (12- and 24-month-old) and control rats (not shown). The pD2 values were: 5.81 ± 0.13 for noradrenaline and 5.91 ± 0.11 for adrenaline in the control group; 6.01 ± 0.04 for noradrenaline and 6.11 ± 0.05 for adrenaline in the 12-month-old rats; and 6.35 ± 0.11 for noradrenaline and 6.32 ± 0.04 for adrenaline in the 24-month-old rats.

**Effect of MCh After Calcium Withdrawal From Nutrient Solution**

The contractile effect of MCh was studied after changing the regular nutrient solution to a calcium-free solution. In general, contractions showed an initial peak phasic component after less than 1 minute, followed by stabilization (tonic component) within about 2 minutes (not shown). Table 1 shows that whereas in controls the contractions were reduced by about 50% after 20 minutes, a higher and faster reduction (44%) was observed just after 3 minutes for organs of 12-month-old rats. No additional time reduction was observed for the 24-month-old groups in relation to the previous group. The effects were practically annulled after
Effects of the L-Type Voltage-Dependent Ca^{2+} Channel Inhibitor PN

The initial phasic component (less than 1 minute) and the second, tonic, component of MCh-induced contraction were measured after adding the blocker of VOC PN (10^{-8} M). Table 2 shows that the effects of MCh were reduced by PN, and that the reduction of the phasic component was more pronounced for the 12-month-old groups after 20- and 30-minute incubation, in relation to controls. Differences between both groups were not detected for the tonic component (Table 2).

Radioligand Binding Assay

The analysis of the binding data with ³H-PN showed that Hill slopes were near unity, whereas the corresponding affinities (Kd) and densities of binding sites (Bmax), obtained through Scatchard plots, were not significantly different, when comparing the jejunum of aged animals with controls (Table 3). These results show that aging did not influence the dihydropyridine binding sites of L-type VOCs.

DISCUSSION

It was shown here that aging caused a decrease of muscarinic receptor activation in jejunum, expressed as a lower pD2 value for MCh, and an enhanced inhibition of
MCh-induced contraction by calcium withdrawal and by the calcium antagonist PN.

The response to the cholinergic agonist MCh decreased in 12- and 24-month-old rats, as indicated by the corresponding pD2 values. Kobashi and colleagues (8) did not detect a decrease of the MCh parameter, although they showed that pD2 values for acetylcholine (ACh) are decreased by 0.72 log units in jejunum of 12-month-old rats, added to an increase of cholinesterase activity. On the contrary, Tezuka and colleagues (7) could not find changes in ACh parameters, nor in cholinesterase activity in jejunum of 24- to 28-month-old rats.

This alteration of pD2 for MCh is most likely not due to a change of receptor affinity, because the pA2 values were not modified. As a matter of fact, other related pA2 determinations showed that aging does not modify the muscarinic receptor affinity (7,8). This finding was recently confirmed through radioligand binding studies.

Figure 3. Concentration-response curves for experiments similar to that shown in the previous figure, except that pirenzepine was used instead of p-fluorohexahydrosiladifenidol (pFHHSiD) in jejunum of 5-month-old controls (A) and 12-month-old rats (B). Bottom: results of similar experiments in which methoctramine was used instead of pFHHSiD in jejunum of 5-month-old controls (C) and 12-month-old rats (D). Insets: Corresponding Schild plots with the related slopes ± standard error of the mean (SEM) and pA2 ± SEM values. Bottom right: molar concentrations for the respective antagonist in the corresponding curves. Symbols in curves represent mean ± SEM of 4–6 experiments. MCh = methacholine.
of muscarinic receptors, showing that \( K_D \) values are not changed in jejunum of 24- to 28-month-old rats (7).

Since the effect of aging on the decrease of jejunum contractility to MCh cannot possibly be ascribed to a reduction of receptor affinity, a change in the signal transduction pathways ought to be considered (11). As a matter of fact, it was shown here that the effect of MCh in the absence of extracellular calcium, or in the presence of PN, was significantly reduced in aged rats, as compared with normal controls. This finding cannot be explained as being due to a change of VOCs, because the \( K_d \) and the \( B_{max} \) of \([^{3}H] \)-PN binding sites were not modified. In contrast, it is known that intestinal contractions related to the activation of M3 receptors are due mainly to IP3-mediated release of Ca\(^{2+}\) ions from the sarcoplasmic reticulum (6). Thus, the possibility exists that the faster decays shown in Tables 1 and 2 are rather due to a faster intracellular removal of Ca\(^{2+}\) by organelles such as mitochondria or sarcoplasmic reticulum, as recently shown for colonic mitochondria of aged rats (13), leading possibly to a fall of free calcium concentration in cytoplasm. It is clear that additional experiments are necessary to clarify this point.

Aging-related changes of muscarinic effects might not be similar in colon and jejunum (7). In fact, Lopes and colleagues (12) showed that differently from jejunum, the contractile response to cholinergic agonist was significantly greater in colon. In other words, tissue- and species-dependent discrepancies can be apparent when comparing pharmacological systems in different parts of gastrointestinal tract.

Although a number of excitatory endogenous agents are present in intestine, such as tachykinins, substance P and ATP, it is known that ACh is the most efficient intestinal excitatory neurotransmitter in terms of stimulus intensity and contraction amplitude (6). However, the fact that ACh is rapidly metabolized by cholinesterase can cause some problems from the experimental standpoint, because the enzymatic inactivation of ACh could lead to changes in the values of pharmacological parameters when this agonist is used for muscarinic receptor characterization. This is particularly meaningful, because it has been shown that the activity of jejunum cholinesterase is increased in aged rats (8), although later results have not been confirmed by Tezuka and colleagues (7). Thus, we have used MCh here instead of ACh when checking for the muscarinic subtype involved and for the differences between aged and normal rats, because this agonist has a much lower affinity for the metabolizing enzyme.

The hierarchy of pA2 values for muscarinic agonists confirmed the predominance of M3 subtype in jejunum. The presence of this subtype in gastrointestinal tract was also advanced by some authors (4,7,27), but the necessary rank order of potency of antagonists was not previously determined, to our knowledge. For instance, the work of Tezuka and colleagues (7) was based solely on the pKB determination for p-F-HHISID, because, according to the authors, pirenzepine and methoctramine presented characteristics of noncompetitive blockers in their experimental conditions. The presence of the M3 subtype in jejunum does not rule out the possibility that other subtypes are involved. As a matter of fact, it has been shown that in knockout mice of M3 subtype, the ileum contractility was impaired by about 77%, whereas the residual contraction was ascribed to the M2 subtype (1,27).

In relation to intestinal relaxation, substances such as NO, VIP, and ATP (interacting with apamin-sensitive receptors), besides sympathetic neurotransmitters, are known to participate in inhibitory effects in intestine (6,28). However, noradrenergic neurons might have a secondary role in the relaxation of longitudinal intestinal muscle, as they innervate notably the enteric muscle of sphincters and arteries within the gut wall (29). This finding seems to be irrelevant.

### Table 1. MCh-Induced Contractions After Ca\(^{2+}\) Withdrawal (%)

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>Incubation (Minutes) in Ca(^{2+})–Free Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (Control)</td>
<td>100 83.0 (\pm) 10.0 62.0 (\pm) 12.4 52.0 (\pm) 11.0 41.0 (\pm) 8.7 10.0 (\pm) 2.5</td>
</tr>
<tr>
<td>12</td>
<td>100 44.0 (\pm) 8.3* 23.0 (\pm) 6.0* 13.0 (\pm) 4.5* 7.0 (\pm) 2.5* 4.0 (\pm) 1.8*</td>
</tr>
<tr>
<td>24</td>
<td>100 49.0 (\pm) 8.7* 15.0 (\pm) 2.3* 13.0 (\pm) 2.5* 0.0 0.0</td>
</tr>
</tbody>
</table>

**Notes:** Data are means \(\pm\) standard error of the mean, \(n = \) at least 6. Contractions were phasic, measured about 1 minute or less after adding methacholine (MCh) \((10^{-5} \text{ M})\) at the corresponding incubation interval.

*\(p < .05\) in relation to controls.

### Table 2. MCh-Induced Contraction (%) in the Presence of Calcium Channel Blocker Isradipine \((10^{-8} \text{ M})\)

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>Contraction Phase</th>
<th>Intervals (Minutes) of Incubation With Isradipine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (Control)</td>
<td>Phasic(^*)</td>
<td>0 100 93.0 (\pm) 3.3 89.0 (\pm) 4.5 86.0 (\pm) 2.6 79.0 (\pm) 3.9 66.0 (\pm) 6.8</td>
</tr>
<tr>
<td></td>
<td>Tonic(^\dagger)</td>
<td>100 85.0 (\pm) 1.8 90.0 (\pm) 3.9 86.0 (\pm) 4.5 83.0 (\pm) 2.2 78.0 (\pm) 1.8</td>
</tr>
<tr>
<td>12</td>
<td>Phasic(^*)</td>
<td>100 73.0 (\pm) 5.3 64.0 (\pm) 9.6 56.0 (\pm) 3.6 50.0 (\pm) 5.5 59.0 (\pm) 9.7</td>
</tr>
<tr>
<td></td>
<td>Tonic(^\dagger)</td>
<td>100 78.0 (\pm) 4.8 83.0 (\pm) 6.1 87.0 (\pm) 4.9 78.0 (\pm) 4.0 78.0 (\pm) 4.9</td>
</tr>
</tbody>
</table>

**Notes:** Data are means \(\pm\) standard error of the mean, for methacholine (MCh) \((10^{-5} \text{ M})\), \(n = \) at least 6.

*\(p < .05\) in relation to corresponding control.

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in relation to our experiments, because we have not observed changes in adrenaline-induced relaxations. However, Baker and colleagues (10) described a decreased relaxation for isoprenaline in ileal muscle, which was ascribed to a reduction of sympathetic innervation in the aged rat.

Our observation that the effects of barium chloride are not influenced by aging in this preparation has been previously shown by Kobashi and colleagues (8). Similar results were found for KCl (7,8), thus indicating a muscarinic specificity for the changes so far reported for MCh. This possibility is corroborated by our finding that the effects of the adrenergic agonists noradrenaline and adrenaline were also not modified by aging.

There is considerable evidence that gastrointestinal motility may be affected by aging, explaining why, from the clinical point of view, dysphagia and chronic functional constipation occur more frequently in elderly people (30). Because the motility of intestinal smooth muscle depends on the clinical point of view, dysphagia and chronic functional constipation occur more frequently in elderly people (30).

ACKNOWLEDGMENTS

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Received May 15, 2006
Accepted September 4, 2006
Decision Editor: LaDora V. Thompson, PhD