Melatonin Treatment Reverts Age-Related Changes in Guinea Pig Gallbladder Neuromuscular Transmission and Contractility

Pedro J. Gomez-Pinilla, Cristina Camello-Almaraz, Rosario Moreno, Pedro J. Camello, and María J. Pozo
Department of Physiology, Nursing School, University of Extremadura, Caceres, Spain
Received June 9, 2006; accepted August 9, 2006

ABSTRACT
The incidence of gallbladder illness increases with age, but the altered mechanisms leading to gallbladder dysfunction are poorly understood. Here we determine the age-related alterations in gallbladder contractility and the impact of melatonin treatment. Isometric tension changes in response to electrical field stimulation and to agonists were recorded from guinea pig gallbladder muscle strips. [Ca^{2+}], was determined by epifluorescence microscopy in fura-2 loaded isolated gallbladder smooth muscle cells, and F-actin content was quantified by confocal microscopy. Aging reduced neurogenic contractions, which was associated with the impairment of nitric innervation and with increased responsiveness of capsaicin-sensitive relaxant nerves, possibly involving calcitonin gene-related peptide. Melatonin treatment for 4 weeks restored neurogenic responses to normal values, with an associated recovery of nitric function and the disappearance of the capsaicin-sensitive component. Aging also reduced the contractile responses to cholecystokinin and Ca^{2+} influx. The impaired contractility only correlated with diminished Ca^{2+} mobilization in response to activation of Ca^{2+} influx. Melatonin improved contractility and increased smooth muscle F-actin content without changing Ca^{2+} homeostasis. In conclusion, aging impairs gallbladder function as the result of changes in the inhibitory neuromodulation of smooth muscle contractility and the reduction in the myogenic response to contractile agonists. Impaired contractility seems to be related to decreased Ca^{2+} influx and damage of contractile proteins. Melatonin significantly ameliorated these age-related changes.

Population aging was one of the most distinctive demographic events of the 20th century, and according to the increase in the mean life expectancy, the elderly population will constitute 20% of the world population in the next 50 years (Centers for Disease Control and Prevention, 2003). Aging is understood to be mainly a slow, gradual, and passive process influenced by the interplay of multiple genetic and environmental factors. It is widely accepted that aging is not a disease, but the borderline between pathological and normal aging is quite narrow. Although there are many kinds of “nondisease” aging manifestations, including blood vessel stiffening, skin slackening, and joint stiffening, the molecular structures of aged organisms are abnormal (Bailey, 2001). These molecular abnormalities could be responsible for the increase in the incidence and prevalence in the elderly of the so-called age-related diseases such as neurodegenerative disorders (Troulinaki and Tavnerarakis, 2005), cardiovascular disease (Ferrari et al., 2003), diabetes (Winer and Sowers, 2004), and incontinence (Nasr and Ouslander, 1998).

Regarding the gastrointestinal (GI) tract, the prevalence of dysfunctions related to motility is higher in older than in younger adults (Camilleri et al., 2000). However, there are very few studies concerning the cellular and molecular mechanisms whose alteration could be responsible for such dysfunctions in aging. Thus, it has been reported that neuronal loss occurs with age in the myenteric plexus of the GI tract in several species including human (Gomes et al., 1997). However, it is unclear whether cell death affects all classes of myenteric neurons nonselectively or is confined to specific phenotypes (Wade and Cowen, 2004).

This work was supported by Junta de Extremadura (2PR03A020) and Ministerio de Educacion y Ciencia (BFU 2004–0827). P.J.G.–P. is the recipient of a doctoral fellowship from Junta de Extremadura.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.106.109256.

ABBREVIATIONS: GI, gastrointestinal; EFS, electrical field stimulation; ACh, acetylcholine; CCK, cholecystokinin; BSA, bovine serum albumin; AM, acetoxymethyl ester; PBS, phosphate-buffered saline; FITC-phalloidin, fluorescein isothiocyanate-labeled phalloidin; CGRP, calcitonin gene-related peptide; L-NNAME, N’-nitro-L-arginine methyl ester; CCK-8, CCK fragment 26 to 33; Bt3-Ins(1,4,5)P3-PM, 2,3,6-tri-O-butyryl-myoinositol-1,4,5-trisphosphate-hexakis(propionoxymethyl) ester; ANOVA, analysis of variance; CCE, capacitative calcium entry; TPS, thapsigargin; IP3, myo-inositol 1,4,5-triphosphate.
anisms of smooth muscle contraction is scantly (Bitar and Patil, 2004). Acetylcholine (ACh)-induced contractile responses are impaired in colon (Roberto et al., 1994), which could be due to the limited cell length distribution found in aged-animals but also to alteration of the intracellular signaling pathways related to activation of protein kinase C and cytoskeleton reorganization (Bitar, 2003). There is little information regarding the possible changes in $\text{Ca}^{2+}$ homeostasis during aging in smooth muscle in general and gastrointestinal smooth muscle in particular, in which it has been described as an aged-related decline in the L-type $\text{Ca}^{2+}$ currents both in rat and human colon smooth muscle cells (Xiong et al., 1993, 1995).

Knowledge of the molecular mechanisms involved in aging is required for development of strategies to preserve the quality of life of the increasingly aging population. Degenerative changes associated with aging have been related to progressive damage by reactive oxygen and nitrogen species in those situations in which the antioxidative defense system fails to eliminate them (Sohal and Allen, 1990). Recently, melatonin, the main hormone of the pineal gland, was proposed as a protective agent against macromolecular destruction associated with longevity (Reiter et al., 1996). The protective effects of melatonin could be related to the ability of the hormone to synchronize circadian rhythms and thereby to reduce biological stress, as well as to its direct free radical scavenging activity and its indirect antioxidants properties (Reiter et al., 2002).

In the current study we explored aged-related alterations of neuromuscular transmission, gallbladder contractility, and smooth muscle $\text{Ca}^{2+}$ homeostasis. Our results show that aging-induced gallbladder impairment is primarily related to changes in the inhibitory innervation of the organ and to a decrease in the myogenic response to excitatory hormones and neurotransmitters. Melatonin treatment reversed the functional impairment caused by aging at the level of both the neuromuscular transmission and the myogenic contractility despite being ineffective in the restoration of $\text{Ca}^{2+}$ homeostasis.

**Materials and Methods**

**Tissue Preparation.** Gallbladders were removed from 4- and 20-month-old female guinea pigs after deep halothane anesthesia and cervical dislocation and were immediately placed in cold Krebs-Henseleit solution (for composition, see Solutions and Drugs) at pH 7.35. The gallbladder was opened from the end of the cystic duct to the base and trimmed of any adherent liver tissue. After the preparation was washed with the nutrient solution to remove residual bile, the mucosa was carefully dissected away.

A group of aged animals was treated orally with melatonin (2.5 mg/kg/day). Melatonin was dissolved in glucose solution (1.5%) and the mucosa was carefully dissected away. After construction of a frequency-response curve and to pharmacologically dissect the neurogenic responses, antagonists were added to the organ bath for 20 min, and then the EFS protocols were repeated. In some experiments, the contractile effects of ACh, cholecystokinin (CCK), KCl, and $\text{Ca}^{2+}$ restoration after intracellular $\text{Ca}^{2+}$ store depletion were tested.

**Cell Isolation.** Gallbladder smooth muscle cells were dissociated enzymatically using a previously described method (Pozo et al., 2002). In brief, the gallbladder was cut into small pieces and incubated for 34 min at $37^\circ\text{C}$ in enzyme solution (for composition see Solutions and Drugs) supplemented with 1 mg/ml bovine serum albumin (BSA), 1 mg/ml papain, and 1 mg/ml 1,4-dithio-DL-threitol. Next, the tissue was transferred to fresh enzyme solution containing 1 mg/ml BSA, 1 mg/ml collagenase, and 100 $\mu$M CaCl$_2$ and incubated for 9 min at $37^\circ\text{C}$. The tissue was then washed three times using cold enzyme solution, and the single smooth muscle cells were isolated by several passages of the tissue pieces through the tip of a fire-polished glass Pasteur pipette. The resultant cell suspension was kept in enzyme solution at 4$^\circ\text{C}$ until use, generally within 6 h. All experiments involving isolated cells were performed at room temperature ($22^\circ\text{C}$). The cell length was similar in the three age groups (young adult 44.68 ± 1.62, aged 45.49 ± 1.83, and melatonin 43.07 ± 1.36 μm; n = 98, 61, and 96 cells, respectively).

**Cell Loading and [Ca$^{2+}$], Determination.** [Ca$^{2+}$], was determined by epifluorescence microscopy using the fluorescent ratiometric Ca$^{2+}$ indicator fura 2 as described previously (Moraes et al., 2004). Isolated cells were loaded with 4 μM fura 2/AM at room temperature for 25 min. An aliquot of cell suspension was placed in an experimental chamber made with a glass poly-d-lysine-treated coverslip (0.17 mm thick) filled with Na$^+$-HEPES solution (for composition see Solutions and Drugs) and mounted on the stage of an inverted microscope (Eclipse TE2000-S; Nikon, Melville, NY). After cell sedimentation, a gravity-fed system was used to perfuse the chamber with Na$^+$-HEPES solution in the absence or presence of the experimental agents. Cells were illuminated at 340 and 380 nm by a computer-controlled monochromator (Optoscan; Cairn Research, Faversham, UK) at 0.3 cycle/s, and the emitted fluorescence was selected by a 510/40-nm bandpass filter. The emitted fluorescence images were captured with a cooled digital charge-coupled device camera (ORCA-ER; Hamamatsu Photonics, Bridgewater, NJ) and recorded using dedicated software (Metamorph; Molecular Devices, Downingtown, PA). The ratio of fluorescence at 340 nm to fluorescence at 380 nm ($\text{F}_{340}/\text{F}_{380}$) was calculated pixel by pixel and used to indicate the changes in [Ca$^{2+}$]. A calibration of the ratio for [Ca$^{2+}$], was not performed in view of the many uncertainties related to the binding properties of fura 2 with Ca$^{2+}$ within smooth muscle cells.

**F-actin Content Measurement.** The F-actin content of gallbladder smooth muscle cells was determined according to a previously published method (Xiong et al., 1993, 1995). Acetylcholine (ACh)-induced contractile responses are impaired in colon (Roberts et al., 1994), which could be due to the limited cell length distribution found in aged-animals but also to alteration of the intracellular signaling pathways related to activation of protein kinase C and cytoskeleton reorganization (Bitar, 2003). There is little information regarding the possible changes in $\text{Ca}^{2+}$ homeostasis during aging in smooth muscle in general and gastrointestinal smooth muscle in particular, in which it has been described as an aged-related decline in the L-type $\text{Ca}^{2+}$ currents both in rat and human colon smooth muscle cells (Xiong et al., 1993, 1995).

**Measurement of Melatonin in Serum.** Melatonin levels were determined in the different age groups by using a commercial radioimmunoassay kit (IBL, Hamburg, Germany), according to the manufacturer’s instructions. The kit consisted of $^{125}$I-labeled melatonin (0.68 μCi/ml), rabbit anti-melatonin serum, melatonin standards, delipidizing agent, assay buffer, precipitating antiserum, and controls (lyophilized plasma samples). Results were expressed in picograms per milliliter.

**Contraction Recording of Guinea Pig Gallbladder Smooth Muscle Strips.** Gallbladder strips (measuring ~3 × 10 mm) were placed vertically in a 10-ml organ bath filled with Krebs-Henseleit solution maintained at 37$^\circ\text{C}$ and gassed with 95% O$_2$-5% CO$_2$. Isoometric contractions were measured using force displacement transducers that were interfaced with a Macintosh computer using a MacLab hardware unit and software (ADInstruments, Colorado Springs, CO). The muscle strips were placed under an initial resting tension equivalent to a 1.5-g load and allowed to equilibrate for 60 min, with solution changes every 20 min. Every strip coming from a given animal was used in a different experimental protocol.

Intrinsic nerves were activated by electrical field stimulation (EFS) with a pair of external platinum ring electrodes (0.7 mm in diameter) connected to a square-wave stimulator (Cibertec CS9/3BO) programmed through Scope software application from MacLab (ADInstruments). Trains of stimuli (0.3-ms duration, 5–40 Hz, 350-mA current strength) were delivered for 10 s at 3-min intervals. After construction of a frequency-response curve and to pharmacologically dissect the neurogenic responses, antagonists were added to the organ bath for 20 min, and then the EFS protocols were repeated. In some experiments, the contractile effects of ACh, cholecystokinin (CCK), KCl, and $\text{Ca}^{2+}$ restoration after intracellular $\text{Ca}^{2+}$ store depletion were tested.
published procedure (Morales et al., 2005b). In brief, samples of gallbladder smooth muscle cell suspensions (200 µl) in Na⁺/HEPES solution were transferred to 200 µl of ice-cold 5% (w/v) formaldehyde in PBS (for composition see Solution and Drugs) for 10 min. Fixed cells were permeabilized by incubation for 10 min with 0.025% (w/v) Nonidet P-40 detergent dissolved in PBS. After incubation, the cells were collected by centrifugation for 2 min at 10,000g and resuspended in PBS. Staining of actin filaments was measured using a confocal laser-scanning system (model MRC-1024; Bio-Rad, Hercules, CA) with an excitation wavelength of 488 nm and emission at 515 nm. The cell F-actin content was quantified as arbitrary units of fluorescence using ImageJ software (National Institutes of Health, Bethesda, MD).

**Solutions and Drugs.** The Krebs-Henseleit solution contained 113 mM NaCl, 4.3 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 25 mM NaHCO₃, and 11.5 mM D-glucose. This solution had a final pH of 7.3 after equilibration with 95% O₂-5% CO₂. The Ca²⁺-free Na⁺-HEPES solution contained 10 mM HEPES, 140 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM D-glucose with pH adjusted to 7.3 with NaOH. The Na⁺-HEPES solution contained 10 mM HEPES, 140 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM D-glucose with pH adjusted to 7.3 with NaOH. The Ca²⁺-free Na⁺-HEPES solution was prepared by substituting EGTA (1 mM) for CaCl₂. The PBS used in F-acitin studies contained 137 mM NaCl, 2.7 mM KCl, 5.62 mM Na₂HPO₄, 1.2 mM KH₂PO₄, and 1.47 mM H₂PO₄ with pH adjusted to 7.2. Drug concentrations are expressed as final bath concentrations.

Drug concentrations are expressed as final bath concentrations. Drugs and chemicals were obtained from the following sources: ACh, atropine, caffeine, CCK fragment 26 to 33 (CCK-8) sulfated 1,4-thiazolidine-3-thione, glibenclamide, 6-methylthio-purine (6-MMP), melatonin, thapsigargin, and FITC-phalloidin were from Sigma Chemical Company (St. Louis, MO); E-capsaicin was from Tocris Cookson (Bristol, UK); Bt3-Ins(1,4,5)P₃-PM was from SiChem (Bremen, Germany); fura 2/AM was from Molecular Probes Europe (Leiden, Netherlands); collagenase was from Fluka (Madrid, Spain); and papain was from Worthington Biochemical (Lakewood, NJ). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain). Stock solutions of atrospine, capsazepine, fura 2/AM, thapsigargin, and 6-methylthio-purine (6-MMP) were prepared in DMSO, and FITC-phalloidin was prepared in ethanol. The solutions were diluted such that the final concentration of DMSO was ≤0.1% (v/v). This concentration of DMSO did not interfere with fura 2 fluorescence. Melatonin was prepared in 80 mM glucose solution.

**Quantification and Statistics.** Results are expressed as means ± S.E.M. of n cells or gallbladder strips. Gallbladder tension is given in millinewtons per milligram of tissue. All results from [Ca²⁺]i determinations are given as ΔF/F₀max. Statistical differences between means were determined by Student's t test. Differences between multiple groups were tested using two-way analysis of variance (ANOVA) followed by a Bonferroni’s post hoc test. Differences were considered significant at P < 0.05.

**Results**

**Age-Related Changes in Efferent Innervation in EFS-Induced Gallbladder Responses.** EFS induced a frequency-dependent gallbladder contraction (a representative trace is shown in Fig. 1A) with a maximal amplitude of 2.23 ± 0.18 mN/mg tissue at the frequency of 40 Hz (n = 26) (Table 1). When gallbladder strips from aged animals were electrically stimulated, a significant decrease in the EFS-induced contraction at all of the frequencies tested was recorded (Fig. 1A; Table 1). The diminished response was reflected by reductions in both the amplitude of the peak and the duration of the contraction (P < 0.001, two-way ANOVA) for both parameters (Fig. 1B; Table 1). In addition, there was a long-lasting off-relaxation (amplitude of 0.245 mN/mg at 40 Hz, t = 22) in the strips from older animals, but not in the younger animals.

A group of old animals was treated orally with melatonin (2.5 mg/kg/day) for 4 weeks, mimicking the circadian rhythm

![Fig. 1](https://example.com/fig1.png)
of the naturally secreted hormone. This treatment increased melatonin blood levels in aged animals when measured at 9:00 AM (young adult 26.24 ± 2.12, aged 19.6 ± 1.48, and melatonin 27.09 ± 2.29 pg/ml; n = 10, 7, and 7 animals, respectively, P < 0.05 aged versus young adult and aged versus melatonin). Melatonin treatment ameliorated age-induced impairment in the EFS response as both the amplitude and duration of EFS-induced contractions were comparable with those in young adult strips, and the off-relaxations were absent (Fig. 1, A and B; Table 1).

The aged-related impairment in gallbladder contractility could involve changes in the intrinsic innervation of the organ, alterations in the smooth muscle contractility itself, or a combination of these mechanisms. The gallbladder contractile response to exogenous ACh was significantly reduced in aged animals (young adult 3.266 ± 0.260, aged 1.911 ± 0.27, and melatonin 4.232 ± 0.538 mN/mg; n = 29, 15, and 11 strips, respectively, P < 0.001 aged versus young adult and aged versus melatonin), suggesting an age-related impairment in the myogenic response to the neurotransmitter released from intrinsic nerves. By taking into account the fact that ACh is the primary excitatory neurotransmitter in the gallbladder (Parkman et al., 1997), the expression of an EFS-induced response to ACh-induced contraction would reflect the amount of ACh released. As shown in Fig. 1C, the EFS-induced response expressed in this way is higher in strips from aged animals (P < 0.001 by ANOVA), which would indicate an increase in the release of ACh or a decrease in the release of inhibitory neurotransmitters. An increase in the release of ACh does not seem probable as the effect of atropine was not different in young adult, aged, or melatonin-treated aged strips (Fig. 2; Table 2).

To explore the possibility of an age-induced change in the release of inhibitory neurotransmitters, we assayed the effects of L-NAME in each experimental group. The inhibitor of the nitric-oxide synthase enhanced EFS-induced contraction in young adult strips (89.3% enhancement at 5 Hz, n = 10, P < 0.01 by ANOVA) but did not have any effects in the aged group (Fig. 3; Table 2). When the old animals were pretreated with melatonin, the nitrergic component was stimulated by EFS and L-NAME enhanced EFS-induced contraction

| Frequency | Young Adult | | | Aged | | | Melatonin | | |
|-----------|-------------|-----------|-----------|-----------|-----------|-----------|
| Hz        | Amplitude   | Duration  | Amplitude | Duration  | Amplitude | Duration  |
|           | mN/mg s     |           | mN/mg s   |           | mN/mg s   |           |
| 5         | 0.988 ± 0.117 | 43.08 ± 1.29 | 0.643 ± 0.063* | 31.01 ± 1.15* | 0.812 ± 0.140 | 42.23 ± 1.66 |
| 10        | 1.315 ± 0.132 | 48.57 ± 1.88 | 0.890 ± 0.078* | 33.73 ± 1.28* | 1.170 ± 0.163 | 45.53 ± 2.65 |
| 15        | 1.609 ± 0.147 | 52.80 ± 1.97 | 1.120 ± 0.099* | 37.26 ± 1.50* | 1.463 ± 0.178 | 45.93 ± 2.43 |
| 25        | 1.956 ± 0.164 | 57.30 ± 2.55 | 1.384 ± 0.120* | 39.14 ± 2.12* | 1.967 ± 0.175 | 52.06 ± 2.97 |
| 40        | 2.232 ± 0.181 | 55.90 ± 2.70 | 1.570 ± 0.139* | 39.47 ± 2.61* | 2.351 ± 0.157 | 52.66 ± 3.24 |

* P < 0.0001 adult vs. aged.

A, B, and C, effects of atropine (1 μM) on the frequency-response curves to EFS (0.3-ms duration pulse, 5–40 Hz, 350 mA, for 10 s every 3 min) applied to gallbladder strips from young adult (n = 24 strips), aged (n = 21 strips), and aged guinea pigs treated with 2.5 mg/kg/day melatonin (n = 10 strips). After EFS was performed in control conditions (——) strips were incubated for 30 min with atropine and EFS was repeated again (●●●). Statistical analysis indicates P < 0.001 (by ANOVA) in all experimental groups. D, atropine-induced inhibition of EFS responses in the three experimental groups. No significant differences between groups were found at any of the frequencies tested.
(193.8% enhancement at 5 Hz, n = 5, P < 0.001, by ANOVA) (Fig. 3; Table 2).

Age-Related Changes in Sensory Innervation. In our model, when the sensory nerves were desensitized by the treatment with a high concentration of capsaicin (10 μM), a transient atropine-sensitive contraction was recorded in both young adult and old strips (adult 0.081 ± 0.016 and aged 0.100 ± 0.016 mN/mg; n = 8 and 6 strips, respectively), but there was no change in EFS-induced responsiveness of young adult strips (Fig. 4; Table 2). However, when aged strips were desensitized by capsaicin a significant increase in the EFS-induced response was recorded (41.9% increase, P < 0.01 by ANOVA) (Fig. 5, A and B; Table 2) and converted the effect of capsaicin to an inhibition of the contractile response (37% inhibition respect to CGRP8–37 alone, n = 4, P < 0.01 by ANOVA) (Fig. 5, A and B; Table 2).

Age-Related Changes in Gallbladder Contractility. To explore how aging affects Ca2+ influx-mediated contractions, we used voltage-activated calcium entry induced by 60 mM KCl, which produced a sustained contraction that was significantly reduced (24.8% reduction) when we tested gallbladder strips from aged animals and recovered by melatonin treatment (young adult 3.77 ± 0.37, aged 2.84 ± 0.26, and melatonin 4.24 ± 0.32 mN/mg; n = 26; 23 and 9 strips, respectively, P < 0.05) (Fig. 6). We have recently shown that

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Young Adults</th>
<th>Aged</th>
<th>Melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine (10 μM)</td>
<td>Abolition</td>
<td>Abolition</td>
<td>Abolition</td>
</tr>
<tr>
<td>l-NAME (100 μM)</td>
<td>Increase</td>
<td>No effect</td>
<td>Increase</td>
</tr>
<tr>
<td>Capsaicin (10 μM)</td>
<td>No effect</td>
<td>Increase</td>
<td>No effect</td>
</tr>
<tr>
<td>Glibenclamide (10 μM)</td>
<td>No effect</td>
<td>Increase</td>
<td>No effect</td>
</tr>
<tr>
<td>Glibenclamide + Capsaicin</td>
<td>No effect</td>
<td>Increase</td>
<td>No effect</td>
</tr>
<tr>
<td>CGRP8–37 (1 μM)</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Decrease</td>
</tr>
<tr>
<td>CGRP8–37 + Capsaicin</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

* Significance by two-way ANOVA.
Capacitative calcium entry (CCE) is another influx route activating contraction in gallbladder smooth muscle (Morales et al., 2004). As previously reported, after depletion of the stores by incubation of control gallbladder strips with 1 μM TPS in Ca^{2+}-free medium for 30 min, reintroduction of extracellular Ca^{2+} induced a sustained contraction (3.69 ± 0.53 mN/mg; n = 12) that was significantly smaller in aged strips (2.68 ± 0.29 mN/mg; n = 9, P < 0.01) but similar in melatonin-treated animals (4.41 ± 0.39 mN/mg; n = 8) (Fig. 6B).

To test whether aging affects Ca^{2+} release from sarcoplas-
mic reticulum, the main intracellular Ca$_{2+}$ store in gallbladder smooth muscle (Morales et al., 2005a), we used CCK as the tool of election. As was the case for Ca$_{2+}$ influx-induced contractions, CCK-induced contraction was impaired in aged strips and melatonin treatment recovered contractility (young adult 3.7 ± 0.38, aged 2.48 ± 0.25, and melatonin 3.67 ± 0.52 mN/mg; n = 30, 23, and 9 strips, respectively, P < 0.01) (Fig. 6C).

**Age-Related Changes in Calcium Handling.** The diminished contractile response to Ca$_{2+}$ influx could be the result of the reduction in the calcium entry as a consequence of age-mediated calcium channel impairment. To test this possibility, we quantified [Ca$_{2+}$]$_i$ in response to 60 mM KCl and to the protocol to activate capacitative calcium entry. As represented in Fig. 7, A and B, a sustained [Ca$_{2+}$]$_i$ plateau due to Ca$_{2+}$ entry from the extracellular medium was reduced in aged gallbladder smooth muscle cells (26.2 and 34.2% reduction for KCl and CCE, respectively; n = 18 to 49 cells, P < 0.05). Interestingly, melatonin did not have any effects on calcium influx (KCl: 0.111 ± 0.008 versus 0.115 ± 0.010 ΔF$_{340/380}$; n = 34 and 13 cells for aged and melatonin-treated aged cells, respectively; CCE: 0.063 ± 0.005 versus 0.067 ± 0.007 ΔF$_{340/380}$; n = 8 and 6 cells).

When we quantified [Ca$_{2+}$]$_i$, in response to a CCK challenge, there was no change in the amplitude of the Ca$_{2+}$ transient peak, indicative of intracellular Ca$_{2+}$ release (n = 28 and 15 cells, P = 0.897) (Fig. 7C). To confirm that Ca$_{2+}$ release from stores through d-myo-inositol 1,4,5-trisphosphate (IP$_3$) and ryanodine channels remained unchanged in aging, we exposed the cells to 10 μM Bt$_3$Ins(1,4,5)P$_3$-PM, a membrane permeable analog of IP$_3$ and to caffeine. Similar to the CCK-induced Ca$_{2+}$ transient, the peak responses to the IP$_3$ analog were not affected by aging (young adult 0.426 ± 0.031 and aged 0.379 ± 0.028 ΔF$_{340/380}$; n = 13 and 7 cells, respectively, P = 0.272). When we used caffeine to induce Ca$_{2+}$ release through ryanodine receptors, we did not detect a difference in the Ca$_{2+}$ transient in young adult versus aged gallbladder smooth muscle cells (n = 31 and 36
cells, \( P = 0.412 \) (Fig. 7D). Collectively, these results suggest that both Ca\(^{2+}\) release channels and calcium content in the stores are not affected by aging. The latter result was confirmed by application of a low level of ionomycin in Ca\(^{2+}\)-free solution to the cells, a treatment that releases the Ca\(^{2+}\) stores while bypassing channels and receptors. This treatment caused a similar increase in \([\text{Ca}^{2+}]_i\) in both adult and aged cells (young adult \(0.188 \pm 0.029\) and aged \(0.180 \pm 0.015\) \(\text{F}_{340/380}\); \(n = 14\) and 24 cells, respectively, \( P = 0.821\)), indicating that the integrity of calcium stores is preserved in aging. Melatonin treatment did not have any effect on Ca\(^{2+}\) release mechanisms nor on Ca\(^{2+}\) store content (data not shown).

**Age-Related Changes in Contractile Machinery.**

Taken together, the results described above indicate that the age impairment in contractility is, at least in part, the result of alterations in Ca\(^{2+}\) influx, but this physiological condition could also alter gallbladder contractility independently of the calcium signal. This possibility was tested by application of 1 \(\mu\)M ionomycin in presence of extracellular Ca\(^{2+}\) (at this concentration ionomycin raises \([\text{Ca}^{2+}]\), independently of channels and receptors). Ionomycin caused similar elevations in adult, aged, and melatonin-treated cells (young adult \(0.355 \pm 0.050\), aged \(0.419 \pm 0.052\), and melatonin \(0.376 \pm 0.057\) \(\text{F}_{340/380}\); \(n = 32, 30,\) and 16 cells, respectively, \( P > 0.05\)) but induced much lower contraction in aged strips than in adult and melatonin-treated aged tissues (young adult \(4.23 \pm 0.57\), aged \(2.12 \pm 0.201\), and melatonin \(5.39 \pm 0.87\) mN/mg; \(n = 7, 14,\) and 8 strips, respectively, \( P < 0.01\) young adult versus aged), suggesting that aging can also alter the contractile machinery independently of the calcium signal.

To investigate one of the possible causes of this impaired contractility, we determined the total amount of the contractile protein F-actin by labeling gallbladder smooth muscle cells with FITC-stained phalloidin. We found that aging resulted in a statistically significant decrease in the F-actin content that was recovered by melatonin treatment (young adult \(33.59 \pm 0.86\), aged \(9.92 \pm 0.49\), and melatonin \(28.95 \pm 0.87\) arbitrary units of fluorescence; \(n = 100, 63,\) and 92 cells, respectively, \( P < 0.05\) young adult versus aged) (Fig. 6D).

**Discussion**

At a time when the world population is aging at a rapid rate, there is an emergent need to understand the biology of the aged GI tract and to translate this knowledge to thera-
peutics to improve GI function and quality of life of elderly persons. The present study was conducted to elucidate functional changes in gallbladder motility during aging and to explore the potential use of melatonin to ameliorate the onslaught of aging on GI structures.

We found that aging impairs muscular contraction and modifies neurological control of muscle. The possibility that altered GI function in elderly persons is related to neurodegeneration of the enteric nervous system is supported by studies conducted both in animals and humans (for review, see Wade and Cowen, 2004). Age-related neuronal loss in the small and large intestines occurs exclusively in the cholinergic population (Roberts et al., 1994; Phillips et al., 2003), with little change, if any, in nitrergic neurons. However, in the gallbladder, cholinergic transmission appears very well maintained with age, because the atropine-sensitive contractile response to EFS remains the same in the adult and aged groups. Our finding of a decrease in the functionality of nitrergic fibers in the aged group can be related to previous reports of morphological alterations in nitrergic neurons (Phillips et al., 2003) and impaired nitrergic function by aging (Smits and Lefebvre, 1996), which is consistent with our functional studies.

Interestingly, in our model, the loss of nitrergic inhibitory function was compensated for by over-reactivity of sensory inhibitory nerves, as demonstrated by the increase in the EFS-induced contraction after desensitization with capsaicin. The ganglionic plexus of the gallbladder is rich in afferent fibers that are immunoreactive for both substance P and CGRP (Mage and Gereshon, 1989). Application of capsaicin induced a contractile response that was the result of the substance P-induced contraction and CGRP-induced relaxation (Maggi et al., 1989), similar to our finding of capsaicin-evoked contraction sensitive to atropine. However, capsaicin desensitization of sensory nerves increased the EFS-induced responses in aged strips without affecting adult strips. The fact that capsaicin effects were mediated by the CGRP1 receptor (blocked by human CGRP(8–37) and its downstream target K<sub>A TP</sub> channels, which are insensitive to capsaicin) clearly suggests that hypersensitivity of inhibitory sensory fibers containing CGRP contribute to gallbladder motility disorders in aging. It is now clear that hypersensitivity of sensory neurons contributes to functional bowel disorders such as irritable bowel syndrome, Hirschsprung's disease, rectal hypersensitivity, and fecal urgency (Grundy, 2006).

Motility disorders during aging can also involve age-dependent changes in the response of smooth muscle to neurotransmitters and hormones (for review, see Bitar, 2003) due to inhibition of transduction pathways or to alterations of contractile proteins (Bitar, 2003; Bitar and Patil, 2004). We report here both changes in Ca<sup>2+</sup> signals and a decrease in the F-actin content in aged gallbladder smooth muscle cells. Our study shows that aging affects Ca<sup>2+</sup> entry through both L-type Ca<sup>2+</sup> channels and store-operated Ca<sup>2+</sup> channels, although the content of intracellular Ca<sup>2+</sup> stores and Ca<sup>2+</sup> release through IP<sub>3</sub> and ryanodine receptors is not influenced. Age-related changes in Ca<sup>2+</sup> homeostasis seem to be tissue-specific. Thus, reduction of voltage-dependent calcium channels has been described in rat brain cortices (Iwamoto et al., 2004), canine atria (Dun et al., 2003), and colon (Xiong et al., 1993, 1995), but voltage-activated Ca<sup>2+</sup> influx is increased in mammalian CA1 hippocampal neurons (Thibault and Landfield, 1996) and heart (Josephson et al., 2002) during aging. There are no published data related to the effects of aging on CCE channels. Our data are consistent with the hypothesis that in gallbladder smooth muscle aging mainly affects membrane L-type and CCE Ca<sup>2+</sup> channels, which could contribute to the reduction in contractility in response to KCl, CCE, but also to CCK, as this hormone induces Ca<sup>2+</sup> entry after releasing Ca<sup>2+</sup> from intracellular stores (Morales et al., 2004, 2005a).

One of the most compelling findings reported here is the beneficial effect of melatonin on gallbladder neuromuscular function and myogenic contractility. Thus, after treating the animals for 4 weeks with melatonin (2.5 mg/kg/day), there was not only a total recovery of the contractile response to Ca<sup>2+</sup> influx activation and EFS but also a reversion of the aged-induced changes in efferent and sensitive innervation, although melatonin did not restore the Ca<sup>2+</sup> influx. The recovery of the contractility without changes in Ca<sup>2+</sup> homeostasis can only be explained by effects of melatonin on Ca<sup>2+</sup>-insensitive contractility steps such as contractile proteins or Ca<sup>2+</sup> sensitization of the contractile machinery. Whereas the former can be related to the increase in the F-actin content described in this study, the later is now under research in our laboratory.

Melatonin is a hormone that is secreted by the pineal gland following a circadian rhythm with minimal blood levels of melatonin during the daytime and maximal levels observed mostly during the middle of the night. Melatonin has special importance in the gastrointestinal tract because the enterochromaffin cells of the gut are the main source of extrapineal melatonin (Kvetnoy et al., 2002). Metabolism of melatonin in liver seems to be the major metabolic pathway for its deactivation (Lane and Moss, 1985), but active melatonin is also secreted in bile and concentrated in the gallbladder (Tan et al., 1999). Although it is well established that pineal melatonin decreases with age (Karasek, 2004), there is no information available regarding gastrointestinal melatonin. If gastrointestinal melatonin content also decreases with age, the hepatobiliary system in general and the gallbladder in particular would because of the lack of melatonin protective effects. The restoration of gallbladder contractility in response to melatonin treatment in aged guinea pigs is consistent with this hypothesis.

Regarding the GI tract, there are numerous lines of evidence demonstrating the protective and healing effects of melatonin in gastric ulcers and experimental colitis. Whereas a direct antioxidative effect, activation of CO<sub>2</sub>H secretion by enterocytes, and release of endogenous prostaglandins mediates these protective effects, the healing effect was associated with production of endogenous nitric oxide and participation of sensory nerves (for review, see Reiter et al., 2003). The effects of melatonin on symptoms of colitis are related to decreases in nitric oxide and prostaglandin E<sub>2</sub> content as the result of down-regulated expression of colonic inducible nitric-oxide synthase and cyclooxygenase-2 (Dong et al., 2003) in addition to an enhancement of the immune response in the gastrointestinal tract (Mei et al., 2002). The present study is the first report on the beneficial effects of melatonin treatment in aged gastrointestinal tract, but more studies are required to establish the cellular mechanisms through which melatonin exerts its effects in the gallbladder and the optimal pattern of melatonin treatment to amelio-
rate the effects of aging. Both lines of research will elucidate the potential use of melatonin in elderly population.

In conclusion, our results indicate that aging causes impairment of gallbladder function as consequence of neurally mediated and neurally independent mechanisms and support a possible beneficial effect of melatonin in gallbladder dysfunction related to aging.

Acknowledgments

We thank Gary M. Mawe for critical reading of the manuscript and Purificacion Delgado for technical assistance.

References


Address correspondence to: Dr. Maria J. Pozo, Department of Physiology, Nursing School, Avda Universidad s/n, 10071 Cáceres, Spain. E-mail: mjpozo@unex.es