Aging impairs Ca^{2+} sensitization pathways in gallbladder smooth muscle

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Abstract Calcium sensitization is an important physiological process in agonist-induced contraction of smooth muscle. In brief, calcium sensitization is a pathway that leads to smooth muscle contraction independently of changes in $[\text{Ca}^{2+}]_i$ by mean of inhibition of myosin light chain phosphatase. Aging has negative impacts on gallbladder contractile response due to partial impairment in calcium signaling and alterations in the contractile machinery. However, information regarding aging-induced alterations in calcium sensitization is scanty. We hypothesized that the calcium sensitization system is negatively affected by age. To investigate this, gallbladders were collected from adult (4 months old) and aged (22–24 months old) guinea pigs. To evaluate the contribution of calcium sensitization pathways we assayed the effect of the specific inhibitors Y-27632 and GF109203X on the “in vitro” isometric gallbladder contractions induced by agonist challenges. In addition, expression and phos-

phorylation (as activation index) of proteins participating in the calcium sensitization pathways were quantified by Western blotting. Aging reduced bethanechol- and cholecystokinin-evoked contractions, an effect associated with a reduction in MLC20 phosphorylation and in the effects of both Y-27632 and GF109203X. In addition, there was a drop in ROCK I, ROCK II, MYPT-1 and PKC expression and in the activation/phosphorylation of MYPT-1, PKC and CPI-17 in response to agonists. Interestingly, melatonin treatment for 4 weeks restored gallbladder contractile responses due to re-establishment of calcium sensitization pathways. These results demonstrate that age-related gallbladder hypocontractility is associated to alterations of calcium sensitization pathways and that melatonin treatment exerts beneficial effects in the recovery of gallbladder contractility.

Keywords Gallbladder · Bethanechol · CCK · Calcium sensitization · Aging · Oxidative stress · Melatonin

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Introduction

Gallbladder functions (bile storage and release) are controlled by the contractile state of the gallbladder smooth muscle. This comprises periods of active and passive relaxations (during which hepatic bile is stored and concentrated) followed by contractions that release the bile in response to the lipids present in the food (Pozo et al. 2004). Smooth muscle contraction is mainly achieved by increases in cytoplasmatic

calcium concentration ($[Ca^{2+}]_i$) that activate myosin light chain kinase (MLCK) to catalyze the phosphorylation of myosin regulatory light chain (MLC₂₀) causing force development (Karaki et al. 1997). An additional signaling pathway involved in force maintenance, termed calcium sensitization pathway, evokes smooth muscle contraction independently of changes in $[Ca^{2+}]_i$ by inhibition of myosin light chain phosphatase (MLCP) and the consequent dephosphorylation of MLC₂₀ in the presence of basal (Ca^{2+} -dependent or Ca^{2+} -independent) or increased MLCK activity (Somlyo and Somlyo 2003). MLCP inhibition occurs mainly by two pathways: Rho A/ROCK/MYPT-1 and PKC/CPI-17. Activation of some G-protein coupled receptors by their agonists induce an increase in membrane-bound Rho A-GTP necessary for the activation of Rho kinases (ROCK I and ROCK II), that phosphorylate, amongst other proteins, the myosin phosphatase targeting 1 (MYPT-1), a subunit of MLCP. p-MYPT-1 inhibits MLCP activity, thus favouring contraction. G proteins cause also activation of phospholipase C (PLC), diacylglycerol (DAG) output and activation of protein kinase-C (PKC). PKC phosphorylates C-kinase-dependent phosphatase inhibitor of 17 kDa (CPI-17), an inhibitor of the MLCP activity via its PP1c subunit, resulting in smooth muscle contraction (Eto et al. 1995; Somlyo and Somlyo 2003). The activity of MLCP can also be regulated by altering its heterotrimeric conformation (Hirano et al. 2004) and by the protein scaffold-myosin phosphatase-Rho interacting protein (M-RIP) (reviewed by Kim et al. 2008). In addition, in the absence of changes in $[Ca^{2+}]_i$, MLC₂₀ can be phosphorylated driving to contraction by Ca^{2+} -independent MLCKs such as ZIP kinase and integrin-linked kinase (Murthy 2006). The impact of aging on calcium sensitization pathways has been poorly explored.

It has been described that aging compromises gallbladder function and drives to an elevated incidence of cholesterol gallstones in old individuals (Chen et al. 1999; van de Heijning et al. 1999; Xu and Shaffer 1996). We found that aging modifies neurological control of guinea pig gallbladder and impairs contractile response as the result of alterations in the contractile machinery without affecting calcium release from intracellular stores (Gomez-Pinilla et al. 2006). The fact that impairment in the contractile capacity does not run in parallel with alterations in calcium signals suggests that calcium-independent mechanisms, such

as calcium sensitization pathways, would be additional targets for the harmful effects of age. Herein we explore this hypothesis to give some insights into the intracellular mechanisms affected. In order to test whether age-related effects in Ca^{2+} -independent contractility could be ameliorated by melatonin we treated a group of aged guinea pig with the indoleamine for 28 days.

Our data indicate that aging decreases the participation of calcium sensitization pathways in guinea pig gallbladder, which is translated into impaired contractile responses to agonists. Melatonin treatment normalizes calcium sensitization pathways and the contractile responses, indicating that both processes run in parallel in the gallbladder.

Material and methods

Animals

Female guinea pigs (Dunkin–Hartley) were divided into two groups according to age: young adults (4 months old, $n=12$) and aged (22–24 months old, $n=10$). Animals were housed in light (12 h light–dark cycles) and temperature (20°C) controlled rooms and had free access to water and food. A group of aged guinea pigs ($n=10$) was treated orally with melatonin (2.5 mg kg⁻¹ day⁻¹) every day 1 h before the dark phase for 28 days. All experiments were performed according to European guidelines for animal research and approved by the Animal Ethics Committees of the University of Extremadura.

Contraction recording of guinea pig gallbladder strips

Deep halothane anesthetized guinea pigs were killed by cervical dislocation, the gallbladder was removed and placed in Krebs–Henseleit solution. The gallbladder was cut in longitudinal full-thickness strips (3 × 8 mm) that were placed vertically in a 10-ml organ bath filled with Krebs–Henseleit solution maintained at 37°C and gassed with 95% O₂–5% CO₂. Isometric contractions were measured using force transducers and digitized using a MacLab system (ADInstruments, Colorado Springs, CO, USA). Gallbladder strips were mounted under an initial resting tension equivalent to 1.5 g load and allowed to equilibrate for 60 min, with solution changes every 20 min. Previous tension recordings, in response to 100 μM bethane-

chol, at different loads showed that 1.5 g load was the optimal stretch for recording the maximal tension in both age groups. Every strip coming from the same animal was used in a different experimental protocol. Force was normalized with respect to the cross section area (CSA) of each muscle strip.

In order to find the contribution of calcium sensitization pathways in the agonist-induced gallbladder smooth muscle contraction a protocol of cumulative dose–response curves for bethanechol (BE) and cholecystokinin (CCK) was designed. The cumulative dose–response curve commenced with the lowest concentration of the agonist and the subsequent doses were not added until the contractile response became constant. After the maximal response was reached, 1 h was required for washing out and returning to the resting tension. Then, the muscle strips were incubated for 30 min with the antagonists (5 μ M Y-27632 or 5 μ M GF109203X, inhibitors of ROCK- and PKC-dependent pathways, respectively) and the cumulative concentration–response curve was repeated.

Analysis of protein expression and phosphorylation by Western blot

Guinea pig gallbladder smooth muscle was cut in three pieces, one was not stimulated (control), one was challenged with BE (0.1 μ M) and the last one with 10 nM of CCK. The stimuli were applied for 1 min. After challenges tissues were quickly frozen, pulverized in liquid nitrogen, extracted in lysis buffer and then sonicated for 5 s. Lysates were centrifuged at 10,000 $\times$ g for 15 min at 4°C to remove nuclei and intact cells and the protein concentration was measured.

Protein extracts (30 μ g) were heat-denatured at 95°C for 5 min with DTT, electrophoresed on 7.5% or 15% polyacrylamide-SDS gels and then transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature using 10% bovine serum albumin (BSA) and incubated overnight at 4°C with affinity-purified polyclonal antibodies against ROCK I and II and COX-2 (1:500; Becton-Dickinson Bioscience, Erembodeggen, Belgium), Rho A, p-CPI-17, CPI-17, p-MYPT-1, MYPT-1, pMLC20 and MLC20 (1:500; 1:750 for pMLC20 and 1:7,000 for MLC20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-PKC (pan antibody, 1:5,000; Cell Signaling Technology, Danvers, MA, USA). A rabbit anti-actin polyclonal antibody (1:5,000; Sigma, St. Louis, MO, USA) was used as control for

protein loading. After washing, the membranes were incubated for 1 h at room temperature with anti-mouse (1:10,000; Amersham Biosciences, Bucks, UK) or anti-rabbit (1:7,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) IgG-horseradish peroxidase conjugated secondary antibodies. Bands were visualized using the supersignal west pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and quantified using the software gel-pro analyzer (4.0, Media Cybernetics, Bethesda, MD, USA) and normalized to actin content.

In protein phosphorylation assays, two similar gels were run and one membrane was incubated with the primary antibody against the total protein and the other with an anti-phosphoprotein of interest.

Solutions and drugs

Krebs–Henseleit solution (in mM): 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃ and 11.5 D-glucose (pH=7.35). Lysis buffer (in mM): 50 Tris–HCl pH 7.4, 150 NaCl, 1% Triton X-100, 1% deoxycholate, 0.5% (w/v) NaN₃, 0.4 EDTA, supplemented with a cocktail of protease and phosphatase inhibitors (Complete, EDTA-free and PhosSTOP; Roche Molecular Biochemicals, Mannheim, Germany). Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: BE, CCK and melatonin are from Sigma and Y-27632 and GF109203X from Calbiochem (La Jolla, CA, USA).

Quantification and statistics

Results are expressed as means $\pm$ SEM of *n* gallbladder strips or blots. Gallbladder tension is given in millinewtons per square millimeter of tissue since data were normalized respect to CSA. Overall comparison between concentration–response curves was performed by a mixed two-way analysis of variance (ANOVA) model using age/treatment $\times$ concentration as fixed and repeated factors, respectively. To compare maximum effects ($E_{\max}$), we used a priori planned contrast. Statistical differences between means were determined by Student's *t*-test. All data were inspected to exclude gross departures from normality or homoskedascity and correlation between mean and variance sizes between groups. The statistical power was adjusted to be at least 0.8 (0.2 type II error). Differences were considered significant at $P<0.05$.

Results

Effects of aging on gallbladder contractile response

In a previous report (Gomez-Pinilla et al. 2006) we showed that aging impaired contraction of the guinea pig gallbladder in response to agonists. In the current study, we give some insights on the age induced alterations of stimulus–contraction coupling mechanisms. To evaluate the effects of aging we exposed guinea pig gallbladder strips to increasing levels of

BE, a muscarinic receptor agonist, or CCK octapeptide, a pro-contraction hormone secreted in response to lipid content of food.

Bethanechol (10 nM to 10 mM) and CCK (10 pM to 1 μ M) produced a dose-dependent contraction with a maximal effect at 1 mM and 1 μ M, respectively (Fig. 1a, b). Analysis by two-way ANOVA showed that age causes a significant decrease in gallbladder contractile responses to both agonists (BE: $F=191.2$ and $P<0.0001$; CCK: $F=58.87$ and $P<0.0001$). Thus, the $E_{\max}$ to BE and CCK were lower in aged

Fig. 1 Aging reduces guinea pig gallbladder myogenic response. Strips were exposed to increasing concentrations of **a** bethanechol (BE, 10 nM to 10 mM) and **b** cholecystikinin (CCK, 10 pM to 1 μ M). Note a significant reduction in the contractile response in gallbladders from aged animals. In (a) and (b), contractile responses are expressed as mean $\pm$ SEM. $n=19$ –27 strips ($***P<0.0001$, adult vs. aged). **c** Original Western blots on gallbladder smooth muscle from adult (Ad) and aged (Ag) guinea pigs for phospho-MLC20 and total MLC20 in unstimulated (control) or agonist-treated gallbladder samples.

After normalization to actin content, the optic density of phosphorylated protein vs. total protein was calculated for control and treated samples in both age groups. Then, the resulting ratio for treated samples was expressed as fold increase respect to unstimulated conditions. Note that protein phosphorylations were decreased in the aged group. The histograms (mean $\pm$ SEM) show summarized data of changes in MLC20 phosphorylation for 8–10 blots ($*P<0.05$, adult vs. aged).

muscles with a 45.62% (BE) and 34.44% (CCK) of reduction associated to aging (BE $E_{\max}$: 4.91 ± 0.28 vs. 2.67 ± 0.25 mN/mg, $n=22$ and 19 , $P<0.001$; CCK $E_{\max}$: 4.21 ± 0.28 vs. 2.76 ± 0.27 mN/mg, $n=27$ and 19 , $P<0.001$; Fig. 1a, b).

The age-induced reduction in the contractile force was associated to a decrease in the ratio of MLC20 phosphorylation as illustrates Fig. 1c. The extent of MLC20 phosphorylation induced by BE and CCK in aged strips was 30% and 36% smaller than in adult strips ($P<0.05$, $n=8$ and 10). Age-related alterations could be explained by alterations in calcium handling, as calcium is the main second messenger controlling contraction in smooth muscle. However, we have previously shown that age-related decreases in gallbladder contraction do not run in parallel with impairment in calcium-dependent signaling (Gomez-Pinilla et al. 2006). Thus, we explored the status of

calcium-independent mechanism leading to contraction or calcium sensitization during aging.

Effects of aging on contractile response sensitivity to Y-27632 and GF109203X

In order to investigate the contribution of calcium sensitization pathways in the contractile response to BE and CCK, concentration–response curves were performed in the absence and presence of Y-27632 (5 μ M) and GF109203X (5 μ M), specific inhibitors of ROCK and PKC, respectively (Toullec et al. 1991; Uehata et al. 1997). The pre-treatment with Y-27632 reduced significantly the dose–response curve to BE in adult and aged strips, (adult: $F=95.90$, $P<0.001$; aged: $F=50.53$, $P<0.001$; Fig. 2a, b) but the reduction in the maximal response to BE was lower in aged tissues (39.1 ± 4.6 vs. $23.9 \pm 5.74\%$ of inhibi-

Fig. 2 Aging reduces the sensitivity of bethanechol-induced gallbladder contractions to Y-27632 and GF109203X, inhibitors of ROCK and PKC, respectively. Effects of 5 μ M Y-27632 (a and b) and 5 μ M GF109203X (c and d) on bethanechol (BE) evoked contractions in gallbladder strips from adult and aged

guinea pigs. Note that the effects of the inhibitors are lower in aged than adult animals indicative of a lower participation of calcium sensitization pathways. Data are expressed as mean $\pm$ SEM. $n=10$ –15 strips (** $P<0.01$ and *** $P<0.001$, adult vs. aged)

tion for adult and aged strips, respectively, $P<0.05$). Similar results were found in the presence of GF109203X (adult: $F=97.21$, $P<0.001$; aged: $F=32.90$, $P<0.01$; Fig. 2c, d) although the inhibition of maximal response to BE in old animals was even lower (42.8 ± 6.8 vs. $16.9\pm8.4\%$ of inhibition for adult and aged strips, respectively, $P<0.01$). When CCK was used as agonists we found a similar picture (Fig. 3), the antagonists reduced the dose response curves of both agonists in both age groups but their effects were less marked in old tissue, especially those of GF109203X.

This pharmacological approach points out that the contribution of calcium sensitization pathways are less prominent in aged animal, which could be responsible for the age-induced impairment of gall-

bladder contractile response. To characterize the age-induced impairment, we assessed by Western blot the expression and activation of the more relevant proteins involved in calcium sensitization pathways.

Effects of aging on expression of members of calcium sensitization pathways

Consistent with the reduced inhibition evoked by Y-27632 and GF109203X on the contractile agonist response in old animals, aging reduced the expression of some proteins participating in calcium sensitization mechanisms. In this regard, aging induced a significant reduction of some components of Rho A/ROCK pathway without affecting the expression of Rho A (ROCK I 50% of adult, ROCK II 33% of adult and

Fig. 3 Aging reduces the sensitivity of CCK-induced contractile response to Y-27632 and GF109203X, inhibitors of ROCK and PKC, respectively. Effects of 5 μ M Y-27632 (a and b) and 5 μ M GF109203X (c and d) on CCK-evoked contractions in gallbladder strips from adult and aged guinea pigs. Note that

the effects of the inhibitors are smaller in aged than adult animals indicative of a lower participation of calcium sensitization pathways. Data are expressed as mean $\pm$ SEM. $n=6-9$ strips (** $P<0.01$ and *** $P<0.001$, adult vs. aged)

Fig. 4 Aging decreases the expression of proteins involved in Rho A/ROCK pathway. Original Western blots on gallbladder smooth muscle from adult (*Ad*) and aged (*Ag*) guinea pigs for **a** Rho A, **b** ROCK I, **c** ROCK II and **d** MYPT-1. There are not differences in the expression of Rho A while aging reduces the expression of ROCK I, ROCK II and MYPT-1. Actin was used as loading control and proteins expression was calculated respect to actin expression. Histograms summarize proteins expression (mean $\pm$ SEM) for 6–8 blots (* P <0.05 and ** P <0.01, adult vs. aged)

Fig. 5 Aging decreases the expression of proteins involved in PKC/CPI-17 pathway. Original Western blots on gallbladder smooth muscle from adult (*Ad*) and aged (*Ag*) guinea pigs for **a** phospho-PKC and **b** CPI-17. Note that aging reduces the

expression of phospho-PKC. Actin was used as loading control and proteins expression was calculated respect to actin expression. Histograms summarize protein expression (mean $\pm$ SEM) for 6–8 blots (** P <0.01, adult vs. aged)

MYPT-1 40% of adult, Fig. 4). In addition, the expression of phospho-PKC was reduced by 40% in aged gallbladder while the expression of its effector CPI-17 was similar in adult and aged tissues (Fig. 5).

Effects of aging on activation of calcium sensitization pathways

Activation of calcium sensitization was explored using phosphoprotein-specific antibodies against the key components of the pathways. Phosphorylation of MYPT-1 (at Thr-853), PKC (at Ser-660) and CPI-17 (at Thr-38) in response to BE and CCK is shown in Fig. 6. In adult gallbladders, the ratios phosphorylated/total proteins after agonist challenge were higher than 1 (the ratios for unstimulated tissues are referred as 1),

indicating an increase in phosphorylation. By the contrary, in aged animals phosphorylation in response to BE and CCK was reduced for the three regulatory proteins tested (Fig. 6). The decreased phosphorylation found in aged animals together with the reduction in protein expression can explain the reduced effects of the inhibitors tested in contractility, and altogether evidence that aging is associated to a partial loss of calcium sensitization which impairs gallbladder contractile response.

Effects of melatonin treatment on gallbladder contractility and calcium sensitization pathways

The beneficial effects of melatonin treatment against age-associated gallbladder hypocontractility has been

Fig. 6 Aging impairs activation of Ca^{2+} sensitization pathways. **a** Western blots using anti-p-Thr853-MYPT-1 and anti-MYPT-1 in unstimulated (control) or CCK-treated gallbladder samples from adult (*Ad*) and aged (*Ag*) guinea pigs. **c** Changes in phospho-PKC expression in response to CCK for both groups of animals. **e** Representative Western blots using anti-p-Thr38-CPI-17 and anti-CPI-17 in unstimulated (control) or CCK-treated tissues. The histograms show summarized data of changes in **b** MYPT-1, **d** PKC and **f** CPI-17 phosphorylation.

After normalization to actin content, the optic density of phosphorylated protein vs. total protein was calculated for control and treated samples in both age groups. Then, the resulting ratio for treated samples was expressed as fold increase respect to unstimulated conditions. Note that protein phosphorylations were decreased in the aged group for all the proteins studied and for both challenges. $n=6-8$ experiments. (* $P<0.05$, ** $P<0.01$, adult vs. aged)

previously described (Gomez-Pinilla et al. 2006), and here we confirm previous result showing that “in vivo” melatonin treatment for 28 days almost normalizes BE and CCK concentration–response curves (Fig. 7, left bars). Given that melatonin exerts potent antioxidant effects at multiple stages of the signal pathways (Pozo et al. 2010), we treated aged animals with melatonin to find if aged-induced alterations in the effects of Y-27632 and GF109203X and in the level of expression and activation of calcium sensitizing proteins could be prevented. In line with the recovery of the contractile capacity, Fig. 7 shows that melatonin treatment normalizes the effects of the inhibitors (Fig. 7, middle and right bars). Melatonin treatment also increases ROCK I, ROCK II, MYPT-1 (Fig. 8a) and phospho-PKC (Fig. 8b) expression and enhances the activation/phosphorylation of MYPT-1, PKC and CPI-17 (Fig. 8c, d) in response to BE and CCK challenge. These results indicate that melatonin-associated recovery of calcium sensitization pathways could be responsible for normalization of gallbladder contractile capacity.

The melatonin-induced normalization of gallbladder contractile capacity correlated with the reestablishment of COX-2 expression that was enhanced in aged gallbladder (Fig. 9).

Discussion

Data presented here support the hypothesis that calcium sensitization signaling is negatively affected by aging in the guinea pig gallbladder. The main finding of this report is the reduction in the contribution of calcium sensitization mechanisms to the contractility of aged guinea pig gallbladder. Here, we describe that the targets for aging are the proteins participating in both Rho A/ROCK/MYPT-1 and PKC/CPI-17 pathways at expression and activation levels. The fact that melatonin treatment restored calcium sensitization contribution to the contractile response and normalized COX-2 expression could point out to an oxidative stress imbalance as cause of the impairment in calcium sensitization participation, although we cannot rule out other mechanisms of action of melatonin.

Aging-associated changes have been described in the gastrointestinal tract, such as reduction in the ability to eat large boluses of food (Rayner et al. 2000), as well as alterations in gastrointestinal function and motility disorders affecting gastric empty and/or colonic transit (Camilleri et al. 2000). However, molecular or cellular mechanisms responsible for these alterations have been poorly explored.

Fig. 7 Melatonin normalizes contractile responses and calcium sensitization contribution to contraction in aged gallbladders. Histograms represent maximal contractile response to **a** bethanechol and **b** cholecystokinin in aged animals (gray bars) and aged animals treated with melatonin (2.5 mg kg⁻¹ day⁻¹)

for 28 days (empty bars). Gallbladder contractility and the inhibitory effects of Y-27632 and GF109203X were increased after melatonin treatment. Data are expressed as percentage of adult values. $n=8-15$ strips (* $P<0.05$ and ** $P<0.01$, aged vs. aged treated with melatonin)

Fig. 8 Melatonin restores the expression and activation of proteins involved in calcium sensitization pathways in aged gallbladders. Histograms represent protein expression of proteins involved in the **a** Rho A/ROCK and **b** PKC/CPI-17 calcium sensitization pathways in aged animals (gray bars) and aged animals treated with melatonin (empty bars). **c** BE-induced and **d** CCK-induced phosphorylation of MYPT-1 (left),

PKC (middle) and CPI-17 (right) in aged animals (gray bars) and aged animals treated with melatonin (empty bars). Note the significant increase in protein expression and activation after melatonin treatment. Data are expressed as percentage of adult values. $n=6-8$ blots (* $P<0.05$ and ** $P<0.01$, aged vs. aged treated with melatonin)

The main contracting signals for gallbladder muscle are acetylcholine and CCK (Pozo et al. 2004). Here, we confirm previous findings (Gomez-Pinilla et al. 2006) about the impairment in myogenic gallbladder responses associated to aging and we further show that a decrease in the phosphorylation of MLC20 is associated to this hypocontractility. In addition, we also unveil the effects of aging in calcium-independent pathways for gallbladder contraction.

Intracellular Ca^{2+} levels play an important role in the modulation of contraction-relaxation cycle of smooth muscle cells. So, increases in $[\text{Ca}^{2+}]_i$ lead to contraction in a calmodulin- and MLCK-dependent way (Horowitz et al. 1996; Karaki et al. 1997).

Classically, disturbances of Ca^{2+} homeostasis and/or signaling are potential targets for intervention in aged tissues. It is well established that aging evokes tissue-specific changes in Ca^{2+} homeostasis in nerves and in skeletal, cardiac and smooth muscles (Gomez-Pinilla et al. 2007; Gomez-Pinilla et al. 2011; Puzianowska-Kuznicka and Kuznicki 2009). In the guinea pig gallbladder, aging reduced calcium influx but not calcium mobilization from internal stores even if contractile impairment is present for both maneuvers (Gomez-Pinilla et al. 2006). These are clear evidences that changes in Ca^{2+} homeostasis can neither fully explain the impairment in gallbladder contractility during aging nor the normalization in contractility

Fig. 9 Aging decreases the expression of COX-2. Original Western blots on gallbladder smooth muscle from adult (*Ad*), aged (*Ag*) and aged animals treated with melatonin (*Mel*) guinea pigs for COX-2. Note that aging increases the expression of COX-2 and that melatonin normalizes this expression pattern. Actin was used as loading control and protein expression was calculated respect to actin expression. Histograms summarize protein expression (mean±SEM) for 10–12 blots (* $P < 0.05$, aged vs. adult and aged treated with melatonin)

induced by melatonin treatment even without the reestablishment of calcium influx (Gomez-Pinilla et al. 2006). In this report, we show that impaired calcium sensitization pathways run in parallel with gallbladder hypomotility and decreased MLC20 phosphorylation. The impairment in calcium sensitization contribution to reduced gallbladder contractility associated to aging is driven by a reduction in the protein expression of ROCK I, ROCK II, MYPT-1 and PKC together with a reduction in the activation of MYPT-1, PKC and CPI-17 in response to agonist challenges. These changes in protein expression and activation were accompanied by an expected reduction of the impact of the specific inhibitors Y-27632 or GF109203X on the contractile responses to agonists. Molecular and pharmacological findings fully explain the reduction in gallbladder contractile capacity associated to aging.

According to our results, calcium sensitization pathways are not static and their contribution can change depending of the environment, indicative of plasticity in those pathways. It has been shown that

increases in calcium-independent contractions play an important role in diseases such as hypertension (Uehata et al. 1997), bronchial asthma (Chiba et al. 1999), erectile dysfunction (Wang et al. 2002) and hyperactive bladder (Persson et al. 1998). Here, we show that aging displays opposite effects since it leads to an impairment of calcium sensitization pathways that is translated into hypocontractility of the gallbladder. These findings, together with results obtained in gallbladders from newborn guinea pigs (Camello-Almaraz et al. 2009), draw the whole picture about the state of calcium sensitization along life. In guinea pig gallbladder, calcium sensitization pathway increases from birth to adult ages (Camello-Almaraz et al. 2009) and after that it suffers a decline. However, calcium handling runs in the opposite direction, with a reduction after birth (Camello-Almaraz et al. 2009) and partial maintenance with aging (Gomez-Pinilla et al. 2006). In the three age points, the state of calcium sensitization matches better with the contractile capacity than calcium signaling does. The effects of aging on calcium sensitization in guinea pig gallbladder described here are similar to those found in aged urinary bladder (Gomez-Pinilla et al. 2008), suggesting that in different contractile organs aging initiates similar harmful effects, which seem to be related to the imbalance in oxidative stress. However, it is likely the presence of species-related differences, since in mouse urinary bladder calcium handling and contractile activity run in parallel during aging (Gomez-Pinilla et al. 2011) in spite that calcium sensitization was not explored.

Melatonin (the main pineal gland hormone but with an important presence also in the gastrointestinal tract) has a substantial direct and indirect antioxidant activity and it can counteract the exacerbated free radicals damage accumulated during aging (Cuesta et al. 2010; Forman et al. 2010; Kireev et al. 2007a, b, 2008; Reiter et al. 1999a, b). Age-related decline in both circulating levels and the amplitude of the rhythm of melatonin are a well documented phenomena in a variety of species (Pang et al. 1990) and leads to an increase in oxidative stress due to an imbalance between pro-oxidants and antioxidant (Reiter et al. 1999b). In fact, we have found that melatonin preserves the membrane potential of “in situ” mitochondria, the main source of intracellular oxidants, in several models (Camello-Almaraz et al. 2008;

Gomez-Pinilla et al. 2008). In addition to its relevant antioxidant activity, melatonin exerts many of its physiological actions by binding to nuclear receptors of the orphan family and by interacting with membrane MT₁ and MT₂ receptors and intracellular proteins such as quinone reductase 2, calmodulin, calreticulin and tubulin (Pandi-Perumal et al. 2008; Reiter et al. 2010), which could underline the beneficial effects reported for melatonin.

Exogenous administration of melatonin to aged guinea pigs restored gallbladder contractility independently of changes in calcium handling (Gomez-Pinilla et al. 2006). In this report, we confirm the beneficial effects of melatonin on gallbladder contractile capacity and describe that it is mediated by an increase in calcium sensitization contribution to the contractile response. Melatonin treatment reestablishes the expression of proteins involved in these pathways and their activation in response to agonist challenges. Accumulated data strongly suggest that continuous (chronic) upregulation of pro-inflammatory mediators are induced during the aging process due to an age-related redox imbalance that activates many pro-inflammatory signaling pathways (reviewed by Chung et al. 2009). We have just reported that aging of gastrointestinal smooth muscle is associated to an increase in oxidative stress that correlates with increased NF- κ B and COX-2 expression indicative of a subtle inflammation pattern in aging (Pascua et al. 2011). Here we show that increased expression of COX-2 in aged gallbladder is normalized by melatonin treatment, which would suggest that the improvement in Ca²⁺ sensitization-mediated contractility could be related to the amelioration of the oxidative insult and subtle inflammation. However, other actions of melatonin can not be ruled out.

These observations are important in several respects. First, they strengthen the parallelism between calcium sensitization pathways and contractility. Second, they suggest that the impairment in calcium sensitization contribution is due to the increase in oxidative stress and subtle inflammation. Finally, this report reinforces the beneficial effects of melatonin treatment on smooth muscle pathological conditions related to oxidative stress imbalance (Pozo et al. 2010).

In summary, we found an age-related decline in the gallbladder contractility due to the reduction of the contribution of calcium sensitization mechanisms, shown at the molecular and pharmacological level.

The administration of exogenous melatonin normalized calcium sensitization participation and gallbladder contractile response and COX expression, suggesting that calcium sensitization pathways could be sensitive to oxidative stress.

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