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Pomolic acid reduces contractility and modulates excitation-contraction coupling in rat cardiomyocytes

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ABSTRACT

Pomolic acid (PA) isolated from *Licania pittieri* has hypotensive effects in rats, inhibits human platelet aggregation and elicits endothelium-dependent relaxation in rat aortic rings. The present study was designed to investigate the effects of PA on cardiomyocytes. Trabeculae and enzymatically isolated cardiomyocytes from rats were used to evaluate the concentration-dependent effects of PA on cardiac muscle tension and excitation-contraction coupling (ECC) by recording Ca^{2+} transients reported with Fluo-3 and Fura-2, as well as L-type Ca^{2+} currents (LTCC). PA reduced the contractile force in rat cardiac trabeculae with an EC_{50} = 14.3 ± 2.4 μM. PA also reduced the amplitude of Ca^{2+} transients in a concentration-dependent manner, with an EC_{50} = 10.5 ± 1.3 μM, without reducing sarcoplasmic reticulum (SR) Ca^{2+} loading. PA decreased the half width of the Ca^{2+} transient by 31.7 ± 3.3% and increased the decay time and decay time constant (τ) by 7.6 ± 2.7% and 75.6 ± 3.7%, respectively, which was associated with increased phospholamban (PLN) phosphorylation. PA also reversibly reduced the macroscopic LTCC in the cardiomyocyte membrane, but did not demonstrate any effects on skeletal muscle ECC. In conclusion, PA reduces LTCC, Ca^{2+} transients and cardiomyocyte force, which along with its vasorelaxant effects explain its hypotensive properties. Increased PLN phosphorylation protected the SR from Ca^{2+} depletion. Considering the effects of PA on platelet aggregation and the cardiovascular system, we propose it as a new potential, multitarget cardiovascular agent with a demonstrated safety profile.

1. Introduction

Hypertension is a medical condition in which arterial blood pressure remains chronically elevated beyond the accepted normal range, which increases the risk of cardiovascular and renal diseases, premature death and disability in millions of people worldwide (Carretero and Oparil, 2000a; Chobanian et al., 2003; Mancia et al., 2013). Despite the increasing availability of effective antihypertensive drugs, the disease remains a public health problem; during the last decade, cardiovascular diseases have been the leading cause of death worldwide (World Health Organization, 2013) and the proportion of hypertensive individuals whose condition is treated or controlled with medication remains low (Carretero and Oparil, 2000b; Chobanian et al., 2003; Ikeda et al., 2014; Mancia et al., 2013).

Ethnobotanical information of medicinal plants and/or plant-derivate remedies used in folk medicine to treat hypertension is increasingly available (Gbolade, 2012; Munir and Karin, 2013); however, there are still very few pharmacological studies that scientifically support the mechanism of action and safety of the use of molecules purified from these medicinal plants.

Previous in vivo studies published by our group demonstrated that pomolic acid (PA), isolated from the leaves of *Licania pittieri*, has hypotensive effects. Intravenous PA at a dose of 0.4 mg/kg reduced both the mean arterial blood pressure and the heart rate of adult rats by 24.1% and 38.7%, respectively (Estrada et al., 2009). In aortic rings precontracted with norepinephrine, PA had a potent (EC_{50} = 2.5 μM),

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endothelium-dependent relaxation effect (Estrada et al., 2011). Further experiments showed that PA selectively inhibited adenosine 5′-diphosphate (ADP) and epinephrine-induced platelet aggregation (IC_{50} = 60 nM) (Estrada et al., 2011, 2009).

In addition to blood vessels, another possible target for this molecule to induce hypotensive effects is the heart. Therefore, in the present study we aimed to deepen the molecular characterization of the effects of PA on the cardiovascular system, by testing whether part of its hypotensive effect is mediated by a direct effect on the heart.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, ten to twelve weeks old (250–300 g), were used for most experiments. Wistar rats were used for the Western blot experiments. A maximum of four rats per acrylic, transparent, rectangular cage, with wood-derived bedding, were housed under conditions of controlled temperature (21 ± 2 °C) and light:darkness (12:00–12:00 h). In addition, the animals had free access to food and tap water in the conventional animal facilities of the Venezuelan Institute for Scientific Research (IVIC), Venezuela, or the University of Antioquia, Colombia. The rats underwent terminal anaesthesia with intraperitoneal injections of sodium pentobarbital (40 mg k-1, SM Pharma, Venezuela), while movement, breathing, response to external stimuli and hindpaw withdrawal reflexes were evaluated. All procedures were approved by the bioethical committees of the IVIC and the University of Antioquia (according to the Law 84 of 1989 and Resolution 8430 of 1993) and were performed in compliance with the Directive 2010/63/EU of the European Parliament guidelines, as well as policies on experimental design and analysis (Curtis et al., 2015; McGrath and Lilley, 2015).

2.2. Pomolic acid

PA was isolated from *Licania pittieri* and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) as previously described (Estrada et al., 2009) (see Supplemental information). The final concentration of DMSO in the experiments typically ranged from 0.1% to 0.3% v/v, and most controls were run in the presence of the same dilution of DMSO.

2.3. Tension measurements

The heart was rapidly removed and bathed in a cold Tyrode solution (see Supplemental material for details of all experimental solutions and buffers). Small strips of either trabeculae or papillary muscles were dissected and mounted horizontally in the experimental chamber, with one end fixed to a force transducer (AE-801, Kronex Technologies Corp., USA). The preparation was allowed to rest and was then stimulated at 0.2 Hz until tension stabilized. Contractions were elicited by supra-threshold, rectangular (3 ms) current pulses through two platinum plate electrodes. The transducer signals were fed into a MiniDigi 1 A acquisition system, and the data were stored and analysed using Axoscope 9.0 software (Axon Instruments, USA). The preparation was continuously bathed at a rate of 1.5 ml/min with Tyrode solution or the experimental compounds dissolved in Tyrode solutions at room temperature (22–24 °C) and was bubbled with O_2.

2.4. Cardiomyocyte isolation

For the enzymatic isolation of cardiomyocytes (Tytgat, 1994), the rat heart was removed and the aorta was rapidly cannulated. Then, it was mounted on a Langendorff perfusion system (Skrzypiec-Spring et al., 2007; Zimmer, 1998). Tyrode solutions with variable amounts of Ca^{2+} at 36.5 °C were used for the procedure (Piper, 2000; Tytgat, 1994). The solutions were constantly bubbled with O_2 and were perfused at a rate of 4–5 ml/min. Tyrode solution without Ca^{2+} and with 2 mg/ml of collagenase 2 (Worthington CLS 2, USA) and 0.2 mg/ml of protease (type XIV, Sigma-Aldrich, USA) was perfused for 10–20 min until isolated cardiomyocytes appeared in the perfusate. Once isolated, ventricular myocytes were kept in 1 mM Ca^{2+} Tyrode solution, which in some cases was supplemented with 0.48 U/ml Apyrase grade I (A6132, Sigma-Aldrich, USA).

2.5. Ca^{2+} measurements in isolated cardiomyocytes

Cardiomyocytes that were Ca^{2+}-tolerant and rod-shaped, did not exhibit spontaneous contraction at any time, and were clearly transversally striated were loaded with either Fura-2 AM or Fluo-3 AM (Life Technologies, USA) at 5–7 μM for 60 or 20–25 min, respectively, at room temperature. The Fluo-3 loaded cells were washed and transferred to an experimental chamber that was mounted on the stage of an inverted Nikon Diaphot TMD (Nikon Co., Japan) microscope equipped for epifluorescence and the fibres were illuminated with a xenon lamp (100 W). Precautions were taken to diminish photodamage and photobleaching of the dye. The light signals were collected with a photomultiplier connected to a Nikon P1 amplifier.

Ca^{2+} transients were elicited by applying supra-threshold, rectangular current pulses (3–4 ms) through two platinum plate electrodes. The amplifier output was fed into a DigiData 1200 interface (Axon Instruments, USA) and the data were acquired and analysed using the pCLAMP 6 software (Axon Instruments, USA).

We present the Ca^{2+} transients as $\Delta F/F =$ ((Fmax-Frest)/Frest), and measured their half width (ms), decay time (ms) and time constant of decay (τ, ms) by fitting the decay phase with a monoexponential function.

Cells loaded with Fura-2 were mounted on an Ionoptix system (IonOptix Co., USA), equipped with a 100-W Xenon lamp and filters to alternatively excite at 340/380 nm (Chroma Tech, USA). Emission fluorescence was collected with a CCD camera at a frequency of 30 Hz. The Ca^{2+} concentration was calculated according to previously published methods (Grynkiewicz et al., 1985; Klein et al., 1988).

Stocks of N(ω)-nitro-L-arginine methyl ester (L-NAME, N5751), caffeine (C0750), epinephrine (E4375), isoproterenol hydrochloride (ISO, I6504) and adenosine 5′-triphosphate magnesium salt (ATP, A9187), all from Sigma, were dissolved in water or Tyrode and added to the preparations at final concentrations indicated in the results section. Caffeine contractures and Na^+/Ca^{2+} exchanger (NCX) activity analyses were carried out as published (Bassani et al., 1994; Boguslavskyi et al., 2014). All experiments were performed at room temperature.

2.6. Western blots for phospholamban phosphorylation studies

The hearts of five male Wistar adult rats sacrificed with CO_2 were rapidly dissected. Each heart was processed as a separate experiment. Small strips from both ventricles of each heart were incubated during 3 min in Tyrode with 1% DMSO (negative control), 30 nM ISO (positive control), or 50 μM PA (experimental condition). Samples were then frozen in liquid nitrogen and conserved at − 80 °C until use. Proteins were extracted using RIPA buffer supplemented with protease and phosphatase inhibitors (cOmplete version 12 and PhosSTOP, Roche, Switzerland). The total amount of proteins in the supernatant was measured with the Bradford method (Bradford, 1976).

Electrophoresis under denaturing conditions was then performed. The stacking gel was 5% polyacrylamide, and the separating gel was 10% polyacrylamide (29:1 acrylamide: bisacrylamide). Typically, 50 μg of total proteins in loading buffer was loaded in each well of a Mini-Protean IV electrophoresis cell (Bio-Rad, USA) and run at 90 V for 150 min at room temperature. A prestained protein ladder (26619, ThermoFisher, USA) guided the run.

Following separation, the gels were transferred to polyvinylidene fluoride (PVDF, 162–0177, Bio-Rad, USA) membranes in transfer buffer

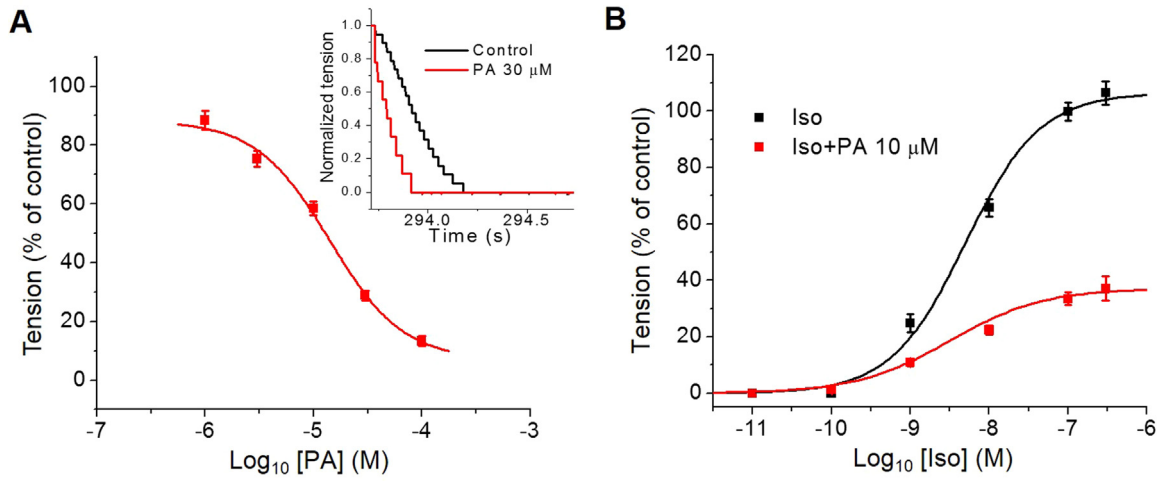

Fig. 1. Effect of pomolic acid (PA) on tension measured in strips of trabeculae or papillary muscles of the rat heart. There was a concentration-dependent reduction in the tension generated after electrical stimulation when the heart strips were incubated with PA (A, red squares). The insert shows a representative record further illustrating the acceleration of the decay phase of the tension records induced by PA. In strips exposed to both PA (10 µM) and increasing concentrations of ISO the negative inotropic effect could not be reversed (B, red squares). Black squares in B correspond to the effect of ISO alone on the heart contraction. All data points include data from six strips of six different rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

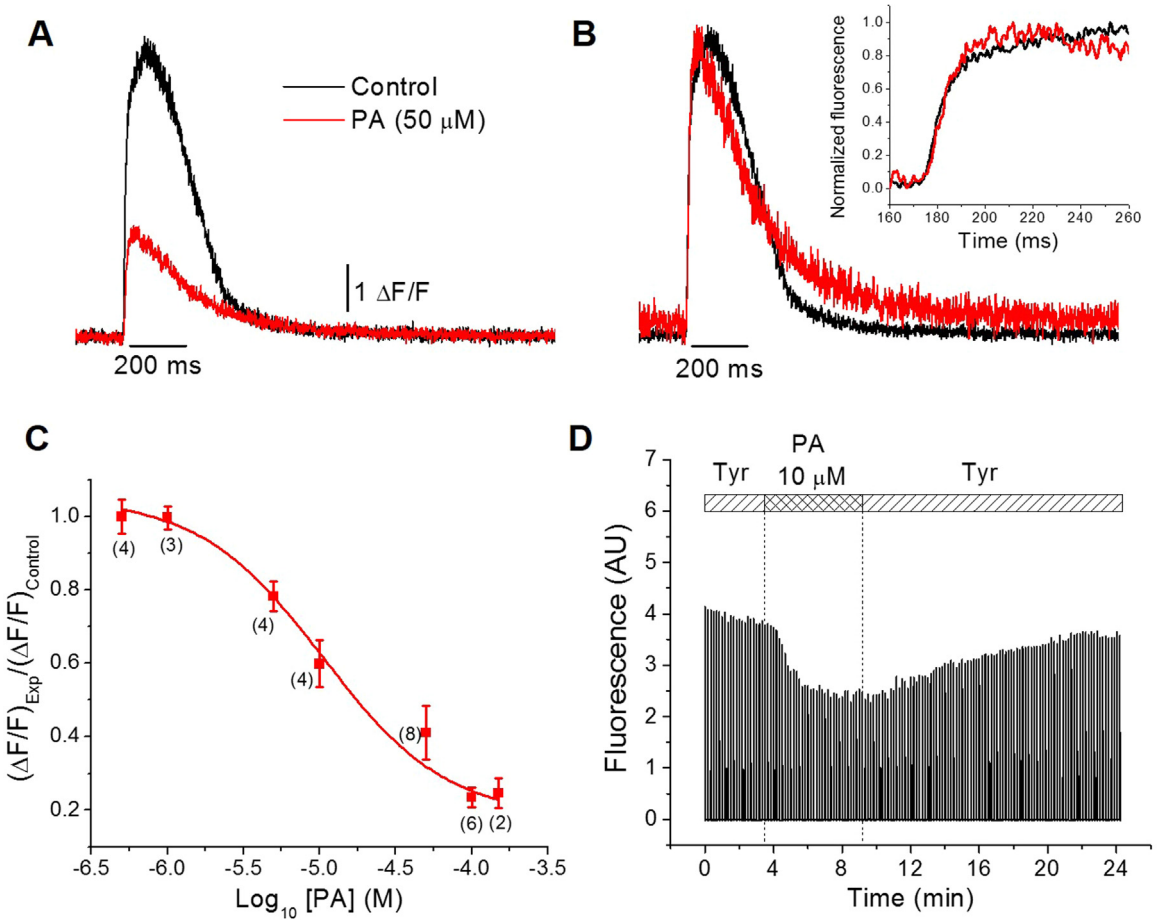

Fig. 2. Effect of pomolic acid (PA) on the kinetics of electrically evoked Ca^{2+} transients recorded in isolated rat cardiomyocytes loaded with Fluo-3 AM. A cardiomyocyte incubated with 50 µM of PA (red signal) reduced the amplitude by 63.1% (A) and changed the kinetics of the decay phase of the Ca^{2+} transient (B, the same records in A after normalization), with minor changes during the rising phase, as shown in the insert. The effect of PA on the amplitude was concentration-dependent with an EC_{50} = 10.5 ± 1.3 µM and a reduction of the Ca^{2+} transient of 82.9 ± 0.1% at a concentration of PA of 100 µM (C). The numbers inside parenthesis indicate the amount of different cells evaluated for each concentration and the red line corresponds to the fit. D reproduces the kinetics of the decrease in amplitude of the crude signal in arbitrary units (AU), established after 2–3 min of 10 µM PA incubation, and also shows the reversibility (84.8%) of the effect after washing out with Tyrode during 15 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

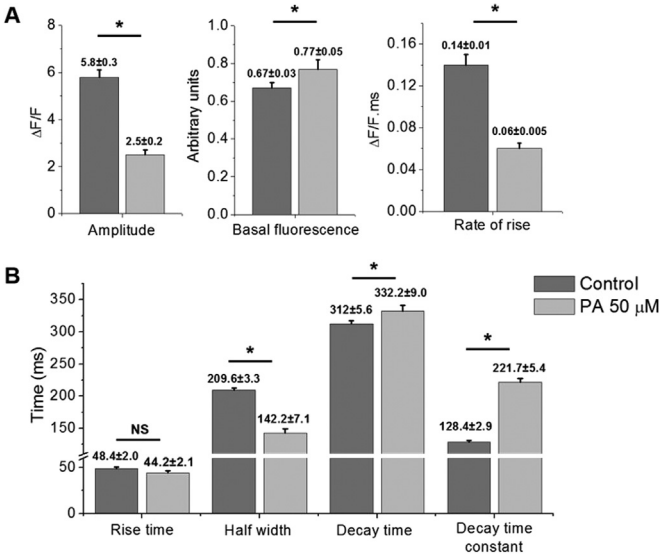

Fig. 3. Changes induced by 50 μM pomolic acid (PA) on kinetic parameters of Ca^{2+} transients obtained from rat cardiomyocytes. Panels in A are variables related to fluorescence, while panel B summarizes changes in variables measured in ms. Dark and light gray bars correspond to control and experimental conditions, respectively. All numbers are absolute values (83 records from 8 cells) as mean ± S.E.M. *P < 0.05 in a paired *t*-test. NS: no statistical difference.

at 4 °C. The membranes were then blocked with TBS-T with 5% bovine serum albumin (BSA) for 1 h at room temperature. Primary antibodies (Cell Signaling, USA, see Supplemental material for further details) to detect total and phosphorylated (Ser16/Thr17) phospholamban (PLN), as well as tubulin, were diluted in TBS-T with 5% BSA and incubated overnight at 4 °C. The secondary antibody was coupled to horseradish peroxidase and incubated during 1 h at room temperature. Protein bands were visualized by a chemiluminescence reaction (7003, Cell Signaling, USA). Pictures were taken directly from the membranes (G: BOX Chemi XRQ, Syngene, UK) and images were analysed for the intensity of both phosphorylated and total PLN bands at 12 and 24 kDa with ImageJ 1.49 free software (National Institutes of Health, USA). Phosphorylated to total intensity ratio (P-PLN/total PLN) was reported for each Western blot.

2.7. Electrophysiology recordings

L-type Ca^{2+} currents (LTCC) through cardiomyocyte dihydropyridine receptors (DHPR, Cav1.2) were recorded in the whole cell configuration of the patch clamp technique as originally described (Hamill et al., 1981), by using an Axopatch 200B amplifier (Axon Instruments, USA). Borosilicate glass pipettes (Garner Glass Co., USA), obtained with a P-80/PC horizontal puller (Sutter Instruments Co., USA), had a resistance of between 2.0 and 3.0 MΩ when filled with internal solution. The external solution was as published (Lacampagne et al., 1995). Voltage protocols were commanded and current signals were acquired with a computer interfaced to a Digidata 1322 A (Axon Instruments, USA) using pCLAMP 10.3 software (Axon Instruments, USA). LTCC were elicited by 100 ms depolarizing pulses from a holding potential of − 50 mV, in 10 mV increments between − 50 and + 70 mV. The maximum amplitude of the current was recorded between 0 and + 10 mV, as published (Hussain and Orchard, 1997). The effect of PA was evaluated on the peak current, applying depolarization steps from − 50 mV to the voltage at which the maximum amplitude of the current was observed, verifying the quality of the seal. Once the effect of PA was established, a new set of depolarizations was applied from − 50 mV to + 70 mV in the presence of the drug. Immediately after that, the PA was washed with the control Tyrode solution and the recovery of the signal was verified following only the steps from − 50 mV to the voltage at which the maximum amplitude of the current was originally seen. A complete new set of depolarizations was then applied from − 50 mV to + 70 mV in some, but not all, cells.

2.8. Statistical analysis

The results are expressed as the mean ± standard error of the mean (S.E.M). Sample sizes of the different experiments were calculated for an expected change of 35% in the studied variable, according to preliminary results, a power of 80% and alpha of 5%. Although the calculations suggested running 4 because the expected change was large and the experiments were paired, in most cases the number of experiments conducted was greater than 4 to add power to the statistical treatment. No sample size was calculated for the concentration-effect curves, instead, a good quality fit was always verified.

Supplemental Table 1 summarizes the number (n) of cells, strips or experimental preparations, as well as the animals, used per figure throughout this work. Unless otherwise stated, n refers to the number of different, independent experiments. Randomization was not needed because paired experiments were designed to look for a mechanism of action of a drug, and not to compare effects between different drugs or treatments *vs* placebo. The EC_{50} and IC_{50} values were calculated from sigmoidal concentration–response curves, which were analysed by nonlinear regression using GraphPad Prism (version 5.0, USA). All final graphs were created with Origin 7.5 or 8.0 software (Microcal Software Inc., Northampton, USA). Scatter plots show individual values as black dots, and mean ± S.E.M values are shown in red. The results obtained in paired experiments were compared with a paired Student's *t*-test, and unpaired experiments were compared with an independent Student's *t*-test. The results of repeated measures were compared with a Friedman test, and if significant, the difference between specific groups was analysed with a Wilcoxon test. Comparisons among three independent groups were performed with a Kruskal-Wallis test, and if significant, posterior contrast between groups was evaluated with the Mann-Whitney test. Statistical comparisons were performed with SPSS 20.0 software (IBM, USA). Statistical significance was set at $P^{<}0.05$.

3. Results

3.1. Effect of PA on cardiac tension

PA reduced the tension-generation capability of ventricular strips in a concentration-dependent manner, with an EC_{50} of 14.3 ± 2.4 μM (Fig. 1A). This negative inotropic effect was still evident in the presence of ISO (Fig. 1B). The concentration-effect curves showed that 10 μM PA diminished by a half the maximum response to ISO, exhibiting a non-competitive antagonism. The insert in Fig. 1A illustrates the lusitropic of PA. The half-time ($t_{1/2}$) of relaxation of tension records shortened from 258 ± 6 ms under control conditions to 78 ± 4 ms after incubation with PA 30 μM (n = 8 strips, paired experiments, P < 0.01).

3.2. Effect of PA on Ca^{2+} transients from rat cardiomyocytes

We next evaluated the effect of PA on electrically evoked Ca^{2+} transients in isolated rat cardiomyocytes loaded with the fluorescent Ca^{2+} dye Fluo-3. The effect on the kinetics was evident (Fig. 2A-B) and a concentration-response curve gave an EC_{50} in the order of the effect of PA on tension (Fig. 2C). Notably, the effect on amplitude was reversible (Fig. 2D). In cells exposed to 50 μM PA, the amplitude, half width and rise time of the Ca^{2+} transients were reduced by 60.8 ± 2.1%, 31.7 ± 3.3% and 8.7 ± 8.8%, respectively. The decay time and decay time constant (τ) increased by 7.6 ± 2.7% and 75.6 ± 3.7%, respectively. Similarly, the basal fluorescence increased by 8.6 ± 2.1%. The proportionally greater reduction in amplitude compared to rise time led

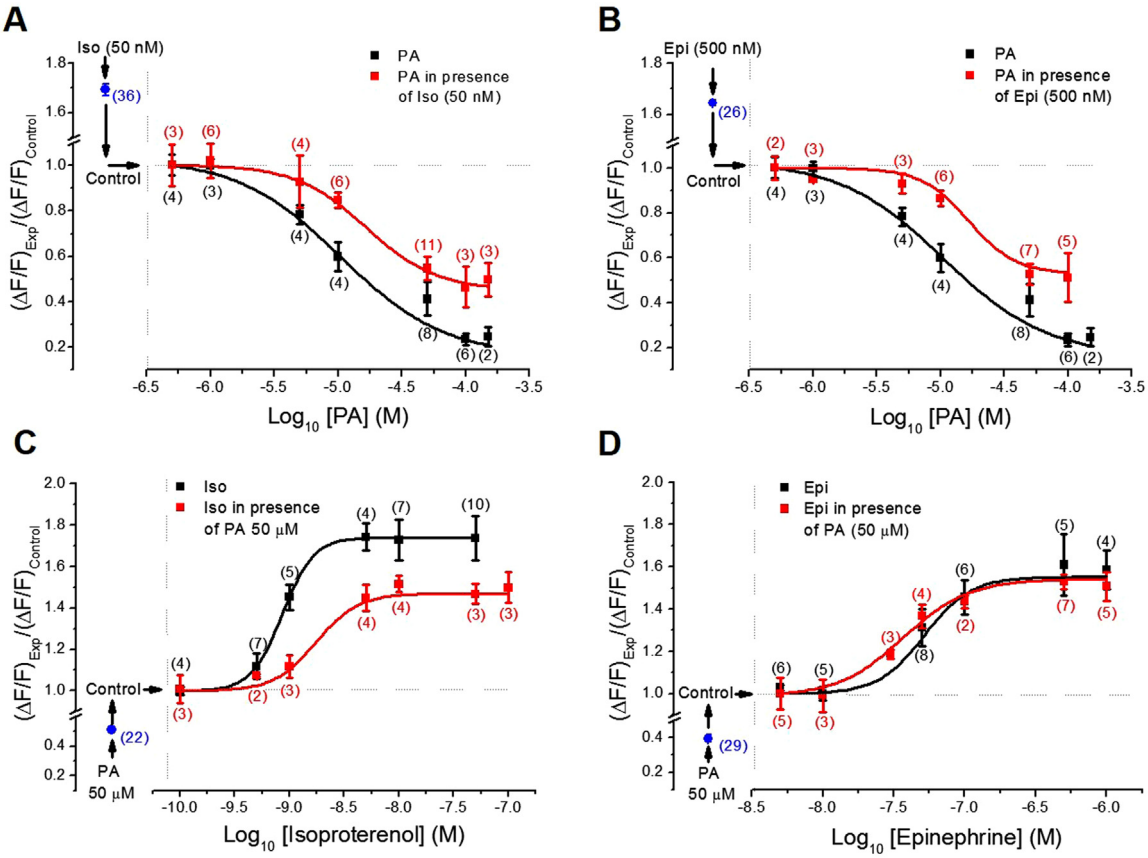

Fig. 4. Effect of pomolic acid (PA) on the amplitude of electrically evoked Ca^{2+} transients recorded in isolated rat cardiomyocytes loaded with Fluo-3 AM and exposed to different adrenergic agonists. In a first set of experiments (A and B) the cardiomyocytes were treated with increasing concentrations of PA (black in both A and B, the same curve presented in Fig. 2C) in presence of adrenergic agonists ISO 50 nM or epinephrine 500 nM (red in both A and B, respectively). As it can be seen in panel A, the pre-treatment of the fibres with 50 nM ISO reduced the inhibitory effect of PA on the amplitude of the Ca^{2+} transients by 30.1%. The IC_{50} of this effect was 16.5 µM. Similar results were observed in experiments using 500 nM epinephrine instead of ISO: a reduction of the inhibitory effect of PA on the amplitude of the Ca^{2+} transients by 33.2% and an IC_{50} of 16.6 µM (B). In a second set of experiments (C and D) the cardiomyocytes were treated with increasing concentrations of ISO or epinephrine (black in C and D, respectively) in presence of 50 µM PA. In this case, when the fibres were first incubated with 50 µM PA, the ISO-induced Ca^{2+} transient potentiation was inhibited by 36.7% (C) and the EC_{50} values increased from 0.86 nM (black curve) to 1.92 nM (red curve). Experiments made exposing the myocytes first to 50 µM PA and then to increasing concentrations of epinephrine (D) showed that pretreatment with PA did not alter the effect of epinephrine on the amplitude of the Ca^{2+} transients, although slightly modified the EC_{50} from 52.1 nM (black curve) to 37 nM (red curve). In all cases, the blue circle corresponds to the value of amplitude measured after exposing to the drug the number of different cells indicated inside parenthesis, but for the sake of comparison with the control, black curve, the red curve was moved as indicated by the vertical arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to a reduction in the rate of rise of the Ca^{2+} transients by 54.2 ± 3.1%. These relative (%) changes were calculated cell by cell and not through the subtraction of mean absolute values, which are shown in Fig. 3. All changes were statistically significant ($P < 0.05$), except for that of the rise time.

The reduction in the amplitude of the Ca^{2+} transients was accompanied by a reduction in sarcomere shortening when both variables were simultaneously recorded, thus demonstrating a negative inotropic effect of PA on isolated cardiomyocytes (Supplemental Fig. 1).

3.3. Effect of PA on Ca^{2+} transients under adrenergic stimulation

As shown in Fig. 4A, pretreatment of the cells with 50 nM ISO reduced the inhibitory effect of PA on the amplitude of the Ca^{2+} transients by 30.1%. The IC_{50} of this effect was 16.5 µM. Similar results were observed in experiments using 500 nM epinephrine instead of ISO: a reduction of the inhibitory effect of PA on the amplitude of the Ca^{2+} transients by 33.2% and an IC_{50} of 16.6 µM (Fig. 4B).

In contrast, when the cells were first incubated with 50 µM PA, the ISO-induced Ca^{2+} transient potentiation was inhibited by 36.7% (Fig. 4C), and the EC_{50} values increased from 0.86 nM (black curve) to 1.92 nM (red curve).

Experiments conducted by exposing the myocytes first to 50 µM PA and then to increasing concentrations of epinephrine (Fig. 4D) showed that pretreatment with PA did not alter the effect of epinephrine on the amplitude of the Ca^{2+} transients, although slightly modified the EC_{50} from 52.1 nM (black curve) to 37 nM (red curve).

Fig. 5 illustrates some of the kinetic changes observed in the previous experiments. In Fig. 5A and B, the cell was exposed to PA and then to ISO plus PA, and in Fig. 5C and D, the cell was first incubated in ISO and then in PA plus ISO. The results show that PA and ISO exert different and independent effects on the kinetics of Ca^{2+} transients. The latter can be noted because ISO induced a reduction in the half width of the Ca^{2+} signals after the PA-induced change was already established. Moreover, the fact that PA exerts a non-competitive inhibition of ISO action suggests that they do not act on the same receptor.

3.4. Effect of PA on tension and Ca^{2+} transients after preincubation with L-NAME

Because PA exerts endothelium-dependent relaxation effects (Estrada et al., 2011) in rat aortic rings and nitric oxide (NO) regulates

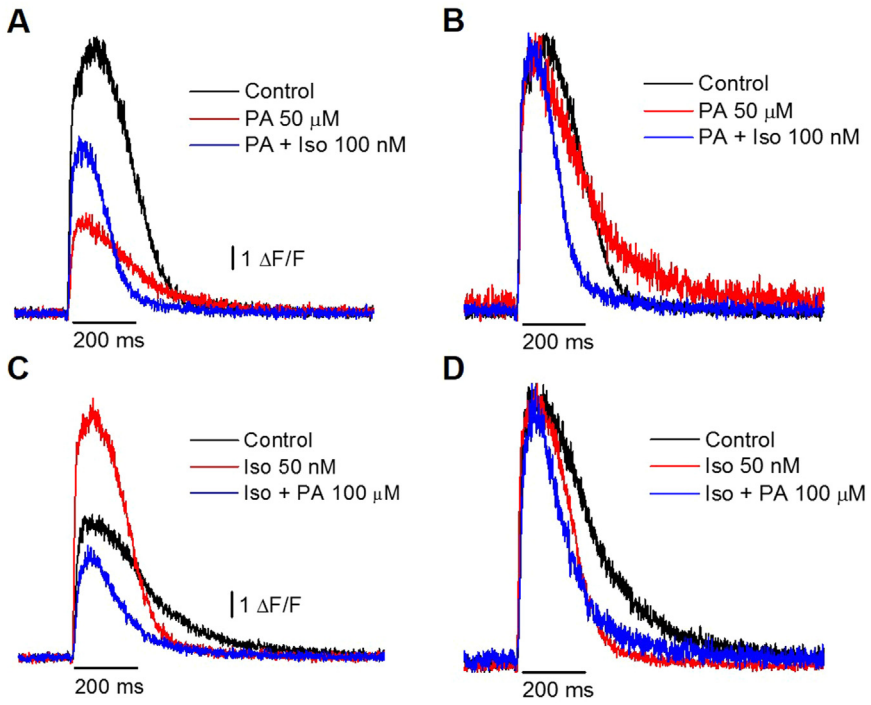

Fig. 5. Effect of pomolic acid (PA) on the kinetics of electrically evoked Ca^{2+} transients recorded in isolated rat cardiomyocytes loaded with Fluo-3 AM and exposed to ISO. The figure illustrates some of the kinetic changes seen in the previous experiments (Fig. 4). In A and B the fibre was exposed to PA and then to ISO plus PA, and in C and D the fibre was first incubated with ISO and then with PA plus ISO. The reduction of 62.2% in the amplitude of the Ca^{2+} transient induced by PA 50 μM could be partially reversed (43.7%) by ISO 100 nM, as shown in A. The treatment with ISO also induced a change in the decay part of the transients (B, the same records in A after normalization) after the PA-induced change was already established. The exposure of the cell in C to ISO 50 nM induced an increase in amplitude of 81.1%, and the subsequent exposure to PA 100 μM reduced the last signal by 56.8% and modified the decay part of the record (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

excitation-contraction coupling (ECC) in mammalian heart (Khan et al., 2003), we evaluated the effect of incubation of papillary muscles and cardiomyocytes with L-NAME on tension (Fig. 4A) and the amplitude of Ca^{2+} transients (Fig. 6B and C) to determine if the negative inotropic effect of PA was mediated by an NO pathway. A 48.3 ± 5.7% reduction in tension was established when 30 μM PA was used, even when the sample was preincubated with 1 mM L-NAME for approximately 60 s (Fig. 6A). A comparable reduction of 54.6 ± 3.7% was measured in Ca^{2+} transients after L-NAME followed by PA incubation in isolated cardiomyocytes (Fig. 6B and C). The quantitative analysis of the Ca^{2+} signals shown in panel C demonstrates significant differences between the control and 30 μM PA and between 1 mM L-NAME and 1 mM L-NAME + 30 μM PA conditions (*$P < 0.05$); however, there was no difference in the amplitude reduction induced by PA before and after L-NAME incubation.

Fig. 6 shows that the effect of PA on tension and Ca^{2+} transient amplitude is NO-independent.

3.5. Effect of PA on sarcoplasmic reticulum Ca^{2+} loading

We used caffeine in strips of heart muscle and isolated cardiomyocytes to rule out the possibility that the negative inotropic effect of PA was mediated by a reduction in sarcoplasmic reticulum (SR) Ca^{2+} loading. For this experiment, we evaluated the effect of 10, 30 and 50 μM PA on tension (Fig. 7A) and amplitude of the Ca^{2+} transients (Fig. 6B, starting at 600 s, and Fig. 7C) of the caffeine contracture. Scatter plots in Fig. 7B and D show the individual and average tension (g) and delta Ca^{2+} concentrations (nM) measured after caffeine application, both before and after PA treatment (results of 10, 30 and 50 μM PA were pooled). The results demonstrate that PA-induced negative inotropism is not explained by SR Ca^{2+} depletion.

To indirectly assess NCX forward mode function, a biexponential function was fitted to the decay phase of the caffeine-induced Ca^{2+} transients. With this approach, the SR Ca^{2+} accumulation is inhibited and then the kinetics of the decay of the Ca^{2+} transient mostly reflects the function of the NCX. This is because the NCX explains up to 90% of the SR-independent Ca^{2+} removal in rat cardiomyocytes (Bassani et al., 1994; Negretti et al., 1993). The values of τ_1 and τ_2 were 1.23 ± 0.33 and 5.32 ± 1.39 s, respectively, under control conditions, and 1.31 ± 0.38 and 7.72 ± 2.21 s, respectively, after PA exposure ($P^{>}0.05$ when comparing within τ_1 or τ_2). These results rule out a change in NCX activity under our experimental conditions.

3.6. Effect of PA on PLN phosphorylation

We evaluated if PLN phosphorylation could partially explain the shortening of the Ca^{2+} transients and the conservation of SR Ca^{2+} content in cells that showed a reduced amplitude of Ca^{2+} transients. The results shown in Fig. 8 suggest that PA induced the phosphorylation of PLN to a degree comparable to that induced by ISO, which in turn is about two and a half times higher than that of the control (uncropped Western blot can be seen in Supplemental Fig. 2).

3.7. Effect of PA on LTCC Ca^{2+} currents

Because one of the targets of ATP signalling inside a cardiomyocyte is the DHPR (Vassort, 2001) and because a direct, inhibitory effect of PA on DHPR would explain the results presented previously, we examined whether PA could block Ca^{2+} currents through DHPR.

Fig. 9 shows that incubating one cardiomyocyte with 1 μM PA caused a 37.9% reduction in the amplitude of the peak current, which was evident after 300 s. A partial recovery of 17.3% was observed after washing out PA for 84 s (Fig. 9A-D). Six cells exposed to 1 μM PA during 8 min showed a 59.2 ± 6.8% reduction in the amplitude of the peak current/cell capacitance. After a mean of 3 min of washing the PA, the signal recovered by 10.4 ± 4.9%. Changes in mean absolute peak current/cell capacitance values for these six cells are shown in panel E. The results demonstrate a partially reversible inhibition of LTCC under the effect of PA in isolated cardiomyocytes.

4. Discussion

We evaluated the effect of PA on tension, kinetics of the Ca^{2+} transients, SR Ca^{2+} loading, PLN phosphorylation and LTCC in rat cardiomyocytes. The main findings were as follows: i) PA reversibly reduced the contraction strength in ventricular muscle, ii) PA reversibly reduced the amplitude and modified the kinetics of electrically elicited Ca^{2+} transients from isolated cardiomyocytes, iii) the effect cannot be

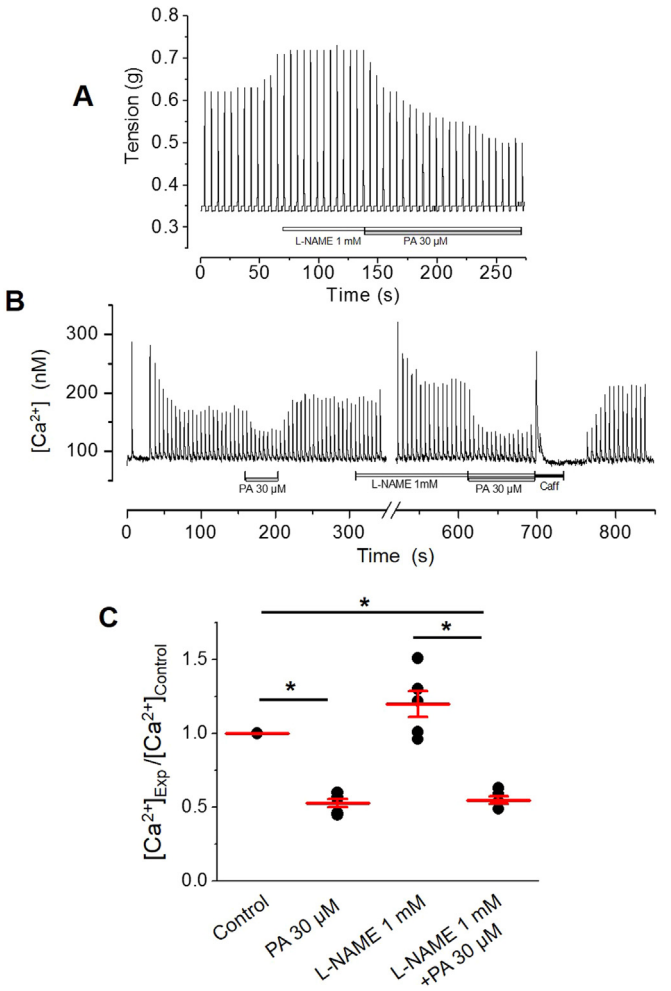

Fig. 6. Effect of L-NAME and pomolic acid (PA) on tension measured in strips of trabeculae or papillary muscles of rat heart and on the amplitude of Ca^{2+} transients recorded in isolated rat cardiomyocytes loaded with Fura-2 AM. Representative trace (out of three experiments) showing a reduction of tension after incubation with 30 µM PA, even when the strip was preincubated with L-NAME 1 mM for about 60 s (A). A comparable reduction was measured in the amplitude of the Ca^{2+} transients when the experiment was done in isolated cardiomyocytes, as shown from seconds 550–690 in the representative cell shown in B. The quantitative analysis of Ca^{2+} signals of similar experiments performed in 5 independent cells is shown in C. Means ± S.E.M for the four experimental conditions indicated in the figure were 1 ± 0, 0.53 ± 0.03, 1.2 ± 0.09 and 0.55 ± 0.02. All values were normalized to the amplitude of the Ca^{2+} transients under control conditions. *P < 0.05 in a Wilcoxon test, performed after a Friedman test.

explained by a reduction in SR Ca^{2+} content but is associated with increased PLN phosphorylation, and iv) PA reversibly reduced the LTCC in isolated cardiomyocytes.

4.1. Mechanism of action of PA in the heart

The reduction in the amplitude of the Ca^{2+} transients found in isolated cardiomyocytes underlies the reduced contractile activity found in the ventricular muscle strips. The reduction of the Ca^{2+} transients induced by PA may have different explanations: i) PA may reduce Ca^{2+} entry through L-type Ca^{2+} channels, ii) PA may inhibit the ryanodine receptors (RyR) or iii) PA may reduce the SR Ca^{2+} content. Options ii and iii were ruled out because the kinetics of the early rising phase of the signals, as well as the kinetics of the caffeine contractures, were slightly altered by PA. To test if option i) was feasible we performed three sets of experiments.

First, we evaluated a possible interaction of PA with adrenergic signalling, known to induce inotropic and lusitropic effects, and increased SR Ca^{2+} content (Hussain and Orchard, 1997) that could be opposed by PA.

In this sense, PA did not affect the epinephrine-induced increase in Ca^{2+} transient amplitude, but inhibited the effect of ISO, an agonist of beta-adrenergic receptors. These results suggest that PA does not act in a competitive fashion with adrenergic receptors. Moreover, the positive inotropic effect of adrenergic agonists was not completely inhibited by PA. Thus, it seems that the effect of PA is mostly independent of adrenergic receptors.

Second, since evidence suggests that PA modulates purinergic receptors and NO signalling in platelets and arteries (Estrada et al., 2011, 2009), we evaluated if PA interacts with these receptors in heart muscle or if its effect was mediated by NO. ATP was able to partially overcome the negative inotropic effect of PA on the Ca^{2+} transients (Supplemental Fig. 3). This could be done in several ways: by increasing IP_3 and then stimulating SR Ca^{2+} release (Balogh et al., 2005; Domeier et al., 2008), by phosphorylating DHPR proteins and increasing LTCC (Vassort, 2001) or by increasing NO (Khan et al., 2003; Vassort, 2001).

The inotropic effect of ATP may depend on both P2X and P2Y receptors (Balogh et al., 2005; Mei and Liang, 2001; Vassort, 2001). Although PA is an antagonist of P2Y12 receptors in platelets (Alvarado-Castillo et al., 2012), these receptors have not been discovered in cardiomyocytes (Balogh et al., 2005). P2Y11 has been proposed to have an inotropic effect (Balogh et al., 2005), but we did not perform a specific experiment to evaluate if PA can antagonize P2Y11 receptors.

The results suggest that some kind of interaction between PA and purinergic signalling in the heart seems feasible (but not mediated by NO), although we did not further explore this interaction, because the effect of PA is not only to antagonize the inotropic effect of ATP, but it has a negative inotropic effect on its own.

Therefore, in a third set of experiments, patch-clamp measurements of LTCC in isolated cardiomyocytes were recorded. The results indicate that the effect of PA on the amplitude of the Ca^{2+} transients can be explained by a reduction in the Ca^{2+} channel function of the DHPR in heart cells. We hypothesize that PA might bind directly to the DHPR and then reduce Ca^{2+} conductance by inhibiting the charge movement or by altering the conformational changes associated with the channel gating. By means of the reduction in its channel function, the expected reduction in both the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism and the SR Ca^{2+} release would affect the heart contractility. We cannot rule out the possibility that PA indirectly inhibits DHPR by modulating the phosphorylation status of the channel. Regardless the mechanism of the PA-DHPR interaction, the fact that the effect of the drug on the LTCC, the Ca^{2+} transients and the tension measurements was partially reversible indicates a modulation, not a damage, of a step of the ECC.

A different sensitivity of Ca^{2+} transients and LTCC inhibition by PA was observed: the EC_{50} for the effect of PA on the amplitude of the Ca^{2+} transients was approximately 10 µM, although for the effect on Ca^{2+} currents was around 1 µM. This can be explained by the fact that NCX can sustain the entry of Ca^{2+} into the cytosol even when L-type Ca^{2+} channels are inhibited (Bers, 2001) and then a higher concentration of PA is needed to noticeably reduce the amplitude of the Ca^{2+} transients.

Besides the reduction in the amplitude, the PA induced kinetic changes in the decay phase of the Ca^{2+} transients. PA produced an acceleration of the early phase, as demostrated by the significant reduction in the half width, and a slowing down of the late phase, as demonstrated when the operation *decay time-(half width-rise time)* was used as an indicator of the decay of the second half of the transient. For the latter case, the values were higher for PA compared to those of the control conditions (234.2 ± 6.1 vs 150.8 ± 3.6 ms, n = 83 transients analysed in Fig. 3, P < 0.01).

The decay phase of a Ca^{2+} transient in the heart comprises the concerted action of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), the NCX, the plasma membrane Ca^{2+} ATPase (PMCA), and

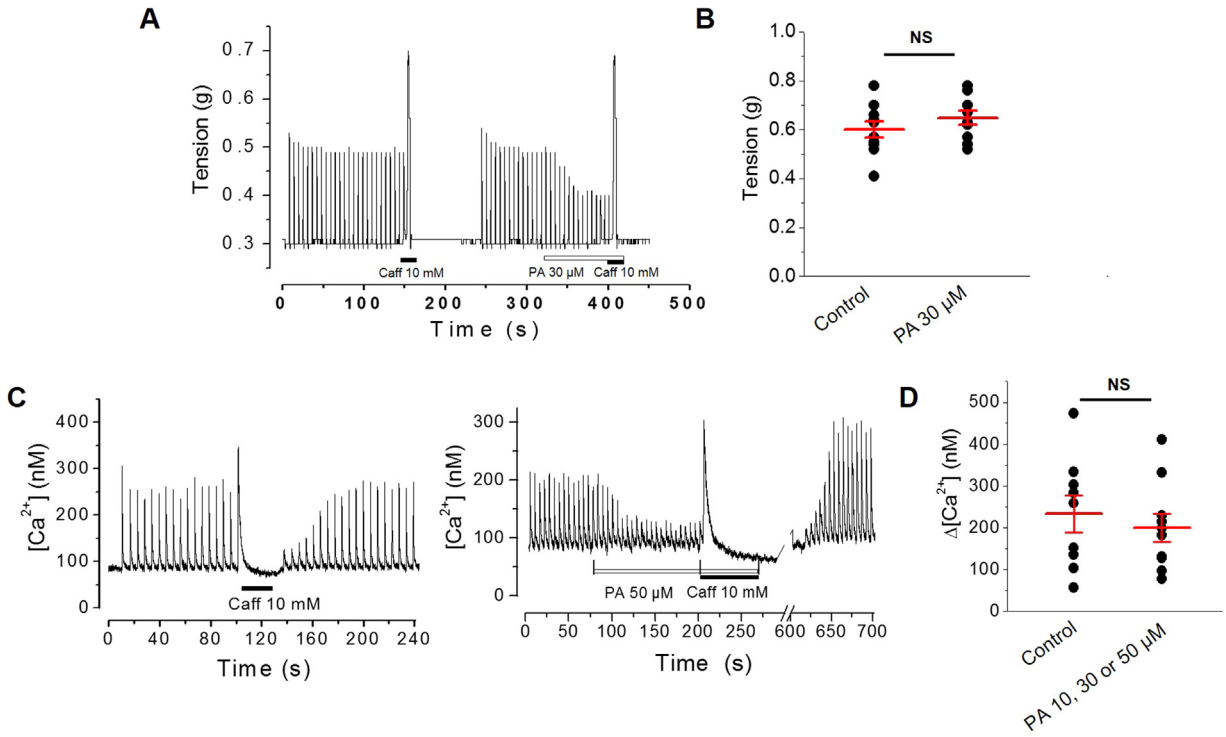

Fig. 7. Effect of caffeine and pomolic acid (PA) on tension measured in strips of trabeculae or papillary muscles of rat heart and amplitude of Ca^{2+} transients recorded in isolated rat cardiomyocytes loaded with Fura-2 AM. A representative record (out of ten) of tension in which there is an equivalence of the amplitude of the caffeine-induced contracture before and after incubation with 30 μM PA (A). A similar finding was observed in Ca^{2+} transients experiments, even when higher PA concentrations were used (C, panel on the right). The panel on the left shows a typical response of control cardiomyocytes to 10 mM caffeine (Caff). In tension, paired experiments, the caffeine contractures elicited before (control 0.6 ± 0.03 g, 63.6 ± 7.4% increase over basal contraction) and after (PA 30 μM 0.64 ± 0.03 g, 54.4% increase over basal contraction) PA exposure were comparable ($P^{>}0.05$, Fig. 5B). In the latter case, caffeine was applied after a PA-induced reduction of tension of 47.5 ± 2.7%. The change in Ca^{2+} concentrations induced by caffeine, measured in non-paired experiments, was not different ($P^{>}0.05$) between control cardiomyocytes (233.2 ± 44.1 nM, n = 9, Fig. 5D) and those exposed to PA (199.7 ± 33.1 nM, n = 10, Fig. 5D). Results in B were compared with a paired *t*-test, while the results in D were compared with a non-paired *t*-test. NS: no statistical difference.

probably the mitochondria (Bassani et al., 1994; Bers, 2001; Eisner et al., 2017; Negretti et al., 1993). The fact that both ISO and PA induced the phosphorylation of PLN supports the idea that the acceleration of the early phase of decay of the Ca^{2+} signal is explained by an increase in SERCA activity (MacLennan and Kranias, 2003). PLN phosphorylation would have led to an increase in SR Ca^{2+} content and a positive inotropic effect (MacLennan and Kranias, 2003), something that was not observed probably because this effect was hindered by the inhibition of the L-type Ca^{2+} channels function. Then, the increase in SERCA function protected the SR from depletion. Consistent with an increase in SERCA activity as a consequence of PLN phosphorylation, we also found that PA reduced the $t_{1/2}$ of relaxation of the tension records. We cannot make sure if PA directly activates PLN or modulates a signalling pathway that then activates PLN.

A certain degree of inhibition of the NCX, mitochondria or PMCA, or an increased SR Ca^{2+} leakage might explain the slowing down of the last part of the transient. However, tau analysis of caffeine transients suggests that the NCX is not affected and the mitochondria are not expected to be altered under acute treatments with PA; furthermore, their role in Ca^{2+} transients decay in heart has not yet been fully established (Eisner et al., 2017). PMCA has a negligible role in removing cytoplasmic Ca^{2+} when it is high, but it is important to keep lower resting Ca^{2+} concentrations in heart (Bers, 2001; Eisner et al., 2017). An increased degree of SR Ca^{2+} leakage as the SERCA reloads the SR may manifest by reducing the rate constant of decay at the end of the decay phase due to the increased strain on SERCA and PMCA functions (Eisner et al., 2017). Then, the lengthening of the very last part of the Ca^{2+} transient might be a consequence of a certain degree of PMCA inhibition, an increased SR Ca^{2+} leakage, or both, which remains to be tested.

With regards to a change in the basal Ca^{2+} concentrations, seemingly contradictory results were found. While Fura-2 traces did not show changes in basal fluorescence, an approximately 8% increase in the basal fluorescence in the Fluo-3 experiments was noted. This agrees with works showing similar increases in basal fluorescence in paced cardiomyocytes loaded with the AM form of Fluo-3 (Hagen et al., 2012). This increase in basal fluorescence was shown to reflect a small increase in the amount of de-esterified Fluo-3 present in the cytosol of the cardiomyocytes towards the end of the experiments. Then, PA does not change the basal Ca^{2+} concentrations in rat cardiomyocytes, a conclusion that argues against a PMCA inhibition and leaves the SR Ca^{2+} leakage as the main mechanism likely explaining the lengthening of the last part of the transient.

4.2. Safety and specificity

Experiments to evaluate toxicity (Alvarado-Castillo *et al.,* unpublished results) showed that intraperitoneal and oral administration of PA in a single dose of up to 2000 mg/kg to BALB-c mice (25 and 30 g) and single oral administration of PA up to 2000 mg/kg to Sprague–Dawley rats did not induce mortality or significant changes in behaviour, general condition, body weight or growth during 14 days of observation compared to controls. Moreover, at day 14, the macroscopic analysis of vital organ appearance did not show any signs of toxicity. Therefore, PA is safe (lethal dose 50 > 2000 mg/kg) in acute toxicity studies (OECD/OCDE, 2001).

Additionally, experiments in vivo showed that up to 1 mg/kg in rats did not change the QTc interval (Supplemental Fig. 4), although these

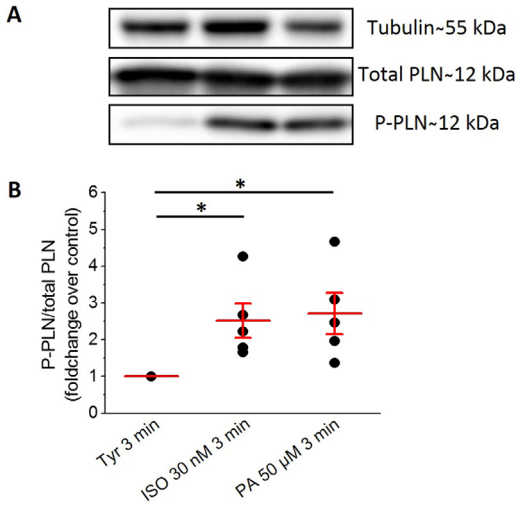

Fig. 8. Effect of pomolic acid (PA) on phospholamban (PLN) phosphorylation (P-PLN). A. Bands of a Western blot from a single, representative experiment (one rat) evaluating the effect of Tyrode (Tyr, negative control, leftmost lane), ISO 30 nM (middle lane) and PA 50 µM (rightmost lane), all during 3 min, on PLN phosphorylation. All bands come from the same gel. Notice that the density of tubulin and total-PLN bands are about the same for all lanes, but the bands of P-PLN obtained after treatment with ISO and PA are more intense compared to the control. B. Average of P-PLN/total PLN ratio of five different Western blot experiments, from five different hearts, evaluating the effect of PA on PLN phosphorylation. The mean ± S.E.M show a clear, higher effect of ISO (2.52 ± 0.47) and PA (2.71 ± 0.57) on PLN phosphorylation in cardiac strips, compared to the normalized control (1 ± 0). *P < 0.05 in a Mann-Whitney test, performed after a Kruskal-Wallis test.

observations must be reproduced in different species to ensure that PA does not induce acute cardiotoxicity (ICH Guideline, 2005).

If we consider that a 250 g rat has approximately 10 ml of plasma volume and 15.8 ml of total blood volume (TBV), as calculated by TBV (ml) = (0.06 *Body weight) + 0.77 (Lee and Blaufox, 1985), then the PA concentrations used in the present work may be up to 135-fold lower than those found in rat plasma that had been treated with 2000 mg/kg of PA.

PA had no effect on the ECC phenomenon in skeletal muscle fibres from mice (Supplemental Fig. 5). This differential result indicates that PA is a specific inhibitor of cardiac Ca^{2+} currents that, through activation of the CICR mechanism, play an important role in cardiac ECC (Fabiato and Fabiato, 1978). In the case of skeletal muscle fibres from mice, it is irrelevant whether PA inhibits or not Ca^{2+} currents, because the mechanism of Ca^{2+} release from skeletal SR is not dependent on the presence of external Ca^{2+} and hence on Ca^{2+} currents or CICR (Armstrong et al., 1972; Caputo and Giménez, 1967; Figueroa et al., 2012).

Finally, it is worth noting that tetrahydropyranyl 3-*O*-ursolic acid has no effect on rat ECC (not shown). It seems that the addition of the hydroxyl group at C19 on the ursane moiety along with the unsubstituted free hydroxyl at C3 (Supplemental Fig. 6) is critical for the effects of PA on the Ca^{2+} transients.

4.3. PA as a new, advantageous cardiovascular agent

There are several problems with the pharmacological treatment of hypertension. Most drugs act on a single target and this is not enough to control hypertension; let alone to control hypertension in patients with other comorbid conditions. In fact, only one third of hypertensive

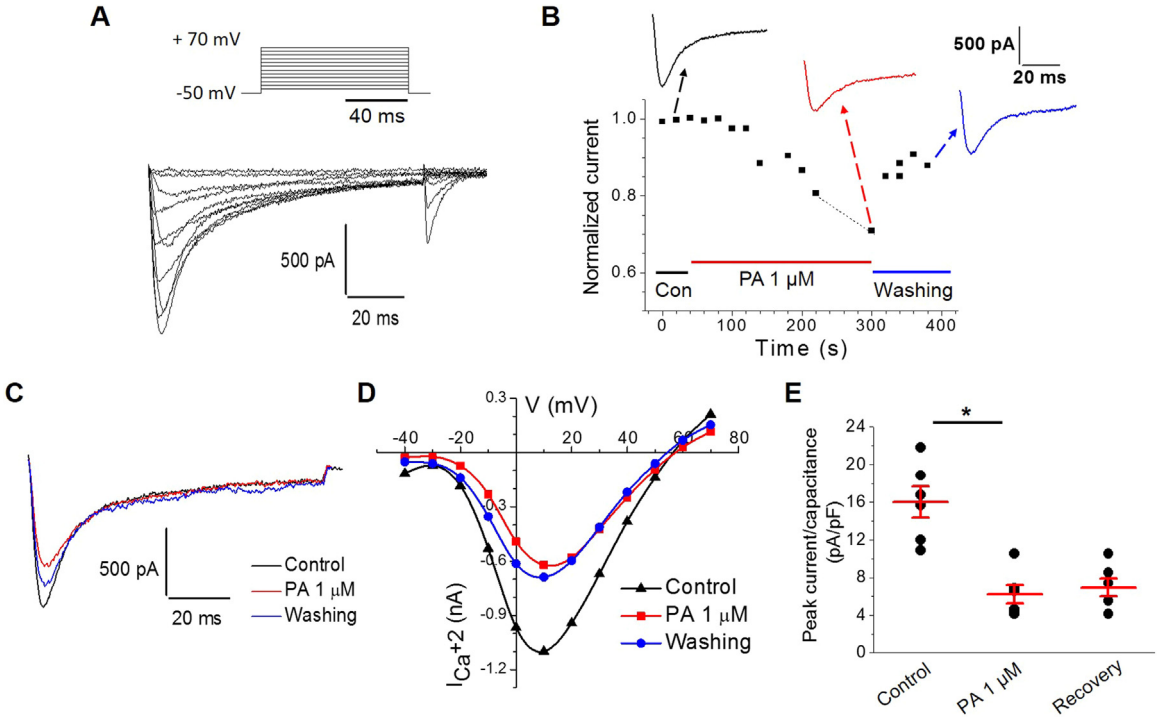

Fig. 9. Effect of pomolic acid (PA) on L-type Ca^{2+} currents in isolated rat cardiomyocytes. Control currents recorded under the indicated protocol are seen in A. Then the cell was incubated with PA 1 µM diluted in the external solution during 300 s and the peak current was followed. B shows the time course of the peak current amplitude during the experiment (black squares) and current traces at three selected moments of the experiment are displayed. The peak current under control conditions is shown in black, the one under the effect of PA is shown in red and after recovery is indicated in blue. In C the peak currents shown in B are superimposed to better see the changes in their kinetics. The complete I-V curves at the selected moments of the experiment with this cell are graphed in D. Average changes in peak currents normalized to cell capacitance obtained in six cardiomyocytes in control (16.0 ± 1.7), PA (6.2 ± 1.0) and recovery (6.9 ± 1.0) conditions are shown in E. *P < 0.05 in a Wilcoxon test, performed after a Friedman test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

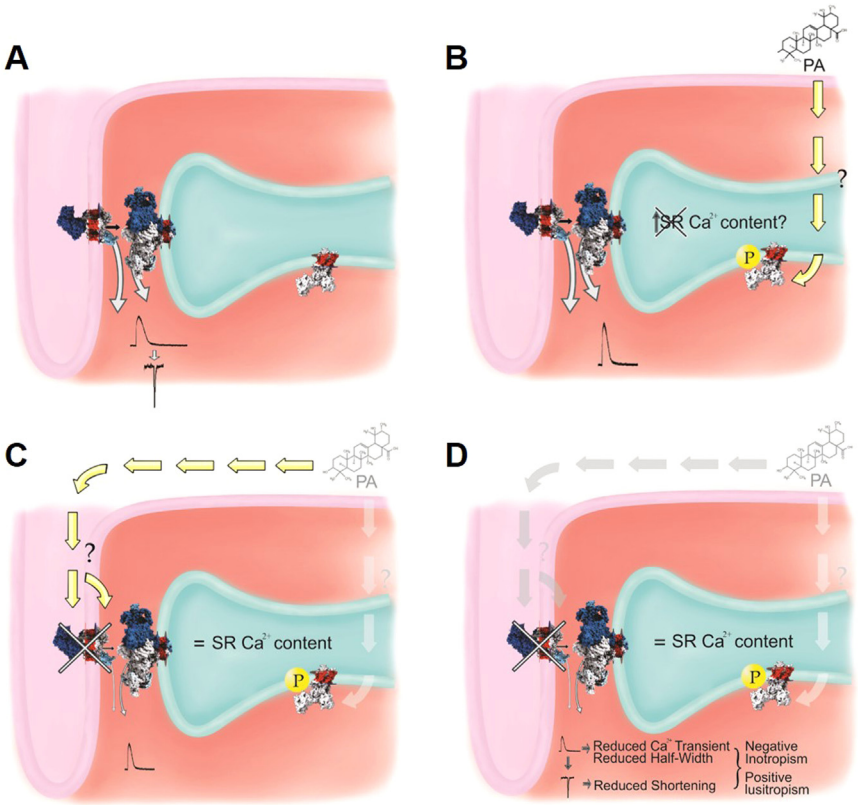

Fig. 10. Sequential model to explain the effect of pomolic acid (PA) on cardiomyocytes. A depicts the normal, control condition in which the dihydropyridine receptors (DHPR), inserted in the T-tubule, interact with the ryanodine receptors (RyR), inserted in the sarcoplasmic reticulum (SR) membrane. Both channels cooperate to increase cytoplasmic Ca^{2+} upon depolarization. The associated Ca^{2+} transient and cell shortening can be measured. PA incubation induces phospholamban phosphorylation (P-PLN) by a mechanism not fully clear, which explains the shortening of the signal at half width, and should have been associated to an increase in SR Ca^{2+} loading (B), which was not actually found because of the, likely direct, partial blocking effect of PA on the DHPR, further explaining the reduction in the Ca^{2+} transient amplitude (C). This finally leads to a reduction in the cell shortening and explains the negative inotropic, positive lusitropic effect of PA (D).

patients are controlled to goal blood pressure (Chobanian et al., 2003). This fact forces physicians to give several medications to their patients or to change the medications frequently (Chobanian et al., 2003; Mancia et al., 2013), which in turn affects adherence to the treatment, and both instances increase the incidence of adverse effects and costs. These limitations of the treatment of hypertension justify seeking drugs with new targets. It would be more interesting to find molecules with several targets that may influence cardiovascular health as a whole, given the trend of approaching hypertensive patients by evaluating their global cardiovascular risk (Carretero and Oparil, 2000b; Mancia et al., 2013). In this regard, PA is a compound with anti-platelet, vasorelaxant and negative inotropic effects, which makes it a good anti-aggregant and antihypertensive drug. Thus, PA may be particularly useful for a large group of hypertensive patients with several morbidities, such as ischaemic heart disease. Tackling several conditions with a single compound would reduce the problems associated with polymedication.

5. Conclusion

PA has a reversible negative inotropic and positive lusitropic effect on cardiomyocytes, by inhibiting DHPR and favouring PLN phosphorylation, which may increase SERCA activity and avoid a change in SR Ca^{2+} loading. In turn, this negative inotropic effect helps explain the hypotensive effect of PA. A model summarizing the effects of PA on rat cardiomyocytes is shown in Fig. 10.

Given its multiple targets in the cardiovascular system, PA may be useful for treating conditions in which there are several comorbidities related to the global cardiovascular risk of a hypertensive patient.

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Declaration of interest

None.

Authors contributions

P Bolaños, O Estrada and JC Calderón designed the experiments and obtained funding; O Estrada isolated the PA; R López, P Bolaños, A Guillén, MC Fernández, M Ramos, S Granados, AF Milán, JC Calderón and O Estrada performed the experiments presented in the results section; C Alvarado-Castillo designed and performed the toxicity experiments described in the discussion section; R López, P Bolaños, MC Fernández, JC Calderón and O Estrada analysed the data; and JC Calderón, O Estrada, C Alvarado-Castillo drafted the manuscript. P Bolaños and C Caputo reviewed the manuscript and made important intellectual contributions. All the authors approved the final version.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2019.02.016.

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