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Mechanism of the negative inotropic effect of naringin in mouse heart

[*Mecanismo del efecto inotrópico negativo de la naringina en el corazón de ratón*]

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Abstract

Context: Naringin (NRG) is the major flavonoid (flavanone glycoside) in grapefruit juice. Its biological activity has been only partially characterized and little is known about the mechanism of the negative inotropic action of this flavonoid.

Aims: To evaluate the effects of NRG on the surface electrogram (ECG) and the force of contraction (FC) of mice hearts as well as on the sodium (I_{Na}), calcium (I_{CaL}) and Na^+ - Ca^{2+} exchange (I_{NaCaX}) currents of enzymatically isolated mouse ventricular cardiomyocytes.

Methods: ECG and FC were recorded on mouse hearts perfused in a Langendorff column. Ventricular cardiomyocytes were enzimatically dissociated and ionic currents recorded with the patch-clamp technique.

Results: NRG increased RR interval and shortened corrected QT only at high concentrations (30-100 µM). However, at a fixed heart rate, it decreased FC with an IC_{50} of 0.4 µM. NRG reduced I_{Na} with an IC_{50} of 0.07 µM but with a maximal inhibition of 60 %. NRG also depressed I_{CaL} with an IC_{50} of 0.013 µM and increased its fast inactivation time constant. The effects on I_{CaL} were not voltage-dependent. I_{NaCaX} was not affected by NRG.

Conclusions: Our results indicate that NRG exerts a negative inotropic effect in mice hearts that could be explained by a decrease in I_{Na} and I_{CaL}. These actions should be taken into account when considering this molecule either as a dietetic supplement or as a template to develop therapeutic agents for human diseases.

Keywords: Calcium; cardiac; flavonoids; naringenin, naringin; sodium.

Resumen

Contexto: La naringina (NRG) es el principal flavonoide (glicósido de flavanona) en el jugo de toronja. Su actividad biológica ha sido solo parcialmente caracterizada y poco se conoce acerca del mecanismo de la acción inotrópica negativa de este flavonoide.

Objetivos: Evaluar los efectos de la NRG sobre el electrograma de superficie (ECG) y la fuerza de contracción (FC) de corazones de ratón, así como sobre las corrientes de sodio (I_{Na}), calcio (I_{CaL}) y del intercambiador Na^+ - Ca^{2+} (I_{NaCaX}) en cardiomiocitos ventriculares de ratón, aislados enzimáticamente.

Métodos: El ECG y la FC se registraron en corazones de ratón perfundidos en una columna de Langendorff. Los cardiomiocitos ventriculares se disociaron enzimáticamente y las corrientes iónicas se registraron con la técnica de patch-clamp.

Resultados: La NRG incrementó el intervalo RR intervalo y acortó el QT solo a altas concentraciones (30-100 µM). No obstante, a frecuencia cardíaca fija, disminuyó la FC con un IC_{50} de 0.4 µM. La NRG redujo I_{Na} con un IC_{50} de 0.07 µM pero con una máxima inhibición de 60 %. La NRG también redujo I_{CaL} con un IC_{50} de 0.013 µM e incrementó su constante de inactivación rápida. Los efectos sobre I_{CaL} no fueron dependientes del potencial. La I_{NaCaX} no fue afectada por la NRG.

Conclusiones: Nuestros resultados indican que la NRG ejerce un efecto inotrópico negativo en corazones de ratón que puede ser explicado por una reducción en I_{Na} e I_{CaL}. Esas acciones deben ser tomadas en cuenta al considerar a esta molécula como suplemento dietético o como plantilla para desarrollar nuevos agentes terapéuticos para tratar las enfermedades en humanos.

Palabras Clave: Calcio; cardíaco; flavonoides; naringenina; naringina; sodio.

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INTRODUCTION

There is evidence for an association between high dietary intake of flavonoids and a reduction of myocardial infarction and stroke (Hertog et al., 2012; Keli et al., 1996). It is widely accepted that these natural compounds could have a potential therapeutic value in the prevention and treatment of cardiovascular diseases (Benavente-García and Castillo, 2008; Habauzit and Morand, 2012) due to their antioxidant, anti-inflammatory, anti-proliferative and anti-thrombotic actions (see for reviews Bharti et al., 2014; Wright et al., 2013). However, the intracellular modulator actions of flavonoids are diverse and complex (Wright et al., 2013) and can be affected by sex, lifestyle, disease states and interactions with drugs thus limiting their impact on human health (Wright et al., 2013; Chanet et al., 2012). Nonetheless, these compounds are extremely interesting as molecular templates to design drugs with better pharmacological profiles for the treatment of human cardiovascular diseases.

The flavanone naringin is the major flavonoid in grapefruit juice, an important dietary source of flavonoids, and gives the grapefruit juice its bitter taste (Peterson et al., 2006). When it is ingested, NRG is transformed and converted to several metabolites (including naringenin) in blood and urine. However, naringin (and naringenin) could be detected in plasma around 5 hours after oral administration (Bharti et al., 2014; Fuhr and Kummert, 1995). Naringin seems to have a cardioprotective action in isoproterenol-induced myocardial infarction in rats (Rajadurai and Prince, 2007). In stroke-prone spontaneously hypertensive rats, orally-administered naringin was reported to suppress the age-related increase in blood pressure, to significantly decrease thrombotic tendency and to increase nitric oxide (NO) production thus improving endothelium-dependent vasodilation (Ikemura et al., 2012). Nevertheless, part of these effects could be also probably due to the actions of naringenin, the aglycone formed during the cleavage of the sugar moiety of naringin after its ingestion (Fuhr and Kummert, 1995). Naringenin, by activating mitochondrial BK potassium channels could protect against ischemia-reperfusion injury (Testai et al., 2013). However, there is still the need of investigations about the possible direct cardiovascular physiological actions of naringin. Saponara et al. (2006) showed that naringin could increase the conductance of vascular smooth muscle calcium-activated potassium ion channels ($BK_{(Ca)}$) but exhibited a poor vasorelaxing action in rat aortic rings compared to its aglycone naringenin. Recently, Saponara et al. (2011) described that a number of flavonoids stimulated or inhibited I_{CaL} in rat tail artery myocytes. They found that naringin and naringenin modestly inhibited I_{CaL}. We have previously reported that naringin, at pharmacological relevant concentrations, induced contraction of rat aortic rings and exerted a modest negative inotropic effect on isolated rat hearts (López-Medina et al., 2014). Since naringin could be used as a dietary supplement, antioxidant, antiinflammatory and even as a template to develop cardiovascular drugs, it is important to investigate its actions on the voltage-dependent Na^+ and Ca^{2+} channels and on the current generated by the Na^+ - Ca^{2+} exchanger in an attempt to elucidate the mechanism of the negative inotropic action of this flavonoid.

MATERIALS AND METHODS

Chemicals

Naringin (4',5,7-trihydroxyflavanone 7-rhamnoglucoside; $C_{27}H_{32}O_{14}$, PubChem CID: 25075; >95% HPLC), nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarbo-xylic acid dimethyl ester; $C_{17}H_{18}N_2O_6$; PubChem CID: 4485; >98% HPLC) and lidocaine (2-diethylamino-N-(2,6-dimethylphenyl)acetamide; $C_{14}H_{22}N_2O$; PubChem CID: 3676; 99.9% HPLC) were purchased from Sigma Aldrich and were prepared in ethanol as 0.1 M (naringin and lidocaine) and 10 µM (nifedipine) stock solutions. All other chemicals were also from Sigma Aldrich.

Animals

Experiments were performed using male adult C57BL/6 (7-8 weeks) mice according to the procedures approved by the Centro Nacional para la Producción de Animales de Laboratorio

(CENPALAB, Santiago de Las Vegas, Habana, Cuba). Prior to experiment animals were adapted for seven days to laboratory conditions (controlled temperature 25 ± 2°C, relative humidity 60 ± 10% and 12 h light/dark cycles). Tap water and standard diet for rodents supplied by CENPALAB were freely provided. All procedures were also conducted according to the European Commission guide-lines for the use and care of laboratory animals and approved by the Committee for Animal Care in Research of the Center. The minimum number of animals and duration of observation required to obtain consistent data were employed.

Recording of electrical and mechanical activities in isolated hearts

Mouse hearts were carefully dissected and mounted on a Langendorff column to record the surface electrogram (ECG) and the force of contraction (FC) as previously described (Galán et al., 1998). ECG and FC values were recorded at the spontaneous heart rate and at a fixed stimulus rate (200 bpm).

Enzymatic isolation of ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated as previously described (Alvarez-Collazo et al., 2012) and were kept in a K^+-Tyrode solution containing 1 mM Ca^{2+} at room temperature (21 ± 2 °C) and used for experiments for 6 h.

Patch-clamp recordings

Whole-cell currents were recorded at room temperature (Alvarez-Collazo et al., 2012). Currents were filtered at 3 kHz and digitized at 50-µs intervals, stored on a computer and analysed off-line with the ACQUIS1 software (version 2.0, CNRS License, France). To study Na^+ and Ca^{2+} currents, K^+ currents were blocked by substituting all potassium by cesium in extracellular and "intracellular" solutions. The extracellular solution contained (in millimolars): 117 NaCl, 20 CsCl, 10 HEPES, 2 $CaCl_2$, 1.8 $MgCl_2$, and 10 glucose, pH 7.4. The standard pipette (intracellular) solution contained (in millimolars): 130 CsCl, 0.4 Na_2GTP, 5 Na_2ATP, Na_2-creatine phosphate, 2.0 $MgCl_2$, 11 EGTA, 4.7 $CaCl_2$ (free Ca^{2+}, 108 nM), and 10 HEPES, with pH adjusted to 7.2 with CsOH.

Pipette resistance was 1.0-1.2 MΩ. Membrane capacitance (Cm) and series resistance (Rs) were calculated on voltage-clamped cardiomyocytes as previously described (Alvarez et al., 2000). Average Cm and uncompensated Rs were 170 ± 10 pF and 3.7 ± 0.3 MΩ, respectively (N = 63). Rs could be electronically compensated up to 50% without ringing and was continually monitored during the experiment. Liquid junction potential was compensated before establishing the gigaseal. No leak or capacitance subtractions were performed in the recordings.

The fast Na^+ current (I_{Na}) was evoked with 50-ms voltage-clamp pulses to -40 mV, applied from a holding potential (HP) of -100 mV (1/4 s). In an attempt to decrease the huge I_{Na} and improve voltage control during patch-clamping, the extracellular Na^+ concentration was reduced to 10 mM keeping osmolarity with (107 mM) tetraethylammonium chloride. Nifedipine (10 µM) was used in these experiments to block the L-type Ca^{2+} current (I_{CaL}). For routine monitoring of I_{CaL} a double pulse voltage-clamp protocol was employed: from a holding potential (HP) of -80 mV every 4s the cell membrane was depolarized to -40 mV for 50 ms to inactivate the fast Na^+ current. From this membrane potential a 300-ms pulse to +10 mV evoked I_{CaL}. The inactivation time courses of I_{Na} and I_{CaL} were fitted to a single (I_{Na}) or a double exponential (I_{CaL}) using the fitting procedures of the ACQUIS1 software.

In other experiments the Na^+/Ca^{2+} exchange current (I_{NaCaX}) was estimated by using 500-ms ramp voltage clamps from +70 to -100 mV (1/15 s; HP = -40 mV). In those experiments nifedipine (10 µM), ouabain (10 µM) and $NiCl_2$ (5 mM) were used to block I_{CaL}, the Na^+-K^+ pump and I_{NaCaX}, respectively.

Statistical analysis

Results are expressed as means and standard errors of means. Statistical significance was evaluated by means of paired or unpaired Student's t test according to the experimental situation.

Differences were considered statistically signify-cant for $p < 0.05$.

RESULTS

Effects of NRG on electrical and mechanical activities in isolated hearts

In six hearts, a wide range of NRG concentrations (0.001 - 100 µM) exerted variable effects on the QRS in such a way that, although there was a tendency to increase its duration and decrease its amplitude, no statistically significant effects could be demonstrated. RR interval was only significantly increased by NRG from 208 ± 2 ms in control to 235 ± 8 ms and 514 ± 17 ms at 30 and 100 µM concentrations respectively ($p < 0.05$). Corrected QT (QTc = QT/√RR) was not significantly affected by low NRG concentrations (up to 10 µM). However, at 30 and 100 µM con-centrations, NRG decreased QTc (from 13 ± 1 ms in control to 8 ± 1.5 ms and 6.5 ± 2 ms, respec-tively; $p < 0.05$). On the other hand, NRG (0.001 - 100 µM) significantly decreased the FC in isolated hearts. Hearts were paced at 200-ms stimulus interval (slightly over the spontaneous RR interval under control condition; 208 ± 2 ms) in order to avoid any frequency-dependent changes in FC. Experimental data were fitted to a Hill function and the estimated IC_{50} for inhibition of contraction was 0.4 ± 0.1 µM (Hill number = 0.6 ± 0.08), comparable to that of nifedipine (IC_{50} = 0.3 ± 0.05 µM; Hill number = 1.5 ± 0.04; n = 5). The action of NRG on FC was reversible upon washout with normal Tyrode solution.

Effects of NRG on sodium current

In control condition (10 mM extracellular Na^+) peak inward I_{Na} at -40 mV was 14.5 ± 2.2 pA/pF (N = 28). Its inactivation time course could be fitted to a single exponential with a time constant of 2.2 ± 0.2 ms. Concentrations of NRG as low as 0.02 µM decreased I_{Na} by ~ 40 %. The decrease in I_{Na} was not use-dependent and occurred in an almost "on-off" fashion (Fig. 1A). Increasing NRG concentration barely increased I_{Na} block. The experimental data were fitted to a Hill function and the estimated IC_{50} for I_{Na} (at -40 mV) inhibi-tion by NRG was 0.07 ± 0.01 µM with a Hill number of 0.83 ± 0.07 but with a maximal I_{Na} inhibition of only 60.1 ± 1.1 % (Fig. 1B). In sharp contrast, the reference compound lidocaine (a classic local anesthetic; 1 - 300 µM), inhibited I_{Na} in a typical use-dependent fashion with an IC_{50} of 23.5 ± 3.1 µM (Hill number, 0.9 ± 0.08) but with a maximal inhibition of 96.3 ± 3.9% (Fig. 1B). The decrease of I_{Na} by NRG was accompanied by variable changes in its inactivation time course. The inactivation time constant of I_{Na} was signify-cantly increased only at concentrations higher than 0.02 µM (Fig. 2). The effects of different lidocaine concentrations on the inactivation time constant of I_{Na} were not statistically significant. As noted above, we used a 10 mM Na^+ extra-cellular solution in an attempt to minimize voltage-clamp errors during the flow of a large I_{Na}. However, even under this condition the re-corded Na^+ current density was large and, due to the presence of a residual series resistance (~ 2 MΩ after compensation), the estimated error factor during the flow of I_{Na} could be large (see Alvarez et al., 2000). This precluded a detailed ana-lysis of I_{Na} kinetics in all studied cells. In cells where the error factor was small (< 4 mV), we studied the effects of 0.06 µM NRG (a concen-tration near the IC_{50}) on I-V, availability and acti-vation curves of I_{Na}. Cardiomyocytes were clam-ped at a HP of -100 mV and a double-pulse volta-ge-clamp protocol was applied at a frequency of 0.125 Hz. A fixed 50-ms test pulse to -40 mV was preceded by 50-ms prepulses applied from -50 to +50 mV. A short 2-ms gap at the HP separated pre- and test pulses. To obtain the availability curve, I_{Na} at each test pulse was normalized to maximal I_{Na} and plotted against the prepulse potential. Activation curve was obtained by calculating the chord conductance at each test potential and normalizing by the maximal chord conductance. Experimental data of availability and activation curves were fitted to Boltzmann functions to obtain mid points for activation and availability ($V_{0.5}$) and the slope factors (s). As can be seen in Fig. 3 (A-C) the action of NRG seemed to be almost voltage-independent. Potentials for half inactivation and activation ($V_{0.5}$) were barely shifted from -73.6 ± 0.3 mV (slope factor, s = 7.4 ±

0.3 mV) to -75.6 ± 0.2 mV (s = 7.1 ± 0.2 mV) and from -37.2 ± 0.4 mV (s = 5.1 ± 0.2 mV) to -36.7 ± 0.5 mV (s = 4.7 ± 0.1 mV), respectively (n = 4). On the other hand, lidocaine (30 μM; near the IC_{50}), had a typical voltage-dependent action and shifted the $V_{0.5}$ for inactivation from -77.3 ± 1.1 mV (s = 7.6 ± 0.4 mV) to -86.5 ± 1.0 mV (s = 7.1 ± 0.2 mV) and the $V_{0.5}$ for activation from -38.8 ± 1.2 mV (s = 5.7 ± 0.3 mV) to -33.8 ± 1.1 mV (s = 5.6 ± 0.5 mV; $p < 0.05$; n = 5).

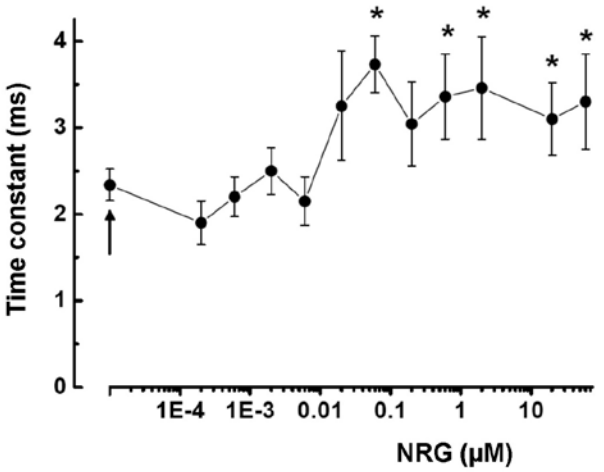

Figure 2. Concentration-dependent effects of NRG on the inactivation time course of I_{Na}. Inactivation time course of I_{Na} could be fitted to one exponential. Although with some variability, the inactivation time constant of I_{Na} was increased by NRG at concentrations of 0.02 μM or greater. The asterisks denote statistically significant effects of NRG ($p < 0.05$) with respect to the value obtained in control condition (arrow).

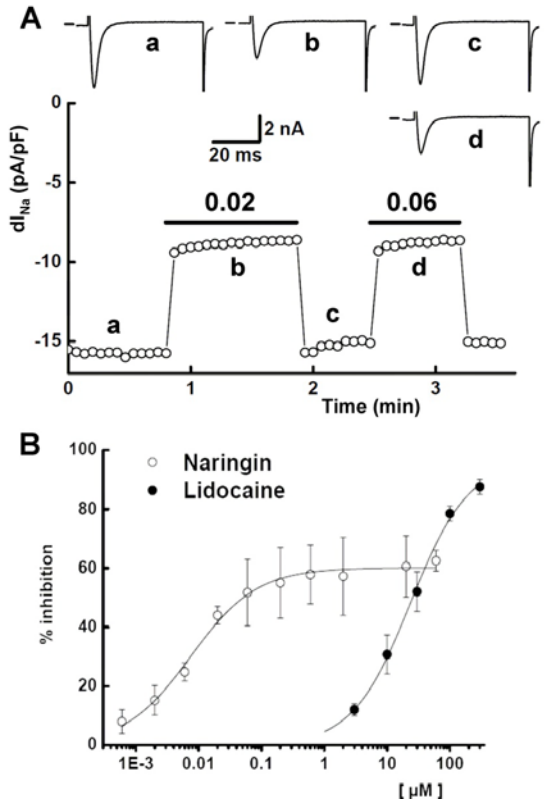

Figure 1. Effects of NRG on the Na^+ current in mouse ventricular cardiomyocytes. **A:** Example of the effects of extracellular application of NRG at two concentrations (0.02 – 0.06 μM) on I_{Na} recorded at -40 mV. The insets show the current traces corresponding to the time points indicated by the labels. **B:** Concentration-response curves for the inhibition of I_{Na} by NRG and lidocaine. Experimental data (n ≥ 4 for each point) were fitted to a Hill function.

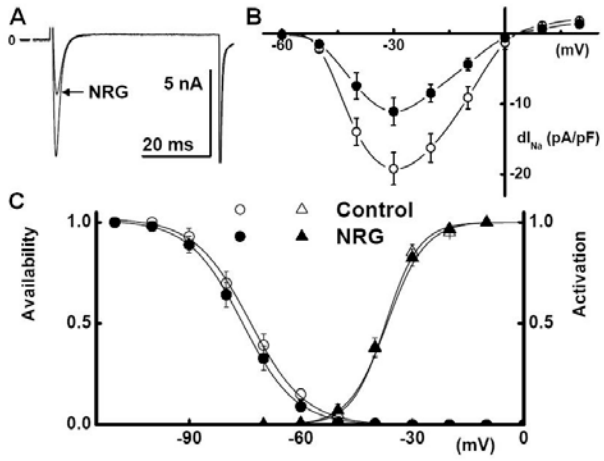

Figure 3. Effects of NRG on the voltage dependence of activation and inactivation of Na^+ current in mouse ventricular cardiomyocytes. **A:** Representative current traces recorded at -40 mV (HP = -100 mV) in control and in the presence of NRG 0.06 μM (a concentration near the IC_{50}). **B:** Effect of NRG (0.06 μM) on the current-voltage (I-V) relationship of I_{Na}. Note that NRG produced no shift on the I-V curve. C: Availability and activation curves of I_{Na} obtained in control and in the presence of 0.06 μM NRG.

Effects of NRG on the L-type calcium current

In control condition mean I_{CaL} density at +10 mV was 6.2 ± 0.3 pA/pF (N = 25). Its inactivation time course was fitted to two exponentials yielding 6.3 ± 0.3 ms and 51.6 ± 2.2 ms for fast (τ_{fast}) and slow (τ_{slow}) components, respectively. Perfusion of cardiomyocytes with NRG resulted in a pulse to pulse decrease of I_{CaL} that reached a steady state in ~ 30 s (Fig. 4A). The effects of NRG were concentration-dependent and were characterized by an IC_{50} of 0.013 ± 0.001 µM, a Hill number (N) of 0.6 ± 0.02 and a maximal inhibition (Bmax) of 100%. This IC_{50} is about one order of magnitude lower than that of nifedipine (0.1 ± 0.07 µM, N = 0.9 ± 0.05, Bmax = 100%; n ≥ 5 cells for each concentration). NRG slowed down I_{CaL} inactivation. This effect was characterized by a small but significant increase of τ_{fast} at low concentrations and a huge increase at 2 and 6 µM concentrations (Fig. 4B). Although τ_{slow} showed a tendency to be increased in the presence of different NRG concentrations, changes were not statistically significant. The relationships of τ_{fast} and τ_{slow} with membrane voltage were "U" shaped with a minimum between 0 and +10 mV. The clear-cut increase in τ_{fast} by NRG was not voltage-dependent as proportionate increases were observed for all imposed membrane potentials at the studied concentrations (data not shown).

To study the effects of NRG on I_{CaL} kinetics cardiomyocytes were clamped at a HP of –80 mV and a double-pulse voltage-clamp protocol was applied at a frequency of 0.125 Hz. A fixed 300-ms test pulse to +10 mV was preceded by 300-ms prepulses applied from -50 to +70 mV. A short 5-ms gap at the HP separated pre- and test pulses. To obtain the availability curve, I_{CaL} at each test pulse was normalized to maximal I_{CaL} and plotted against the prepulse potential. Activation curve was obtained by calculating the chord conductance at each test potential and normalizing by the maximal chord conductance.

Experimental data of availability and activation curves were fitted to Boltzmann functions to obtain mid points for activation and availability ($V_{0.5}$) and the slope factors (s). The action of NRG on I_{CaL} was barely voltage-dependent (Fig. 5A). At 0.02 µM concentration (near the IC_{50}) NRG shifted the $V_{0.5}$ of I_{CaL} inactivation from -28.0 ± 1.7 mV to -30.04 ± 0.4 mV (not significant; n = 5). I_{CaL} activation was also scarcely affected, $V_{0.5}$ was shifted from -16.6 ± 0.2 mV to -13.6 ± 0.3 mV (not significant; Fig. 5B).

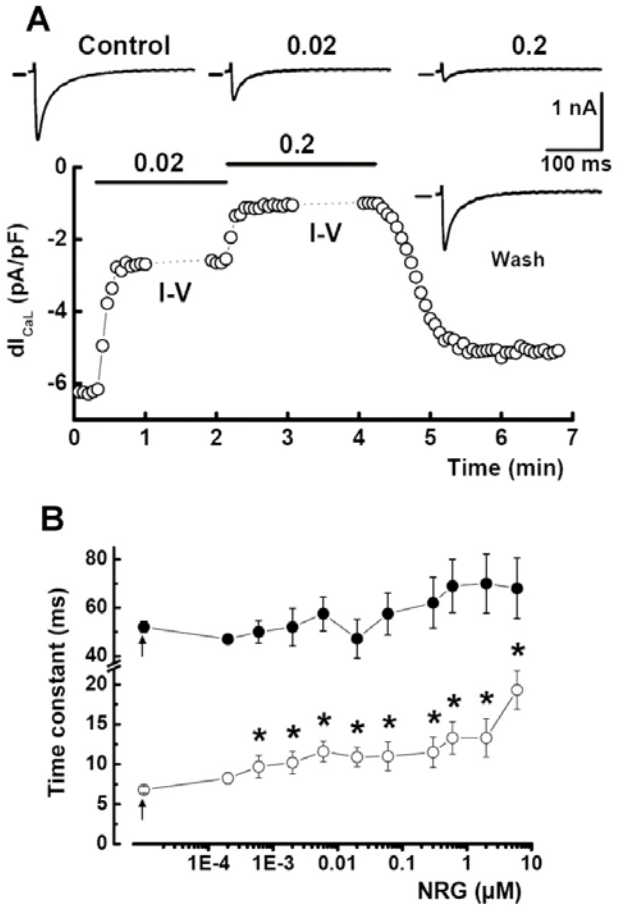

Figure 4. Effects of NRG on the L-type Ca^{2+} current in mouse ventricular cardiomyocytes. **A:** Example of the effects of extracellular application of NRG at concentrations of 0.02 and 0.2 µM on I_{CaL} recorded at +10 mV. The insets show the current traces corresponding to the time points indicated by the labels. I-V labels indicate periods at which current-to-voltage relationships were constructed. **B:** Concentration-dependent effects of NRG on the time constants of fast (τ_{fast}) and slow (τ_{slow}) inactivation of I_{CaL} evoked at +10 mV. The asterisks denote statistically significant effects of NRG ($p < 0.05$) with respect to τ_{fast} and τ_{slow} obtained in control condition (arrows).

Higher concentrations of NRG resulted in similar changes in $V_{0.5}$ of both activation and availability. No significant changes were observed in the corresponding slope factors or on the I_{CaL}

availability at positive prepulse potentials. The reference compound nifedipine exhibited a marked voltage-dependent action with significant ($p < 0.05$; $n = 5$) shifts in $V_{0.5}$ for availability and activation from -27.1 ± 2.0 mV to -38.2 ± 0.5 mV and from -17.2 ± 0.3 mV to -12.5 ± 0.4 mV, respectively. The corresponding slope factors were 6.9 ± 0.5 mV and 6.5 ± 0.4 mV for availability and 6.3 ± 0.2 mV and 6.5 ± 0.3 mV for activation.

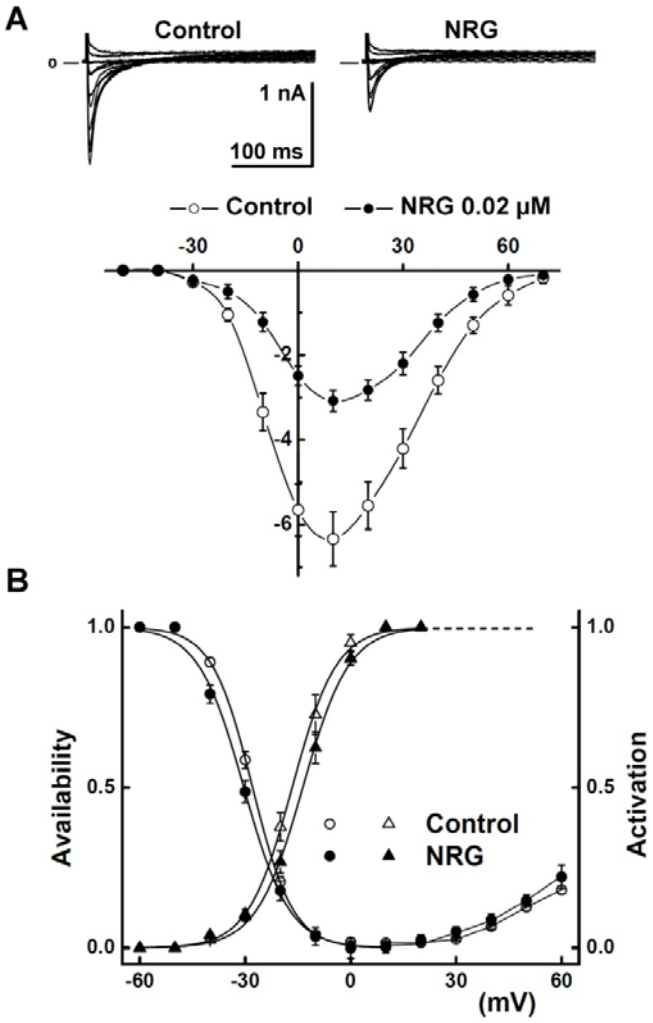

Figure 5. Effects of NRG on the voltage dependence of activation and inactivation of L-type Ca^{2+} current in mouse ventricular cardiomyocytes. **A:** Effect of NRG, at a concentration near the IC_{50} on the current-voltage (I-V) relationship of I_{CaL}. Note that NRG produced no shift on the I-V curve. The inset shows representative current traces recorded in control and in the presence of 0.02 µM NRG. **B:** Availability and activation curves of I_{CaL} obtained in control and in the presence of 0.02 µM NRG.

Lack of effect of NRG on the Na^+ - Ca^{2+} exchange current

Taking advantage of the voltage-dependency of the Na^+ - Ca^{2+} exchanger (Bers, 2001), we used 500-ms ramp voltage clamps from +70 to -100 mV from an HP of -40 mV in an experimental condition where I_{CaL} and the Na^+-K^+ pump were blocked. I_{NaCaX} was estimated using $NiCl_2$ (5 mM) a known blocker of the Na^+ - Ca^{2+} exchanger. In six cardiomyocytes, NRG at high concentration (30 µM) had no effect on the Ni^{2+} sensitive current. Under control condition I_{NaCaX} densities at +60 (I_{+60}) and -90 mV (I_{-90}) were 1.22 ± 0.12 pA/pF and -0.91 ± 0.7 pA/pF, respectively. The reversal potential was -15.3 ± 1.2 mV. In the presence of NRG I_{+60} and I_{-90} were 1.17 ± 0.11 and -0.96 ± 0.09 pA/pF, respectively and the reversal potential was -15.1 ± 1.1 mV.

DISCUSSION

The present results show that NRG possesses "Ca^{2+}-antagonist" properties. This flavanone glycoside exerts a negative inotropic action in mouse heart and decreased both Na^+ and Ca^{2+} currents. We thus confirm that NRG exerts a negative inotropic action on rodent hearts (c.f. López-Medina et al., 2014). However, to our surprise, in mouse heart, NRG was as potent as the classical Ca^{2+}-antagonist nifedipine. As shown in Results, the concentration-dependent negative inotropic action of NRG was accompanied by minor changes in electrical activity at concentrations around the IC_{50} for the inhibition of contractile force. Physiologically significant shortening of QTc and an increase in RR interval were seen only at high concentrations well over the effective plasma concentrations found by Xiao-Hong et al. (2010). It should be considered that NRG could exert multiple actions on different ionic channels that balance to each other, resulting in scarce effects on the cardiac surface electrogram. The negative inotropic effect of NRG was more evident and this prompted us to study the effects of NRG on three major protagonists of cardiac contraction, the fast Na^+ current, the L-type Ca^{2+} current and the Na^+ - Ca^{2+} exchange current.

NRG decreased I_{Na} in a concentration-dependent manner with an IC_{50} (0.07 μM) much smaller than that of the classic local anesthetic lidocaine (see also Tan and Saint, 2000) but with a lower potency since maximal block achieved was ~ 60%. Further experiments are needed to understand this specific feature of NRG action on Na^+ current (potent but incomplete inhibition). As discussed above, I_{Na} was recorded in a Na^+-poor extracellular solution. We cannot rule out that the IC_{50} for NRG inhibition of I_{Na} could be different at physiological extracellular Na^+ concentrations. It has been reported for Na^+ channels that the permeant ion might influence channel block by antiarrhythmic drugs by the so-called "knock out" effect (Barber et al., 1992). NRG block of I_{Na} was not use-dependent; channel block occurred in an almost "on-off" fashion and was not voltage-dependent (activation and availability curves were barely modified). Although not explored in detail, the results suggest that NRG blocks Na^+ channels in the open state. Due to the lack of voltage dependency, it can be assumed that NRG blockade of Na^+ channel could take place by simply plugging the channel and not by interfering with channel gating process but this requires further investigation. In any case, blockade of I_{Na} could decrease Na^+ load and have some effect in cardiac contraction (Bers, 2001). However, it is evident that most of the negative inotropic effect of NRG could be due to its blocking action on I_{CaL}.

Blockade of I_{CaL} by NRG was concentration-dependent and was achieved in a pulse-to-pulse manner, an action that is reminiscent of that of the classic phenylalkylamine Ca^{2+} channel blocker verapamil (Rubio et al., 1993). NRG blocked I_{CaL} with an IC_{50} of 0.013 μM, about one order of magnitude lower than that of nifedipine (0.1 μM) but unlike nifedipine's classic action, blockade of I_{CaL} by NRG was poorly voltage-dependent with only minor changes in availability and activation curves. The inhibition of I_{CaL} was accompanied by an increase in fast inactivation time constant τ_{fast} with maximal increases at 2-6 μM concentrations for maximal I_{CaL} evoked at +10 mV. Although τ_{slow} showed a tendency to increase in the presence of different NRG concentrations, changes were not statistically significant. Our results confirm in part those of Saponara et al. (2011) who showed that naringin and its aglycone naringenin modestly inhibited I_{CaL} in rat tail artery myocytes. However, besides that cell type and experimental conditions were different, it is difficult to explain why NRG was more potent on I_{CaL} in mouse ventricular cardiomyocytes than in rat arterial smooth muscle cells. As Saponara et al. (2011) suggested, the flavonoid scaffold could be a valuable template for the design of novel drugs acting on vascular smooth muscle Cav1.2 channels for the treatment of hypertension and stroke. On the other hand, in experiments on recombinant human $K_{IR}3.1$-3.4 and $K_{IR}3.1$-3.2 expressed in *Xenopus* oocytes or HAC15 cells (Oki et al., 2012; Yow et al., 2011), naringin (but not naringenin) was shown to be a direct activator of inward rectifying K^+ currents, an effect that, together with its action on I_{CaL}, could have an impact on heart rate at least in isolated heart or spontaneous activity in single cell preparations. Since we found no evidence of a NRG action on I_{NaCaX}, the inhibition of L-type Ca^{2+} channels in mouse ventricular cardiomyocytes can easily explain the negative inotropic effect we found in isolated mouse hearts. However, we cannot rule out effects of NRG on other systems (e.g. ryanodine receptor, Ca^{2+}-ATPase) that could also affect contractile force.

NRG is an important active ingredient in citrus fruits, a major source of dietetic flavonoids. As stated before, there is an interest on flavonoids (and NRG) not only as dietetic supplements but also as templates to develop therapeutic agents for the treatment of several human diseases. Besides its cardiovascular effects described here, it has been reported that NRG possesses antioxidant and neuroprotective (Choi et al., 2010) actions. It also acts as an anti-inflammatory (Jain and Parmar, 2011). NRG is reported to be able to decrease total cholesterol levels (Lee et al., 2001) and to inhibit high glucose-induced apoptosis by attenuating mitochondrial dysfunction (Huang et al., 2013). It is thus important to study its pharmacological properties in order to identify the beneficial profile and as well its undesirable effects (Bharti et al., 2014). Here we show that NRG

could have a negative inotropic action in heart due to a blocking action on Na^+ and Ca^{2+} channels. Whether these effects are relevant or not to human health require further investigation.

CONCLUSIONS

We may conclude that NRG exerts a negative inotropic effect in mouse heart. This effect could be explained by its inhibitory action on sodium and calcium currents. These actions should be taken into account when considering this molecule either as a dietetic supplement or as a template to develop therapeutic agents for human diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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