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A novel dihydropyridine with 3-aryl *meta*-hydroxyl substitution blocks L-type calcium channels in rat cardiomyocytes

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ABSTRACT

Rationale: Dihydropyridines are widely used for the treatment of several cardiac diseases due to their blocking activity on L-type Ca^{2+} channels and their renowned antioxidant properties.
Methods: We synthesized six novel dihydropyridine molecules and performed docking studies on the binding site of the L-type Ca^{2+} channel. We used biochemical techniques on isolated adult rat cardiomyocytes to assess the efficacy of these molecules on their Ca^{2+} channel-blocking activity and antioxidant properties. The Ca^{2+} channel-blocking activity was evaluated by confocal microscopy on fluo-3AM loaded cardiomyocytes, as well as using patch clamp experiments. Antioxidant properties were evaluated by flow cytometry using the ROS sensitive dye 1,2,3 DHR.
Results: Our docking studies show that a novel compound with 3-OH substitution inserts into the active binding site of the L-type Ca^{2+} channel previously described for nitrendipine. In biochemical assays, the novel *meta*-OH group in the aryl in C4 showed a high blocking effect on L-type Ca^{2+} channel as opposed to *para*-substituted compounds. In the tests we performed, none of the molecules showed antioxidant properties.
Conclusions: Only substitutions in C2, C3 and C5 of the aryl ring render dihydropyridine compounds with the capacity of blocking LTCC. Based on our docking studies, we postulate that the antioxidant activity requires a larger group than the *meta*-OH substitution in C2, C3 or C5 of the dihydropyridine ring.

Introduction

Voltage-dependent calcium (Ca^{2+}) channels (VDCC) are widely expressed throughout different tissues and importantly in the cardiovascular system, where they represent the main route for cellular Ca^{2+} entry that couples electrical excitation to contraction. These channels are multimeric proteins that consist of a principal α_1 subunit forming the permeation pore, which associates with the auxiliary subunits $\alpha_2\delta$, β and γ. Among the different types of VDCC, predominantly the L-type calcium channel (LTCC) is found in cardiomyocytes, where it is a key component of the contractile cycle (Ertel et al., 2000). Due to the relevance of the LTCC in cardiac diseases, this channel is the primary target of several pharmacological agents. There are different families of LTCC inhibitors; dihydropyridines such as nifedipine and nitrendipine are the most widely used, followed by phenylalkylamines such as verapamil, or benzothiazepines like diltiazem (Colecraft et al., 2002). The amino acid sequence of the α_1 subunit is organized in four repeated domains (I to IV). Each one of them contains six transmembrane segments (S1 to S6), a membrane-associated loop between S5 and S6, as well as both N- and C-termini facing the intracellular side. In each domain, S4 constitutes the voltage sensor. Domains III and IV contain the specific binding sites for all Ca^{2+} channel blockers

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found so far; these are located in III S5, III S6, and IV S6 for dihydropyridines, and III S6 and IV S6 for the other members of the Ca^{2+} channel blocker family (Ertel et al., 2000; Walsh et al., 2009). The 1,4-dihydropyridines (DHPs) are the most important of these therapeutic agents (Krzeminski et al., 2011; Triggle, 2007). By binding to and blocking the α_{1C} subunit, they display a negative inotropic effect, which is characterized by a decrease in the force of contraction of the myocardium (Schramm et al., 1983). They also slow down the conduction of electrical activity during the plateau phase of the action potential of the heart, resulting in a negative chronotropic effect that lowers the heart rate (Millard et al., 1983). This negative chronotropic effect of Ca^{2+} channel blockers (CCBs) makes them useful drugs for patients with atrial fibrillation or flutter. Additionally, the resulting lower heart rates represent lower cardiac oxygen requirements and promote better adaptability to adrenergic tone (Scholz, 1997).

The relevance of reactive oxygen species (ROS) in cardiovascular disease has been well established. They are an aggravating and accelerating factor in the progress of cardiac pathologies. Changes in the cellular redox environment can affect the gating properties of ion channels, and because cardiac contraction is highly dependent on the regulation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), redox modification of Ca^{2+} channels and transporters has a profound effect on cardiac function (Kourie, 1998). Key components of the cardiac excitation–contraction (EC) coupling machinery such as the sarco–endoplasmic reticulum Ca^{2+} ATPase (SERCA) and LTCC are subject to redox modulation, which is directly involved in cardiac pathologies. Significant bursts of reactive oxygen species (ROS) generation occur during reperfusion of the ischemic heart, and changes in the activity of the major components of $[Ca^{2+}]_i$ regulation, such as ryanodine receptors, Na^+–Ca^{2+} exchangers and Ca^{2+} ATPases, are likely to play an important role in ischemia-related Ca^{2+} overload (Kourie, 1997; Waring, 2005).

In this regard, dihydropyridines have been the subject of intensive research over the last four decades. Their discovered antioxidant activity gave birth to very versatile molecules, which are classified as third generation DHPs with strong LTCC blocking effects and also with attributed antioxidant properties. We have been investigating new DHPs that could combine both properties. In our studies we synthesized six differently substituted DHP molecules, we studied their LTCC blocking activity and their antioxidant properties and found that only a novel 3-OH substituted DHP displayed a strong LTCC blocking activity but did not exhibit antioxidant effects as well as the rest of these newly synthesized DHPs.

Materials and methods

Isolation and culture of adult rat cardiomyocytes. Adult rats were bred in the Animal Breeding Facility from the Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile (Santiago, Chile). We performed all studies with the approval of the institutional bioethical committee from Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago. This investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health. Adult rat ventricular myocytes (ARVMs) were prepared from the hearts of male Sprague Dawley rats (2–3 months, 250–300 g). Animals were anesthetized by an intraperitoneal (i.p.) injection of ketamine:xilazine 2:1. The heart was removed via thoracotomy and transferred to a Gerard ice-cold solution. The aorta was cannulated and the heart mounted into a Langendorff apparatus then successively perfused with the following oxygenated solutions: Gerard buffer (Ca^{2+} 2.6 mM), to allow recovery of spontaneous activity; nominally Ca^{2+}-free Gerard buffer supplemented with EGTA (2.5 mM) until contraction ceased and then digestion solution supplemented with collagenase type A and 2,3-butanedione monoxime (BDM), for 30 min. Once flaccid, the heart was rinsed for 2 min without collagenase. Ventricles were removed and finely minced and gently triturated, then placed in 15 mL of digestion solution, at 37 °C under constant and soft agitation for 10 min; after which the supernatant was transferred to a Falcon tube and centrifuged at 500 rpm for 2 min. The pellet was gently resuspended in a Gerard buffer supplemented with BDM. For microscopy and patch clamp experiments, ARVMs were then seeded in coverslips precoated with laminin (5 μg/mL). After 20–30 min the buffer was changed and replaced with M199/HEPES/Ca^{2+} 2 mM, supplemented with penicillin–streptomycin. Further BDM supplementation was used only for patch clamp experiments. ARVMs were used on the same day of isolation (Communal et al., 1998; Snabaitis et al., 2005). Neonatal rat ventricular cardiomyocytes (NRVM) were prepared as previously established (Foncea et al., 1997). Briefly, NRVM were isolated from the hearts from one- to three-day-old Sprague Dawley rats by enzymatic digestion. Cells were pre-plated to discard non-myocyte cells. NRVM were maintained in DMEM:M199 (4:1) medium with 10% (w/v) FBS and penicillin–streptomycin before experiments. NRVM were either used in suspension, shortly (2 h) after isolation for ROS measurements and analysis by flow cytometry, or were plated on gelatin-precoated dishes and treated according to different experimental conditions; cells were then trypsinized and used in suspension for flow cytometry analyses to measure cell viability.

Dynamic Ca^{2+} measurements. Dynamic Ca^{2+} measurements were performed on an inverted confocal microscope (Carl Zeiss Axiovert 200 Pascal5-LSM Microsystems). Experiments were performed on the same day as the cardiomyocytes were isolated. Cardiac myocytes were washed three times with Ca^{2+}-containing resting media (Krebs buffer (mM): 145 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Na, 5.6 glucose, pH 7.4), to remove M199 culture medium, and loaded with 5.4 μM Fluo 3-AM (coming from a stock in 20% pluronic acid, DMSO) for 30 min at room temperature. After loading, ventricular myocytes were washed with the same buffer and used immediately thereafter (Pravdic et al., 2009). The cell-containing coverslips were mounted in a 1 mL capacity plastic chamber and placed in the confocal microscope for fluorescence measurements after excitation with a 488-nm wavelength argon laser beam or filter system. Fluorescence measurements were performed on individual cardiomyocytes. Line-scan images were acquired at a sampling rate of 15.8 msec per line, along the longitudinal axis of the cell, avoiding crossing the nucleus. An objective lens Plan Apo 63X (numerical aperture 1.4) and a pinhole of 1 Airy unit disk were used. In all acquisitions, the image dimension was 512 × 120 pixels. Intracellular Ca^{2+} was expressed as a percentage of fluorescence intensity. All line scan images of Ca^{2+} transients were background subtracted (Pearl et al., 2011). Compounds were added after 30 s of initiating the recordings. The action peak was defined at 240 s. We used linescan analysis for the assessment of chronotropy, by quantifying the frequency of fluorescence spots obtained from the linescan pictures and inotropy from the fluorescence intensity.

ROS measurements. All tests were performed on a FACSCanto flow cytometer (BD Biosciences) with a 2-laser configuration, 488/633 nm. To study the antioxidant capacity of our different DHPs, we used the fluorescent dye dihydrorhodamine 1 2 3 (DHR 1,2,3) at 25 μM, a probe to study various species of ROS. Flow cytometry allows the analysis of cells in suspension, but the particle size ranges from 0.2 to 50 μm. Large cells such as ARVM (>100 μm) cannot be analyzed by flow cytometry, therefore we used NRVM, as they provide the closest validated model for cardiac cell-based studies. To measure ROS levels we used a previously described protocol (Mashimo and Ohno, 2006). Briefly, neonatal rat cardiomyocytes suspended at a density of 3×10^5/mL with either Krebs-Ringer (mM: 145 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Na, 5.6 glucose, pH 7.4) or Krebs-Ringer plus the compounds. After 30 min of incubation, the cells were centrifuged again and the supernatant medium replaced with Krebs-Ringer containing the ROS producing stimulus H_2O_2 (100 μM) for 1 h. After this, the probe DHR