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MDIMP, a novel cardiac Ca^{2+} channel blocker with atrial selectivity

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ABSTRACT

In cardiac muscle cells both T-and L-type Ca^{2+} channels (TTCCs and LTCCs, respectively) are expressed, and the latter are relevant to a process known as excitation-contraction coupling (ECC). Evidence obtained from docking studies suggests that isoindolines derived from α-amino acids bind to the LTCC $Ca_V1.2$. In the present study, we investigated whether *methyl (S)-2-(1,3-dihydroisoindol-2-yl)-4-methyl-pentanoate* (MDIMP), which is derived from L-leucine, modulates both Ca^{2+} channels and ECC. To this end, mechanical properties, as well as Ca^{2+} transients and currents, were all investigated in isolated cardiac myocytes. The effects of MDIMP on $Ca_V1.2$ (transiently expressed in 293T/17 cells) were also studied. In this system, evidence was found for an inhibitory action that develops and recovers in min, with an IC_{50} of 450 μM. With respect to myocytes: atrial-TTCCs, atrial-LTCCs, and ventricular-LTCCs were also inhibited, in that order of potency. Accordingly, Ca^{2+} transients, contractions, and window currents of LTCCs were all reduced more strongly in atrial cells. Interestingly, while the modulation of LTCCs was state-independent in these cells, it was state-dependent, and dual, on the ventricular ones. Furthermore, practically all of the ventricular LTCCs were closed at resting membrane potentials. This could explain their resistance to MDIMP, as they were affected in only open or inactivated states. All these features in turn explain the preferential down-regulation of the atrial ECC. Thus, our results support the view that isoindolines bind to Ca^{2+} channels, improve our knowledge of the corresponding structure-function relationship, and may be relevant for conditions where decreased atrial activity is desired.

1. Introduction

The functional diversity of voltage-gated Ca^{2+} channels (VGCCs) depends on not only the identity of the gene encoding the principal subunit (α) but also on many splice variants and interactions of α with accessory subunits. These channels have been classified as low- and high-threshold activated (LVA and HVA) and are also known as T-and L-type; from now on referred to as TTCCs and LTCCs, respectively. $Ca_V1.2$ represents the principal subunit of LTCCs in the heart; it is highly sensitive to dihydropyridines (DHPs) and exists in both atrial and ventricular myocytes (Catterall, 2011; Chu et al., 2004).

Concerning the cardiac TTCCs, they arise from both $Ca_V3.1$ and $Ca_V3.2$, which are preferentially expressed in the atrium. Their physiological role is to contribute to pace-making activity and may also be relevant to the diseased heart (Cribbs, 2010; Vassort et al., 2006).

LTCCs are crucial in a process known as excitation-contraction coupling (ECC). During this process, action potentials rise the intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$) via a feedback cycle known as Ca^{2+}-induced Ca^{2+} release (CICR). Briefly, the influx of Ca^{2+} through LTCCs activates ryanodine receptors (RyR2, located in the sarcoplasmic reticulum or SR), and the ensuing release of Ca^{2+} stimulates the contractile machinery. The rise in $[Ca^{2+}]_i$ is transitory because this ion activates Ca^{2+} extrusion systems, such as sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA), and Na^+/Ca^{2+} exchanger (NCX) (Bers, 2001; Voigt et al., 2012).

The isoindolines are considered the reduced form of the isoindole and their skeleton is found in several natural and synthetic compounds. The isoindole consists of two fused rings, one aromatic, and an aliphatic five-membered ring with one nitrogen atom. While various isoindolines have been investigated and are known to exert a broad range of biological activities, many remain unexplored (Speck and Magauer, 2013). A previous *in silico* work

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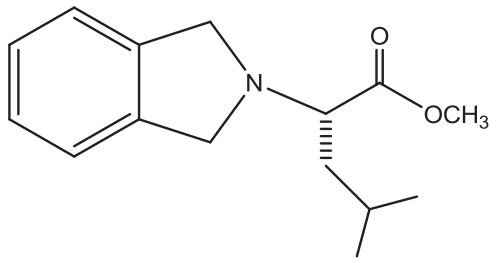

Fig. 1. Chemical structure and stereochemistry of the isoindoline MDIMP. The structure displays two fused rings, one aromatic, and an aliphatic five-membered ring with one nitrogen atom, where the nitrogen atom belongs to ester derived from the L-leucine amino acid. Chemical name: *methyl (S)-2-(1,3-dihydroisoindol-2-yl)-4-methylpentanoate*.

suggests that isoindolines-2-substituted derived from α-amino acids may interact with $Ca_V1.2$ (Mancilla-Percino et al., 2010). In particular, the isoindoline MDIMP (Fig. 1) is proposed to bind to the Glu736 (which belongs to the selectivity filter), as well as to the respective pore-lining segments (see the third figure of Mancilla-Percino et al., 2010).

Here, we investigated whether MDIMP, in fact, modulates VGCCs. The results show a clear inhibition on $Ca_V1.2$ expressed heterologously, an effect that develops and reverts in min with an IC_{50} of 452 μM. Moreover, the compound also inhibits VGCCs of both atrial and ventricular myocytes, being more potent in the former than in the latter, with IC_{50} values of (μM): 115 (atrial TTCC), 233 (atrial LTCC), and 1070 (ventricular LTCC). Apparently, the MDIMP resistance of ventricular LTCCs arises from a state-dependent mechanism that precludes binding of the compound to the close state of the channels. Finally, the impact in both Ca^{2+} transients and contractions was investigated, leading to the conclusions that MDIMP also inhibits ECC and the inhibition of contractions is much more potent in atrial myocytes –similar to the effects on VGCCs; which is in keeping with the notion that MDIMP is a new calcium channel blocker with atrial selectivity.

2. Materials and methods

2.1. Heterologous expression of $Ca_V1.2$

The 293T/17 cell line was acquired from the American Type Cell Culture Collection and kept in culture according to instructions of the provider. In brief, cells were passaged in T-25 flasks twice a week and small aliquots were plated into glass coverslides, pre-treated with poli-L-lysine. These cells were transfected with cDNAs encoding both the principal and accessory subunits of LTCCs, using FuGENE HD (Invitrogen). As a reporter gene, the cDNA encoding EGFP was added, and equimolar amounts of cDNAs were used. The following cDNA clones for channel subunits were used (with their respective GenBank accession numbers): β2a, M80545; α2δ-1, M21948; and $Ca_V1.2$, AY728090. Two to three days after transfection, the cells were observed with epifluorescence and calcium currents were investigated in fluorescent cells, as described in a later section (Ca^{2+} currents).

2.2. Cardiac myocytes

Atrial and ventricular myocytes were obtained from male Wistar rats (~230 g), as described previously (Ramos-Mondragón et al., 2012). In short, after the animals were anaesthetized with a mixture of ketamine-xylazine (100:10 mg Kg^{-1} of body weight), the heart was excised and perfused retrogradely using a digestion buffer (DB) added with collagenase type 2 (1.5 mg/ml) and protease XIV (40 μg/ml). The tissue of interest was dissected, mechanically dispersed, and the resulting myocytes were stepwise resuspended in DB containing rising levels of $CaCl_2$. Finally, the myocytes were plated into laminin-coated glass coverslips, and kept at 37° C in a CO_2 incubator, in the presence of culture medium: Dulbecco's modified Eagle's medium, supplemented with heat-inactivated foetal bovine serum (10%), penicillin (100 μml^{-1}), streptomycin (100 $\mu g ml^{-1}$), and L-glutamine (4 mM). Experiments were performed within the next two days. This protocol was carried out in accord with both the EU Directive 2010/63/EU for animal experiments and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011), NRC 2011. It complies with the Mexican Official Norm NOM-062-ZOO-1999 and was approved by the Institutional Animal Care and Use Committee (Cinvestav, 0259–05).

2.3. Ca^{2+} currents

The activity of Ca^{2+} channels was investigated with the whole-cell patch-clamp technique, using an Axopatch 200B amplifier, as described elsewhere (Ramos-Mondragón et al., 2012). For 293T/17 cells, I_{Ca} was assessed in absence, presence, and after washout of MDIMP, using external recording solutions applied with a fast perfusion system (Warner, SF-77B). Test pulses were delivered from a holding potential (HP) of −50 mV, every 15 s. In the case of cardiac myocytes, they were immersed during 10–50 min in a fixed external recording solution, which contained or not MDIMP. We used this bath application because a severe run down of Ca^{2+}channel activity precluded confident comparisons of I_{Ca} between control and MDIPM conditions in the same cell. Thus, in myocytes, I_{Ca} was recorded within the first two min of breaking into the whole-cell configuration, prior to the beginning of run down. For these reasons, the corresponding data were compared between cell groups, following normalization of the absolute value of I_{Ca} by the respective cell membrane capacitance (C_m). The latter was estimated by the integral of capacitative currents elicited by voltage steps of −10 mV, applied from a HP of −80 mV. For the isolation of T-type ($I_{Ca,T}$) and L-type ($I_{Ca,L}$) currents, the HP set to −90 mV and −50 mV, respectively. Once C_m was estimated, it was cancelled by the capacitance cancelation feature of the amplifier. The remaining linear components were digitally eliminated with P/N leak subtraction.

The current signal was analogically filtered with a 4-pole lowpass Bessel filter (set at 2 KHz), and stored on the hard disk of a PC for off-line analysis. Two sampling rates were used; 5 KHz and 50 KHz, for 200-Ms and 30-ms step depolarisations, respectively. An Axon interface (either Digidata 1200A or 1322A) communicated the PC with the amplifier, and all hardware was under the control of the software suite p-Clamp (v9. 2 and v10. 2).

The current-to-voltage relationships or I-V curves were fitted according to the following function:

$$I_{Ca} = G_{max}(V_m - V_r)/(1 + exp[(V_m - V_{G,l/2})/k]) \quad (1)$$

where G_{max} is the maximal conductance, V_r represents the apparent reversal potential, $V_{G1/2}$ is the potential to activate 50% of G_{max}, and k is the slope factor.

With the idea of investigating the voltage-dependence of inactivation of LTCCs, 400-ms conditioning pre-pulses to different potentials preceded a test pulse to +20 mV. The resulting inactivation curves were mathematically described by the following Boltzmann equation:

$$I_{Ca} = I_{max}/(1 + exp[-(V_m - V_{i,l/2})/k]) \quad (2)$$

where I_{max} represents the maximum current recruited at very negative potentials, $V_{i,l/2}$ is the potential required to inactivate 50%