British Journal of Pharmacology

British Journal of Pharmacology (2018) **175** 2635–2652 2635

RESEARCH PAPER

Late sodium current inhibitors to treat exercise-induced obstruction in hypertrophic cardiomyopathy: an *in vitro* study in human myocardium

Correspondence Dr Raffaele Coppini, MD, PhD, Department NeuroFarBa, Division of Pharmacology, University of Florence, Viale G. Pieraccini 6, 50139 Florence, Italy. E-mail: raffaele.coppini@unifi.it

Received 4 July 2017; **Revised** 7 March 2018; **Accepted** 8 March 2018

Cecilia Ferrantini[1,2], Josè Manuel Pioner[1], Luca Mazzoni[3], Francesca Gentile[1], Benedetta Tosi[1], Alessandra Rossi[2], Luiz Belardinelli[4], Chiara Tesi[1], Chiara Palandri[3], Rosanna Matucci[3], Elisabetta Cerbai[3], Iacopo Olivotto[2], Corrado Poggesi[1], Alessandro Mugelli[3] and Raffaele Coppini[3]

[1]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy, [2]Cardiomyopathy Unit, Careggi University Hospital, Florence, Italy, [3]Department NeuroFarBa, University of Florence, Florence, Italy, and [4]Gilead Sciences, Inc., Foster City, CA, USA

BACKGROUND AND PURPOSE

In 30–40% of hypertrophic cardiomyopathy (HCM) patients, symptomatic left ventricular (LV) outflow gradients develop only during exercise due to catecholamine-induced LV hypercontractility (inducible obstruction). Negative inotropic pharmacological options are limited to β-blockers or disopyramide, with low efficacy and tolerability. We assessed the potential of late sodium current (I_{NaL})-inhibitors to treat inducible obstruction in HCM.

EXPERIMENTAL APPROACH

The electrophysiological and mechanical responses to β-adrenoceptor stimulation were studied in human myocardium from HCM and control patients. Effects of I_{NaL}-inhibitors (ranolazine and GS-967) in HCM samples were investigated under conditions simulating rest and exercise.

KEY RESULTS

In cardiomyocytes and trabeculae from 18 surgical septal samples of patients with obstruction, the selective I_{NaL}-inhibitor GS-967 (0.5 μM) hastened twitch kinetics, decreased diastolic [Ca^{2+}] and shortened action potentials, matching the effects of ranolazine (10μM). Mechanical responses to isoprenaline (inotropic and lusitropic) were comparable in HCM and control myocardium. However, isoprenaline prolonged action potentials in HCM myocardium, while it shortened them in controls. Unlike disopyramide, neither GS-967 nor ranolazine reduced force at rest. However, in the presence of isoprenaline, they reduced Ca^{2+}-transient amplitude and twitch tension, while the acceleration of relaxation was maintained. I_{NaL}-inhibitors were more effective than disopyramide in reducing contractility during exercise. Finally, I_{NaL}-inhibitors abolished arrhythmias induced by isoprenaline.

CONCLUSIONS AND IMPLICATIONS

Ranolazine and GS-967 reduced septal myocardium tension during simulated exercise *in vitro* and therefore have the potential to ameliorate symptoms caused by inducible obstruction in HCM patients, with some advantages over disopyramide and β-blockers.

Abbreviations

LV, left ventricle; LVOT, left ventricular outflow tract; HCM, hypertrophic cardiomyopathy; I_{NaL}, late sodium current; I_{CaL}, L-type calcium current; EAD, early after-depolarization; DAD, delayed after-depolarization; APD, action potential duration

DOI:10.1111/bph.14223

Introduction

Symptoms related to obstruction occurring at the left ventricular outflow tract (LVOT) are present in approximately 65% of hypertrophic cardiomyopathy (HCM) patients (Gersh *et al.,* 2011; Authors/Task Force *et al.*, 2014). Marked upper septal hypertrophy and LV hypercontractility lead to accelerated blood flow in the LVOT, exerting a drag force on the elongated anterior mitral valve leaflet that moves towards the septum, ultimately determining a significant pressure gradient (>30 mmHg) in the LVOT (Maron *et al.,* 2006). While a significant gradient is present at rest in about half of patients with obstructive HCM, obstruction develops only during stress or exercise in the other 50% ('dynamic' or 'inducible' obstruction) (Maron *et al.,* 2006; Pozios *et al.,* 2015).

To date, pharmacological options to treat inducible obstruction are limited to **β-adrenoceptor** antagonists (β-blockers) or **disopyramide**. High doses of non-selective β-blockers, such as 80–100 mg·day^{-1} of **nadolol**, are often required to reduce dynamic gradients (Nistri *et al.,* 2012) but a large number of patients with exertional symptoms do not tolerate such aggressive β-blocker treatments and are left with residual symptomatic gradients. Disopyramide is an non-selective compound that primarily behaves as a **Na^+ channel** and **hERG** K^+ channel blocker and is extensively used in HCM patients with rest obstruction to relieve symptoms associated with LVOT gradients or intraventricular gradients. The clinical efficacy of disopyramide on rest obstruction has been attributed to its negative inotropic effect. Through the reduction of LV contractility, disopyramide slows down the acceleration of flow in the LVOT during systole, thus delaying or abolishing mitral-septal contact (Sherrid *et al.,* 2005). However, the use of disopyramide for inducible obstruction is limited by its unpredictable efficacy on dynamic gradients, particularly those elicited by exercise (Maron *et al.,* 2006), and by the important side effects of the drug (e.g. the anticholinergic effects). Thus, there is a strongly felt need to identify more effective and better tolerated agents for patients with obstructive HCM. However, the identification of new pharmacological options to reduce dynamic gradients is limited by the lack of studies on the cellular pathophysiology of exercise-induced obstruction. In particular, the response to β-adrenoceptor stimulation has never been studied in human myocardium from HCM patients.

We previously analysed the electromechanical profile of cardiomyocytes isolated from myectomy samples of patients with obstructive HCM (Coppini *et al.,* 2013). When compared with control cells, HCM cardiomyocytes showed prolonged action potentials (APs), slower Ca^{2+} transients and elevated diastolic Ca^{2+}, largely determined by a marked overexpression of the late sodium current ($\mathbf{I_{NaL}}$). Such electro-mechanical abnormalities were reversed *in vitro* by the I_{NaL}-inhibitor **ranolazine**, with beneficial effects on diastolic function and cellular arrhythmias (Coppini *et al.,* 2013). Ranolazine is available in the clinic to treat stable angina and is being employed in HCM patients with angina symptoms (Ammirati *et al.,* 2016). However, ranolazine is a non-selective I_{NaL}-inhibitor, with several potentially relevant pleiotropic effects, such as the inhibition of K^+ and Ca^{2+} currents (Antzelevitch *et al.,* 2004), the reduction of myofilament Ca^{2+} sensitivity (Lovelock *et al.,* 2012) and the stabilization of **ryanodine receptors** (Parikh *et al.,* 2012). Novel, highly selective I_{NaL}-inhibitors (GS-967 and **GS-6615**) (Belardinelli *et al.,* 2013; Rajamani *et al.,* 2016) have been recently developed. GS-967 (6-[4-(trifluoromethoxy) phenyl]-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine) is a potent inhibitor of I_{NaL} (Belardinelli *et al.,* 2013), with an IC_{50} of 0.2 to 0.5 μM (IC_{50} of ranolazine is 2–4 μM). Moreover, GS-967 has no significant effects on either peak I_{Na} or hERG K^+ current in the effective range of concentrations (Belardinelli *et al.,* 2013). GS-967 has been shown to suppress experimentally induced ventricular arrhythmias in rat and rabbit cardiomyocytes, *via* shortening of AP duration and reduction of intracellular Na^+ and Ca^{2+} overload (Belardinelli *et al.,* 2013; Sicouri *et al.,* 2013; Pezhouman *et al.,* 2014). This, in turn, abolished the occurrence of arrhythmogenic early and delayed after-depolarizations (EADs and DADs) (Belardinelli *et al.,* 2013; Sicouri *et al.,* 2013; Pezhouman *et al.,* 2014). Moreover, GS-967, used in pigs, suppressed ischaemia-induced and catecholamine-triggered ventricular arrhythmias (Bonatti *et al.,* 2014; Alves Bento *et al.,* 2015), as well as atrial fibrillation triggered by autonomous stimulation (Carneiro *et al.,* 2015). Notably, GS-967 did not exert any effects on the mechanical and electrical function of the heart in healthy rats (Fernandes *et al.,* 2014). This result is in line with the negligible effects of ranolazine in human myocardium from non-hypertrophic, non-failing patients (Coppini *et al.,* 2013), due to the absence of I_{NaL} overexpression, which appears to be a specific, disease-related target.

In the present study, we first used GS-967 as a pharmacological tool to assess whether the previously observed effects of ranolazine in human HCM myocardium can be entirely attributed to I_{NaL} inhibition rather than its pleiotropic effects. We then compare the mechanical and electrophysiological responses of HCM myocardium to the β-adrenoceptor agonist **isoprenaline** with those observed in control cardiac samples, in order to characterize the features and possible abnormalities of β-adrenoceptor signalling in the HCM heart. Finally, the main objective of this study was to investigate the *in vitro* effects of ranolazine and GS-967 under β-adrenoceptor stimulation, the latter used to simulate stress and exercise in the myocardium of patients with obstructive HCM. With this approach, we aimed to assess whether the *in vitro* pharmacological profile of I_{NaL}-inhibitors supports their use to treat inducible obstruction in HCM patients as an alternative to disopyramide and β-blockers or in combination with these commonly used compounds.

Methods

Details are available online ('Expanded Methods' section of the Online Data Supplement).

Patients

The study follows the principles of WMA Declaration of Helsinky for medical research involving human subjects. The experimental protocols were approved by the ethical committee of Careggi University-Hospital of Florence (2006/0024713, renewed May 2009; 2013/0035305). Each enrolled patient gave written informed consent. We enrolled 22 HCM patients who were followed by the Cardiomyopathy

Unit in Florence, consecutively referred to surgical septal myectomy, for relief of drug-refractory symptoms related to LVOT obstruction. Among the 22 patients, 15 agreed to undergo mutational screening in sarcomeric genes. Clinical data are found in Table 1.

The control cohort comprised five patients aged <65 years undergoing heart surgery for aortic stenosis or regurgitation and who required a septal myectomy operation due to the presence of a bulging septum causing symptomatic obstruction. All control patients had septal thickness <14 mm and preserved left ventricular systolic function (ejection fraction >55%). Clinical data are found in Supporting Information Table S1.

Tissue processing and cell isolation

Surgical septal specimens from HCM and control patients were washed with standard cardioplegic solution and processed within 30 min from excision. Endocardial trabeculae suitable for mechanical measurements were dissected, and the remaining tissue was minced and subjected to enzymic dissociation to obtain viable single myocytes, as previously described (Coppini *et al.*, 2014a).

Table 1

HCM patient characteristics

HCM Patients (*n* = 22)	
Age at surgery	51 ± 8 years
Gender	Female 11/22 (50%)
NYHA Class II	13/22 (59%)
NYHA Class III	9/22 (41%)
Genotype	15/21 (7 MYBPC, 4 MYH, 4 SARCOMERE-NEG.)
Arrhythmic risk	
Syncope	7/22 (32%)
Non-sustained ventricular tachycardia	11/22 (50%)
History of atrial fibrillation	10/22 (45%)
ICD implanted	3/22 (13%)
Echo features	
Maximal septal thickness	27 ± 6 mm
Ejection fraction	66 ± 6%
LVOT gradient at rest >30 mmHg	22/22 (100%)
LVOT gradient at rest	81 ± 9 mmHg
LA end-systolic volume	101 ± 37 mL
Severe diastolic dysfunction (pseudonormalized, restrictive)	10/22 (45%)
Pharmacological therapy	
β-blockers	22/22 (100%)
Disopyramide	11/22 (50%)
Amiodarone	4/22 (18%)
Diuretics/ACE-inhibitors	11/22 (50%)

Clinical features of the HCM patients enrolled in the study. Clinical data refers to visits performed less than 1 month before the myectomy operation. Categorical data are expressed as proportion of patients; continuous values are expressed as mean ± SD. NYHA, New York Heart Association; MYPBC, myosin-binding protein C; MYH, myosin heavy chain; ICD, implantable cardioverter defibrillator; LA, left atrium.

Single cell studies

Perforated patch whole-cell current-clamp was used to measure membrane potential, as previously described (Coppini *et al.*, 2013). $[Ca^{2+}]$ variations were simultaneously monitored using the Ca^{2+}-sensitive fluorescent dye Fluoforte (Enzo Life Sciences, Farmingdale, NY, USA), by measuring fluorescence at 515 ± 10 nm during excitation at 490 ± 8 nm. Cells were mechanically permeabilized at the end of the experiment, and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from emitted fluorescence as previously described (Voigt *et al.*, 2012), using 389 nmol·L^{-1} as Fluoforte dissociation constant. Late I_{NaL} and L-type Ca^{2+} current ($\mathbf{I_{CaL}}$) were measured using whole-cell voltage clamp as previously described (Coppini *et al.*, 2013). I_{CaL} was also recorded under AP-clamp conditions, using representative AP traces, recorded from HCM cardiomyocytes under the same conditions, as command voltage. The inward current recorded under AP-clamp conditions was dihydropyridine-sensitive (see Supporting Information Figure S6).

Intact trabeculae studies

Ventricular trabeculae were mounted between a force transducer and a motor for muscle length control, as previously described (Coppini *et al.*, 2013); isometric force was recorded under different experimental conditions and stimulation protocols. Isometric force was recorded at 35 ± 2°C under various conditions including various pacing rates (0.1–2.5 Hz) and acute drug administration (see below). The occurrence of premature beats was evaluated during steady-state stimulation (0.5 Hz) and during stimulation pauses.

Drug studies

For experiments on isolated cardiomyocytes and trabeculae, Ranolazine was used at the concentration of 10 μM, disopyramide at 5 μM and GS-967 at 0.5 μM, unless otherwise specified. Each cell or trabecula was randomly assigned to be treated with one of the three test drugs (disopyramide, ranolazine or GS-967). Test recordings in the presence of the drug were performed after >3 min from the beginning of drug exposure. Afterwards, the drug was washed out for >5 min, and measurements were repeated. Isoprenaline (10^{-7} M) was used to mimic β-adrenoceptor stimulation during exercise in single cells and trabeculae.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Raw traces were analysed by a blinded operator, different from the one who performed the experiments. For each trace, we performed measurements in at least five subsequent events and averaged them to obtain a single value for a given cell/trabecula in a certain condition. Clinical data from patients are expressed as mean ± SD. Data from cells and muscles are expressed as mean ± SEM. Figures 1G and 2D, F show the effects of drugs in a normalized fashion: we calculated the drug-induced variation of a given

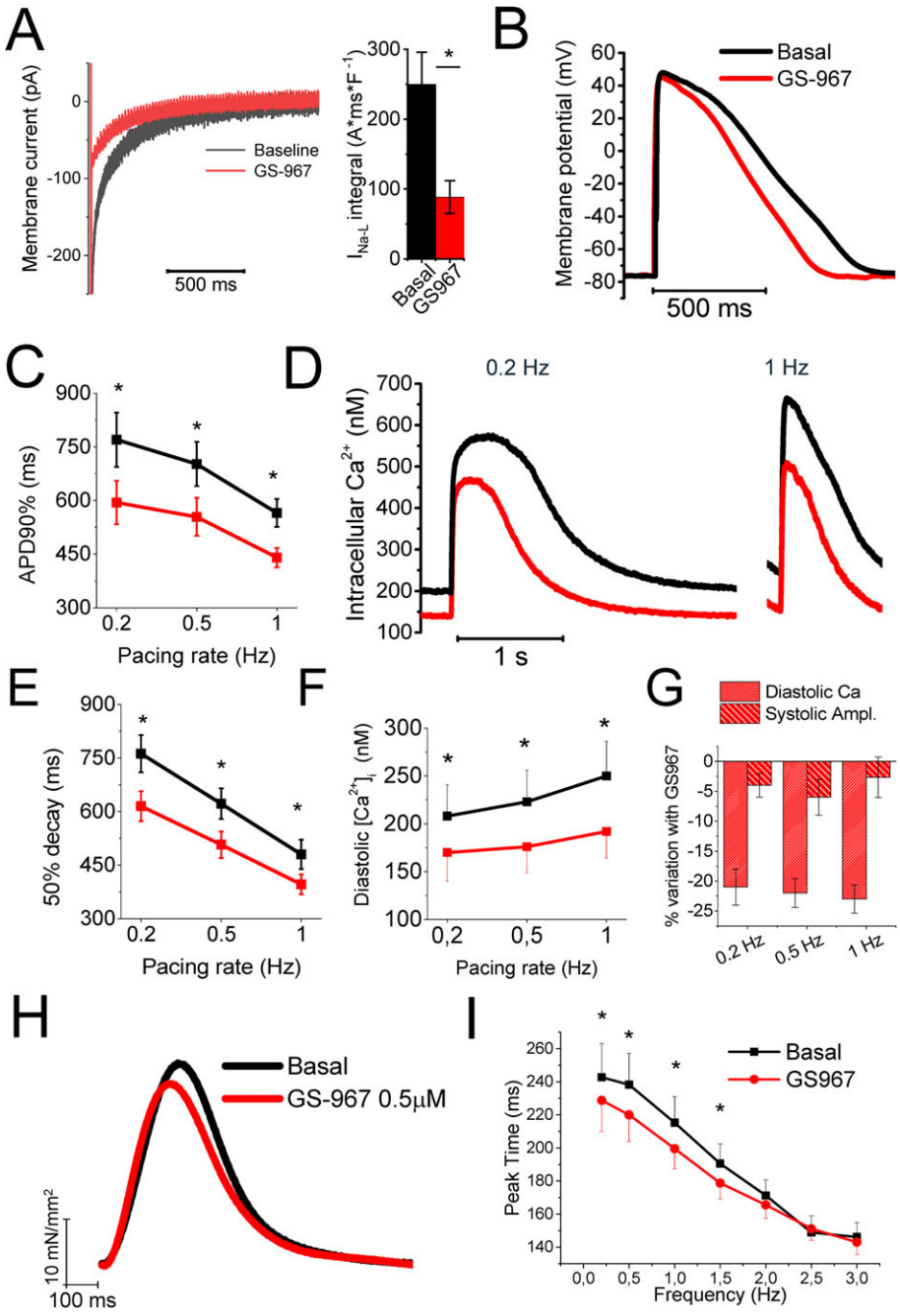

Figure 1

GS-967 shortens action potentials, hastens Ca transients and accelerates contraction in HCM myocardium. (A) Left: representative I_{NaL} traces from an HCM cardiomyocyte during depolarization to −20 mV in the absence (Basal) or presence of GS-967 (0.5 μmol·L^{-1}). Right: Average integrals of I_{NaL} calculated between 50 and 750 ms from the onset of depolarization. Means ± SE from 14 HCM myocytes (five patients). (B) Representative superimposed APs at baseline and in the presence of GS-967 (0.5 μM), elicited at 0.2 Hz. (C) APD at 90% of repolarization (APD90%) at baseline and in the presence of GS-967 (0.5 μM). Means ± SE from 37 HCM cardiomyocytes from nine HCM patients. (D) Representative superimposed Ca transients at baseline and in the presence of GS-967 (0.5 μM), elicited at 0.2 Hz (left) and 1 Hz (right). (E) Time from peak to 50% decay of Ca transients (50% decay) at baseline and in the presence of GS-967 (0.5 μM), elicited at 0.2, 0.5 and 1 Hz. (F) Diastolic intracellular Ca-concentration ($[Ca^{2+}]_i$) at baseline and in the presence of GS-967 (0.5 μM), during regular stimulation at 0.2, 0.5 and 1 Hz at steady state. (G) % variation of diastolic $[Ca^{2+}]_i$ and Ca-transient amplitude (Systolic Ampl.) with the application of GS-967 with respect to baseline in HCM cardiomyocytes. (E–G) Means ± SE from 26 cardiomyocytes from seven HCM patients. (H) Representative superimposed force twitches elicited at 0.5 Hz in HCM trabeculae in the absence and presence of GS-967. (I) Time from stimulus to peak of force twitches elicited at different frequencies (0.1 to 3 Hz) at baseline, in the presence of GS-967. Means ± SE from 10 trabeculae from seven patients. *$P < 0.05$, significant differences; linear mixed models corrected for paired comparisons.

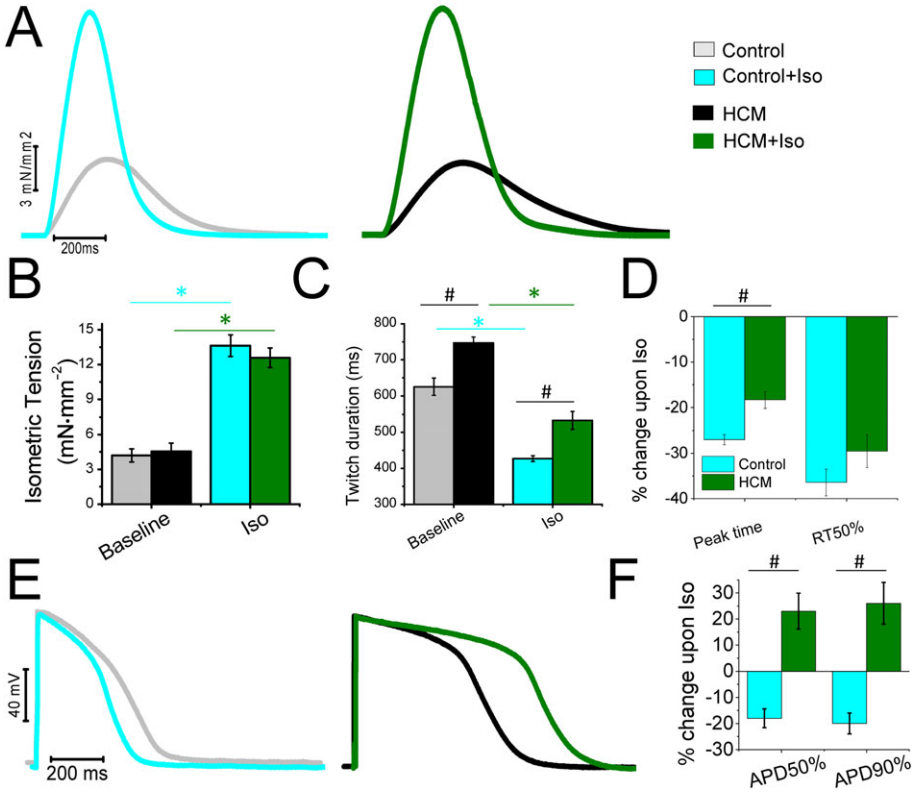

Figure 2

Mechanical and electrical response to β-adrenoceptor stimulation of HCM myocardium. (A) Representative superimposed force twitches elicited at 0.5 Hz in control (left) and HCM (right) trabeculae in the absence and presence of isoprenaline (Iso; 0.1μM). (B) Isometric tension during steady-state stimulation at 0.5 in the absence and presence of isoprenaline in control and HCM trabeculae. (C) Duration of force twitches (from stimulus to 90% of relaxation) elicited at 0.5 Hz in the absence and presence of isoprenaline (Iso; 0.1μM). (D) Percentages of change in the parameters of twitch kinetics (0.5 Hz) upon exposure to isoprenaline in control and HCM trabeculae: time from stimulus to peak contraction (peak time) and time from peak to 50% of relaxation (RT50%). (B–D) Means ± SE from six trabeculae from five control patients and 30 trabeculae from 20 HCM patients. (E) Representative superimposed APs elicited at 0.5 Hz in control (left) and HCM (right) cardiomyocytes, in the absence and presence of isoprenaline. (F) Percentages of change in the parameters of AP kinetics upon exposure to isoprenaline in control and HCM cardiomyocytes: time from stimulus to 50% repolarization (APD50%) and time from peak to 90% of repolarization (APD90%). (F) Means ± SE from 13 cardiomyocytes from five control patients and 31 cardiomyocytes from nine HCM patients. #$P < 0.05$, significantly different as indicated; linear mixed models, unpaired comparisons; *$P < 0.05$, significantly different as indicated; linear mixed models corrected for paired comparisons.

parameter from the baseline [(drug-baseline)/baseline, expressed as percentage] in each cell/trabecula and used these values (percentages of variation) to calculate averages and perform statistical analyses. The number of cells/trabeculae and the number of different patient samples from which cells/trabeculae were isolated are indicated for each group/condition in the respective figure legends. For each group, we included data points from at least five patient samples, obtained from an even number of cells/trabeculae for each sample. Statistical analysis was performed as previously described using linear mixed models (Coppini *et al.*, 2013), taking into account non-Gaussian distribution, inequality of variances and within-subject correlation. In brief, in order to reduce the risk of type I errors resulting from the closer interrelationship between cells/trabeculae isolated from the same patient sample, we used hierarchical statistics including two nested levels (patients and cells) (Sikkel *et al.*, 2017); a third hierarchical level was added when drugs were tested in a cell/trabecula, in order to allow paired comparisons. This approach was implemented using linear mixed models in Stata 12.0 (StataCorp LLC, USA). For categorical data (e.g. occurrence of cellular arrhythmias), we used the Fisher exact test. $P < 0.05$ was considered statistically significant. Due to the lesser availability of control samples, the number of control cells/trabeculae included in the unpaired comparisons (Figure 2) is lower than that of HCM cells/trabeculae.

Materials

The compounds used in these studies were supplied as follows: disopyramide by Sigma-Aldrich (St. Louis, Missouri, USA), ranolazine by Menarini International (Luxembourg, Luxembourg) and GS-967 from Gilead Sciences (Foster City, California, USA).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*,

2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d).

Results

GS-967 shortens action potentials and accelerates the kinetics of Ca^{2+} transients and twitches in HCM cardiomyocytes

GS-967 was tested at 0.5 μM in isolated cardiomyocytes from HCM patients during patch-clamp and Ca^{2+}-fluorescence measurements (Figure 1A). GS-967 consistently reduced I_{NaL} (Figure 1A). Moreover, GS-967 shortened the duration of APs at all frequencies studied by about 20% (Figure 1B, C). Moreover, GS-967 reduced the rate of arrhythmogenic EADs occurring spontaneously during regular stimulation for 3 min. The number of cells showing EADs during 3 min of stimulation was halved by GS-967 (Supporting Information Figure S2). Notably, GS-967 had no effects on the upstroke time nor on the amplitude of the APs (Supporting Information Table S3). The kinetics of intracellular Ca^{2+} transients was hastened by GS-967 at all frequencies investigated (Figure 1D, E): both the rise and decay times of Ca^{2+} transients were shortened by GS-967 application (Supporting Information Table S3). GS-967 reduced diastolic $[Ca^{2+}]$, while the amplitude of Ca^{2+} transients was unaffected (Figure 1F, G). Accordingly, GS-967 reduced the occurrence of delayed after-depolarizations (Supporting Information Figure S2). GS-967 was then tested in intact trabeculae from HCM patients, while recording force under isometric conditions (Figure 1H) during stimulation at different frequencies (0.1 to 3 Hz). GS-967 shortened twitch duration mainly by reducing time to peak contraction (Figure 1I). GS-967 (0.5 μM) had no effects on APs and Ca^{2+} transients recorded from cardiomyocytes isolated from control patients (Supporting Information Figure S3).

Stimulation of β-adrenoceptors exerts comparable mechanical effects in HCM and control myocardium but causes a paradoxical prolongation of action potentials in HCM cardiomyocytes

The effects of β-adrenoceptor stimulation with isoprenaline (0.1μM) on isometric twitches were tested in trabeculae from control and HCM septal samples (Figure 2A–D). This β-adrenoceptor agonist exerted qualitatively and quantitatively similar effects on twitch amplitude and kinetics in control and HCM trabeculae. In particular, isoprenaline increased peak force by about three-fold in both control and HCM trabeculae (Figure 2B). Twitch kinetics was slower in HCM versus control trabeculae both at baseline and during β-adrenoceptor stimulation (Figure 2C). Indeed, isoprenaline accelerated the kinetics of relaxation by a similar amount in HCM and control trabeculae, although the acceleration of twitch force development was less pronounced in HCM trabeculae (Figure 2D). All in all, the kinetics of contraction and relaxation, despite being accelerated by isoprenaline, was still slower in HCM than in control trabeculae, even during maximal β-adrenoceptor stimulation.

In contrast, the effects of isoprenaline on APs were profoundly different in HCM with respect to control cardiomyocytes (Figure 2E). While isoprenaline shortened the duration of APs in control cardiomyocytes, this β-adrenoceptor agonist prolonged repolarization in all cardiomyocytes from HCM patients (Figure 2F).

Ranolazine and GS-967 decrease isometric force following β-adrenoceptor stimulation

We tested the effects of disopyramide (5 μM), ranolazine(10 μM), GS-967 (0.5 μM) and **propranolol** (0.1 μM) on isometric twitch amplitude and kinetics in trabeculae from HCM patient samples, in the absence and presence of β-adrenoceptor stimulation with isoprenaline(0.1 μM), used to simulate exercise or stress conditions. Under baseline conditions, disopyramide reduced twitch amplitude by approximately 50%, while force was unaffected by ranolazine, GS-967 and propranolol (Figure 3A, C). Activation of β-adrenoceptors with isoprenaline led to the expected increase of twitch amplitude and hastening of twitch kinetics (Figure 3A, B, D). Interestingly, disopyramide, ranolazine, GS-967 and propranolol reduced twitch amplitude when added to isoprenaline (Figure 3A–C). The reduction of twitch amplitude under isoprenaline was quantitatively less pronounced with disopyramide (16 ± 7%) compared with ranolazine (56 ± 23%, $P < 0.05$) or GS-967 (32 ± 21%, $P < 0.05$; Figure 3A, C). The reduction of twitch force in the presence of ranolazine or GS-967 was comparable with that obtained by blocking β-adrenoceptors with propranolol (Figure 3C). Nonetheless, the acceleration of twitch kinetics by isoprenaline was not antagonized by the application of disopyramide, ranolazine or GS-967 (Figure 3A, B, D). Indeed, after the addition of these drugs on top of isoprenaline, both time to peak and 50% relaxation time remained as fast as in the presence of isoprenaline alone (Figure 3D). However, propranolol completely antagonized the lusitropic effect of isoprenaline (Figure 3D).

Cellular mechanisms underlying the effects of I_{NaL}-inhibitors in the presence of isoprenaline

Ranolazine and GS-967 were similarly tested in isolated HCM cardiomyocytes (Figure 4, Table 2). In line with the effects observed on twitch amplitude in trabeculae, ranolazine and GS-967 did not affect Ca^{2+}-transient amplitude at baseline (Figure 4A). Ca^{2+}-transient amplitude increased in response to isoprenaline by 14 ± 3%. The application of either ranolazine or GS-967 in the presence of isoprenaline significantly reduced Ca^{2+}-transient amplitude (Figure 4B). Interestingly, while isoprenaline did not affect intracellular diastolic $[Ca^{2+}]$, ranolazine and GS-967, applied in the presence of isoprenaline, significantly reduced diastolic $[Ca^{2+}]$ (Figure 4C). Interestingly, isoprenaline accelerated Ca^{2+}-transient decay but prolonged Ca^{2+}-transient rise time in HCM cardiomyocytes (Figure 4A, Table 2). Accordingly, isoprenaline slowed time-to-peak shortening in HCM cardiomyocytes but accelerated relaxation (Supporting Information Figure S1). Ranolazine and GS-967 shortened Ca^{2+}-transient rise when applied with β-adrenoceptor stimulation with isoprenaline but did not affect Ca^{2+}-transient decay kinetics (Figure 4A, Table 2). In line with that, ranolazine and GS-967 reversed the prolongation of APs induced by isoprenaline (Figure 4A, D). However,

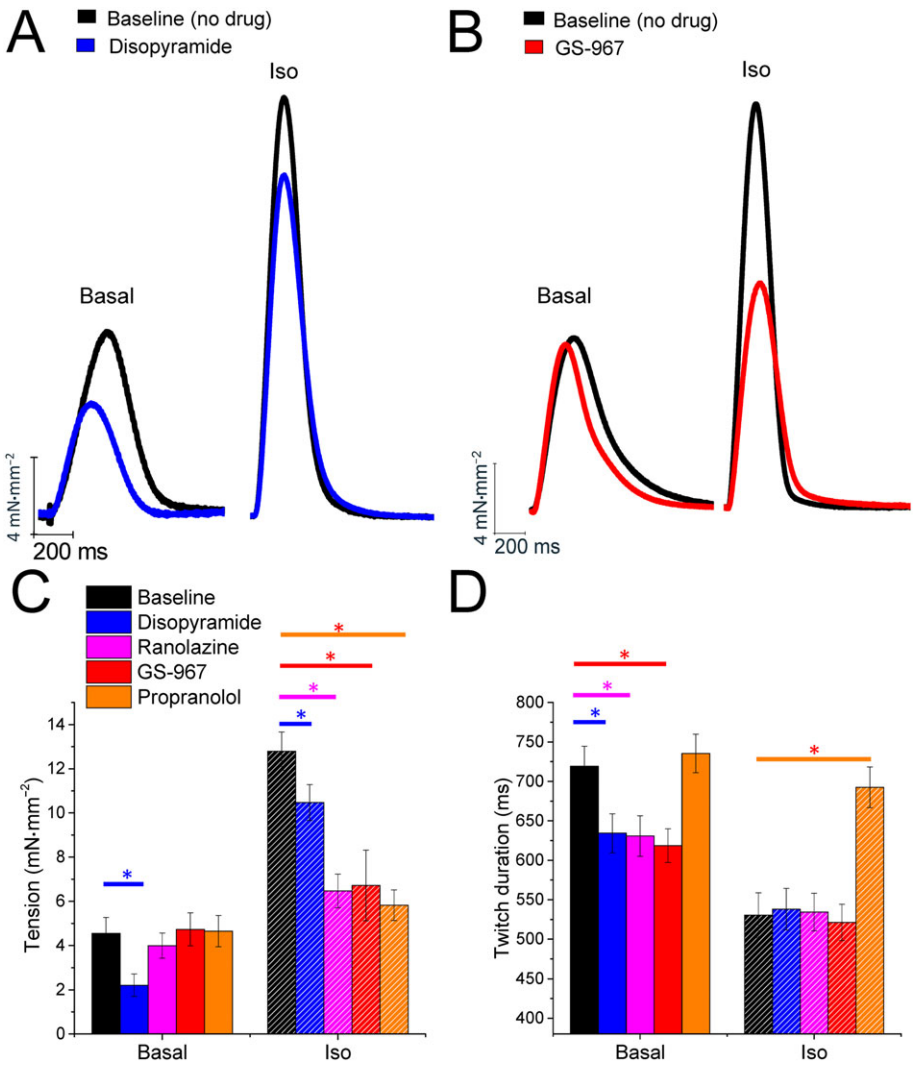

Figure 3

GS-967 and ranolazine reduce contractile force only during β-adrenoceptor stimulation in HCM myocardium. (A) Representative superimposed force twitches elicited at 1 Hz in HCM trabeculae in the absence and presence of disopyramide (5 μM), at basal conditions (left) and in the presence of isoprenaline (Iso; 0.1 μM). (B) Representative superimposed force twitches elicited at 1 Hz in HCM trabeculae in the absence and presence of GS-967 (0.5 μM), at basal conditions (left) and in the presence of isoprenaline. (C) Amplitude of force twitches elicited at 1 Hz at baseline, in the presence of disopyramide, ranolazine, GS-967 or propranolol, at basal conditions (left) or in the presence of isoprenaline(right). (D) Duration of force twitches (from stimulus to 90% repolarization) elicited at 1 Hz at baseline (black), in the presence of disopyramide, ranolazine , GS-967 or propranolol, at basal conditions (left) or in the presence of isoprenaline(right). (C, D) Means ± SE from 10 trabeculae from seven HCM patients. *$P < 0.05$, significantly different as indicated; linear mixed models corrected for paired comparisons.

ranolazine (added to isoprenaline) did not affect the duration of APs in control myocytes (Supporting Information Figure S4A, B). In line with that, GS-967 did not reduce twitch force during β-adrenoceptor stimulation in control trabeculae (Supporting Information Figure S4C, D). We excluded that the reduction of Ca^{2+} transients and force by I_{NaL}-inhibitors under β-stimulation depends on a partial block of β-adrenoceptors by these compounds (Supporting Information Figure S5). The binding studies showed that ranolazine has a very mild interaction with **β_1** and **β_2** receptors at the concentration used in this work (10 μM), while GS-967 does not bind β-receptors, even at very high concentrations. In further support of this hypothesis, we found that ranolazine exerted a negative inotropic effect also when the positive inotropic effect of β-adrenoceptor stimulation was mimicked by **forskolin**, thereby bypassing the β-receptors (Supporting Information Figure S4E, F). Finally, we investigated whether inhibition of L-Type Ca^{2+} current by I_{NaL}-inhibitors contributed to the observed effects (Figure 5). We found that neither ranolazine nor GS-967 affected the peak density of I_{CaL} in HCM cardiomyocytes (Figure 5A, B). Interestingly, we noticed that isoprenaline not only increased the amplitude of I_{CaL} but also markedly slows down the current's inactivation in HCM cells (Figure 5C, D). Using the AP-clamp technique, we found that the prolongation of AP by isoprenaline increased the total inward flow of Ca^{2+} during the plateau of the AP (Figure 5E, F): this is mainly determined by the direct

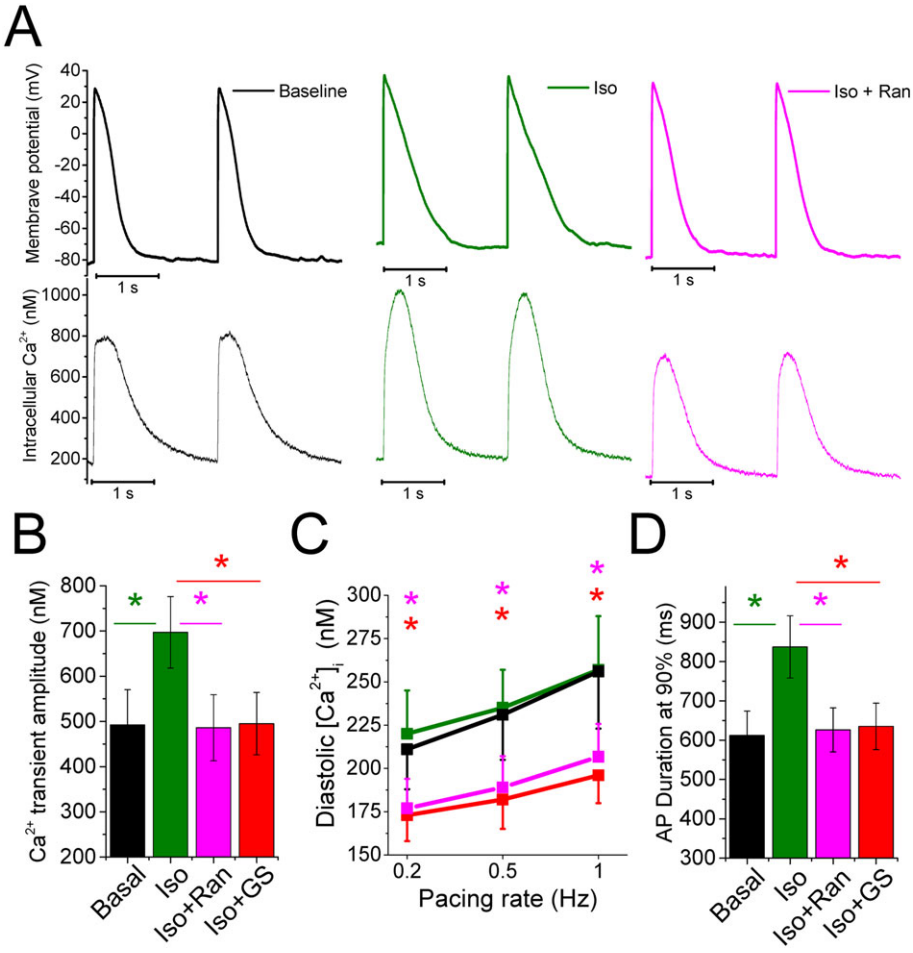

Figure 4

GS-967 and ranolazine shorten action and Ca transients during β-adrenoceptor stimulus. (A) Representative simultaneously recorded APs (above) and Ca transients (below) at baseline (left), in the presence of isoprenaline (Iso; 0.1 μM; centre) and in the presence of ranolazine (10 μM) added to isoprenaline (right), elicited at 0.5 Hz in an HCM cardiomyocyte. (B) Ca-transient amplitude (0.5 Hz) at basal conditions, in the presence of isoprenaline, in the presence of ranolazine (10 μM) added to isoprenaline (Iso + Ran) or with GS-967 (0.5 μM) added to isoprenaline (Iso + GS). (C) Diastolic intracellular Ca-concentration ($[Ca^{2+}]_i$) at basal conditions, in the presence of isoprenaline, in the presence of (Iso + Ran) or with (Iso +- GS), during regular stimulation at 0.2, 0.5 and 1 Hz (steady state). (D) APD at 90% repolarization (0.5 Hz) at baseline, with isoprenaline alone, with (Iso + Ran) or with (Iso + GS). (B–D) Means ± SE from 21 cardiomyocytes from seven HCM patients. *$P < 0.05$, significantly different as indicated; linear mixed models, corrected for paired data.

effects of β-adrenoceptor stimulation on I_{CaL} (i.e. the increased I_{CaL} amplitude and prolonged inactivation) rather than being a consequence of AP prolongation (Supporting Information Figure S6). Ranolazine, added to isoprenaline, markedly reduced the total inward flow of Ca^{2+} in HCM myocytes (Figure 5E, F), as a consequence of the marked shortening of APs caused by I_{NaL} inhibition during β-adrenoceptor stimulation (Supporting Information Figure S6).

I_{NaL}-inhibitors abolish catecholamine-induced arrhythmia in HCM myocardium

We then evaluated the effects of ranolazine and GS-967 on cellular arrhythmias evoked by β-adrenoceptor stimulation (Figure 6A, B). Isoprenaline markedly increase the occurrence of both early and delayed after-depolarizations in HCM cardiomyocytes (Figure 6). Interestingly, both ranolazine (10 μM) and GS-967 (0.5 μM) reduced the occurrence of EADs and DADs.

Finally, we evaluated the frequency of premature spontaneous beats and sustained triggered activity occurring in HCM trabeculae during stimulation pauses in the presence of isoprenaline (Figure 7A, B). When added to isoprenaline, ranolazine and GS-967 markedly reduced the occurrence of arrhythmic events (Figure 7C), in keeping with the reduction of EADs and DADs observed in isolated HCM cardiomyocytes.

Discussion

Selective I_{NaL} inhibition: a disease-specific pharmacological option for HCM treatment

I_{NaL} is three times larger in the myocardium of HCM patients compared to that of non-failing non-hypertrophic subjects,

Table 2

Effects of ranolazine (Ran) and GS-967 on APs and intracellular Ca^{2+} in the presence of isoprenaline (Iso)

Kinetics 0.5 Hz (ms)	Ca^{2+} transients				Action potentials	
	TTP	50%	90%	TOT. T.	APD50	APD90
Baseline[1]	163 ± 41	568 ± 54	1010 ± 71	1178 ± 78	699 ± 47	792 ± 50
Iso (0.1 μM)[1]	315 ± 58	445 ± 58	904 ± 61	1219 ± 79	854 ± 59	981 ± 68
Iso + Ran[2]	207 ± 29	435 ± 41	903 ± 53	1132 ± 61	706 ± 34	796 ± 40
Iso + GS[3]	209 ± 28	432 ± 38	894 ± 56	1128 ± 60	708 ± 32	799 ± 38
P (Baseline vs. Iso)	<0.05	<0.05	<0.05	n.s.	<0.05	<0.05
P (Iso vs. Iso + Ran)	<0.05	n.s.	n.s.	n.s.	<0.05	<0.05
P (Iso vs. Iso + GS)	<0.05	n.s.	n.s.	n.s.	<0.05	<0.05

Data are expressed as means ± SEM. *P* values were calculated using linear mixed models corrected for paired comparisons. TTP, time from stimulus to peak; 50%, time from peak to 50% decay of Ca transients; 90%, time from peak to 90% decay of Ca transients; TOT. T., Total Ca^{2+}-transient duration (from stimulus to 95% decay); APD20, APD at 20% of repolarization; APD50, APD at 50% of repolarization; APD90, APD at 90% of repolarization.
[1]Data from 25 HCM cardiomyocytes isolated from seven HCM patient samples;
[2]13 cells, 6 pts.
[3]12 cells, 5 pts.

leading to a prolonged APD that we observed consistently in myocardial samples from over 30 HCM myectomy patients (Coppini *et al.*, 2013). In our view, these results highlight I_{NaL} inhibition as a disease-specific pharmacological strategy to treat HCM. We previously confirmed this hypothesis by acutely applying ranolazine on myocardial samples from myectomy HCM patients, where we observed marked beneficial effects on diastolic function and arrhythmogeneicity (Coppini *et al.*, 2013). In this work, a novel, more selective I_{NaL}-inhibitor, GS-967, was tested in intact cardiomyocytes and trabeculae from 18 HCM patients, in order to confirm that the beneficial effects previously observed with ranolazine (Coppini *et al.*, 2013) were indeed consequence of I_{NaL} inhibition and were not mediated by the other pleiotropic effects of the drug [such as inhibition of K^+ and Ca^{2+} currents (Antzelevitch *et al.*, 2004), reduction of myofilament Ca^{2+} sensitivity (Lovelock *et al.*, 2012), stabilization of ryanodine receptors (Parikh *et al.*, 2012) or a mild β-blocker effect (Flenner *et al.*, 2016)]. Here, we found that all the effects of GS-967 observed in HCM myocardium are qualitatively and quantitatively similar to those previously obtained with ranolazine, but they are achieved at a 20 times lower concentration (0.5 μM GS-967 vs. 10 μM ranolazine), owing to the increased potency and selectivity for I_{NaL}. As an example, 0.5 μM GS-967 reduced I_{NaL} in HCM myocytes by 67% (Figure 1), while ranolazine inhibited 72% of I_{NaL} at 10 μM (Coppini *et al.*, 2013). Moreover, GS-967, just like ranolazine, shortened AP duration, reduced the risk of arrhythmogenic EADs (Figure 1), lowered diastolic [Ca^{2+}], suppressed the risk of DADs, accelerated Ca^{2+}-transient kinetics and hastened twitch relaxation in HCM myocardium (Figure 1). All in all, our results indicate that GS-967 may exert the same possible beneficial effects as ranolazine on arrhythmias and diastolic function, the main determinants of symptoms and outcome in HCM patients (Coppini *et al.*, 2014b; Olivotto *et al.*, 2015).

These results also have important mechanistic implications. Because GS-967 does not affect any other relevant target besides I_{NaL} at this concentration (Belardinelli *et al.*, 2013), our results confirm that the reduction of arrhythmogenicity and the amelioration of diastolic function, previously observed with ranolazine (Coppini *et al.*, 2013), are a direct consequence of I_{NaL} inhibition. Of note, we observed no major effects of GS-967 in cardiomyocytes from control patients (Supporting Information Figure S3), as previously observed with ranolazine (Coppini *et al.*, 2013), suggesting that the efficacy of I_{NaL}-inhibitors is specific to disease conditions where a pathological increase of I_{NaL} is observed.

In addition to the acute effects on myocardial function, I_{NaL} inhibition may lead to additional benefits during long-term treatment. Work from our group showed that lifelong I_{NaL} inhibition in a mouse model of HCM prevents the development of hypertrophic phenotype and LV dysfunction (Coppini *et al.*, 2017) and suggested that I_{NaL} inhibition with ranolazine or more selective agents may be an effective disease-modifying strategy in patients with HCM.

I_{NaL}-inhibitors: novel options to treat inducible obstruction?

The principal aim of this work is to investigate the potential of I_{NaL}-inhibitors in HCM, beyond the two already demonstrated effects, that is, reduction of the arrhythmic risk and the amelioration of symptoms related to diastolic dysfunction. Here, we tested these compounds as negative inotropic agents to decrease exercise-induced LV septal hypercontractility, which is considered a direct cause of obstruction-related symptoms in patients with obstructive HCM.

The negative inotropic agents that are currently used to treat obstructive HCM are limited, particularly for the cases of inducible obstruction. Despite its widespread use, disopyramide is far from being the perfect drug to control obstructive symptoms. Although it has been successfully

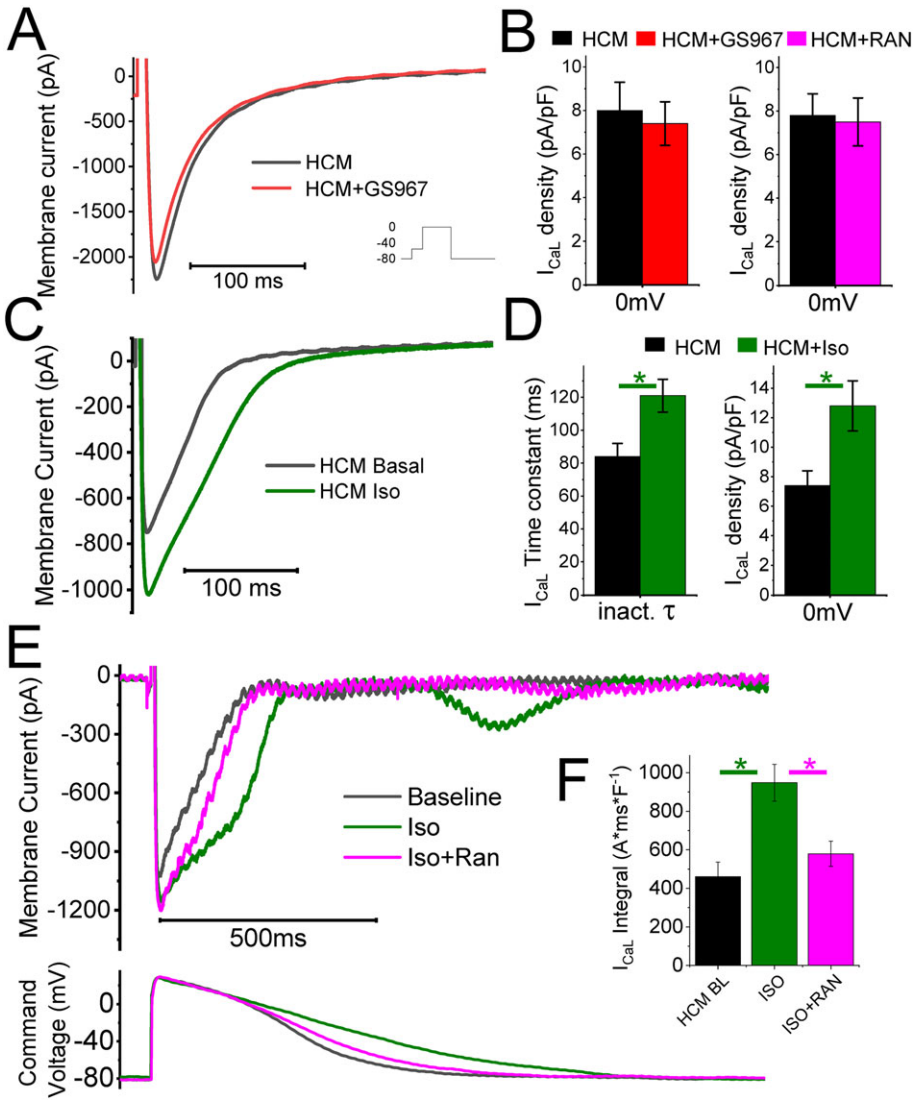

Figure 5

Ranolazine and GS967 reduced total Ca^{2+} entry through I_{CaL} during β-adrenoceptor stimulation. (A) Representative superimposed I_{CaL} traces at baseline and in the presence of GS-967 (0.5 μM) from an HCM cardiomyocyte (the pre-pulse at −40 mV is omitted from the traces). (B) I_{CaL} density at baseline, in the presence of GS-967 (0.5 μM) or ranolazine (Ran; 10 μM). Means ± SE from 15 HCM cardiomyocytes from five patients. (C) Representative superimposed I_{CaL} traces at baseline and in the presence of isoprenaline (Iso). (D) I_{CaL} inactivation time-constant (left) and density (right) at baseline and with isoprenaline in HCM cells (13 myocytes, five patients). (E) Representative I_{CaL} traces recorded during AP-clamp at baseline, with isoprenaline and with (Iso + Ran); command voltage is shown below. (F) Integral of Ca-current during AP-clamp. Means ± SE from 14 cells (five patients). *$P < 0.05$, significantly different as indicated; linear mixed models, corrected for paired data.

employed for more than 40 years to treat rest obstruction, this drug has a very limited efficacy on exercise-induced LVOT gradients (Maron *et al.*, 2006). In addition, undesired anticholinergic side-effects (mouth dryness, constipation, drowsiness) are relatively common and may require discontinuation of the drug unless they may be controlled by pyridostigmine (Sherrid, 2016). β-blockers, the only available therapeutic option to treat inducible obstruction, are often insufficient to control symptoms, and the high dosages required to significantly affect dynamic LVOT gradients may be poorly tolerated (Nistri *et al.*, 2012).

Our results show that the selective I_{NaL}-inhibitors reduced myocardial force only during intense β-adrenoceptor stimulation, mimicking stress or exercise, while leaving baseline ('rest') contractility unaffected. Interestingly, the lusitropic effect of β-adrenoceptor stimulation (i.e. acceleration of relaxation) is maintained, while only the positive inotropic effect is lost. This is at variance with β-blockers which, by acting at receptor level, antagonize the full spectrum of β-adrenoceptor-mediated effects, including the acceleration of relaxation (Figure 3) and the increase in heart rate, both essential for tolerating intense exercise activity.

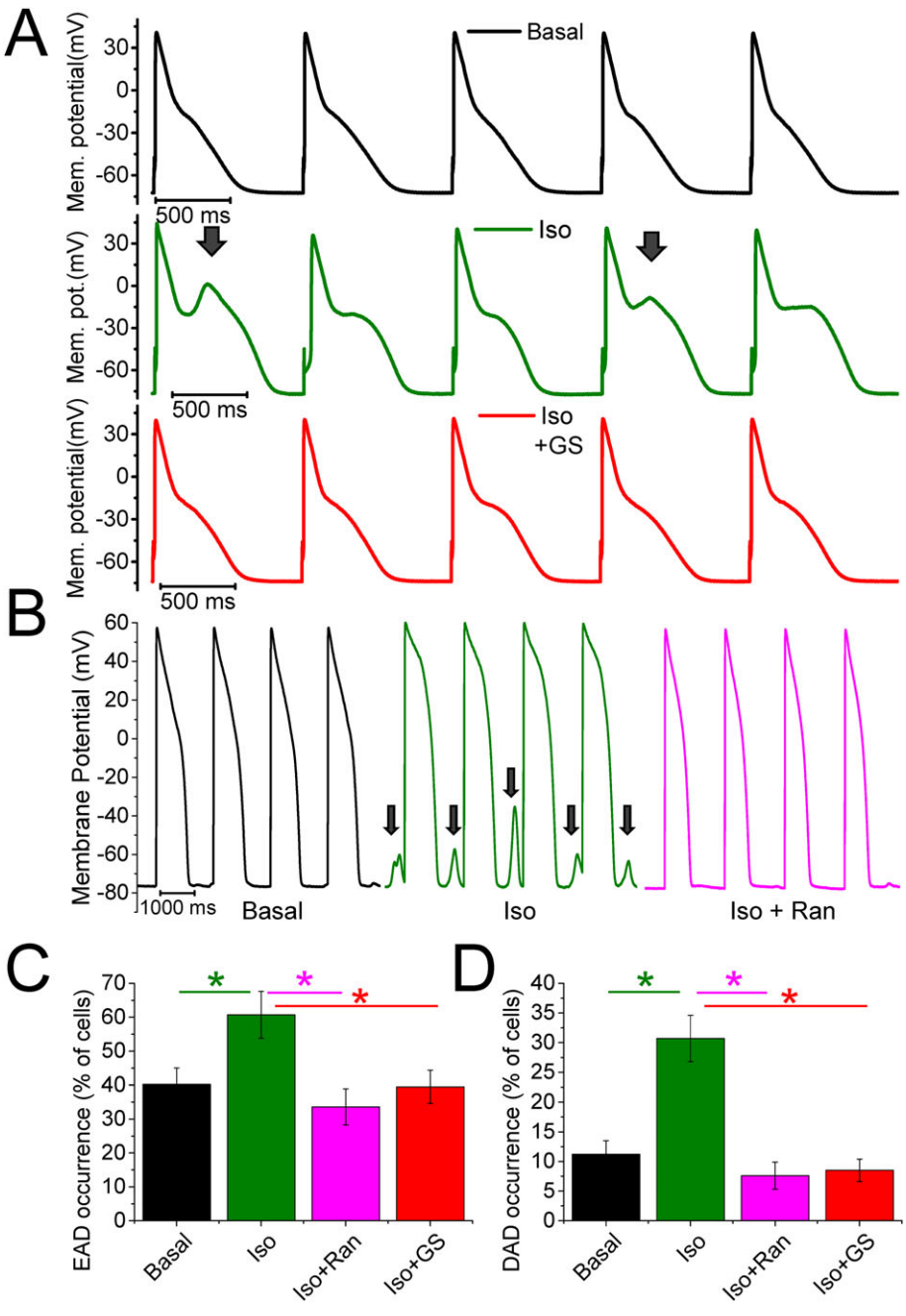

Figure 6

GS-967 and ranolazine reduce the occurrence of EAD during β-adrenoceptor stimulation. (A) Representative action traces at baseline, in the presence of isoprenaline (Iso; 0.1μM) and in the presence of GS-967 (0.5 μM) added to isoprenaline, elicited at 1 Hz pacing rate. Ranolazine (Ran) suppresses EADs (marked by black arrows) that occur under isoprenaline. (B) Representative AP traces at baseline, with isoprenaline alone or with (Iso + Ran), 0.5 Hz pacing rate. Ranolazine suppresses DADs (marked by black arrows) that occur under isoprenaline. (C, D) Percentage of HCM cardiomyocytes showing at least two EADs (EADs, in C) or two DADs (in D) during 3 min of continuous stimulation, at baseline, in the presence of isoprenaline alone, with (Iso+Ran) and with (Iso+GS-967). Means ± SE from 31 HCM cardiomyocytes from nine HCM patients. *$P < 0.05$, significantly different as indicated; linear mixed models corrected for paired comparisons.

Interestingly, this effect appears to be specific of HCM myocardium, as the reduction of contractile force by I_{NaL}-inhibitors during β-adrenoceptor stimulation is not observed in myocardial samples from control patients (Supporting Information Figure S4). The cellular mechanisms underlying the unexpected negative inotropic action of I_{NaL}-inhibitors under β-adrenoceptor stimulation in HCM myocardium have been extensively investigated.

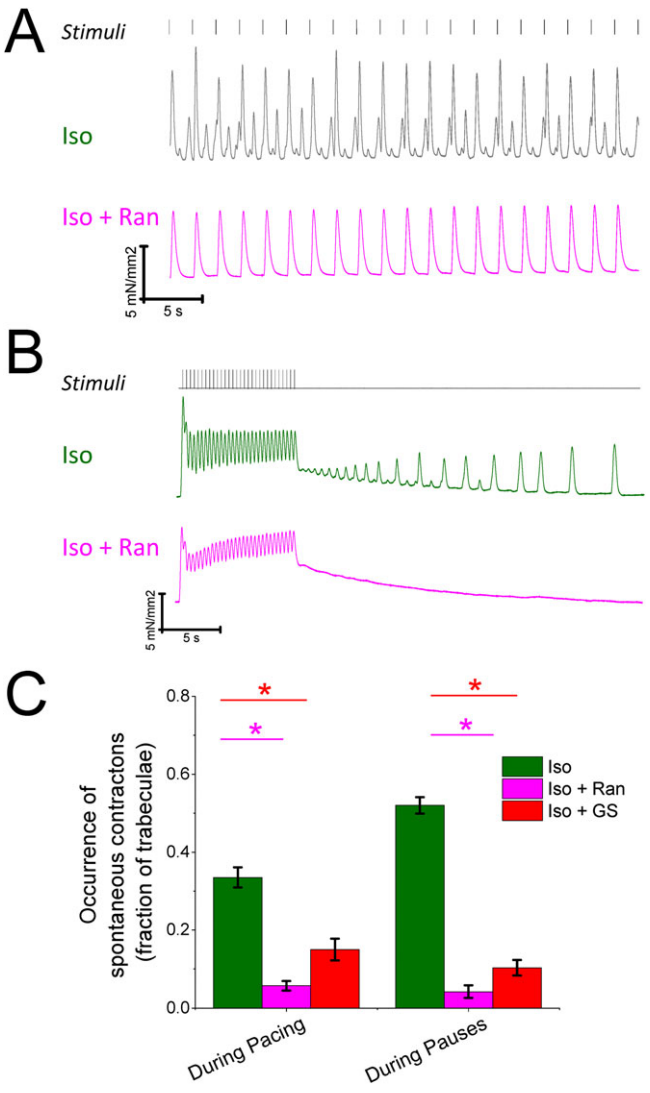

Figure 7

GS-967 and ranolazine reduce the occurrence of spontaneous contractions during β-adrenoceptor stimulus. (A) Representative force traces at baseline, in the presence of isoprenaline (Iso; 0.1μM) and in the presence of (Iso+Ran), elicited at 0.5 Hz pacing rate. Ranolazine (Ran) suppresses the spontaneous activity that occur with isoprenaline. (B) Representative force traces at baseline, in the presence of isoprenaline alone and in the presence of (Iso+Ran). After 10 s of burst pacing at 3 Hz, the stimulation was abruptly interrupted to induce spontaneous activity. (C) Summary data from isoprenaline alone, ((Iso + Ran) and (Iso + GS). Means ± SE from 15 HCM trabeculae from 12 HCM patients. *$P < 0.05$, significantly different as indicated; linear mixed models, corrected for paired data.

Mechanisms underlying the negative inotropic action of I_{NaL}-inhibitors under β-adrenoceptor stimulation in HCM myocardium

1) Altered electrical response to β-adrenoceptor stimulation in HCM myocardium

By directly comparing the response of HCM myocardium β-adrenoceptor stimulation with that of control cardiac muscle (Figure 2), we here provided the first characterization of the features and abnormalities of β-adrenoceptor signalling in human hearts from HCM patients. In particular, we observed that the mechanical response to isoprenaline in terms of increased force amplitude and relaxation velocity was comparable in HCM and control trabeculae, but contractile kinetics remained slower in HCM, than in control trabeculae, even during maximal β-adrenoceptor stimulation. These results indicate that the subcellular mechanisms responsible for the lusitropic effects of the β-adrenoceptor cascade are essentially preserved in HCM myocardium: HCM myofilaments retain the ability to decrease Ca^{2+} sensitivity upon phosphorylation of troponin-I by **PKA** (Sequeira *et al.*, 2013), and PKA-mediated phosphorylation of phospholamban occurs normally in human HCM myocardium (Coppini *et al.*, 2013; Helms *et al.*, 2016).

On the contrary, the electrophysiological response to isoprenaline in isolated HCM cardiomyocytes was profoundly abnormal (Figure 2E, F). Among the many different effects on sarcolemmal and SR targets, isoprenaline enhances both L-Type Ca^{2+} current and the slow delayed rectifier K^+ current (I_{Ks}), with the latter prevailing in healthy human cells (Terrenoire *et al.*, 2005), thus leading to a net reduction of the APD (Taggart *et al.*, 2003) in control myocardium (Figure 2E, F). In HCM cardiomyocytes, hypertrophic remodelling leads to the unbalanced changes in the expression of Ca^{2+} and K^+ currents (Coppini *et al.*, 2013), that is, the expression of all K^+ channels (including those responsible for I_{Ks}) is greatly reduced in the presence of a normal (or even slightly increased) density of I_{CaL}, as compared with control myocardium. In HCM myocytes, in response to β-adrenoceptor stimulation, the potentiation of I_{CaL} prevails over the increase of K^+ currents, thus leading to a net increase of depolarizing currents during the plateau of the AP, causing the observed 'paradoxical' prolongation of APD (Figure 2E, F). Moreover, in HCM cardiomyocytes, β-adrenoceptor stimulation not only increases peak I_{CaL} amplitude but also markedly slows down I_{CaL} inactivation (Figure 5). The marked slowing of I_{CaL} inactivation is likely to contribute to the prolongation of APs by β-adrenoceptor agonists. Additionally, it was previously shown that β-adrenoceptor stimulation of ventricular cardiomyocytes with isoprenaline leads to I_{NaL} enhancement (Dybkova *et al.*, 2014) through activation of CaMKII. A further increase of I_{NaL} following β-adrenoceptor stimulation in HCM myocytes may have contributed to the paradoxical prolongation of APs observed in these cells. This paradoxical response might be relevant for the pathophysiology of exercise or stress-induced arrhythmias in HCM patients, as AP prolongation increased the risk of arrhythmogenic early-afterdepolarizations and triggered activity in HCM myocardium (Figures 5 and 6).

2) Altered calcium response to β-adrenoceptor stimulation in HCM myocardium

In HCM cardiomyocytes, unlike control myocardium, the β-adrenoceptor-dependent increase of Ca^{2+} release and force may greatly rely on the increase in the duration of Ca^{2+} entry *via* L-Type Ca^{2+} channels caused by the slower I_{CaL} inactivation. In addition to the enhancement of I_{CaL}, increased Ca^{2+} entry *via* the reverse-mode action of the **Na/Ca exchangers (NCX)** contributes to augment Ca^{2+} transients upon β-

adrenoceptor stimulation (Perchenet *et al.*, 2000). The latter effect is likely to be a consequence of the elevation of intracellular [Na^+] in response to the enhancement of I_{NaL} and the prolongation of AP plateau that follows β-adrenoceptor stimulation (Dybkova *et al.*, 2014; Coppini *et al.*, 2013). Indeed, we found that the rise time of Ca^{2+} transients is lengthened in response to isoprenaline in HCM cardiomyocytes (Figure 4, Table 2). The prolongation of Ca^{2+}-transient rise time may have direct mechanical consequences: indeed, we observed that the acceleration of twitches' rising phase (time to peak) upon β-adrenoceptor stimulation was significantly less pronounced in HCM versus control trabeculae (Figure 2D). The idea that the increase of force in response to β-adrenoceptor stimulation mainly depends on the increase of sarcolemmal Ca^{2+} entry is in apparent contrast with the commonly accepted idea that the positive inotropic effects of β-adrenoceptor stimulation derive from the increase of sarcoplasmic reticulum Ca^{2+} content (Desantiago *et al.*, 2008), due to enhancement of **SERCA** function by PKA-mediated phospholamban phosphorylation. The observed acceleration of Ca-transient decay by isoprenaline in HCM cardiomyocytes (Figure 4 and Table 2) suggests that the β-adrenoceptor-induced increase of SERCA function is preserved in HCM myocardium. The increase of SR Ca^{2+} content following β-stimulation may be limited by the increased diastolic leakage from hyper-phosphorylated ryanodine receptors (due to increased calmodulin-kinase activity, see Coppini *et al.*, 2013 and Ferrantini *et al.*, 2016), thus rendering inotropic responses more dependent on the increase of Ca^{2+} entry through the sarcolemma. Another possible contributor to this phenomenon is the reduced density of T-tubules (Orchard and Brette, 2008). We performed a preliminary investigation of the density of T-tubules in cells isolated from the 10 HCM patient samples included in the study (Supporting Information Figure S7): in all the cells observed, T-tubule density was extremely low much lower than the expected for healthy myocardium (Lyon *et al.*, 2009). The functional consequences of β-adrenoceptor activation may be radically different in disease myocytes with a markedly reduced density of T-tubules. In the near-absence of T-tubules and with a large redistribution of L-Type Ca^{2+} channels to the surface sarcolemma (Coppini *et al.*, 2013), modulation of myocardial inotropism becomes largely dependent on the amplitude of Ca^{2+} trigger (Ferrantini *et al.*, 2014), that is, the density and duration of I_{CaL} plus the rate of NCX-mediated Ca^{2+} entry (reverse mode). Due to the reduced density of T-tubules in HCM cardiomyocytes, the prolongation of APs is likely to be essential for the positive inotropic effect of β-adrenoceptor activation. All in all, the abnormal Ca^{2+} response to β-adrenoceptor agonists may contribute to impair relaxation during exercise, leading to exercise intolerance and exertional symptoms in HCM patients, regardless of obstruction.

3) Effects of I_{NaL}-inhibitors on APs during β-adrenoceptor stimulation

The abnormal response of HCM myocardium to stimulation of β-adrenoceptors in terms of AP duration and Ca^{2+} handling underlies the unexpected negative inotropic action of I_{NaL}-inhibitors. We found that both ranolazine and GS-967, when applied with isoprenaline, cause shortening of AP and Ca^{2+}-transient duration and reduce Ca^{2+}-transient amplitude (Figure 4). On the contrary, AP duration does not change in response to ranolazine in control cardiomyocytes in the presence of isoprenaline (Supporting Information Figure S4). Interestingly, AP shortening by I_{NaL}-inhibitors occurs also in the absence of β-adrenoceptor stimulation (see Figure 1 for GS-967 and Coppini *et al.*, 2013, for ranolazine). However, when we compare the degree of APD shortening by I_{NaL}-inhibitors at baseline (in a total of >50 cells combined) with that obtained with isoprenaline (in a total of >30 cells), the effect is significantly more pronounced in the presence of the β-adrenoceptor agonist. At 0.5 Hz pacing rate, reduction of APD was −27.1 ± 3.7% in the presence of isoprenaline , while it was −17.2 ± 2.4% at baseline ($P < 0.05$, linear mixed models). When APD is prolonged and repolarizing K^+ currents are reduced, the duration of AP plateau is more dependent on I_{NaL} (Wu *et al.*, 2011; Shattock *et al.*, 2017): therefore, the reduction of APD following a proportionally comparable inhibition of I_{NaL} is larger when APD is longer and I_{NaL} is further increased (i.e. in the presence of isoprenaline). The prolongation of APD in response to isoprenaline leads to an increase in the frequency of EADs in HCM cardiomyocytes. Interestingly, when I_{NaL}-inhibitors are added with β-adrenoceptor agonists, the shortening of APD brings the rate of EADs back to baseline, suggesting a possible efficacy of I_{NaL}-inhibitors in preventing stress-induced arrhythmias in HCM.

4) Effects of I_{NaL}-inhibitors on Ca^{2+} transients and diastolic [Ca^{2+}] under β-adrenoceptor stimulation

I_{NaL}-inhibitors, *via* shortening of APD, antagonize the increase of the duration of I_{CaL} during the plateau of APs induced by isoprenaline in HCM cardiomyocytes, as demonstrated here by AP-clamp experiments (Figure 5 and Supporting Information Figure S6), ultimately leading to a marked reduction of total Ca^{2+} entry *via* I_{CaL} (−65 ± 12% with respect to Iso). This effect is a direct consequence of the shortening of APs and does not depend by any direct interactions of I_{NaL}-inhibitors with Ca^{2+} channels (Figure 5). Moreover, in the presence of β-adrenoceptor stimulation, I_{NaL} inhibition with ranolazine may reduce intracellular [Na^+] by a greater extent as compared with the 'rest' condition. The marked reduction in intracellular [Na^+] due to I_{NaL} inhibition (Coppini *et al.*, 2013; Kornyeyev *et al.*, 2015) during β-adrenoceptor stimulation may lead to a pronounced decrease in reverse-mode NCX activity, which in turn may contribute to diminish Ca^{2+} entry during AP plateau.

In line with the reduction of Ca^{2+} entry *via* I_{CaL} and reverse-mode NCX, time to peak Ca^{2+} transients (markedly prolonged by isoprenaline) were brought back to baseline levels by the addition of I_{NaL}-inhibitors (Figure 4 and Table 2). The reduction of isoprenaline-induced AP prolongation by I_{NaL} inhibition combined with the decrease of [Na^+] may prevent the increase of Ca^{2+} entry through I_{CaL} and reverse-mode NCX contractility by β-adrenoceptor stimulation in HCM myocardium. The marked reduction of Ca^{2+} entry appears to be large enough to counteract the isoprenaline-induced increase of Ca^{2+}-transient amplitude and force in HCM myocardium, while I_{NaL}-inhibitors are

unable to significantly reduce Ca^{2+} transient and twitch amplitude in the absence of isoprenaline (Figures 1, 3 and 4). In parallel with the decrease of reverse-mode NCX, the marked reduction of $[Na^+]$ by I_{NaL} inhibition also leads to increased forward activity of the exchanger (Coppini *et al.*, 2013), thus increasing the rate of Ca^{2+} efflux through the sarcolemma during the diastolic period. This may have directly contributed to the marked reduction of diastolic $[Ca^{2+}]$ observed when ranolazine or GS-967 are added to isoprenaline (Figure 4A–C). The increased sarcolemmal efflux combined with the decreased Ca^{2+} influx through I_{CaL} is likely to cause a reduction of SR Ca^{2+} content (Trafford *et al.*, 2001; Eisner *et al.*, 2013) in response to I_{NaL} inhibition during isoprenaline. A decrease of SR Ca^{2+} load may reduce diastolic leakage, contributing to the decrease of diastolic cytosolic $[Ca^{2+}]$ (Ferrantini *et al.*, 2016). In line with that, the frequency of diastolic Ca^{2+} waves leading to DADs (markedly increased by isoprenaline) is reduced by I_{NaL}-inhibitors in HCM cardiomyocytes during β-adrenoceptor stimulation.

The weak β-blocker action of ranolazine is not responsible for its negative inotropic effect in the presence of isoprenaline

Ranolazine was previously shown to behave as a weak β-adrenoceptor blocker (Letienne *et al.*, 2001; Flenner *et al.*, 2016), albeit at higher concentrations than that used in our experiments (20 μM or higher). We here confirmed that ranolazine mildly binds to β_1 and β_2 adrenoceptors at the concentration used in our experiments (10 μM). In contrast, GS-967 does not interact with β-adrenoceptors, even at high concentrations (Supporting Information Figure S5). The mild β-blocking effect of ranolazine is unlikely to play a major role in the observed response of HCM muscle to ranolazine in the presence of isoprenaline, for the following reasons: (i) the acceleration of relaxation by isoprenaline was not antagonized by 10 μM ranolazine , only the increase in force amplitude is reduced. (ii) The same negative inotropic effect as ranolazine was observed with 0.5 μM GS-967 (Figure 3E), which does not have any β-blocking capabilities (Supporting Information Figure S5), in line with previous observations in different healthy and diseased animal models (Belardinelli *et al.*, 2013; Fernandes *et al.*, 2014; Alves Bento *et al.*, 2015). (iii) Finally, when we mimicked the inotropic and lusitropic response to β-adrenoceptor stimulation with forskolin in HCM trabeculae (thereby bypassing β-receptors), ranolazine, added in the presence of forskolin, exerted the same negative inotropic effect that was observed in the presence of β-adrenoceptor stimulation with isoprenaline (Supporting Information Figure S4). These observations confirm that the interaction of I_{NaL}-inhibitors with the physiological consequences of β-adrenoceptor stimulation occurs downstream of the β-adrenoceptor and of adenylyl cyclase activation and is a sole consequence of the inhibition of I_{NaL}.

Clinical implications and conclusions

In HCM myocardium, disopyramide exerts a potent negative inotropic effect at rest but is less effective under β-adrenoceptor stimulation (Figure 3). This provides a clear explanation of why disopyramide effectively reduces resting gradients but is inadequate to control symptoms in patients with inducible obstruction. Conversely, I_{NaL}-inhibitors (ranolazine and GS-967) reduce myocardial force only during β-adrenoceptor stimulation, used here to simulate exercise and stress. Because of this effect, ranolazine and selective I_{NaL}-inhibitors (Justo *et al.*, 2016; Rajamani *et al.*, 2016) may be effective in reducing septal tension during exercise and therefore have a theoretical potential to ameliorate symptoms caused by inducible obstruction in HCM. Furthermore, by limiting Ca^{2+} overload during stress, I_{NaL}-inhibitors may also play a protective role against stress-induced arrhythmias (Lyon *et al.*, 2009; Maron *et al.*, 2006). Intriguingly, both ranolazine and GS-967 abolished the increase in spontaneous contractions and triggered activity induced by isoprenaline (Figures 5 and 6). By acting downstream of the receptor, I_{NaL}-inhibitors only counteract the positive inotropic effect of β-adrenoceptor stimulation on septal tension and do not reduce the lusitropic and the chronotropic effects of noradrenaline, which are essential to tolerate exercise. High doses of non-selective β-blockers, such as nadolol, are often required to reduce dynamic gradients in patients with exertional symptoms due to inducible obstruction (Nistri *et al.*, 2012): a large number of patients do not tolerate such aggressive β-blocker treatments and are left with residual symptomatic gradients. In other patients, residual dynamic gradients persist even at the maximal target dose of β-blockers (e.g. 80–100 $mg \cdot day^{-1}$ of nadolol; see Nistri *et al.*, 2012). Based on our results, we can envision the use of I_{NaL}-inhibitors in addition to β-blockers in patients with residual obstruction-related symptoms at the maximal tolerated dose of β-blockers, with the purpose of abolishing the remaining inducible gradients.

In conclusion, the novel selective I_{NaL}-inhibitor GS-967 improved diastolic function and reduced the arrhythmogenic potential of HCM myocardium *in vitro*, matching the effects of ranolazine at 20 times lower concentrations. I_{NaL}-inhibitors (ranolazine and GS-967) appeared to reduce septal contractility at peak exercise without affecting resting performance. These data suggest improved efficacy of I_{NaL}-inhibitors over disopyramide, and a synergistic action with β-adrenoceptor antagonists, in HCM patients with exercise-induced obstruction.

Acknowledgements

This work was supported by Fondazione Telethon (GGP13162), by the European Commission (STREP Project 241577 'BIG HEART', 7th European Framework Program), by the Italian Ministry of Health (RF 2010-2313451, RF-2013-02356787 and GR-2011-02350583) and by Regione Toscana (FAS-Salute 2014, project ToRSADE).

Author contributions

C.F. devised the project, performed intact trabeculae experiments, analysed the data and wrote the manuscript. J.M.P. contributed to intact trabeculae experiments and to analyse the data. L.M. collected and processed cardiac samples and contributed to single cell experiments. F.G. and B.T. contributed to intact trabeculae experiments. A.R. collected clinical

data from patients. L.B. contributed to devise the project and critically reviewed the manuscript. R.M. performed β-receptor binding experiments. C.P. contributed to single cell experiments. C.T. and E.C. contributed to data analysis and interpretation and edited the manuscript. I.O. recruited HCM patients and reviewed the manuscript. C.P. and A.M. contributed to conceive the project and critically edited the manuscript. R.C. devised the experiments, performed single cell experiments, analysed and interpreted the results and wrote the manuscript.

Conflict of interest

L.B. was employed by Gilead Sciences, Inc., till 2016.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Alexander SPH, Striessnig J, Kelly E, Marrion NV, Peters JA, Faccenda E *et al.* (2017a). The Concise Guide to PHARMACOLOGY 2017/18: Voltage-gated ion channels. Br J Pharmacol 174: S160–S194.

Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA *et al.* (2017b). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. Br J Pharmacol 174: S17–S129.

Alexander SPH, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD *et al.* (2017c). The Concise Guide to PHARMACOLOGY 2017/18: Transporters. Br J Pharmacol 174: S360–S446.

Alexander SPH, Fabbro D, Kelly E, Marrion NV, Peters JA, Faccenda E *et al.* (2017d). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. Br J Pharmacol 174: S272–S359.

Alves Bento AS, Bacic D, Saran Carneiro J, Nearing BD, Fuller H, Justo FA *et al.* (2015). Selective late INa inhibition by GS-458967 exerts parallel suppression of catecholamine-induced hemodynamically significant ventricular tachycardia and T-wave alternans in an intact porcine model. Heart Rhythm 12: 2508–2514.

Ammirati E, Contri R, Coppini R, Cecchi F, Frigerio M, Olivotto I (2016). Pharmacological treatment of hypertrophic cardiomyopathy: current practice and novel perspectives. Eur J Heart Fail 18: 1106–1118.

Antzelevitch C, Belardinelli L, Zygmunt AC, Burashnikov A, Di Diego JM, Fish JM *et al.* (2004). Electrophysiological effects of ranolazine, a novel antianginal agent with antiarrhythmic properties. Circulation 110: 904–910.

Authors/Task Force m, Elliott PM, Anastasakis A, Borger MA, Borggrefe M, Cecchi F *et al.* (2014). 2014 ESC Guidelines on diagnosis and management of hypertrophic cardiomyopathy: the Task Force for the Diagnosis and Management of Hypertrophic Cardiomyopathy of the European Society of Cardiology (ESC). Eur Heart J 35: 2733–2779.

Belardinelli L, Liu G, Smith-Maxwell C, Wang WQ, El-Bizri N, Hirakawa R *et al.* (2013). A novel, potent, and selective inhibitor of cardiac late sodium current suppresses experimental arrhythmias. J Pharmacol Exp Ther 344: 23–32.

Bonatti R, Silva AF, Batatinha JA, Sobrado LF, Machado AD, Varone BB *et al.* (2014). Selective late sodium current blockade with GS-458967 markedly reduces ischemia-induced atrial and ventricular repolarization alternans and ECG heterogeneity. Heart Rhythm 11: 1827–1835.

Carneiro JS, Bento AS, Bacic D, Nearing BD, Rajamani S, Belardinelli L *et al.* (2015). The selective cardiac late sodium current inhibitor GS-458967 suppresses autonomically triggered atrial fibrillation in an intact porcine model. J Cardiovasc Electrophysiol 26: 1364–1369.

Coppini R, Ferrantini C, Aiazzi A, Mazzoni L, Sartiani L, Mugelli A *et al.* (2014a). Isolation and functional characterization of human ventricular cardiomyocytes from fresh surgical samples. J Vis Exp JoVE.

Coppini R, Ho CY, Ashley E, Day S, Ferrantini C, Girolami F *et al.* (2014b). Clinical phenotype and outcome of hypertrophic cardiomyopathy associated with thin-filament gene mutations. J Am Coll Cardiol 64: 2589–2600.

Coppini R, Ferrantini C, Yao L, Fan P, Del Lungo M, Stillitano F *et al.* (2013). Late sodium current inhibition reverses electromechanical dysfunction in human hypertrophic cardiomyopathy. Circulation 127: 575–584.

Coppini R, Mazzoni L, Ferrantini C, Gentile F, Pioner JM, Laurino T *et al.* (2017). Ranolazine prevents phenotype development in a mouse model of hypertrophic cardiomyopathy. Circ Heart Fail 10: e003565.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

Desantiago J, Ai X, Islam M, Acuna G, Ziolo MT, Bers DM *et al.* (2008). Arrhythmogenic effects of beta2-adrenergic stimulation in the failing heart are attributable to enhanced sarcoplasmic reticulum Ca load. Circ Res 102: 1389–1397.

Dybkova N, Wagner S, Backs J, Hund TJ, Mohler PJ, Sowa T*et al.* (2014). Tubulin polymerization disrupts cardiac beta-adrenergic regulation of late INa. Cardiovasc Res 103: 168–177.

Eisner D, Bode E, Venetucci L, Trafford A (2013). Calcium flux balance in the heart. J Mol Cell Cardiol 58: 110–117.

Fernandes S, Hoyer K, Liu G, Wang WQ, Dhalla AK, Belardinelli L *et al.* (2014). Selective inhibition of the late sodium current has no adverse effect on electrophysiological or contractile function of the normal heart. J Cardiovasc Pharmacol 63: 512–519.

Ferrantini C, Coppini R, Scellini B, Ferrara C, Pioner JM, Mazzoni L *et al.* (2016). R4496C RyR2 mutation impairs atrial and ventricular contractility. J Gen Physiol 147: 39–52.

Ferrantini C, Coppini R, Sacconi L, Tosi B, Zhang ML, Wang GL *et al.* (2014). Impact of detubulation on force and kinetics of cardiac muscle contraction. J Gen Physiol 143: 783–797.

Flenner F, Friedrich FW, Ungeheuer N, Christ T, Geertz B, Reischmann S *et al.* (2016). Ranolazine antagonizes catecholamine-induced dysfunction in isolated cardiomyocytes, but lacks long-term therapeutic effects in vivo in a mouse model of hypertrophic cardiomyopathy. Cardiovasc Res 109: 90–102.

Gersh BJ, Maron BJ, Bonow RO, Dearani JA, Fifer MA, Link MS *et al*. (2011). 2011 ACCF/AHA guideline for the diagnosis and treatment of hypertrophic cardiomyopathy: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation 124: e783–e831.

Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S *et al*. (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res 46: D1091–D1106.

Helms AS, Alvarado FJ, Yob J, Tang VT, Pagani F, Russell MW*et al*. (2016). Genotype-dependent and -independent calcium signaling dysregulation in human hypertrophic cardiomyopathy. Circulation 134: 1738–1748.

Justo F, Fuller H, Nearing BD, Rajamani S, Belardinelli L, Verrier RL (2016). Inhibition of the cardiac late sodium current with eleclazine protects against ischemia-induced vulnerability to atrial fibrillation and reduces atrial and ventricular repolarization abnormalities in the absence and presence of concurrent adrenergic stimulation. Heart Rhythm 13: 1860–1867.

Kornyeyev D, El-Bizri N, Hirakawa R, Nguyen S, Viatchenko-Karpinski S, Yao L *et al*. (2015). Contribution of the late sodium current to intracellular sodium and calcium overload in rabbit ventricular myocytes treated by anemone toxin. Am J Physiol Heart Circ Physiol ajpheart 00520 02015.

Letienne R, Vie B, Puech A, Vieu S, Le Grand B, John GW (2001). Evidence that ranolazine behaves as a weak beta1- and beta2-adrenoceptor antagonist in the rat [correction of cat] cardiovascular system. Naunyn Schmiedebergs Arch Pharmacol 363**:** 464–471.

Lovelock JD, Monasky MM, Jeong EM, Lardin HA, Liu H, Patel BG *et al*. (2012). Ranolazine improves cardiac diastolic dysfunction through modulation of myofilament calcium sensitivity. Circ Res 110: 841–850.

Lyon AR, MacLeod KT, Zhang Y, Garcia E, Kanda GK, Lab MJ *et al*. (2009). Loss of T-tubules and other changes to surface topography in ventricular myocytes from failing human and rat heart. Proc Natl Acad Sci U S A 106: 6854–6859.

Maron MS, Olivotto I, Zenovich AG, Link MS, Pandian NG, Kuvin JT *et al*. (2006). Hypertrophic cardiomyopathy is predominantly a disease of left ventricular outflow tract obstruction. Circulation 114: 2232–2239.

Nistri S, Olivotto I, Maron MS, Ferrantini C, Coppini R, Grifoni C *et al*. (2012). Beta blockers for prevention of exercise-induced left ventricular outflow tract obstruction in patients with hypertrophic cardiomyopathy. Am J Cardiol 110: 715–719.

Olivotto I, d'Amati G, Basso C, Van Rossum A, Patten M, Emdin M *et al*. (2015). Defining phenotypes and disease progression in sarcomeric cardiomyopathies: contemporary role of clinical investigations. Cardiovasc Res 105: 409–423.

Orchard C, Brette F (2008). t-Tubules and sarcoplasmic reticulum function in cardiac ventricular myocytes. Cardiovasc Res 77: 237–244.

Parikh A, Mantravadi R, Kozhevnikov D, Roche MA, Ye Y, Owen LJ *et al*. (2012). Ranolazine stabilizes cardiac ryanodine receptors: a novel mechanism for the suppression of early afterdepolarization and torsades de pointes in long QT type 2. Heart Rhythm 9: 953–960.

Perchenet L, Hinde AK, Patel KC, Hancox JC, Levi AJ (2000). Stimulation of Na/Ca exchange by the beta-adrenergic/protein kinase A pathway in guinea-pig ventricular myocytes at 37 degrees C. Pflugers Archiv 439: 822–828.

Pezhouman A, Madahian S, Stepanyan H, Ghukasyan H, Qu Z, Belardinelli L *et al*. (2014). Selective inhibition of late sodium current suppresses ventricular tachycardia and fibrillation in intact rat hearts. Heart Rhythm 11: 492–501.

Pozios I, Corona-Villalobos C, Sorensen LL, Bravo PE, Canepa M, Pisanello C *et al*. (2015). Comparison of outcomes in patients with nonobstructive, labile-obstructive, and chronically obstructive hypertrophic cardiomyopathy. Am J Cardiol 116: 938–944.

Rajamani S, Liu G, El-Bizri N, Guo D, Li C, Chen XL *et al*. (2016). The novel late Na+ current inhibitor, GS-6615 (eleclazine) and its anti-arrhythmic effects in rabbit isolated heart preparations. Br J Pharmacol 173: 3088–3098.

Sequeira V, Wijnker PJ, Nijenkamp LL, Kuster DW, Najafi A, Witjas-Paalberends ER *et al*. (2013). Perturbed length-dependent activation in human hypertrophic cardiomyopathy with missense sarcomeric gene mutations. Circ Res 112: 1491–1505.

Shattock MJ, Park KC, Yang HY, Lee AWC, Niederer S, MacLeod KT *et al*. (2017). Restitution slope is principally determined by steady-state action potential duration. Cardiovasc Res 113: 817–828.

Sherrid MV (2016). Drug therapy for hypertrophic cardiomypathy: physiology and practice. Curr Cardiol Rev 12: 52–65.

Sherrid MV, Barac I, McKenna WJ, Elliott PM, Dickie S, Chojnowska L *et al*. (2005). Multicenter study of the efficacy and safety of disopyramide in obstructive hypertrophic cardiomyopathy. J Am Coll Cardiol 45: 1251–1258.

Sicouri S, Belardinelli L, Antzelevitch C (2013). Antiarrhythmic effects of the highly selective late sodium channel current blocker GS-458967. Heart Rhythm 10: 1036–1043.

Sikkel MB, Francis DP, Howard J, Gordon F, Rowlands C, Peters NS *et al*. (2017). Hierarchical statistical techniques are necessary to draw reliable conclusions from analysis of isolated cardiomyocyte studies. Cardiovasc Res 113: 1743–1752.

Taggart P, Sutton P, Chalabi Z, Boyett MR, Simon R, Elliott D *et al*. (2003). Effect of adrenergic stimulation on action potential duration restitution in humans. Circulation 107: 285–289.

Terrenoire C, Clancy CE, Cormier JW, Sampson KJ, Kass RS (2005). Autonomic control of cardiac action potentials: role of potassium channel kinetics in response to sympathetic stimulation. Circ Res 96: e25–e34.

Trafford AW, Diaz ME, Eisner DA (2001). Coordinated control of cell Ca(2+) loading and triggered release from the sarcoplasmic reticulum underlies the rapid inotropic response to increased L-type Ca(2+) current. Circ Res 88: 195–201.

Voigt N, Li N, Wang Q, Wang W, Trafford AW, Abu-Taha I *et al*. (2012). Enhanced sarcoplasmic reticulum Ca2+ leak and increased Na+-Ca2+ exchanger function underlie delayed afterdepolarizations in patients with chronic atrial fibrillation. Circulation 125: 2059–2070.

Wu L, Ma J, Li H, Wang C, Grandi E, Zhang P *et al*. (2011). Late sodium current contributes to the reverse rate-dependent effect of IKr inhibition on ventricular repolarization. Circulation 123: 1713–1720.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

https://doi.org/10.1111/bph.14223

Table S1 Control Patients Characteristics. Clinical features of the control patients enrolled in the study. Clinical data refers to visits performed less than 1 month before the operation. Categorical data is expressed as proportion of patients; continuous values are expressed as mean ± SD. NYHA = New York Heart Association; LVOT = Left-Ventricular Outflow Tract; LA = Left Atrium.

Table S2 Effects of GS-967 on action potentials and intracellular Ca2+ in HCM cardiomyocytes. Data are expressed as means ± standard error of mean. Action potentials: data from 37 HCM cardiomyocytes isolated from 9 HCM patient samples. Ca-transients = data from 26 myocytes from 7 HCM patients. P values were calculated using linear mixed models corrected for paired comparisons. T. to Peak = time to peak; 50% decay = time from peak to 50% decay of Catransients; 90% decay = time from peak to 90% decay of Ca-transients; MDP = mean diastolic potential; Ampl. = amplitude of action potentials; Upstr. = maximal upstroke speed; APD20 = action potential duration at 20% of repolarization; APD50 = action potential duration at 50% of repolarization.

Figure S1 Human HCM cardiomyocytes and trabeculae: representative images and cell shortening. (A) Representative videomicroscopy images of a HCM cardiomyocyte suspension right after isolation and reintroduction of calcium. Of note, a large amount of debris as well as dead cells are visible at this stage. White bar is 15 μm. (B) Representative videomicroscopy images of HCM trabeculae mounted between the tip of a force transducer and a length controlling motor. Wire is used to tie the trabecula's end to the attachments at both sides. White bar is 1 mm. (C) Representative images of a contracting HCM myocyte at end diastole (above) and at peak shortening (below). White bar is 15 μm. A video is also provided as online supplement. (D) Left: representative superimposed sarcomere shortening traces from a HCM myocyte, recorded in the absence (black trace) and presence (dark green) of isoproterenol 10–7 M. Top right: average sarcomere shortening during stimulation at 0.5 Hz in HCM myocytes. Bottom right: kinetics of sarcomere shortening in HCM cardiomyocytes; TTP = time from stimulus to peak, RT50 = time from peak to 50% relaxation. Means ± SEM from 14 myocytes from 3 HCM patients.

Figure S2 GS-967 suppresses cellular arrhythmias in HCM cardiomyocytes. (A) Representative superimposed trains of action potentials elicited at 0.5 Hz at baseline (black traces) and in the presence of GS-967 0.5 μM (red traces). Early after-depolarizations (EADs) are marked by arrows. (B) Representative superimposed trains of action potentials elicited at 0.5 Hz. Delayed afterdepolarizations (DADs) are marked by arrows (C) Fraction of HCM cardiomyocytes showing at least 2 early after-depolarizations (EADs) during 3 min of continuous stimulation, at baseline (black) and in the presence of GS-967 0.5 μM (red). (D) Fraction of HCM cardiomyocytes showing at least 2 delayed after-depolarizations (DADs) during 3 minutes of pacing, at baseline (black) and in the presence of GS-967 0.5 μM (red). (C-D) Means ± standard error from 37 HCM cardiomyocytes from 9 HCM patients. * = $P < 0.05$, linear mixed models corrected for paired comparisons.

Figure S3 Effects of GS-967 in control cardiomyocytes. (A) Representative superimposed action potentials at baseline (grey trace) and in the presence of GS-967 0.5 μM (orange trace), elicited at 0.2 Hz (left) and 1 Hz (rught) from a control cardiomyocyte. (B) Action potential duration at 90% of repolarization (APD90%) at baseline (grey) and in the presence of GS-967 0.5 μM (orange) at different frequencies of stimulation. Means ± standard error from 10 control cardiomyocytes from 3 patients. (C) Representative superimposed Ca-transients at baseline (grey traces) and in the presence of GS-967 0.5 μM (orange traces), elicited at 0.5 Hz (left). (D) Ca-transient amplitude (Δ[Ca2+]i) at baseline (grey) and with the application of GS-967 (orange) in control cardiomyocytes. (E) Time to peak (TP), Time from peak to 50% decay of Ca-transients (T50%) and time from peak to 90% decay (T90%) at baseline (grey) and in the presence of GS-967 0.5 μM (orange), elicited at 0.5 Hz. (D-E) Means ± standard error from 9 cardiomyocytes from 3 patients.

Figure S4 Late Na current blockers under β-stimulation in control samples and effects of forskolin (A) Representative action potentials at baseline (top, grey), in the presence of Isoproterenol 10–7 M (Iso, centre, green traces) and in the presence of Ran 10 μM added on top of Iso (right, purple traces), elicited at 1 Hz in a CTR cardiomyocyte. Means ± standard error from 8 cardiomyocytes from 3 HCM patients. (B) AP duration at 90% repolarization. Means± SEM from 7 control myocytes (2 pts.) (C) Representative twitches recorded at 0.5 Hz at basal conditions (grey), in the presence of GS (green), in the presence of ran+GS (purple). (D)Twitch (0.5 Hz) amplitude (top) and duration (bottom, from stimulus to 90% relax.) at baseline (grey), with Iso (green), with Iso + GS (plum). Means ± standard error from 3 trabeculae from 2 control patients. (E) Superimposed twitches at 0.5 Hz at baseline (black), with 1 μM forskolin (ochra) and with GS-967 added on top of forskolin (pink) in a HCM trabecula. (F) Twitch amplitude and duration in response to forskolin and forskolin+ GS-967 Means ± standard error from 4 trabeculae from 2 HCM patients.

Figure S5 Binding studies with β1 and β2-adrenergic receptors. (A) Attenuation of 3H-CGP12177 bound to HEK cell membranes expressing β1-adrenergic radioactivity by different concentrations of ranolazine, GS-967 and propranolol. Curves of ranolazine and propranolol are fitted with hill equations. (B) Attenuation of 3H-CGP12177 bound to HEK cell membranes expressing β2- adrenergic radioactivity by different concentrations of ranolazine, GS-967 and propranolol. Curves of ranolazine and propranolol are fitted with hill equations. (C) Normalized comparison of the binding curves of ranolazine with membranbes expressing β1 or β2-adrenergic receptors. Calculated IC_{50} values are indicated. All experiments were conducted in triplicate and the average value is shown with the calculated standard error.

Figure S6 Additional AP-Clamp results. (A) Representative L-type Ca-current traces recorded during AP-clamp at baseline (black), with Iso-AP as Command Voltage but in the absence of Iso in the solution (light green) and after the addition of Iso to the bathing solution (green); command voltage APtraces are shown in figure E (main text). (B) Integral of L-Type Ca-current recorded during AP-clamp in the conditions of panel A. Notably, the application of a prolonged AP (Iso-AP) as a command voltage does not

significantly increase the total amount of Ca-entry; the actual addition of isoproterenol to the solution is necessary. (C) Representative Ca-current traces recorded during AP-clamp with Iso (green), with Iso + Ran- AP as Command Voltage but in the absence of Ran in the solution (light pink) and after the addition of Ran (on top of Iso) to the bathing solution (magenta). (D) Integral of L-Type Ca-current recorded in the conditions of panel C. Notably, the application of a shortened AP (Iso + Ran-AP) as a command voltage significantly reduces the total amount of Ca-entry; the actual addition of ranolazine to the solution does not significantly increase the reduction of Ca-entry. (B-D) Means ± standard error from 14 cells (5 patients). (E-F) Ca-current traces recorded during AP-clamp in the absence and presence of lacidipine (10–5 M): representative traces (E) and average values (F) from 7 cells (2 patients).

Figure S7 The density of T-tubules is markedly low in HCM cardiomyocytes. Representative confocal images of single cardiomyocytes isolated from the same HCM samples used for the functional experiment. Each cell derives from a different patient sample (ID of the patient is indicated next to the cell in each respective image). Cells were stained with Di-3-ANEPPDHQ (Thermo-Fisher) and imaged with a Leica Confocal microscope using the 488 nm laser line. Sections were taken at mid cell. While the outer sarcolemma is well stained in all myocytes, T-tubules are barely visible in most of them and some cells are completely devoid of T-tubules. White bars equal 10 μm.