DATA SUPPLEMENT TO MANUSCRIPT

β_3-adrenergic receptor activation increases human atrial tissue contractility and stimulates the L-type Ca^{2+} current

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Nonstandard abbreviations used: β-AR, β-adrenergic receptor; $I_{Ca,L}$, L-type Ca^{2+} channel current; HAM, human atrial myocyte; ISO, isoprenaline.

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Results

β_3-AR agonists do not stimulate $I_{Ca,L}$ in frog ventricular myocytes. The effects of the three β_3-AR agonists tested on $I_{Ca,L}$ in HAMs were also tested in frog ventricular myocytes, where all the β-adrenergic stimulation of the current is mediated by β_2-ARs.(1) As shown in Supplemental Figure 1A, SR58611, at concentrations ranging from 1 nM to 1 μM, had no effect on basal $I_{Ca,L}$. Moreover, it dose-dependently inhibited $I_{Ca,L}$ prestimulated by the nonselective β-AR agonist ISO (1 μM). This indicates that, at least in frog heart, SR58611 does not activate but instead inhibits β_2-ARs, possibly due to a very low agonistic efficacy for this β-AR subtype. In contrast, BRL37344, at concentrations ranging from 10 nM to 100 μM, produced a dose-dependent stimulation of $I_{Ca,L}$ (Supplemental Figure 1B). However, the effect of BRL37344 on frog $I_{Ca,L}$ developed with an EC_{50} of 536±93 nM (n=3), i.e. at concentrations >30-fold larger than those required to stimulate $I_{Ca,L}$ in HAMs (Figure 1D in main article) and compatible with an activation of β_2-ARs.(2) Finally, CGP12177, at concentrations ranging from 1 nM to 10 μM, had no effect on basal $I_{Ca,L}$, but dose-dependently antagonized the stimulatory effect of ISO (1 μM, Supplemental Figure 1C), as expected from its β_2-AR antagonistic properties. Altogether, these results indicate that β_3-ARs are either not expressed or not positively coupled to $I_{Ca,L}$ in frog ventricle. Moreover, none of the three β_3-AR agonists tested increased $I_{Ca,L}$ in frog ventricle at concentrations at which they produced a clear effect in HAMs. Although differences must exist between human and frog β_2-AR subtype in their affinities and selectivities for the various pharmacological agents used here, this result lowers the likeliness that the stimulatory effect observed in HAMs was due to activation of β_2-ARs.

β_3-AR agonists do not stimulate $I_{Ca,L}$ in rat ventricular myocytes. We next examined the possibility that the stimulatory effects of the three β_3-AR agonists on $I_{Ca,L}$ in HAMs were mediated by activation of β_1-ARs. For this, we tested their effects on $I_{Ca,L}$ in rat ventricular myocytes, where β_1- and β_2-ARs are co-expressed.(3) Neither CGP12177 (n=4, Supplemental Figure 2A) nor SR58611 (n=4, not shown), in the range of 1 nM to 1 μM concentrations, had any significant effect on basal $I_{Ca,L}$ in rat, but they both dose-dependently inhibited the stimulatory effect of ISO (100 nM) on $I_{Ca,L}$ (Supplemental Figure 2B &C). Therefore, these two β_3-AR agonists behaved in rat ventricular myocytes just like in frog, and very differently from HAMs. As seen in Supplemental Figure 2D, BRL37344 exerted a slight <10% stimulation on basal $I_{Ca,L}$ (n=4), but that effect was only seen at micromolar concentrations, i.e. at concentrations 50-fold higher than necessary to affect $I_{Ca,L}$ in HAMs. As seen in Supplemental Figure 2D, that effect was negligible compared to the effect produced by 100 nM ISO. Altogether, these results indicate that β_3-ARs are either not expressed or not positively coupled to $I_{Ca,L}$ in rat ventricle. Moreover, none of the three β_3-AR agonists tested increased $I_{Ca,L}$ in rat ventricle at concentrations at which they produced an effect in HAMs. Although differences must exist between human and rat β_1-AR subtype in their affinities and selectivities for the various pharmacological agents used here, this result lowers the likeliness that the stimulatory effect observed in HAMs was due to activation of β_1-ARs.

Methods

Our investigations conform with the European Community guiding principles in the care and use of animals (86/609/CEE), *CE Off J* nº L358, 18 December 1986) and the French decree nº87-848 of October 19, 1987 (*J of République Française*, 20 October 1987, pp. 12245-12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture, de la Pêche et de l'Alimentation (nº7475, May 27, 1997).

Frog ventricular myocytes. Hearts from frog (*Rana esculenta*) were enzymatically dispersed by a combination of collagenase (Yakult) and trypsin (type XIII, Sigma) as described.(4) Frogs were killed by decapitation, the spinal cord was destroyed with a steel rod and the heart was then excised. The isolated cells were stored in storage Ringer solution, and kept at 4^{o}C until use (2-48 hours after dissociation). In some isolations, amino acids were omitted from the dissociation and storage solutions, with no change in the results.

Rat ventricular myocytes. Adult rat ventricular myocytes were obtained by retrograde perfusion from hearts of male Wistar rats (160-180 g) as previously described.(5,6) The myocytes were maintained at 37^{o}C until use.

Solutions. For electrophysiology, the control external solution contained (in mM): NaCl 107; HEPES 10; CsCl 20; $NaHCO_3$ 4; NaH_2PO_4 0.8; $MgCl_2$ 1.8; $CaCl_2$ 1.8; D-glucose 5; sodium pyruvate 5; tetrodotoxin $3x10^{-4}$ (for frog) or $6x10^{-3}$ (for rat); pH 7.4 adjusted with NaOH. Patch electrodes (0.6-1.5 Mohms) were filled with control internal solution which contained (in mM): CsCl 119.8; EGTA (acid form) 5; $MgCl_2$ 4; creatine phosphate disodium salt 5; Na_2ATP 3.1; Na_2GTP 0.42; $CaCl_2$ 0.062 (pCa 8.5); HEPES 10; pH 7.1 (frog) or 7.3 (rat)

adjusted with CsOH. Collagenase type A for rat cardiac myocyte dissociation and fetal calf serum were from Boehringer Mannheim (Germany). Collagenase for frog ventricular myocyte dissociation was from Yakult (Tokyo, Japan). DMEM was obtained from Gibco-BRL. Tetrodotoxin (TTX) was from Latoxan (Rosans, France). SR58611 was a generous gift of Sanofi Recherche (Montpellier, France). BRL37344 was a generous gift of SmithKine & Beecham (Saint-Grégoire, France). CGP12177 was a generous gift from Novartis Pharma (Rueil-Malmaison, France). L-748,337 was a generous gift from Merck & Co. Inc. (Rahway, NJ, USA). All other drugs were from Sigma Chemical Co. (St. Louis, USA). All drugs tested in patch-clamp experiments were solubilized in experimental solutions just before application onto the cell studied, i.e. only fresh solutions were tested.

Whole-cell current recording. The whole-cell configuration of the patch-clamp technique was used to record the high-threshold L-type Ca^{2+} current $I_{Ca,L}$ in Ca^{2+}-tolerant frog and rat ventricular myocytes. In the routine protocols the cells were depolarized every 8 s from a holding potential of -80 to 0 mV for 200 or 400 ms. In rat cardiomyocytes, the test pulse to 0 mV was preceded by a short pre-pulse (50 ms) to -50 mV. The prepulse and/or the application of TTX were used to eliminate fast sodium currents. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. Voltage-clamp pulses were generated and currents recorded using either a single (VP-500) or two separate patch-clamp amplifiers (RK400, Bio-Logic, Claix, France). In the latter case, when possible, two different nearby cells in the same dish were successively attached to two patch pipettes, whole-cell configuration was applied to both and two different $I_{Ca,L}$ currents were simultaneously recorded. Visual-Patch v.1.30 (Bio-Logic) or a home-made computer software were used to control all experimental parameters, cell stimulation and current recording. Recordings were low-pass filtered at 2 kHz and stored on the hard disc of an IBM-compatible computer.

Control and drug containing solutions were applied to the exterior of the cell by placing the cell at the opening of 300-μm inner diameter capillary tubes flowing at a rate of about 50 μl/min. Changes in extracellular solutions were automatically achieved using a rapid solution changer (RSC-200, Bio-Logic). All experiments were done at room temperature (19-25^{o}C), and the temperature did not vary by more than 1°C in a given experiment.

Data analysis. The maximal amplitude of whole-cell $I_{Ca,L}$ was measured as previously described.(4) Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was done for each membrane depolarization, peak and steady-state current values. The results are expressed as mean ± S.E.M. For statistical evaluation the paired and unpaired Student's *t*-test were used, and a difference was considered statistically significant when p was <0.05.

Supplementary References

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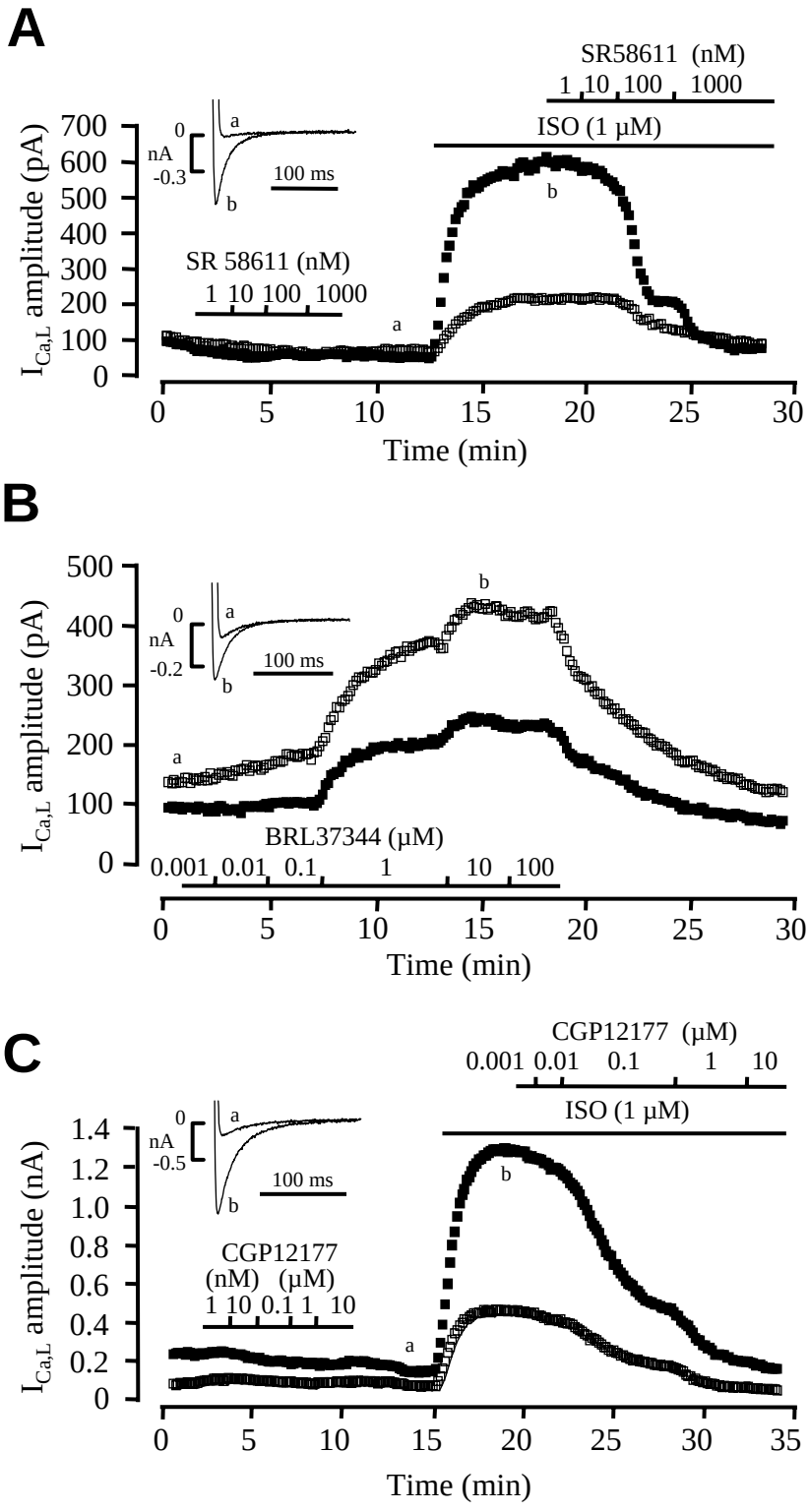
Supplementary Figure Legends

Supplementary Figure 1

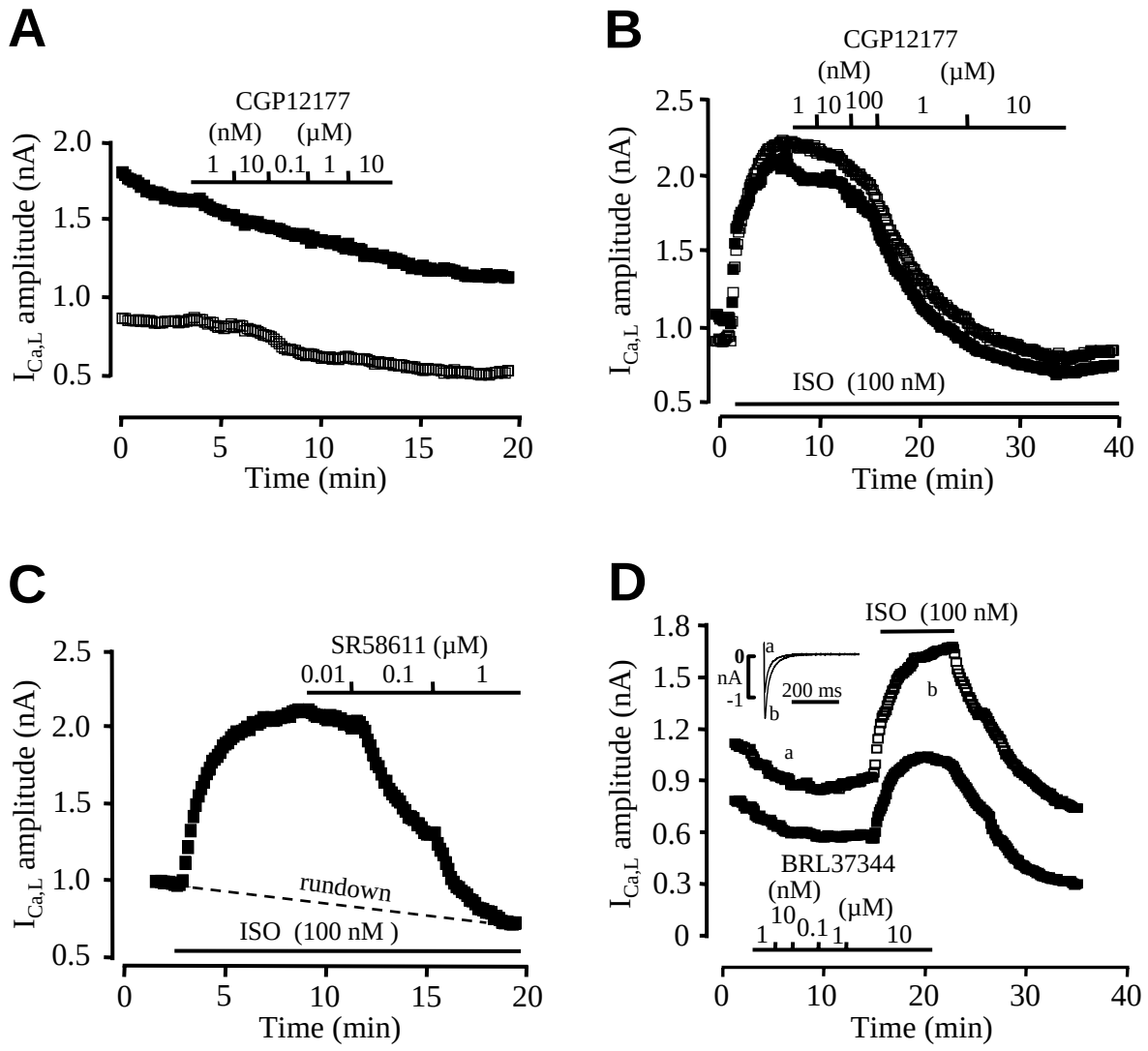
β_3-AR agonists do not stimulate $I_{Ca,L}$ in frog ventricular myocytes. Each experiment shows the time course of $I_{Ca,L}$ recorded in two frog myocytes which were simultaneously exposed to increasing concentrations of SR58611 **(A)**, BRL37344 **(B)** or CGP12177 **(C)**, or to the drugs after application of ISO (1 μM, **A** and **C**). The individual current traces were obtained in each experimental condition at the times indicated by the corresponding letters in the main graph.

Supplementary Figure 2

β_3-AR agonists do not stimulate $I_{Ca,L}$ in rat ventricular myocytes. In each experiment, $I_{Ca,L}$ was recorded in one **(C)** or two **(A, B, D)** rat myocytes which were simultaneously exposed to increasing concentrations of CGP12177 **(A, B)**, SR58611 **(C)** or BRL37344 **(D)** applied either in basal conditions **(A, D)** or in the presence of ISO (100 nM, **B** and **C**). The individual current traces in **D** were obtained at the times indicated by the corresponding letters in the main graph.



Supplemental Figure 1



Supplemental Figure 2