

## **$\beta_3$ -adrenergic receptor activation increases human atrial tissue contractility and stimulates the L-type $\text{Ca}^{2+}$ current**

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Research Article

Cardiology

$\beta_3$ -adrenergic receptor ( $\beta_3$ -AR) activation produces a negative inotropic effect in human ventricles. Here we explored the role of  $\beta_3$ -AR in the human atrium. Unexpectedly,  $\beta_3$ -AR activation increased human atrial tissue contractility and stimulated the L-type  $\text{Ca}^{2+}$  channel current ( $I_{\text{Ca,L}}$ ) in isolated human atrial myocytes (HAMs). Right atrial tissue specimens were obtained from 57 patients undergoing heart surgery for congenital defects, coronary artery diseases, valve replacement, or heart transplantation. The  $I_{\text{Ca,L}}$  and isometric contraction were recorded using a whole-cell patch-clamp technique and a mechanoelectrical force transducer. Two selective  $\beta_3$ -AR agonists, SR58611 and BRL37344, and a  $\beta_3$ -AR partial agonist, CGP12177, stimulated  $I_{\text{Ca,L}}$  in HAMs with nanomolar potency and a 60%–90% efficacy compared with isoprenaline. The  $\beta_3$ -AR agonists also increased contractility but with a much lower efficacy (~10%) than isoprenaline. The  $\beta_3$ -AR antagonist L-748,337,  $\beta_1$ -/ $\beta_2$ -AR antagonist nadolol, and  $\beta_1$ -/ $\beta_2$ -/ $\beta_3$ -AR antagonist bupranolol were used to confirm the involvement of  $\beta_3$ -ARs (and not  $\beta_1$ -/ $\beta_2$ -ARs) in these effects. The  $\beta_3$ -AR effects involved the cAMP/PKA pathway, since the PKA inhibitor H89 blocked  $I_{\text{Ca,L}}$  stimulation and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) strongly increased the positive inotropic effect. Therefore, unlike in ventricular tissue,  $\beta_3$ -ARs are positively coupled to L-type  $\text{Ca}^{2+}$  channels and contractility in human atrial tissues through a cAMP-dependent pathway.

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# $\beta_3$ -adrenergic receptor activation increases human atrial tissue contractility and stimulates the L-type $\text{Ca}^{2+}$ current

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**$\beta_3$ -adrenergic receptor ( $\beta_3$ -AR) activation produces a negative inotropic effect in human ventricles. Here we explored the role of  $\beta_3$ -AR in the human atrium. Unexpectedly,  $\beta_3$ -AR activation increased human atrial tissue contractility and stimulated the L-type  $\text{Ca}^{2+}$  channel current ( $I_{\text{Ca,L}}$ ) in isolated human atrial myocytes (HAMs). Right atrial tissue specimens were obtained from 57 patients undergoing heart surgery for congenital defects, coronary artery diseases, valve replacement, or heart transplantation. The  $I_{\text{Ca,L}}$  and isometric contraction were recorded using a whole-cell patch-clamp technique and a mechanoelectrical force transducer. Two selective  $\beta_3$ -AR agonists, SR58611 and BRL37344, and a  $\beta_3$ -AR partial agonist, CGP12177, stimulated  $I_{\text{Ca,L}}$  in HAMs with nanomolar potency and a 60%–90% efficacy compared with isoprenaline. The  $\beta_3$ -AR agonists also increased contractility but with a much lower efficacy (~10%) than isoprenaline. The  $\beta_3$ -AR antagonist L-748,337,  $\beta_1$ -/ $\beta_2$ -AR antagonist nadolol, and  $\beta_1$ -/ $\beta_2$ -/ $\beta_3$ -AR antagonist bupranolol were used to confirm the involvement of  $\beta_3$ -ARs (and not  $\beta_1$ -/ $\beta_2$ -ARs) in these effects. The  $\beta_3$ -AR effects involved the cAMP/PKA pathway, since the PKA inhibitor H89 blocked  $I_{\text{Ca,L}}$  stimulation and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) strongly increased the positive inotropic effect. Therefore, unlike in ventricular tissue,  $\beta_3$ -ARs are positively coupled to L-type  $\text{Ca}^{2+}$  channels and contractility in human atrial tissues through a cAMP-dependent pathway.**

## Introduction

In cardiac myocytes,  $\text{Ca}^{2+}$  current through the L-type  $\text{Ca}^{2+}$  channels ( $I_{\text{Ca,L}}$ ) provides  $\text{Ca}^{2+}$  for the activation of the contractile apparatus and is a crucial determinant of cardiac contractile activity (1). Among several regulatory pathways that control cardiac  $\text{Ca}^{2+}$  channel activity, the best described is the  $\beta$ -adrenergic stimulation of  $I_{\text{Ca,L}}$ , which contributes to the positive inotropic and part of the positive chronotropic effects of catecholamines (1, 2). Although to date, 3 types of  $\beta$ -adrenergic receptors ( $\beta$ -ARs) have been cloned,  $\beta_1$ -,  $\beta_2$ -, and  $\beta_3$ -ARs, respectively, the effect of catecholamines in human heart is generally attributed to  $\beta_1$ - and  $\beta_2$ -ARs, with opposite effects on hypertrophy (3, 4) and apoptosis (5, 6) and with a respective contribution of each receptor subtype that varies significantly depending on the cardiac tissue, the pathophysiological state, the age, or the developmental stage (7). However, expression of a  $\beta_3$ -AR in human myocardium was also demonstrated both at the mRNA (8, 9) and protein level (9–12).

Like  $\beta_1$ - and  $\beta_2$ -ARs,  $\beta_3$ -ARs are positively coupled to adenylyl cyclase in fat and colon as well as in CHO cells transfected with the cloned receptor (13–15). Therefore, activation of  $\beta_3$ -ARs in human heart would be expected to stimulate  $I_{\text{Ca,L}}$  and increase contractility. However,  $\beta_3$ -AR agonists were shown to inhibit contractility in human endomyocardial biopsies from transplanted hearts (8, 16) and in left ventricular samples from failing and

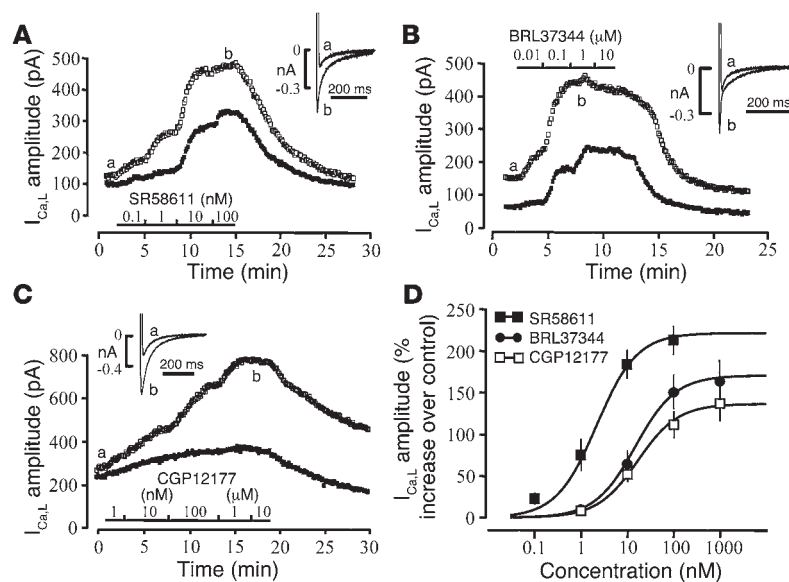
nonfailing explanted hearts (11), an effect mediated by  $\text{G}_{\text{i/o}}$  proteins and NO production via activation of endothelial type-3 NO synthase (8, 16, 17). This effect was accompanied by a shortening of the action potential (8, 18). A similar finding was obtained with BRL37344, a  $\beta_3$ -AR agonist, in Langendorff-perfused isolated guinea pig hearts (19) and was accompanied by a decrease in  $\text{Ca}^{2+}$  transients. The NO dependency of  $\beta_3$ -AR action was also found in mice, using animals with a homozygous deletion mutation of the  $\beta_3$ -AR ( $\text{ADRB3}^{-/-}$ ) (20). While NOS3 inhibitors (arginine analogues) potentiated the positive inotropic effect of isoprenaline (ISO) in wild-type mice, this effect was absent in either  $\text{ADRB3}^{-/-}$  or  $\text{NOS3}^{-/-}$  mice (20). In another study, ISO applied to a culture of neonatal cardiomyocytes isolated from mice with a double homozygous deletion mutation of the  $\beta_1$ -AR and  $\beta_2$ -AR ( $\text{ADRB1}^{-/-}/\text{ADRB2}^{-/-}$ ) was found to slightly decrease spontaneous beating frequency, a phenomenon mimicked by CL316243, a  $\beta_3$ -AR agonist (21). Moreover, this negative chronotropic effect of ISO turned into a positive one after treatment of the myocytes with Bordetella pertussis toxin (21), confirming the original finding that  $\beta_3$ -AR signaling involves activation of  $\text{G}_{\text{i/o}}$  proteins (8).

Although the case seems rather strong for a negative inotropic and chronotropic effect of  $\beta_3$ -ARs, a number of studies are inconsistent with such hypothesis. For instance, several reports demonstrate the absence of a functional  $\beta_3$ -AR in mouse heart (22–24). Cardiac overexpression of human  $\beta_3$ -ARs in mice results in  $\text{G}_{\text{s}}$  activation of adenylyl cyclase and positive inotropy on stimulation with a  $\beta_3$ -AR agonist (25). In human atrium and failing ventricle, several  $\beta_3$ -AR agonists were found to be devoid of any cardiodepressant effect (26–28). These latter findings are of importance, since the amount of  $\beta_3$ -AR proteins was shown to increase up to 3-fold in different models of heart failure (11, 29, 30).

**Nonstandard abbreviations used:**  $\beta$ -AR,  $\beta$ -adrenergic receptor; HAM, human atrial myocyte; IBMX, 3-isobutyl-1-methylxanthine;  $I_{\text{Ca,L}}$ , L-type  $\text{Ca}^{2+}$  channel current;  $I_{\text{Ks}}$ , delayed rectifier potassium current; ISO, isoprenaline; L-NMMA,  $\text{N}^{\text{G}}$ -monomethyl-L-arginine; PDE, phosphodiesterase.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

$\beta_3$ -AR stimulation of  $I_{Ca,L}$  in HAMs. Each experiment shows the time course of  $I_{Ca,L}$  amplitude recorded in 2 HAMs that were simultaneously exposed to increasing concentrations of SR58611 (A), BRL37344 (B), and CGP12177 (C). Each symbol indicates a net amplitude of  $I_{Ca,L}$  measured every 8 seconds at 0 mV membrane potential. The individual current traces were obtained in each experimental condition at the times indicated by the corresponding letters in the main graph. (D) Concentration-response curve for the effects of the 3 agonists on  $I_{Ca,L}$ . The points show the mean  $\pm$  SEM of 6 (SR58611, filled square), 10 (BRL37344, filled circle), or 8 (CGP12177, open square) cells.

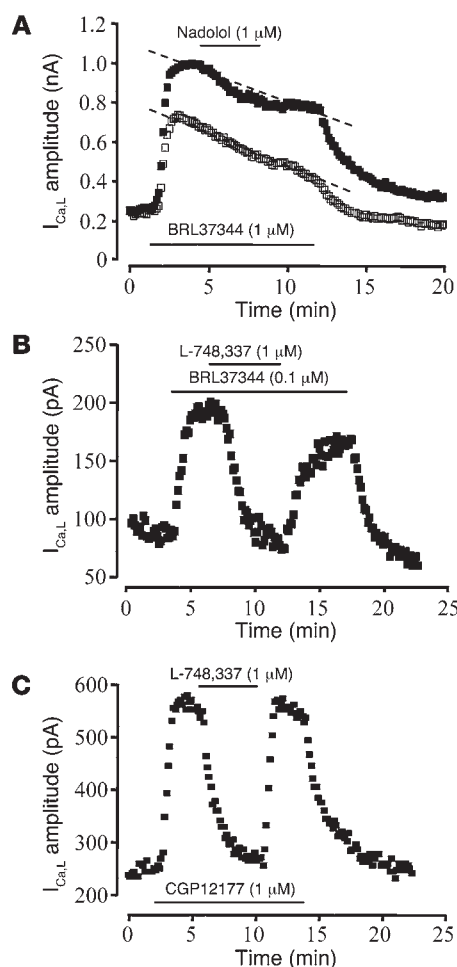
In this study, our aim was to get further insights into the role of  $\beta_3$ -ARs on human cardiac function at the single-cell level. For this, we examined the effects of several  $\beta_3$ -AR ligands on  $I_{Ca,L}$  recorded in isolated human atrial myocytes (HAMs): SR58611 (31) and BRL37344 (32), which are 2  $\beta_3$ -AR agonists; CGP12177, which is a partial  $\beta_3$ -AR agonist (33) as well as a  $\beta_2$ -AR antagonist and a partial agonist at  $\beta_1$ -AR through a low-affinity site (34, 35); and L-748,337, which is a  $\beta_3$ -AR antagonist (36). These effects were compared with those obtained on the contractility of human atrial strips.

## Results

**$\beta_3$ -AR agonists stimulate  $I_{Ca,L}$  in HAMs.** To examine the regulation of  $I_{Ca,L}$  by  $\beta_3$ -ARs in HAMs, we used 2  $\beta_3$ -AR agonists, SR58611 (31) and BRL37344 (32), as well as CGP12177, which is a  $\beta_3$ -AR partial agonist (33), a  $\beta_2$ -AR antagonist, and a partial agonist at  $\beta_1$ -AR through a low-affinity site (34, 35). Typical experiments are shown in Figure 1. In each example, 2 cells were simultaneously exposed to increasing concentrations of SR58611 (Figure 1A), BRL37344 (Figure 1B), and CGP12177 (Figure 1C). Such double-cell experiments provided a simple way to evaluate cell-to-cell variability, since all external parameters (temperature, perfusion, time after isolation, etc.) were strictly identical during the recordings obtained in the 2 cells. As can be seen in Figure 1, all compounds produced a dose-dependent and reversible increase in  $I_{Ca,L}$ . The cumulative dose-response curves shown in Figure 1D were fitted to the Michaelis equation to derive the maximal stimulation ( $E_{max}$ ) of  $I_{Ca,L}$  and the concentration required for half-maximal effect ( $EC_{50}$ ) for each agonist. Thus,  $E_{max}$  and  $EC_{50}$  values, respectively, were as follows:  $221\% \pm 20\%$  and  $2.5 \pm 0.8$  nM ( $n = 6$ ) for SR58611;  $172\% \pm 26\%$  and  $15.2 \pm 0.6$  nM ( $n = 10$ ) for BRL37344; and  $136\% \pm 21\%$  and  $17.1 \pm 3.0$  nM ( $n = 8$ ) for CGP12177 (Figure 1D). The efficacy of SR58611 and BRL37344 on  $I_{Ca,L}$  was comparable to that of ISO ( $241\% \pm 47\%$  at 1  $\mu$ M ISO;  $n = 5$ ), while that of CGP12177 was somewhat smaller. None of the 3  $\beta_3$ -AR agonists tested had any stimulatory effect in the nanomolar range of concentrations on  $I_{Ca,L}$  measured in ventricular myocytes isolated from frog and rat hearts (Supplemental Results; supplemental material available online with this article; doi:10.1172/JCI32519DS1).

**$\beta_3$ -AR agonists increase  $I_{Ca,L}$  in HAMs via  $\beta_3$ -AR activation.** The subtype selectivity of  $\beta$ -AR ligands is always questionable. For instance, SR58611 and BRL37344 were shown to also activate  $\beta_1$ - and  $\beta_2$ -ARs in the micromolar range of concentrations (13), and CGP12177 was shown to be an agonist of  $\beta_1$ -AR at a low-affinity site (34, 35). Since human cardiomyocytes also possess  $\beta_1$ - and  $\beta_2$ -ARs, we examined the possibility that the stimulatory effects of the 3 agonists on  $I_{Ca,L}$  in HAMs were caused by activation of  $\beta_1$ - and/or  $\beta_2$ -ARs. To do this, we examined whether the  $\beta_1$ -AR/ $\beta_2$ -AR antagonist nadolol was able to antagonize the stimulatory effect of SR58611 and BRL37344. Because the  $K_d$  for nadolol on the human  $\beta_3$ -AR is more than 600 nM as compared with 14–40 nM on the  $\beta_1$ - and  $\beta_2$ -ARs (13), most experiments were performed using 200 nM nadolol. At this concentration, nadolol antagonized some of the stimulatory effect of SR58611 (1  $\mu$ M; data not shown) on  $I_{Ca,L}$ , indicating that the effect of this agonist was at least partly mediated by activation of  $\beta_1$ - and/or  $\beta_2$ -ARs. However, even a 5-fold higher concentration of nadolol (1  $\mu$ M) had no effect on BRL37344-induced (1  $\mu$ M) stimulation of  $I_{Ca,L}$  ( $5.4\% \pm 3.9\%$ ;  $n = 4$ ;  $P > 0.05$ ; Figure 2A). In contrast, the effect of BRL37344 was almost completely blocked (by  $88.5\% \pm 5.5\%$ ;  $n = 5$ ;  $P < 0.001$ ) by 1  $\mu$ M of the  $\beta_3$ -AR antagonist L-748,337 (36, 37) (Figure 2B). Interestingly, L-748,337 also almost completely antagonized the stimulatory effect of CGP12177 on  $I_{Ca,L}$  (by  $87.9\% \pm 4.4\%$ ;  $n = 4$ ;  $P < 0.01$ ; Figure 2C), which excludes the possibility that CGP12177 was acting via the low-affinity site of the  $\beta_1$ -AR (34, 35). As seen in Figure 2, B and C, the effect of L-748,337 was fully reversible. In another series of experiments, bupranolol (1  $\mu$ M), which combines  $\beta_1$ -/ $\beta_2$ -/ $\beta_3$ -AR antagonistic properties (38), was found to strongly antagonize the stimulatory effect of 1  $\mu$ M CGP12177 (by  $79.3\% \pm 1.6\%$ ;  $n = 5$ ;  $P < 0.05$ ). Altogether, these results indicate that at the concentrations used the stimulatory effects of BRL37344, CGP12177, and, to a large extent, SR58611 on  $I_{Ca,L}$  in HAMs result from activation of  $\beta_3$ -ARs.

**NO pathway is not involved in the  $\beta_3$ -AR regulation of  $I_{Ca,L}$ .** In human ventricle,  $\beta_3$ -AR agonists were shown to produce a negative inotropic effect as a consequence of NOS3 activation (17). Through an activation of the soluble guanylyl cyclase and accumulation of



intracellular cGMP, NO can lead to opposite effects on human atrial  $I_{Ca,L}$ : a stimulation at low concentrations due to inhibition of the cGMP-inhibited cAMP-phosphodiesterase (PDE3) (39, 40) and an inhibition at higher concentrations due to activation of the cGMP-stimulated phosphodiesterase (PDE2) (39, 40). To evaluate the contribution of the NO/cGMP pathway in the regulation of  $I_{Ca,L}$  by  $\beta_3$ -AR agonists, we examined the effect of CGP12177 in the presence of the NOS inhibitor N<sup>G</sup>-monomethyl-L-arginine (L-NMMA). As shown in Figure 3A, the presence of L-NMMA (1 mM) in both the extracellular and pipette solutions did not modify the stimulatory effect of CGP12177 (1  $\mu$ M) on  $I_{Ca,L}$  (with L-NMMA, 65%  $\pm$  11% over control,  $n = 4$ ; without L-NMMA, 58%  $\pm$  4% over control,  $n = 4$ ). This indicates that the NO/cGMP pathway is not required for the  $\beta_3$ -AR stimulation of  $I_{Ca,L}$ . To examine whether  $\beta_3$ -AR stimulation can lead to inhibitory effects via this pathway, HAMs were first exposed to serotonin (100 nM), which strongly increased  $I_{Ca,L}$  (by 154%  $\pm$  64%;  $n = 3$ ; Figure 3B) via

### Figure 3

NO pathway is not involved in the stimulation of  $I_{Ca,L}$  by  $\beta_3$ -AR agonists. In each experiment,  $I_{Ca,L}$  was recorded in a single HAM exposed to CGP12177 in the presence of either 1 mM L-NMMA in intracellular and extracellular solutions (A) or serotonin (100 nM, B). The dotted line in B indicates spontaneous rundown.

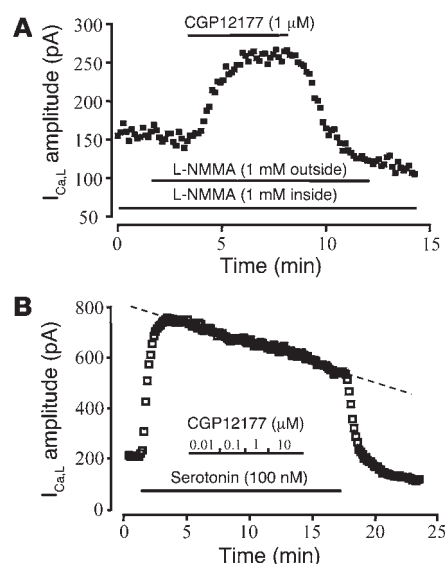
### Figure 2

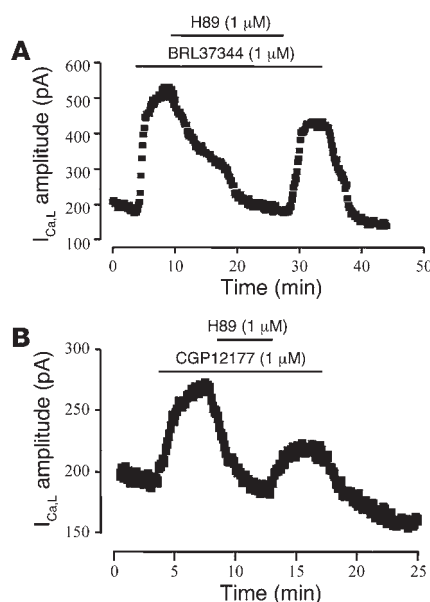
Effects of  $\beta_3$ -AR and  $\beta_1$ -/ $\beta_2$ -AR antagonists on  $\beta_3$ -AR stimulation of  $I_{Ca,L}$  in HAMs. In each experiment,  $I_{Ca,L}$  was recorded in 1 (B and C) or 2 (A) HAMs, which were simultaneously exposed to BRL37344 (1  $\mu$ M, A; 0.1  $\mu$ M, B) or CGP12177 (1  $\mu$ M, C) and then to the  $\beta_1$ -/ $\beta_2$ -AR antagonist nadolol (1  $\mu$ M, A) or to the  $\beta_3$ -AR antagonist L-748,337 (1  $\mu$ M, B and C). The dotted lines in A indicates spontaneous rundown.

activation of the 5-HT<sub>4</sub> receptor (41), and then to CGP12177 in the presence of serotonin. As shown in Figure 3B, CGP12177, at concentrations ranging from 0.01 to 10  $\mu$ M, had no inhibitory effect on prestimulated  $I_{Ca,L}$  (101%  $\pm$  1% of serotonin level;  $n = 3$ ). These experiments indicate that the NO/cGMP pathway is not involved in the activation of  $I_{Ca,L}$  by  $\beta_3$ -AR agonists, and that  $\beta_3$ -AR stimulation does not activate a parallel inhibitory pathway regulating  $I_{Ca,L}$ .

$\beta_3$ -AR activation of  $I_{Ca,L}$  involves cAMP-dependent protein kinase. Because cAMP-dependent PKA is a classic activator of L-type Ca<sup>2+</sup> channels and accounts for the stimulatory effects of  $\beta_1$ - and  $\beta_2$ -AR agonists on  $I_{Ca,L}$ , we examined whether PKA was also involved in the  $\beta_3$ -AR activation of  $I_{Ca,L}$ . Figure 4 shows 2 individual experiments in which the cell permeant PKA inhibitor H89 (42) was applied to HAMs on top of BRL37344 (1  $\mu$ M; Figure 4A) or CGP12177 (1  $\mu$ M; Figure 4B). In both cases, H89 (1  $\mu$ M) completely inhibited the stimulatory effect of the  $\beta_3$ -AR agonist. The inhibitory effect of H89 was mostly reversible upon washout. On average, BRL37344 and CGP12177 increased  $I_{Ca,L}$  by 297%  $\pm$  84% ( $n = 4$ ) and 45%  $\pm$  6% ( $n = 4$ ), respectively, in the absence of H89, and the current amplitude differed from basal level by only 3%  $\pm$  6% ( $n = 4$ ) and -7%  $\pm$  3% ( $n = 4$ ) in the presence of the PKA inhibitor. These experiments indicate that  $\beta_3$ -AR agonists increase  $I_{Ca,L}$  in HAMs through activation of the cAMP/PKA pathway.

$\beta_3$ -AR agonists increase force of contraction in human atrial trabeculae. In cardiac myocytes,  $I_{Ca,L}$  provides Ca<sup>2+</sup> for the activation of the contractile proteins and is a crucial determinant of the cardiac contractile activity. Therefore, any modulation of  $I_{Ca,L}$  amplitude might be expected to induce parallel changes in contractile amplitude. Since our results so far indicate that  $\beta_3$ -ARs are positively coupled to  $I_{Ca,L}$  in HAMs, additional experiments were performed





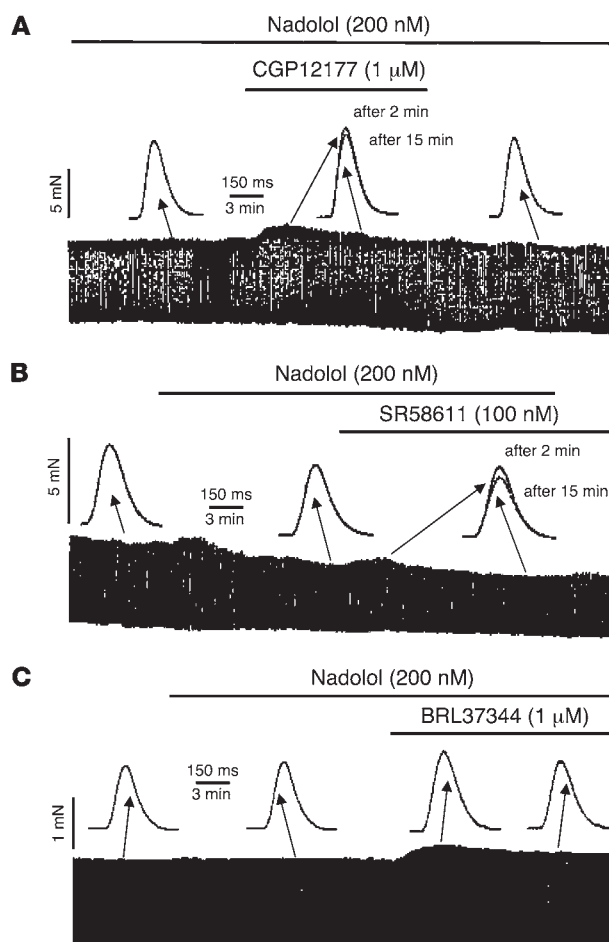
**Figure 4**

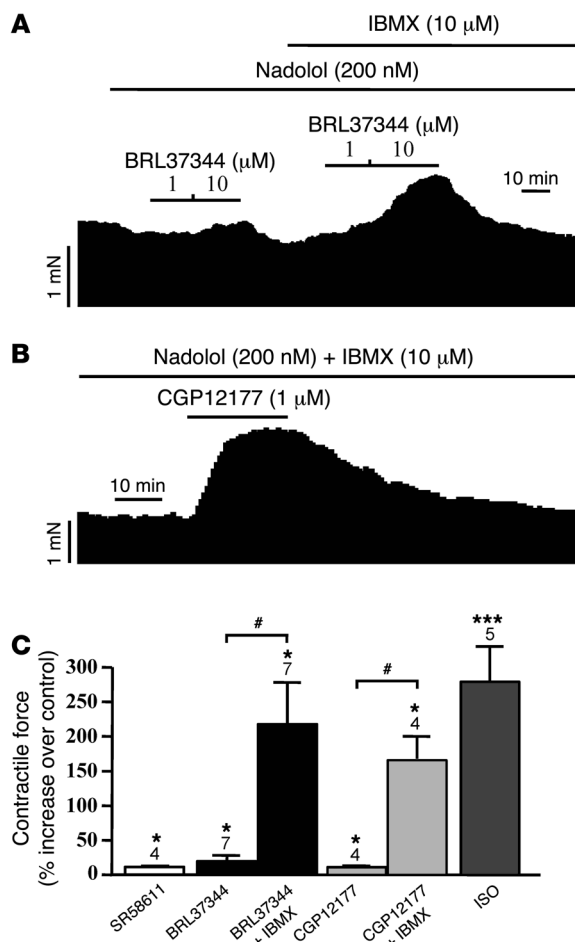
PKA mediates the stimulation of  $I_{Ca,L}$  by  $\beta_3$ -AR agonists.  $I_{Ca,L}$  was recorded in a single HAM exposed to either 1  $\mu$ M BRL3744 (A) or 1  $\mu$ M CGP12177 (B). When the current was at its maximal stimulation, the PKA inhibitor H89 (1  $\mu$ M) was added to the extracellular solutions in the presence of the  $\beta_3$ -AR agonists, and the current returned to basal levels.

to evaluate the effects of  $\beta_3$ -AR agonists on the force of contraction in trabeculae isolated from human atrial tissues. To eliminate any possible interaction of the agonists with  $\beta_1$ - and/or  $\beta_2$ -ARs (43), the experiments were performed in the presence of nadolol (200 nM), which alone reduced atrial basal contractile amplitude to  $82\% \pm 9\%$  ( $n = 8$ ) of control level. The individual experiments shown in Figure 5 indicate that CGP12177 (1  $\mu$ M; Figure 5A), SR58611 (100 nM; Figure 5B), and BRL37344 (1  $\mu$ M; Figure 5C) induced a modest and transient positive inotropic effect in human atrial muscle. A 10%–20% peak stimulation was observed with all 3 compounds at approximately 2 minutes after drug application, and a steady state was reached at approximately 15 minutes. The summary data in Figure 6C indicate that all 3  $\beta_3$ -AR agonists induced a significant increase in contractile amplitude at 2 minutes in human atrium, but only BRL37344 maintained a significant positive inotropic effect at 15 minutes ( $11.7\% \pm 1.7\%$ ;  $n = 6$ ;  $P < 0.05$ ). In contrast, the effect of ISO (1  $\mu$ M) was more prominent (Figure 6C). Because the  $\beta_3$ -AR stimulation of  $I_{Ca,L}$  was mediated by PKA, and because different  $G_s$ -coupled receptors generate heterogeneous cAMP signals due to specific functional coupling of phosphodiesterase (PDE) isoforms (44, 45), we questioned whether the modest effect on contractility was due to a limited diffusion of cAMP that might result from a high PDE activity. If this is the case, then PDE inhibition should potentiate the contractile effects of  $\beta_3$ -AR agonists. We tested this hypothesis by reevaluating the contractile effects of  $\beta_3$ -AR agonists in the presence of 10  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX), a wide-spectrum PDE inhibitor. As shown in Figure 6,

**Figure 5**

Effect of  $\beta_3$ -AR ligands on contractility in human atrial trabeculae. CGP12177 (A), SR58611 (B), or BRL37344 (C) were applied to human atrial preparations at the concentrations indicated, after 15 minutes of perfusion with 200 nM nadolol followed by the continuous presence of nadolol. The individual contractile traces were obtained in each experimental condition at the times indicated by the corresponding arrows. All 3 compounds significantly ( $P < 0.05$ ) increased force of contraction.





large increase in  $I_{Ca,L}$  (49), which is very unlikely at the nanomolar concentrations of  $\beta_3$ -AR agonists used here. Besides, the effects of SR58611, BRL37344, and CGP12177 on  $I_{Ca,L}$  resembled their effects in  $\beta_3$ -AR adenylyl cyclase assays (13), with SR58611 and BRL37344 behaving as full agonists (by comparison with ISO) and CGP12177 only as partial agonist (Figure 1D) (13, 33). With respect to CGP12177, some caution may be required because the drug is not only a  $\beta_3$ -AR agonist and a  $\beta_2$ -AR antagonist, but is also a partial agonist at  $\beta_1$ -AR through a low-affinity site (34, 35). However, the stimulatory effect of CGP12177 on  $I_{Ca,L}$  (like that of BRL37344) was blocked by L-748,337, a highly potent and selective  $\beta_3$ -AR antagonist (36), at a concentration that had no effect on the stimulation of the current by ISO (data not shown). Moreover, the effect of CGP12177 on  $I_{Ca,L}$  was inhibited by the  $\beta_1$ -/ $\beta_2$ -/ $\beta_3$ -AR antagonist bupranolol but not by the  $\beta_1$ -/ $\beta_2$ -AR antagonist nadolol. Therefore, we are confident that the effects of the agonists tested involved  $\beta_3$ -AR stimulation rather than nonselective  $\beta_1$ - or  $\beta_2$ -AR stimulation and conclude that  $\beta_3$ -AR activation leads to stimulation of the  $I_{Ca,L}$  and contractile activity in human atrium.

Although a number of studies have demonstrated the expression of  $\beta_3$ -ARs in human ventricular (9–12) and atrial tissue (10), little is known about their function in human heart at the single-cell level. A single abstract reports on the lack of  $\beta_3$ -AR modulation of contractile function in human ventricular myocytes (28). Reports on the effects of  $\beta_3$ -AR agonists in isolated cardiomyocytes from other species are limited to 6 studies (29, 50–54). In 3 of these, the

**Figure 6**

PDE inhibition potentiates the effects of  $\beta_3$ -AR agonists on contractility in human atrial trabeculae. BRL37344 (**A**) or CGP12177 (**B**) were applied to human atrial preparations at the concentrations indicated, in the presence of 200 nM nadolol and in the absence (**A**) or presence (**A** and **B**) of the PDE inhibitor IBMX (10  $\mu$ M). (**C**) Summary of the effects of the  $\beta_3$ -AR agonists on contractility. The effects of SR58611 (100 nM), BRL37344 (10  $\mu$ M), and CGP12177 (1  $\mu$ M), with or without IBMX (10  $\mu$ M), are compared with that of ISO (1  $\mu$ M). The bars show the mean  $\pm$  SEM of the number of experiments indicated above the bars. \* $P$  < 0.05; \*\*\* $P$  < 0.005 versus control. ANOVA followed by post-hoc Bonferroni's test was used to compare the effects of the  $\beta_3$ -AR agonists in the absence or presence of IBMX. # $P$  < 0.05.

effect of a  $\beta_3$ -AR activation was investigated on  $I_{Ca,L}$  in ventricular myocytes isolated from dog (29), rat (53), and guinea pig hearts (50) using BRL37344 as a  $\beta_3$ -AR agonist. In these studies, the drug induced a small, approximately 20%, maximal reduction of basal  $I_{Ca,L}$ , and the effect was increased to approximately 30% when the myocytes were isolated from failing hearts (29, 53). Surprisingly, the effects of BRL37344 persisted after washout of the drug, which is a concern when considering that  $I_{Ca,L}$  is subjected to substantial spontaneous rundown in mammalian cardiomyocytes (55, 56). However, the inhibitory effects of BRL37344 were attenuated by preincubation of the cells with the NOS inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) (50, 53) or after treatment of the myocytes with Bordetella pertussis toxin (53), which is consistent with earlier findings that a  $\beta_3$ -AR signaling cascade involved  $G_{i/o}$  protein and NOS3 activation (8, 16, 17). In our experiments, after rundown subtraction, neither BRL37344, SR58611, or CGP12177, in the range of concentrations at which these ligands act selectively via  $\beta_3$ -ARs (13), had any significant effect on basal  $I_{Ca,L}$  in rat ventricular myocytes (Supplemental Figure 2), while they all stimulated the current in HAMs.

The  $\beta_3$ -AR stimulatory effect on  $I_{Ca,L}$  was not due to NOS activation, because the effect persisted in the presence of L-NMMA. The effect was not mediated by  $G_{i/o}$  proteins, since none of the  $\beta_3$ -AR agonists tested had any effect on  $I_{Ca,L}$  prestimulated by either serotonin or forskolin, while  $G_{i/o}$  protein activation via muscarinic M2 receptors inhibits the current under similar conditions (57). On the contrary, the  $\beta_3$ -AR stimulatory effect on  $I_{Ca,L}$  involved PKA activation, since it was blocked by H89, a cell-permeant PKA inhibitor (42). Therefore, when combining our results in human, rat, and frog cardiomyocytes (Supplemental Figure 1) with those from earlier studies in rat, guinea pig, and dog heart,  $\beta_3$ -AR regulation of  $I_{Ca,L}$  appears clearly different in human heart from any other species explored so far.

Our results raise an interesting possibility that the  $\beta_3$ -AR can change its signaling pathway from a stimulatory,  $G_s$ -mediated cAMP/PKA mechanism to an inhibitory,  $G_{i/o}$ -mediated one, depending on the cellular context. Some support for this hypothesis comes from heterologous  $\beta_3$ -AR expression studies. For instance, in CHO cells (14, 58) and murine 3T3-F442A preadipocytes (59) transfected with rat  $\beta_3$ -ARs, the receptors are coupled to  $G_s$  proteins, leading to an increase in intracellular cAMP level. However, in rat and mice adipocytes, in addition to their coupling to  $G_s$ ,  $\beta_3$ -ARs couple to  $G_{i/o}$  proteins and inhibit adenylyl cyclase (60, 61). In the human A549 lung epithelial cell line and in the human colonic carcinoma cell line T84,  $\beta_3$ -ARs appear to be exclusively linked to  $G_{i/o}$  proteins (18, 62). Functional response to  $\beta_3$ -AR



**Table 1**  
Clinical characteristics of patients

|                                              |       |
|----------------------------------------------|-------|
| <b>Patient data</b>                          |       |
| Age range, years                             | 32–81 |
| Mean age, years                              | 62    |
| Female, <i>n</i>                             | 15    |
| Male, <i>n</i>                               | 42    |
| Total, <i>n</i>                              | 57    |
| <b>Surgical intervention</b>                 |       |
| Aortic valve surgery <sup>A</sup> , <i>n</i> | 13/57 |
| Mitral valve surgery <sup>A</sup> , <i>n</i> | 14/57 |
| CABG surgery <sup>A</sup> , <i>n</i>         | 42/57 |
| <b>Treatment regimens</b>                    |       |
| Beta blockers                                | 33/57 |
| Calcium antagonists                          | 3/57  |
| ACE inhibitors/angiotensin receptor blockers | 25/57 |
| Antiarrhythmic agents                        | 2/57  |
| Diuretics                                    | 10/57 |
| Nitrates                                     | 21/57 |

<sup>A</sup>Some patients underwent both valve surgery and coronary artery bypass graft (CABG) surgery.

activation may also depend on the amount of expressed receptors. Indeed, cardiac overexpression of human  $\beta_3$ -ARs in mice results in  $G_s$  activation of adenylyl cyclase and positive inotropy on stimulation with a  $\beta_3$ -AR agonist (25). Thus, if the human atrium expresses a higher amount of  $\beta_3$ -ARs than the ventricle (which would go in line with the higher amount of spare  $\beta_1$ -/ $\beta_2$ -ARs in the atrium; ref. 49), it might favor a  $G_s$ - over  $G_{i/o}$ -mediated signaling pathway. Finally, depending on its cellular environment, the  $\beta_3$ -AR might either activate or inhibit the same ion channel, such as the L-type  $Ca^{2+}$  channel. Such a situation has been reported for the  $\beta_3$ -AR regulation of the slow component of the delayed rectifier potassium current ( $I_{Ks}$ ), a current which like  $I_{Ca,L}$  is activated by cAMP/PKA cascade (63). Indeed, in isolated guinea pig ventricular myocytes,  $\beta_3$ -AR activation by BRL37344 inhibits  $I_{Ks}$  by 20%–40% (51). However, when KvLQT1/MinK channels, which underline  $I_{Ks}$  current, are heterologously coexpressed with the human  $\beta_3$ -AR in *Xenopus* oocytes, activation of the receptor increases  $I_{Ks}$  (64).

Although  $\beta_3$ -AR activation increased  $I_{Ca,L}$  in HAMs, the positive inotropic effects in human atrium were modest as compared with  $\beta_1$ -/ $\beta_2$ -AR activation with ISO. Because contractility of a trabeculum is a more integrated response than  $I_{Ca,L}$  amplitude in a single myocyte, many other parameters will participate in the inotropic response to  $\beta_3$ -AR agonists. One of these is the action potential duration, which is affected not only by  $\beta_3$ -AR regulation of  $I_{Ca,L}$  but also by the effect of the receptor on other ion channels, such as  $I_{Ks}$  (51), the rapid component of the delayed rectifier potassium current ( $I_{Kr}$ ) (52), the hyperpolarization-activated pacemaker current  $I_f$  (54), and the cystic fibrosis transmembrane conductance regulator (CFTR)  $Cl^-$  current (18). Other downstream factors include intracellular  $Ca^{2+}$  transient and sarcoplasmic reticulum function and the phosphorylation status of key proteins such as phospholamban, ryanodine receptor, and contractile proteins. Finally, human cardiac trabeculae are multicellular preparations composed of myocytes and nonmyocytes such as endothelial cells, which also express  $\beta_3$ -ARs (65, 66). Of note,  $\beta_3$ -ARs in endothelial cells activate NO production that may reduce cardiac contractil-

ity (67, 68) and counterbalance a stimulatory effect of  $\beta_3$ -ARs in myocytes. We tested this hypothesis in 4 human atrial trabeculae exposed to CGP12177 (100 nM) in the presence or absence of L-NMMA and found that NOS inhibition increased approximately 2-fold the positive inotropic effect of the agonist in atrium ( $P < 0.05$ ; data not shown). Therefore, the overall effect of a  $\beta_3$ -AR activation on the contractility of human atrium may vary from a stimulation to an inhibition, depending on the chamber, the amount of nonmyocyte endothelial and endocardial cells, and the activity of NOS enzymes. Another level of complexity may result from the ability of the myocyte to distinguish among different stimuli acting on a common signaling cascade. Indeed, even though  $\beta_1$ -,  $\beta_2$ -, and  $\beta_3$ -ARs may all activate the cAMP/PKA pathway, the signaling cascade generated by each receptor may be confined to distinct intracellular compartments, which may result in different functional responses (45). A number of studies, including our own (44, 69), have underlined the importance of cAMP PDEs in generating and maintaining intracellular cAMP domains (45). Our findings that PDE inhibition with IBMX transformed an unimpressively small contractile response to  $\beta_3$ -AR agonists to a large, positive inotropic effect similar to a  $\beta_1$ -AR response strongly suggest that not all cAMP/PKA effectors are normally activated by  $\beta_3$ -AR activation. A similar observation was recently reported for the serotonin 5-HT<sub>4</sub> receptors in failing human ventricular muscle, where these receptors increased contractility only when PDE activity was blocked (70).

In summary, while  $\beta_3$ -AR activation produces negative inotropic effects in human endomyocardial biopsies from transplanted hearts (8, 16) and in left ventricular samples from failing and non-failing explanted hearts (11, 16) (see also refs. 26–28), our study demonstrates that  $\beta_3$ -AR activation increases contractility in human atrial tissue. This is reminiscent of the contractile effects of the serotonin 5-HT<sub>4</sub> receptors (71), which are also coupled to increases in force of contraction (72) or  $I_{Ca,L}$  (41) in atria but not in healthy ventricles. In human ventricle, the negative inotropic effect of  $\beta_3$ -AR activation was proposed to spare the heart of excessive oxygen demand and may therefore play a role as a “safety-valve” during intense adrenergic stimulation (16). The identification of an opposite effect of  $\beta_3$ -ARs in human atrium is therefore of importance, particularly in the scope of the development of new therapeutic agents acting on these receptors for the control of the adrenergic system in cardiovascular diseases. Finally, the positive coupling of  $\beta_3$ -ARs to  $I_{Ca,L}$  in human atrium, with relatively modest consequences on force of contraction, might confer to these receptors a role in the control of beat frequency. During heart failure, the expression level of  $\beta_3$ -ARs increases (11, 29, 30), and this might change their beneficial effect into a deleterious one, with a persistent negative inotropic effect enhancing myocardial depression. It remains to be determined whether the level of  $\beta_3$ -ARs and their functional role are also modified in human atrial pathologies such as atrial dilation, arrhythmias, or fibrillation. This is particularly relevant, since congestive heart failure and atrial fibrillation are commonly encountered together.

## Methods

Our investigations conform with the local ethics committee (Comité Régional d’Ethique en matière d’Expérimentation Animale [CREEA], Ile-de-France, Sud, France) guidelines and French decree no. 87-848 of October 19, 1987 (Journal Officiel De La Republique Francaise, pp. 12245–12248, 20 October 1987). All protocols for obtaining human cardiac tissue were approved by the ethics committee of our institutions (Groupe



de Réflexion Ethique Biomédicale de Bicêtre [GREBB], Hôpital de Bicêtre, Université de Paris-Sud and Kaunas University Hospital); informed consent was obtained before cardiac surgery. Whole-cell patch-clamp experiments performed in ventricular myocytes isolated from frog and rat hearts are detailed in the Supplemental Methods.

**HAMs.** Specimens of right atrial trabeculae were obtained from 57 patients (42 males aged  $61 \pm 2$  years and 15 females aged  $66 \pm 3$  years) undergoing heart surgery for congenital defects, coronary artery diseases, valve replacement, or heart transplantation at the Hôpital Marie-Lannelongue, Le Plessis-Robinson, France or at the Department of Cardio-surgery of Kaunas University Hospital, Kaunas, Lithuania. Most patients received a pharmacological pretreatment ( $\text{Ca}^{2+}$ -channel blockers, digitalis,  $\beta$ -AR antagonists, diuretics, ACE inhibitors, NO donors, and/or antiarrhythmic drugs) that was stopped 24 hours before surgery. In addition, all patients received sedatives, anesthesia, and antibiotics. Details regarding the clinical characteristics of the patients and their drug regimens are shown in Table 1.

Dissociation of the cells was performed immediately after surgery as described previously (39, 73). The cell suspension was filtered, centrifuged, and the pellet resuspended in Dulbecco minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, 1 nM insulin, and antibiotics (100 IU/ml penicillin and 0.1  $\mu\text{g}/\text{ml}$  streptomycin).

**Solutions.** For electrophysiology, the control external solution contained (in mM): 107 NaCl, 10 HEPES, 40 CsCl, 4  $\text{NaHCO}_3$ , 0.8  $\text{NaH}_2\text{PO}_4$ , 1.8  $\text{MgCl}_2$ , 1.8  $\text{CaCl}_2$ , 5 D-glucose, 5 sodium pyruvate,  $6 \times 10^{-3}$  tetrodotoxin, pH 7.4 adjusted with NaOH. Patch electrodes (0.6–1.5 Mohms) were filled with control internal solution, which contained (in mM): 119.8 CsCl; 5 EGTA (acid form); 4  $\text{MgCl}_2$ ; 5 creatine phosphate disodium salt; 3.1  $\text{Na}_2\text{ATP}$ ; 0.42  $\text{Na}_2\text{GTP}$ ; 0.062  $\text{CaCl}_2$  (pCa 8.5); 10 HEPES; pH 7.3 adjusted with CsOH. Collagenase type IV and protease type XXIV were purchased from Sigma-Aldrich. DMEM was obtained from Gibco-BRL. Tetrodotoxin was from Latoxan. SR58611 was a generous gift from Sanofi Recherche. BRL37344 was a generous gift from SmithKline & Beecham. CGP12177 was a generous gift from Novartis Pharmaceutical. L-748,337 was a generous gift from Merck & Co. Inc. All other drugs were from Sigma-Aldrich. 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  $I_{\text{Ca,L}}$  in  $\text{Ca}^{2+}$ -tolerant HAMs as described previously (39, 57, 74). In the routine protocols, the cells were depolarized every 8 seconds from a holding potential of  $-80$  mV by a short prepulse (50 milliseconds) to  $-50$  mV and then to 0 mV for 200 or 400 milliseconds. The prepulse and the application of tetrodotoxin were used to eliminate fast sodium currents.  $\text{K}^+$  currents were blocked by replacing all  $\text{K}^+$  ions with intracellular and extracellular  $\text{Cs}^+$ . Voltage-clamp pulses were generated and currents recorded using either a single (VP-500) or 2 separate patch-clamp amplifiers (RK400; Bio-Logic). In the latter case, when possible, 2 different, nearby cells in the same dish were successively attached to 2 patch pipettes, whole-cell configuration was applied to both, and 2 different  $I_{\text{Ca,L}}$  currents were simultaneously recorded. Visual-Patch version 1.30 (Bio-Logic) or computer software that was generated in-house 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- $\mu\text{m}$  inner diameter capillary tubes that flow at a rate of about 50  $\mu\text{l}/\text{min}$ . Changes in extracellular solutions were automatically achieved using a rapid solution changer (RSC-200; Bio-Logic). All experiments were done at room temperature ( $19^\circ\text{C}$ – $25^\circ\text{C}$ ), and the temperature did not vary by more than  $1^\circ\text{C}$  in a given experiment.

**Mechanoelectrical measurements.** Experiments were performed on human trabeculae isolated from the right atrium. During transport from hospital to the laboratory, atrial tissues were placed in a cold ( $10^\circ\text{C}$ ) St. Thomas cardioplegic solution composed of (in mM) 110 NaCl, 16 KCl, 1.2  $\text{CaCl}_2$ , 16  $\text{MgCl}_2$ , 5 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. Trabeculae were then placed in an experimental chamber and superfused at a flow rate of 6 ml/min with oxygenated (100%  $\text{O}_2$ ) Tyrode solution ( $\text{pO}_2$  580–600 mmHg) composed of (in mM) 137 NaCl, 5.4 KCl, 1.8  $\text{CaCl}_2$ , 0.9  $\text{MgCl}_2$ , 5 glucose, 10 HEPES at  $36.0^\circ\text{C} \pm 0.5^\circ\text{C}$ , pH 7.4. Trabeculae were subjected to field stimulation with the following characteristics: stimulus pulse width was 5 milliseconds, stimulation rate was 1.0 Hz, and amplitude was twice the diastolic threshold. Isometric contraction was recorded using a mechanoelectrical force transducer. The  $\beta_3$ -AR agonists and other drugs were applied after steady-state conditions were established, i.e. after 40–50 minutes perfusion with control Tyrode solution. All experiments were done at  $37^\circ\text{C}$ .

**Statistics.** The maximal amplitude of whole-cell  $I_{\text{Ca,L}}$  was measured as previously described (39, 74). Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was done for each membrane depolarization to determine peak and steady-state current values. The changes in contractile force were expressed as percentage variation versus control. The results are expressed as mean  $\pm$  SEM. For statistical evaluation the paired and unpaired 1-tailed Student's *t* test were used, and a difference was considered statistically significant when  $P$  was  $< 0.05$ . ANOVA followed by post-hoc Bonferroni's test was used for contractile experiments when comparing the effects of the  $\beta_3$ -AR agonists in the absence or presence of IBMX.

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