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Murine ventricular L-type Ca^{2+} current is enhanced by zinterol *via* β_1-adrenoceptors, and is reduced in TG4 mice overexpressing the human β_2-adrenoceptor

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1 The functional coupling of β_2-adrenoceptors (β_2-ARs) to murine L-type Ca^{2+} current ($I_{Ca(L)}$) was investigated with two different approaches. The β_2-AR signalling cascade was activated either with the β_2-AR selective agonist zinterol (myocytes from wild-type mice), or by spontaneously active, unoccupied β_2-ARs (myocytes from TG4 mice with 435 fold overexpression of human β_2-ARs). Ca^{2+} and Ba^{2+} currents were recorded in the whole-cell and cell-attached configuration of the patch-clamp technique, respectively.

2 Zinterol (10 μM) significantly increased $I_{Ca(L)}$ amplitude of wild-type myocytes by $19 \pm 5\%$, and this effect was markedly enhanced after inactivation of Gi-proteins with pertussis-toxin (PTX; $76 \pm 13\%$ increase). However, the effect of zinterol was entirely mediated by the β_1-AR subtype, since it was blocked by the β_1-AR selective antagonist CGP 20712A (300 nM). The β_2-AR selective antagonist ICI 118,551 (50 nM) did not affect the response of $I_{Ca(L)}$ to zinterol.

3 In myocytes with β_2-AR overexpression $I_{Ca(L)}$ was not stimulated by the activated signalling cascade. On the contrary, $I_{Ca(L)}$ was lower in TG4 myocytes and a significant reduction of single-channel activity was identified as a reason for the lower whole-cell $I_{Ca(L)}$. The β_2-AR inverse agonist ICI 118,551 did not further decrease $I_{Ca(L)}$. PTX-treatment increased current amplitude to values found in control myocytes.

4 In conclusion, there is no evidence for β_2-AR mediated increases of $I_{Ca(L)}$ in wild-type mouse ventricular myocytes. Inactivation of Gi-proteins does not unmask β_2-AR responses to zinterol, but augments β_1-AR mediated increases of $I_{Ca(L)}$. In the mouse model of β_2-AR overexpression $I_{Ca(L)}$ is reduced due to tonic activation of Gi-proteins.

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Abbreviations: β-AR, β-adrenoceptor; LM, non-transgenic littermate control mouse; PKA, protein kinase A; PTX, pertussis toxin; TG4, transgenic mouse with heart specific overexpression of the human β_2-adrenoceptor

Introduction

The active conformation of the β_1-adrenoceptor (β_1-AR) initiates a signalling cascade which includes activation of stimulatory heterotrimeric G-proteins (Gs), increases of adenylyl cyclase activity, elevation of cyclic AMP, and stimulation of protein kinase A (PKA), resulting in phosphorylation of effector proteins (Kaumann & Molenaar, 1997). L-type Ca^{2+} channels are known targets for PKA-mediated phosphorylation which, at the whole-cell level, increases L-type Ca^{2+} current ($I_{Ca(L)}$) and shifts the voltage-dependence of activation and steady-state inactivation to hyperpolarized membrane potentials. At the single-channel level, phosphorylated channels are characterized by increased open probability and availability, and by a shift of gating towards prolonged openings (reviewed by McDonald *et al*., 1994).

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The effects of stimulation of β_2-adrenoceptors (β_2-ARs) on $I_{Ca(L)}$ appear to be heterogeneous across species. For example, agonist stimulation of β_2-ARs enhances $I_{Ca(L)}$ in frog and man (Skeberdis *et al*., 1997), but not in adult mouse ventricular myocytes, where the β_2-AR agonist zinterol failed to stimulate $I_{Ca(L)}$ (Xiao *et al*., 1999). However, following pertussis toxin-treatment of myocytes to inactivate inhibitory G-proteins (Gi), zinterol increased $I_{Ca(L)}$. This effect was attributed to the β_2-AR subtype, however, high concentrations of zinterol can also stimulate β_1-ARs (Kuznetsov *et al*., 1995; Nagykaldi *et al*., 1999). Therefore, we examined the effects of zinterol in the absence and presence of β-AR subtype-selective blockers.

Besides agonist stimulation of $I_{Ca(L)}$ *via* β_2-ARs, transgenic technology might offer an alternative means of determining whether stimulation of the β_2-AR signalling cascade alters $I_{Ca(L)}$ function in mice. Overexpression of human β_2-ARs in cardiomyocytes of transgenic mice (TG4)

leads to a functional phenotype similar to that caused by β-AR agonist stimulation in control mice (Milano *et al*., 1994). The enhanced contractility was explained by a sufficient fraction of the overexpressed receptor pool spontaneously existing in an active conformation, enabling activation of the post-receptor signalling cascade. Inverse agonists of the β_2-AR, such as ICI 118,551, reduce spontaneous activity by stabilizing the inactive conformation of the receptor (Bond *et al*., 1995). Therefore, if spontaneously active β_2-ARs in overexpressed β_2-AR systems cause phosphorylation of L-type calcium channels *via* a PKA-dependent pathway, the properties of $I_{Ca(L)}$ in TG4 myocytes should resemble those outlined above for agonist stimulation of β-ARs. Indeed, elevation of basal $I_{Ca(L)}$ amplitude was demonstrated in myocytes from late foetal and neonatal TG4 mice (An *et al*., 1999). In myocytes from adult TG4 animals $I_{Ca(L)}$ amplitude was not different from control (Zhou *et al*., 1999) or was significantly reduced (Heubach *et al*., 1999). However, an unaltered or even reduced current amplitude alone does not exclude stimulation of the current, if the possibility of a reduced channel number is taken into account.

In the present work we investigated whether the β_2-AR is functionally coupled to $I_{Ca(L)}$ of ventricular myocytes from adult mice. The effects of zinterol on $I_{Ca(L)}$ of wild-type mouse ventricular myocytes was studied with the whole-cell voltage-clamp technique. $I_{Ca(L)}$ of TG4 myocytes was studied using whole-cell and single channel recordings, for evidence of β-AR stimulation, and the β_2-AR selective antagonist ICI 118,551 was employed to test for inverse agonism. The role of inhibitory G-proteins (Gi) in reduction of $I_{Ca(L)}$ was studied by treating myocytes with PTX to inactivate Gi. We found that zinterol enhanced $I_{Ca(L)}$ in ventricular myocytes from adult wild-type mice through β_1-, but not β_2-ARs. Overexpression of human β_2-ARs suppressed $I_{Ca(L)}$ which was restored by inactivation of PTX-sensitive G-proteins. No evidence of cardiostimulatory β_2-ARs was found in adult mouse ventricle.

Methods

Characterization of mice

All studies complied with the German home office regulations governing the care and use of laboratory animals. Heparinization of the mice was approved by the Regierungspräsidium Dresden (Az 75-9168.11-1/12/98). Male C57BL6 wild-type mice were 3 to 5 months of age and used to study the effects of zinterol. The transgenic mice used in this study descended from the TG4 line of β_2-AR overexpressing mice originally described by Milano *et al.* (1994). The TG4 mice and their non-transgenic littermates (LM), which were used as controls, were of either sex, 4 to 8 months of age, and had a mixed genetic background. The genotype of the offsprings was tested by PCR using the following primers: 5′-AGTGCGCTTACCTGCCAGA; 3′-TAAAATACCCCG-TGTGAGCAA. One hundred ng of tail DNA-isolate (High Pure PCR Template Preparation Kit, Boehringer Mannheim, Germany) were amplified in 25 μl buffer containing (mM): KCl 50, Tris-HCl 10 (pH 8.3), $MgCl_2$ 1.3, 200 μM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer, and 1.25 U Taq DNA-polymerase (Boehringer Mannheim). Cycling conditions were set to 94°C for 60 s, 65°C for 60 s, and 72°C for 120 s. After 35 cycles, an 8-μl aliquot of each amplification mixture was examined by agarose gel electrophoresis for presence or absence of the amplified human β_2-AR sequence.

Isolation of myocytes

Ventricular myocytes were isolated by enzymatic dissociation using the method described previously (Heubach *et al*., 1999). Briefly, hearts were perfused with a collagenase solution (Worthington type I or II, 75 U l^{-1}) on a Langendorff setup and subsequently cut into small chunks. Myocytes were harvested by pouring the suspension through a cheese cloth. The tissue that was retained in the cloth was frozen in liquid nitrogen for radioligand binding experiments. A fraction of cells was incubated either with pertussis toxin (1.5 μg ml^{-1}) or buffer for 3 h at 37°C, and was then kept at room temperature until use.

Radioligand binding studies

For membrane preparation the ventricular tissue was thawed and homogenized in ice-cold assay buffer (composition in mM; pH 7.4): Tris-HCl 50, EGTA 5, EDTA 1, $MgCl_2$ 4, ascorbic acid 1, phenylmethylsulphonylfluoride (PMSF) 0.5, then centrifuged for 10 min at $175 \times g$ (4°C). The supernatant was centrifuged at $50{,}000 \times g$ (4°C) for 15 min and the pellet resuspended in ice-cold assay buffer to give a solution of 1 : 50 (w v^{-1}) and further diluted to obtain a ratio of total radioligand bound (specific + non-specific) : total radioactivity added that was less than 0.1. Protein was determined (Lowry *et al*., 1951) using bovine serum albumin as a standard. Saturation binding curves to β-AR binding sites were constructed using 0.75 – 175 pM (−)-[^{125}I]-cyanopindolol in the absence or presence of 200 μM (−)-isoproterenol to define non-specific binding. Following 120 min of incubation at 37°C in assay buffer containing 100 μM GTP the assays were filtered by a cell harvester (Brandel M-30R) over Whatman GF/B filters. Radioactivity retained on filter paper was counted in a Packard gamma-counter. Saturation binding experiments were analysed for one binding site by non-linear curve fitting of the equation:

$$\text{Beq} = (\text{Bmax}_{\beta\text{-AR}} * [(-)\text{-}[^{125}\text{I}]\text{-cyanopindolol}])/(\text{K}_{\text{D}\beta\text{-AR}} + [(-)\text{-}[^{125}\text{I}]\text{-cyanopindolol}]),$$

where Beq is specific binding at equilibrium, $\text{Bmax}_{\beta\text{-AR}}$ is the maximal density of β-AR binding sites and $\text{K}_{\text{D}\beta\text{-AR}}$ is the equilibrium dissociation constant of (−)-[^{125}I]-cyanopindolol. Saturation binding isotherms were analysed with PRISM (GraphPad Software, San Diego, CA, U.S.A.).

Measurement of whole-cell calcium current $I_{Ca(L)}$

Whole-cell voltage-clamp technique (Hamill *et al*., 1981) was applied using a List EPC-7 amplifier to measure membrane currents. Command pulse timing and amplitude was controlled using pCLAMP (version 5.5; Axon Instruments, Foster City, CA, U.S.A.) while acquiring current data.

pCLAMP (version 6.0.3) was used for data analyses. Myocytes were transferred to a small perspex chamber (volume 0.5 ml) placed on the stage of an inverted microscope (Olympus IMT-2). The chamber was continuously perfused at a constant rate (1.8 ml min^{-1}). Electrodes were fabricated from filamented borosilicate glass (Hilgenberg, Malsfeld, Germany; outer diameter 1.5 mm) using a fully controlled, programmable horizontal puller (DMZ universal puller, Zeitz, München, Germany). When filled with pipette solution (see below), the microelectrodes had tip resistances of 1.5–3 MΩ. Gigaohm seals were formed by gentle suction. The seal resistances were usually between 2 and 5 GΩ.

Before series resistance compensation membrane capacitance was measured by means of fast depolarizing ramp pulses (from −40 to −45 mV, duration 5 ms) at the beginning of each experiment. Since the membrane conductance is constant in this range, a change in current level is due to the capacitive properties of the cell membrane. Compensated access resistance was regularly checked and maintained below 5 MΩ. Series resistance was routinely compensated by 50–70%. Membrane currents were low-pass filtered at 2 kHz.

$I_{Ca(L)}$ was measured at room temperature (23±1°C). Only rod-shaped myocytes with clear striations were used. The stimulation frequency was 0.2 Hz. For isolation of $I_{Ca(L)}$ from contaminating currents, I_{Na} and T-type I_{Ca} (if present) were inactivated by a 50 ms long prepulse to −40 mV (holding potential = −80 mV), and K^+ currents were reduced by replacing K^+ with Cs^+. The composition of the bath solution was (in mM): NaCl 137.0, CsCl 5.4, $CaCl_2$ 2.0, $MgCl_2$ 1.25, HEPES 10.0, glucose 10.0; the pH was adjusted to 7.4 with NaOH. The pipette solution had the following composition (in mM): CsCl 140.0, $MgCl_2$ 4.0, HEPES 10.0, EGTA 10.0, Na_2ATP 4.0; the pH was adjusted to 7.3 with CsOH. Peak current amplitude was determined as the difference between the peak inward current and the current at the end of the depolarizing pulse. In order to account for variabilities in cell size, absolute current amplitudes (in pA) were divided by the respective cell capacitance (in pF) and are expressed as membrane current I in pA pF^{-1}. Fits of theoretical equations to the experimental data were performed using Prism (GraphPad Software).

Steady-state inactivation curves for $I_{Ca(L)}$ were obtained by plotting the normalized peak membrane current at the test potential as a function of the conditioning potential (V_m). A Boltzmann function was fitted to the normalized values: $I/I_{max} = 1/(1+\exp((V_m - V_{0.5\ inact.})/k_{inact.}))$, where $V_{0.5\ inact.}$ and $k_{inact.}$ are the potentials of half-maximum inactivation and the slope factor, respectively.

Activation curves were fitted to current-voltage relations (I–Vs) using the equation: $G = I/(V_m - E_{rev})$, where G and I are peak Ca^{2+} conductance and current at the test potential V_m, respectively. The apparent reversal potential E_{rev} was obtained by linear regression of four data points close to E_{rev} (two points positive and two points negative to the expected reversal potential). The relation between normalized peak conductance G/G_{max} and membrane potential V_m could be described by the Boltzmann equation: $G/G_{max} = 1/(1+\exp((V_{0.5\ act.} - V_m)/k_{act.}))$, where $V_{0.5\ act.}$ is the half-activation potential and $k_{act.}$ is the slope factor.

Single-channel recordings

To measure Ba^{2+} currents through single calcium channels (Schröder & Herzig, 1999), cells were placed in disposable perfusion chambers (3 ml). Pipettes (7–11 MΩ) for cell-attached recordings (Hamill *et al*., 1981) contained (in mM): $BaCl_2$ 70, sucrose 110, and HEPES 10, with pH adjusted to 7.4 with tetraethylammonium hydroxide. The composition of the bath solution was (in mM): K-glutamate 120, KCl 25, $MgCl_2$ 2, HEPES 10, EGTA 2, Na_2-ATP 1, $CaCl_2$ 1, dextrose 10, pH 7.3. Ba^{2+} currents were elicited by voltage steps (150 ms at 1.66 Hz) from −100 mV to +20 mV (⩾120 sweeps per experiment). Data were sampled at 10 kHz and filtered at 2 kHz (−3 dB, 4-pole Bessel) by using an Axopatch 200 A amplifier (Axon Instruments). pCLAMP software (version 6.0) was used for data acquisition and analysis of openings and closures (half-height criterion). Linear leak and capacity currents (averaged nonactive sweeps) were digitally subtracted. Channel availability (avl.; percentage of sweeps containing ⩾1 channel opening, i.e. fraction of active sweeps per total number of test pulses), open probability (P_{open}; fractional occupancy of the open state during active sweeps), and the maximum current of the ensemble average (I_{max}) were corrected by the number of channels in the patch (n) in case of double-channel patches. n was derived from the maximum current amplitude observed, divided by the unitary current amplitude. Experiments with $n>2$ were entirely rejected from analysis. Maximum current was normalized by division through n. The availability was corrected by the square root method: (1-availability$_{corr}$) is the n^{th} root of (1-availability$_{uncorr}$), where availability$_{corr}$ and availability$_{uncorr}$ are corrected and uncorrected availability, respectively. The corrected P_{open} was calculated on the basis of the corrected number of active sweeps, i.e. total open time (in ms) within all sweeps of the ensemble, divided by (150 ms * n * availability$_{corr}$ * number of test pulses). Open and closed times were analysed from experiments with only one channel present.

Chemicals

All chemicals were purchased from commercial suppliers and were of analytical grade. (−)-Isoproterenol-HCl (Sigma, Deisenhofen, Germany), ICI 118,551 (Tocris, Bristol, U.K.) and CGP 20712A methanesulfonate (RBI Natick, MA, U.S.A.) were dissolved in H_2O. Zinterol was dissolved in DMSO and was a gift of Bristol-Myers Squibb. Stock solutions of 10 mM were aliquoted and stored at −20°C until use. Pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA, U.S.A.).

Statistics

The results are expressed as mean values±s.e.mean. Numbers in brackets indicate the number of myocytes/number of animals. Significance of differences between means of groups was tested by the two-tailed Alternate t-test (Welch-test) or by Ordinary ANOVA followed by Bonferroni multiple comparisons test. Statistics were performed using n = number of myocytes, except for binding studies, where n = number of

animals. Differences between means were considered significant if $P<0.05$.

Results

Zinterol increases $I_{Ca(L)}$ via the β_1-AR subtype

The effects of zinterol on $I_{Ca(L)}$ were studied in ventricular myocytes from wild-type mice incubated for 3 h at 37°C either with buffer or PTX. Figure 1 shows original current recordings and current-voltage relations (I–Vs) in the absence and presence of 10 μM zinterol. Zinterol slightly increased $I_{Ca(L)}$ and shifted the I–V towards more negative potentials (Figure 1). The effects of zinterol were more pronounced on PTX-treated myocytes. PTX-treatment alone had no effect on current amplitude or voltage-dependence of $I_{Ca(L)}$. Figure 2 summarizes the concentration-dependent effects of zinterol at a potential of +10 mV and also shows the spontaneous reduction of $I_{Ca(L)}$ in the absence of zinterol due to run-down. The increases of $I_{Ca(L)}$ after application of 10 μM zinterol amounted to 19±5% in buffer-incubated (Figure 2A; $n=11/4$) and 76±13% in PTX-treated myocytes (Figure 2B; $n=14/8$). The effects were significant ($P<0.01$ and $P<0.001$, respectively), when compared to time-matched controls, where current decreased due to run-down.

To address the question which β-AR subtype is responsible for the increase of $I_{Ca(L)}$ we investigated zinterol effects in the presence of either 300 nM CGP 20712A, a β_1-AR selective antagonist, or 50 nM ICI 118,551, a β_2-AR selective antagonist. With buffer-incubated myocytes the zinterol-induced increase of $I_{Ca(L)}$ was absent in the presence of CGP 20712A (Figure 2A), indicating that it was mediated by β_1-ARs, rather than β_2-ARs. The same conclusion was obtained from PTX-treated myocytes (Figure 2B), where ICI 118,551 did not affect the zinterol-induced increase of $I_{Ca(L)}$, which was completely blocked by CGP 20712A. There was no evidence for β_2-AR mediated stimulation of $I_{Ca(L)}$ even after PTX-incubation of myocytes. Thus, PTX-incubation did not unmask β_2-AR responses to zinterol, but augmented β_1-AR mediated increases of $I_{Ca(L)}$.

Overexpression of β-ARs in TG4 mice

The phenotype and consequences of β_2-AR overexpression strongly depend on expression level (Liggett *et al*., 2000). In order to characterize the TG4 mice used for this study we measured the degree of overexpression of ventricular β-ARs by (−)-[^{125}I]-cyanopindolol saturation binding. The experiments confirmed a 435 fold overexpression of binding sites (maximal density of binding sites, LM 13.3±1.3 fmol mg^{-1} protein, $n=4$ animals; TG4 5790±460 fmol mg^{-1} protein, $n=8$ animals). The equilibrium dissociation constant of the radioligand (K_D) was not different (9.8±2.5 and 6.2±1.1 pM in LM and TG4, respectively).

Reduced effectiveness of isoproterenol on $I_{Ca(L)}$ in TG4 compared to LM myocytes

The characteristic changes of $I_{Ca(L)}$ upon agonist stimulation of the β-adrenergic signalling cascade were studied in LM myocytes using isoproterenol (ISO) (Figure 3). Application of

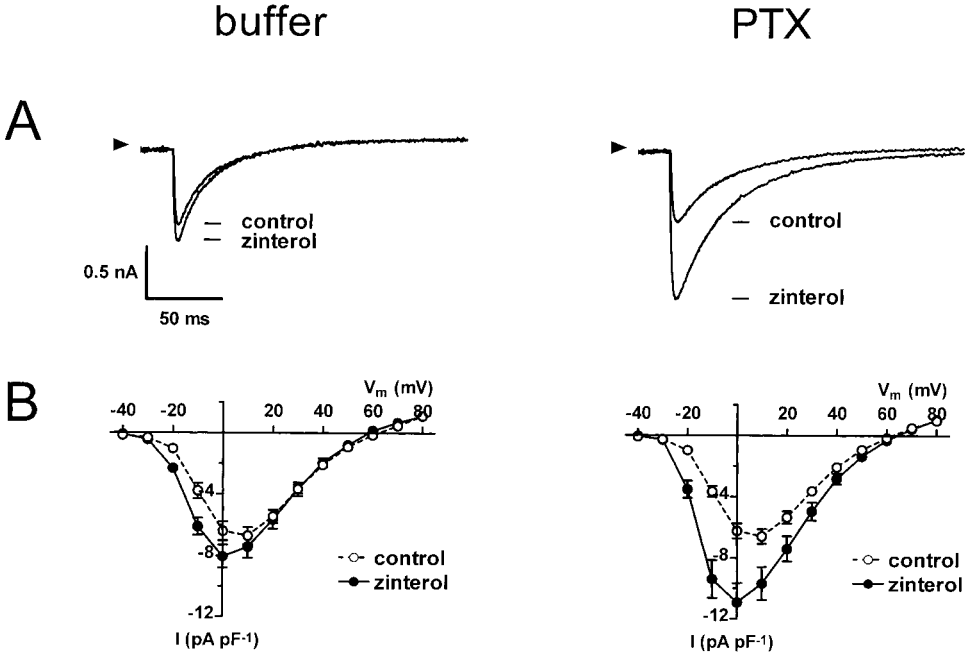

Figure 1 Effects of the β_2-AR selective agonist zinterol (10 μM) on $I_{Ca(L)}$ of ventricular myocytes from wild-type C57BL6 mice. Myocytes were incubated with buffer (left) or pertussis toxin (PTX, right) prior to experimentation. (A) Representative original current traces under control conditions and in the presence of zinterol. Holding potential = −80 mV; test potential = +10 mV, preceeded by a 50 ms prepulse to −40 mV in order to inactivate Na^+ current (not shown). Arrowheads indicate zero current. (B) Current-voltage relations under control conditions and after application of zinterol ($n=9/4$ buffer-incubated myocytes; $n=8/4$ PTX-incubated myocytes).

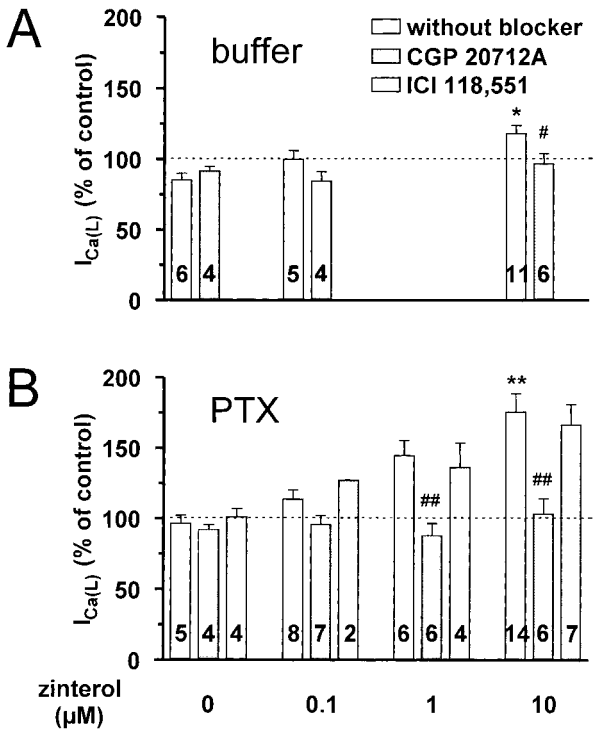

Figure 2 Effects of zinterol on $I_{Ca(L)}$ of wild-type mouse ventricular myocytes without blocker and in the presence of either 300 nM CGP 20712A or 50 nM ICI 118,551. Voltage protocol as for Figure 1A. Myocytes were exposed to the blockers for at least 10 min prior to application of zinterol, which was added 8 min after rupture of the membrane. Currents were recorded 6 to 8 min after application of zinterol and normalized to the respective pre-zinterol current amplitudes. An individual myocyte was either used as a time-matched control (TMC; depicted at 0 μM zinterol) or exposed to a single concentration. Numbers of myocytes are indicated within the bars. (A) Buffer-incubated myocytes. Zinterol significantly increased $I_{Ca(L)}$ (*$P<0.01$; 10 μM *vs* TMC; ANOVA), however, the increase was not observed in the presence of CGP 20712A (#$P<0.05$, Welch-test). (B) PTX-incubation augmented the response to zinterol (**$P<0.001$; 10 μM *vs* TMC; ANOVA). Again, CGP 20712A abolished the increase (##$P<0.01$; CGP 20712A *vs* without blocker; ANOVA), whereas ICI 118,551 did not.

1 μM ISO increased peak current amplitude by $61\pm6\%$, and the maximum of the I–V curve was shifted by ≈10 mV to more negative potentials. ISO shifted the voltage-dependence of activation by -6.8 ± 0.9 mV ($P<0.0001$) and steady-state inactivation by -8.6 ± 1.5 mV ($n=9/2$; $P<0.0001$) towards more negative potentials.

In TG4 myocytes, $I_{Ca(L)}$ was significantly smaller when compared to LM controls (-5.46 ± 0.42 pA pF^{-1} in TG4 *vs* -7.19 ± 0.68 pA pF^{-1} in LM; $P<0.05$), and the potential values for threshold, peak current and reversal were the same as in LM myocytes under control conditions. Addition of 1 μM ISO had no major effect on I-V curve, and caused smaller shifts than in LM controls of the activation (-2.2 ± 0.4 mV, $n=11/5$; n.s.) and steady state inactivation curves (-3.3 ± 1.3, $n=8/3$; n.s.) towards hyperpolarized potentials. Nisoldipine abolished the current as in LM controls. Taken together, the reduced current amplitude and the absence of shifts in voltage-dependences of control $I_{Ca(L)}$ argues against stimulation of the current *via* spontaneously active β_2-ARs in TG4 myocytes. The average capacitance of myocytes from 4–5-month-old TG4 mice (184 ± 10 pF; $n=18/6$) was similar to average values of control myocytes (wild-type and LM; 175 ± 7 pF; $n=84/17$).

Lack of inverse agonism by ICI 118,551

In TG4 mice, the β_2-AR selective antagonist ICI 118,551 has been shown previously to reduce contractility of isolated left atria (Bond *et al*., 1995) and ventricular myocytes (Xiao *et al*., 1999; Gong *et al*., 2000) due to inverse agonism. Therefore, we investigated whether this inverse agonist is able to further reduce $I_{Ca(L)}$ as an additional test for stimulation of basal $I_{Ca(L)}$ by spontaneously active β_2-ARs (Figure 4). The acute application of 10–1000 nM ICI 118,551 had no effect on $I_{Ca(L)}$ amplitude and did not affect the I-V relation (inset). This implies that the effects of the inverse agonist on contractility are independent of $I_{Ca(L)}$ changes.

Single-channel recordings

L-type Ca^{2+} current was investigated at the single-channel level by measuring unitary Ba^{2+} currents. In TG4 myocytes, open probability of the Ca^{2+} channels was significantly reduced (Figure 5A and Table 1), instead of being elevated as expected for β-AR stimulation. In line with whole-cell recordings of $I_{Ca(L)}$, maximum current of the ensemble average was lower than in LM myocytes (Figure 5B). The individual gating parameters revealed a trend of increased mean closed times for TG4-derived channels (Table 1). As seen in Figure 5C, channel closed state distributions were characterized by the typical two closed time components, and the slow component appeared shifted to the right in TG4 channels. Indeed, the two peak τ values of the closed time histograms were different by 1.34 ± 0.08 log units in the case of LM myocytes ($n=13/7$), and by 1.67 ± 0.15 log units in the case of TG4 myocytes ($n=8/4$; $P<0.05$). This accounts for most of the difference in open probability between TG4 and LM, and therefore in maximum current of the ensemble average. In addition, the waiting time from beginning of the pulse to the first opening (first latency) was significantly ($P<0.05$) prolonged in TG4 myocytes (37.2 ± 6.3 ms; $n=10/5$) compared with LM myocytes (16.2 ± 2.1 ms; $n=23/10$).

$I_{Ca(L)}$ of PTX-treated myocytes

The possible contribution of Gi-protein activation to the reduction of $I_{Ca(L)}$ from TG4 myocytes was assessed indirectly by comparing $I_{Ca(L)}$ amplitude of myocytes incubated with normal buffer and that containing PTX (Figure 6 and Table 2). As observed in the experiments on wild-type myocytes (Figure 1), PTX-treatment did not have any significant effect on $I_{Ca(L)}$ of LM myocytes, i.e. neither amplitude nor voltage-dependences of I-V curves, activation and steady-state inactivation were affected. In buffer-incubated TG4 myocytes, $I_{Ca(L)}$ amplitude was lower and $V_{0.5\ act.}$ was more positive than in LM.

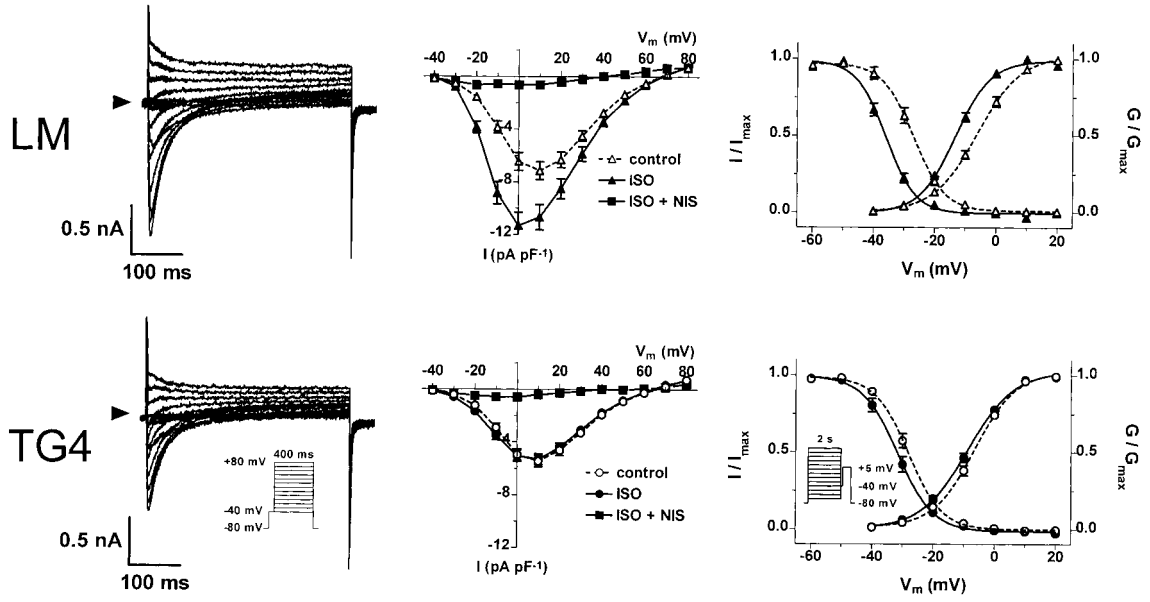

Figure 3 Effects of 1 μM isoproterenol (ISO) on voltage-dependence of $I_{Ca(L)}$ in ventricular myocytes from transgenic (TG4) and non-transgenic (LM) mouse hearts. Left: Original current traces under control conditions. Arrowheads indicate zero current. Middle: A comparison of the control I–Vs from LM ($n=13/3$) and TG4 myocytes ($n=11/5$) shows, that both I–V curves peak at +10 mV, however, the peak current amplitude was significantly smaller in TG4. Application of ISO increased $I_{Ca(L)}$ of LM myocytes and shifted the peak of the I–V curve from +10 mV to 0 mV. In contrast, myocytes from TG4 hearts showed little response to ISO. Addition of 1 μM nisoldipine (NIS) blocked the current almost completely. Right: Voltage-dependence of activation (G/G_{max} *vs* V_m) and steady-state inactivation (I/I_{max} *vs* V_m) of $I_{Ca(L)}$ were very similar in myocytes from TG4 and LM under control conditions. Control potentials for half-maximum activation and slope factors were -6.56 ± 0.86 mV and 5.86 ± 0.27 mV in LM, and -6.98 ± 0.88 mV and 6.06 ± 0.25 mV in TG4. The $V_{0.5}$ values for steady-state inactivation and the corresponding slope factors were -27.4 ± 1.5 mV and 4.82 ± 0.22 mV in LM ($n=9/2$), and -28.3 ± 1.0 mV and 5.10 ± 0.16 mV in TG4 ($n=8/3$); differences not significant; symbols as for I–V curves). In LM myocytes application of ISO significantly shifted both $I_{Ca(L)}$ activation and steady-state inactivation by 5–10 mV towards more negative potentials, whereas these effects were much smaller in TG4 myocytes. Changes in slope were not observed. Voltage protocol for steady-state inactivation as indicated.

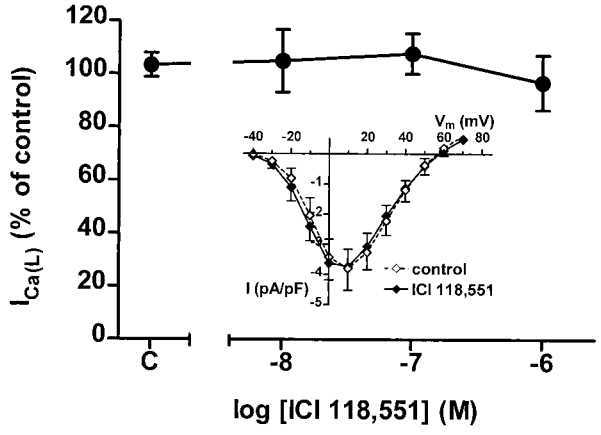

Figure 4 Lack of inverse agonism of ICI 118,551 on $I_{Ca(L)}$ of ventricular myocytes from TG4 mice. Current amplitude in the presence of an individual ICI 118,551 concentration (10 min application) was normalized to the predrug amplitude. The data points depicted at 'C' represent time-matched controls and show absence of run-down between the 8th and 18th minute after rupture of the membrane ($n=5-10$ cells per concentration). Inset: 1 μM ICI 118,551 was without effect on the I–V.

In marked contrast to LM, PTX-incubation increased $I_{Ca(L)}$, but peak amplitude did not exceed the values found in LM. PTX-incubated TG4 myocytes had a $V_{0.5\,act.}$ value similar to those of LM myocytes, but the effect of PTX was not quite significant ($P=0.08$). Other parameters were not significantly affected by PTX-treatment (Table 2).

Discussion

Lack of β_2-AR mediated stimulation of $I_{Ca(L)}$ in adult mouse ventricle

The present work addresses the question, whether the β_2-AR subtype is able to stimulate $I_{Ca(L)}$ of ventricular myocytes from adult mice. The β_2-AR selective compound zinterol was used as an agonist in order to compare our results with previous work on wild-type mice where zinterol at a concentration of 10 μM (Xiao *et al*., 1999) did not stimulate $I_{Ca(L)}$. PTX-treatment rescued a zinterol-mediated increase of $I_{Ca(L)}$ (Zhou *et al*., 1999; Xiao *et al*., 1999) and this increase was attributed to the β_2-AR subtype. However, the interpretation of these studies may have been confounded by the use of zinterol in the absence of β_1-AR blockade. We detected a significant response to 10 μM zinterol with myocytes incubated with buffer for 3 h at 37°C. Similar to the findings of Xiao *et al*. (1999) the response to zinterol was enhanced by PTX-treatment. The effects, however, were mediated by the

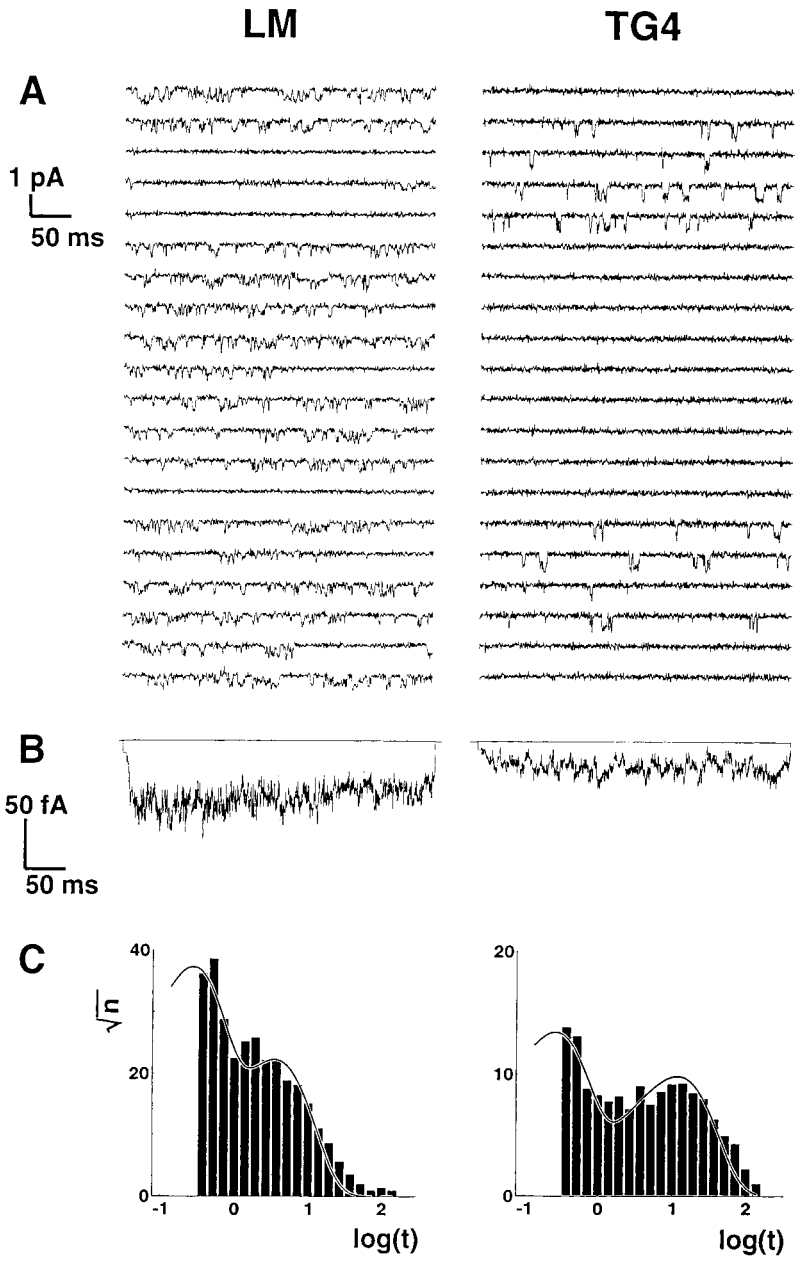

Figure 5 Unitary Ba^{2+} currents through single L-type Ca^{2+} channels of ventricular myocytes from LM and TG4 hearts. (A) Original current traces, where voltage steps from -100 to $+20$ mV were delivered every 600 ms for 150 ms. Single-channel activity of TG4 myocytes was markedly reduced when compared to LM myocytes. (B) Ensemble average currents from the respective channels. (C) Closed time distribution of single-channels. Closed times (in ms) were binned on a logarithmic scale, and the number of events in each bin, n, was square-root-transformed. Note the biexponential distribution which appears double-bell shaped after this transformation. The larger closed times seen in TG4 experiments was due to a larger time constant of the slow component in this group. A detailed analysis of single-channel gating is provided in Table 1.

β_1-AR subtype, because they were abolished in the presence of the β_1-AR selective blocker CGP 20712A and unaffected in the presence of the β_2-AR selective blocker ICI 118,551. Therefore, there was no evidence for a β_2-AR mediated, stimulatory zinterol effect in myocytes from wild-type mice, even after PTX-treatment. Our data on mouse ventricular myocytes add evidence that zinterol is able to stimulate $I_{Ca(L)}$ *via* the β_1-AR subtype as previously shown for cardiomyocytes from dog (Nagykaldi *et al*., 1999), guinea-pig (Hool & Harvey, 1997) and rat (Laflamme & Becker, 1998).

Lack of $I_{Ca(L)}$ stimulation by spontaneously active β_2-ARs

We used the TG4 mouse as an alternative model to study the effects of an activated β_2-AR signalling cascade on $I_{Ca(L)}$. Based on the following findings, $I_{Ca(L)}$ of adult TG4 myocytes is not stimulated by the spontaneously activated β_2-AR signalling cascade: (i) Current amplitude was reduced rather than increased in TG4 myocytes; (ii) characteristic

shifts of basal voltage-dependences of I–V relation, activation and steady-state inactivation, similar to the ones observed with ISO in LM myocytes, were absent (iii) acute application of the inverse agonist ICI 118,551 (Bond *et al*., 1995; Xiao *et al*., 1999) was without effect, and (iv) single channel activity was markedly reduced, which is sufficient to explain the lower $I_{Ca(L)}$ without necessity to assume a reduced number of channels.

Our results contrast with recently published data from myocytes of late foetal and neonatal TG4 mice where $I_{Ca(L)}$ showed typical characteristics of β-adrenergic stimulation, i.e. increased peak amplitude and shift of the I–V relation and activation curve towards more negative potentials (An *et al*., 1999). Furthermore, our data contrast with the results of Zhou *et al*. (1999), who did not find any differences between $I_{Ca(L)}$ from TG4 and LM myocytes from young adult mice (2–3 months of age). However, the mice used in our previous study (3–8 months; Heubach *et al*., 1999) and in this study (4–8 months) were older. Therefore, $I_{Ca(L)}$ could be progressively downregulated with age in an adaptive manner to cope with permanent adrenergic stimulation. Although average cell capacitance values in our studies on strongly overexpressing TG4 mice do not provide evidence for significant hypertrophy ($\approx$435 fold overexpression; Heubach *et al*., 1999; this study), reduction of $I_{Ca(L)}$ due to Gi-protein activity could also be associated with the onset of heart failure. Liggett *et al*. (2000) found $I_{Ca(L)}$ to be reduced by 44% in a similar, but separately generated transgenic mouse model already at 3 months of age. The mice used for $I_{Ca(L)}$ measurements in that study had a similarly high degree of overexpression (350 fold) and demonstrated impaired left ventricular function with ventricular enlargement as a sign of hypertrophy.

Table 1 Single-channel gating parameters of unitary Ba^{2+} currents through L-type calcium channels

	LM	*TG4*
P_{open} (%)	15.1±2.74	6.17±3.28*
$<t_{open}>$ (ms)	0.53±0.06	0.46±0.05
$<t_{close}>$ (ms)	2.80±0.60	7.35±2.55
avl (%)	56.3±4.0	38.1±9.9
I_{max} (fA)	68±11	33±12*
n (P_{open}, avl., I_{max})	23/10	10/5
n ($<t_{open}>$, $<t_{close}>$)	13/7	8/4

P_{open}: open probability; $<t_{open}>$: mean open time; $<t_{close}>$: mean close time; avl.: channel availability; I_{max}: maximum current of the ensemble average (for a detailed definition of parameters see Methods section). *n*: number of myocytes/number of animals. For analysis of $<t_{open}>$ and $<t_{close}>$ only one-channel experiments were considered. *$P<0.05$ TG4 *vs* LM, Welch-test.

A Gi-protein tone reduces L-type calcium current in adult TG4 mice

PTX-treatment of the myocytes for disruption of Gi-protein function increased $I_{Ca(L)}$ amplitude to values found in LM

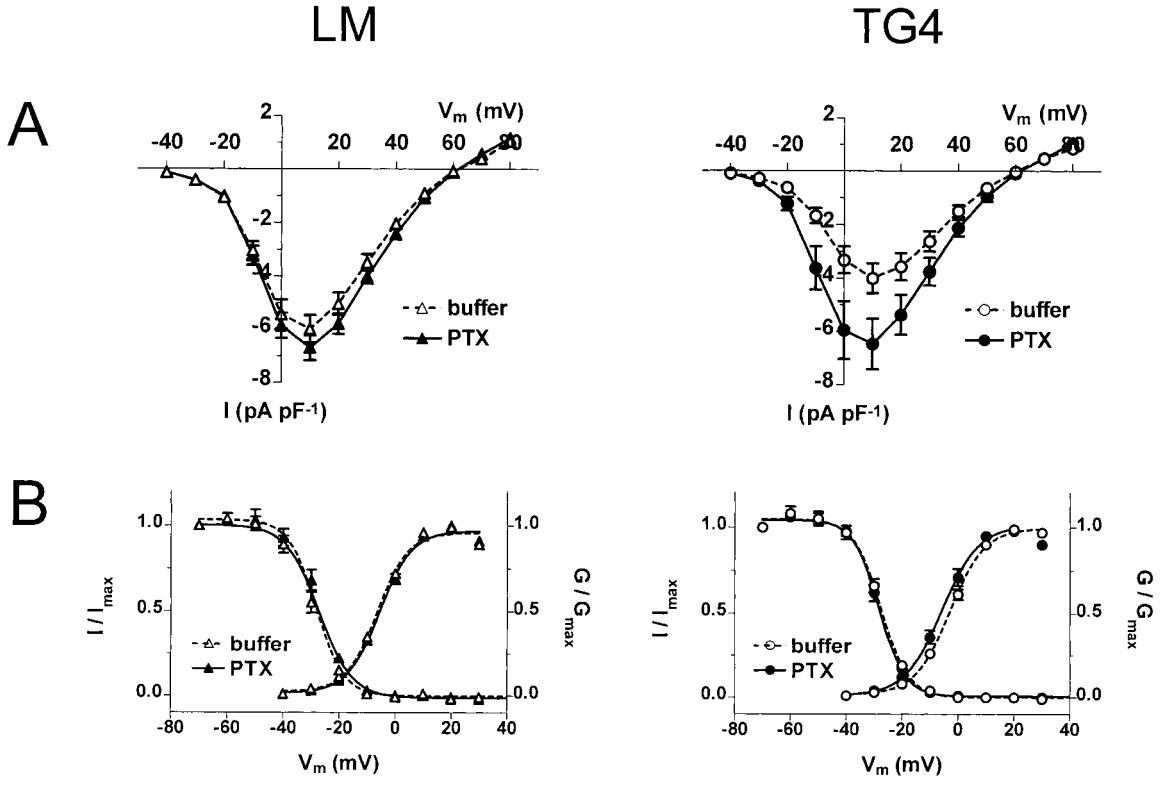

Figure 6 Effects of PTX-treatment on I–V-relation (A) and voltage-dependence of activation and steady-state inactivation of $I_{Ca(L)}$ (B). In LM myocytes, incubation with PTX was without significant effect on $I_{Ca(L)}$ amplitude. Furthermore, the I-V peak potential, and the voltage-dependences of activation (G/G_{max} *vs* V_m) and steady-state inactivation (I/I_{max} *vs* V_m) were unaffected. However, in TG4 myocytes PTX-treatment significantly increased $I_{Ca(L)}$ amplitude to values found in LM myocytes. Other parameters were not significantly affected by PTX-treatment. Results are summarized in Table 2.

Table 2 Effect of PTX-treatment on amplitude and voltage-dependence of $I_{Ca(L)}$

	LM (buffer)	*LM (PTX)*	*TG4 (buffer)*	*TG4 (PTX)*
Amplitude (pA pF^{-1})	-6.21 ± 0.49	-6.67 ± 0.40	-4.23 ± 0.35**	-6.70 ± 0.51#
$V_{0.5\ act.}$ (mV)	-6.20 ± 0.46	-5.87 ± 0.55	-3.93 ± 0.84*	-5.70 ± 0.55
$k_{act.}$ (mV)	5.49 ± 0.14	5.55 ± 0.12	5.88 ± 0.16	5.48 ± 0.15
n	24/11	34/11	25/9	44/9
$V_{0.5\ inact.}$ (mV)	-28.7 ± 0.79	-26.8 ± 0.72	-27.1 ± 0.47	-27.8 ± 0.54
$k_{inact.}$ (mV)	5.14 ± 0.24	5.61 ± 0.21	4.86 ± 0.16	4.70 ± 0.12
n	6/3	6/3	12/5	17/5

$V_{0.5\ act.}$ and $V_{0.5\ inact.}$ are the potentials for half-maximum activation and steady-state inactivation, and k values are the respective slope factors from Boltzmann curve fits of the curves shown in Figure 6. Activation curves were deduced from I–V relations (see Methods section). *n*: number of myocytes/number of animals. *$P<0.05$, **$P<0.005$ TG4 (buffer) *vs* LM (buffer); #$P<0.0005$ TG4 (PTX) *vs* TG4 (buffer), Welch-test.

myocytes. This finding indicates that basal $I_{Ca(L)}$ of myocytes from adult TG4 mice is suppressed by tonic Gi-protein activity. We have previously shown that Giα1/2 is upregulated in hearts of our TG4 mice (Gong *et al*., 2000). Gi-protein coupling of β_2-ARs has been demonstrated in expression systems to occur after PKA-dependent phosphorylation of the receptor (Daaka *et al*., 1997). Since adenylate cyclase activity was found to be increased in TG4 hearts (Milano *et al*., 1994), a fraction of β_2-ARs might subsequently be phosphorylated by PKA, which would allow Gi-protein coupling to occur. However, in TG4 hearts Gi-protein coupling has only been demonstrated for the agonist-stimulated β_2-AR (Xiao *et al*., 1999), but not for the unoccupied, spontaneously active β_2-AR (Gürdal *et al*., 1997; Xiao *et al*., 1999). The results of our present work support these findings. If the tonic Gi-protein activation, that suppressed $I_{Ca(L)}$ of TG4 myocytes, was mainly due to coupling by unoccupied, spontaneously active β_2-ARs, one would expect an increase of $I_{Ca(L)}$ after application of the inverse agonist ICI 118,551, which forces the receptor into an inactive conformation. Such an increase was not observed upon acute application of the inverse agonist. This implies that there is an inhibition of $I_{Ca(L)}$ by Gi unrelated to spontaneous β_2-AR coupling. The Gi-protein dependent tonic inhibition of $I_{Ca(L)}$ may be due to Gi-mediated activation of channel-associated protein phosphatase activity as already suggested by Kuschel *et al*. (1999), and found for a number of other examples of channel regulation by Gi/o proteins (Herzig & Neumann, 2000). This tonic activity of PTX-sensitive G-proteins also seems to reduce the responsiveness of $I_{Ca(L)}$ to stimulation with isoproterenol (Figure 3), since PTX-treatment of the myocytes restored the response to agonist stimulation in TG4 myocytes (Xiao *et al*., 1999).

Single-channel gating in TG4 myocytes

At the single-channel level, myocytes from TG4 hearts did not show the phenomenology known for β-AR stimulation (see Yue *et al*., 1990). On the contrary, based on slightly shorter open times and longer closed times, open probability was significantly reduced. In addition, the maximum current of the ensemble average was significantly lower in TG4 myocytes when compared to LM controls, and channel availability tended to be reduced, too. All of these differences were at least partially reversed by PTX-treatment (data not shown). The PTX-sensitivity of $I_{Ca(L)}$ reduction is compatible with a PTX-sensitive mechanism reducing channel phosphorylation in the transgenic animals. Comparing LM and TG4 regarding closed times seems particularly interesting for the following reason: we noted previously (Schröder & Herzig, 1999) that stimulation of native β_2-ARs in rat cardiomyocytes exerted an otherwise typical stimulatory response of single calcium channels, but failed to shorten closed times. In the present study, closed times from TG4 mice appeared prolonged. Possibly, a Gi-protein inhibits calcium channels preferentially by antagonizing phosphorylation-dependent shortening of closed times.

What is the functional role of β_2-ARs in the adult mouse heart?

There is no doubt that overexpression of the human β_2-AR induces an altered phenotype of the transgenic mouse, providing evidence that the human β_2-AR is able to couple to the murine β-AR signalling cascade. However, stimulatory coupling of native murine β_2-ARs to excitation-contraction coupling and contractility in adult hearts is less obvious. Xiao *et al*. (1999) studied the effects of zinterol on contraction amplitude of isolated myocytes from wild-type mice and observed no effect at concentrations up to 10 μM. After PTX-treatment zinterol markedly increased contractility and this effect was interpreted to be mediated by the β_2-AR subtype. The lack of effect with non-PTX-treated myocytes was explained by dual coupling of the β_2-AR to Gs and Gi, with Gi-protein activation masking the stimulatory effects. However, Oostendorp & Kaumann. (2000) could not detect any effect of β_2-AR stimulation on contractility of isolated left atria even after robust PTX-treatment of isolated atria (*in vitro*) or mice (*in vivo*), and by using the physiological β_2-AR agonist (−)-adrenaline. These discrepancies might reflect, that β_2-AR mediated stimulation of contractility in the adult mouse heart is restricted to PTX-treated ventricular tissue.

Conclusions

In ventricular myocytes from adult wild-type mice zinterol caused enhancement of $I_{Ca(L)}$ through stimulation of β_1-ARs but not β_2-ARs. Furthermore, inactivation of PTX-sensitive G-proteins did not uncover β_2-AR mediated stimulatory effects on $I_{Ca(L)}$. Overexpression of human β_2-ARs in the

adult mouse heart did not stimulate, but did suppress $I_{Ca(L)}$ in a PTX-sensitive manner. In summary, our data provide no evidence for a β_2-AR mediated stimulation of $I_{Ca(L)}$ in the adult mouse ventricle.

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References

AN, R.-H., HEATH, B.M., HIGGINS, J.P., KOCH, W.J., LEFKOWITZ, R.J. & KASS, R.S. (1999). β_2-adrenergic receptor overexpression in the developing mouse heart: evidence for targeted modulation of ion channels. *J. Physiol. (Lond.),* **516.1,** 19–30.

BOND, R.A., LEFF, P., JOHNSON, T.D., MILANO, C.A., ROCKMAN, H.A., MCMINN, T.R., APPARSUNDARAM, S., HYEK, M.F., KENAKIN, T.P., ALLEN, L.F. & LEFKOWITZ, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoceptor. *Nature,* **374,** 272–276.

DAAKA, Y., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1997). Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature,* **390,** 88–91.

GONG, H., ADAMSON, D.L., RANU, H.K., HEUBACH, J.F., KOCH, W.J., RAVENS, U. & HARDING, S.E. (2000). The effect of Gi-protein inactivation on basal, and β_1- and β_2AR-stimulated contraction of myocytes from transgenic mice overexpressing the β_2-adrenoceptor. *Br. J. Pharmacol.,* **131,** 594–600.

GÜRDAL, H., BOND, R.A., JOHNSON, M.D., FRIEDMAN, E. & ONARAN, H.O. (1997). An efficacy-dependent effect of cardiac overexpression of β_2-adrenoceptor on ligand affinity in transgenic mice. *Mol. Pharmacol.,* **52,** 187–194.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch clamp technique for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.,* **391,** 85–100.

HERZIG, S. & NEUMANN, J. (2000). Effects of serine/threonine protein phosphatases on ion channels in excitable membranes. *Physiol. Rev.,* **80,** 173–210.

HEUBACH, J.F., TREBEß, I., WETTWER, E., HIMMEL, H.M., MICHEL, M.C., KAUMANN, A.J., KOCH, W.J., HARDING, S.E. & RAVENS, U. (1999). L-type calcium current and contractility in ventricular myocytes from mice overexpressing the cardiac β_2-adrenoceptor. *Cardiovasc. Res.,* **42,** 173–182.

HOOL, L.C. & HARVEY, R.D. (1997). Role of β_1- and β_2-adrenergic receptors in regulation of Cl^- and Ca^{2+} channels in guinea pig ventricular myocytes. *Am. J. Physiol.,* **273,** H1669–H1676.

KAUMANN, A.J. & MOLENAAR, P. (1997). Modulation of human cardiac function through 4 β-adrenoceptor populations. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **355,** 667–681.

KUSCHEL, M., ZHOU, Y.-Y., CHENG, H., ZHANG, S.-J., CHEN, Y., LAKATTA, E.G. & XIAO, R.-P. (1999). G_i protein-mediated functional compartimentalization of cardiac β_2-adrenergic signalling. *J. Biol. Chem.,* **274,** 22048–22052.

KUZNETSOV, V., PAK, E., ROBINSON, R.B. & STEINBERG, S.F. (1995). β_2-Adrenergic receptor actions in neonatal and adult rat ventricular myocytes. *Circ. Res.,* **76,** 40–52.

LAFLAMME, M.A. & BECKER, P.L. (1998). Do β_2-adrenergic receptors modulate Ca^{2+} in adult rat ventricular myocytes? *Am. J. Physiol.,* **274,** H1308–H1314.

LIGGETT, S.B., TEPE, N.M., LORENZ, J.N., CANNING, A.M., JANTZ, T.D., MITARAI, S., YATANI, A. & DORN II, G.W. (2000). Early and delayed consequences of β_2-adrenergic receptor overexpression in mouse hearts. *Circulation,* **101,** 1707–1714.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MCDONALD, T.F., PELZER, S., TRAUTWEIN, W. & PELZER, D.J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol. Rev.,* **74,** 365–507.

MILANO, C.A., ALLEN, L.F., ROCKMAN, H.A., DOLBER, P.C., MCMINN, T.R., CHIEN, K.R., JOHNSON, T.D., BOND, R.A. & LEFKOWITZ, R.J. (1994). Enhanced myocardial function in transgenic mice overexpressing the β_2-adrenergic receptor. *Science,* **264,** 582–586.

NAGYKALDI, Z., KEM, D., LAZZARA, R. & SZABO, B. (1999). Canine ventricular myocyte β_2-adrenoceptors are not functionally coupled to L-type calcium current. *J. Cardiovasc. Electrophysiol.,* **10,** 1240–1251.

OOSTENDORP, J. & KAUMANN, A.J. (2000). Pertussis toxin suppresses carbachol-evoked cardiodepression but does not modify cardiostimulation mediated through β_1- and putative β_4-adrenoceptors in mouse left atria: no evidence for β_2- and β_3-adrenoceptor function. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **361,** 134–145.

SCHRÖDER, F. & HERZIG, S. (1999). Effects of β_2-adrenergic stimulation on single-channel gating of rat cardiac L-type Ca^{2+} channels. *Am. J. Physiol.,* **276,** H834–H843.

SKEBERDIS, V.A., JUREVICIUS, J. & FISCHMEISTER, R. (1997). Beta-2 adrenergic activation of L-type Ca^{++} current in cardiac myocytes. *J. Pharmacol. Exp. Ther.,* **283,** 452–461.

XIAO, R.-P., AVDONIN, P., ZHOU, Y.-Y., CHENG, H., AKHTER, S.A., ESCHENHAGEN, T., LEFKOWITZ, R.J., KOCH, W.J. & LAKATTA, E.G. (1999). Coupling of β_2-adrenoceptor to G_i proteins and its physiological relevance in murine cardiac myocytes. *Circ. Res.,* **84,** 43–52.

YUE, D.T., HERZIG, S. & MARBAN, E. (1990). β-Adrenergic stimulation of calcium channels occurs by potentiation of high-activity gating modes. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 753–757.

ZHOU, Y.-Y., CHENG, H., SONG, L.-S., WANG, D.-J., LAKATTA, E.G. & XIAO, R.-P. (1999). Spontaneous β_2-adrenergic signaling fails to modulate L-type Ca^{2+} current in mouse ventricular myocytes. *Mol. Pharm.,* **56,** 485–493.

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