Functional adenylyl cyclase inhibition in murine cardiomyocytes
by 2’(3’)-O-(N-methylantraniloyl)-guanosine 5’-[γ-thio]triphosphate

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Running Title: Inhibition of cardiac adenylyl cyclase by MANT-GTPγS

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Non-Standard Abbreviations:
AC: adenylyl cyclase
MANT-GTPγS: 2'(3')-O-(N-methylanthraniloyl)-guanosine 5-[(γ-thio)triphosphate
I_{Ca,L}: L-type Calcium current
AC5−/−: type 5 adenylyl cyclase knockout
WT: wild-type
IV-curves: current-voltage-curves
β1-AR: β1-adrenoreceptor
GαS: α-subunit of stimulating G-protein

**Recommended Section Assignment:** Cardiovascular
Abstract

β₁-Adrenergic receptor activation stimulates cardiac L-type Ca^{2+} channels via adenylyl cyclases (AC), with AC5 and AC6 being the most important cardiac isoforms. Recently, we have identified 2'(3')-O-(N-methylantraniloyl)-guanosine 5-[γ-thio]triphosphate (MANT-GTPγS) as a potent competitive AC inhibitor. Intriguingly, MANT-GTPγS inhibits ACs 5 and 6 more potently than other cyclases. These data prompted us to study the effects of MANT-GTPγS on L-type Ca^{2+} currents (I_{Ca,L}) in ventricular myocytes of wildtype (WT) and AC5-deficient (AC5⁻/⁻) mice by whole-cell recordings. In wildtype myocytes MANT-GTPγS attenuated I_{Ca,L} stimulation following isoproterenol application in a concentration-dependent manner (control: +77 ± 13 %; 100 nM MANT-GTPγS: +43 ± 6 %; 1 µM MANT-GTPγS: +21 ± 9 %, p < 0.05). The left-ward shift of IV-curves was abolished by 1 µM, but not by 100 nM MANT-GTPγS. In myocytes from AC5⁻/⁻ mice the residual stimulation of I_{Ca,L} was not further attenuated by the nucleotide, indicating AC5 to be the major AC isoform mediating acute β-adrenergic stimulation in WT mice. Interestingly, basal I_{Ca,L} was lowered by 1 µM, but not by 100 nM MANT-GTPγS. The decrease was less pronounced in myocytes from AC5⁻/⁻ mice compared to wildtypes (-23 ± 1 % vs. -40 ± 7 %), indicating basal I_{Ca,L} to be partly driven by AC5. Collectively, we found a concentration-dependent inhibition of I_{Ca,L} by MANT-GTPγS, both under basal conditions and following β-adrenergic stimulation. Comparison of data from wildtype and AC5-deficient mice indicates that AC5 plays a major role in I_{Ca,L} activation and that MANT-GTPγS predominantly acts via AC5 inhibition.
Introduction

β₁-Adrenoreceptor (β₁-AR) activation via the stimulatory G-protein Gαₛ leads to enhanced cAMP generation catalyzed by adenylyl cyclases (AC). cAMP mediates diverse cellular responses, e.g. by activating protein kinase A. One target for phosphorylation by protein kinase A is the cardiac L-type Ca²⁺ channel (Kamp and Hell, 2000). At least 10 mammalian AC isoforms with a tissue-specific distribution have been identified (Hanoune and Defer, 2001). In mammalian heart the Ca²⁺-dependent isoforms AC5 and AC6 are the two main cyclase isoforms at the mRNA level. Besides AC5 and AC6, several other AC isoforms (types 2, 3, 4, 7 and 9) were found in murine hearts (Okumura et al., 2003a), but are thought to play a negligible functional role due to low expression levels or low enzymatic activity (Defer et al., 2000; Hanoune and Defer, 2001).

Although AC5 represents the isoform with the highest catalytic activity in the adult heart (Ishikawa et al., 1992), it remains unclear whether it is the functionally cardiac isoform, in particular regarding acute regulation of L-type Ca²⁺ channel activity and thus modulation of Ca²⁺ influx triggering contraction. Nevertheless AC5 is an interesting potential drug target in the therapy of heart disease. In this regard transgenic overexpression of type 6 AC did not induce abnormal histological findings or deleterious changes in the heart (Gao et al., 1999), while overexpression of type 5 AC affects basal AC activity and cardiac function (Tepe et al., 1999). These findings indicate that AC5, in contrast to type 6 AC, plays an important role under pathological conditions. Furthermore in AC5-knockout (AC5⁻/⁻) mice, contractile properties and L-type Ca²⁺-currents (I_{Ca,L}) were impaired in a Ca²⁺-dependent manner (Okumura et al., 2003a), while cardiac function was protected against pressure overload induced by aortic banding (Okumura et al., 2003b). Particularly these findings on AC5-deficiency underline the important role of AC5 in murine hearts and suggest AC inhibitors to be promising pharmacological tools and perhaps even therapeutics. This notion is supported
by the finding that excessive activation of the β₁-AR-Gαₛ-AC cascade is detrimental for cardiac function (Engelhardt et al., 1999; Lohse et al., 2003).

Most AC inhibitors known so far are either non-competitive or non-specific regarding isoforms (Hanoune and Defer, 2001; Iwatsubo et al., 2004). With respect to therapeutic use, however, both competitiveness and isoform-specificity would be helpful properties in minimizing the risk of adverse effects. Recently, we have identified 2′(3′)-O-(N-methylantraniloyl) (MANT) nucleotides as a novel class of potent competitive AC inhibitors (Gille and Seifert, 2003). Intriguingly, MANT-nucleotides inhibit AC5 and AC6 more potently than other cyclases (Gille et al., 2004). Crystallographic, biophysical and biochemical studies have provided in-depth insights into the molecular interactions of MANT-nucleotides with AC, providing a rational basis for the development of even more potent and selective AC inhibitors than those currently available (Mou et al., 2005, 2006).

To assess the pharmacological effects of MANT-nucleotides in murine cardiomyocytes, we took advantage of the pivotal position of AC linking β-adrenergic signaling to L-type Ca²⁺ current stimulation. We used the hydrolysis-resistant MANT-nucleotide MANT-GTPγS as a prototypical competitive AC inhibitor. During whole-cell patch clamp recordings, the membrane-impermeant compound was delivered by intracellular dialysis. Freshly isolated murine cardiomyocytes were exposed to isoproterenol to study AC5 inhibition under basal conditions and β-adrenergic stimulation. We repeated these experiments in AC5⁻/⁻ cardiomyocytes to determine the AC5-specificity of the effects of MANT-GTPγS.
Methods

Animals. Mice (3-9 months) with type 5 adenylyl cyclase deficiency (AC5+/−) (Okumura et al., 2003b) were fully backcrossed (>5 generations) into the C57/Bl6 strain and bred with their respective non-transgenic littermates. Wildtype C57/Bl6 mice (3-9 months) served as control. Experiments complied with respective laws and local regulations regarding animal care.

Genotyping of knockout mice. A tail clip analysis was performed at 3-4 weeks of age. After preparation of genomic DNA, a PCR was run. To genotype AC5−/−, we used the following primer pairs: wild type (+), forward, 5'-CGC TAC TTC TTC CAC CTG AAC CAG-3'; reverse, 5'-TGA TAA GGA TCA CGC CCA CAG C-3' and knockout (-), forward, 5'-TCG TGC TTT ACG GTA TCG CCG CTC CCG ATT-3'; reverse, 5'-TGA TAA GGA TCA CGC CCA CAG C-3'. Both reactions were run over 40 cycles (saturation). Amplified sequences were 157 bp for the WT allele and 443 bp for the targeting construct.

Isolation of cardiac myocytes. Single ventricular myocytes were isolated by enzymatic dissociation using the method previously described (Foerster et al., 2003). In brief, hearts were perfused with a collagenase solution (Worthington type I and II, 75 units per milliliter; Cell Systems, St. Katharinen, Germany) in a Langendorff setup and subsequently cut into small chunks. Myocytes were harvested by pouring the suspension through cheesecloth.

Whole-cell recording. L-type calcium channel currents were measured at room temperature using the whole-cell configuration of the patch-clamp technique as described previously (Heubach et al., 2001). Whole-cell experiments were performed in an external solution containing (mM): NaCl 137, CsCl 5.4, CaCl2 2, MgCl2 1.25, HEPES 10, glucose 10 (pH 7.4 with NaOH). Pipettes (borosilicate glass, 1.5-3 MΩ) were filled with (mM) CsCl 140, MgCl2
4, HEPES 10, EGTA 10, Na$_2$ATP 4 (pH 7.3 with CsOH). Cells were continuously superfused with drug-free bath solution and then switched to a solution containing 100 nM (-)-isoproterenol. 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. 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. Only rod shaped myocytes with clear striation were used. Whole-cell Ca$^{2+}$ currents were elicited by 200 ms depolarizing voltage steps from a holding potential of -60 mV. The stimulation frequency was 0.2 Hz (EPC-9, HEKA, Lambrecht, Germany). For each cell a current-voltage relationship was established at the beginning and the end of the experiment. The test potential was +10 mV. For isolation of $I_{Ca,L}$ from contaminating currents, $I_{Na}$ and T-type $I_{Ca}$ were inactivated by a 50 ms long prepulse to -40 mV (holding potential: -60 mV), and K$^+$ currents were minimized by replacing K$^+$ with Cs$^+$. In order to account for variability 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. The software PULSE (version 9.12; HEKA) was used for data acquisition.

**Drugs.** MANT-GTP$_{\gamma}$S (Jena Bioscience, Jena, Germany) and (-)-isoproterenol-HCl (Sigma, Deisenhofen, Germany) were prepared as 10 mM stock solutions in double-distilled H$_2$O. Isoproterenol was further diluted in bath solution and superfused the cardiomyocytes using a rapid solution changer (RSC 200, Bio-Logic, Claix, France). MANT-GTP$_{\gamma}$S was diluted in pipette solution immediately before use.

**Statistics and data analysis.** Results are given as mean values ± s.e.m.. Differences between mean values were tested by alternate t-test and considered statistically significant for $p < 0.05.$
Results

**Effect of MANT-GTPγS on basal $I_{Ca,L}$ in wildtype myocytes.**

We first wanted to assess the effect of MANT-GTPγS on basal currents mediated by L-type Ca$^{2+}$ channels in wildtype ventricular myocytes. Under control conditions $I_{Ca,L}$ showed a slight decrease that did not exceed 8% of the initial current within 10 minutes and reached stable values for the remaining recording time (from $-4.8 \pm 0.3$ pA/pF to $-4.4 \pm 0.25$ pA/pF after 10 minutes, n.s., n = 6; Fig. 2B). When 1 µM MANT-GTPγS was present in the pipette solution, current density was not instantaneously different compared to control recordings, but decreased significantly over time (Fig. 2A and B). After 10 minutes of recording with MANT-GTPγS in the pipette current density was lowered by 40 ± 7% (Fig. 2A and B; from $-4.0 \pm 0.24$ pA/pF to $-2.5 \pm 0.3$ pA/pF, $p < 0.05$, n = 6). This demonstrates that 1 µM MANT-GTPγS reduced basal L-type Ca$^{2+}$ current as a function of time, due to intracellular dialysis via the patch pipette. The reduction of basal L-type Ca$^{2+}$ channel activity reached a plateau after 12-15 minutes of recording, suggesting that the reduction of L-type Ca$^{2+}$ current is not due to continuous “run-down”.

**Effects of MANT-GTPγS on $I_{Ca,L}$ stimulation by isoproterenol in wildtype myocytes.**

Isoproterenol is known to stimulate currents mediated by L-type Ca$^{2+}$ channels due to increased protein kinase A activity following enhanced cAMP production catalyzed by AC. We therefore obtained $I_{Ca,L}$ stimulated by isoproterenol in the absence and the presence of the AC inhibitor MANT-GTPγS (1 µM). Under control conditions 100 nM isoproterenol increased maximum $I_{Ca,L}$ by 77%. This increase was attenuated to only +21% in the presence of MANT-GTPγS (+77 ± 13% vs. +21 ± 9%, $p < 0.01$; n = 10; Figs. 3A and B; Fig. 5D). When MANT-GTPγS was applied, the leftward-shift of I/V-curves typically caused by
isoproterenol was absent (Fig. 3C and D). Of note, after 15 minutes of isoproterenol perfusion currents still were significantly increased above pre-drug values in the absence of MANT-GTPγS. In contrast, in the presence of the AC inhibitor current density declined below values obtained before drug application (Fig. 3B). This could be due to an ongoing reduction of basal current, of isoproterenol-stimulated current, or both. To discriminate between these possibilities, washout experiments were performed. Isoproterenol was applied early in the course of the experiment and washed out 10 minutes later (Fig. 4A and B). Compared to controls (Fig. 4B) calcium current decrease by isoproterenol washout was less pronounced in the presence of MANT-GTPγS, indicating smaller remaining agonist effect at that time. These data match our findings showing a decrease in current density by MANT-GTPγS even in the absence of β-adrenergic stimulation (see above). In summary, MANT-GTPγS significantly and almost completely diminished the increase of ICa,L due to isoproterenol.

**Concentration-dependence of MANT-GTPγS effects on ICa,L in wildtype myocytes.**

MANT-GTPγS inhibited isoproterenol effects in a concentration-dependent way. A lower concentration of the MANT-nucleotide (100 nM) still reduced the increase in ICa,L due to isoproterenol (100 nM) stimulation (Fig. 5A), albeit to a lower extent (control: +77 ± 13 %; 100 nM MANT-GTPγS: +43 ± 6 %; 1 µM MANT-GTPγS: +21 ± 9 %, Fig. 5D). Of note, in the presence of the lower MANT-GTPγS concentration, isoproterenol still caused the typical leftward-shift of the I/V-curve as observed under control conditions (Fig. 5C). Interestingly, 8 minutes after starting isoproterenol (100 nM) perfusion, stimulated ICa,L remained stable in the presence of 100 nM MANT-GTPγS, suggesting that no further decrement of the isoproterenol effect was caused by the low nucleotide concentration (Fig. 5A and B). These findings also suggest that lower concentrations of the nucleotide did not affect basal ICa,L. To evaluate the effect of 100 nM MANT-GTPγS on basal ICa,L a comparison of current values immediately before starting isoproterenol perfusion (at 2.5 min) was performed: While under control
conditions (+4 ± 2 %) and in the presence of 100 nM MANT-GTP\(\gamma\)S (+3 ± 2 %, n.s. vs. control) currents were unaffected, 1 \(\mu\)M MANT-GTP\(\gamma\)S decreased current by already -19 ± 4 % (\(p < 0.01\) vs. control, \(n = 5\)). This confirms that lower concentrations of MANT-GTP\(\gamma\)S did not affect basal \(I_{\text{Ca,L}}\). In summary, basal and isoproterenol-stimulated inhibition of \(I_{\text{Ca,L}}\) in the presence of MANT-GTP\(\gamma\)S is concentration-dependent.

**Effect of MANT-GTP\(\gamma\)S on basal \(I_{\text{Ca,L}}\) in AC5\(^{-/-}\) myocytes.**

To determine AC5 specific effects of MANT-nucleotides and to characterize the functional role of AC5, experiments obtained with WT cardiomyocytes were repeated in ventricular myocytes from mice lacking AC5 (AC5\(^{-/-}\)). In ventricular myocytes from AC5\(^{-/-}\) mice 1 \(\mu\)M MANT-GTP\(\gamma\)S decreased \(I_{\text{Ca,L}}\) (after 10 minutes: -23 ± 1 %, vs. 8 ± 2 % without MANT-GTP\(\gamma\)S, \(p < 0.01\); \(n = 6\); Fig. 6A and B). This reduction of basal \(I_{\text{Ca,L}}\) in AC5\(^{-/-}\) myocytes was significantly less pronounced than in WT myocytes (after 10 minutes: -41 ± 7 %, \(p < 0.05\); \(n = 6\)), indicative for an AC5-specific effect of MANT-GTP\(\gamma\)S.

**Effects of MANT-GTP\(\gamma\)S on \(I_{\text{Ca,L}}\) stimulation by isoproterenol in AC5\(^{-/-}\) myocytes.**

In accordance with previous findings (Okumura et al., 2003b) isoproterenol effects on \(I_{\text{Ca,L}}\) were mitigated in ventricular myocytes of AC5-deficient (AC5\(^{-/-}\)) mice. Of note, MANT-GTP\(\gamma\)S (1 \(\mu\)M) did not further attenuate \(I_{\text{Ca,L}}\) stimulated by isoproterenol in AC5\(^{-/-}\) myocytes (32 ± 3 % vs. 29 ± 3 %, n.s.; \(n=5\); Figs. 7A, B and C). Towards the end of incubation, isoproterenol effects appeared to decrease in the presence of MANT-GTP\(\gamma\)S. However, subtraction of the basal \(I_{\text{Ca,L}}\) time course (cp. Fig. 6B) from isoproterenol stimulated \(I_{\text{Ca,L}}\) revealed that this effect is entirely explained by the reduction in basal L-type Ca\(^{2+}\) current activity (data not shown). The MANT-GTP\(\gamma\)S-resistance of isoproterenol effects in AC5\(^{-/-}\)-myocytes was confirmed by nearly unaffected I/V-curves (Fig. 7D and E).
Discussion

In our present study we characterized the effect of the novel competitive AC inhibitor MANT-GTPγS on murine ventricular Icai. MANT-GTPγS is well suited for electrophysiological studies using intracellular dialysis since it is hydrolysis-resistant. Our findings indicate AC5 to be the major functional isoform mediating acute β1-adrenergic stimulation of Icai. In the presence of MANT-GTPγS we find an attenuation of Icai increase following β-adrenergic stimulation via isoproterenol thus confirming previous findings obtained with AC5-deficient mice (Okumura et al., 2003a). Most importantly, we found that in AC5-deficient mice 1 µM MANT-GTPγS did not further attenuate maximum Icai response to isoproterenol. Considering the similar affinities of MANT-GTPγS to the cardiac isoforms AC5 and AC6 (Gille et al., 2004), we conclude that AC5 plays a major role in mediating immediate β-adrenergic stimulation of L-type Ca2+-channels in ventricular cardiomyocytes.

Using the lower concentration of MANT-GTPγS (100 nM), a moderate inhibition of isoproterenol effects was obtained without concomitant inhibition of basal currents. Furthermore, Icai increase following isoproterenol was maintained here, while in the presence of 1 µM of the nucleotide, Icai decreased continuously during the course of experiments. This indicates that the isoproterenol-stimulated current is more sensitive towards the AC inhibitor than the basal current. The only minimal effect of MANT-GTPγS on basal current in AC5-deficient mice reveals that part of the inhibition of basal current in WT mice is due to AC 5 inhibition. Additional mechanisms, such as involvement of a less sensitive functional AC isoform in basal current regulation, or other non-specific effects of the nucleotide cannot be further elucidated by our experiments. Thus, our data reveal AC5 to activate tonically basal Ca2+ current activity. This interpretation might at first glance contrast to the unchanged basal current values reported for AC−/− mice by group comparison (Okumura et al., 2003a), but long-term compensatory mechanisms are to be expected in such genetic models. Considering the
lower isoproterenol effect on $I_{Ca,L}$ in AC$^{-/-}$ myocytes one might also speculate on decreased abundance of potential phosphorylation sites, e.g. by lower expression of the pore-forming, PKA-sensitive $\alpha 1C$ subunit coded by exon 1a (van der Heyden et al., 2005) or altered composition of the heteromeric channel complex by pore-forming $\alpha 1C$ and auxiliary $\beta$-subunits (Bünemann et al., 1999).

MANT-nucleotides represent novel experimental tools in the search for potential therapeutic compounds since they are competitive AC inhibitors (Gille and Seifert, 2003). Recent high-resolution crystallographic data underline their eminent role as lead compounds for the development of potent and isoform-specific AC inhibitors (Mou et al., 2005, 2006). Our present data show that isoproterenol effects are reduced by MANT-GTP$\gamma$S via concentration-dependent inhibition of AC5. Considering the sympathetic overdrive in heart failure this indicates MANT-nucleotides as promising starting points regarding innovative therapeutic strategies. MANT-GTP$\gamma$S as a prototypical competitive AC inhibitor shows AC5-mediated effects, as proven by its lack of effect on isoproterenol-stimulation of $I_{Ca,L}$ in AC5$^{-/-}$ cardiomyocytes. Since AC5 deficiency seems to be protective against heart failure due to pressure overload (Okumura et al., 2003b) this demonstrates that MANT-nucleotides could serve as a novel approach in the therapy of cardiac diseases. This is supported by the known benefits of $\beta$-adrenoceptor antagonists in the treatment of heart failure. $\beta$-Adrenoceptor antagonists have become standard treatment of heart failure although it is unclear what the exact underlying mechanism is. Some data suggest that signaling via $\beta_2$-adrenoceptors would be beneficial (Dorn et al., 1999; Du et al., 2000), while $\beta_1$-adrenoceptor signaling obviously is detrimental (Engelhardt et al., 1999; Lohse et al., 2003). This divergence is supported by findings on the differential signaling cascades and L-type Ca$^{2+}$ channel regulation by $\beta_1$- and $\beta_2$-adrenoceptors (Foerster et al., 2003; 2004; Xiao et al., 2006). A major difference is dual signaling via inhibitory (G$_i$) and stimulatory (G$_s$) G-proteins by $\beta_2$-adrenoceptors, and only G$_s$
in case of β₁-adrenoceptors. Hence, adenylyl cyclase inhibitors might be an attractive alternative to β-adrenoceptor antagonists due to their selective prevention of an (excessive) increase of cAMP.

In future studies, we will have to design potent cell-permeable AC5 inhibitors since the currently available AC inhibitors are not cell membrane-permeable. A feasible approach is the design of pronucleotides that can be deprotected and phosphorylated within cells (Laux et al., 2004). In fact, we have already shown that MANT-nucleoside 5’-diphosphates are phosphorylated to the corresponding triphosphates by cellular kinases (Gille et al., 2004). It should be emphasized that the concept of AC5 inhibition for the treatment of cardiovascular disease is quite different from the current concept of β₁-adrenoceptor blockade since ACs integrate the input from multiple receptor systems (Hanoune and Defer, 2001). Our present study shows that the combination of pharmacological studies with gene knock-out studies is very powerful at delineating signal transduction pathways and establishing novel therapeutic concepts.

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Footnotes

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Legends for Figures

Figure 1  **Structural formula of MANT-GTPγS.** Note the spontaneous isomerization between the 2´ and 3´-MANT-substituted structure.

Figure 2  **Effect of MANT-GTPγS (1 µM) on basal L-type Ca^{2+}-current in wildtype murine cardiomyocytes.** (A) Original traces in the absence and presence of 1 µM MANT-GTPγS at the time points 0 and 10 minutes. (B) Averaged time course of whole-cell L-type Ca^{2+} current density in the absence (filled symbols, n = 6) and in the presence of 1µM MANT-GTPγS (open symbols, n = 6). Starting from a holding potential of -60 mV the current was elicited by a test potential of +10mV preceded by a prepulse to -40mV.

Figure 3  **Effect of MANT-GTPγS (1 µM) on isoproterenol-stimulated L-type Ca^{2+}-current in wildtype murine cardiomyocytes.** Cells were superfused by isoproterenol containing bath solution for 12.0 ± 0.1 min (control) and 13.0 ± 0.2 min (MANT-GTPγS), respectively. (A) Original traces before and after 100 nM (-)-isoproterenol stimulation and in the absence and presence of 1 µM MANT-GTPγS at the time point 0 minutes and at the maximum increase of L-type Ca^{2+} current. (B) Averaged time course of whole-cell L-type Ca^{2+} current density from experiments with murine ventricular myocytes before and after 100 nM (-)-isoproterenol stimulation and in the absence (filled symbols, n = 5) and in the presence of 1 µM MANT-GTPγS (open symbols, n = 5). Starting from a holding potential of -60 mV the current was elicited by a test potential of +10mV preceded by a prepulse to -40mV. (C) Control current density-voltage relationship in the absence (filled symbols, n = 5) and in the presence (open symbols, n = 5) of 100 nM (-)-isoproterenol, measured before and 10 minutes after start of isoproterenol application. (D) Current density-voltage relationship with 1 µM
MANT-GTPγS diluted in the pipette solution in the absence (filled symbols, n = 5) and in the presence (open symbols, n = 5) of 100 nM (-)-isoproterenol measured at the beginning and at the end of the experiments. * indicates \( p < 0.05 \) (alternate t-test).

**Figure 4** Reversibility of the effect of isoproterenol on L-type Ca\(^{2+}\)-current in wildtype murine cardiomyocytes. (A) Averaged time course of whole-cell L-type Ca\(^{2+}\) current density from experiments with myocytes before and after 100 nM (-)-isoproterenol stimulation, followed by isoproterenol washout in the presence of 1 µM MANT-GTPγS (filled symbols, n = 5). Open symbols show basal L-type Ca\(^{2+}\) current time course (cf. Fig. 2B) to illustrate reversibility of isoproterenol stimulation in the presence of MANT-GTPγS. Starting from a holding potential of -60 mV the current was elicited by a test potential of +10mV preceded by a prepulse to -40mV. (B) Filled symbols represent the averaged (n = 5) time course of similar washout-experiments, performed in the absence of MANT-GTPγS.

**Figure 5** Effect of MANT-GTPγS (100 nM) on isoproterenol-stimulated L-type Ca\(^{2+}\)-current in wildtype murine cardiomyocytes. Cells were superfused by isoproterenol containing bath solution for 8.4 ± 0.1 min. (A) Original traces before and after 100 nM (-)-isoproterenol stimulation and in the presence of 100 nM MANT-GTPγS at the time point 0 minutes and at the maximum increase of L-type Ca\(^{2+}\) current. (B) Averaged time course of whole-cell L-type Ca\(^{2+}\) current density from experiments with murine ventricular myocytes before and after 100 nM (-)-isoproterenol stimulation in presence of 100 nM MANT-GTPγS (filled symbols, n = 5). Starting from a holding potential of -60 mV the current was elicited by a test potential of +10mV preceded by a prepulse to -40mV. (C) Current density-voltage relationship with 100 nM MANT-GTPγS diluted in the pipette solution in the absence (filled symbols, n = 5) and in the presence (open symbols, n = 5) of 100 nM (-)-isoproterenol measured at the beginning and at the end of the experiments, respectively. * indicates \( p < 0.05 \).
(alternate t-test). (D) Maximum increase of L-type Ca\(^{2+}\) current density by isoproterenol (% of pre-drug values) compared with basal current in the absence (black bar, n = 10) and in the presence of 100 nM (grey bar, n = 5) or 1 µM (white bar, n = 11) MANT-GTP\(\gamma\)S, respectively. * indicates \(p < 0.05\) vs. control (alternate t-test).

**Figure 6** Effect of MANT-GTP\(\gamma\)S (1 µM) on basal L-type Ca\(^{2+}\)-current in AC5\(^{-/-}\) murine cardiomyocytes. (A) Original traces in the absence and presence of 1 µM MANT-GTP\(\gamma\)S at the time points 0 and 10 minutes. (B) Averaged time course of whole-cell L-type Ca\(^{2+}\) current density from experiments with murine ventricular myocytes lacking type 5 AC (AC5\(^{-/-}\)) in the absence (filled symbols, n = 6) and in the presence of 1 µM MANT-GTP\(\gamma\)S (open symbols, n = 6). Starting from a holding potential of -60 mV the current was elicited by a test potential of +10mV preceded by a prepulse to -40mV.

**Figure 7** Effect of MANT-GTP\(\gamma\)S (1 µM) on isoproterenol-stimulated L-type Ca\(^{2+}\)-current in AC5\(^{-/-}\) murine cardiomyocytes. Cells were superfused by isoproterenol containing bath solution for 8.2 ± 0.1 min (control) and 8.2 ± 0.1 min (MANT-GTP\(\gamma\)S), respectively. (A) Original traces before and after 100 nM (-)-isoproterenol stimulation and in the absence and presence of 1 µM MANT-GTP\(\gamma\)S at the time point 0 minutes and at the maximum increase of L-type Ca\(^{2+}\) current. (B) Averaged time course of whole-cell L-type Ca\(^{2+}\) current density from experiments with murine ventricular myocytes lacking type 5 AC (AC5\(^{-/-}\)) before and after 100 nM (-)-isoproterenol stimulation and in the absence (filled symbols, n = 5) and in the presence of 1 µM MANT-GTP\(\gamma\)S (open symbols, n = 5). Starting from a holding potential of -60 mV the current was elicited by a test potential of +10mV preceded by a prepulse to -40mV. (C) Maximum increase of L-type Ca\(^{2+}\) current density (%) compared with basal current (= 0) in the absence (black bar, n = 5) and in the presence (white bar, n = 5) of 1 µM MANT-GTP\(\gamma\)S. (D) Control current density-voltage relationship from
AC5<sup>-/-</sup> myocytes in the absence (filled symbols, n = 5) and in the presence (open symbols, n = 5) of 100 nM (-)-isoproterenol measured at the beginning and at the end of the experiments.

(E) Current density-voltage relationship from AC5<sup>-/-</sup> myocytes with 1 µM MANT-GTPγS diluted in the pipette solution in the absence (filled symbols, n = 5) and in the presence (open symbols, n = 5) of 100 nM (-)-isoproterenol measured at the beginning and at the end of the experiments, respectively.
5A

MANT-GTP$_\gamma$S $10^{-7}$M

ISO $10^{-7}$M

400pA

50ms
5B

![Graph showing the effect of Isoproterenol and MANT-GTPγS on current density over time.](image)

- **Isoproterenol 10^{-7}M**
- **MANT-GTPγS 10^{-7}M**
Isoproterenol $10^{-7}$ M

$I_{Ca,L}$ density (pA/pF)

- $AC5^{-/-}$
- $AC5^{-/-} + MANT-GTP\gamma S \ 10^{-6}$ M
Iso-Effect on I_{Ca,L} (%)

AC5^/-

AC5^/- + MANT-GTP\gamma S
10^{-6}M
7D

Test potentials (mV)

-40 -20 0 20 40

-2

-4

-6

-8

$I_{Ca,L}$ (pA/pF)

- M

- AC5$^{-/-}$

- AC5$^{-/-}$ + ISO $10^{-7}$ M