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Functional Adenylyl Cyclase Inhibition in Murine Cardiomyocytes by 2′(3′)-*O*-(*N*-Methylanthraniloyl)-Guanosine 5′-[γ-Thio]triphosphate

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ABSTRACT

β_1-Adrenergic receptor activation stimulates cardiac L-type Ca^{2+} channels via adenylyl cyclases (ACs), with AC5 and AC6 being the most important cardiac isoforms. Recently, we have identified 2′(3′)-*O*-(*N*-methylanthraniloyl)-guanosine 5′-[γ-thio-]triphosphate (MANT-GTPγS) as a potent competitive AC inhibitor. Intriguingly, MANT-GTPγS inhibits AC5 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 wild-type (WT) and AC5-deficient ($AC5^{-/-}$) mice by whole-cell recordings. In wild-type 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 leftward shift of current-voltage 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 with wild types (−23 ± 1 versus −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 wild-type 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.

β_1-Adrenoreceptor activation via the stimulatory G protein $G\alpha_S$ leads to enhanced cAMP generation catalyzed by adenylyl cyclases (ACs). 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 Ca^{2+} 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 Ca^{2+}-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 they 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 dominant cardiac isoform, in particular regarding acute regulation of L-type Ca^{2+} channel activity and thus modulation of Ca^{2+} 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), whereas 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 Ca^{2+} currents ($I_{Ca,L}$) were impaired in a Ca^{2+}-dependent manner (Okumura et al.,

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ABBREVIATIONS: AC, adenylyl cyclase; $I_{Ca,L}$, L-type calcium current; MANT, 2′(3′)-*O*-(*N*-methylanthraniloyl); GTPγS, guanosine 5′-[γ-thio]-triphosphate; WT, wild-type; I/V, current-voltage.

2003a), whereas 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 β_1-adrenoreceptor-$G\alpha_S$-AC cascade is detrimental for cardiac function (Engelhardt et al., 1999; Lohse et al., 2003).

Most AC inhibitors known so far are either noncompetitive or nonspecific regarding isoforms (Hanoune and Defer, 2001; Iwatsubo et al., 2004). However, with respect to therapeutic use, both competitiveness and isoform specificity would be helpful properties in minimizing the risk of adverse effects. Recently, we have identified 2′(3′)-*O*-(*N*-methylanthraniloyl) (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 insight 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 Ca^{2+} current stimulation. We used the hydrolysis-resistant MANT-nucleotide MANT-GTPγS (Fig. 1) 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.

Materials and Methods

Animals. Mice (3–9 months old) with type 5 adenylyl cyclase deficiency ($AC5^{-/-}$) (Okumura et al., 2003b) were fully backcrossed (more than five generations) into the C57/Bl6 strain and bred with their respective nontransgenic littermates. Wild-type C57/Bl6 mice (3–9 months old) served as control. Experiments complied with respective laws and local regulations regarding animal care.

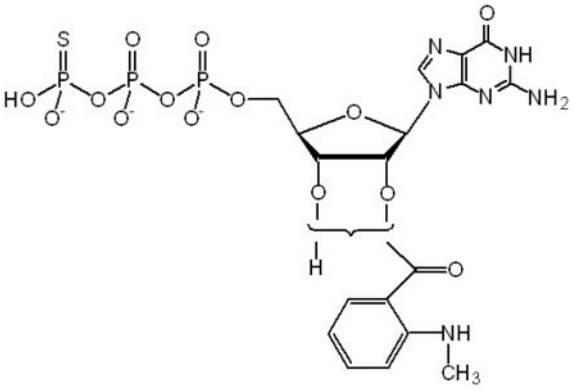

Fig. 1. Structural formula of MANT-GTPγS. Note the spontaneous isomerization between the 2′- and 3′-MANT-substituted structure.

Genotyping of Knockout Mice. A tail-clip analysis was performed at 3 to 4 weeks of age. After preparation of genomic DNA, a polymerase chain reaction 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 base pairs for the WT allele and 443 base pairs for the targeting construct.

Isolation of Cardiac Myocytes. Single ventricular myocytes were isolated by enzymatic dissociation using the method described previously (Foerster et al., 2003). In brief, hearts were perfused with a collagenase solution (Worthington type I and II, 75 U/ml; Cell Systems, St. Katharinen, Germany) in a Langendorff setup, and they were 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 137 mM NaCl, 5.4 mM CsCl, 2 mM $CaCl_2$, 1.25 mM

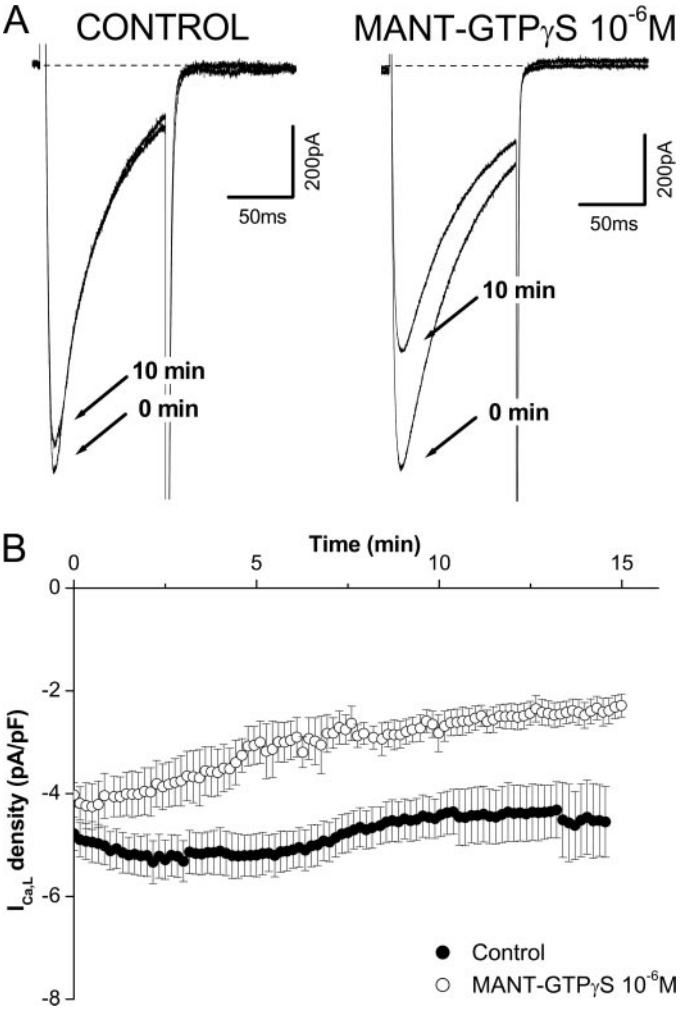

Fig. 2. Effect of 1 μM MANT-GTPγS on basal L-type Ca^{2+} current in wild-type murine cardiomyocytes. A, original traces in the absence and presence of 1 μM MANT-GTPγS at the time points 0 and 10 min. B, averaged time course of whole-cell L-type Ca^{2+} current density in the absence (filled symbols; $n = 6$) and in the presence of 1 μM 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 −40 mV.

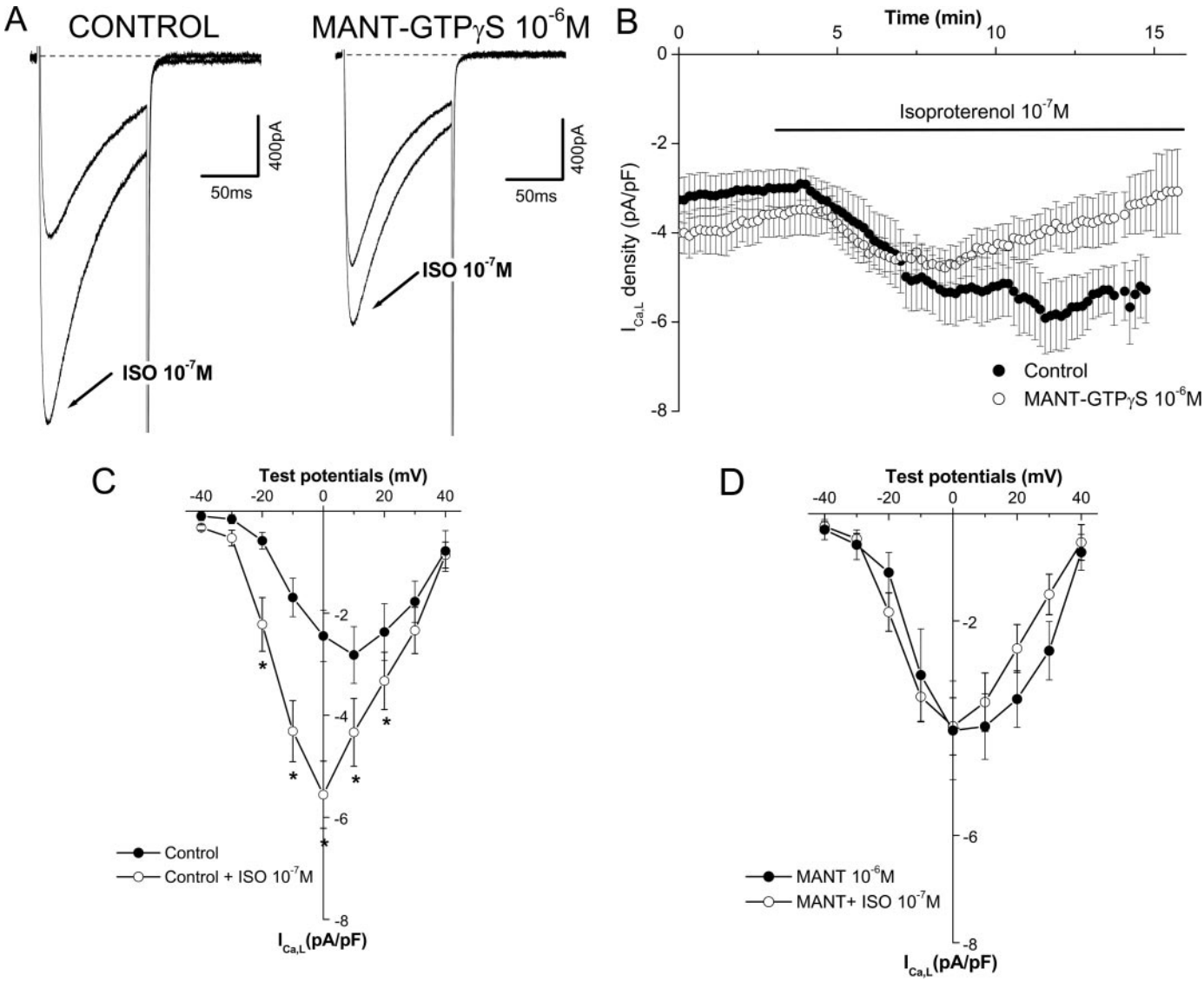

Fig. 3. Effect of 1 μM MANT-GTPγS on isoproterenol-stimulated L-type Ca^{2+} current in wild-type murine cardiomyocytes. Cells were superfused by isoproterenol containing bath solution for 12.0 ± 0.1 min (control) and 13.0 ± 0.2 min (MANT-GTPγS), respectively. A, original traces before and after 100 nM (−)-isoproterenol stimulation and in the absence and presence of 1 μM MANT-GTPγS at the time point 0 min and at the maximal 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 +10 mV preceded by a prepulse to −40 mV. 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 min 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. ∗, $p < 0.05$ (alternate t test).

$MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4 with NaOH. Pipettes (1.5 to 3-MΩ borosilicate glass) were filled with 140 mM CsCl, 4 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, and 4 mM Na_2ATP, pH 7.3 with CsOH. Cells were continuously superfused with drug-free bath solution, and then they were 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 −35 mV; 5-ms duration) at the beginning of each experiment. Compensated access resistance was regularly checked and maintained below 5 MΩ. Series resistance was routinely compensated by 50 to 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/Pfalz, 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, sodium and T-type calcium currents 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^+. To account for variability in cell size, absolute current amplitudes [in picoamperes (pA)] were divided by the respective cell capacitance [in picofarads (pF)], and values are expressed as membrane current I in picoamperes per picofarads. The software PULSE, version 9.12 (HEKA) was used for data acquisition.

Drugs. MANT-GTPγS (Jena Bioscience, Jena, Germany) and (−)-isoproterenol HCl (Sigma Chemie, Deisenhofen, Germany) were prepared as 10 mM stock solutions in double-distilled H_2O. Isoproterenol was further diluted in bath solution and superfused the cardiomyocytes using a rapid solution changer (RSC 200; Bio-Logic,

Claix, France). MANT-GTPγS 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 Wild-Type Myocytes. We first wanted to assess the effect of MANT-GTPγS on basal currents mediated by L-type Ca^{2+} channels in wild-type ventricular myocytes. Under control conditions, $I_{Ca,L}$ showed a slight decrease that did not exceed 8% of the initial current within 10 min, and it reached stable values for the remaining recording time (from −4.8 ± 0.3 pA/pF to −4.4 ± 0.25 pA/pF after 10 min; N.S., $n = 6$; Fig. 2B). When 1 μM MANT-GTPγS was present in the pipette solution, current density was not instantaneously different compared with control recordings, but it decreased significantly over time (Fig. 2, A and B). After 10 min of recording with MANT-GTPγS in the pipette, current density was lowered by 40 ± 7% (Fig. 2, A and B; from −4.0 ± 0.24 pA/pF to −2.5 ± 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 to 15 min 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 Wild-Type 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 at 1 μM. Under control conditions, 100 nM isoproterenol increased maximal $I_{Ca,L}$ by 77%. This increase was attenuated to only +21% in the presence of MANT-GTPγS (+77 ± 13 versus +21 ± 9%; $p < 0.01$, $n = 10$; Figs. 3, A and B, and 5D). When MANT-GTPγS was applied, the leftward-shift of I/V curves typically caused by isoproterenol was absent (Fig. 3, C and D). It is noteworthy that after 15 min of isoproterenol perfusion, currents still were significantly increased above predrug 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 min later (Fig. 4, A and B). Compared with 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 $I_{Ca,L}$ due to isoproterenol.

Concentration Dependence of MANT-GTPγS Effects on $I_{Ca,L}$ in Wild-Type 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 $I_{Ca,L}$ due to 100 nM isoproterenol stimulation (Fig. 5A), albeit to a lower extent (control, +77 ± 13%; 100 nM MANT-GTPγS, +43 ± 6%; and 1 μM MANT-GTPγS, +21 ± 9%; Fig. 5D). It is noteworthy that 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 min after starting 100 nM isoproterenol perfusion, stimulated $I_{Ca,L}$ 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. 5, A and B). These findings also suggest that lower concentrations of the nucleotide did not affect basal $I_{Ca,L}$. To evaluate the effect of 100 nM MANT-GTPγS on basal $I_{Ca,L}$, a comparison of current values immediately before starting isoproterenol perfusion (at 2.5 min) was performed. Under control conditions (+4 ± 2%) and in the presence of 100 nM

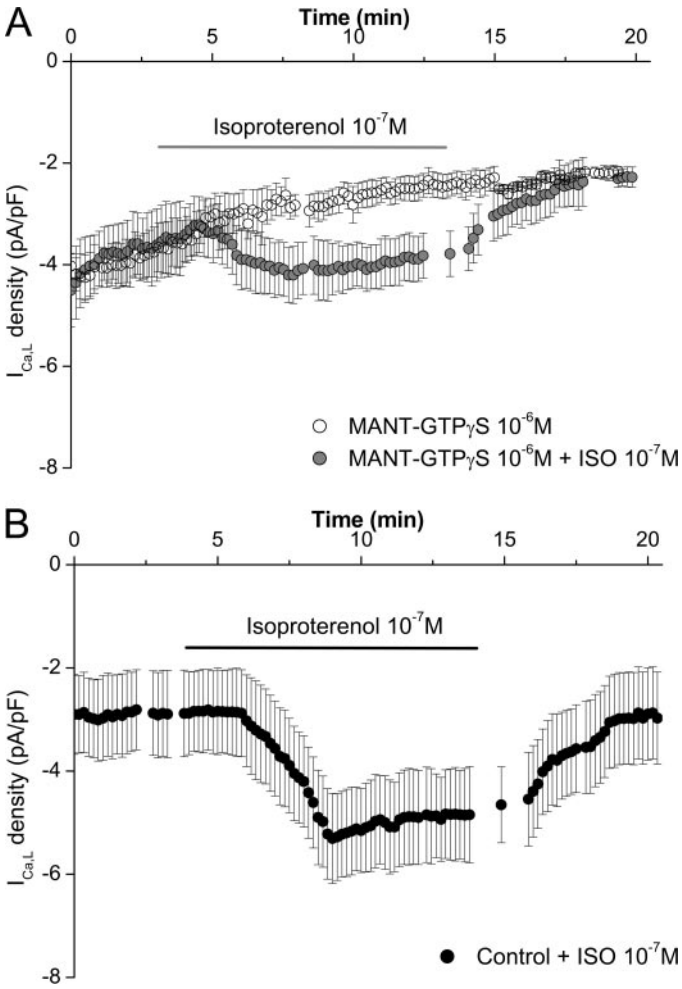

Fig. 4. Reversibility of the effect of isoproterenol on L-type Ca^{2+} current in wild-type murine cardiomyocytes. A, averaged time course of whole-cell L-type Ca^{2+} current density from experiments with myocytes before and after 100 nM (−)-isoproterenol stimulation, followed by isoproterenol washout 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 +10 mV preceded by a prepulse to −40 mV. B, filled symbols represent the averaged ($n = 5$) time course of similar washout experiments, performed in the absence of MANT-GTPγS.

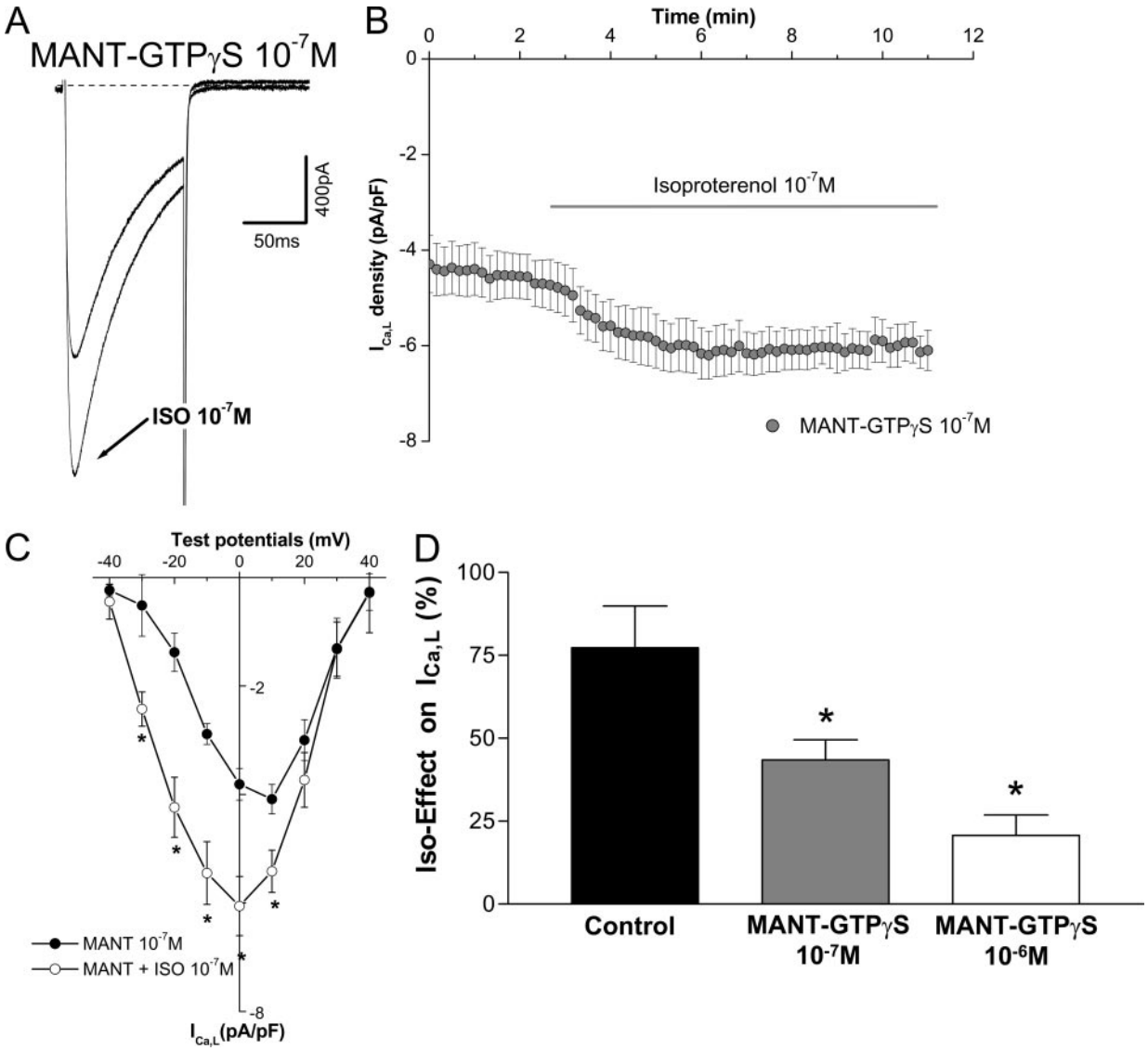

Fig. 5. Effect of 100 nM MANT-GTPγS on isoproterenol-stimulated L-type Ca^{2+} current in wild-type murine cardiomyocytes. Cells were superfused by isoproterenol containing bath solution for 8.4 ± 0.1 min. A, original traces before and after 100 nM (−)-isoproterenol stimulation and in the presence of 100 nM MANT-GTPγS at the time point 0 min and at the maximal 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 +10 mV preceded by a prepulse to −40 mV. 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. *, $p < 0.05$ (alternate t test). D, maximal increase of L-type Ca^{2+} current density by isoproterenol (percentage of predrug values) compared with basal current in the absence (black bar; $n = 10$) and in the presence of 100 nM (gray bar; $n = 5$) or 1 μM (white bar; $n = 11$) MANT-GTPγS, respectively. *, $p < 0.05$ versus control (alternate t test).

MANT-GTPγS (+3 ± 2%; N.S. versus control), currents were unaffected; 1 μM MANT-GTPγS decreased current by already −19 ± 4% ($p < 0.01$ versus control; $n = 5$). This confirms that lower concentrations of MANT-GTPγS did not affect basal $I_{Ca,L}$. In summary, basal and isoproterenol-stimulated inhibition of $I_{Ca,L}$ in the presence of MANT-GTPγS is concentration-dependent.

Effect of MANT-GTPγS on Basal $I_{Ca,L}$ 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 μM MANT-GTPγS decreased $I_{Ca,L}$ (after 10 min, −23 ± 1 versus 8 ± 2% without MANT-GTPγS; $p < 0.01$, $n = 6$; Fig. 6, A and B). This reduction of basal $I_{Ca,L}$ in $AC5^{-/-}$ myocytes was significantly less pronounced than in WT myocytes (after 10 min, −41 ± 7%; $p < 0.05$, $n = 6$), indicative for an AC5-specific effect of MANT-GTPγS.

Effects of MANT-GTPγS on $I_{Ca,L}$ Stimulation by Isoproterenol in $AC5^{-/-}$ Myocytes. In accordance with previous findings (Okumura et al., 2003a) isoproterenol effects on $I_{Ca,L}$ were mitigated in ventricular myocytes of $AC5^{-/-}$ mice. It is noteworthy that MANT-GTPγS at 1 μM did not further attenuate $I_{Ca,L}$ stimulated by isoproterenol in $AC5^{-/-}$ myocytes (32 ± 3 versus 29 ± 3%; N.S., $n = 5$; Fig. 7, A–C). Toward the end of incubation, isoproterenol effects seemed to decrease in the presence of MANT-GTPγS. How-

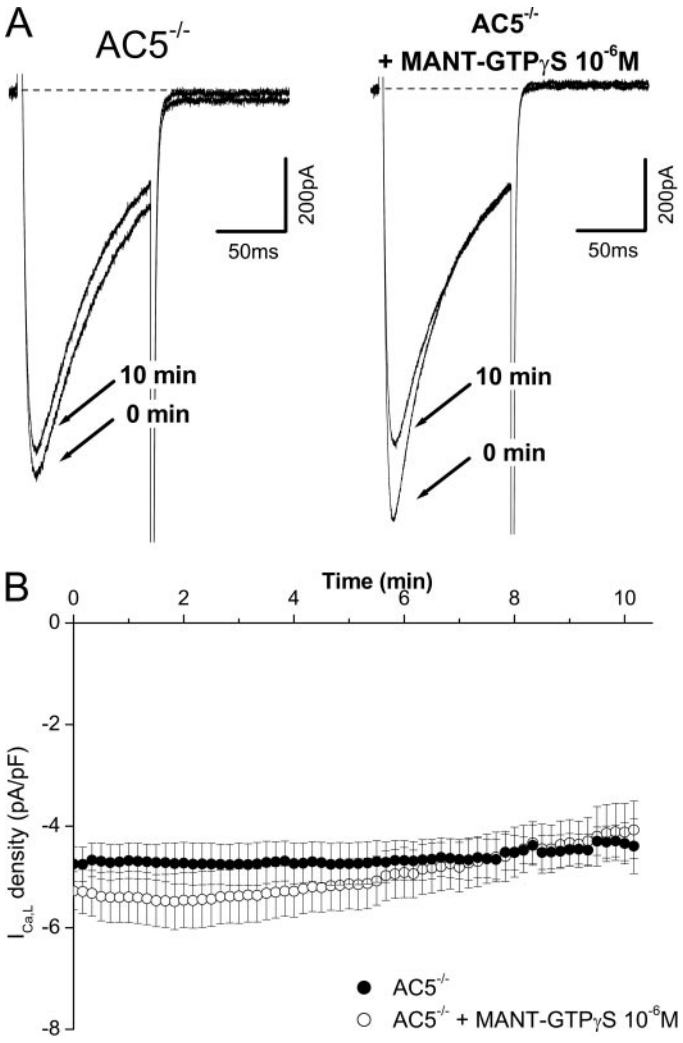

Fig. 6. Effect of 1 μM MANT-GTPγS on basal L-type Ca^{2+} current in $AC5^{-/-}$ murine cardiomyocytes. A, original traces in the absence and presence of 1 μM MANT-GTPγS at the time points 0 and 10 min. B, averaged time course of whole-cell L-type Ca^{2+} current density from experiments with murine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

ever, subtraction of the basal $I_{Ca,L}$ time course (cf. Fig. 6B) from isoproterenol-stimulated $I_{Ca,L}$ revealed that this effect is entirely explained by the reduction in basal L-type Ca^{2+} current activity (data not shown). The MANT-GTPγS resistance of isoproterenol effects in $AC5^{-/-}$ myocytes was confirmed by nearly unaffected I/V curves (Fig. 7, D and E).

Discussion

In our present study, we characterized the effect of the novel competitive AC inhibitor MANT-GTPγS on murine ventricular $I_{Ca,L}$. MANT-GTPγS is well suited for electrophysiological studies using intracellular dialysis because it is hydrolysis-resistant. Our findings indicate AC5 to be the major functional isoform mediating acute β_1-adrenergic stimulation of $I_{Ca,L}$. In the presence of MANT-GTPγS, we find an attenuation of $I_{Ca,L}$ increase following β-adrenergic stimulation via isoproterenol, 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 maximal $I_{Ca,L}$ 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 Ca^{2+} 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, $I_{Ca,L}$ increase following isoproterenol was maintained here, whereas in the presence of 1 μM nucleotide, $I_{Ca,L}$ decreased continuously during the course of experiments. This indicates that the isoproterenol-stimulated current is more sensitive toward 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 AC5 inhibition. Additional mechanisms, such as involvement of a less sensitive functional AC isoform in basal current regulation, or other nonspecific effects of the nucleotide cannot be further elucidated by our experiments. Thus, our data reveal AC5 to activate tonically basal Ca^{2+} current activity. This interpretation might at first glance contrast to the unchanged basal current values reported for $AC5^{-/-}$ 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 $AC5^{-/-}$ myocytes, one might also speculate on decreased abundance of potential phosphorylation sites, e.g., by lower expression of the pore-forming, protein kinase A-sensitive α1C subunit coded by exon 1a (van der Heyden et al., 2005) or altered composition of the heteromeric channel complex by pore-forming α1C and auxiliary β-subunits (Bünemann et al., 1999).

MANT-nucleotides represent novel experimental tools in the search for potential therapeutic compounds because 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-GTPγS 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-GTPγS 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. Because 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 β-adrenoceptor antagonists in the treatment of heart failure. β-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 β_2-adrenoceptors would be beneficial (Dorn et al., 1999; Du et al., 2000), whereas β_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

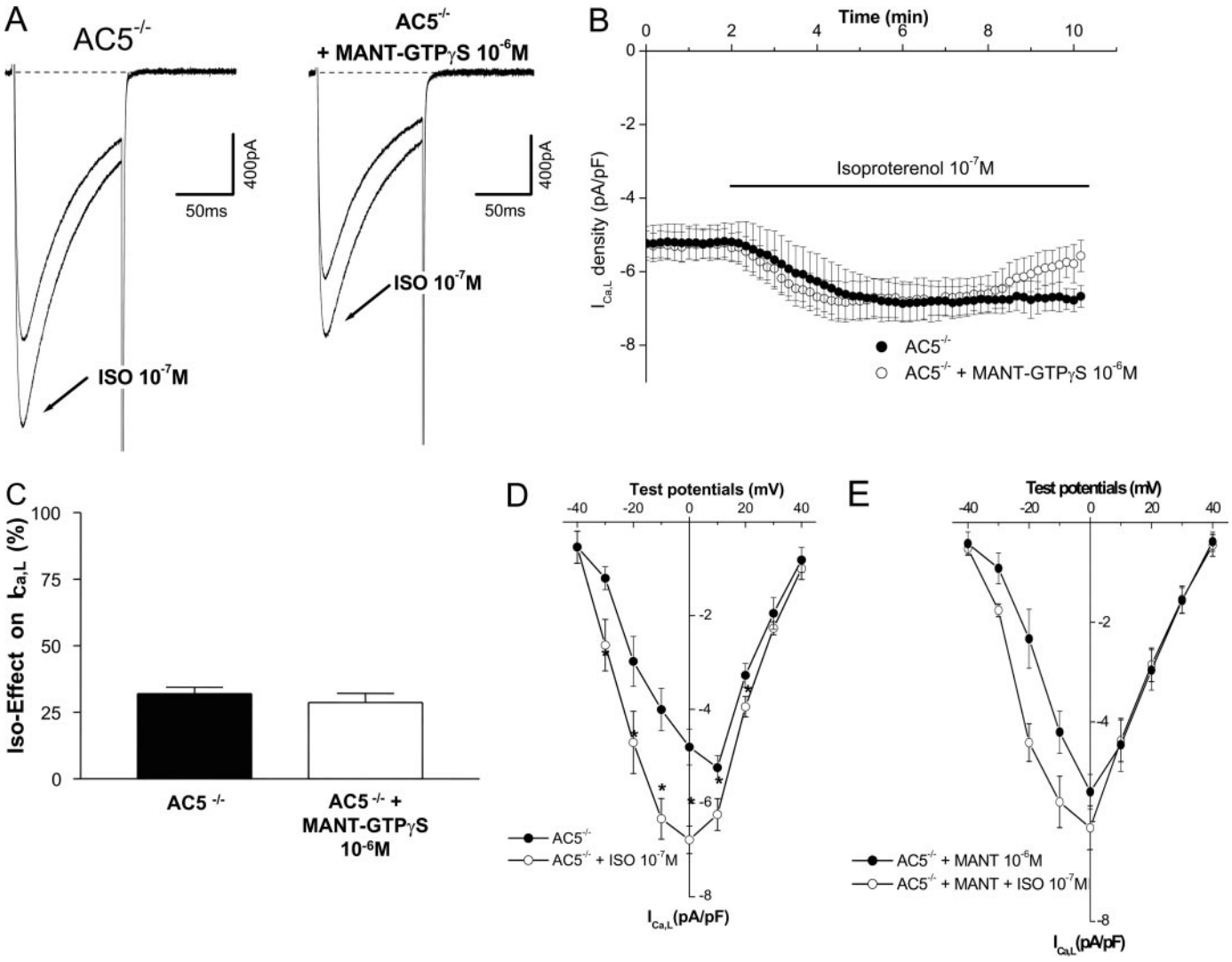

Fig. 7. Effect of 1 μM MANT-GTPγS on isoproterenol-stimulated L-type Ca^{2+} current in $AC5^{-/-}$ murine cardiomyocytes. Cells were superfused by isoproterenol containing bath solution for 8.2 ± 0.1 min (control) and 8.2 ± 0.1 min (MANT-GTPγS), respectively. A, original traces before and after 100 nM (−)-isoproterenol stimulation and in the absence and presence of 1 μM MANT-GTPγS at the time point 0 min and at the maximal 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-GTPγS (open symbols; $n = 5$). Starting from a holding potential of −60 mV, the current was elicited by a test potential of +10 mV preceded by a prepulse to −40 mV. C, maximal increase of L-type Ca^{2+} current density (percentage) compared with basal current (=0) in the absence (black bar; $n = 5$) and in the presence (white bar; $n = 5$) of 1 μM MANT-GTPγS. D, control current density-voltage relationship from $AC5^{-/-}$ 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 $AC5^{-/-}$ 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.

Ca^{2+} channel regulation by β_1- and β_2-adrenoceptors (Foerster et al., 2003, 2004; Xiao et al., 2006). A major difference is dual signaling via G_i and G_s proteins by β_2-adrenoceptors, and only G_s in case of β_1-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 because 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). Moreover, 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 β_1-adrenoceptor blockade because ACs integrate the input from multiple receptor systems (Hanoune and Defer, 2001). Our present study shows that the combination of pharmacological studies with gene knockout studies is very powerful at delineating signal transduction pathways and establishing novel therapeutic concepts.

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