β_2-Adrenergic receptor overexpression in the developing mouse heart: evidence for targeted modulation of ion channels

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1. We studied the effect of overexpression of the β_2-adrenergic receptor (β_2-AR) in the heart on ion channel currents in single cells isolated from hearts of fetal and neonatal transgenic and wild-type mice. The β_2-AR transgene construct was under the control of the murine α-myosin heavy chain (α-MHC) promoter, and ion channel activity was measured at distinct developmental stages using whole-cell and perforated patch clamp techniques.

2. We found no change in L-type Ca^{2+} channel current (I_{Ca}) density in early embryonic stages (E11–13) of β_2-AR transgenic positive (TG+) mice, but significant increases in I_{Ca} density in intermediate (E14–16, 152%) and late (E17–19, 173·7%) fetal and neonatal (1 day post partum, 161%) TG+ compared with transgenic negative (TG−) mice. This increase in I_{Ca} was accompanied by a negative shift in the peak of the current–voltage relationship in TG+ mice.

3. Transient (< 3 min) or prolonged (16–24 h) exposure of TG− neonatal stage myocytes to 8-Br-cAMP (300 μM) increased I_{Ca} density and caused a shift in the current–voltage relationship to a similar extent to that seen in TG+ mice. In TG+ myocytes, 8-Br-cAMP had no effect. Exposure of TG+ cells to Rp-cAMPS reversed both the shift in voltage dependence and reduced the peak current density observed in these myocytes. We concluded from these results that the L-type Ca^{2+} channel is maximally modulated by cAMP-dependent protein kinase (PKA) in TG+ mice and that the α-MHC promoter is functional in the ventricle as early as embryonic day 14.

4. In contrast, we found that slow delayed rectifier K^+ channels were not changed significantly at any of the developmental stages studied by the overexpression of β_2-ARs compared with TG− mice. The sensitivity of murine slow delayed rectifier K^+ channels to cAMP was tested by both transient and prolonged exposure to 8-Br-cAMP (300 μM), which increased the slow delayed rectifier K^+ channel current ($I_{K,s}$) density to a similar extent in both TG− and TG+ neonatal myocytes. In addition, we found that there was no difference in the concentration dependence of the response of I_{Ca} and $I_{K,s}$ to 8-Br-cAMP.

5. Thus, overexpression of the β_2-AR in the heart results in distinct modulation of I_{Ca}, but not $I_{K,s}$, and this is not due to differences in the 8-Br-cAMP sensitivity of the two channels. Instead, these results are consistent with both compartmentalization of β_2-AR-controlled cAMP and distinct localization of L-type Ca^{2+} and slow delayed rectifier K^+ channels. This cAMP is targeted preferentially to the L-type Ca^{2+} channel and is not accessible to the slow delayed rectifier K^+ channel.

During the development of the murine heart, a variety of electrophysiological changes takes place: both the levels of expression of ion channels and their regulatory properties are altered (Kojima *et al.* 1990; Maki *et al.* 1996). During early murine embryogenesis, it has been shown that the L-type Ca^{2+} channel current (I_{Ca}) plays a dominant role in excitation, whereas the slow component of the delayed rectifier K^+ channel current ($I_{K,s}$) is not so apparent until the later stages of embryonic development, or even after birth (Honore *et al.* 1991; Davies *et al.* 1995). Also, during development of the mouse heart, the β-adrenergic signalling pathway has been found to be functionally incomplete until

the later stages of embryogenesis suggesting a possible link between this pathway and expression of $I_{K,s}$ (Chen *et al.* 1982; Kojima *et al.* 1990; An *et al.* 1996).

The influence of catecholamines acting through β-adrenergic receptors (β-ARs) on the activity and expression of ion channels is of particular interest in the heart as it is well known that in chronic heart failure the myocardial β-AR system is defective. This functional impairment is associated with a decrease in agonist-induced inotropy, and is thought to be caused by a receptor defect, since the adenylyl cyclase response remains intact (Bristow *et al.* 1982). Specifically, it has been found that there is a selective downregulation of β_1-ARs which increases substantially the percentage of total β-ARs that are of the β_2-subtype (Bristow *et al.* 1986; Ungerer *et al.* 1993). Additionally, a population of remaining receptors (both β_1- and β_2-ARs) is functionally uncoupled, possibly due to increased homologous desensitization as the levels of β-adrenergic receptor kinase are increased in heart failure (Ungerer *et al.* 1993). Thus, β-AR agonists used to treat heart failure are not effective chronically and patients are at a higher risk of mortality as a result of the elevated levels of catecholamines (Ginsburg *et al.* 1983).

Recently, a transgenic mouse model has been developed in which β_2-ARs are overexpressed specifically in the heart (Milano *et al.* 1994). In the hearts of the adult transgenic mice, there is a >100-fold increase in β_2-AR density accompanied by apparent maximal heart rate and cardiac contractility. The physiological changes in heart rate and contractility are not believed to arise simply as a result of stimulation of the increased number of β_2-ARs by circulating catecholamines. Instead, they are thought to be due specifically to an increase in the number of β_2-ARs present in the active conformation, which are able to activate adenylyl cyclase in the absence of agonist (Milano *et al.* 1994). This novel transgenic model provides a unique opportunity to investigate the effects of the β-AR pathway on the expression of ionic channels in the heart during development and to determine directly whether increased numbers of receptors by themselves activate functionally relevant steps in the β-AR signal cascade. In addition, it affords an opportunity to study modulatory properties that may be unique to the β_2-AR and hence relevant to heart failure when the relative importance of this receptor subtype increases.

Here, we used this mouse model to study the effects of overexpression of the β_2-AR on I_{Ca} and $I_{K,s}$ in the developing mouse heart. This study had multiple goals. First, we wanted to determine directly whether β_2-AR overexpression modulates ion channel activity in the developing mouse heart in the absence of exogenous agonist and whether the effects of β_2-AR overexpression could be detected functionally during embryonic development. Second, we used this model to test directly for an inter-relationship between the β_2-AR signalling pathway and expression of $I_{K,s}$. Our results indicate that β_2-AR overexpression enhances I_{Ca} in a cAMP-dependent manner as early as day 14 of embryogenesis (E14). Surprisingly however, despite a robust sensitivity to exogenous 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), $I_{K,s}$ was not enhanced at any stage of development in β_2-AR transgenic positive (TG+) animals. These results clearly indicate unique electrophysiological consequences of β_2-AR-induced liberation of cAMP, and are consistent both with compartmentalization of β_2-AR-controlled cAMP and distinct localization of L-type Ca^{2+} and slow delayed rectifier K^+ channels.

METHODS

Embryonic cardiomyocyte isolation and culture

Pregnant female mice were killed by cervical dislocation and embryos, at different stages of development (11–13 days post coitus: early stage; 14–16 days: intermediate stage; or 17–20 days: late stage), were removed. For neonatal stage myocytes, mice at 1 day post partum were killed by decapitation, just prior to the removal of cardiac tissue as described by Sturm & Tam (1993) and in accordance with the guidelines of the local ethics committee (Institutional Animal Care and Use Committee, Columbia University College of Physicians and Surgeons). Cardiac myocytes were isolated from embryonic and neonatal hearts as described previously (Kubalak *et al.* 1995). In brief, hearts were dissected from embryos and neonates and placed in normal Tyrode solution (Kass *et al.* 1989). Atrial and ventricular tissues were separated under a dissecting microscope and placed in an Eppendorf tube with 0·5 ml Tyrode solution containing 0·5 mg ml^{-1} collagenase Type II (Worthington) and 1·0 mg ml^{-1} pancreatin (Gibco) for a 15 min digestion at 37 °C. Cells from the enzymatic digestion were placed in culture medium (modified Eagle's medium; Gibco) containing 10% fetal bovine serum, plated into plastic Petri dishes and cultured in a 10% CO_2 incubator at 37 °C. Electrophysiological recordings were carried out approximately 18–24 h after plating of the cells and could be carried out for periods up to 48 h following plating. Unless specified for individual experiments, cells were maintained in agonist-free media for this entire period.

Electrophysiology

Experimental results shown in this paper were obtained using patch clamp procedures in conventional whole-cell (Hamill *et al.* 1981) or in the perforated patch configuration (Horn & Marty, 1988). In experiments to study $I_{K,s}$, E4031 (5 μM; obtained as a gift from Eisai Limited, Tokyo, Japan) was added to the external solution to block the rapid component of the delayed rectifier K^+ channel current (Sanguinetti & Jurkiewicz, 1990) and nisoldipine (1 μM) was added to block the L-type Ca^{2+} channel current. For the measurement of $I_{K,s}$, the external solution contained (mM): KCl, 5; *N*-methyl-glucamine, 125; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; glucose, 5 (pH 7·4 with KCl). The internal solution for recording whole-cell $I_{K,s}$ contained (mM): potassium aspartate, 110; $CaCl_2$, 1; Hepes, 10; EGTA, 11; $MgCl_2$, 1; K_2ATP, 5; pH 7·3 (KOH). Whole-cell I_{Ca} was recorded using external solution containing (mM): CsCl, 5; Hepes, 10; *N*-methyl-glucamine, 125; glucose, 5; $MgCl_2$, 1. $BaCl_2$ (20 mM) was added to this solution as charge carrier and the internal solution contained (mM): aspartic acid, 50; K_2ATP, 5; CsCl, 60; EGTA, 11; Hepes, 10; $CaCl_2$, 1 (pH 7·2 with CsOH). For perforated patch recordings of L-type Ca^{2+} channel currents, amphotericin B was dissolved in DMSO at a concentration of 30 mg ml^{-1}, and then added to the above internal solution to yield a

final concentration of 120–250 μg ml^{-1}. Both the amphotericin B stock solution and the amphotericin B-containing pipette solution were subjected to 5–10 min of ultrasonication before use. Capacity transients were monitored as a function of time after attaining a high resistance seal with the surface membrane. Electrical access to the cell was judged by the time course of the capacity transient, and adequate access was usually attained within 10 min of seal formation.

In order to measure the time course of regulatory responses, Ca^{2+} channel currents were measured during test pulses (40 ms) to +10 mV applied once every 10 s. Holding potentials of −40 mV were used for both Ca^{2+} and K^+ channel current recordings, and isochronal (2 s pulses applied at 10 s intervals) activation curves were used to measure the activation of $I_{K,s}$. Similarly, I_{Ca} activation was measured by 40 ms test pulses to a series of potentials (10 mV increments) applied at 10 s intervals from a holding potential of −40 mV to +60 mV. I_{Ca} inactivation curves were obtained by measuring peak current at a test potential of +10 mV after application of a series of 5 s conditioning pules (−80 to +30 mV, 10 mV increments). A 10 ms return to the holding potential (−40 mV) separated each test and conditioning pulse. Patch pipettes (Clay Adams glass) were pulled to resistances of 2·5–5·0 MΩ when filled with internal solution. Total cell membrane capacitance was used as a measure of membrane area and was determined either by analog capacity compensation or by integration of current transients in response to 10 mV test pulses. Electrophysiological recordings were carried out at room temperature (20–22 °C) except for the experiments in which the slow delayed rectifier K^+ channel was transiently exposed to 8-Br-cAMP, which were conducted at 30–32 °C.

Chemicals were obtained from the following suppliers: amphotericin B (Sigma); 8-chlorophenylthio-cAMP (8-CPT-cAMP; Boehringer Mannheim); 8-Br-cAMP (Sigma); adenosine 3′,5′-cyclic monophosphothioate, R_P-isomer, triethylammonium salt (Rp-cAMPS; CalBiochem). Stock (20 mM) solutions of 8-CPT-cAMP or 8-Br-cAMP (dissolved in H_2O) were mixed and diluted to 300 μM for each experiment. Stock Rp-cAMPS solution (10 mM dissolved in H_2O) was mixed and diluted daily to 300 μM.

Data were collected, stored and analysed on IBM (486)-compatible computers interfaced to Axopatch (200A) amplifiers (Axon Instruments) under the control of pCLAMP (version 6.0) software (Axon Instruments). Graphics and statistical data analysis were carried out using Origin software (Microcal, Northampton, MA, USA). Averaged data are shown as means ± S.E.M. and were compared using Student's t test with a P value of $< 0{\cdot}05$ taken to indicate statistical significance.

Cell capacitance was measured and compared for TG+ and transgenic negative (TG−) cells as a function of developmental stage. For each stage there was no significant difference at the 0·05 level between TG+ and TG− cell capacitance; data obtained were (TG−, TG+, means ± S.E.M.): early stage: 25·4 ± 3·3 pF ($n = 10$), 29·9 ± 6·5 pF ($n = 8$); intermediate stage: 23·4 ± 7·5 pF ($n = 26$), 26·0 ± 9 pF ($n = 34$); late stage: 26·5 ± 11·5 pF ($n = 32$), 31·4 ± 3·2 pF ($n = 15$); neonatal: 25·7 ± 1·8 pF ($n = 10$), 28·0 ± 3·2 pF ($n = 20$).

Transgenic mice

The transgenic mice line utilized (TG4) has been described in detail (Milano *et al.* 1994). These mice (TG4) possess cardiac overexpression of the human β_2-AR at >100-fold over endogenous myocardial levels. Myocardial specificity was targeted by the use of the murine α-myosin heavy chain (α-MHC) gene promoter (Milano *et al.* 1994). Breeding pairs used were heterozygous for the transgene as determined by Southern blotting (Milano *et al.* 1994) and pregnancies were timed for the purpose of these studies in order to isolate embryonic myocytes. Embryos and neonatal pups were genotyped by PCR analysis with primers specific for the α-MHC gene promoter and the β_2-AR (Milano *et al.* 1994).

RESULTS

L-type Ca^{2+} channel activity is enhanced in TG+ mice

We first focused on possible modulation of I_{Ca} by β_2-AR overexpression because we had previously found that the L-type Ca^{2+} channel was expressed at robust levels and could be modulated in a cAMP-dependent manner at early stages in the developing mouse heart (Davies *et al.* 1995; An *et al.* 1996). As in our previous studies, we focused on distinct stages of development and compared I_{Ca} density at each stage between cells isolated from TG− and TG+ hearts. It should be pointed out that we also compared data obtained in cells isolated from wild-type (WT) and TG− hearts, and found no difference in stage-dependent current densities or responses to 8-Br-cAMP for either I_{Ca} or $I_{K,s}$ (data not shown). Figure 1 shows that I_{Ca} was increased by β_2-AR overexpression, and that the increase in channel activity could be detected clearly during embryogenesis. As seen in Fig. 1, at the earliest stage studied (days 11–13), no effect of the transgene was observed. However, by the intermediate embryonic period studied (days 14–16), we were able to detect significantly larger currents in TG+ compared with TG− hearts and this difference was maintained throughout development. In the remainder of this study, we focused on channel activity in cells isolated from neonatal hearts because the effect of the transgene was clear at that stage, and baseline channel activity was more pronounced than at earlier developmental stages.

Functional consequences of β_2-AR overexpression: a role for elevated cAMP

In order to assess the functional consequences of β_2-AR overexpression we compared the voltage dependence of activation and inactivation in cells isolated from TG+ and TG− mice. Figure 2 summarizes these experiments and shows that there were clear differences in the voltage dependence of L-type Ca^{2+} channel activation that were reflected both in the peak current–voltage relationships (Fig. 2*A*) and in the conductance–voltage relationships extracted from these data (Fig. 2*B*). These effects occurred with no change in the voltage dependence of steady-state inactivation (Fig. 2*C*).

The shift in the voltage dependence of activation and the increase in peak current density observed in TG+ cells are consistent with well-known modulatory effects of cAMP-dependent protein kinase A (PKA) on cardiac L-type Ca^{2+} channels (reviewed by McDonald *et al.* 1994; Hove-Madsen *et al.* 1996). The results are consistent with the view that increased cAMP production caused by β_2-AR overexpression-induced stimulation of adenylyl cyclase, even in the absence of agonist, modulates L-type Ca^{2+}

channel activity in these cells (Rockman *et al.* 1996). In order to test more directly for this possibility, we studied the effects of 8-Br-cAMP on I_{Ca} recorded in TG− cells to determine whether or not cAMP causes similar changes in the voltage dependence and density of I_{Ca} in these cells.

We found that 8-Br-cAMP did increase peak current density and shifted the peak of the current–voltage relationship in the negative direction in TG− cells in a manner that strikingly resembled the effects of overexpression of the β_2-AR in the absence of exogenous 8-Br-cAMP application. Figure 3*A* illustrates the effects of transient exposure of a TG− cell to 8-Br-cAMP, which is a cAMP analogue. In this and the following experiments, the perforated patch arrangement of the patch clamp technique was used to minimize disruption of key intracellular signalling molecules. The response to 8-Br-cAMP reached a steady state within 1 min, was maintained in the presence of 8-Br-cAMP and was reduced completely within minutes of return to 8-Br-cAMP-free solution (Fig. 3*A*). We found similar results with 8-CPT-cAMP, another membrane-permeant cAMP analogue (data not shown). Figure 3*B* summarizes the effects of 8-Br-cAMP on the current–voltage relationship of several cells. These data were obtained after steady state had been reached in the presence of 8-Br-cAMP. Peak current was increased and the peak of the current–voltage relationship was shifted in the negative direction. The increase in peak current in TG− myocytes measured in response to 8-Br-cAMP was remarkably similar to the increase in current density measured as a result of overexpression of the transgene. It was also of interest to study the effect of 8-Br-cAMP on I_{Ca} in TG+ cells since it is possible that the increased I_{Ca} observed in TG+ cells was due to an influence other than cAMP, such as a direct membrane-delimited pathway that caused functional changes in channels which simply resemble those induced by cAMP (Brown & Birnbaumer, 1988, 1990). Such experiments would also help to establish whether I_{Ca} was maximally modulated in the TG+ animals. Therefore, TG+ cells were exposed overnight to 8-Br-cAMP and Ca^{2+} current density was measured. In contrast to I_{Ca} in TG− cells, TG+ I_{Ca} was not enhanced by exposure to exogenous 8-Br-cAMP (Fig. 3*C*), providing further evidence that not only is [cAMP] elevated in the vicinity of L-type Ca^{2+} channels in TG+ cells but also that it is sufficiently high to saturate the modulatory response of L-type Ca^{2+} channels.

A summary of the Ca^{2+} current density in TG− and TG+ myocytes, a comparison of these data with the effect of 8-Br-cAMP on I_{Ca} density in TG− myocytes (both transient and overnight exposure), and finally data on the lack of effect of 8-Br-cAMP (overnight exposure) on the Ca^{2+} current density in TG+ cells are illustrated in Fig. 4. When the increased I_{Ca} density found in TG+ myocytes was compared with the effect of transient exposure of TG− myocytes to 8-Br-cAMP on I_{Ca} density in a large number of cells, no significant difference between the two groups was found (unpaired t test, $P > 0{\cdot}05$; Fig. 4). Further, we found that sustained exposure to 8-Br-cAMP (16–24 h) produced

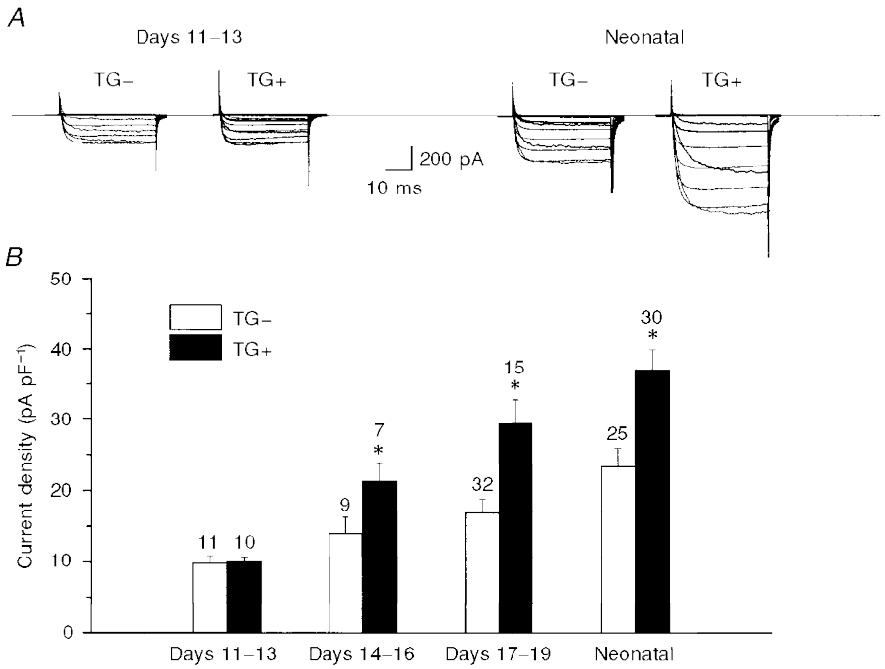

Figure 1. β_2-AR overexpression increases I_{Ca} in the developing mouse heart

A, current traces illustrating families of currents (for voltages, see Methods) measured in cells from TG− and TG+ early stage embryonic (left) and neonatal (right) hearts. *B*, bar graph summarizing peak I_{Ca} density recorded at +10 mV from a large number of cells (indicated above each bar) for three embryonic stages and for neonatal cells. * Significant difference compared with TG−, $P < 0{\cdot}01$.

changes in current density and I_{Ca} voltage dependence that were not significantly different from changes caused by transient exposure to 8-Br-cAMP or overexpression of β_2-ARs (Fig. 4). Lastly, Fig. 4 also shows that not only did 8-Br-cAMP fail to enhance I_{Ca} further in TG+ cells, but also that the current density in these cells was of a similar level to that observed in TG− cells only after they were exposed to 8-Br-cAMP.

Although these results strongly suggest that the functional changes in I_{Ca} caused by β_2-AR overexpression are due to increased cAMP, it is still possible that the effects are due to a direct membrane-delimited pathway that causes functional changes in channels that simply resemble those induced by increased cAMP (Brown & Birnbaumer, 1988, 1990). In order to distinguish between these possibilities, we exposed TG+ cells to the inhibitor of PKA, Rp-cAMPS (Rothermel & Parker, 1988). Rp-cAMPS was applied to TG+ cells, in which I_{Ca} was again measured in the perforated patch configuration. We reasoned that, if the voltage-dependent changes in current density caused by β_2-AR overexpression in TG+ cells were due to a membrane-delimited pathway, this inhibitor would have no effect on the measured channel activity. Instead, we found that Rp-cAMPS reduced I_{Ca} density and shifted the peak of the current–voltage relationship in the positive direction such that it now occurred over the same voltage range as that observed for I_{Ca} in TG− cells (Fig. 5; compare with the TG− current–voltage relationship shown in Fig. 2*A*). The data summarized in Fig. 5 were obtained after steady state was reached in the presence of Rp-cAMPS (300 μM), which typically took 5 min. The properties of currents measured

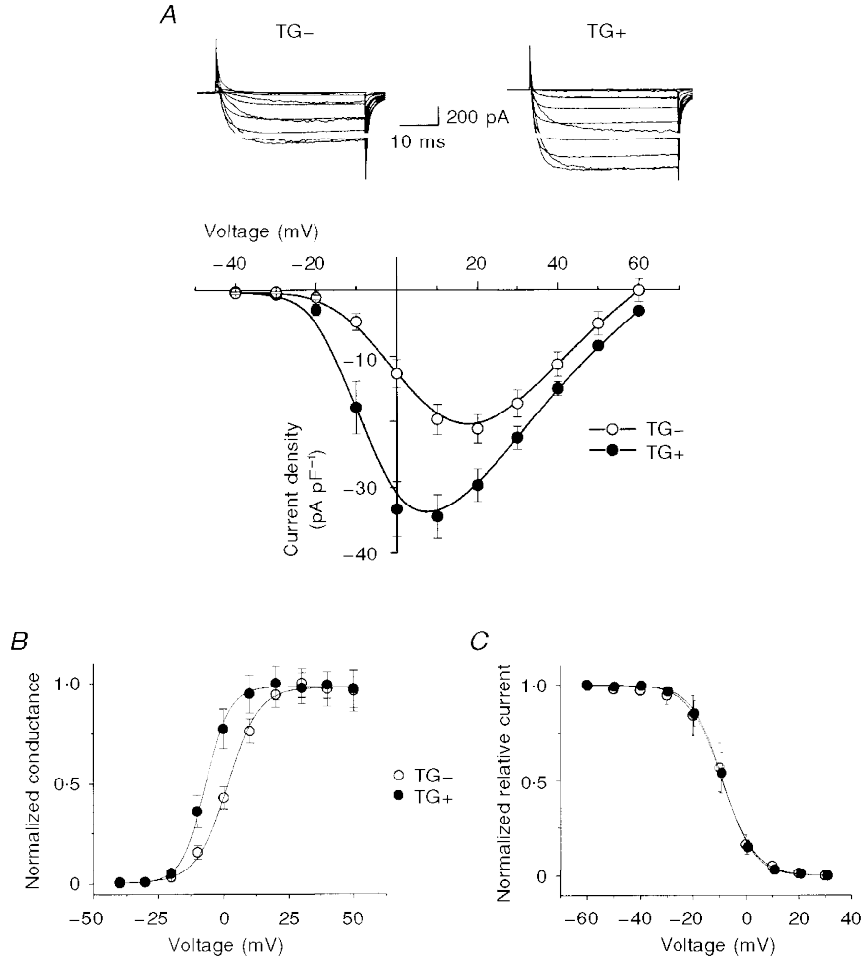

Figure 2. Voltage-dependent changes in I_{Ca} in TG+ cells

A, mean peak current–voltage relationships for TG+ ($n = 25$) and TG− ($n = 20$) cells. Note that the peak of the relationship occurred near +5 mV for TG+ and near +15 mV for TG− cells. The current traces (top) illustrate typical TG− (left) and TG+ (right) recordings (see Methods for voltage protocol). *B*, conductance–voltage relationships determined from *A* by normalizing currents to driving force. The smooth curves are Boltzmann fits to the data with the following parameters: potential at half-maximal activation ($V_{½}$) = −7 mV, slope factor (V_K) = 5·2 mV (TG+); $V_{½}$ = 1·8 mV, V_K = 6·5 mV (TG−). *C*, β_2-AR overexpression did not affect I_{Ca} steady-state inactivation. Steady-state inactivation was measured using 5 s conditioning pulses for TG+ and TG− cells. The smooth curves are Boltzmann relationships: potential at half-maximal inactivation ($V_{½}$) = −8·6 mV; V_K = 5·8 mV.

in the presence of Rp-cAMPS strongly resembled those of I_{Ca} in TG− cells, consistent with the conclusion that the effects of the transgene on I_{Ca} are due to elevation of cAMP.

Delayed K^+ current ($I_{K,s}$) is not enhanced by β_2-AR overexpression

Our experiments up to this point have provided strong evidence that I_{Ca} is enhanced in the TG+ cells by elevation of cAMP. Since $I_{K,s}$ is also known to be modulated by cAMP in the heart, we investigated the effects of β_2-AR overexpression on $I_{K,s}$ by measuring its activation as a function of voltage at each developmental stage. In contrast to I_{Ca}, we found no statistically significant difference in the expression of $I_{K,s}$ or its voltage dependence at any developmental stage between cells isolated from TG+ and TG− mice. Figure 6 shows the results obtained from the neonatal stage of development. As in the case of our study of I_{Ca}, we focused on currents in cells isolated from neonatal hearts because expression of $I_{K,s}$ is most pronounced at this stage (Davies *et al.* 1995).

Because there was no difference in the properties of $I_{K,s}$ measured in TG+ and TG− cells, as summarized in Fig. 6, and because we have demonstrated that β_2-AR overexpression increases [cAMP] in the vicinity of L-type Ca^{2+} channels, the results in Fig. 6 raise the possibility that murine $I_{K,s}$ is insensitive to intracellular cAMP. In fact, species differences in modulatory properties of recombinant $I_{K,s}$ have been reported (Varnum *et al.* 1993). In order to test for this possibility, we studied the effects of 8-Br-cAMP on $I_{K,s}$ measured in cells from neonatal hearts using the same protocols previously applied to the measurement of

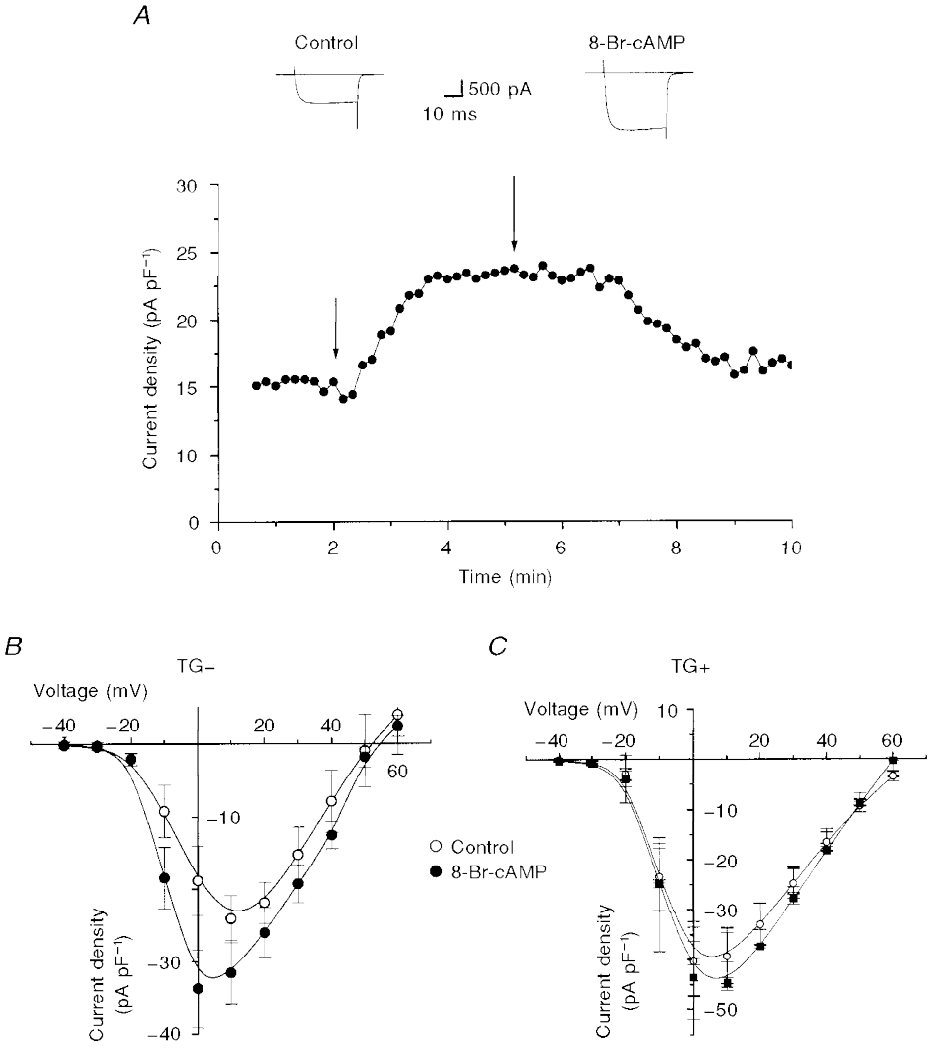

Figure 3. Influence of 8-Br-cAMP on I_{Ca} in TG− and TG+ cells

A, transient exposure to 8-Br-cAMP (300 μM) reversibly increased the peak I_{Ca} (activated by repeated 40 ms depolarizations to +10 mV) in a TG− cell. Typical Ca^{2+} channel currents shown above the plot were sampled at the points indicated by the arrows. *B*, mean current–voltage relationships for I_{Ca} in TG− cells before (Control) and after overnight (16–24 h) exposure to 8-Br-cAMP ($n = 4$–6). *C*, mean current–voltage relationships for I_{Ca} in TG+ cells in which overnight exposure to 8-Br-cAMP had no significant effect ($P > 0{\cdot}05$; $n = 6$–18).

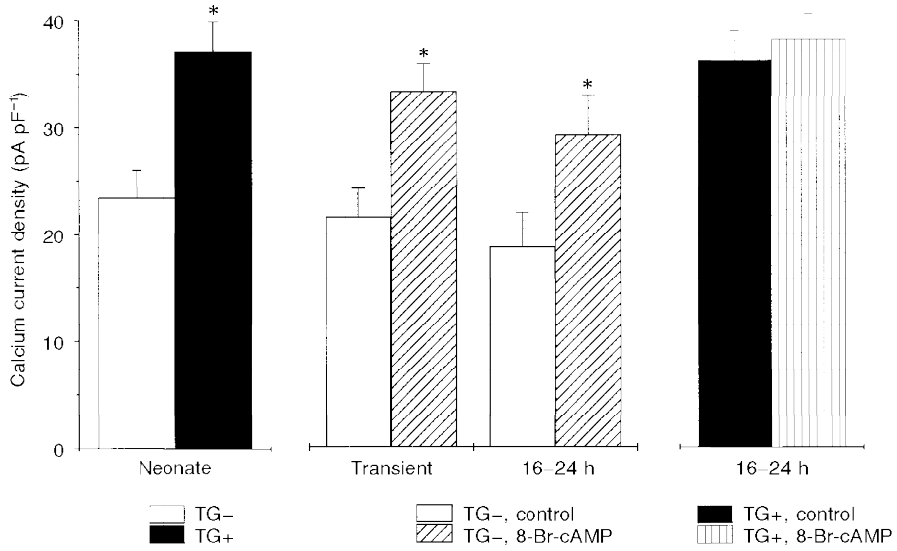

Figure 4. Comparison of the effects of β_2-AR overexpression and 8-Br-cAMP (300 μM) on peak I_{Ca}

Bar graphs summarizing peak current densities recorded at +10 mV. Left, peak currents in TG− ($n = 26$) *vs.* TG+ ($n = 30$) cells. Middle, peak current density in TG− cells before and after transient (< 3 min; middle left, $n = 6$ each) and prolonged (16–24 h; middle right, $n = 8$ each) exposure to 8-Br-cAMP. * There was no significant difference between the enhancement of peak I_{Ca} in the TG+ cells (left) and that produced by 8-Br-cAMP in TG− cells (middle; unpaired t test, $P > 0{\cdot}05$). Right, peak current density before and after prolonged exposure of TG+ cells to the same concentration of 8-Br-cAMP.

I_{Ca}. We incubated TG− cells for 16–24 h in the presence 8-Br-cAMP in order to study the effects of maintained cAMP elevation, similar to maintained elevation of cAMP caused by overexpression of β_2-AR as detected in our studies of I_{Ca}. Under these conditions, we found that the slow delayed rectifier K^+ channel activity in TG− myocytes was enhanced by 8-Br-cAMP and that the effect was statistically significant ($P < 0{\cdot}01$; Fig. 7*A*). Thus, as is the case for many other species, $I_{K,s}$ in neonatal murine heart is potentiated by cAMP.

If prolonged exposure to elevated intracellular 8-Br-cAMP enhances $I_{K,s}$ in TG− (and WT) hearts, then why is the current not affected by β_2-AR overexpression in the TG+ cells in which we have shown that I_{Ca} is enhanced in a cAMP-dependent manner? One possibility is that β_2-AR overexpression also stimulates an inhibitory pathway that is specific for $I_{K,s}$ and not I_{Ca}. If this were the case, then exposure of cells obtained from TG+ hearts to 8-Br-cAMP might not be expected to enhance $I_{K,s}$. We tested for this possibility by incubating TG+ cells in 300 μM 8-Br-cAMP but found that $I_{K,s}$ was enhanced to the same extent as currents in TG− cells ($P < 0{\cdot}01$; Fig. 7*A*). This finding is consistent with the hypothesis that there is no inhibitory factor preventing the cAMP-dependent modulation of $I_{K,s}$.

It is possible, however, that the increases in $I_{K,s}$ observed following prolonged exposure of the TG− and TG+ cells to 8-Br-cAMP might have resulted from cAMP-mediated changes in channel expression rather than a direct effect of

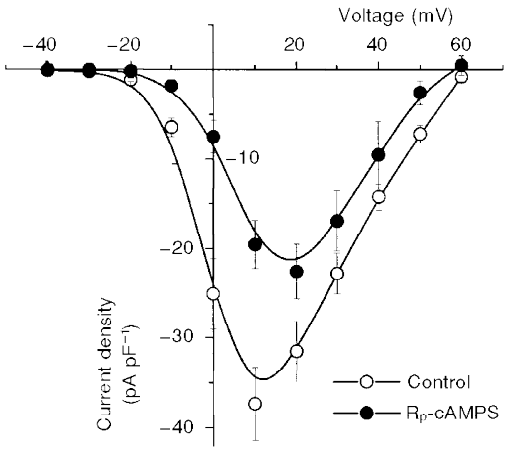

Figure 5. Effect of Rp-cAMPS on I_{Ca} recorded from TG+ cells

Mean ($n = 6$) peak current–voltage relationships for currents measured in TG+ cells before (Control) and after exposure to Rp-cAMPS (300 μM). Steady-state effects which were obtained within 3–5 min of exposure to the PKA inhibitor are shown. Note that the peak of the current–voltage relationship was shifted to positive voltages and reduced in amplitude.

cAMP on the channels themselves. Therefore, we also transiently exposed both TG− and TG+ cells to 8-Br-cAMP and found that, under these conditions, $I_{K,s}$ was also enhanced in both TG− and TG+ cells (Fig. 7*B*). These results provide further evidence to support the hypothesis that $I_{K,s}$ in TG− and TG+ cells is sensitive to cAMP and are consistent with the lack of an inhibitory pathway or factor in the TG+ cells preventing a cAMP-dependent regulation of $I_{K,s}$.

Finally, it might be possible that L-type Ca^{2+} channels are significantly more sensitive to cAMP than slow delayed rectifier K^+ channels. If this were the case, then perhaps β_2-AR overexpression might increase cAMP in regions near both slow delayed rectifier K^+ and L-type Ca^{2+} channels, but to a concentration that saturates the response of I_{Ca} but which is not sufficiently high to increase $I_{K,s}$. To test this, the concentration dependence of both $I_{K,s}$ and I_{Ca} to cAMP was investigated by exposure of TG− myocytes to 8-Br-cAMP at different concentrations. In Fig. 8*A*, the relative increase in I_{Ca} and $I_{K,s}$ at different 8-Br-cAMP concentrations is shown. There was no significant difference between the effect of 8-Br-cAMP on I_{Ca} and $I_{K,s}$ at any 8-Br-cAMP concentration tested. This can be seen more clearly in Fig. 8*B*, where the concentration– response curves for I_{Ca} and $I_{K,s}$ to [8-Br-cAMP] are shown. The EC_{50} values for 8-Br-cAMP for $I_{K,s}$ and I_{Ca} were determined: for $I_{K,s}$ (overnight exposure), 1·52 μM; I_{Ca} (transient exposure), 1·47 μM; and I_{Ca} (overnight exposure), 1·56 μM. Thus, the EC_{50} values were broadly similar and therefore a difference in the cAMP sensitivity is unlikely to explain the observed distinction between $I_{K,s}$ and I_{Ca} enhancement in TG+ cells.

DISCUSSION

In this paper we report the first cellular measurements of ion channel activity and its modulation by the β-AR signalling cascade in developing hearts of mice engineered to overexpress β_2-AR. We found that β_2-AR overexpression enhanced L-type Ca^{2+} channel currents in a cAMP-dependent manner, but that the slow delayed rectifier K^+ channel current was not affected by the transgene. Our results, based on functional assays of expressed channel activity, provide new insights into mechanisms by which receptor activation can direct modulatory enzymes to distinct targets (ion channels).

Evidence for embryonic activity of the α-MHC promoter

In cells isolated from TG+ hearts, we found significant effects of the β_2-AR transgene at very early developmental stages. I_{Ca} was enhanced in TG+ cells as early as day 14 of embryogenesis indicating that the α-MHC promoter must be active in the ventricle at this stage of development, in contrast to previous reports suggesting activation after birth (Milano *et al.* 1994). Transgene activity in the ventricle is thus likely to be much more important during embryogenesis than previously estimated.

cAMP-dependent modulation of I_{Ca} in TG+ mice in the absence of exogenous agonist

In their studies of *in vivo* heart function in β_2-AR TG+ mice, Milano *et al.* (1994) suggested that changes in heart rate and contractility detected in these hearts were not due to circulating catecholamines, but instead were due specifically to an increase in the number of β_2-ARs present

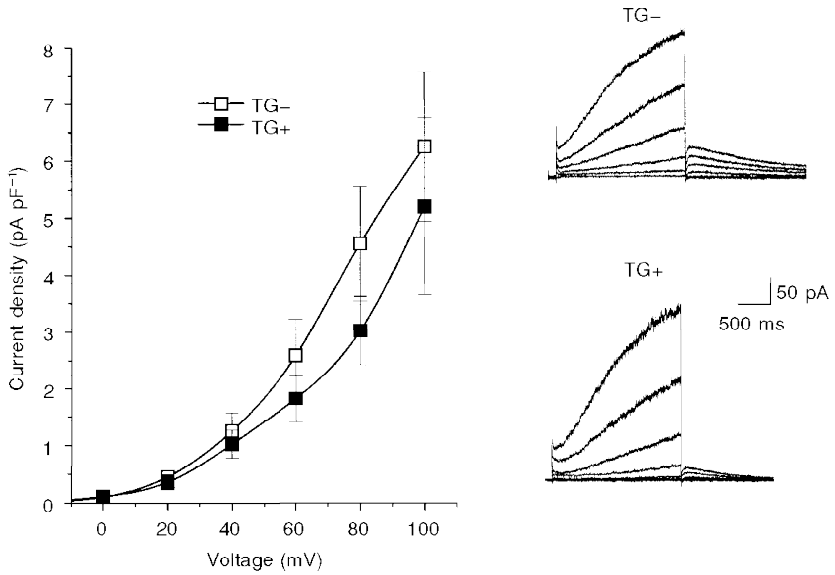

Figure 6. β_2-AR overexpression did not affect $I_{K,s}$

Right, representative traces of $I_{K,s}$ (activated by isochronal 2 s depolarizations from −40 to +60 mV in 20 mV increments from a holding potential of −40 mV) recorded from TG− (top) and TG+ (bottom) cells. Left, the mean current–voltage relationships for time-dependent $I_{K,s}$ activated during depolarization for TG− ($n = 19$) and TG+ ($n = 20$) cells (neonatal stage). There was no significant difference between TG− and TG+ currents at any voltage.

in the active conformation. This increase in active receptors is thought subsequently to activate adenylyl cyclase in the absence of agonist (Bond *et al.* 1995; Bond, 1997). Our data confirm this interpretation. Cells in our experiments were enzymatically isolated and then plated and kept under cell culture conditions in agonist-free media for 24 to 36 h before experiments were carried out. Before measuring ion channel activity, plates of cells were washed with agonist-free Tyrode solution and then cells which were studied with patch clamp procedures were continuously perfused with agonist-free solutions before and during the measurement of channel activity. Hence it is very unlikely that any of the effects on channel activity that we report caused by the transgene are due to stimulation of expressed receptor by exogenous catecholamines.

The observed functional differences in Ca^{2+} channel activity between TG− and TG+ cells as well as the effects of Rp-cAMPS on the voltage dependence and current density of I_{Ca} in TG+ cells presents strong evidence in favour of a cytoplasmic cAMP-dependent pathway for modulation of I_{Ca} in TG+ cells, consistent with activation of adenylyl cyclase and subsequent production of cAMP caused directly by an increased number of β_2-ARs in the active conformation (Bond *et al.* 1995; Bond, 1997). This hypothesis is consistent with regulation of the L-type Ca^{2+} channel current by cytoplasmic pathways in the presence of β-AR stimulation in the heart previously shown by Hartzell *et al.* 1991.

cAMP discrimination between $I_{K,s}$ and I_{Ca} in TG+ mice: evidence for cAMP compartmentalization

Despite the cAMP-induced enhancement of I_{Ca} we found no effect of β_2-AR overexpression on $I_{K,s}$ activity, either in its voltage dependence or in the level of expression. This suggested to us that the β_2-AR-induced change in intracellular cAMP was not uniform throughout the cell, but was localized to regions near the L-type Ca^{2+} channel. This was supported by the finding that in both TG− and TG+ cells, exogenous 8-Br-cAMP application (both transient and prolonged exposure) increased peak $I_{K,s}$ density to a similar extent in both TG− and TG+ cells, demonstrating that $I_{K,s}$ was sensitive to cAMP in the mouse cells and that in the TG+ cells, this is consistent with the absence of any inhibitory pathway that might have prevented cAMP-dependent regulation. A comparison of the concentration dependence of I_{Ca} and $I_{K,s}$ for 8-Br-cAMP-mediated changes in current showed that the two channel types share a similar sensitivity to 8-Br-cAMP. We measured the change in each current caused by exposure to 8-Br-cAMP and the results were consistent with previous reports in other cell types, which have shown similar cAMP sensitivities for the two channel types (Kass & Wiegers,

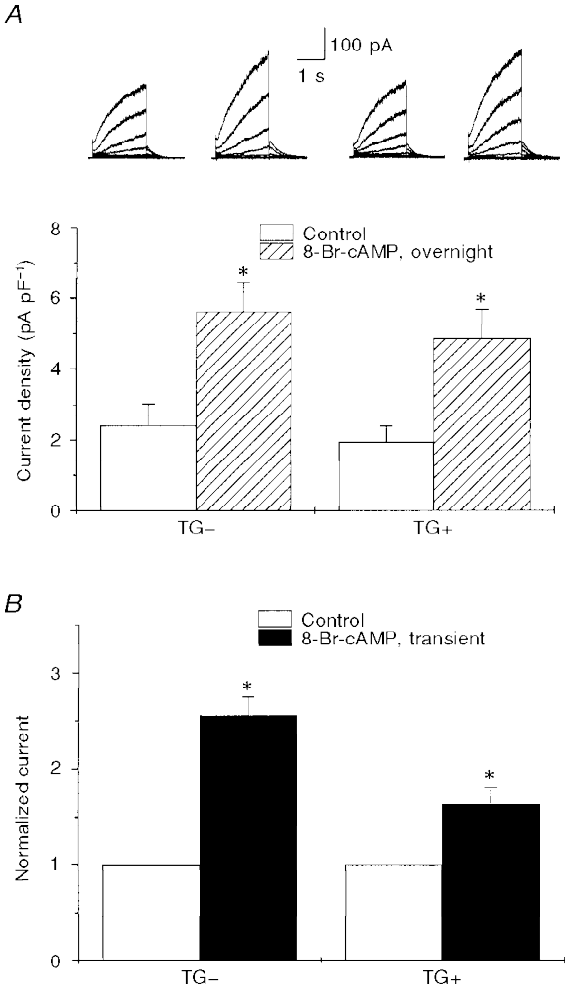

Figure 7. Effects of 8-Br-cAMP on $I_{K,s}$ in TG+ and TG− cells

A shows mean $I_{K,s}$ density measured at +40 mV in TG− and TG+ cells in the absence of 8-Br-cAMP (Control) and after 16–24 h incubation in the presence of 8-Br-cAMP (300 μM), $n = 5$–18. Representative $I_{K,s}$ traces (at +60 mV) before and after exposure to cAMP in TG− and TG+ cells are shown above the bars. * Significant difference compared with control, $P < 0{\cdot}01$. *B* shows the relative $I_{K,s}$ amplitude before (Control) and after transient (< 3 min) exposure to 300 μM 8-Br-cAMP in TG− and TG+ cells. Currents were normalized to those in control ($n = 2$–6). * Significant difference compared with control, $P < 0{\cdot}01$.

1982; Giles *et al.* 1989; Iijima *et al.* 1990; Yazawa & Kameyama, 1990). Taken together, these results support the view that in the TG+ cells, the basal cAMP concentrations differ in regions near these two channel types consistent with targeting or compartmentalization of the β_2-AR-induced increases in cAMP.

Although this is the first report of cAMP compartmentalization in cardiac cells in a manner that distinguishes target ion channel regulation by β_2-AR overexpression, our results complement those of others who have focused on calcium entry and calcium transients in cardiac myocytes using pharmacological approaches (Xiao *et al.* 1994; Jurevicius & Fischmeister, 1996, 1997; Zhou *et al.* 1997). Previous studies have linked β_2-AR-induced changes in contraction, cytosolic Ca^{2+}, and L-type Ca^{2+} channel currents to activation of adenylyl cyclase and subsequent increases in intracellular cAMP (Skeberdis *et al.* 1997). Distinct functional differences have been reported in modulation of these parameters by stimulation of β_1- or β_2-receptors (Xiao & Lakatta, 1993), and these differences have been suggested to be due in part to β-receptor subtype-specific compartmentalization of cAMP (Aass *et al.* 1988; Hohl & Li, 1991; Xiao *et al.* 1994; Jurevicius & Fischmeister, 1996; Rockman *et al.* 1996; Skeberdis *et al.* 1997; Zhou *et al.* 1997). Our results support this view. The data presented in this paper strongly suggest that the modulation of I_{Ca} in the TG+ hearts is indeed due to increased cAMP activity in the vicinity of the L-type Ca^{2+} channels, and that the discrimination between modulation of I_{Ca} and $I_{K,s}$ is consistent with localization of this increased cAMP pool. Furthermore, the results of this study indicate that this mouse model in which β_2-ARs are overexpressed will make an excellent system in which to study the molecular mechanism by which cAMP is targeted to L-type Ca^{2+} rather than slow delayed rectifier K^+ channels. Future studies will be directed at determining whether distinct anchoring proteins are involved in this targeting.

Relationship to arrhythmias and heart failure

Electrical activity of the mammalian myocardium is maintained by a fine balance of a large number of ionic currents carried by ion channel and transport proteins (Kass, 1995). Sympathetic stimulation, a key regulatory pathway in the cardiovascular system, modulates contractile activity, but also, importantly, electrical activity at the single cell level. In order to ensure adequate diastolic filling time in the face of increased heart rate in the presence of elevated sympathetic tone, it is essential that the ventricular action potential duration be controlled by the sympathetic nervous system such that, concomitant with elevated heart rate, the ventricular action potential shortens. Key to this regulation is a balance between β-AR modulation of $I_{K,s}$

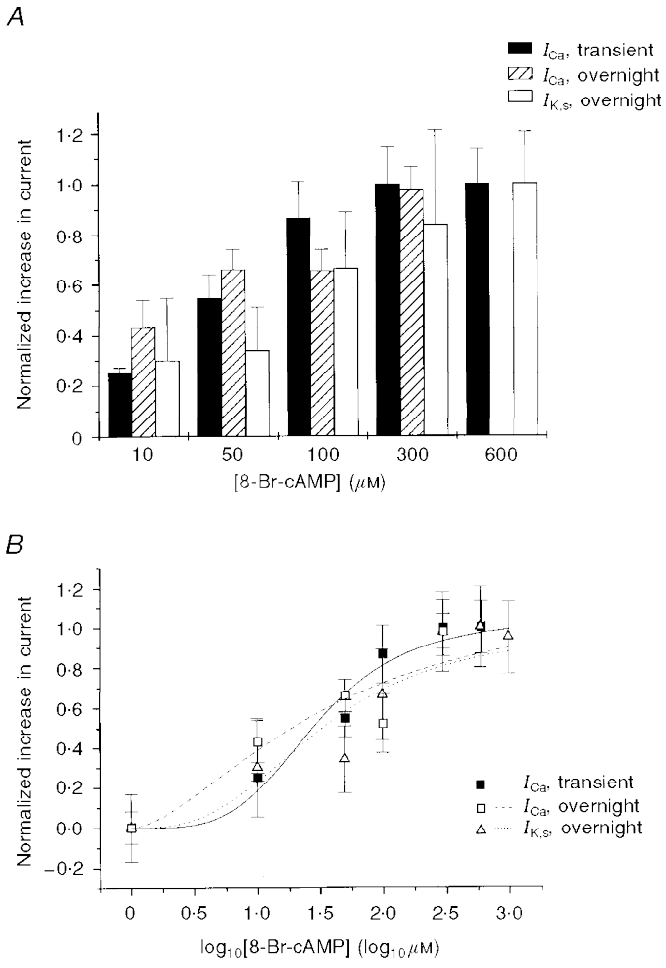

Figure 8. Concentration dependence of $I_{K,s}$ and I_{Ca} response in TG− cells to 8-Br-cAMP

A, bar graph summarizing the normalized change in I_{Ca} (measured at +10 mV) following transient and 16–24 h (overnight) exposures to 8-Br-cAMP and the change in $I_{K,s}$ following 16–24 h exposure to 8-Br-cAMP (measured at +40 mV) as a function of [8-Br-cAMP]. There was no significant difference between groups at each 8-Br-cAMP concentration ($P > 0{\cdot}3$). *B*, log concentration–response curves for I_{Ca} and $I_{K,s}$ (normalized change in peak I_{Ca} for transient and 16–24 h exposures and in peak $I_{K,s}$ for 16–24 h exposure plotted against [8-Br-cAMP]).

and I_{Ca} (Kass & Wiegers, 1982; Kass, 1994). This regulation occurs in a cAMP-dependent manner (Hove-Madsen *et al.* 1996). Dysfunction in slow delayed rectifier K^+ channel activity has been shown to be linked to at least one inherited human cardiac arrhythmia, the long QT syndrome (Sanguinetti *et al.* 1996; Splawski *et al.* 1997). Interestingly, patients with defects in the genes encoding either of the two subunits of the slow delayed rectifier K^+ channel, KvLQT1 or minK, are prone to arrhythmias in the face of elevated sympathetic tone, providing key clinical evidence for the importance of the regulation of this ion channel by the sympathetic nervous system for the maintenance of normal cardiac function (Moss, 1997). Because the relative contribution of β_2-receptors to AR stimulation increases both in heart failure (Bristow *et al.* 1985, 1986) and in a recent experimental transgenic model in which nerve growth factor was overexpressed in a cardiac-specific manner (Heath *et al.* 1998), it is possible that cAMP-dependent regulation of the slow delayed rectifier K^+ channel also may change in failure. If this were the case, action potential duration would not shorten sufficiently with increased levels of circulating catecholamines promoting conditions that resemble LQT1-type dysfunction. Investigation of conditions that promote arrhythmias in models of the failing heart should thus clearly include studies of the control of slow delayed rectifier K^+ channels by β-AR stimulation.

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