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$G\alpha_q$ and $G\alpha_{11}$ contribute to the maintenance of cellular electrophysiology and Ca^{2+} handling in ventricular cardiomyocytes

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Aims $G\alpha_q$ and $G\alpha_{11}$ signalling pathways contribute to cardiac diseases such as hypertrophy and arrhythmia, but their role in cardiac myocytes from healthy hearts has remained unclear. We aimed to investigate the contribution of $G\alpha_q$ and $G\alpha_{11}$ signalling to the basal properties of ventricular myocytes.

Methods and results We created a conditional $G\alpha_q$ knockout (KO) after tamoxifen injection into $gnaq^{flox/flox}$ $gna11^{-/-}$ α-MHC $Cre^{tg/0}$ mice and found alterations in the electrophysiological and Ca^{2+} handling properties of ventricular myocytes using patch-clamp and Fura-2 video imaging. To reveal the genuine effects of protein KO, we investigated the individual contributions of (i) tamoxifen injection, (ii) Cre recombinase expression, (iii) $G\alpha_{11}$ KO, and (iv) $G\alpha_q$ KO. Profound and persistent alterations in myocyte properties occurred following the tamoxifen injection alone. Consequently, we used the presence or absence of Cre recombinase expression as the determinant for the $G\alpha_q$ KO. Myocytes from the $G\alpha_q$ and/or $G\alpha_{11}$ KO mice displayed genuine alterations in the action potentials, membrane capacitance, membrane currents, and Ca^{2+} handling (amplitude, post-rest behaviour, and Ca^{2+} removal processes).

Conclusions We conclude that, in a transgenic model, the role of $G\alpha_q$ can be best studied using Cre recombinase expression as the molecular determinant for $G\alpha_q$ KO rather than tamoxifen/miglyol injection. While excessive hormonal stimulation of the $G\alpha_q/G\alpha_{11}$ signalling pathways plays an essential role in cardiac diseases, we propose that the persistent low-level stimulation of these pathways by $G\alpha_q/G\alpha_{11}$ activation is instrumental in the physiological behaviour of ventricular myocytes.

Keywords Gαq/Gα11 • Ca^{2+} homoeostasis • Cellular electrophysiology • Tamoxifen • Cre recombinase

1. Introduction

Members of the heterotrimeric G-protein family mediate both acute and chronic cardiac responses.[1,2] Within the G-protein family, the role of $G\alpha_q$ and $G\alpha_{11}$ ($G\alpha_{q/11}$) in the acute physiological response of cardiomyocytes is not fully understood.[3–7] Moreover, an involvement of $G\alpha_{q/11}$ in the regulation of myocyte contractility via cross talk to β-adrenergic $G\alpha_s$ signalling has been proposed.[8–11] The role of $G\alpha_{q/11}$ in chronic pathological responses, including the development of cardiac hypertrophy, has been established using genetically modified mouse models.[12,13] Transgenic mice over-expressing $G\alpha_q$ showed an increased expression of hypertrophic marker genes.[14] In contrast, the inhibition of $G\alpha_{q/11}$ via the expression of a 54-amino acid $G\alpha_{q/11}$-inhibitory peptide diminished pressure-overload-induced cardiac hypertrophy.[15] A heart-specific inactivation of $G\alpha_{q/11}$ abolished the hypertrophic responses to pressure overload.[13] The over-expression of the GTPase activating protein RGS4, which accelerates $G\alpha_q$ and $G\alpha_{11}$ inactivation, reduced pressure-overload-induced cardiac hypertrophy.[16] Previously, the knockout (KO) of $G\alpha_q$ and/or $G\alpha_{11}$ was achieved by non-inducible approaches. Even though the KO might have been tissue-specific, the gene deletion occurred early in development enabling compensatory mechanisms to prevail and possibly obscure the KO effect.

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Surprisingly, few studies took into consideration that the modulation of $G\alpha_q$ or $G\alpha_{11}$ levels itself might affect the physiology of cardiac myocytes, including Ca^{2+} handling and electrophysiological properties.[17,18] Nevertheless, it appears imperative to understand such interactions to fully appreciate $G\alpha_{q/11}$ KO in disease models. In the current study, we investigated the contribution of $G\alpha_{q/11}$ proteins to the basic physiological properties of myocytes, such as cellular electrophysiology and Ca^{2+} transients. The combination of a tissue-specific and inducible Cre/loxP system[19] offered the possibility for a tissue- or cell-type specific gene modulation at any developmental stage. Because Offermanns *et al.*[20] reported overlapping effects of $G\alpha_{11}$ and $G\alpha_q$ proteins, our approach used conditional $G\alpha_q$ KO mice and constitutive $G\alpha_{11}$ KO mice to address the following questions: (i) what are the best combinations of genotypes/treatments to study the role of $G\alpha_q$ and $G\alpha_{11}$? (ii) Are there cellular phenotypes following $G\alpha_q$ KO and/or $G\alpha_{11}$ KO? And (iii) if so what are the contributions of $G\alpha_q$ and $G\alpha_{11}$?

2. Methods

2.1 Generation of transgenic mice and isolation of ventricular myocytes

Animal care and isolation procedure were approved by the animal Ethics Committee of the Saarland University and were performed according to the European directive on Laboratory Animals (86/609/EEC) and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). For detailed information on the generation of the transgenic mice and the sequence information of the used primers, see Supplementary material online.

The transgenic mice, their genotype, and the abbreviations used in this study are listed in *Table 1*.

2.2 Preparation of isolated ventricular myocytes

The isolation protocol was adapted from a procedure for rat cardiomyocytes.[21] Animals were anaesthetised (10 mL/kg body weight) i.p. with a mixture of ketamine hydrochloride (10 mg/kg body weight) and xylazine hydrochloride (98 mg/kg body weight) dissolved in NaCl. Anaesthesia of the mice was considered sufficient when the paw pinch test was negative. Afterwards, we injected citrate (117 mg/kg body weight) i.p. to reduce blood clotting. The animal was killed by decapitation. Solution A^+ (in mmol/L: NaCl 134, KCl 4, glucose 11, $MgSO_4$ 1.2, Na_2HPO4 1.2, HEPES 10, EGTA 0.2, pH adjusted to 7.35 using NaOH) was injected in both ventricles to arrest the heart and to remove blood. Then the heart was cannulated to allow a retrograde Langendorff perfusion with solution A^+ at room temperature for 5 min. Following a Liberase TM^{TM} perfusion [1 mg in solution A (Roche Diagnostics Corp.)] at 37°C for 12 min, the heart was placed in a petri dish with solution A (in mmol/L: NaCl 134, KCl 4, glucose 11, $MgSO_4$ 1.2, Na_2HPO4 1.2, HEPES 10, pH adjusted to 7.35 using NaOH, sterile filtered, oxygenated). The ventricles were cut and triturated in the wells of the 12-well plate (with coverslips coated with extracellular matrix, Sigma-Aldrich). Thereafter, the extracellular Ca^{2+} concentration was increased by repetitive addition of 150 µL solution B (in mmol/L: NaCl 134, KCl 4, glucose 11, $MgSO_4$ 1.2, Na_2HPO4 1.2, HEPES 10, $CaCl_2$ 0.2, DNAse 0.1%, pH adjusted to 7.35 using NaOH) for 10 times in 5 min intervals. The supernatant was discarded and finally 1 mL medium M199 was added. The myocytes were incubated at 37°C with 5% CO_2 and saturated humidity. Cells were used for the experiments up to 5 h after isolation.

2.3 Western blot analysis

Freshly isolated cells were used for preparation of protein extracts. Proteins were separated on 10% SDS–PAGE and electrophoretically transferred to PVDF membrane. Blots were probed with corresponding primary and secondary antibodies to detect proteins of interest. For details, see Supplementary material online.

Table 1 Transgenic mice used in this study

Label	*gnaq*	*gna11*	Cre status	Tamoxifen	Appearance
Gq^{fl} $G11^{-}$ Cre^{+} Tam^{-}	flox/flox	–/–	tg/0	-	Figure 1
Gq^{fl} $G11^{-}$ Cre^{+} Tam^{+}	flox/flox	–/–	tg/0	+	Figures 1 and 4
Gq^{wt} $G11^{wt}$ Cre^{-} Tam^{-}	wt/wt	wt/wt	0/0	-	Figures 2 and 3
Gq^{wt} $G11^{wt}$ Cre^{-} Tam^{+}	wt/wt	wt/wt	0/0	+	Figure 2
Gq^{fl} $G11^{-}$ Cre^{-} Tam^{-}	flox/flox	–/–	0/0	-	Figures 2 and 4
Gq^{fl} $G11^{-}$ Cre^{-} Tam^{+}	flox/flox	–/–	0/0	+	Figures 2 and 4
Gq^{wt} $G11^{wt}$ Cre^{+} Tam^{-}	wt/wt	wt/wt	tg/0	-	Figure 3
Gq^{fl} $G11^{wt}$ Cre^{-} Tam^{+}	flox/flox	wt/wt	0/0	+	Figure 4
Gq^{fl} $G11^{wt}$ Cre^{+} Tam^{+}	flox/flox	wt/wt	tg/0	+	Figure 4

wt, wild-type; tg, transgenic (Cre expression); 0, no Cre expression; colour-code in figures is identical.

2.4 Electrophysiological measurements

Action potentials (APs) and a transient outward current were recorded in the whole-cell configuration by using an EPC10 patch-clamp amplifier. For details, see Supplementary material online.

2.5 Ca^{2+} transient measurements

Ca^{2+} transient measurements were performed on a video imaging setup after loading the cells with Fura-2 AM. A description of the stimulation protocol is provided in Supplementary material online, *Figure S2*. For more details, see Supplementary material online.

2.6 Statistical analysis

Bar graphs display the mean ± SEM for the Gaussian distributed data. Box plots show the median and the 25/75 percentiles (lower/upper end of the boxes) for non-Gaussian distributions. For details, see Supplementary material online.

3 Results

3.1 $G\alpha_q$ KO modulates the electrophysiological properties and Ca^{2+} handling in ventricular myocytes

We used the Cre mouse described in the method section (see also Supplementary material online, *Figure S1*) to generate a cardiac-specific $G\alpha_q$ KO. We performed the $G\alpha_q$ KO on a $G\alpha_{11}$-deficient background because $G\alpha_q$ and $G\alpha_{11}$ might have overlapping functions.[20] We accomplished this using $Gq^{fl}G11^{-}Cre^{+}$ mice (for abbreviations see *Table 1*) and compared animals that were injected with tamoxifen ($G\alpha_{q/11}$ double knockout, DKO) to those without tamoxifen induction ($G\alpha_{11}$ KO). Ventricular myocytes were characterised by measuring basic electrophysiological properties with patch-clamp techniques and their global Ca^{2+} handling with an established post-rest protocol (*Figure 1* and Supplementary material online *Figure S2*).

When analysing APs, we found that in the $G\alpha_{q/11}$ DKO, the resting membrane potential (V_R) was unchanged (*Figure 1B*) while the amplitude of the APs (*Figure 1C*) was significantly increased, and cells displayed a lower membrane capacitance (C_M, *Figure 1A*). *Figure 1Da* details two exemplified APs from each population and indicates that, in addition to the amplitude, the time course of repolarisation appeared prolonged in the $Gq^{fl}G11^{-}Cre^{+}Tam^{+}$ cells. This observation was obtained by measuring and analysing APD_{30} (early phase of repolarization) and APD_{70} (late phase of repolarization). *Figure 1Db* illustrates the significant prolongation of APD_{70}. The repolarisation of the AP is determined by a multitude of potassium currents of which a few might reportedly be under the control of $G\alpha_{q/11}$-dependent signalling.[22,23] It was indicated recently that the $G\alpha_{11}$ protein might be essential in the regulation of the transient outward potassium current (I_{to}).[17] We therefore investigated I_{to} (*Figure 1E*). Due to our experimental approach, I_{to} might be contaminated with L-type Ca^{2+} current (I_{Ca}), therefore we refer to it as I_{toc} (see Supplementary material online). Because I_{toc} was significantly reduced in the $G\alpha_{q/11}$ DKO cells, we concluded that $G\alpha_q$ KO altered the electrophysiological properties of $G\alpha_{11}$-deficient cardiomyocytes.

In a second set of experiments, we analysed the global Ca^{2+} transients to study the properties of global Ca^{2+} homoeostasis (*Figure 1F*). We decided to use a post-rest protocol (see Supplementary material online, *Figure S2*) because such a stimulation regime allows for the simultaneous investigation of many parameters involved in Ca^{2+} handling. The amplitude of the Ca^{2+} transients was significantly increased in $Gq^{fl}G11^{-}Cre^{+}Tam^{+}$ myocytes directly after rest (A^{1st}) and during steady-state (A^{stst}, *Figure 1Fb*), post-rest behaviour was unaltered (PRB in *Figure 1Fb*). As a global indicator for the alterations in the apparent activity of the Ca^{2+} removal mechanisms, we compared the duration of the Ca^{2+} transient at 80% recovery (CTD_{80}, *Figure 1Fc*) and found a slight (13%) but significant prolongation of the Ca^{2+} transient under steady-state conditions.

Despite these results, we were concerned whether all the effects were genuinely caused by $G\alpha_q$ KO or whether tamoxifen injection, a lack of $G\alpha_{11}$ and/or Cre expression *per se* might contribute to the observed phenotypes. Therefore, we designed experiments that specifically addressed: (i) the impact of tamoxifen, (ii) the effects of Cre expression, (iii) the effects of $G\alpha_{11}$-KO, and (iv) which genotypes are the best for studying the effect of $G\alpha_q$ KO?

3.2 Tamoxifen and $G\alpha_{11}$ KO alter the properties of myocytes

Cre recombinase-induced changes in the protein expression profiles of transgenic animals have become an invaluable tool in many areas of research,[24] but the possible interactions with the tamoxifen injection were rarely taken into account. This appeared surprising because changes in gene expression have already been described for chronic treatments in small rodents.[25] We used injections of tamoxifen on five consecutive days followed by 30 days without tamoxifen treatment (the $G\alpha_q$ protein was virtually absent after 25 days as probed by western blot analysis, see Supplementary material online, *Figure S1*). All experiments were carried out in the absence of the drug. Therefore, we studied the putative effects of our standard tamoxifen injection regime on a $G\alpha_{11}$ wild-type (wt) background ($Gq^{wt}G11^{wt}Cre^{-}$). To further investigate whether the tamoxifen effects might depend on the genetic background, we also included $Gq^{fl}G11^{-}Cre^{-}$ animals in our study (*Figure 2*). In control experiments, we found no alterations caused by the insertion of the loxP sites for all parameters we studied (data not shown). Therefore, there was no difference between the Gq^{fl} and Gq^{wt} animals, which allowed us to directly compare the two groups.

While in $G\alpha_{11}$ KO animals, C_M was reduced (*Figure 2A*), V_R and AP amplitude were unaltered (*Figure 2B* and *C*, respectively). When we analysed the time course of cellular APs, the findings became rather complex (*Figure 2D*). In wt animals ($Gq^{wt}G11^{wt}Cre^{-}$), tamoxifen injection did not change the AP properties (compare the brown and grey APs/bars in *Figure 2D*). In contrast, following tamoxifen injection in the $G\alpha_{11}$ KO myocytes ($Gq^{fl}G11^{-}Cre^{-}$), AP repolarization was significantly prolonged both in the early and later phases (APD_{30} and APD_{70}, compare the violet and green APs/bars in *Figure 2D*). Moreover, the KO of the $G\alpha_{11}$ protein resulted in a shortening of the AP repolarisation (compare the brown and violet APs/bars in *Figure 2D*). We also investigated I_{toc} (*Figure 2E*) and found alterations among the experimental conditions. These changes were in agreement with the time course of the AP repolarisation. The observed AP shortening following the $G\alpha_{11}$ KO (the brown vs. violet) and the AP prolongation after tamoxifen injection in $Gq^{fl}G11^{-}Cre^{-}$ mice (the violet vs. green) were accompanied by an increase and decreased in the I_{toc} activity, respectively (*Figure 2Eb*).

Ca^{2+} signalling also underwent complex changes (*Figure 2F*). Tamoxifen injection into wt animals ($Gq^{wt}G11^{wt}Cre^{-}$) increased

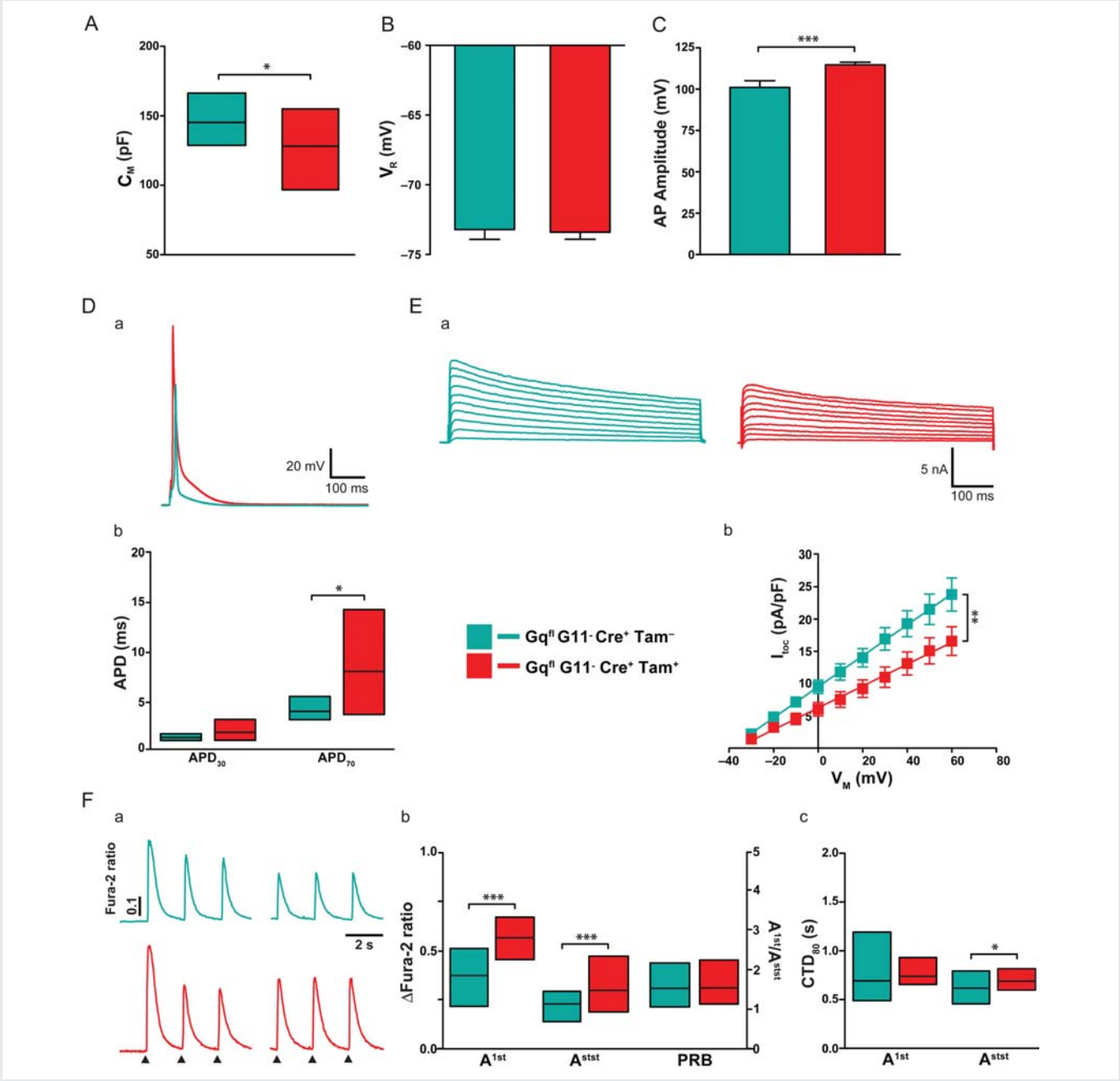

Figure 1 Modulation of $G\alpha_q$ KO on electrophysiological and Ca^{2+} handling properties in ventricular myocytes. (*A*–*C*) Membrane capacitance (C_M), resting membrane potential (V_R), and action potential amplitude (AP amplitude). (*D*) Representative APs (Da) and action potential duration (Db) after 30 and 70% repolarisation (APD_{30} and APD_{70}, respectively). (*E*) Typical traces acquired for I_{toc} recordings (Ea) and corresponding IV-relationships (Eb). (*F*) Representative Ca^{2+} transients (Fa), corresponding statistical analysis including first amplitude after rest (A^{1st}, Fb), steady-state amplitude (A^{stst}, Fb), post-rest behaviour (PRB = A^{1st}/A^{stst}, Fb), and calcium transient duration at 80% decay (CTD_{80}) for A^{1st} and A^{stst} (Fc). Number of animals and cells are shown in Supplementary material online *Table S2*.

the first amplitude after rest (A^{1st} in *Figure 2Fb*, compare the brown and grey boxes), while the steady-state amplitudes and post-rest behaviour were unaffected. Tamoxifen injection into $Gq^{fl}G11^{-}Cre^{-}$ mice did not change the amplitude of the Ca^{2+} transients. The duration of the Ca^{2+} signals was significantly reduced after tamoxifen injection into $Gq^{wt}G11^{wt}Cre^{-}$ but not in $Gq^{fl}G11^{-}Cre^{-}$ myocytes (*Figure 2Fc*; compare the brown vs. grey and the violet vs. green). The duration of steady-state Ca^{2+} transients was also dependent on the genotype: in the $G\alpha_{11}$ KO animals ($Gq^{fl}G11^{-}Cre^{-}$), it was reduced, while tamoxifen injection resulted in a prolongation (see the grey and green bars in *Figure 2Fc*).

From these findings we concluded: (i) tamoxifen injection resulted in a rather complex, genotype-dependent alteration of electrophysiological properties and Ca^{2+} handling even though its injection had been terminated at least 30 days prior to the actual experiment; and (ii) $G\alpha_{11}$ KO altered the electrophysiological properties of ventricular myocytes and their Ca^{2+} handling.

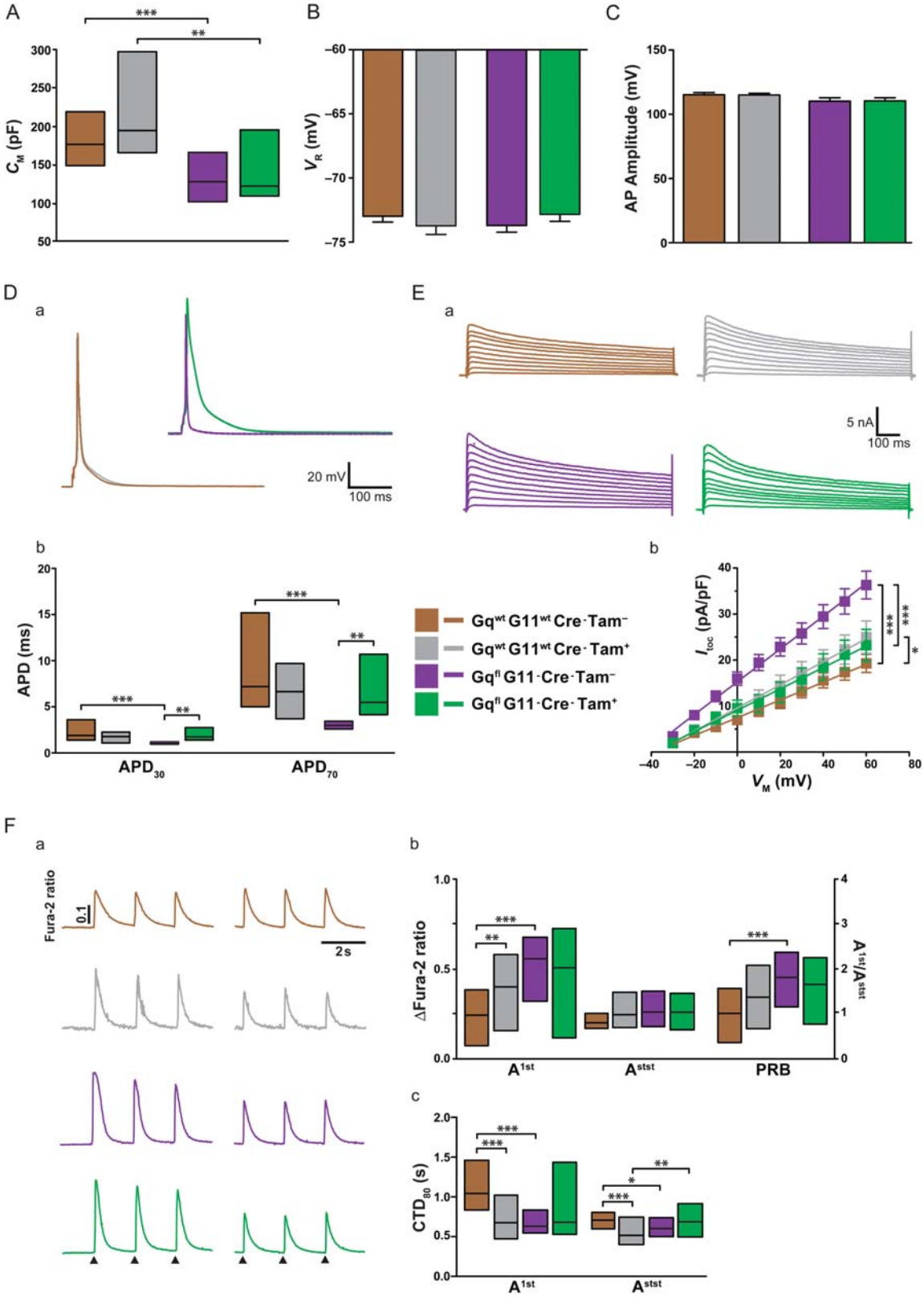

Figure 2 Effect of tamoxifen application on electrophysiological characteristics and Ca^{2+} homoeostasis of ventricular myocytes. (*A*–*C*) Membrane capacitance (C_M), resting membrane potential (V_R), and action potential amplitude (AP Amplitude). (*D*) Representative APs (Da) and action potential duration (Db) after 30 and 70% repolarisation (APD_{30} and APD_{70}, respectively). (*E*) Typical traces acquired for I_{toc} recordings (Ea) and corresponding IV relationships (Eb). (*F*) Representative Ca^{2+} transients (Fa), corresponding statistical analysis including first amplitude after rest (A^{1st}, Fb), steady-state amplitude (A^{stst}, Fb), post-rest behaviour (PRB = A^{1st}/A^{stst}, Fb), and calcium transient duration at 80% decay (CTD_{80}) for A^{1st} and A^{stst} (Fc). The numbers of animals and cells are shown in Supplementary material online, *Table S2*.

3.3 Cre recombinase expression causes only minor changes in myocyte properties

We then investigated whether the Cre expression exerted effects on properties of the wt ($Gq^{wt}G11^{wt}$) and $G\alpha_{11}$ KO ($Gq^{fl}G11^{-}$) myocytes, since sole effects of Cre expression have been reported.[26] The results are summarised in *Figure 3*. It has to be mentioned that the boxes or bars with identical colours represent the same data set and were replotted if necessary (the brown and violet in *Figures 2* and *3* and the red in *Figures 1* and *4*).

The only electrophysiological parameter that was affected by the Cre expression was the apparent magnitude of the I_{toc} in the $G\alpha_{11}$-deficient mice (compare the turquoise and violet in *Figure 3E*). Interestingly, this Cre-induced decrease in I_{toc} did not change the APD_{30} and APD_{70} (*Figure 3Db*).

Similarly, the Ca^{2+} handling was also only slightly affected by the Cre expression (*Figure 3F*). The presence of the Cre recombinase shortened the duration of the first Ca^{2+} transient after rest (compare the brown and orange group in *Figure 3Fc*). All other significant changes were due to the $G\alpha_{11}$ KO rather than the Cre expression.

From these results, we concluded that the Cre expression did not greatly alter the properties of ventricular myocytes.

3.4 Two sets of genotypes are sufficient to study the $G\alpha_{q/11}$ KO in ventricular myocytes

The experimental series introduced in *Figures 2* and *3* were conducted to evaluate the putative effects of tamoxifen injection, the $G\alpha_{11}$ KO and Cre expression on the basic electrophysiological properties as well as the Ca^{2+} handling of ventricular myocytes. The major conclusions were that tamoxifen injection and $G\alpha_{11}$ KO resulted in severe alterations in these properties, but Cre expression only demonstrated small changes. Additionally, in control experiments, we found that the insertion of the loxP sites did not alter the properties studied at all (data not shown). Therefore, we concluded that the tamoxifen/miglyol injection on a constant genetic background, similar to what we introduced in *Figure 1*, was not the best approach to study the effects of $G\alpha_q$ KO on ventricular myocytes. Rather, Cre or loxP insertion would better serve that purpose.

Therefore, we designed two genetic approaches to address the role of $G\alpha_q$; they are summarised in *Tables 2* and *3*. The basic idea behind *Table 2* was to generate $G\alpha_q$ KO by inserting the loxP sites, while *Table 3* introduces the idea of Cre expression. While loxP insertion would require the breeding of four separate mouse lines with an increasing genetic distance over time, the genotypes introduced in *Table 3* (Cre expression with tamoxifen injection) would require the breeding of only two mouse lines, enabling us to compare the $G\alpha_q$ KO effects in litter mates. Therefore, we decided to perform the final set of experiments using the genotypes detailed in *Table 3*, which all included tamoxifen injection.

3.5 $G\alpha_q$ KO results in altered properties of cardiomyocytes, but its consequences depend on $G\alpha_{11}$ expression

When using the genotypes shown in *Table 3*, we found that a plethora of electrophysiological properties were changed (*Figure 4A–E*). In the presence of $G\alpha_{11}$ (*Figure 4A–C*, the black and blue groups), $G\alpha_q$ KO resulted in an increase in AP amplitude and more negative V_R. Such changes could not be observed in the $G\alpha_{11}$-deficient myocytes (*Figure 4A–C*, the green and red groups).

In contrast to most of the interventions introduced so far, when investigating myocytes from our optimized genotype cohort, we did not notice any significant changes in the AP time course (*Figure 4D*) despite significant but minor alterations in I_{toc} (*Figure 4E*). While $G\alpha_{11}$ KO alone resulted in an increased I_{toc} (*Figure 4E*, the black vs. green group), the additional KO of $G\alpha_q$ shifted the activity of I_{toc} back to 'control' levels, i.e. towards the currents found in the $Gq^{fl}G11^{wt}Cre^{-}Tam^{+}$ mice (*Figure 4E*, the red vs. black groups).

The detailed analysis of Ca^{2+} handling in these genotypes revealed a rather complex picture (*Figure 4F*). All genetic interventions, $G\alpha_q$ KO in the presence (*Figure 4F*, the green vs. red) and absence of $G\alpha_{11}$ (*Figure 4F*, the black vs. blue) as well as the $G\alpha_{11}$ KO alone (*Figure 4F*, the black vs. green), altered Ca^{2+} handling. For some parameters, the interventions even altered parameters in opposite directions. KO of both the $G\alpha_{11}$ and the $G\alpha_q$ proteins resulted in an increase in the amplitudes of the Ca^{2+} transients, but the steady-state parameter was affected much less than the post-rest values (*Figure 4Fb*). These responses resulted in a severe increase in post-rest potentiation (*Figure 4Fb*, right). The recovery of the Ca^{2+} transients (CTD_{80}, *Figure 4Fc*) displayed a speed-up after $G\alpha_q$ was deleted in the presence of $G\alpha_{11}$ (*Figure 4Fc*, the black vs. blue), but a slowdown or no changes were observed after $G\alpha_q$ deletion in the absence of $G\alpha_{11}$ (*Figure 4Fc*, the green vs. red).

From these results, we concluded that $G\alpha_q$ and $G\alpha_{11}$ KO were important determinants of the electrophysiological properties of ventricular myocytes but play an even more exaggerated role in Ca^{2+} handling under basic, physiological conditions.

4. Discussion

4.1 A novel heart-specific Cre recombinase mouse line

The creation of transgenic mice expressing Cre recombinase under the control of the α-MHC promoter was a milestone in cardiac research.[27,28] The employed Cre/loxP technology allowed the control of defined genetic alterations in a temporal and tissue-specific manner. Recent publications emphasized the need of proper controls when using the Cre/loxP technology due to system-inherent pitfalls.[25,26,29] The most popular Cre mice in cardiac research studies was created by Sohal *et al.*[28] But these Cre mice showed abnormalities such as a decreased fractional shortening, increased end-diastolic diameter, and decreased SERCA expression.[25,26]

Here, we employed a novel Cre mouse (Takefuji *et al.*, 2012, submitted for publication) to induce a conditional $G\alpha_q$ KO. To induce $G\alpha_q$ KO, we administered tamoxifen intraperitoneally for five consecutive days (40 mg/kg body weight). The expression of the $G\alpha_q$ protein diminished after 25 days as verified by western blot analysis (Supplementary material online, *Figure S1*). Although Sohal *et al.*[28] typically used lower tamoxifen doses (20 mg/kg body weight), Koitabashi *et al.* reported this to be inefficient for protein knockdown.[25] Considering that 50% of the initial tamoxifen concentration is eliminated from the mouse within around 5 days,[30] we assume that at the end of our waiting period of at least 30 days the tamoxifen concentration was below 2% of its starting value.

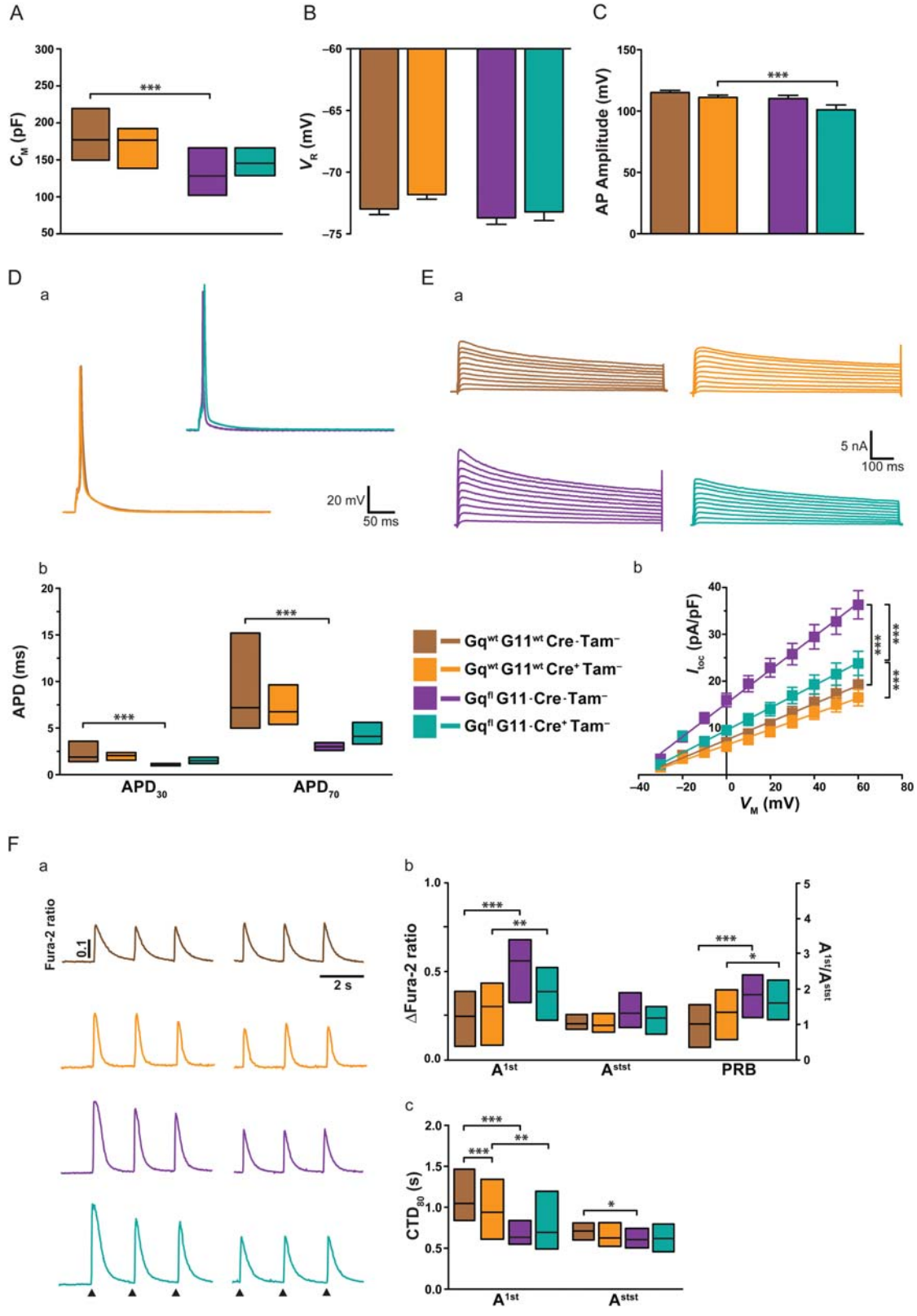

Figure 3 Effect of Cre expression on electrophysiological characteristics and Ca^{2+} homoeostasis of ventricular myocytes. (*A*–*C*) Membrane capacitance (C_M), resting membrane potential (V_R), and action potential amplitude (AP Amplitude). (*D*) Representative APs (Da) and action potential duration (Db) after 30 and 70% repolarisation (APD_{30} and APD_{70}, respectively). (*E*) Typical traces acquired for I_{toc} recordings (Ea) and corresponding IV relationships (Eb). (*F*) Representative Ca^{2+} transients (Fa), corresponding statistical analysis including first amplitude after rest (A^{1st}, Fb), steady-state amplitude (A^{stst}, Fb), post-rest behaviour (PRB = A^{1st}/A^{stst}, Fb), and calcium transient duration at 80% decay (CTD_{80}) for A^{1st} and A^{stst} (Fc). The numbers of animals and cells are shown in Supplementary material online, *Table S2*.

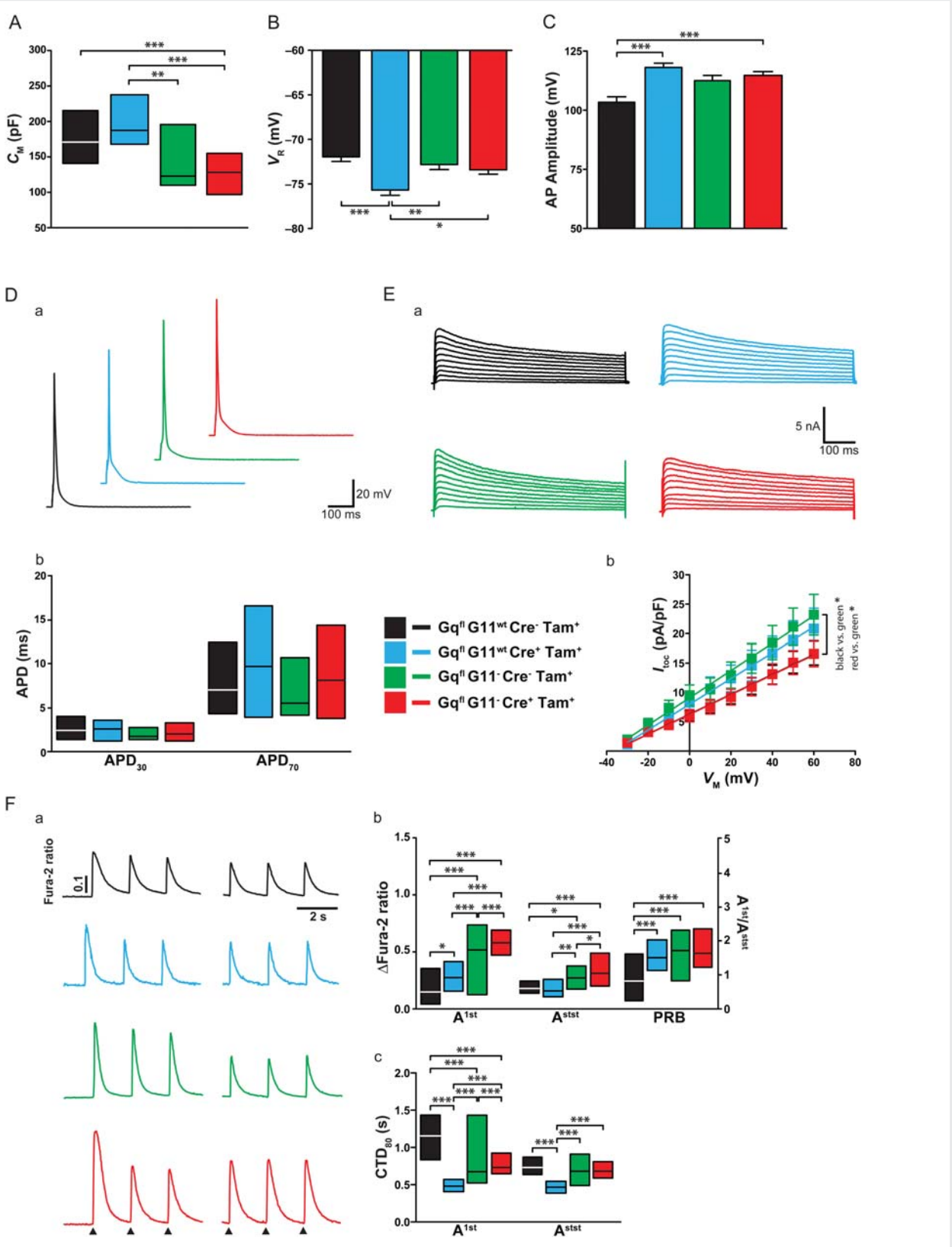

Figure 4 Effect of $G\alpha_q$ KO on electrophysiological characteristics and Ca^{2+} homoeostasis of ventricular myocytes. (*A*–*C*) Membrane capacitance (C_M), resting membrane potential (V_R), and action potential amplitude (AP Amplitude). (*D*) Representative APs (Da) and action potential duration (Db) after 30 and 70% repolarisation (APD_{30} and APD_{70}, respectively). (*E*) Typical traces acquired for I_{toc} recordings (Ea) and corresponding IV relationships (Eb). (*F*) Representative Ca^{2+} transients (Fa), corresponding statistical analysis including first amplitude after rest (A^{1st}, Fb), steady-state amplitude (A^{stst}, Fb), post-rest behaviour (PRB = A^{1st}/A^{stst}, Fb), and calcium transient duration at 80% decay (CTD_{80}) for A^{1st} and A^{stst} (Fc). The numbers of animals and cells are shown in Supplementary material online, *Table S2*.

4.2 The impact of tamoxifen treatment and Cre recombinase expression

Using this novel Cre mouse, we generated the basic genotype for studying the $G\alpha_q$ KO, a $Gq^{fl}G11^{-}Cre^{+}$ mouse. For a comprehensive analysis, we decided to investigate two largely integrative cellular phenomena for isolated ventricular cardiomyocytes: the AP and electrically evoked global Ca^{2+} transients.

The shape of the AP reflects the integration of all ion currents; therefore, it served as an indicator for changes in ion channel contributions, which could be the result of altered protein expression or altered channel properties. Electrically evoked Ca^{2+} transients after a resting period enabled us to investigate individual Ca^{2+} transients and their post-rest behaviour as a valuable tool to screen alterations in global Ca^{2+} handling, including Ca^{2+} release and Ca^{2+} removal processes.[31]

For both parameters, we initially found significant alterations (*Figure 1*), but we were concerned whether our results could be tempered by the possible genomic effects of tamoxifen injection and/or Cre expression.

We investigated these interventions and found that tamoxifen injection, besides its well-known acute effect,[32] did cause long-lasting alterations in APs and Ca^{2+} handling (*Figure 2*).

Tamoxifen and its important metabolite 4-hydroxytamoxifen exert both the genomic and non-genomic effects. Alterations of numerous ionic currents as well as inhibition of sarcoplasmic reticulum (SR) Ca^{2+} uptake have been associated with acute (non-genomic) effects of tamoxifen application.[33–35] Genomic tamoxifen effects result in long-lasting modulations of gene expression of ion channels, including potassium channels, e.g. I_{to}, the rapid delayed rectifier current (I_{Kr} aka HERG), the steady-state potassium current (I_{SS}), and the inward rectifier current (I_{K1}).[33] It has to be mentioned here that the latter report applied a chronic tamoxifen treatment (60 days). This does not reflect the usual tamoxifen treatment for inducible KO studies, including our own. The effects we describe here originate from a relatively brief tamoxifen treatment and prevail more than four weeks after termination of the treatment. At this time point, the tamoxifen concentration is believed to be less than 2% of its initial concentration (see above). Tamoxifen injection also caused opposite effects depending on the particular genetic background (e.g. APD in *Figure 2Db*). Although we did not investigate this further, we found that such changes were in good agreement with the current density of I_{toc} (*Figure 2E*).

We also found profound changes in Ca^{2+} handling extending to both their amplitude and recovery phase. Both parameters displayed a rather complex relationship with respect to $G\alpha_q/_{11}$-KO. The KO of $G\alpha_q$ resulted in an acceleration of recovery (decrease in CTD_{80}) in the presence of $G\alpha_{11}$ (*Figure 4Fc*), while the same KO displayed reversed effects in the absence of $G\alpha_{11}$ (*Figure 4Fc*). The major processes responsible for the Ca^{2+} removal are the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump and the Na^{+}/Ca^{2+} exchanger (NCX). Since acute tamoxifen application inhibited SERCA,[35] we might speculate that changes in SERCA activity or expression might underlie the alterations in CTD_{80}. Therefore, we suggest that tamoxifen caused long-term changes in gene expression that also manifested in altered Ca^{2+} handling (*Figure 2F*). This requires additional studies to determine the underlying mechanism.

Although tamoxifen injection *per se* altered myocyte behaviour, its administration was obligatory for the generation of the conditional $G\alpha_q$ KO. Other methods to bypass the tamoxifen treatment including siRNA or adenoviral transfections are no alternatives. In the *in-vivo* situation, the efficiency of the transfection and the resulting protein knockdown is limited. Despite the fact that single cell culture has majorly advanced recently,[36,37] whole animal genetic approaches allow the investigation of the intervention in the context of the entire animal, an invaluable advantage.

Table 2 $G\alpha_q$ KO by insertion of loxP sites

Label	*gnaq*	*gna11*	Cre status	Tamoxifen
Gq^{wt} $G11^{wt}$ Cre^{+} Tam^{+}	wt/wt	wt/wt	tg/0	+
Gq^{fl} $G11^{wt}$ Cre^{+} Tam^{+}	flox/flox	wt/wt	tg/0	+
Gq^{wt} $G11^{-}$ Cre^{+} Tam^{+}	wt/wt	−/−	tg/0	+
Gq^{fl} $G11^{-}$ Cre^{+} Tam^{+}	flox/flox	−/−	tg/0	+

Wt, wild-type; tg, transgenic (Cre expression); 0, no Cre expression.

4.3 Sets of genetic combinations

We decided not to compare animals in which KO was primarily induced by tamoxifen injection, but instead used a different molecular determinant for the induction of the KO while maintaining the tamoxifen injection for all experimental settings. We suggested two sets of genetic combinations that are suited to study the role of

Table 3 $G\alpha_q$ KO by insertion of Cre

Label	*gnaq*	*gna11*	Cre status	Tamoxifen
Gq^{fl} $G11^{wt}$ Cre^{-} Tam^{+}	flox/flox	wt/wt	0/0	+
Gq^{fl} $G11^{wt}$ Cre^{+} Tam^{+}	flox/flox	wt/wt	tg/0	+
Gq^{fl} $G11^{-}$ Cre^{-} Tam^{+}	flox/flox	−/−	0/0	+
Gq^{fl} $G11^{-}$ Cre^{+} Tam^{+}	flox/flox	−/−	tg/0	+

Wt, wild-type; tg, transgenic (Cre expression); 0, no Cre expression.

$G\alpha_q$ and $G\alpha_{11}$ (*Tables 2* and *3*). From those studies, we conclude that basic signalling of $G\alpha_q$ and $G\alpha_{11}$ has to be considered when studying their pathological significance.

The effects of a $G\alpha_q$-KO could either be studied using Cre^- and Cre^+ animals on a $gnaq^{flox/flox}$ background or by employing $gnaq^{flox/flox}$ and $gnaq^{wt/wt}$ animals while maintaining the tamoxifen injections (*Tables 2* and *3*). Although the insertion of the loxP sites did not alter the myocytes' behaviour (data not shown), we decided to employ the Cre expression as the genetic determinant. Using the former approach would have required breeding of four separate mouse lines. In contrast, Cre expression requires only two separate mouse lines and, most importantly, allowed us to utilise litter-mates (the black vs. blue and the red vs. green, *Table 3*). Regular back-crossing of two mouse lines could be performed with greater ease when compared with four separate mouse lines.

Utilizing the combination of mouse genotypes shown in *Table 3*, we re-visited our initial question of the possible contributions of normal $G\alpha_{q/11}$ signalling to the physiological behaviour of ventricular myocytes (*Figure 4*).

4.4 Physiological relevance of $G\alpha_q$ in ventricular myocytes

Transgenic mouse models employing gene KO or knockin of $G\alpha_q$ strongly underpinned its role for cardiac diseases.[13–16] The hypertrophic response of the heart is accompanied by the growth of the individual myocyte. The size of the myocytes can be estimated by evaluating their cross-sectional dimension in histological samples, by measuring their cell area in wide field microscopy or by assessing their plasma membrane capacitance in electrophysiological experiments. Various papers have reported that the hypertrophic response of the heart is under the control of $G\alpha_q/G\alpha_{11}$-coupled signalling pathways.[13–15,38] Our results of the cellular capacitance were in agreement with such studies even without any treatment and interventions. The KO of $G\alpha_q$ and $G\alpha_{11}$ resulted in a reduced capacitance, whereby $G\alpha_q$-deficient myocytes displayed the largest effect (*Figure 4A*).

Furthermore, we report that basic $G\alpha_q$ and $G\alpha_{11}$ signalling are important contributors to the basic physiological properties of cardiac myocytes (*Figure 4*). Additionally, we also demonstrated that $G\alpha_{11}$ KO resulted in altered APs and Ca^{2+} handling (*Figures 2* and *3*).

Sah *et al.*[39] described that prolonged AP repolarisation leads to decreased I_{Ca} and decreased cellular Ca^{2+} transients. Using slightly different approaches, the same authors also reported opposite effects.[40] This might indicate that the relationship between AP repolarisation duration, I_{Ca} and SR Ca^{2+} release is rather complex, which is supported by our results: in *Figures 2* and *3*, APD_{30} was decreased but the amplitude of steady-state Ca^{2+} transients remained unchanged (*Figure 2Db* vs. *Fb* and *Figure 3Db* vs. *Fb*). In *Figure 4*, APD_{30} was unchanged for all phenotypes but the amplitude of Ca^{2+} transients was greatly increased (*Figure 4Db* vs. *Fb*). By analysing I_{Ca} in future studies, we might be able to shed light on possible underlying contributors.

How do $G\alpha_q$ and $G\alpha_{11}$ proteins mediate changes in gene expression and regulation resulting in altered electrophysiology and Ca^{2+} homoeostasis? We believe that the changes were not brought about by the proteins per se, but instead, the results reflect the importance of a constant, possibly low-level stimulation of the upstream G-protein-coupled receptors with agonists coupling to $G\alpha_q$ and/or $G\alpha_{11}$ such as endothelin-1 or angiotensin-II.[41] When stimulated chronically at high levels, G-proteins contribute to cardiac diseases and associated changes in gene expression.[42,43] In contrast, with persistent stimulation at a much lower hormone level, $G\alpha_q$- and $G\alpha_{11}$-coupled signalling pathways appear to be an important contributor to the maintenance of the myocyte's homoeostasis.

One of the best known signalling pathway coupled to $G\alpha_q$-proteins involves $G\alpha_q$-induced phospholipase C_β (PLC_β) activation. This leads to a breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (a major activator of PKCs)[44] and inositol 1,4,5 triphosphate ($InsP_3$; a major activator of $InsP_3$ receptors)[45]. $InsP_3$ itself is discussed as an important mediator of cardiac pathologies.[4,5,7] In cardiac myocytes, the plasma membrane levels of PIP_2 itself are potent modulators of ion channels or transporters, such as K_{ATP} channels and NCX[46] (for a recent review see[47]). Taking this into account, we hypothesise that the reduced metabolism of PIP_2 in $G\alpha_q$-deficient myocyte might lead to PIP_2-dependent deregulation of NCX and thus might contribute to the altered Ca^{2+} handling described here.

The identification of important homoeostatic hormones and their downstream signalling appears important but lies well outside of the scope of these reports. Further studies will provide such information that is vital for our understanding of processes that are essential for maintaining a 'physiological' gene expression pattern.

We thus conclude that both $G\alpha_q$ and $G\alpha_{11}$ are instrumental to the physiological properties of cardiac myocytes by mediating the continuance of a basic rate of gene expression.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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