

Coxsackie and Adenovirus Receptor Is a Modifier of Cardiac Conduction and Arrhythmia Vulnerability in the Setting of Myocardial Ischemia

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Objectives

The aim of this study was to investigate the modulatory effect of the coxsackie and adenovirus receptor (CAR) on ventricular conduction and arrhythmia vulnerability in the setting of myocardial ischemia.

Background

A heritable component in the risk of ventricular fibrillation during myocardial infarction has been well established. A recent genome-wide association study of ventricular fibrillation during acute myocardial infarction led to the identification of a locus on chromosome 21q21 (rs2824292) in the vicinity of the *CXADR* gene. *CXADR* encodes the CAR, a cell adhesion molecule predominantly located at the intercalated disks of the cardiomyocyte.

Methods

The correlation between CAR transcript levels and rs2824292 genotype was investigated in human left ventricular samples. Electrophysiological studies and molecular analyses were performed using CAR haploinsufficient (CAR^{+/-}) mice.

Results

In human left ventricular samples, the risk allele at the chr21q21 genome-wide association study locus was associated with lower *CXADR* messenger ribonucleic acid levels, suggesting that decreased cardiac levels of CAR predispose to ischemia-induced ventricular fibrillation. Hearts from CAR^{+/-} mice displayed slowing of ventricular conduction in addition to an earlier onset of ventricular arrhythmias during the early phase of acute myocardial ischemia after ligation of the left anterior descending artery. Expression and distribution of connexin 43 were unaffected, but CAR^{+/-} hearts displayed increased arrhythmia susceptibility on pharmacological electrical uncoupling. Patch-clamp analysis of isolated CAR^{+/-} myocytes showed reduced sodium current magnitude specifically at the intercalated disk. Moreover, CAR coprecipitated with Na_v1.5 in vitro, suggesting that CAR affects sodium channel function through a physical interaction with Na_v1.5.

Conclusions

CAR is a novel modifier of ventricular conduction and arrhythmia vulnerability in the setting of myocardial ischemia. Genetic determinants of arrhythmia susceptibility (such as CAR) may constitute future targets for risk stratification of potentially lethal ventricular arrhythmias in patients with coronary artery disease. (J Am Coll Cardiol 2014;63:549–59) © 2014 by the American College of Cardiology Foundation

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**Abbreviations
 and Acronyms**

- AP** = action potential
- CAR** = coxsackie and adenovirus receptor
- CVL** = longitudinal conduction velocity
- CVT** = transversal conduction velocity
- Cx** = connexin
- GWAS** = genome-wide association study
- I_{Na}** = sodium current
- LAD** = left anterior descending
- MI** = myocardial infarction
- mRNA** = messenger ribonucleic acid
- VF** = ventricular fibrillation
- VT** = ventricular tachycardia
- WT** = wild-type

Ventricular fibrillation (VF) is a frequent and potentially lethal complication of acute myocardial infarction (MI). In this setting, VF is the consequence of disturbed electrical properties of the ischemic myocardium, which includes a decrease in cardiomyocyte excitability and cell-to-cell coupling. These factors result in conduction slowing of the cardiac electrical impulse, which is a prerequisite for the occurrence of life-threatening arrhythmias such as VF in MI (1).

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Traditional cardiovascular risk factors do not identify which patient with MI is at risk for VF, and specific and sensitive risk

predictors are currently lacking. A heritable component in the determination of risk of VF and sudden cardiac death during MI has been well established (2,3), but progress in understanding the molecular and genetic determinants of ischemia-induced VF has been limited. To identify susceptibility genes, our group recently conducted a genome-wide association study (GWAS) that led to the identification of common genetic variants at the chromosome 21q21 locus associated with the risk of VF in the setting of acute MI (4). The most significant association signal for VF on the chromosome 21q21 locus comprised the single nucleotide polymorphism rs2824292, located immediately upstream of 2 genes, *CXADR* and *BTG3* (the only genes within a region of 1 megabase spanning the association signal) (4). The *BTG3* gene encodes B-cell translocation gene 3, a member of the antiproliferative BTG/Tob protein family, known to regulate cell cycle progression, gene expression, tumorigenesis, and cancer (5). The *CXADR* gene encodes the coxsackie and adenovirus receptor (CAR), a transmembrane cell adhesion molecule predominantly located at the intercalated disk between cardiomyocytes (6–9). CAR has been recognized primarily for its involvement in virus-mediated myocarditis,

but recent studies in CAR knockout mice revealed atrioventricular conduction slowing due to loss of expression of the gap junction protein connexin (Cx) 45 (9,10). Moreover, expression levels of Cx43, the predominant gap junction molecule in ventricular myocardium, were decreased in hearts from CAR knockout mice (9), raising the possibility that CAR may play a role in ventricular conduction. Of note, CAR is known to be differentially regulated in various cardiac disease states, including dilated cardiomyopathy and MI (6,11).

We hypothesized that CAR affects ventricular conduction and susceptibility to ventricular arrhythmia in the setting of MI and show that the rs2824292 risk genotype is associated with decreased expression of *CXADR* in the human heart. Furthermore, we show that mice haploinsufficient for CAR display ventricular conduction slowing and an earlier onset of ventricular arrhythmias during MI, at least in part mediated by a reduced sodium current (I_{Na}) magnitude at the intercalated disk.

Methods

The methods are provided in the [Online Appendix](#).

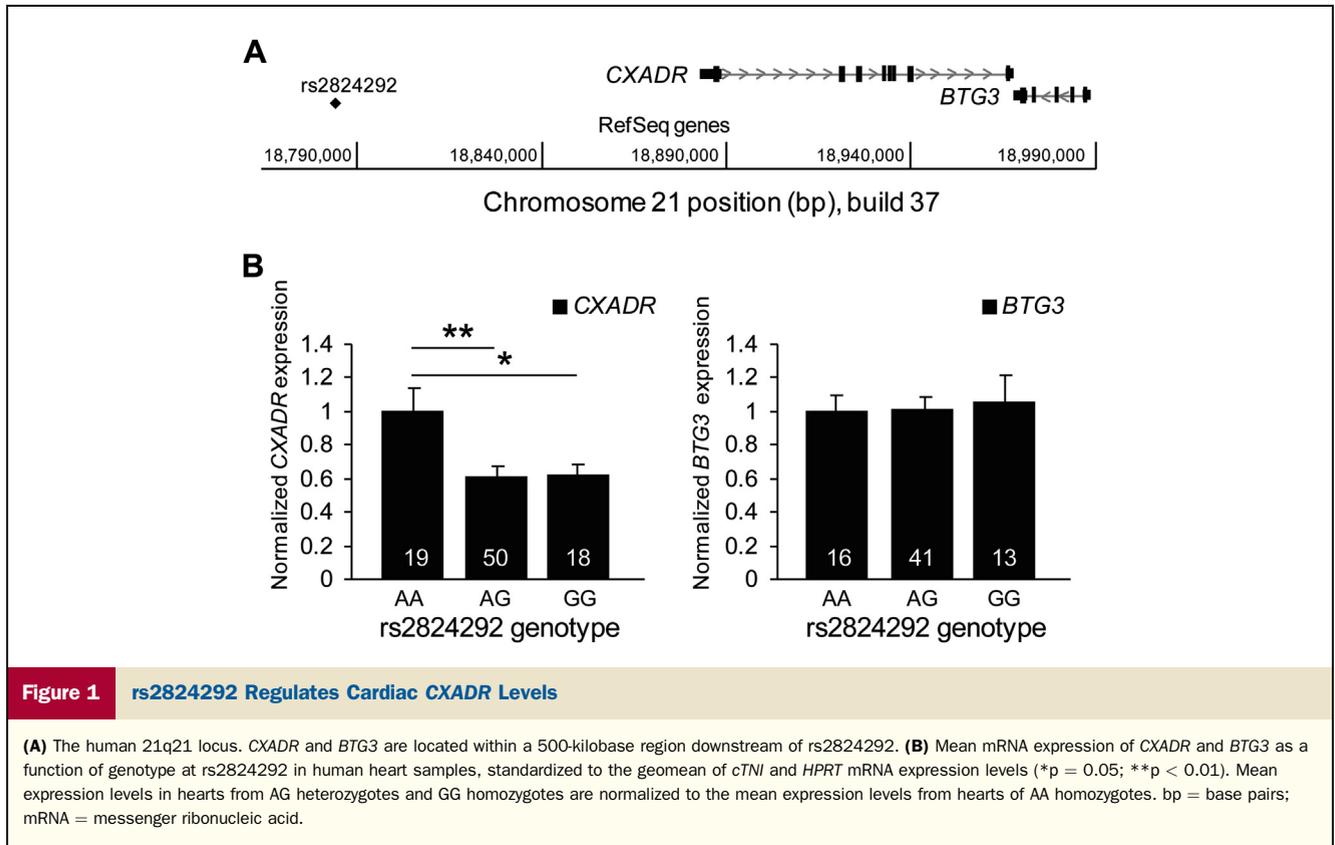
Results

rs2824292 regulates cardiac expression of *CXADR*, the gene encoding CAR. Because the risk haplotype tagged by rs2824292 occurs in a noncoding region of chr21q21, it is likely to affect the risk of VF through effects on gene expression. We therefore investigated whether the rs2824292 genotype correlates to transcript expression levels of the only 2 neighboring genes within 1 megabase, namely *CXADR* (located 100 kilobases downstream of rs2824292) and *BTG3* (located 179 kilobases downstream). Assessment of transcript abundance for these genes in normal donor heart myocardium (n = 129) uncovered an association between the genotype at rs2824292 and cardiac *CXADR* messenger ribonucleic acid (mRNA) levels (Fig. 1). Subjects carrying 1 or 2 copies of the risk allele (AG or GG genotype) displayed significantly lower (0.6-fold) *CXADR* mRNA expression compared with subjects with the nonrisk (AA) genotype (dominant model: p = 0.002; additive model: p = 0.006). No such relationship was observed with cardiac expression of *BTG3*. Thus, the VF risk allele at rs2824292 is associated with decreased cardiac expression of *CXADR*, suggesting that decreased levels of CAR predispose to VF during ischemia.

CAR haploinsufficient mice. To investigate the effects of reduced CAR expression on cardiac conduction and arrhythmogenesis, we generated mice deficient for CAR (Online Fig. 1) (12). Homozygous CAR-deficient mice were not viable and died midgestation between embryonic days 10.5 and 12.5 (Online Fig. 2, Online Table 1). Heterozygous CAR-deficient mice (CAR^{+/-}) were born at the expected Mendelian ratio (Online Table 1) and had a normal life span.

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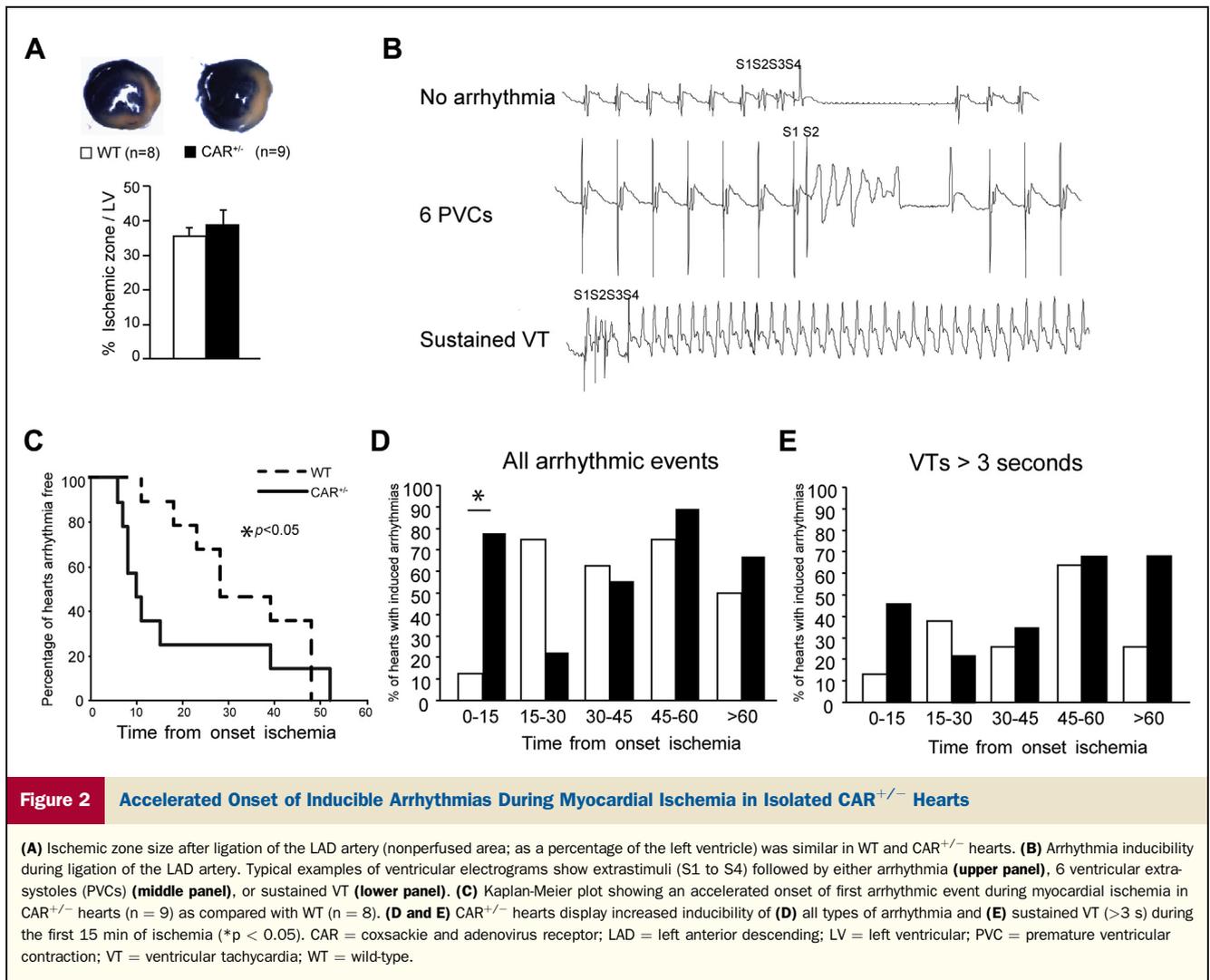
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Earlier onset of inducible ventricular arrhythmias during acute myocardial ischemia in $CAR^{+/-}$ mouse hearts. To investigate whether reduced CAR levels predispose to arrhythmia during acute myocardial ischemia, we induced regional ischemia in Langendorff-perfused wild-type (WT) and $CAR^{+/-}$ hearts by ligating the left anterior descending (LAD) artery and tested arrhythmia inducibility. Ischemic zone size was not significantly different between WT and $CAR^{+/-}$ hearts (Fig. 2A). Hearts were stimulated from the nonischemic area at a basic cycle length of 120 ms (S1), and arrhythmia inducibility was tested before ligation of the LAD artery and at different intervals during ischemia using up to 3 extrastimuli (S2-S3-S4) and burst pacing. Spontaneous sustained arrhythmias did not occur in WT or $CAR^{+/-}$ hearts at baseline or during the ischemic period. Arrhythmias were never induced before ligation of the LAD artery but were induced in all WT and $CAR^{+/-}$ hearts during ischemia. These included premature ventricular contractions, nonsustained ventricular tachycardia (VT), and sustained VT (Fig. 2B). During the first 15 min of ischemia, we observed inducible arrhythmias (nonsustained and sustained) in 7 of 9 $CAR^{+/-}$ hearts. In contrast, arrhythmias were induced in only 1 of 8 WT hearts ($p < 0.05$) (Figs. 2C and 2D). Similarly, in the first 15 min of ischemia, there was a trend toward a higher prevalence of inducible sustained VT (>3 s) in $CAR^{+/-}$ compared with WT hearts (Fig. 2E). During later stages of the ischemic episode, arrhythmia inducibility was not different between WT

and $CAR^{+/-}$ hearts. Thus, $CAR^{+/-}$ hearts displayed an advanced onset of ventricular arrhythmia inducibility during the early phase of acute myocardial ischemia.

Ventricular conduction slowing in isolated $CAR^{+/-}$ hearts. We hypothesized that altered electrophysiological properties secondary to CAR deficiency render $CAR^{+/-}$ hearts more susceptible to arrhythmia inducibility in the setting of myocardial ischemia. Surface electrocardiographic analysis showed no significant differences in electrocardiographic parameters (RR, PQ, QRS, and QTc intervals) between $CAR^{+/-}$ and WT mice (Online Fig. 3). We therefore studied ventricular conduction in more detail by electrical mapping of isolated WT and $CAR^{+/-}$ hearts. A 247-point electrode was placed on the left ventricular epicardial surface, and hearts were stimulated from the center of the electrode. Local electrograms were used to construct activation maps (Fig. 3A), from which longitudinal conduction velocity (CVL) and transversal conduction velocity (CVT) were determined. The stimulation threshold and shortest coupled intervals of S2, S3, and S4 (effective refractory period) were not significantly different between $CAR^{+/-}$ and WT hearts (Online Table 3). During basic stimulation (S1), CVL ($CAR^{+/-}$: 63.7 ± 4.7 cm/s; WT: 77.8 ± 2.8 cm/s; $p = 0.02$) and CVT ($CAR^{+/-}$: 28.6 ± 2.0 cm/s; WT: 38.0 ± 2.5 cm/s; $p = 0.01$) were significantly reduced in $CAR^{+/-}$ compared with WT hearts (Figs. 3B and 3C). These differences in conduction velocity were also observed after application of 3 extrastimuli (S4; $CAR^{+/-}$:

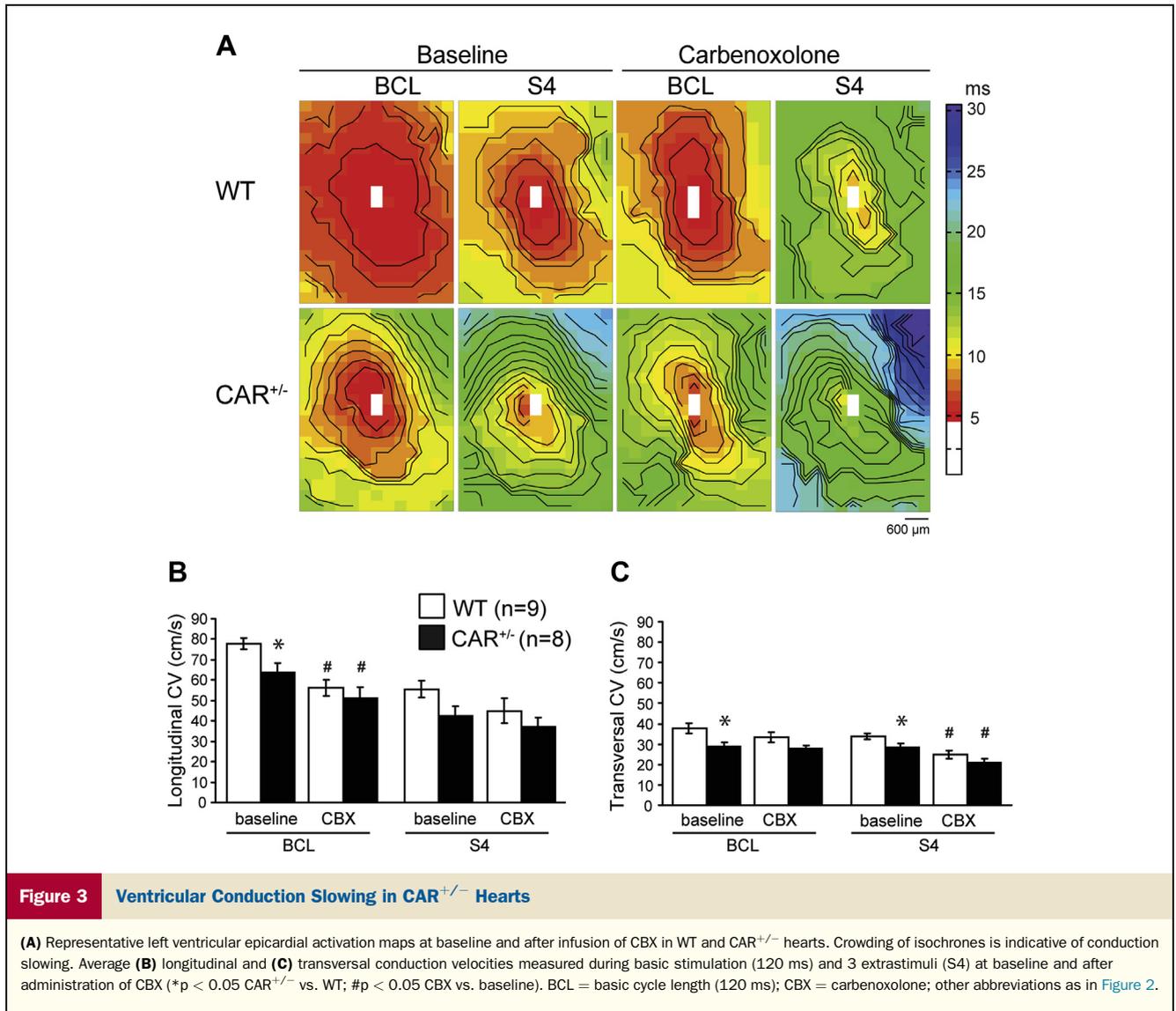


42.5 ± 4.6 cm/s; WT: 55.5 ± 4.2 cm/s; $p = 0.054$) and CVT ($CAR^{+/-}$: 28.6 ± 1.8 cm/s; WT: 33.9 ± 1.5 cm/s; $p = 0.04$) (Figs. 3B and 3C).

Pharmacological electrical uncoupling induces ventricular arrhythmias in $CAR^{+/-}$ hearts. Conduction slowing in $CAR^{+/-}$ hearts may stem from altered tissue architecture, cell-to-cell coupling, and/or cardiomyocyte excitability. No evidence for myocardial hypertrophy or fibrosis was found in $CAR^{+/-}$ hearts (Online Fig. 4). In addition, transmission electron microscopy did not reveal ultrastructural changes in $CAR^{+/-}$ hearts (Online Fig. 5). To evaluate whether the conduction slowing in $CAR^{+/-}$ hearts is mediated by alterations in gap junction composition and/or function, we investigated the expression and distribution of connexins and challenged WT and $CAR^{+/-}$ hearts with the gap junction uncoupler carbenoxolone. Carbenoxolone is known to decrease conduction velocity both in the longitudinal and transversal direction without affecting action potential (AP) characteristics and underlying ionic currents (13). During stimulation at basic cycle length (S1), carbenoxolone

significantly reduced CVL in both WT and $CAR^{+/-}$ hearts (Fig. 3B). Similarly, CVT was decreased by carbenoxolone in WT and $CAR^{+/-}$ hearts after application of 3 extrastimuli (S4) (Fig. 3C).

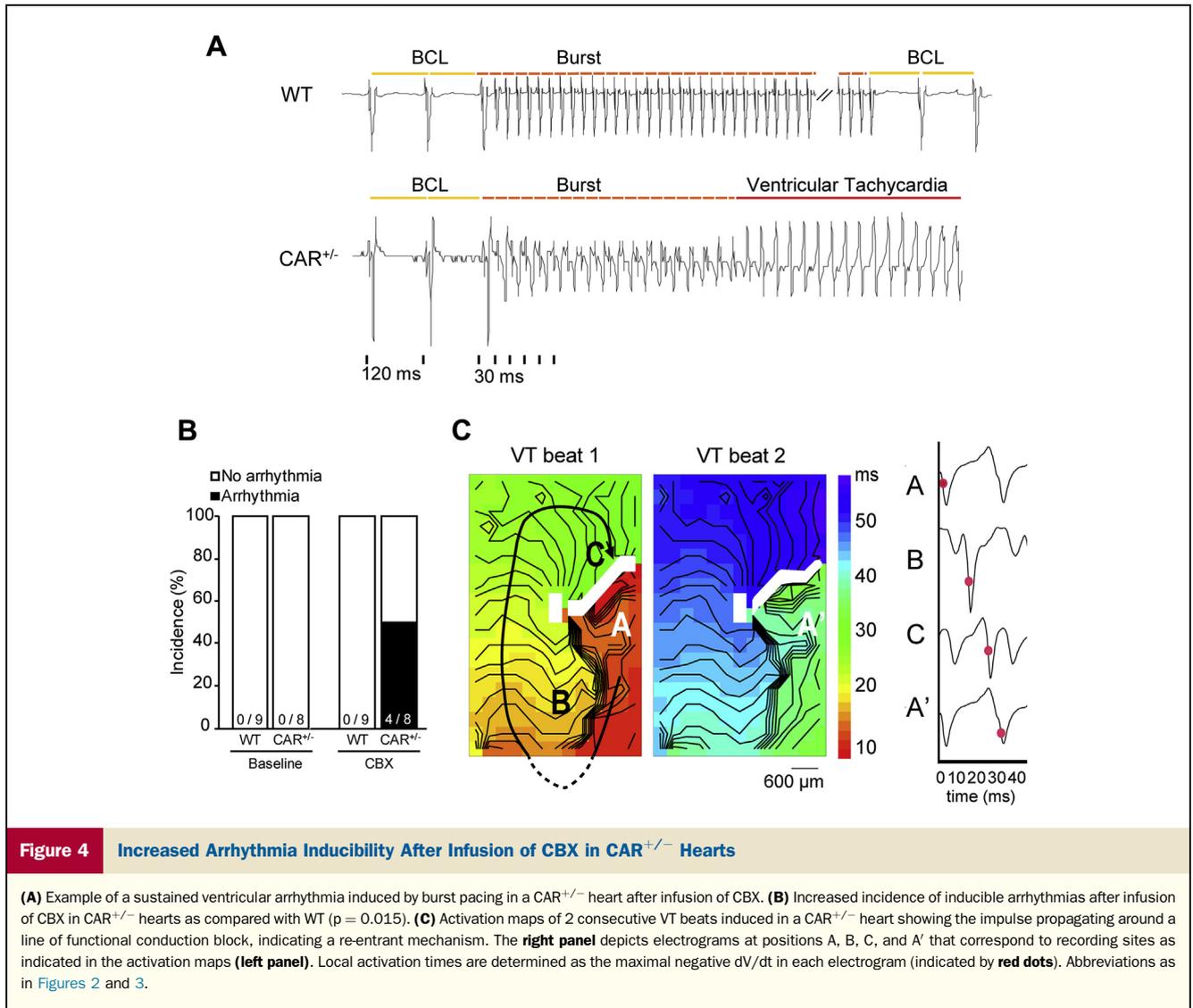
The consequences of the observed conduction slowing after infusion of carbenoxolone with respect to arrhythmia inducibility were tested using up to 3 extrastimuli (S2-S3-S4) and burst pacing. The shortest possible coupling intervals of S2-S3-S4 and burst trains were not significantly different between $CAR^{+/-}$ and WT hearts at baseline and after infusion of carbenoxolone (Online Table 3). Spontaneous arrhythmias did not occur in either experimental group, and we were unable to induce arrhythmias in WT or $CAR^{+/-}$ hearts at baseline. Although none of the WT hearts showed induced arrhythmias on infusion of carbenoxolone, 50% of the $CAR^{+/-}$ hearts developed sustained ventricular arrhythmias during burst pacing after administration of carbenoxolone ($CAR^{+/-}$: 4/8; WT: 0/9; $p = 0.015$) (Figs. 4A and 4B). VTs induced in $CAR^{+/-}$ hearts after infusion of carbenoxolone were reproducible



and lasted for more than 5 s to 1 min. Activation maps of 2 consecutive beats of an induced ventricular tachycardia in a $CAR^{+/-}$ heart show the impulse propagating around a line of block, indicating a re-entrant mechanism underlying the arrhythmia (Fig. 4C). Ventricular mRNA and protein levels of the gap junction proteins Cx43 and Cx45 were not different between WT and $CAR^{+/-}$ hearts (Figs. 5A to 5C), and there were no differences in distribution of Cx43 (in particular, no lateralization or redistribution) in ventricular cryosections (Fig. 5D) and isolated myocytes (Online Fig. 6). Protein expression levels of other intercalated disk proteins, namely β -catenin, zonula occludens 1, N-cadherin, and Cx45, were not different between WT and $CAR^{+/-}$ hearts (Online Fig. 7, Online Table 2).

Reduced sodium channel availability in $CAR^{+/-}$ mice and cardiomyocytes. Because the cardiac sodium channel protein $Nav1.5$ is enriched in the intercalated disk region, CAR may also have an impact on conduction by affecting

sodium channel expression and/or function. *Scn5a* mRNA and $Nav1.5$ protein levels were not different between $CAR^{+/-}$ and WT ventricular tissue (Figs. 5A to 5C), and there were no differences in $Nav1.5$ localization at the intercalated disk or lateral membrane (Fig. 5D). Functionally, however, AP upstroke velocity (dV/dt_{max} , a measure of sodium channel availability under near-physiological conditions [14]) was altered in cardiomyocytes isolated from $CAR^{+/-}$ hearts (Figs. 6A and 6B). On average, dV/dt_{max} was $\approx 15\%$ lower in $CAR^{+/-}$ myocytes as compared with WT, indicating a decrease in functional sodium channel availability secondary to CAR haploinsufficiency. Resting membrane potential, AP amplitude, and AP duration were unchanged in $CAR^{+/-}$ cardiomyocytes (Fig. 6B). In vivo short-term administration of the sodium channel blocker flecainide in anesthetized mice revealed a significantly larger increase in PR interval and QRS duration in $CAR^{+/-}$ versus WT mice, underlining the functional relevance of the

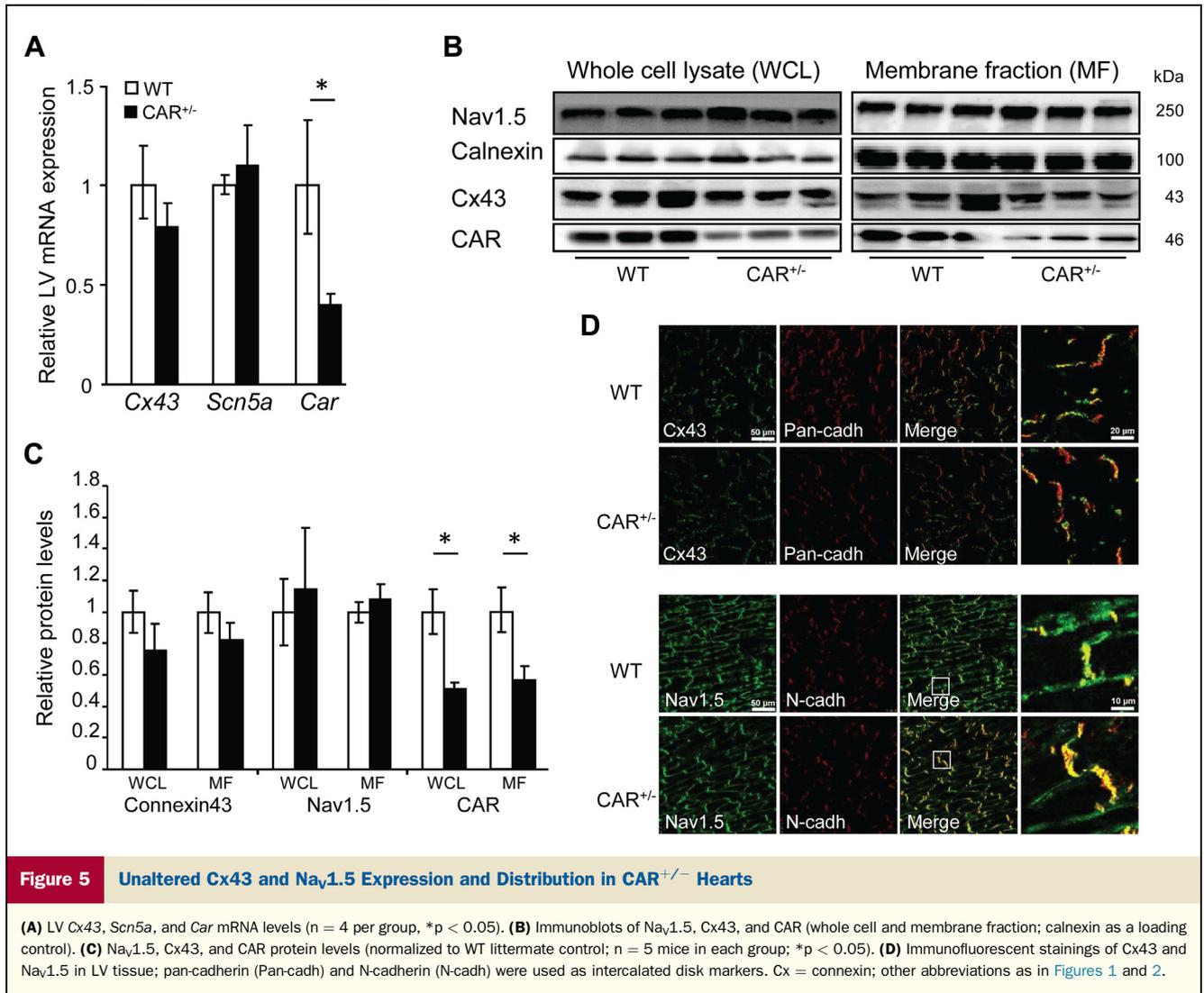


reduced sodium channel availability for cardiac conduction (**Online Figs. 3C and 3D**).

CAR haploinsufficiency affects I_{Na} magnitude specifically at the intercalated disk. Sodium channels are located both at the lateral membrane and at the intercalated disk of the cardiomyocyte (15,16). Because CAR is localized predominantly at the intercalated disk, we hypothesized that CAR deficiency affects I_{Na} preferentially in this region. We therefore investigated I_{Na} characteristics at the lateral membrane and at the intercalated disk of isolated $CAR^{+/-}$ and WT ventricular myocytes using the macropatch cell-attached mode of the patch-clamp technique, which allows for regional assessment of I_{Na} amplitude and gating properties at a physiological temperature (36°C) (17). At the lateral membrane, the number of functional sodium channels (I_{Na} amplitude) was similar in WT and $CAR^{+/-}$ cardiomyocytes (**Fig. 6C**). However, a significant decrease in I_{Na} amplitude at the intercalated disk was observed in $CAR^{+/-}$ cardiomyocytes as compared with WT (**Fig. 6D**).

Voltage dependence of I_{Na} activation and inactivation was not significantly different between WT and $CAR^{+/-}$ at the lateral membrane or at the intercalated disk (**Figs. 6E and 6F**). These findings indicate that CAR haploinsufficiency leads to reduced sodium channel availability secondary to a decreased I_{Na} amplitude at the intercalated disk.

Physical interaction between $Nav1.5$ and CAR. To determine if CAR affects $Nav1.5$ function through a physical association, we performed coimmunoprecipitation studies. FLAG-tagged human $Nav1.5$ and/or human CAR was transiently overexpressed in HEK293 cells, and cell lysates were incubated with anti-FLAG affinity beads. Overexpressed CAR was not found to precipitate with anti-FLAG beads in the absence of $Nav1.5$ (**Fig. 7**). In contrast, immunoblotting of eluates from HEK293 cells overexpressing both $Nav1.5$ and CAR revealed that CAR coprecipitates with $Nav1.5$ (**Fig. 7**), indicating a direct or indirect physical interaction between the 2 proteins.



Discussion

Our current work follows up on the discovery by a GWAS of a genetic locus on chromosome 21q21 associated with vulnerability for VF in the setting of acute MI (4). We show that in human cardiac tissue, the risk allele at this locus is associated with decreased cardiac expression of *CXADR*, suggesting that reduced levels of CAR predispose to VF during ischemia. Reduced expression of CAR in CAR haploinsufficient mice leads to slowing of ventricular conduction and earlier onset of inducible ventricular arrhythmias during acute regional myocardial ischemia, mediated (at least in part) through a reduction of I_{Na} magnitude specifically at the intercalated disk. Our findings thus establish CAR as a novel modifier of cardiac conduction and arrhythmia vulnerability in myocardial ischemia and indicate the usefulness of GWAS for the identification of novel determinants of complex phenotypes such as arrhythmia.

The rs2824292 single nucleotide polymorphism at the arrhythmia susceptibility locus on 21q21 is associated with reduced cardiac *CXADR* expression. In a recent GWAS, we identified a susceptibility locus for VF during a first acute MI at chromosome 21q21 (4). The risk haplotype at this locus, tagged by rs2824292, occurred at an intergenic region and thus likely modulates the risk of VF through effects on gene expression (18). Of the 2 genes at this locus (*CXADR* and *BTG3*), *CXADR* appears to be the most biologically plausible because previous studies have shown its myocardial localization at the intercalated disk and its modulatory effect on connexin expression and atrioventricular conduction (9,10). Our finding that carriage of the risk (G) allele at rs2824292 is associated with reduced expression of the *CXADR* gene encoding CAR in human heart samples supports the proposition that the effect of the 21q21 locus on ischemia-induced arrhythmia is mediated by CAR and suggests that decreased cardiac

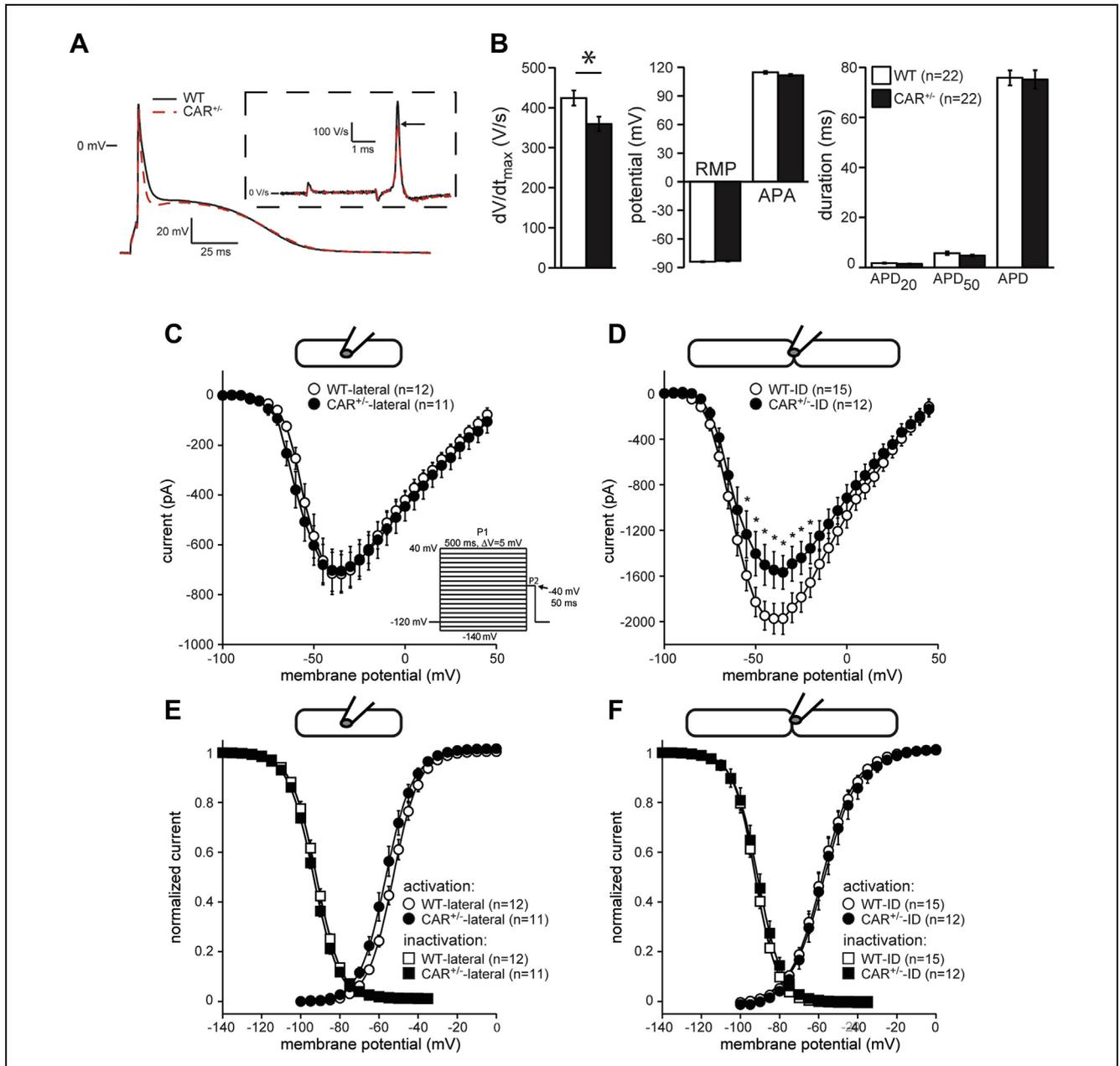
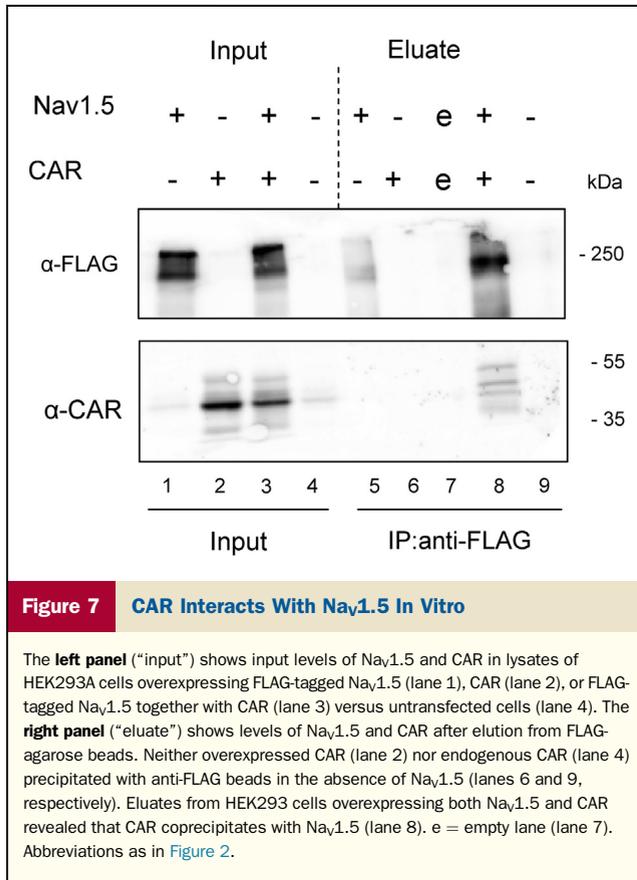


Figure 6 Reduced Sodium Channel Availability and Decreased I_{Na} Amplitude at the Intercalated Disk in $CAR^{+/-}$ Cardiomyocytes

(A) APs measured from isolated LV cardiomyocytes of WT and $CAR^{+/-}$ hearts (inset: dV/dt of the AP upstroke). (B) Average AP characteristics. Maximal AP upstroke velocity (dV/dt_{max}) was significantly lower in $CAR^{+/-}$ myocytes as compared with WT ($*p < 0.05$). Resting membrane potential, APA, and APD at 20%, 50%, and 90% of repolarization (APD_{20} , APD_{50} , and APD_{90} , respectively) were not different between WT and $CAR^{+/-}$ myocytes. (C and D) Current-voltage (I-V) relationships show similar I_{Na} amplitude at the lateral membrane of WT and $CAR^{+/-}$ cardiomyocytes (C) but reduced I_{Na} amplitude in $CAR^{+/-}$ versus WT at the intercalated disk (assessed by 2-way repeated-measures analysis of variance followed by Holm-Sidak post-hoc testing; $p = 0.002$ for overall effect) (D). Asterisks denote membrane potentials at which I_{Na} amplitude is significantly different between WT and $CAR^{+/-}$. (E and F) Average voltage dependencies of activation and inactivation show no significant difference between WT and $CAR^{+/-}$ at the lateral membrane (E) or at the intercalated disk (ID) (F). AP = action potential; APA = action potential amplitude; APD = action potential duration; I_{Na} = sodium current; RMP = resting membrane potential; other abbreviations as in Figure 2.

levels of CAR predispose to VF. The GWAS for ischemia-induced VF, which identified the chr21q21 risk locus, is thus far the only study of this specific clinical presentation. Genetic studies of this phenotype are hindered by the unavailability of large patient sets; indeed, a study by Bugert et al. (19) in a small case-control set did

not detect the association of rs2824292 with VF. Thus, although the effect of the variant/haplotype that we detected on *CXADR* expression is in line with our previous GWAS findings, the robustness of this signal must be further validated in future association studies in larger patient sets.



Mechanism(s) of ventricular conduction slowing and arrhythmia vulnerability in CAR^{+/-} mice. Conduction slowing in CAR^{+/-} hearts may stem from altered cardiomyocyte excitability (i.e., sodium channel function), cell-to-cell coupling, tissue architecture, and combinations thereof. We found no evidence for myocardial hypertrophy or fibrosis in CAR^{+/-} hearts, making electrical remodeling secondary to structural abnormalities unlikely. Nav1.5 protein levels were unchanged in CAR^{+/-} hearts, but decreased AP upstroke velocity was observed in CAR^{+/-} myocytes. Moreover, prolongation of electrocardiographic conduction indexes after administration of flecainide was more pronounced in CAR^{+/-} mice, further indicating the functional relevance of the reduced sodium channel availability secondary to CAR haploinsufficiency. In cardiac-specific inducible CAR knockout mice (i.e., post-natal CAR knockout mice), Cx43 protein levels in ventricular myocardium were reduced by approximately 40% (9). Although we did not detect a significant reduction of Cx43 expression in CAR^{+/-} hearts, this does not preclude the possibility that subtle alterations in Cx43 expression and/or function may be of relevance for conduction, especially during perturbed conditions such as ischemia, as discussed in the following text.

Decreased membrane excitability and electrical uncoupling are key mechanisms for conduction slowing and conduction block during ischemia and provide a proarrhythmic substrate for the development of re-entrant ventricular

arrhythmias (20,21). The ventricular conduction slowing in CAR^{+/-} hearts likely exacerbates conduction slowing during ischemia, thereby contributing to arrhythmogenesis in this setting. The degree of conduction slowing observed at baseline in CAR^{+/-} hearts was modest and not sufficient to facilitate spontaneous or induced ventricular arrhythmias. Thus, for (re-entrant) arrhythmias to occur, additional electrical and/or structural perturbations appear to be necessary, such as intercellular uncoupling or ischemia. Infusion of carbenoxolone, a gap junction uncoupler that does not affect AP characteristics or underlying ion currents (13), further decreased conduction velocity in both the longitudinal and the transversal direction and increased arrhythmia susceptibility in CAR^{+/-} but not in WT hearts. Of note, carbenoxolone also decreased conduction velocity in WT hearts, and although the magnitude of the effects of carbenoxolone was similar in both WT and CAR^{+/-} hearts, the absolute values of both CVL and CVT were lowest in CAR^{+/-} hearts after treatment with carbenoxolone. The concomitant reduction in sodium channel availability in CAR^{+/-} hearts (but not in WT hearts) makes these hearts more susceptible to arrhythmias in the presence of the uncoupler carbenoxolone. Interestingly, an earlier onset of ventricular arrhythmias during ex vivo regional ischemia was also observed in hearts from Cx43 haploinsufficient mice (22), similar to our current observation in CAR^{+/-} hearts. Because infusion of carbenoxolone is arrhythmogenic in CAR^{+/-} mice, subtle alterations in gap junction proteins (undetected in our study) may contribute to the observed ischemia-induced proarrhythmia in CAR^{+/-} hearts. On the other hand, arrhythmias in the early phase of ischemia are predominantly associated with conduction delay (which can facilitate re-entry) secondary to decreased membrane excitability in depolarized tissue. Thus, reduced sodium channel availability secondary to decreased I_{Na} (as shown in CAR^{+/-} myocytes) may also contribute to increased arrhythmia susceptibility.

Several animal models have been developed to study CAR. Of these, the complete (germline) constitutive knockout results in early embryonic lethality characterized by severe cardiac morphological defects (23,24), cardiac-specific knockout mice develop cardiomyopathy and/or atrioventricular conduction disturbance (10), and the cardiac-specific inducible knockout develops progressive atrioventricular block (9). We chose to investigate the effects of CAR in heterozygous knockout mice to assess the impact of a less drastic reduction in CAR in the absence of remodeling, which may better reflect the variability in CAR levels occurring in human health and disease. Our observation that CAR haploinsufficiency results in ventricular conduction slowing appears to be in contrast to findings in cardiac-specific knockout hearts, in which atrioventricular conduction was abnormal but ventricular conduction velocities remained unaffected (10). However, this discrepancy may be attributed to differences in measuring techniques, targeting strategies, or genetic backgrounds (25).

CAR affects cardiac sodium channels at the intercalated disk. CAR is a cell adhesion molecule with multiple binding partners, including the scaffolding protein zonula occludens 1, which in turn interacts with Cx43 (8,26). Sodium channels interact with Cx43 and are regulated by cell adhesion molecules, including the sodium channel β -subunit and ankyrin-G (27,28). Thus, CAR may exert its effects on sodium channel availability by both direct and indirect mechanisms, which are likely not mutually exclusive. Crucially, I_{Na} was reduced at the intercalated disk of CAR^{+/-} myocytes, whereas I_{Na} at the lateral myocyte membrane was unaffected. Because sodium channels at the intercalated disk are considered essential for proper conduction (15), a decrease in I_{Na} in this region is likely to impair function. Indeed, our findings show that a relatively modest reduction in I_{Na} at the intercalated disk is sufficient for sodium channel availability to be decreased. Moreover, we found that CAR interacts with Nav1.5 in vitro, suggesting that CAR affects sodium channel function through a physical interaction with Nav1.5. The observation that CAR affects I_{Na} specifically at the intercalated disk is in line with the emerging concept that cross talk exists between structural and electrical components in the intercalated disk region (29–31).

Study limitations. Extrapolation from studies with animal models to humans should be done with caution. We have shown increased arrhythmia inducibility in CAR^{+/-} hearts after carbenoxolone and earlier onset of arrhythmia inducibility in CAR^{+/-} hearts after LAD ligation but did not observe spontaneous arrhythmias in these settings. Arrhythmia-inducing triggers in patients with ischemia-induced arrhythmias may not be present in our model and vice versa, and patients may differentially compensate CAR deficiency.

Despite the observed CAR-dependent differences in AP upstroke velocity and I_{Na} at the intercalated disk, we did not detect changes in Nav1.5 protein. Nevertheless, small yet functionally relevant differences in amount and/or localization of Nav1.5 may be present, albeit below the detection limit of our Western blot and immunohistochemistry assays.

Conclusions

Our expression studies in the human heart, combined with electrophysiological studies in CAR^{+/-} mice, establish a novel role for CAR in mediating ventricular conduction and arrhythmogenesis during conditions such as gap junctional uncoupling or myocardial ischemia. CAR may have similar modulatory effects on cardiac electrical activity in other pathophysiological conditions, including dilated cardiomyopathy and myocarditis, which require further investigation. The regulatory effects of CAR on sodium channel availability and cardiac conduction provide additional insight into the complex mechanisms underlying arrhythmogenesis during myocardial ischemia. Furthermore, genetic determinants of arrhythmia susceptibility during myocardial ischemia

(such as CAR) may constitute future targets for risk stratification and prevention of potentially lethal ventricular arrhythmias in patients with coronary artery disease.

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REFERENCES

1. Rubart M, Zipes DP. Mechanisms of sudden cardiac death. *J Clin Invest* 2005;115:2305–15.
2. Dekker LRC, Bezzina CR, Henriques JPS, et al. Familial sudden death is an important risk factor for primary ventricular fibrillation: a case-control study in acute myocardial infarction patients. *Circulation* 2006;114:1140–5.
3. Kaikkonen KS, Kortelainen M-L, Linna E, Huikuri HV. Family history and the risk of sudden cardiac death as a manifestation of an acute coronary event. *Circulation* 2006;114:1462–7.
4. Bezzina CR, Pazoki R, Bardai A, et al. Genome-wide association study identifies a susceptibility locus at 21q21 for ventricular fibrillation in acute myocardial infarction. *Nat Genet* 2010;42:688–91.
5. Winkler GS. The mammalian anti-proliferative BTG/Tob protein family. *J Cell Physiol* 2010;222:66–72.
6. Noutsias M, Fechner H, de Jonge H, et al. Human coxsackie-adenovirus receptor is colocalized with integrins $\alpha\beta 3$ and $\alpha\beta 5$ on the cardiomyocyte sarcolemma and upregulated in dilated cardiomyopathy: implications for cardiotropic viral infections. *Circulation* 2001;104:275–80.
7. Shaw CA, Holland PC, Sinnreich M, et al. Isoform-specific expression of the Coxsackie and adenovirus receptor (CAR) in neuromuscular junction and cardiac intercalated discs. *BMC Cell Biol* 2004;5:42.
8. Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A* 2001;98:15191–6.
9. Lisewski U, Shi Y, Wrackmeyer U, et al. The tight junction protein CAR regulates cardiac conduction and cell-cell communication. *J Exp Med* 2008;205:2369–79.
10. Lim B, Xiong D, Dorner A, et al. Coxsackievirus and adenovirus receptor (CAR) mediates atrioventricular-node function and connexin 45 localization in the murine heart. *J Clin Invest* 2008;118:2758–70.
11. Fechner H, Noutsias M, Tschoepe C, et al. Induction of coxsackievirus-adenovirus-receptor expression during myocardial tissue formation and remodeling: identification of a cell-to-cell contact-dependent regulatory mechanism. *Circulation* 2003;107:876–82.
12. Shi Y, Chen C, Lisewski U, et al. Cardiac deletion of the Coxsackievirus-adenovirus receptor abolishes Coxsackievirus B3

- infection and prevents myocarditis in vivo. *J Am Coll Cardiol* 2009;53:1219–26.
13. De Groot JR, Veenstra T, Verkerk AO, et al. Conduction slowing by the gap junctional uncoupler carbenoxolone. *Cardiovasc Res* 2003;60:288–97.
 14. Berecki G, Wilders R, De Jonge B, Van Ginneken ACG, Verkerk AO. Re-evaluation of the action potential upstroke velocity as a measure of the Na⁺ current in cardiac myocytes at physiological conditions. *PLoS One* 2010;5:e15772.
 15. Kucera JP, Rohr S, Rudy Y. Localization of sodium channels in intercalated disks modulates cardiac conduction. *Circ Res* 2002;91:1176–82.
 16. Petitprez S, Zmoos A-F, Ogrodnik J, et al. SAP97 and dystrophin macromolecular complexes determine two pools of cardiac sodium channels Nav1.5 in cardiomyocytes. *Circ Res* 2011;108:294–304.
 17. Verkerk AO, Van Ginneken ACG, Van Veen TAB, Tan HL. Effects of heart failure on brain-type Na⁺ channels in rabbit ventricular myocytes. *Europace* 2007;9:571–7.
 18. Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M. Mapping complex disease traits with global gene expression. *Nat Rev Genet* 2009;10:184–94.
 19. Bugert P, Elmas E, Stach K, et al. No evidence for an association between the rs2824292 variant at chromosome 21q21 and ventricular fibrillation during acute myocardial infarction in a German population. *Clin Chem Lab Med* 2011;49:1237–9.
 20. Janse MJ, Wit AL. Electrophysiological mechanisms of ventricular arrhythmias resulting from myocardial ischemia and infarction. *Physiol Rev* 1989;69:1049–69.
 21. Coronel R, Wilms-Schopman FJ, Opthof T, Van Capelle FJ, Janse MJ. Injury current and gradients of diastolic stimulation threshold, TQ potential, and extracellular potassium concentration during acute regional ischemia in the isolated perfused pig heart. *Circ Res* 1991;68:1241–9.
 22. Lerner DL, Yamada KA, Schuessler RB, Saffitz JE. Accelerated onset and increased incidence of ventricular arrhythmias induced by ischemia in Cx43-deficient mice. *Circulation* 2000;101:547–52.
 23. Dorner AA, Wegmann F, Butz S, et al. Coxsackievirus-adenovirus receptor (CAR) is essential for early embryonic cardiac development. *J Cell Sci* 2005;118:3509–21.
 24. Chen J-W, Zhou B, Yu Q-C, et al. Cardiomyocyte-specific deletion of the coxsackievirus and adenovirus receptor results in hyperplasia of the embryonic left ventricle and abnormalities of sinuatrial valves. *Circ Res* 2006;98:923–30.
 25. Fischer R, Poller W, Schultheiss H-P, Gotthardt M. CAR-diology—a virus receptor in the healthy and diseased heart. *J Mol Med (Berl)* 2009;87:879–84.
 26. Giepmans BN, Moolenaar WH. The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. *Curr Biol* 1998;8:931–4.
 27. Malhotra JD, Thyagarajan V, Chen C, Isom LL. Tyrosine-phosphorylated and nonphosphorylated sodium channel beta1 subunits are differentially localized in cardiac myocytes. *J Biol Chem* 2004;279:40748–54.
 28. Mohler PJ, Rivolta I, Napolitano C, et al. Nav1.5 E1053K mutation causing Brugada syndrome blocks binding to ankyrin-G and expression of Nav1.5 on the surface of cardiomyocytes. *Proc Natl Acad Sci U S A* 2004;101:17533–8.
 29. Sato PY, Musa H, Coombs W, et al. Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes. *Circ Res* 2009;105:523–6.
 30. Sato PY, Coombs W, Lin X, et al. Interactions between ankyrin-G, Plakophilin-2, and Connexin43 at the cardiac intercalated disc. *Circ Res* 2011;109:193–201.
 31. Rizzo S, Lodder EM, Verkerk AO, et al. Intercalated disc abnormalities, reduced Na⁺ current density, and conduction slowing in desmoglein-2 mutant mice prior to cardiomyopathic changes. *Cardiovasc Res* 2012;95:409–18.

Key Words: arrhythmia ■ ion channels ■ ischemia ■ single nucleotide polymorphism genetics ■ ventricular fibrillation.

 **APPENDIX**

For the supplemental appendix, figures, and tables, please see the online version of this article.