Phosphodiesterase 4B in the cardiac L-type Ca\(^{2+}\) channel complex regulates Ca\(^{2+}\) current and protects against ventricular arrhythmias in mice

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Introduction

During the cardiac action potential, Ca\textsuperscript{2+} influx through sarcoplasmic L-type Ca\textsuperscript{2+} channels (LTCCs) triggers Ca\textsuperscript{2+} release from juxtaposed ryanodine receptor 2 (RyR2) located in the sarcoplasmic reticulum (SR). This allows a rapid and synchronous Ca\textsuperscript{2+} elevation throughout the cell, which activates contraction. During cardiac relaxation, Ca\textsuperscript{2+} is rapidly extruded by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and re-sequestered into the SR by the Ca\textsuperscript{2+}-ATPase, SERCA2A (1). This process is highly regulated, in particular, by the sympathetic nervous system. β-Adrenergic receptors (β-ARs) exert strong inotropic and lusitropic effects by increasing intracellular cAMP levels and activating PKA. PKA increases Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release via phosphorylation of L-type Ca\textsuperscript{2+} channels (LTCCs) and ryanodine receptor 2. Multiple cyclic nucleotide phosphodiesterases (PDEs) regulate local cAMP concentration in cardiomyocytes, with PDE4 being predominant for the control of β-AR-dependent cAMP signals. Three genes encoding PDE4 are expressed in mouse heart: Pde4a, Pde4b, and Pde4d. Here we show that both PDE4B and PDE4D are tethered to the LTCC in the mouse heart but that β-AR stimulation of the L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}) is increased only in Pde4b\textsuperscript{−/−} mice. A fraction of PDE4B colocalized with the LTCC along T-tubules in the mouse heart. Under β-AR stimulation, Ca\textsuperscript{2+} transients, cell contraction, and spontaneous Ca\textsuperscript{2+} release were increased in Pde4b\textsuperscript{−/−} and Pde4d\textsuperscript{−/−} myocytes compared with those in WT myocytes. In vivo, after intraperitoneal injection of isoprorenaline, catheter-mediated burst pacing triggered ventricular tachycardia in Pde4b\textsuperscript{−/−} mice but not in WT mice. These results identify PDE4B in the Ca\textsubscript{1.2} complex as a critical regulator of I\textsubscript{Ca,L} during β-AR stimulation and suggest that distinct PDE4 subtypes are important for normal regulation of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in cardiomyocytes.

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Association note: Jérôme Leroy and Wito Richter contributed equally to this work.

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and PDE4D are expressed in cardiac tissue (21). These 3 genes give rise to multiple isoforms generated through alternative splicing and by the use of different promoters (22). So far, of these genes, most of our knowledge in the heart concerns the PDE4D gene. Long variants of this gene expressed in the heart include the isoforms PDE4D3, PDE4D5, PDE4D8, and PDE4D9 (23, 24). PDE4D5, PDE4D8, and PDE4D9 were shown to differentially interact with β1-AR and β2-AR subtypes in neonatal cardiomyocytes, either directly or by binding to β-arrestin (25–27). The PDE4D3 isoform was found in macromolecular signaling complexes, regulating 2 major players of ECC: KCNQ1/KCNE1 potassium channels (28) and RyR2 (29). In Pde4d–/– mice, RyR2 is hyperphosphorylated by β2-adrenergic receptor (β2-AR) stimulation of cardiac function. PDE4B, together with an increase in cell contraction and Ca2+ transients. In vivo, upon β-AR stimulation, catheter-mediated burst pacing triggered ventricular tachycardia (VT) in Pde4b–/– mice but not in WT mice. We conclude that not only PDE4D, but also PDE4B, plays a key role during β-AR stimulation of cardiac function. PDE4B, by limiting the amount of Ca2+ that enters the cell via the LTCCs, prevents Ca2+ overload and arrhythmias.

Results

β-AR stimulation of Ica,L and effect of PDE4 inhibition in adult mouse ventricular myocytes. We previously showed that the β-AR stimulation of Ica,L is under the control of PDE4 in adult rat ventricular myocytes (18). To determine whether the same was true in mice, Ica,L was recorded in adult mouse ventricular myocytes (AMVMs) using the whole-cell patch-clamp technique, and the response of this current to a short application of the nonselective β-AR agonist isoproterenol (Iso) was studied. As shown in Figure 1A, a 15-second pulse of 100 nM Iso elicited a rapid and transient increase of Ica,L amplitude that returned to baseline in less than 10 minutes. Selective PDE4 inhibition with Ro 20-1724 (10 μM) 2 minutes prior to the 15-second pulse of Iso (100 nM, 15 seconds) application alone (N = 62 myocytes) or when PDE4 was inhibited by Ro 20-1724 (gray squares; N = 37 myocytes). All curves represent the mean ± SEM. *P < 0.05 indicates the first of the statistically significant points on the graph.

Figure 1

PDE4 modulates β-AR stimulation of Ica,L in mouse ventricular myocytes. (A) Typical time course of Ica,L amplitude in a cell stimulated by a 15-second pulse of Iso (100 nM). Each square represents the amplitude of Ica,L recorded every 8 seconds during a depolarization from –50 to 0 mV. (B) Ica,L was further increased when the cell was preincubated with the PDE4 inhibitor Ro 20-1724 (Ro; 10 μM) 2 minutes prior to the 15-second pulse of Iso (100 nM) and maintained throughout the experiment. The individual current traces shown on top of A and B were recorded at the times indicated by the corresponding roman numerals in the graphs below. (C) Mean variation of Ica,L after Iso (100 nM, 15 seconds) application alone (black diamonds; N = 62 myocytes) or when PDE4 was inhibited by Ro 20-1724 (gray squares; N = 37 myocytes). We believe that until now, the molecular identity of the PDE4 gene. Long variants of this gene include the isoforms PDE4A and PDE4B, and found that PDE4B, but not PDE4A, was also associated with the LTCC. In Pde4b–/– mice, the β-AR response of Ica,L was increased, together with an increase in cell contraction and Ca2+ transients. In vivo, upon β-AR stimulation, catheter-mediated burst pacing triggered ventricular tachycardia (VT) in Pde4b–/– mice but not in WT mice. We conclude that not only PDE4D, but also PDE4B, plays a key role during β-AR stimulation of cardiac function. PDE4B, by limiting the amount of Ca2+ that enters the cell via the LTCCs, prevents Ca2+ overload and arrhythmias.

Characterization of PDE4 subtypes in mouse heart. To evaluate the contribution of PDE4 to cAMP hydrolysis in the mouse heart, total cAMP hydrolytic activity was measured in ventricle homogenates, and the fraction corresponding to PDE4 was assessed with the specific inhibitor rolipram. As shown in Figure 2A, PDE4 represented about 33% of the total activity. To further dissect the contributions of single PDE4 subtypes to the total PDE4 activity, heart extracts were immunoprecipitated using subtype-specific antibodies.
against PDE4A, PDE4B, and PDE4D (23), and the PDE activity recovered in the IP pellets was assayed. As shown in Figure 2B, the activities of PDE4A, PDE4B, and PDE4D were comparable in the mouse heart. Western blot experiments indicated that each of these activities results from the expression of long PDE4 variants detected as one major immunoreactive band. The absence of signal in mice deficient for the corresponding gene demonstrates that the antibodies used are specific (Figure 2C).

**PDE4B and PDE4D are part of the L-type calcium channel complex.** To determine whether any of these PDE4 associate with the LTCC, we performed IP experiments using mouse heart extracts. The pore-forming subunits of the channel, Ca$\text{V}_{1.2}$, were immunoprecipitated with an anti-Ca$\text{V}_{1.2}$ antibody (31). A cAMP-hydrolyzing PDE4 activity was associated with the IP Ca$^{2+}$ channel subunit from WT cardiac tissue and was unaffected in heart extracts from Pde4a$^{-/-}$ mice. In contrast, this PDE4 activity was significantly decreased in ventricular tissues from Pde4b$^{-/-}$ and Pde4d$^{-/-}$ mice (Figure 3A). This suggests that PDE4B and PDE4D are part of a macromolecular complex that includes the LTCC, a finding confirmed by Western blot analysis of Ca$\text{V}_{1.2}$ IP pellets. Using specific isoform antibodies, both PDE4B (Figure 3B) and PDE4D (Figure 3C) were detected in the cardiac LTCC complex. These interactions were specific, because both enzymes were excluded from IPs using control IgGs (Figure 3, B and C). Furthermore, PDE4B could not be detected in the Ca$\text{V}_{1.2}$ complex in hearts from Pde4b$^{-/-}$ mice, and the same was true for PDE4D when the coimmunoprecipitation experiment was performed in hearts from Pde4d$^{-/-}$ mice. This demonstrates that both PDE4B and PDE4D isoforms associate with Ca$\text{V}_{1.2}$ in the heart and are part of a macromolecular signaling complex, including the cardiac LTCC.

**Figure 2** Characterization of PDE4 activity and subtypes in the adult mouse heart. (A) Heart protein extracts were assayed using 1 $\mu$M cAMP as substrate. PDE4 activity is defined as the fraction of total PDE activity inhibited by the specific PDE4 inhibitor rolipram (10 $\mu$M). (B) Cardiac extracts were immunoprecipitated with control IgG or using antibodies specific for PDE4A, PDE4B, or PDE4D. The graph shows the PDE activity in the IP pellet using 1 $\mu$M cAMP as substrate. Data represent the mean ± SEM of experiments performed at least 3 times. (C) Heart protein extracts from WT mice, Pde4a$^{-/-}$ mice, and Pde4d$^{-/-}$ mice were separated on SDS/PAGE and revealed with the corresponding PDE4 subtype-specific antibodies. In the case of PDE4D, cardiac extracts from WT and Pde4d$^{-/-}$ were first immunoprecipitated with the specific PDE4D antibody and then revealed by Western blot.

**Figure 3** PDE4B and PDE4D are part of the LTCC complex. (A) Ca$\text{V}_{1.2}$ was immunoprecipitated from cardiac homogenates of WT mice, Pde4a$^{-/-}$, Pde4b$^{-/-}$, and Pde4d$^{-/-}$ mice. The associated PDE4 activity was assessed using rolipram. **$P < 0.01$. (B) PDE4B was detected in cardiac lysates from WT animals and coimmunoprecipitated with Ca$\text{V}_{1.2}$. (C) PDE4D was also detected in cardiac lysates from WT animals and was associated with Ca$\text{V}_{1.2}$. IP IgG, negative control. Data are representative of at least 3 independent experiments.
β-AR stimulation of I_{Ca,L} and effect of PDE4 inhibition in PDE4-deficient mice. To assess the respective contribution of PDE4A, PDE4B, and PDE4D in the β-AR regulation of I_{Ca,L}, patch-clamp experiments were performed in AMVMs isolated from mice deficient in the corresponding genes (Pde4a−/−, Pde4b−/−, and Pde4d−/− mice). Cell capacitance and I_{Ca,L} amplitude at baseline were similar in ventricular myocytes from each genotype. As shown in Figure 4A, I_{Ca,L} potentiation by Iso (100 nM, 15 seconds) was unchanged in Pde4a−/− compared to that in WT AMVMs. Unexpectedly, the same was true for myocytes isolated from Pde4d−/− mice (Figure 4C). In line with these results, the PDE4 inhibitor Ro 20-1724 (10 μM) strongly enhanced the effect of Iso on I_{Ca,L} in Pde4a−/− and Pde4d−/− mice (Figure 4D, E, and F), and this effect was not different from that obtained in WT mice (compare Figure 4, D and F, with Figure 1C), suggesting that these isoforms do not modulate the β-AR stimulation of I_{Ca,L}. In contrast, the response of I_{Ca,L} to a transient β-AR stimulation was significantly enhanced in ventricular myocytes isolated from Pde4b−/− mice compared with that from WT mice (Figure 4B, maximal I_{Ca,L} potentiation by Iso was 78.3% ± 3.6% in Pde4b−/− mice, n = 46; P < 0.001 vs. WT). Moreover, the effect of Ro 20-1724 was severely blunted in Pde4b−/− myocytes (Figure 4E). These findings indicate that PDE4B is the predominant PDE4 isoform involved in I_{Ca,L} regulation by short β-AR stimulation in mice. However, since the effect of Ro 20-1724 was not completely abolished in Pde4b−/− mice, another PDE4 isoform must be involved in the β-AR regulation of I_{Ca,L} at least when PDE4B is absent. This is most likely PDE4D, since this isoform was also found to coimmunoprecipitate with the pore-forming subunit of Cav1.2 (Figure 3, A and C). The biophysical properties of I_{Ca,L} were compared in WT and Pde4b−/− myocytes. As shown in Figure 5A, the current-voltage relationship revealed a similar I_{Ca,L} density measured in WT and Pde4b−/− myocytes under basal conditions. I_{Ca,L} exhibited similar voltage dependence in both genotypes: the half-time for activation and inactivation remained unchanged (Figure 5B). When measured at 0 mV, I_{Ca,L} amplitude, upon continuous application of Iso at a submaximal concentration (3 nM), was significantly increased in WT AMVMs, from 5.9 ± 0.4 pA/pF to 7.3 ± 0.6 pA/pF (P < 0.05), but the β-AR stimulation induced a greater increase of I_{Ca,L} amplitude in Pde4b−/− myocytes than in WT myocytes (Figure 5C). This increase was accompanied by a pronounced leftward shift of the activation curve of I_{Ca,L} in Pde4b−/− myocytes, whereas steady-state inactivation was not modified (Figure 5D). As a consequence, under β-AR stimulation, the "window" current between −30 mV and 0 mV was enhanced in myocytes lacking PDE4B.

Colocalization of PDE4B and Cav1.2 channels at the Z-line in AMVMs. We next determined the subcellular localization of PDE4B in AMVMs and examined whether PDE4B and Cav1.2 colocalize in vivo by using the double-labeling immunofluorescence technique. In agreement with previous studies (7, 32), the staining for Cav1.2 obtained with the anti-Cav1.2 antibody was observed as a regularly spaced, punctuated pattern, which overlapped with the Z-line marker α-actinin (Figure 6A). Immunolabeling of PDE4B with an antibody that specifically recognizes this isoform (Figure 2C) revealed a strong fluorescent signal throughout the cell and a tight, striated pattern, with the main intensity stripe overlapping with α-actinin (Figure 6B). In AMVMs isolated from Pde4b−/− hearts, the overall staining intensity for PDE4B was reduced, and, notably, the striation that had overlapped with α-actinin in WT
Iso (100 nM, 15 seconds) strongly increased SL shortening and amplitude was increased by about 25% and 28% more in isolated myocytes at the peak of Iso stimulation was increased by about 50% in WT [n = 47]. In Pde4b−/− and Pde4d−/− myocytes (n = 47 each) relaxation was further accelerated (t1/2off) values were decreased by about 81% and 78% of basal value, respectively; P < 0.001 vs. WT), whereas decay kinetics of the Ca2+ transients in the presence of Iso were not significantly different from those of WT myocytes (Figure 7C). However, the decay phase of Ca2+ transients tended to be accelerated in Pde4b−/− myocytes compared with WT myocytes (P = 0.059), and the difference was significant compared with Pde4d−/− myocytes (P < 0.01). As shown in Figure 7, D and E, Pde4b−/− and Pde4d−/− myocytes also displayed increased spontaneous Ca2+ release (SCR) events upon Iso stimulation. On average, during a 20-second period after the maximal effect of Iso stimulation, 4.5 ± 1.1 SCR events occurred in Pde4b−/− myocytes (n = 47), and 4.5 ± 0.9 SCR events occurred in Pde4d−/− myocytes (n = 47), whereas in WT cells, only 1.4 ± 0.5 SCR events were observed (n = 51; P < 0.01). These results show that both PDE4B and PDE4D are required for normal regulation of ECC during β-AR stimulation. To further examine the consequences of PDE4B and PDE4D ablation on SR function, we measured SR Ca2+ load by fast application of 10 mM caffeine in basal conditions and at the peak of Iso (100 nM, 15 seconds) stimulation. As shown in the Supplemental Figure 1A, C, and B (supplemental material available online with this article; doi:10.1172/JCI44747DS1), SR Ca2+ load was not significantly different in the 3 genotypes. However, there was a tendency for a decreased SR Ca2+ load in Pde4d−/− myocytes in basal conditions (−20%). As a consequence, the percentage increase in SR Ca2+ load induced by Iso was significantly higher in Pde4d−/− myocytes versus WT myocytes (P < 0.05, n = 18 each; Supplemental Figure 1C). We thus examined PLB phosphorylation at Ser-16 (PKA site) in isolated myocytes from the 3 genotypes. PLB phosphorylation was not different in the 2 groups under basal conditions. After Iso challenge (100 nM, 15 seconds), PLB phosphorylation was significantly increased in Pde4d−/− myocytes compared with that in WT myocytes, and a nonsignificant increase was observed in Pde4b−/− myocytes (Supplemental Figure 1D).

**ECG, intracardiac recording, and pacing.** The arrhythmic behavior of Pde4b−/− myocytes and the increased susceptibility of Pde4d−/− mice to exercise-induced cardiac arrhythmias (29) prompted us to evaluate the propensity to ventricular arrhythmias in Pde4b−/− mice. Six-lead ECGs were recorded in 10 WT and 11 Pde4b−/− mice (mean age of 17 ± 1 weeks in both groups) in baseline conditions. No significant differences were observed in RR interval (WT, 128 ± 7 ms; Pde4b−/−, 114 ± 7 ms), P wave (WT, 14 ± 1 ms; Pde4b−/−, 11 ± 1 ms), and QRS duration (WT, 24 ± 3 ms; Pde4b−/−, 24 ± 4 ms). The QRS interval was significantly increased in WT mice compared with Pde4b−/− mice (P < 0.01). The QRS duration was significantly increased in Pde4b−/− mice compared with WT mice (P < 0.01). The QRS interval was significantly increased in Pde4b−/− mice compared with WT mice (P < 0.01).

**Figure 5**
Pde4b ablation increases LTCC window current upon β-AR stimulation. (A) Mean I_{Ca,L} density elicited by depolarizing steps from a holding potential of −50 mV, using a double-pulse protocol (inset), was similar in WT (n = 15 cells) and Pde4b−/− (n = 16 cells) AMVMs under control conditions. (B) Combined activation and steady-state inactivation curves under control conditions illustrate window current of I_{Ca,L} (half-time for activation [V1/2, act] = −6.1 ± 0.9 mV, k = 5.4 ± 0.1 mV in Pde4b−/− AMVMs and V1/2, act = −5.6 ± 0.7 mV, k = 5.4 ± 0.2 mV in WT [NS]; half-time for inactivation [V1/2, inact] = −21.7 ± 0.8 mV, k = 7.1 ± 0.3 mV in Pde4b−/− AMVMs and −21.9 ± 0.9 mV, k = 6.4 ± 0.3 mV in WT [NS]). (C) I_{Ca,L} density recorded during depolarizing pulses from −50 mV was further potentiated in Pde4b−/− (n = 14) versus WT (n = 16) AMVMs upon sustained β-AR stimulation (3 nM Iso). (D) Increased I_{Ca,L} window current upon β-AR stimulation in Pde4b−/− AMVMs. Activation curve of I_{Ca,L} was more hyperpolarized upon Iso (3 nM) application in Pde4b−/− AMVMs (V1/2, act = −12.2 ± 1.2 mV, k = 5.3 ± 0.2 mV) than in WT AMVMs (V1/2, act = −7.5 ± 1.3 mV, k = 5.4 ± 0.2 mV, P < 0.05); inactivation was not affected by β-AR stimulation in either genotype (V1/2, inact = −21.7 ± 0.4 mV, k = 6.2 ± 0.4 mV in Pde4b−/− and V1/2, inact = −21.3 ± 0.7 mV, k = 6.3 ± 0.2 mV in WT).
ms), PQ interval (WT, 39 ± 1 ms; Pde4b−/−, 37 ± 1 ms), QRS complex (WT, 12 ± 1 ms; Pde4b−/−, 11 ± 1 ms), and QTc interval (WT, 47 ± 2 ms; Pde4b−/−, 50 ± 3 ms) durations. These mice were then used for catheter-mediated ventricular pacing in baseline conditions and after intraperitoneal injections of Iso (0.02 mg/kg and 0.2 mg/kg). Two mice (1 per group) died during the experimental procedure (one each per group). Two mice at 0.02 mg/kg Iso responded to baseline conditions and were excluded from the statistical analysis. In baseline conditions, intracardiac ECG recordings in WT and Pde4b−/− mice were similar. Pde4b− inactivation had no significant effect on the ventricular effective refractory period (VERP) measured at a basic cycle length of 75 ms (WT, 44 ± 2 ms; Pde4b−/−, 40 ± 2 ms; NS). Iso decreased spontaneous RR interval from 118 ± 5 ms in baseline conditions to 93 ± 4 ms and 90 ± 1 ms, respectively, in WT mice (P < 0.001 vs. baseline for both doses) and from 109 ± 5 ms to 88 ± 2 ms and 87 ± 1 ms, respectively, in Pde4b−/− mice (P < 0.001 vs. baseline for both doses; P = NS vs. WT). VERP was reduced to 38 ± 3 ms and 34 ± 3 ms after injection of Iso at, respectively, 0.02 mg/kg and 0.2 mg/kg Iso, respectively, in WT mice (P < 0.001 vs. baseline for both doses) and from 109 ± 5 ms to 88 ± 2 ms and 87 ± 1 ms, respectively, in Pde4b−/− mice (P < 0.001 vs. baseline for both doses; P = NS vs. WT). Other ECG parameters were similar in the 2 groups (data not shown). In baseline conditions, burst pacing did not induce arrhythmias either in WT or in Pde4b−/− mice. However, after pre-treatment with Iso, Pde4b−/− mice responded differently from WT mice. Indeed, burst pacing induced arrhythmias in Pde4b−/− mice in 1 out of 10 mice at 0.02 mg/kg Iso (P = NS vs. WT) and 4 out of 10 mice at 0.2 mg/kg Iso (P < 0.05 vs. WT; Figure 8, A and B) but not in WT mice. VT was induced in the 4 responding Pde4b−/− mice. Two of them had sustained VT. Thus, after treatment with Iso, Pde4b gene inactivation favored the occurrence of arrhythmias induced by burst pacing. In contrast, paced extrasystoles induced arrhythmias in both WT and Pde4b−/− mice (Figure 8C), with a moderately, though not significantly, higher incidence in Pde4b−/− mice.

**Discussion**

PDE4 is the predominant PDE family that controls cAMP and LTCC in rodent cardiomyocytes. In the present study, we show that Pde4a, Pde4b, and Pde4d are expressed in mouse heart, but only PDE4B and PDE4D are associated with Cav1.2 channels. Whereas β-AR stimulation of I_{Ca,L} is normal in Pde4a−/− and Pde4d−/− myocytes, the β-AR responses of I_{Ca,L}, Ca2+ transients, and contraction are enhanced in myocytes from Pde4b−/− mice, and this is accompanied by an increased propensity for arrhythmia. β-AR stimulation of Ca2+ transients and contraction are also enhanced in myocytes from Pde4d−/− mice independently of I_{Ca,L}. For the first time to our knowledge, our results identify PDE4B as a major regulator of I_{Ca,L} and cardiac function and suggest that PDE4B and PDE4D regulate ECC by different mechanisms.

In mouse ventricular myocytes, pharmacological inhibition of PDE4 does not affect basal I_{Ca,L} but strongly enhances the effect of β-AR stimulation (Figure 1). A similar regulation of I_{Ca,L} by PDE4 was found previously in human atrial and rat ventricular myocytes, thus clearly indicating that PDE4 is a major negative regulator of I_{Ca,L} in the heart (14, 20, 33). Similar to what was reported earlier in the rat (34), PDE4 activity in the mouse ventricle was due to the expression of PDE4A, PDE4B, and PDE4D variants. The migra-
tion on SDS/PAGE of these variants corresponds to long forms, and although only one immunoreactive band was detected in each case, this band may correspond to the comigration of several long forms, as demonstrated earlier for PDE4D (23, 24, 29).

Specific variants of PDE4D have been shown to be associated with cardiac ion channels. Indeed, PDE4D3 was found to be coupled to RyR2 (29) and to be included in a macromolecular complex together with the KCNQ1 subunit of a potassium channel regulated by cAMP (28). Here, we found that PDE4D is also associated with the CaV1.2 subunit of the LTCC but, surprisingly, does not seem to regulate the channel activity, at least under normal conditions, because inactivation of the Pde4d gene modified neither ICa,L nor its β-AR regulation. Since PDE4B was also found in the complex that includes the CaV1.2 subunit, and β-AR stimulation of ICa,L is upregulated in Pde4b−/− mice, it is possible that PDE4B is more tightly coupled with the LTCC, hence controlling its phosphorylation, while PDE4D is more remote. This hypothesis is supported by the observation that the PDE4 inhibitor Ro 20-1724 had a small but significant effect on the β-AR response of ICa,L in Pde4b−/− mice (Figure 4E), as if the effect of PDE4D were unmasked in the absence of PDE4B. PDE4D isoforms have been shown previously to interact with β-ARs (25–27). Since β-ARs are part of a signalosome, including the LTCCs (35), it is conceivable that the 2 PDE4 isoforms that immunoprecipitated with CaV1.2 are in fact located at different ends of the signalosome, with PDE4D associated with the β-AR and PDE4B associated with the LTCC. Alternatively, PDE4D and PDE4B may control different pools of CaV1.2 (36), the latter being responsible for Iso-dependent ICa,L regulation.

Since LTCCs are predominantly expressed in the T-tubules (7, 32), PDE4B should be located in the same compartment. Consistent with this idea, immunocytochemical experiments with a specific PDE4B antibody revealed a strong staining at the Z-line in WT myocytes but not in Pde4b−/− myocytes (Figure 6). This indicates that PDE4B is enriched in the T-tubules, at variance with that found in neonatal rat cardiomyocytes (16). This discrepancy could be due to either interspecies differences or to the higher level of differentiation of adult cardiomyocytes.

ICa,L constitutes the trigger for Ca2+-induced Ca2+ release and cardiac contraction, and its size dictates the amplitude of Ca2+ transients and contraction (37, 38). Accordingly, Ca2+ transients

Figure 7
Enhanced ECC and increased arrhythmias upon transient β-AR stimulation in Pde4b−/− and Pde4d−/− mice. (A) Representative traces of sarcomere shortening (top) and Ca2+ transients (bottom) recorded in electrically paced (0.5 Hz) AMVMs from WT, Pde4b−/−, and Pde4d−/− mice. The black traces correspond to control conditions, and the gray traces correspond to the maximal effect produced by a 15-second pulse of Iso (100 nM). (B) Mean data for sarcomere shortening (expressed as the percentage of resting length) and Fura-2 ratio (expressed as the percentage of diastolic ratio) variation measured in control conditions (black) and at the maximum of Iso stimulation (gray) measured in AMVMs from WT (n = 51), Pde4b−/− (n = 47), and Pde4d−/− (n = 47) mice. (C) Average t1/2off values for relaxation (top graph) and Ca2+ transient decay (bottom graph) in control conditions (black) and after Iso stimulation (gray) measured in AMVMs from WT (n = 51), Pde4b−/− (n = 47), and Pde4d−/− (n = 47) mice. (D) A representative trace of SCR events (arrows) induced by Iso in a Pde4b−/− AMVM paced at 0.5 Hz. (E) Bar graph representing the average number of SCR events during a 20-second period preceding the application of Iso (black) and an equally long period after the peak of the Iso effect (gray) in WT (n = 51), Pde4b−/− (n = 47), and Pde4d−/− (n = 47) AMVMs. Bar graphs represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
Supplemental Figure 2, PDE4B was anchored in macromolecular signaling complexes to Ca, which may not be sufficient to explain an increase in Ca transient amplitude (38). A recent study indicates that PDE4D may also control PLB phosphorylation (30). Consistent with this idea, PLB phosphorylation was increased in Pde4d–/– myocytes under β-AR stimulation, and Iso more potently increased SR Ca load in Pde4d–/– myocytes than in WT myocytes (Supplemental Figure 1). We propose that in Pde4d–/– myocytes both SR Ca release and SR Ca uptake are increased, leading to enhanced Ca transients, while trigger activity remains unaltered. In this scheme, the unchanged decay kinetics of the Ca transients in Pde4d–/– myocytes compared to those of WT myocytes under β-AR stimulation could be due to the balancing effect of increased diastolic Ca leak and increased Ca reuptake by the SR. Altogether, these results provide strong evidence that PDE4B and PDE4D isoforms differentially regulate cardiac ECC.

Alterations in Ca handling are linked to cardiac arrhythmias (43). SCR events were increased in Pde4d–/– cells upon β-AR stimulation. These events were likely due to “leaky” hyperphosphorylated RyR2 channels that promote arrhythmias in mice invalidated for the Pde4d gene (29). Interestingly, inactivation of the Pde4d gene also favored SCR events in isolated cardiomyocytes upon β-AR stimulation, likely by a different mechanism. Therefore, PDE4B deficiency could lead to cardiac dysfunction and arrhythmias. This was further demonstrated in vivo using catheter-based ventricular stimulation that reliably induces ventricular arrhythmias (44). The susceptibility of WT and Pde4b–/– mice to the development of VTs increased susceptibility to VT upon β-AR stimulation in Pde4b–/– mice. (A) Representative examples of simultaneous lead 1 ECG and intraventricular electrogram recordings obtained in WT and Pde4b–/– mice under Iso (0.2 mg/kg) after a burst of 30 stimuli at a cycle length (CL) of 55 ms. (B and C) Bar graphs showing the percentage of mice with arrhythmias induced (B) by burst pacing and (C) by 1 to 3 extrasystoles under baseline conditions and after intraperitoneal injections of Iso (0.02 mg/kg and 0.2 mg/kg). PES, paced extrasystole. *P < 0.05.

and contraction were enhanced in Pde4b–/– myocytes upon β-AR stimulation. This was accompanied by an accelerated relaxation and Ca transient decay, although the latter did not quite reach statistical significance (P = 0.059 vs. WT). Accordingly, PLB phosphorylation and SR Ca load tended to be increased during Iso stimulation in Pde4b–/– myocytes, but the differences with WT were not significant (Supplemental Figure 1). In addition, as shown in Supplemental Figure 2, PDE4B was anchored in macromolecular signaling complexes to Cav1.2 but not PLB or RyR2, and neither PLB phosphorylation at Ser-16 nor RyR2 phosphorylation at Ser-2808 were increased in Pde4b–/– hearts compared with that in WT hearts. Altogether, these results suggest that LTCCs constitute the primary target of PDE4B, although Pde4b ablation may not exclusively impact LTCC regulation. Interestingly, the absence of PDE4D also led to increased Ca transients and contraction in mouse ventricular myocytes (Figure 7). Since, in these cells Ica,L regulation was normal, the augmented ECC in Pde4d–/– myocytes could be related to PKA hyperphosphorylation of RyR2 during β-AR stimulation and increased open probability of the Ca release channel (8, 29). However, increasing RyR activity alone may not be sufficient to explain an increase in Ca transient amplitude (38), especially because such phenomenon can result ultimately in a decreased SR Ca content (39, 40). PLB mediates part of the positive inotropic effect of β-AR stimulation by increasing SR Ca load (41, 42). SR calcium content can be itself determine calcium transient amplitude independently from Ica,L.
potential. Recovery from inactivation of $I_{\text{Ca,L}}$ within its window may provoke EADs during the action potential plateau (45–47). In addition, increased Ca$^{2+}$ entry in cardiomyocytes could lead to Ca$^{2+}$ overload and increase spontaneous SR Ca$^{2+}$ release, thus promoting the occurrence of DADs. Future studies will be required to distinguish between these 2 possibilities.

In conclusion, our data identify PDE4B as an integral component of the LTCC complex and as the major PDE isofrom regulating $I_{\text{Ca,L}}$ during β-AR stimulation. Consequently, PDE4B is crucial for the regulation of cardiac ECC and arrhythmogenesis. Interestingly, we recently reported that PDE4B expression and activity were decreased during cardiac hypertrophy (34). This could lead to increased phosphorylation of LTCC, as reported in failing human hearts (35). Frozen adult mouse hearts or isolated ventricular myocytes were either used directly for PDE activity assays or Western blotting or were homogenized in ice-cold buffer containing 150 mM NaCl, 10 mM HEPES (pH 7.4), 2 mM EDTA, and 0.2 mM EGTA and supplemented with 10% glycercol, 1% Triton X-100, 1 μM microcystin-LR, and Complete Protease Inhibitor Tablets (Roche Diagnostics). Tissue lysates were centrifuged at 20,000 g and 4°C for 20 minutes, and supernatants were either used directly for PDE activity assays or Western blotting or were first subjected to IPs as described below.

Methods
All experiments performed conformed to the European Community guidelines in the care and use of animals (86/609/CEE), the local ethics committee (Comité Régional d’Ethique en matière d’Expérimentation Animale [CREAA] Ile-de-France Sud) guidelines, and the French decree no. 87–848. Approval for animal experiments according to this decree was obtained from the French Ministère de l’Agriculture, de la Pêche et de l’Alimentation (no. 92–283). Details concerning reagents, immunostaining, confocal imaging, electrophysiological experiments, ECG recording, intracardiac recording, and pacing can be found in the Supplemental Methods.

Preparation of mouse ventricular myocytes. Generation of Pde4a, Pde4b, and Pde4d homozygous null mice has been described previously (50). Ventricular myocytes were obtained from 113 mM NaCl, 4.7 mM KCl, 4 mM MgSO$_4$, 1.8 mM CaCl$_2$, 10 mM HEPES, 10 mM HEPES, 30 mM Tau-rine, and 20 mM d-glucose, adjusted to pH 7.4. The ascending aorta was cannulated, and the heart was perfused with oxygenated Ca$^{2+}$-free Tyrode’s solution at 37°C for 4 minutes using retrograde Langendorff perfusion. For enzymatic dissociation, the heart was perfused with Ca$^{2+}$-free Tyrode’s solution containing Liberase TM Research Grade (Roche Diagnostics) for 10 minutes at 37°C. Then the heart was removed and placed into a dish containing Tyrode’s solution supplemented with 0.2 mM CaCl$_2$ and 5 mM bovine serum albumin, and stored at 4°C for 10 minutes. The supernatant was removed, and cells were suspended in Tyrode’s solution supplemented with 0.5 mM CaCl$_2$ and 5 mM bovine serum albumin. The procedure was repeated once, and cells were suspended in Tyrode’s solution with 1 mM CaCl$_2$. Freshly isolated ventricular myocytes were plated in 35-mm culture dishes coated with laminin (10 μg/ml) and stored at room temperature until use. All experiments were performed at room temperature within 6 hours after cell isolation.

Preparation of protein extracts. Frozen adult mouse hearts or isolated AMVMs were homogenized in ice-cold buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA, and 0.2 mM EGTA and supplemented with 10% glycerol, 1% Triton X-100, 1 μM microcystin-LR, and Complete Protease Inhibitor Tablets (Roche Diagnostics). Tissue lysates were centrifuged at 20,000 g and 4°C for 20 minutes, and supernatants were either used directly for PDE activity assays or Western blotting or were first subjected to IPs as described below.

IP. IP of PDE4 subtypes was performed as described previously (24). In brief, cell and tissue extracts, prepared as described in “Preparation of protein extracts,” were precltered for 1 hour using 50 μl protein G-Sepharose, followed by a second centrifugation at 20,000 g and 4°C for 10 minutes. Preclered detergent extracts were then immunoprecipitated using 10 μg of the respective anti-PDE4 antibody or 10 μg of control IgG for 2 hours at 4°C. IP of Ca$^{2+}$, RyR2, and PLC was performed under similar conditions as described in more detail previously (7, 24, 29).

PDE assay. Cyclic AMP-PDE activity was measured according to the method of Thompson and Appleman as described previously (51). In brief, samples were assayed in a 200-μl reaction mixture containing 40 mM Tris-HCl (pH 8.0), 1 mM MgCl$_2$, 1.4 mM β-mercaptoethanol, 1 μM cAMP, 0.75 mg/ml bovine serum albumin, and 0.1 μCi of [3H]cAMP for 30 minutes at 33°C. The reaction was terminated by heat inactivation in a boiling water bath for 1 minute. The PDE reaction product 5′-AMP was then hydrolyzed by incubation of the assay mixture with 50 μg Crotalus atrox snake venom for 20 minutes at 33°C, and the resulting adenosine was separated by anion exchange chromatography using a 1 ml AG1-X8 resin (Bio-Rad) and quantified by scintillation counting. PDE4 activity was defined as the fraction of CAMP-PDE activity inhibited by 10 μM rolipram.

Electrophysiological experiments. The whole-cell configuration of the patch-clamp technique was used to record $I_{\text{Ca,L}}$. Patch electrodes with 1–2 MΩ resistance when filled with internal solution contained 118 mM CsCl, 5 mM EGTA, 4 mM MgCl$_2$, 5 mM sodium phosphocreatine, 3.1 mM Na$_2$ATP, 0.42 mM Na$_2$GTP, 0.062 mM CaCl$_2$ (pCa 8.5), and 10 mM HEPES, adjusted to pH 7.3. External Cs$^+$-Ringer solution contained 107.1 mM NaCl, 20 mM CsCl, 4 mM NaHCO$_3$, 0.8 mM Na$_2$PO$_4$, 5 mM t-glucose, 5 mM sodium pyruvate, 10 mM HEPES, 1.8 mM MgCl$_2$, and 1.8 mM CaCl$_2$, adjusted to pH 7.4. The cells were depolarized every 8 seconds from −50 mV to 0 mV for 400 ms. The use of −50 mV as holding potential allowed the inactivation of voltage-dependent sodium currents. Potassium currents were blocked by replacing all K$^+$ ions with external and internal Cs$^+$. The current-voltage relationship and steady-state inactivation properties were measured by applying a 200-ms pulse from −60 to +60 mV, followed by a 3-ms repolarization to −50 mV, before the 200-ms test pulse to 0 mV.

Measurements of Ca$^{2+}$ transients and cell shortening. Isolated cardiomyocytes were loaded with 5 μM Fura-2 AM (Invitrogen) at room temperature for 15 minutes and then washed with external Ringer solution containing 121.6 mM NaCl, 5.4 mM KCl, 4.013 mM NaHCO$_3$, 0.8 mM Na$_2$PO$_4$, 10 mM HEPES, 5 mM glucose, 5 mM Na pyruvate, 1.8 mM MgCl$_2$, and 1 mM CaCl$_2$, pH 7.4. The loaded cells were field stimulated at (5 V, 4 ms) at a frequency of 0.5 Hz. SL and Fura-2 ratio (measured at 512 nm upon excitation at 340 nm and 380 nm) were simultaneously recorded using an IonOptix System (IonOptix). Cell contractility was assessed by the percentage of sarcomere shortening, which is the ratio of twitch amplitude (difference of end-diastolic and peak systolic SL) to end-diastolic SL. Ca$^{2+}$ transients were assessed by the percentage of variation of the Fura-2 ratio by dividing the twitch amplitude (difference of end-diastolic and peak systolic ratios) to end-diastolic ratio. The $I_{\text{Ca,L}}$ was used as an index of relaxation and Ca$^{2+}$ transient decay kinetics. All parameters were calculated offline using a dedicated software (IonWizard 6x).

Immunocytochemistry. Cardiomyocytes were permeabilized after fixation with 0.25% Triton X-100 and probed with rabbit polyclonal anti-Ca$^{2+}$-II antibody (CNC1, ref. 52) and mouse monoclonal anti-α-actinin or rabbit antibody (113-4 raised against the C terminus of PDE4B and mouse monoclonal anti-α-actinin).

ECG recording and intracardiac recording and pacing. ECG recording was performed as previously described on mice anesthetized with intraperitoneal injection of etomidate (32 mg/kg; Janssen-Cilag) (53). After ECG recording, the extremity of a 2F octopolar catheter (Biosense Webster) was placed
in the right ventricle through the right internal jugular vein. Standard pac- ing protocols were used to determine the VERPs and to induce ventricular arrhythmias. The inducibility of ventricular arrhythmias was assessed in baseline condition and after intraperitoneal infusions of Isop (0.02 mg/kg and 0.2 mg/kg) by using the programmed electrical stimulation method with 1 to 3 extrastimuli (performed twice) and burst pacing. VT was defined as the occurrence after the last paced beat of at least 4 consecutive QRS complexes with a different morphology from that seen in normal sinus rhythm. VT of more than 10 cycles was defined as sustained VT.

Statistics. Data are represented as mean ± SEM. Statistics were performed using Excel and the StatXact 8 software. Statistical significance was evaluated using Student’s 2-tailed unpaired t test and Barnard’s exact test. A difference was considered statistically significant when P < 0.05.

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