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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β-Adrenergic receptors (β-ARs) enhance cardiac contractility by increasing cAMP levels and activating PKA. PKA increases Ca$^{2+}$-induced Ca$^{2+}$ release via phosphorylation of L-type Ca$^{2+}$ 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$^{2+}$ current (I$_{Ca,L}$) is increased only in Pde4b$^{-/-}$ mice. A fraction of PDE4B colocalized with the LTCC along T-tubules in the mouse heart. Under β-AR stimulation, Ca$^{2+}$ transients, cell contraction, and spontaneous Ca$^{2+}$ release events were increased in Pde4b$^{-/-}$ and Pde4d$^{-/-}$ myocytes compared with those in WT myocytes. In vivo, after intraperitoneal injection of isoprenaline, catheter-mediated burst pacing triggered ventricular tachycardia in Pde4b$^{-/-}$ mice but not in WT mice. These results identify PDE4B in the Ca$_{1,2}$ complex as a critical regulator of I$_{Ca,L}$ during β-AR stimulation and suggest that distinct PDE4 subtypes are important for normal regulation of Ca$^{2+}$-induced Ca$^{2+}$ release in cardiomyocytes.

Introduction

During the cardiac action potential, Ca$^{2+}$ influx through sarclemmal L-type Ca$^{2+}$ channels (LTCCs) triggers Ca$^{2+}$ release from juxtaposed ryanodine receptor 2 (RyR2) located in the sarcoplasmic reticulum (SR). This allows a rapid and synchronous Ca$^{2+}$ elevation throughout the cell, which activates contraction. During cardiac relaxation, Ca$^{2+}$ is rapidly extruded by the Na$^+$/Ca$^{2+}$ exchanger and re-sequestered into the SR by the Ca$^{2+}$-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 cAMP-dependent PKA. PKA then phosphorylates the key proteins of the excitation-contraction coupling (ECC) process, including LTCC and RyR2 but also phospholamban (PLB), which controls Ca$^{2+}$ reuptake by SERCA2A, as well as the myofilament proteins troponin I and myosin binding protein C (1).

The cardiac LTCC consists of the central pore-forming subunit α$_{1C}$ (Ca$_{1,2}$) and auxiliary β and α$_{2-δ}$ subunits that modulate its function (2). Upon β-AR stimulation, phosphorylation of Ca$_{1,2}$, the auxiliary β$_2$ subunit, or the closely associated protein AHNAK by PKA increases channel activity, thus enhancing the L-type Ca$^{2+}$ current (I$_{Ca,L}$) (3–5). This regulation involves physical association of PKA with Ca$_{1,2}$ via an A-kinase anchoring protein, AKAP15/18 (6, 7). Similarly, different AKAPs are responsible for the localization of PKA in the immediate vicinity of ECC proteins: the muscle-specific AKAP targets PKA to RyR2 (8, 9), and AKAP188 localizes PKA to PLB (10).

PKA targeting by AKAPs would not be sufficient to explain hormonal specificity if cAMP could diffuse freely inside cells (11). In addition to discrete production sites, cAMP spreading is restricted by cyclic nucleotides phosphodiesterases (PDEs), the enzymes that degrade cAMP and cGMP into their inactive counterparts, 5′-AMP and 5′-GMP, respectively. Classically, 4 different PDE families (PDE1–PDE4) hydrolyze cAMP in heart: PDE1, which is activated by Ca$^{2+}$-calmodulin; PDE2, which is stimulated by cGMP; PDE3, which is inhibited by cGMP; and PDE4. PDE1 and PDE2 can hydrolyze both cAMP and cGMP, PDE3 preferentially hydrolyzes cAMP, and PDE4 is specific for cAMP (12). A fifth PDE, PDE8A, has been recently shown to modulate cAMP signaling in mouse cardiomyocytes (13). Association of individual PDE families to G$_i$-coupled receptors enables cardiac cells to generate heterogeneous cAMP signals in response to different hormones and specific control of I$_{Ca,L}$ (14).

PDE4 is one of the main PDEs expressed in heart. PDE4 becomes predominantly active upon β-AR stimulation and regulates global cAMP levels in cardiac cells (14–19). Particularly, PDE4 is the main PDE modulating I$_{Ca,L}$ in cardiomyocytes (18, 20). The PDE4 family is encoded by 4 genes (PDE4A–PDE4D), but only PDE4A, PDE4B,
PDE4D is part of a CaV1.2 signaling complex, but, to our surprise, inactivation of the Pde4d gene had no consequence on basal or β-AR stimulated I_{Ca,L}, although Ca^2+ transients and contraction were enhanced. We therefore explored the role of the 2 other cardiac PDE4 isoforms, PDE4A and PDE4B, and found that PDE4B, but not PDE4A, was also associated with the LTCC. In Pde4b^−/− mice, but not in Pde4a^−/− mice, the β-AR response of I_{Ca,L} was increased, together with an increase in cell contraction and Ca^2+ 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 Ca^2+ that enters the cell via the LTCCs, prevents Ca^2+ overload and arrhythmias.

**Results**

β-AR stimulation of I_{Ca,L} and effect of PDE4 inhibition in adult mouse ventricular myocytes. We previously showed that the β-AR stimulation of I_{Ca,L} is under the control of PDE4 in adult rat ventricular myocytes (18). To determine whether the same was true in mice, I_{Ca,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 isoprenaline (Iso) was studied. As shown in Figure 1A, a 15-second stimulation with 100 nM Iso elicited a rapid and transient increase of I_{Ca,L} of 58.8% ± 3.3% (n = 62; Figure 1C). When I_{Ca,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 I_{Ca,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). All curves represent the mean ± SEM. *P < 0.05 indicates the first of the statistically significant points on the graph. All points that occur after this time point are significant.

Figure 1

PDE4 modulates β-AR stimulation of I_{Ca,L} in mouse ventricular myocytes. (A) Typical time course of I_{Ca,L} amplitude in a cell stimulated by a 15-second pulse of Iso (100 nM). Each square represents the amplitude of I_{Ca,L} recorded every 8 seconds during a depolarization from −50 to 0 mV. (B) I_{Ca,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 I_{Ca,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). All curves represent the mean ± SEM. *P < 0.05 indicates the first of the statistically significant points on the graph. All points that occur after this time point are significant.

We believe that until now, the molecular identity of the PDE4 regulating the LTCC was unknown. Because of the prominent role of PDE4 variants in the β-AR cascade, we anticipated that a PDE4 variant might also regulate the LTCC activity. Our initial goal here was to test this hypothesis. We indeed found that 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 of 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 βAR cascade, we anticipated that...
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\(_{\text{2+}}\) 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 Pde4b\(^{-/-}\) 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 4, D 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). 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...
myocytes was absent (Figure 6C). Our interpretation of these results is that Ca_{T1,2} and a fraction of PDE4B are colocalized along T-tubule membranes in AMVMs.

β-AR regulation of ECC in AMVMs from Pde4b^{−/−} and Pde4d^{−/−} mice. The fact that PDE4B modulates the β-AR stimulation of I_{Ca,L} and PDE4D regulates RyR2 (29) prompted us to compare the ECC process in WT, Pde4b^{−/−}, and Pde4d^{−/−} mice. For this, sarcomere length (SL) and intracellular Ca^{2+} were recorded simultaneously in Fura-2-loaded ventricular myocytes paced at 0.5 Hz. As shown in Figure 7, A and B, under nonstimulated basal conditions, SL shortening and Ca^{2+} transient amplitude were similar in the 3 genetic backgrounds. In WT, Pde4b^{−/−}, and Pde4d^{−/−} cells, pulse stimulation with Iso (100 nM, 15 seconds) strongly increased SL shortening and Ca^{2+} transient amplitude. However, these effects were more pronounced in PDE4-deficient myocytes: maximal shortening measured at the peak of Iso stimulation was increased by about 50% in Pde4b^{−/−} (n = 47) and Pde4d^{−/−} (n = 47) myocytes compared with that in WT myocytes (n = 51, P < 0.001), whereas Ca^{2+} transient amplitude was increased by about 25% and 28% more in Pde4b^{−/−} (n = 47) and Pde4d^{−/−} myocytes (n = 47), respectively, than in WT myocytes (n = 51; P < 0.01). Figure 7C shows the corresponding kinetics of relaxation and decay of the Ca^{2+} transients. In basal conditions, there was no difference in time to half maximal decay (t_{1/2off}) values of relaxation and Ca^{2+} transient decay among the different genotypes. Iso strongly accelerated relaxation (t_{1/2off} values were decreased by about 65%) and Ca^{2+} transient decay (t_{1/2off} values were decreased by about 57%) in WT myocytes (n = 51).

In Pde4b^{−/−} and Pde4d^{−/−} myocytes (n = 47 each) relaxation was further accelerated (t_{1/2off} values were decreased by about 81% and 78% of basal value, respectively; P < 0.001 vs. WT), whereas decay kinetics of the Ca^{2+} transients in the presence of Iso were not significantly different from those of WT myocytes (Figure 7C). However, the decay phase of Ca^{2+} 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 < 0.05). As shown in Figure 7, D and E, Pde4b^{−/−} and Pde4d^{−/−} myocytes also displayed increased spontaneous Ca^{2+} 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 Ca^{2+} 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 1, A and B (supplemental material available online with this article; doi:10.1172/JCI44747DS1), SR Ca^{2+} load was not significantly different in the 3 genotypes. However, there was a tendency for a decreased SR Ca^{2+} load in Pde4d^{−/−} myocytes in basal conditions (−20%). As a consequence, the percentage increase in SR Ca^{2+} 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).
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 that 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} transients, and contraction are enhanced in myocytes from Pde4b−/− mice, and this is accompanied by an increased propensity for arrhythmia. β-AR stimulation of Ca^{2+} transients and contraction are also enhanced in myocytes from Pde4b−/− 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 migration of the 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.

**Figure 6**

Localization of PDE4B and LTCC at the transverse tubules in AMVMs. (A) Confocal images of a WT AMVM double labeled with anti-Cav1.2 antibody (CNC1, green) and anti–α-actinin antibody (α-Act, red). (B) Confocal images of a WT AMVM double labeled with anti-PDE4B antibody (green) and anti–α-actinin antibody (red). (C) Confocal images of a Pde4b−/− AMVM double labeled with anti-PDE4B antibody (green) and anti–α-actinin antibody (red). Scale bar: 20 μm. The graph below each panel indicates the relative fluorescence intensities in the green and red channels measured along a 20-μm distance, as indicated by the yellow line on the enlarged boxed area from the merged images.

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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 Ca\textsuperscript{V1.2} 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 I\textsubscript{Ca,L} nor its \(\beta\)-AR regulation. Since PDE4B was also found in the complex that includes the Ca\textsuperscript{V1.2} subunit, and \(\beta\)-AR stimulation of I\textsubscript{Ca,L} is upregulated in \(\text{Pde4b}^{-/-}\) 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 \(\beta\)-AR response of I\textsubscript{Ca,L} in Pde4b\textsuperscript{-/-} 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 \(\beta\)-ARs (25–27). Since \(\beta\)-ARs are part of a signalingosome, including the LTCCs (35), it is conceivable that the 2 PDE4 isoforms that immunoprecipitated with Ca\textsuperscript{V1.2} are in fact located at different ends of the signalosome, with PDE4D associated with the \(\beta\)-AR and PDE4B associated with the LTCC. Alternatively, PDE4D and PDE4B may control different pools of Ca\textsuperscript{V1.2}, the latter being responsible for \(\beta\)-AR-dependent I\textsubscript{Ca,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\textsuperscript{-/-} 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. I\textsubscript{Ca,L} constitutes the trigger for Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release and cardiac contraction, and its size dictates the amplitude of Ca\textsuperscript{2+} transients and contraction (37, 38). Accordingly, Ca\textsuperscript{2+} transients

Figure 7
Enhanced ECC and increased arrhythmias upon transient \(\beta\)-AR stimulation in Pde4b\textsuperscript{-/-} and Pde4d\textsuperscript{-/-} mice. (A) Representative traces of sarcomere shortening (top) and Ca\textsuperscript{2+} transients (bottom) recorded in electrically paced (0.5 Hz) AMVMs from WT, Pde4b\textsuperscript{-/-}, and Pde4d\textsuperscript{-/-} 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\textsuperscript{-/-} (n = 47), and Pde4d\textsuperscript{-/-} (n = 47) mice. (C) Average \(t_{1/2}\text{off}\) values for relaxation (top graph) and Ca\textsuperscript{2+} transient decay (bottom graph) in control conditions (black) and after Iso stimulation (gray) measured in AMVMs from WT (n = 51), Pde4b\textsuperscript{-/-} (n = 47), and Pde4d\textsuperscript{-/-} (n = 47) mice. (D) A representative trace of SCR events (arrows) induced by Iso in a Pde4b\textsuperscript{-/-} 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\textsuperscript{-/-} (n = 47), and Pde4d\textsuperscript{-/-} (n = 47) AMVMs. Bar graphs represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
and contraction were enhanced in Pde4b−/− myocytes upon β-AR stimulation. This was accompanied by an accelerated relaxation and Ca2+ transient decay, although the latter did not quite reach statistical significance (P = 0.059 vs. WT). Accordingly, PLB phosphorylation and SR Ca2+ 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 Ca2+ 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 Ca2+ release channel (8, 29). However, increasing RyR activity alone may not be sufficient to explain an increase in Ca2+ transient amplitude (38), especially because such phenomenon can result ultimately in a decreased SR Ca2+ content (39, 40). PLB mediates part of the positive inotropic effect of β-AR stimulation by increasing SR Ca2+ load (41, 42). SR calcium content can by itself determine calcium transient amplitude independently from ICa,L.

Figure 8
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 by burst pacing and (C) by 1 to 3 extrastimuli under baseline conditions and after intraperitoneal injections of Iso (0.02 mg/kg and 0.2 mg/kg). PES, paced extrasystole. *P < 0.05.

results provide strong evidence that PDE4B and PDE4D isoforms differentially regulate cardiac ECC.

Alterations in Ca2+ 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 after paced extrasystoles or burst pacing was very low in baseline conditions. Interestingly, under β-AR stress, Pde4b inactivation significantly increased the incidence of arrhythmias induced by burst pacing but not by extrastimuli. In addition, no difference was detected between ECGs recorded from WT and Pde4b−/− hearts. 

These results are consistent with triggered arrhythmias due to altered Ca2+ homeostasis rather than reentries or altered conduction. Arrhythmias triggered by burst pacing during β-AR stimulation could be due to early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs) (43). Interestingly, increased β-AR stimulation also resulted in a larger shift of the activation curve of ICa,L in Pde4b−/− myocytes. As a consequence, a larger window current would occur, promoting Ca2+ entry during the action potential.
potential. Recovery from inactivation of I_{Ca,L} within its window may provoke EADs during the action potential plateau (45–47). In addition, increased Ca\textsuperscript{2+} entry in cardiomyocytes could lead to Ca\textsuperscript{2+} overload and increase spontaneous SR Ca\textsuperscript{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 isoform regulating I_{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 ventricular myocytes (48, 49), and therefore provide a mechanistic link to arrhythmias and sudden death in heart failure.

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 [CREEA] 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\textdblash, Pde4b\textdblash, and Pde4c\textdblash homozygous null mice has been described previously (50). Ventricular myocytes were obtained from 5- to 6-month-old males with a mixed background. Animals were anesthetized by intraperitoneal injection of pentothal (150 mg/kg), and the heart was quickly removed and placed into a cold Ca\textsuperscript{2+}-free Tyrode’s solution containing 113 mM NaCl, 4.7 mM KCl, 4 mM NaHCO\textsubscript{3}, 1.6 mM MgSO\textsubscript{4}, 10 mM HEPES, 4 mM HEHPO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, and 5 mM Mg\textsuperscript{2+}, adjusted to pH 7.4. The ascendency aorta was cannulated, and the heart was perfused with oxygenated Ca\textsuperscript{2+}-free Tyrode’s solution at 37°C for 4 minutes using retrograde Langendorff perfusion.

For enzymatic dissociation, the heart was perfused with Ca\textsuperscript{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\textsubscript{2} and 5 mg/ml BSA (Sigma-Aldrich). The ventricles were separated from the atria, cut into small pieces, and trituated with a pipette to disperse the myocytes. Ventricular myocytes were filtered on gauze and allowed to sediment by gravity for 10 minutes. The supernatant was removed, and cells were suspended in Tyrode’s solution supplemented with 0.5 mM CaCl\textsubscript{2} and 5 mg/ml BSA.

The procedure was repeated once, and cells were suspended in Tyrode’s solution with 1 mM CaCl\textsubscript{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 precleared for 1 hour using 50 μl protein G-Sepharose, followed by a second centrifugation at 20,000 g and 4°C for 10 minutes. Precleared 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\textsubscript{1.2}, RyR2, and PLB 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\textsubscript{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 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_{Ca,L}. Patch electrodes with 1–2 MΩ resistance when filled with internal solution contained 118 mM CsCl, 5 mM EGTA, 4 mM MgCl\textsubscript{2}, 5 mM sodium phosphocreatine, 3.1 mM Na\textsubscript{2}ATP, 0.42 mM Na\textsubscript{2}GTP, 0.062 mM CaCl\textsubscript{2} (pCa 8.5), and 10 mM HEPES, adjusted to pH 7.3. External Cs\textsuperscript{+}-Ringer solution contained 107.1 mM NaCl, 20 mM CsCl, 4 mM NaHCO\textsubscript{3}, 0.8 mM NaHPO\textsubscript{4}, 5 mM t-glucose, 5 mM sodium pyruvate, 10 mM HEPES, 1.8 mM MgCl\textsubscript{2}, and 1.8 mM CaCl\textsubscript{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\textsuperscript{+} ions with external and internal Cs\textsuperscript{+}.

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\textsuperscript{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\textsubscript{3}, 0.8 mM NaHPO\textsubscript{4}, 10 mM HEPES, 5 mM glucose, 5 mM Na pyruvate, 1.8 mM MgCl\textsubscript{2}, and 1 mM CaCl\textsubscript{2}, pH 7.4. The loaded cells were field stimulated (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\textsuperscript{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 t_{1/2off} was used as an index of relaxation and Ca\textsuperscript{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\textsubscript{1.2} 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 by 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 interventricular jugular vein. Standard pac- ing protocols were used to determine the VERs and to induce ventricular arrhythmias. The inducibility of ventricular arrhythmias was assessed in baseline condition and after intraperitoneal infusion of ISO (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. Statistical analysis was performed using Excel and the StatXact 8 software. Statistical significance was evaluated using Student’s t-test or Bartlett’s exact test. A difference was considered statistically significant when P < 0.05.

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