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Cardiac myocyte–secreted cAMP exerts paracrine action via adenosine receptor activation

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Acute stimulation of cardiac β-adrenergceptors is crucial to increasing cardiac function under stress; however, sustained β-adrenergic stimulation has been implicated in pathological myocardial remodeling and heart failure. Here, we have demonstrated that export of cAMP from cardiac myocytes is an intrinsic cardioprotective mechanism in response to cardiac stress. We report that infusion of cAMP into mice averted myocardial hypertrophy and fibrosis in a disease model of cardiac pressure overload. The protective effect of exogenous cAMP required adenosine receptor signaling. This observation led to the identification of a potent paracrine mechanism that is dependent on secreted cAMP. Specifically, FRET-based imaging of cAMP formation in primary cells and in myocardial tissue from murine hearts revealed that cardiomyocytes depend on the transporter ABCC4 to export cAMP as an extracellular signal. Extracellular cAMP, through its metabolite adenosine, reduced cardiomyocyte cAMP formation and hypertrophy by activating A1 adenosine receptors while delivering an antifibrotic signal to cardiac fibroblasts by A2 adenosine receptor activation. Together, our data reveal a paracrine role for secreted cAMP in intercellular signaling in the myocardium, and we postulate that secreted cAMP may also constitute an important signal in other tissues.

Introduction
During the fight-or-flight response, activation of the sympathetic nervous system leads to a release of adrenaline and noradrenaline, which mediate their effects through the activation of adrenoceptors (1). Within the heart, β-adrenergceptors (βARs) couple mainly to stimulatory G proteins (Gs), thereby activating adenylyl cyclase to generate the second messenger cAMP from ATP. Intracellular cAMP formation represents the strongest mechanism for increasing cardiac function, but continuous activation of the cAMP pathway can promote cardiac hypertrophy and fibrosis (i.e., myocardial remodeling) and thereby contribute to cardiac disease (2). To prevent such detrimental consequences of sustained cAMP signaling, the extent and duration of cAMP formation underlie feedback control mechanisms, which include desensitization of βARs or rapid degradation of cAMP (3). In addition, stimulated cells invest a substantial amount of energy into transporting cAMP outside cells, a process that is mediated in mammals by members of the group of ATP-binding cassette (ABC) transporters, in particular, ABCC4, ABCC5, and ABCC11 (also termed MRP4, MRP5, and MRP8, respectively) (4–7).

A pivotal finding was that infusion of extracellular cAMP into mice exerted physiological effects in the kidney (8). These responses are conferred by adenosine receptors (adenosine receptor 1 [A1R], A2aR, A2bR, and A3R), which are expressed in most organs (9). A potential link between cAMP in the bloodstream and these receptors on renal cells was postulated based on the knowledge that 2 enzymes, ectonucleotide pyrophosphatase/phosphodiesterase 1 (ecto-PDE, alias ENPP1) and ectonucleotide 5′-nucleotidase (NTSE, the enzyme that converts AMP to adenosine, also known as CD73), convert cAMP to AMP and AMP to adenosine, respectively (10).

Interestingly, the heart is also equipped with the protein repertoire to export intracellular cAMP (11) and the enzymes to metabolize it to adenosine (12). This raises the intriguing question of whether the myocardium, potentially exemplary for many tissues, provides for its own source of extracellular adenosine by secretion of cAMP and whether this cAMP promotes cell-to-cell communication within the same tissue.

We addressed these issues by combining experiments in vivo with optical and biochemical analysis in vitro. Our data suggest an important regulatory role of secreted cAMP that serves to dampen the detrimental consequences of prolonged βAR-cAMP signaling.

Results
Extracellular cAMP prevents adrenergically induced cardiac hypertrophy and fibrosis. To test for a role of extracellular cAMP in the heart, we chose chronic adrenergic stimulation of mice as an in vivo model for cardiac hypertrophy and fibrosis. Ten-week-old C57BL/6 N mice were infused for 7 days with isoproterenol (Iso) (βAR agonist) and phenylephrine (PE) (α1-adrenerceptor agonist)
(30 mg/kg/d each) in the presence or absence of cAMP (30 mg/kg/d). After 7 days, animals were sacrificed for analysis of the hearts. Mice treated with Iso/PE alone developed cardiac hypertrophy and fibrosis (Figure 1, A–C). Importantly, cAMP infusion significantly prevented these structural changes, as determined by morphometry and histology (Figure 1, A–C) and by quantification of the mRNAs that encode Myh7, Col1a2, and Col3a1 (Figure 1D and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI74349DS1). The presence of extracellular cAMP reduced 1H-thymidine incorporation in cardiac fibroblasts (CFs) (Supplemental Figure 1C), indicating that cAMP impairs their activation. Apoptosis of cardiac cells, in contrast, was not significantly altered by exogenous cAMP (Supplemental Figure 2).

Since the heart, like other organs, is equipped with membrane-anchored ENPP1 and NT5E (13, 14), exogenously applied cAMP is expected to be rapidly degraded to adenosine, which, upon receptor activation, may have induced the observed effects. Support for this idea came from experiments in which we inhibited the first enzymatic step in cAMP metabolism, that is, the conversion of cAMP to AMP by ENPP1. We identified SYL-001 as a potent and highly selective inhibitor of ENPP1 (K, 26.9 nM, human ENPP1, > 1000-fold selective versus other ectonucleotidases) (Supplemental Figure 3, A–C). The presence of SYL-001 prevented extracellular cAMP from exerting its antihypertrophic and antifibrotic effects in Iso/PE-treated mice (Supplemental Figure 4). Furthermore, the myocardium appears to be equipped with all components of an endogenous cAMP/adenosine pathway, since cAMP accumulated in the pericardial fluid of mice subjected to transverse aortic constriction (TAC) and since ENPP1 inhibition exacerbated cardiac myocyte (CM) hypertrophy and cardiac fibrosis induced by pressure overload (Supplemental Figure 3, D–F). Consistent with our findings, other studies reported that deficiency of ENPP1 or NT5E impaired cardiac function or cardioprotective mechanisms, respectively (15, 16).

To determine which adenosine receptor was involved in extracellular cAMP signaling, we applied adenosine receptor antagonists in vivo. PSB16-P, a specific antagonist for A1R (see refs. 17, 18 for this and following antagonists), abolished the antihypertrophic effect of extracellular cAMP in Iso/PE-treated mice (Figure 1, A–C), whereas fibrosis remained suppressed (Figure 1, A–C). Another drug that we tested in parallel was MSX-3, an antagonist of A2AR. With this, we observed a reciprocal effect, that is, it failed to reduce cardiac hypertropy, but inhibited the ability of cAMP to prevent cardiac fibrosis (Figure 1, A–C). Histological analysis of cardiac tissue confirmed that extracellular cAMP prevented Iso/PE-induced CM hypertrophy and that PSB16-P reverted this reduction (Figure 1, E and F). Since A1R antagonization did not completely inhibit the cAMP effect on CFs, we suspected that receptor subtype A2AR may also contribute to extracellular cAMP/adenosine actions (moreover, A2AR shows significant expression in CMs and CFs) (Figure 2, A and E). Similar results were obtained from adult mouse CMs (AMCMs) or adult mouse CFs (AMCFs) (Supplemental Figure 5, A and B).

We then determined in what way distinct adenosine receptor profiles of cardiac cells affect their response to exogenously added cAMP. Since adenosine receptors couple to G proteins, the activity of adenylyl cyclase, quantitation of intracellular cAMP, synthesis of collagen (Col1a2 and Col3a1), and intercellular CM-CF cAMP crosstalk.

Exogenous cAMP confers diametral changes of intracellular cAMP in CMs and CFs. A quantitative PCR (qPCR) analysis of adenosine receptor expression in primary rat neonatal CMs or CFs (purity of isolates >90% each) revealed that the A1R subtype is virtually the exclusive adenosine receptor in CMs (Figure 2A), whereas A2AR and A2BAR dominate in CFs (ref. 19 and Figure 2E). A1R expression was marginal in CMs and CFs (Figure 2, A and E). These data suggest that extracellular cAMP protects the heart from adrenergically induced hypertrophy and fibrosis and that this is mediated through its metabolite adenosine acting mainly on CM A1R and CF A2AR.

Another drug that we tested in parallel was MSX-3, an antagonist of extracellular cAMP with A2AR in CMs and A2B R in CFs (Figure 2A and Supplemental Figure 1C). Consistent with our findings, other studies reported that deficiency of ENPP1 or NT5E impaired cardiac function or cardioprotective mechanisms, respectively (15, 16).
CMs showed basal cAMP export activity, which increased 5-fold when Iso was present (Figure 3A). Isolated CFs showed substantially less cAMP efflux, both under basal and Iso-induced conditions (Figure 3A). This points toward CMs as the primary cardiac source of secreted cAMP. Of note, extracellular levels of ATP or cGMP did not change in response to Iso stimulation in CMs (Supplemental Figure 7), thus emphasizing the specific role of cAMP as an extracellular signal upon βAR stimulation.

We then asked whether export of cAMP from isolated CMs under adrenergic stimulation is able to condition the growth medium such that it elicits an intracellular response when transferred to CFs (see schematic drawing in Figure 3B). Since the recipient cells must be insensitive to adrenergic stimulation, CMs were derived from mice with homozygous deletion of the β₁ and β₂-adenoreceptors (Adrb1−/− Adrb2−/− mice; ref. 20). These Adrb1−/− Adrb2−/− CFs were then infected with an adenovirus encoding the cAMP sensor. The “sensor fibroblasts” indeed did not show intracellular cAMP formation in the presence of Iso, in contrast to exogenously added cAMP, which promoted intracellular cAMP formation via the activity of type 2 adenosine receptors (Supplemental Figure 6, A and B). We then stimulated adult WT CMs with Iso (10 μM for 1 hour), and the cell-free medium (hereafter termed conditioned medium [coM]) was transferred to the sensor CFs (Figure 3B). FRET detection in single sensor fibroblasts showed that coM from Iso-treated CMs significantly increased intracellular cAMP formation in CFs (Figure 3, C–E). This response was sensitive to the AR antagonist DPCPX (10 nM), indicating that adenosine was involved in this signaling.

Since the ABCC transporter family comprises the only active cAMP exporters known in the mammalian system, an inhibitor (MK571, 50 μM) was applied to test for their contribution to signaling of cAMP secreted by CMs. Indeed, MK571 potently prevented Iso-treated CMs from activating CFs (Supplemental Figure 8). Primary candidates for cAMP export activity among ABCC proteins are ABCC4 and ABCC5, given that cardiac expression of ABCC1 (the third potential cyclic nucleotide exporter, see ref. 7) is low (21). Intriguingly, coM from Iso-stimulated Abcc4−/− CMs completely failed to induce intracellular cAMP formation in sensor fibroblasts (Figure 3, C–E). The extent of this effect indicates that ABCC4 has a prominent role in CM-cAMP export. The link between ABCC4 and catecholamine-induced cAMP efflux from CMs is further supported by the preferential expression of Abcc4 in this cell type (Supplemental Figure 5C) and by the finding that Abcc4−/− mice showed exacerbated cardiac hypertrophy and fibrosis in response to catecholamine infusion (Supplemental Figure 9, A and B).

cAMP-dependent communication between cardiac cells in cocultures and intact cardiac tissue. To simulate more closely the cardiac tissue context, primary CMs were directly layered onto preadhered sensor fibroblasts (Adrb1−/− Adrb2−/− with CAMP sensor; see Figure 4A). One day later, a fraction of these CMs had come into direct contact with the sensor fibroblasts, as judged by passive movements of the latter through their attachment to beating CMs. β-Adrenergic stimulation of the cocultured CMs induced an increase of intracellular cAMP in attached sensor fibroblasts, thus confirming the observations made in experiments using coM (Figure 4, B and C).

To investigate native cardiac tissue for cAMP-dependent communication between cells, we devised a near-vivo setup based on intact slices of myocardium that maintain organotypic functions for several days (22). Slices from hearts of 12-week-old mice were taken into culture, and sensor fibroblasts were transferred onto them. Confocal microscopic imaging showed embedding of sensor fibroblasts (green in Figure 4D) into the slices. FRET determination of intracellular cAMP formation in sensor fibroblasts revealed that they reacted to β-adrenergic stimulation of the myocardial slice (Figure 4, E and F). In contrast, β-adrenergic stimulation of Abcc4−/− slices failed to induce a FRET response in integrated sensor fibroblasts (Figure 4, E and F), suggesting dependence of the observed effects on secreted cAMP. Released endogenous cAMP was also sufficient to inhibit contractility of myocardial slices. Treatment of slices from WT mice with an A₁R antagonist (DPCPX, 100 nM) led to a significant increase of Iso-induced contractility (Supplemental Figure 9, C and D). This positive inotropic effect of A₁R blockade was entirely abolished in myocardial slices deficient for Abcc4 (Supplemental Figure 9, C and D). These data demonstrate a key role of secreted cAMP for intercellular communication in the myocardium and substantiate the critical involvement of ABCC4 in this regulatory mechanism. Together, our results demonstrate that β-adrenergic stimulation causes CMs to secrete cAMP as a source of adenosine, which then signals to CFs (preventing fibrosis) and to vicinal CMs (preventing hypertrophy) (Figure 5).

Discussion

The results of this study indicate that cAMP secreted into the extracellular space is an important paracrine factor in the myocardium. Upon β-adrenergic stimulation, CMs actively export their intracellular second messenger cAMP, which, through its metabolite adenosine, controls the development of cardiac hypertrophy and fibrosis.

We provide the first evidence, to our knowledge, of the cardioprotective effects extracellular cAMP has on the myocardium, and we resolve the physiological impact of secreted cAMP at the cellular and molecular level.

The diametrically opposing effects of extracellular cAMP observed in CMs and CFs are in agreement with a cardioprotective role of secreted cAMP (see Figure 5), since reduced intracellular cAMP levels (after G inhibition of adenylyl cyclase) interfere with prohypertrophic signaling in CMs (23) and elevated intracellular cAMP concentrations prevent activation of CFs (12, 24).

Inspired by the early finding that systemic infusion of cAMP alters renal activity (8), exogenously added cAMP was shown to also elicit effects in various cells and tissues in vitro (25, 26), yet the physiological relevance of the latter remained unresolved. We provide several lines of evidence that the myocardium as a sympathetically innervated organ can supply extracellular cAMP to establish a local paracrine signal: first, increased cAMP efflux was detected after Iso stimulation of primary CMs in vitro (Figure 2A) and in the pericardial fluid of mice subjected to TAC (Supplemental Figure 3D). Second, Iso-stimulated CMs conditioned their medium to promote protective signaling in CFs, a mechanism that requires the cAMP exporter ABCC4 on CMs and functional adenosine receptors on CFs (Figure 3, D and E). And third, block-
Figure 1. Extracellular cAMP prevents cardiac hypertrophy and fibrosis. (A–C) Mice were chronically infused with Iso/PE (30 mg/kg/d each) and, where denoted, with exogenous cAMP (30 mg/kg/d), an A₁R antagonist (PSB-16P; 5 mg/kg/d), or an A₃R antagonist (MXS-3, 5 mg/kg/d). After 7 days, mice were sacrificed to assess cardiac remodeling. (A) Representative myocardial tissue sections after staining with Sirius Red (for collagen) and Fast Green counterstaining. Images at higher magnification are shown below. Scale bars: 2 mm (top row); 200 μm (bottom row). (B) Ratio of heart weight-to-tibia length (HW/TL) and (C) quantification of myocardial fibrosis. n = 9–16 mice/group. (D) qPCR analysis of β-myosin heavy chain (Myh7) mRNA in myocardial tissue from the indicated groups. n = 5–9 mice/group. (E) Representative WGA staining of left ventricle tissue after the indicated treatments and (F) quantitative analysis (n = 6–8 mice/group). Scale bar: 50 μm. (G and H) NRCMs were treated with Iso/PE, and where denoted, cAMP (100 μM) was added in the presence or absence of antagonists against A₁R (DPCPX; 100 nM), A₂AR (SCH-442416; 100 nM), A₃R (PSB-1115; 500 nM), or A₃R (VUF-5574; 100 nM). (G) Processed images after immunofluorescent staining of primary CMs by an α-actinin antibody (green cells). Nonmyocyte cells (marked in red) were defined as such if they yielded a DAPI signal, but not a signal for α-actinin. Scale bar: 100 μm. (H) Quantitative analysis of the data (n = 3 experiments in triplicate). *P < 0.05; **P < 0.01; ***P < 0.001.

The data presented here agree well with the antihypertrophic effect of adenosine (36) and A₁R activation (23). In line with this, overexpression of A₁R protected mice against myocardial infarction (37). Individual studies on both A₁Rs point toward a cardioprotective role (32–34) through vasodilation and immunostimulation (32, 33) or the reduction of cardiac fibrosis after myocardial infarction (32, 35). With respect to the latter, it was shown that extracellular adenosine accumulation was inversely correlated with CF proliferation in vitro (38). Our findings now substantiate a functional link between cAMP secretion and A₁R activation, based on the following: (a) secreted cAMP has an antifibrotic effect on the heart, (b) antagonists against A₁R interfere with this effect in vivo, and (c) this subtype induces intracellular cAMP formation in CFs (thus activating antifibrotic pathways).

The protective effects of A₁R and A₃R agonists and early approval of adenosine as an antiarrhythmic drug supported a series of agonist-based clinical studies (18, 39). Our results also contribute to our understanding of the therapeutic efficacy of adenosine receptor agonists. By mimicking the action of CM-derived cAMP, these drugs exploit an intrinsic mechanism that protects the myocardium from the detrimental consequences of prolonged βAR stimulation. Since earlier studies had indicated that exogenously added cAMP can alter the morphology or physiology of other organs (40), cell-to-cell communication via cAMP may be of broader relevance and represent a common principle.

Methods
Reagents. ZM-241385, PSB-1115, SCH-442416 and POM-1 were purchased from Tocris Biosciences. 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), HEPES, and Tris were obtained from Applichem. Disodium hydrogenophosphate was purchased from Carl Roth. 4-Aminopyrine, AMP, ATP, calcium chloride, choline oxidase, DMSO, p-nitrophenyl phosphate, magnesium chloride, 1-oleoyl-sn-glycero-3-phosphocholine (LPC) (18:1), peroxidase from horseradish, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydroxide, sodium tetraborate (borax), 3-(N-ethyl-3-methylamino)-2-hydroxypropyl sulfonic acid (TOOS), and uridine were obtained from Sigma-Aldrich. Human recombinant soluble ENTPDase3 and human recombinant soluble alkaline phosphatase (TNAP), expressed in CHO cells, were also obtained from Applichem. Human recombinant soluble ENTPDase1 and -2, and human recombinant soluble NT5E, expressed in CHO cells, were also obtained from R&D Systems GmbH. Human recombinant soluble ENTPDase1 and -2, and human recombinant soluble NT5E, expressed in CHO cells, were also obtained from R&D Systems GmbH.

Human recombinant soluble ENTPDase3 and human recombinant soluble alkaline phosphatase (TNAP), expressed in NSO cells from murine myeloma, were obtained from R&D Systems GmbH. Human recombinant soluble ENTPDase1 and -2, and human recombinant soluble NT5E, expressed in CHO cells, were also obtained from R&D Systems GmbH. Human recombinant soluble ENTPDase3 and human recombinant soluble alkaline phosphatase (TNAP), expressed in NSO cells from murine myeloma, were obtained from R&D Systems GmbH. The synthesis of PSB-16P (see Supplemental Figure 10 for structural information and numbering) started from 3-propyl-5-cyclopentane-carboxamide-6-aminouracil (41), which was obtained
Figure 2. Exogenous cAMP confers opposing changes of intracellular cAMP in CMs and CFs. (A) Quantification of endogenous adenosine receptor subtype expression in NRCMs (by qPCR, n = 3). (B) Real-time measurement of intracellular (intrac.) cAMP formation (shown as CFP/YFP ratio) in NRCMs transduced with a FRET-based sensor for cAMP. Cells were stimulated with Iso and cAMP in the absence (top row) or presence (bottom row) of the A1R antagonist DPCPX (100 nM). Scale bar: 10 μm. (C) Intracellular cAMP formation in NRCMs in the presence of Iso, with further addition of cAMP to the medium (100 μM, black), and in the absence or presence of antagonists against A1R (100 nM DPCPX, yellow), A2AR (100 nM SCH-442416, green), A2BR (500 nM PSB-1115, green), A3R (100 nM VUF-5574, blue), and the nonspecific adenosine receptor antagonist DPPX (10 nM, red). Representative tracings for the different treatment groups. (D) Quantitative analysis of the results. (E) Quantification of endogenous adenosine receptor subtype expression in isolated NRCF (by qPCR, n = 4, 2 days of culture). (F–H) Data derived from NRCF in an order analogous to that in the series above. In NRCF, exogenously added cAMP promoted the formation of intracellular cAMP (F and G). Scale bar: 10 μm. Inhibition of A2AR or of A2BR (green tracings/bar) prevented the intracellular response to extracellular cAMP. (H) Quantitative FRET data were obtained from at least 9 cells/group. *P < 0.05; **P < 0.01; ***P < 0.001.
by standard procedures. Amidite 1 was dissolved in dry dimethylformamide (DMF) in the presence of dry potassium carbonate, and alkylation in position 1 was performed with 3-iodo-1-propyl acetate as in previously described procedures (42). The subsequent ring closure to obtain xanthine derivative 3 was carried out in refluxing 1,1,1,3,3,3-hexamethyldisilazane (HMDS) in the presence of a catalytic amount of ammonium sulfate, followed by deacetylation in potassium hydroxide/methanol at room temperature to obtain compound 4.

Finally, compound 4 was phosphorylated at the hydroxy group using phosphorus oxychloride in trimethyl phosphate (43). SYL-001 was identified as a potent and highly selective inhibitor of ENPP1 (Ki 26.9 nM, human ENPP1, > 1000-fold selective versus other ectonucleotidases). It displayed a noncompetitive mechanism of inhibition (for details, see Supplemental Figure 3, A–C). All other agonists, antagonists, and cAMP were purchased from Sigma-Aldrich. Reagents were dissolved as suggested by the manufacturer.

Figure 3. CM-derived secreted cAMP promotes intracellular cAMP formation in CFs. (A) Quantitation of extracellular cAMP in supernatants from cultured cardiomyocytes and CFs treated with 10 μM Iso or PBS. CMs from mice deficient in β1- and β2-adrenoceptors (Adrb1−/− Adrb2−/−) did not show Iso-induced extracellular cAMP accumulation. A phosphodiesterase inhibitor (IBMX, 300 μM) was added to prevent cAMP degradation. n = 4–6 experiments in duplicate. (B) FRET-based study design to assess cell-to-cell communication via cAMP. CFs from Adrb1−/− Adrb2−/− mice (thus nonresponsive to Iso) were infected with an adenovirus encoding a FRET sensor for intracellular cAMP formation. To these CFs (green), coM from Iso-treated CMs (yellow) was added, and adenylyl cyclase activation in these CFs was monitored by the change in FRET signal of the cAMP sensor. (C–E) Intracellular cAMP formation in sensor-infected fibroblasts after addition of conditioned or control medium from WT and Abcc4-deficient CMs. (C) A time course of cAMP formation in CFs in the presence of coM from Iso-treated CMs with WT or Abcc4−/− background. Scale bar: 10 μm. (D) Representative FRET recordings from CFs after incubation with coM from WT CMs without or with Iso treatment. Data shown in red or orange, respectively, were obtained using medium from CMs treated with Iso and the adenosine receptor antagonist DPCPX or from Iso-treated Abcc4−/− CMs. Arrow indicates application of Iso or PBS. (E) Quantitative analysis of the results. n = 3–6 experiments with 2–4 cells each. **P < 0.01.
and repeatedly backcrossed to FVB mice to greater than 99% FVB (46). *Adrb1*–/– *Adrb2*–/– mice were generated by Rohrer et al. (20). All genotypes were verified by PCR analysis.

Isolation of CMs and CFs from mouse and rat hearts. Neonatal rat CMs (NRCMs) and neonatal rat CFs (NRCFs) were isolated from 1- to 2-day-old Sprague-Dawley rats after decapitation, as described previously (47). Cells were cultured in MEM with 5% FCS on uncoated culture dishes. For coculture experiments, NRCMs were layered onto preadhered *Adrb1*–/– *Adrb2*–/– adult CFs that had been infected 48 hours earlier with the Adv cAMP sensor. AMCMs and AMCFs were isolated from WT, *Adrb1*–/– *Adrb2*–/–, or *Abcc4*–/– mice. Briefly, hearts were extracted and coronary arteries were perfused with buffer A (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄, 12 mM NaHCO₃, 10 mM KHCO₃, 10 mM HEPES, and 30 mM taurine) in a retrograde fashion by cannulation of the aorta. Collagenase type II (Worthington) was added to the buffer to enzymatically dissociate ventricular cells. The disintegrated tissue was allowed to settle for 10 minutes at 37°C before the CF-enriched supernatant (supernatant A) and the pellet-containing CMs were resuspended in buffer B (47.5 ml

Animal models. As a model for chronic adrenergic stimulation, miniosmotic pumps (Alzet) containing (–)– Iso and PE (delivering 30 mg/kg/d each) were implanted subcutaneously into 10-week-old FVB male mice (animals were randomly assigned with parallel group design). Animals were continuously infused for 7 days with Iso/PE alone or together with CAMP (30 mg/kg/d), the A₁R antagonist (PSB-16P, 5 mg/kg/d), the A₂R antagonist (MSX-3, 5 mg/kg/d), or the ENPP1 and adenosine receptor antagonist (DPSPX 10 mg/kg/d). Then mice were sacrificed to determine parameters of cardiac hypertrophy and fibrosis. For the analysis of collagen deposition, paraffin sections of left ventricular myocardium were stained with Sirius Red and Fast Green. Collagen content was calculated as the percentage of the area in each section that was stained with Sirius Red. Thoracic aortic constriction was performed on 8-week-old male C57BL/6 N mice essentially as described previously (44). In sham surgery, only the chest was opened, but no ligation of the aorta was carried out.

*Abcc4*–/– mice were established by the John Schuetz laboratory (45) (St. Jude Children’s Research Hospital, Memphis, Tennessee, USA) and repeatedly backcrossed to FVB mice to greater than 99% FVB (46). *Adrb1*–/– *Adrb2*–/– mice were generated by Rohrer et al. (20). All genotypes were verified by PCR analysis.

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**Figure 4.** Intercellular communication through secreted cAMP between CMs and CFs in cocultures and living cardiac tissue. (A) Experimental scheme to assess the role of secreted cAMP in cocultures of CMs and CFs. Primary CFs from *Adrb1*–/– *Adrb2*–/– mice (green) were infected with an adeno virus for the expression of a FRET-cAMP sensor (termed sensor CFs). WT NRCMs (yellow) were plated on top of the cultured primary CFs. The response of CFs to CM-derived cAMP (after stimulation with 10 μM Iso) was monitored by FRET. (B) Representative FRET recordings in CFs after iso-induced cAMP export from CMs. PBS served as a negative control. (C) Quantification of the results. n = 10–15 cells (3–6 independent experiments). **P < 0.01, determined by unpaired t test with Welch’s correction. (D) Multicolor 2-photon images of a slice from cultured mouse myocardium to which sensor CFs had been transplanted. Histochemical stainings were for phalloidin (red), adenovirus-encoded cAMP-sensor (YFP, green), and propidium iodide (PI, blue). Image perspectives are overhead (large), horizontal (top), and vertical (right) cross sections. Arrowheads indicate the addition of isoproterenol or PBS. Scale bar: 20 μm. (E) cAMP formation in individual CFs after incubation of WT myocardial slices with Iso (black circles) or PBS (white circles, black outline). Data from an analogous experiment with slices from *Abcc4*–/– mice are displayed in red (red circles, Iso; white circles, red outline, PBS). (F) Quantification of the results. n = 7–15 cells from 3–5 independent experiments. **P < 0.001, NS, not significant versus WT; Iso determined by 2-way ANOVA with Bonferroni’s post hoc analysis.
perfusion buffer A, 2.5 ml FCS, 62.5 ml 10 μM CaCl$_2$). CaCl$_2$ was gradually added back to yield a final concentration of 100 μM, and the isolated cells were preplated in MEM (5% FCS, 10 mM 2,3-butanedione monoxime, 2 mM L-glutamine, and 1% penicillin/streptomycin) for 1 hour at 37°C and 1% CO$_2$. For CFs, supernatant was centrifuged for 5 minutes (225 g) and the pellet was resuspended in 5% FCS MEM culture medium, followed by plating on a 6-cm culture dish.

Assessment of CM hypertrophy. NRCMs were plated onto optically optimized 96-well plates (ibidi) in MEM medium containing 1% FCS. Twenty-four hours later, the medium was exchanged to 0.1% FCS in MEM medium in the presence or absence of 50 μM PE, 10 μM Iso, 100 μM cAMP, 100 nM DPCPX, 100 nM ZM-241385, or 100 μM Iso, thereby engaging Gs to inhibit intracellular cAMP formation. On CFs (right), adenosine activates its predominant receptor subtypes, A$_1$R and A$_2$BR. Their coupling to Gi activates adenyl cyclase, thus enhancing cAMP formation, which, in this cell type, inhibits proliferation and extracellular matrix deposition (thus preventing cardiac fibrosis).

Figure 5. Model of signal transmission between adrenergically stimulated CMs and CFs through secreted cAMP. In CMs (left), stimulation of βARs activates adenylate cyclase (AC) via Gs, causing rapid cAMP formation. This second messenger has the potential to elicit inotropic effects and CM hypertrophy and apoptosis. Alternatively, ABC proteins, in particular ABC4, export cAMP, which is stepwise metabolized by ENPP1 and NTSE to adenosine. Adenosine feeds back onto vical CMs through binding to its predominant adenosine receptor subtype, A$_1$R, thereby engaging Gi to inhibit intracellular cAMP formation. On CFs (right), adenosine activates its predominant receptor subtypes, A$_1$R and A$_2$BR. Their coupling to Gi, activates adenyl cyclase, thus enhancing cAMP formation, which, in this cell type, inhibits proliferation and extracellular matrix deposition (thus preventing cardiac fibrosis).
isolated from adult mouse hearts, either from WT or the \textit{Abcc4}–/– genotype (as a source for CMs) or from the \textit{Adrb1}–/– \textit{Adrb2}–/– genotype (for CFs). The latter were seeded onto coverslips and infected with adenovirus to express the Epac1-cAMP sensor (see above). The CM pellet was resuspended in 10 ml buffer, then split into 2 aliquots, and centrifuged again (1 minute, 100 g). Aliquots were resuspended in 500 \mu l buffer D (137 mmols/l NaCl, 5.4 mmols/l KCl, 2 mmols/l CaCl\textsubscript{2}, 1 mmols/l MgCl\textsubscript{2}, 10 mmols/l HEPES, pH 7.3), one containing Iso (10 \mu M), the other un-supplemented as a control. After incubation for 1 hour (37°C, 5% CO\textsubscript{2}), cells were centrifuged and the conditioned media (Iso-coM and control-coM, respectively) were superfused to the aforementioned CFs during FRET recording. An alternative approach without intermediate cell centrifugation employed CMs and CFs from neonatal rat hearts: NRCMs were isolated from 1- to 2-day-old Sprague-Dawley rats as above and seeded (1.5 x 10\textsuperscript{5} cells/cm\textsuperscript{2}) after 24 hours, 300 \mu l of buffer D was added, either including Iso (10 \mu M) or without Iso addition. After 1 hour at 37°C, 5% CO\textsubscript{2}, the Iso-coM and control-coM were collected and the conditioned media (Iso-coM and control-coM, respectively) were superfused to the sensor CFs. For adenosine receptor antagonists, CFs were isolated from \textit{Adrb1}–/– \textit{Adrb2}–/– mice and were infected with adenovirus to express the Epac1-cAMP sensor (see above).

**Ectonucleotide pyrophosphatase/phosphodiesterase assays.** ENPP1 and ENPP3 inhibition assays were carried out at 37°C in a final volume of 100 \mu l. The reaction mixture contained 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 10 mM CHES, pH 9.0, and 400 \mu M ATP as substrate. Solutions (20 \mu l) of SYL-001 (various concentrations) in enzyme assay buffer were added, and the reaction was initiated by the addition of 20 \mu l of human ENPP1 (1.7 \mu g) or human ENPP3 (43 \mu g), respectively. The mixture was incubated for 30 minutes (ENPP1) or 60 minutes (ENPP3), respectively, and terminated by heating at 90°C for 3 minutes. After cooling the reaction samples on ice, they were transferred into CE vials and injected into the CE instrument. The operation conditions in CE analyses were as follows: all experiments were carried out using a P/ACE MDQ Capillary Electrophoresis System (Beckman Instruments) equipped with a DAD Detection System. Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT obtained from Beckman Coulter (Fullerton). The electrophoretic separations were carried out using a polyacrylamide-coated capillary (60 cm [50 cm effective length], 50 \mu m [20 psi]; obtained from CS-Chromatography). Electrophoretic injections were performed using a voltage of -6 kV for 60 seconds, and separations were carried out by a voltage of -20 kV. Analytes were detected using direct UV absorbance at 260 nm. The capillary temperature was kept constant at 15°C, and the temperature of the storing unit was adjusted to 15°C. The running buffer consisted of 100 mM phosphate buffer (pH 6.5). Between separations, the capillary was washed with water for 2 minutes (20 psi) and subsequently with running buffer for 2 minutes (20 psi) before each injection. All experiments were performed twice in triplicate. The Ki values were determined by curve fitting of the data using Prism (GraphPad Software).

**Inhibitory activity at ectonucleoside triphosphate diphosphohydrolase assays.** Inhibitory activity of human and human NT5E activity was carried out in a mixture containing 4 mM CaCl\textsubscript{2}, 4 mM MgCl\textsubscript{2}, 40 mM HEPES, pH 7.4, and 400 \mu M ATP as substrate. Solutions (20 \mu l) of SYL-001 (various concentrations) in enzyme assay buffer were added, and the reaction was initiated by the addition of 20 \mu l of human ENTPD1 (0.3 \mu g), human ENTPD2 (0.5 \mu g), or human ENTPD3 (1.2 \mu g), respectively. The mixture was incubated for 30 minutes and terminated by heating at 99°C for 5 minutes. After cooling the reaction samples on ice, they were transferred into capillary electrophoresis (CE) vials and injected into the CE instrument. The operation conditions in CE analyses were as follows: all experiments were carried out using a P/ACE MDQ Capillary Electrophoresis System (Beckman Instruments) equipped with a DAD Detection System. Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT obtained from Beckman Coulter (Fullerton). The electrophoretic separations were carried out using a polyacrylamide-coated capillary (60 cm [50 cm effective length], 50 \mu m [20 psi]; obtained from CS-Chromatography). Electrophoretic injections were performed using a voltage of -6 kV for 60 seconds, and separations were carried out by a voltage of -20 kV. Analytes were detected using direct UV absorbance at 260 nm. The capillary temperature was kept constant at 15°C, and the temperature of the storing unit was adjusted to 15°C. The running buffer consisted of 100 mM phosphate buffer (pH 6.5). Between separations, the capillary was washed with water for 2 minutes (20 psi) and subsequently with running buffer for 2 minutes (20 psi) before each injection. All experiments were performed twice in triplicate. The Ki values were determined by curve fitting of the data using Prism (GraphPad Software).
quenty terminated by heating at 99°C for 5 minutes. Finally, 50 µl of the reaction mixture was transferred into mini-CE vials containing 50 µl of the internal standard uridine (final concentration, 6.25 µM).

The operation conditions in CE were as follows: P/ACE MDQ capillary electrophoresis system, fused silica capillary (40 cm [30 cm effective length] × 75.5 µm id); obtained from Polymicro Technologies), hydrodynamic injection (0.5 psi, 5 s), separation voltage of 15 kV, running buffer (40 mM borax and 100 mM SDS at pH 9.0), and detection at 260 nm. Between separations, the capillary was washed with 0.1 N aqueous (aq.) NaOH solution for 2 minutes (30 psi) and subsequently with running buffer for 1 minute (30 psi) before each injection. All experiments were performed twice in triplicate. The Ki values were determined by curve fitting of the data using Prism.

**Tissue-nonspecific alkaline phosphatase assays.** Reactions for tissue-nonspecific alkaline phosphatase (ALPL) inhibition studies were performed in a 96-well plate in a total volume of 100 µl. Assay buffer containing 1 mM CaCl₂, 2 mM MgCl₂, 10 mM CHES, pH 10.5, various concentrations of SYL001, and 400 µM p-nitrophenyl phosphate as a substrate were used. The reaction was initiated with 0.12 µg of human ALPL, and after 30 minutes of incubation at 37°C, the liberated p-nitrophenolate was measured colorimetrically at 400 nm. All experiments were performed twice in triplicate. The Kᵢ values were determined by curve fitting of the data using Prism 5.0.

**Immunohistochemical analyses.** The cross-sectional area in CMs was determined on 6-µm-thick paraffin-embedded tissue sections of left ventricular myocardium stained with Alexa Fluor 647-labeled wheat-germ agglutinin (WGA) (Life Technologies) to determine cell borders and SYTOX Green (Life Technologies) to detect nuclei. Images were taken from areas of transversely cut muscle fibers by confocal microscopy (Leica TCS SP5 II, ×20 objective; laser lines, 488 nm for SYTOX Green and 633 nm for WGA). Individual cells were analyzed in an automated manner using morphology filters of MetaMorph software (Molecular Devices) to draw lines separating individually cells based on WGA staining and to exclude cells with nuclei touching a cell border. Thresholding was applied to exclude regions of background (no cells) or extensive fibrosis. The average area of myocytes with centralized nucleus in 1 section (n = 59–200) was calculated using the MetaMorph integrated morphometry analysis function. Apoptosis was assessed by TUNEL staining using the In Situ Cell Death Detection Kit, TMR Red (Roche), according to the manufacturer’s instructions for treatment of paraffin-embedded tissue. Briefly, sections of paraffin-embedded mouse hearts (6–8 µm) were rehydrated, followed by 5 minutes of microwave irradiation in antigen-retrieval solution (Dako). The samples were incubated with TUNEL reaction mixture for 1 hour at 37°C in the dark. SYTOX Green (Life Technologies) was added for nuclear counterstaining. Both a negative control (without enzyme solution) and a positive control (pretreatment with DNase for 10 minutes at room temperature) were included. Images of whole-heart sections were acquired by a confocal microscope (×20 objective, laser lines, 488 nm for SYTOX Green and 633 nm for TMR). Red (TUNEL positive) and green (210,000–340,000 per group) nuclei were automatically counted using an image analysis algorithm (MetaMorph). To visualize the embedding of sensor CFs into myocardial slices, tissue was fixed in 4% PFA overnight at 4°C. After permeabilization with 1% Triton-X 100 for 30 minutes at room temperature and RNase treatment (100 µg/ml, 20 minutes, 37°C), slices were stained overnight at 4°C with Alexa Fluor 647 phallolidin (1:100, Life Technologies), followed by 2 hours incubation at room temperature with secondary antibody Alexa Fluor 488 goat anti-rabbit (Life Technologies). Nuclei were stained with propidium iodide (1:200, Life Technologies) for 15 minutes at 37°C. Slices were mounted in Aquatex (Merck), and confocal imaging was performed.

**Quantitative real-time PCR.** Total RNA was prepared with RNeasy Mini Kits (QiAGEN), and 500 ng was reverse transcribed using a standard protocol (Superscript II, Invitrogen). Quantitative real-time PCR amplification of adenosine receptor mRNA was performed with the primers listed below, using the FastStart universal SYBR Green Master Mix (Roche). The specificity of each primer set was monitored by analyzing the dissociation curve. The sample volume was 12.5 µl, containing 1× SYBR Green Master Mix, 400 nM gene-specific primers, and a 2.5 µl template. Sequences of primers used for real-time PCR were as follows (gene symbols and species followed by sequences of forward and reverse primers): Adora1 rat (5′-ATTCGTGGTGGGATGATACCC-3′, 5′-GAATCCAGCAGCCGACCTAT-3′); Adora2a rat (5′-GCAAGATCTTCTTATTTACG-3′, 5′-CGCCTCTACAACCTGTCA-3′); Adora2b rat (5′-TCTCATCTT-TAGCTCTTGG-3′, 5′-TCTCTTCTGCTGTGTTC-3′); Adora3 rat (5′-CTCGCGTCAACGGTAG-3′, 5′-GTCGCCACCAAGAAAGCA-3′); Abcc4 rat (5′-CAACAGAGATCCGGGAGAA-3′, 5′-TCTT-GGACGAAGGACATCG-3′); Gapdh rat (5′-TGCAACATCTCCT-CAAGATTGTC-3′, 5′-GCCATGGGCTGTGATCA-3′); RPL32 rat (5′-TCTTGTCACAAATGTCAGG-3′, 5′-TGTCGCTTCTTACGG-3′); Col1a2 mouse (5′-AGCTCTTCGAGCTGATG-3′, 5′-ACCACACGCTTCTTTCAT-3′); Col3a1 mouse (5′-ACGACAAATCTACTACAGCTTC-3′, 5′-CTCATT-GCCCTTGCTGTT-3′); Myh7 mouse (5′-ACTGTCACACTAGAAGGTC-3′, 5′-TGCGATATGGTTACTCTCAGG-3′); Adora1 mouse (5′-GTATGGTGGGGTGTAAGG-3′, 5′-AGTACGTTCTGAGGCTGG-3′); Adora2a mouse (5′-TGCAAGAAGGTCAACACT-3′, 5′-ACAACGGCCAGAGAGGAG-3′); Adora2b mouse (5′-GCTATGATTGGGCTGTGAG-3′, 5′-GACAACGTAGATTGGGGTT-3′); Adora3 mouse (5′-TCTCTGTATTACACGGGACT-3′, 5′-TCTACTGCTTGCCTTCACAGCTTC-3′); and Gapdh mouse (5′-GTGAAGGTCGCTGTAAGG-3′, 5′-TCCTGTAGGAGGACATCTC-3′).

**Statistics.** All quantitative data are reported as mean ± SEM. Statistical analysis was performed with the Prism software package (GraphPad version 6). Data distribution was assessed by a Shapiro-Wilk test for normality. Common variance was tested using the F-test or Bartlett’s test. Differences between 2 means were assessed by a 2-tailed paired or unpaired t test. Differences among multiple means were assessed, as indicated, by 1-way or 2-way ANOVA followed by Bonferroni’s test analysis. If the sample number did not suffice to test for normality (p < 0.05 per group), nonparametric tests (Mann-Whitney U test or 1-way ANOVA followed by Holm-Sidak’s test analysis) were used. A P value of less than 0.05 was considered significant.

**Study approval.** Animal care and experimental procedures were approved by the local authorities (Regierung von Oberbayern, Munich, Germany).

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