Corrections

MEDICAL SCIENCES
Correction for “Regulation of bone remodeling by vasopressin explains the bone loss in hyponatremia,” by Roberto Tamma, Li Sun, Concetta Cuscito, Ping Lu, Michelangelo Corcelli, Jianhua Li, Graziana Colaianni, Surinder S. Moonga, Adriana Di Benedetto, Maria Grano, Silvia Colucci, Tony Yuen, Maria I. New, Alberta Zallone, and Mone Zaidi, which appeared in issue 46, November 12, 2013, of Proc Natl Acad Sci USA (110:18644–18649; first published October 28, 2013; 10.1073/pnas.1318257110).

The authors note that Fig. 1 appeared incorrectly. The corrected figure and its legend appear below.

![Fig. 1. Bone cells express Avprs. Immunofluorescence micrographs (A) and Western immunoblotting (B) show the expression of Avpr1α in osteoblasts and osteoclasts, and as a function of osteoblast (mineralization) and osteoclast (with Rankl) differentiation. The expression of Avp (ligand) and Avpr1α (receptor) in osteoblasts is regulated by 17β-estradiol, as determined by quantitative PCR (C) and Western immunoblotting (D). (Magnification: A, 63×.) Because Avp is a small peptide, its precursor neurophysin II is measured. Statistics: Student t test, P values shown compared with 0 h. Stimulation of Erk phosphorylation (p-Erk) as a function of total Erk (t-Erk) by Avp (10−8 M) in osteoclast precursors (preosteoclasts), osteoclasts (OC), and osteoblasts establishes functionality of the Avpr1α in the presence or absence of the receptor inhibitor SR49059 (10−8 M) (E). Western immunoblotting showing the expression of Avpr2 in preosteoclasts, OCs (F), and osteoblasts (G) isolated from Avpr1α−/− mice, as well as in MC3T3.E1 osteoblast precursors (G). Functionality of Avpr2 was confirmed by the demonstration that cells from Avpr1α−/− mice remained responsive to AVP in reducing the expression of osteoblast differentiation genes, namely Runx2, Osx, Bsp, Atf4, Opn, and Osteocalcin (quantitative PCR, P values shown) (H). Only relevant bands from Western blots are shown, with gaps introduced where empty lanes are excised to conserve space.

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PHARMACOLOGY

The authors note that an additional affiliation should be listed for Torsten Christ and Alberto Kaumann. The new affiliation should appear as Department of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf. The corrected author and affiliation lines appear below. The online version has been corrected.

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PHYSIOLOGY

The authors note that the author name Timo Myöhänen should instead appear as Timo Myöhänen. The corrected author line appears below. The online version has been corrected.

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Arrhythmias, elicited by catecholamines and serotonin, vanish in human chronic atrial fibrillation

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Edited* by Lutz Birnbaum, National Institute of Environmental Health Sciences, Research Triangle Park, NC, and approved June 23, 2014 (received for review December 30, 2013)

Atrial fibrillation (AF) is the most common heart rhythm disorder. Transient postoperative AF can be elicited by high sympathetic nervous system activity. Catecholamines and serotonin cause arrhythmias in atrial trabeculae from patients with sinus rhythm (SR), but whether these arrhythmias occur in patients with chronic AF is unknown. We compared the incidence of arrhythmic contractions caused by norepinephrine, epinephrine, serotonin, and forskolin in atrial trabeculae from patients with SR and patients with AF. In the patients with AF, arrhythmias were markedly reduced for the agonists and abolished for forskolin, whereas maximum inotropic responses were markedly blunted only for serotonin. Serotonin and forskolin produced spontaneous diastolic Ca\textsuperscript{2+} releases in atrial myocytes from the patients with SR that were abolished or reduced in myocytes from the patients with AF. For matching L-type Ca\textsuperscript{2+}-current (I\textsubscript{Ca,L}) responses, serotonin required and produced ~100-fold less cAMP/PKA at the Ca\textsuperscript{2+} channel domain compared with the catecholamines and forskolin. Norepinephrine-activated I\textsubscript{Ca,L} responses were decreased by inhibition of Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) in myocytes from patients with SR, but not in those from patients with AF. Agonist-evoked phosphorylation by CaMKII at phospholamban (Thr-17), but not of ryanodine2 (Ser-2814), was reduced in trabeculae from patients with AF. The decreased CaMKII activity may contribute to the blunting of agonist-evoked arrhythmias in the atrial myocardium of patients with AF.

Catecholamines and serotonin elicit arrhythmias in atrial trabeculae and arrhythmogenic diastolic Ca\textsuperscript{2+} releases in myocytes from patients with normal sinoatrial rhythm (SR). Arrhythmic events are greatly blunted in the myocardium of patients with chronic atrial fibrillation (AF). The mediation by cyclic AMP-dependent protein kinase of L-type Ca\textsuperscript{2+}-current (I\textsubscript{Ca,L}) responses to agonists, lusitropy, and protein phosphorylation are preserved in AF. The vanishing of agonist-evoked arrhythmias in AF is associated with the disappearance of an I\textsubscript{Ca,L} response, facilitated by Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII), in myocytes from patients with SR, and decreased phosphorylation of phospholamban, but not ryanodine, by this enzyme. The disappearance of arrhythmias and associated reduction of CaMKII functions questions the use of CaMKII inhibitors in AF, as has been proposed by other groups.


The authors declare no conflict of interest.

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stimulate both kinases would be expected to evoke more arrhythmias in patients with AF. However, in vitro induction of arrhythmias and activation of PKA and CaMKII by catecholamines and 5-HT were not assessed in tissues from patients with AF. Thus, we compared the effects of endogenous agonists on force and arrhythmias in intact trabeculae, as well as the $I_{\text{Ca,L}}$, Ca$^{2+}$ transients (CaTs), and diastolic Ca$^{2+}$ release, in atrial myocytes obtained from patients with SR and patients with AF. Functional measurements, including relaxation, were supplemented by Western blot analysis of relevant targets of PKA and CaMKII. Chronic AF caused a marked decrease in agonist-evoked arrhythmias, associated with a decrease in some CaMKII-catalyzed functions but unchanged PKA functions.

Results

In AF Arrhythmias Elicited by Catecholamines, 5-HT, and Forskolin Are Markedly Reduced, and Inotropic Responses to 5-HT Are Selectively Depressed. The maximum inotropic response to 5-HT, but not to norepinephrine (NE), epinephrine (EPI), or forskolin (FSK), was markedly reduced in trabeculae of patients with AF. The inotropic potencies (−log EC$_{50}$ values) of the four drugs were decreased (Fig. 1 and SI Appendix, Table S2). NE, EPI, 5-HT, and FSK caused concentration-dependent arrhythmic contractions with maximum incidences of 17%, 14%, 4%, and 14%, respectively, in trabeculae of patients with SR. In trabeculae of patients with AF, the incidence of arrhythmic contractions (Fig. 1) was markedly reduced (≤2% for the agonists, abolished for FSK). Our results with 5-HT are in line with previous findings in atrial myocytes from patients with AF compared with patients with SR (15).

Biphasic CaTs and Spontaneous Diastolic Ca$^{2+}$ Releases Induced by 5-HT and FSK in Myocytes from Patients with SR Are Blunted in Patients with AF. In the patients with AF, the inotropic and arrhythmic responses to 5-HT were markedly decreased, and the inotropic potency to FSK was reduced by sevenfold. FSK failed to elicit arrhythmias in AF (Fig. 1). Because $I_{\text{Ca,L}}$ responses to 5-HT were similar to the responses of catecholamines and FSK in myocytes from patients with SR and patients with AF (see below), the blunted arrhythmias in AF suggest a decrease in the cytoplasmic arrhythmogenic Ca$^{2+}$ surges in AF produced by 5-HT and FSK. CaTs in the absence of 5-HT and FSK were monophasic. In myocytes from patients with SR, both 5-HT and FSK increased CaTs, which became biphasic and often associated with monophasic spontaneous diastolic Ca$^{2+}$ releases (SDCRs) (Fig. 2, and SI Appendix, Figs. S1–S3). Interestingly, ISO (1 μM) caused similar results with biphasic CaTs and SDCRs (SI Appendix, Fig. S4). In AF, the CaTs generated by 5-HT and FSK were reduced and lost their biphasic shape (SI Appendix, Figs. S1–S3). SDCRs were absent (5-HT) or reduced (FSK) in AF (Fig. 2), in line with the failure of 5-HT and FSK to elicit arrhythmias.

Maximum $I_{\text{Ca,L}}$ Responses to NE, 5-HT, and FSK Are Similarly Reduced in AF, but Control by PKA Is Preserved. $I_{\text{Ca,L}}$ is the initial step in electromechanical coupling, believed to contribute to increased contractile force and generation of arrhythmias (16). The maximum $I_{\text{Ca,L}}$ responses to NE, EPI, 5-HT, and FSK were similar in patients with SR and similarly reduced by approximately one-third in patients with AF, but the potencies appeared unchanged in AF (Fig. 3 and SI Appendix, Table S3), consistent with preserved function of the receptors and adenylyl cyclase (AC). The reduced $I_{\text{Ca,L}}$ responses in myocytes from patients with AF could be related to reduced channel density or reduced PKA-catalyzed Ca$^{2+}$ channel phosphorylation and/or reduced access of cAMP to the channel domain.

To quantitatively assess the cAMP/PKA-dependent regulation of $I_{\text{Ca,L}}$ by the agonists and FSK, we diazoylated the cardiomyocytes with the nonhydrolyzable analog Rp-8-Br-cAMPS, which competes
with cAMP for binding to the regulatory unit of PKA (17). The more cAMP produced, the greater the amount of Rp-8-Br-cAMPS needed to block PKA activation. I_{Ca,L} was not modified by Rp-8-Br-cAMPS in patients with SR or those with AF (Fig. 4A) in the absence of agonists, inconsistent with regulation of basal I_{Ca,L} by cAMP-activated PKA. Rp-8-Br-cAMPS caused concentration-dependent reductions in the I_{Ca,L} responses to the agonists. The corresponding –log IC_{50} values of Rp-8-Br-cAMPS were not different for the agonists and FSK in myocytes from patients with SR and from patients with AF, respectively. (C and D) Examples of original CaT traces and calculated frequency of spontaneous CaTs before and after 1 and 2 min of FSK application in cardiomyocytes from patients with SR and patients with AF, respectively. 

**Fig. 2.** Effects of 5-HT and FSK on spontaneous Ca^{2+}\textsuperscript{2+} release in cardiomyocytes from patients with SR and AF. (A) Examples of original CaT traces before and after exposure to 5-HT (100 μM) in a cardiomyocyte, from a patient with SR (Upper) and a patient with AF (Lower). (Insets) Enlarged part of the traces. Arrows indicate spontaneous Ca^{2+}\textsuperscript{2+} release. (B) Calculated frequency of spontaneous CaTs before and after 1 and 2 min of 5-HT application in cardiomyocytes from patients with SR and patients with AF, respectively. (C and D) Examples of original CaT traces and calculated frequency of spontaneous CaTs before and after 1 and 2 min of FSK (10 μM) application in cardiomyocytes from patients with SR and patients with AF. *P < 0.05; **P < 0.01, 1 min vs. mean basal values; #P < 0.05, 2 min vs. basal values; all paired t tests. Numbers in the columns indicate myocytes/patients.

Lusitropy and PKA-Catalyzed Phosphorylation of Proteins by the Agonists and FSK Are Not Different Between Patients with SR and Patients with AF. The expression of proteins involved in lusitropy, PLB, troponin-I (Tn-I), and myosin binding protein C (Prot-C), as well as SERCA2, did not differ between patients with SR and patients with AF (SI Appendix, Fig. S5). Basal phosphorylation of PLB-Ser-16 was hardly detectable in trabeculae contracting for 1 h in the organ bath used for the relaxation measurements (SI Appendix, Figs. S6 and S7), suggesting that in the absence of agonists, this protein contributes little to relaxation. In contrast, basal phosphorylation of Tn-I-Ser23/24 and Prot-C-Ser-282 was detectable under these conditions, but did not differ between patients with SR and patients with AF (SI Appendix, Figs. S6 and S7). Our data are at variance with a report of increased phosphorylation of PLB-Ser-16 and decreased phosphorylation of Prot-C-Ser-282 in atria from patients with AF, frozen in the operating theater (11). Thus, we compared the phosphorylation of PLB at Ser-16 in trabeculae frozen in the operating theater with trabeculae from the same patient that was frozen in the operating theater (11)....
In contrast to a previous report (11), here the phosphorylation of PLB at Ser-16 and Thr-17 was consistently lower in trabecular frozen in the operating theater from patients with AF than in those from patients with SR. The high basal phosphorylation levels in trabecular frozen in the operating theater likely reflect catecholamine surges during surgery. After contracting for 1 h in the organ bath, the endogenous catecholamines dissipated from the trabeculae, as revealed by the marginal phosphorylation levels. The decreased phosphorylation of basal PLB at Ser-16 and Thr-17 in AF correspond well to the rightward shift in the concentration-effect curves for agonists and FSK in trabeculae of patients with AF (Fig. 1 and SI Appendix, Table S2). The hastening of trabecular relaxation caused by 10 μM of the agonists and FSK did not differ between patients with SR and those with AF (SI Appendix, Fig. S9). Accordingly, the agonists and FSK increased phosphorylation of PLB-Ser-16, Tn-I-Ser-23/24, and Prot-C-Ser-282 similarly in trabeculae of patients with SR and patients with AF (Fig. 4B and SI Appendix, Fig. S7). In both groups, stimulation of PLB-Ser-16 by 5-HT was less pronounced than stimulation by NE, EPI, or FSK. Our results are consistent with an early report indicating that ISO-evoked relaxation and AC stimulation do not differ between patients with SR and patients with AF (18).

**KN-93 Reduces NE-Evoked Increases of I_{Ca,L} in Patients with AF, but Not in Patients with AF.** We next compared the role of CaMKII on basal I_{Ca,L} and the activation of I_{Ca,L} by NE in myocytes from patients with SR and patients with AF. As reported previously (13, 19), the CaMKII inhibitor KN-93, but not its inactive analog KN-92, reduced basal I_{Ca,L} in myocytes from patients with SR but not in those from patients with AF. In addition, KN-93 reduced the NE-evoked I_{Ca,L} responses in myocytes from patients with SR but not in those from patients with AF, consistent with a loss in AF of the NE-facilitated phosphorylation of the L-type Ca^{2+} channel by CaMKII (Fig. 5 A and B).

**Reduced Agonist-Evoked Phosphorylation of PLB by CaMKII, but No Change in RyR2 Phosphorylation Catalyzed by CaMKII and PKA in AF.** NE, EPI, and FSK induced the phosphorylation of PLB at Thr-17 in contracting trabeculae of both patients with SR and patients with AF, but to a lesser degree in the latter AF (Fig. 5C and SI Appendix, Fig. S7). The effects of 5-HT were small in patients with SR, but even smaller in those with AF (Fig. 5C and SI Appendix, Fig. S7B). Increased phosphorylation of RyR2 channels at Ser-2808, catalyzed by PKA (11, 12), and Ser-2814, catalyzed by CaMKII, has been implicated in the arrhythmogenic release of Ca^{2+} through leaky RyR2 channels (13, 20); however, in contrast to previous reports, neither basal phosphorylation (SI Appendix, Figs. S6 and S7) nor phosphorylation stimulated by agonists of RyR2 at Ser-2808 (Fig. 4B and SI Appendix, Fig. S7A) and Ser-2814 (Fig. 5C and SI Appendix, Fig. S7B) were different between patients with SR and those with AF.

**Discussion**

Surprisingly, we hardly detected arrhythmic contractions in atrial trabeculae of the patients with AF. This finding is inconsistent with the idea of leaky sarcoplasmic reticulum in AF. The agonists must have produced an additional Ca^{2+} load, as has been reported under βAR stimulation (21). The increased Ca^{2+} load facilitates the release process (22), consistent with the increased CaTs observed with 5-HT, FSK, and ISO in cardiomyocytes from patients with SR. βAR stimulation increases the Ca^{2+} sensitivity of the RyR2 channels and reportedly increases the likelihood of RyR2 clusters opening and triggering release from neighboring clusters.
CaTs are reportedly biphasic at room temperature (23) in the absence of agonists in human atrial myocytes. The early phase is apparently due to Ca\(^{2+}\)-induced Ca\(^{2+}\) release by I\textsubscript{Ca,L} from the longitudinal junctional sarcoplasmic reticulum. The late phase appears to occur through Ca\(^{2+}\) release from corbular reticulum, by Ca\(^{2+}\) released from neighboring RyR2 channels, independent of I\textsubscript{Ca,L}, as suggested by recent modeling (24). In myocytes from our patients with SR, Ca\(^{2+}\) transients were monophasic at 37 °C; however, a late phase became conspicuous with 5-HT, FSK, and ISO at 37 °C, suggesting that these agents cause propagation of Ca\(^{2+}\) release from the periphery into the myocyte interior through collection of RyR2 channels, resulting in increased contractility and propensity to arrhythmias.

Arhythmogenic, delayed afterdepolarizations (DADs) have been reported with ISO via spontaneous diastolic Ca\(^{2+}\) releases, with a subsequent increase in inward depolarizing current induced by the Na\(^+-\)-Ca\(^{2+}\) exchanger (25), which in turn could elicit DADs. 5-HT, FSK, and ISO caused SDCRs in myocytes of patients with SR. In patients with AF, SDCRs were absent with 5-HT and reduced with FSK, suggesting that the blunting of arrhythmias elicited by 5-HT and FSK in AF are related, at least in part, to the reduction in SDCRs.

CaMKII participates in the generation of catecholamine-evoked arrhythmias in animal models. Arrhythmogenic effects of ISO were found to be antagonized by CaMKII inhibitors in rabbit ventricle (26). Increased Ca\(^{2+}\) leaking and DADs have been reported in myocytes from mice overexpressing CaMKII and ISO elicited ventricular arrhythmias that were prevented by KN-93 (27). Particularly relevant to AF, KN-93 prevented ISO-evoked arrhythmic activity in rabbit pulmonary veins (28).

We have demonstrated that the agonists and FSK caused PLB phosphorylation at Thr-17 by CaMKII, conceivably activated by preceding increased cytoplasmic Ca\(^{2+}\) levels owing to PKA- and CaMKII-mediated increased I\textsubscript{Ca,L}, as well as to Ca\(^{2+}\) release from the sarcoplasmic reticulum (29). SERCA, activated by the preceding PKA-catalyzed phosphorylation of PLB at Ser-16, would increase the Ca\(^{2+}\) load of the sarcoplasmic reticulum. SERCA’s Ca\(^{2+}\) pumping activity then may be further enhanced by phosphorylation of PLB at Thr-17 (29), resulting in additional Ca\(^{2+}\) overload of the sarcoplasmic reticulum and release and thus facilitating the appearance of SDCRs and arrhythmias observed with agonists and FSK.

Increased CaMKII-catalyzed phosphorylation of PLB and RyR2 in the absence of agonists have been reported in AF and implicated in the triggering and/or maintenance of this arrhythmia in human atrium (11, 13, 20). The reports of CaMKII-catalyzed hyperphosphorylation have suggested the therapeutic use of CaMKII inhibitors in AF (13, 30); however, our evidence indicates that CaMKII-dependent phosphorylation is either reduced or unchanged in human AF. Phosphorylation of PLB at Thr-17 in trabeculae in the operating theater (SI Appendix, Fig. S8) and in contracting atria by the agonists and FSK (Fig. 5) was reduced in AF. These findings, together with the loss of the CaMKII-facilitated component of both basal I\textsubscript{Ca,L} and agonist-evoked I\textsubscript{Ca,L} response, may contribute to the decrease in arrhythmias. Phosphorylation by CaMKII of RyR2 at Ser-2814 by the agonists and FSK was unchanged in patients with AF compared with patients with SR (Fig. 5), suggesting unchanged modulation of Ca\(^{2+}\) release through this RyR2 phosphorylation site.

The marked blunting or disappearance of atrial arrhythmias elicited by the agonists and FSK in AF also could be related in part to the more negative resting membrane potential. This hyperpolarization is caused by a twofold increase in the inward rectifying current (I\textsubscript{K1}) reported in myocytes of patients with AF compared with myocytes of patients with SR (31). Hyperpolarization in AF would reduce the likelihood of depolarizing currents reaching the threshold for arrhythmogenic depolarizations.

The maximum response of I\textsubscript{Ca,L} to the catecholamines, 5-HT, and FSK was similarly reduced by one-third in the patients with AF. But although the inotropic response to the catecholamines
and FSK was similar in patients with SR and those with AF, it was markedly reduced for 5-HT in patients with AF, suggesting uncoupling between the L-type Ca$^{2+}$ current and contractile processes. The low 5-HT$_R$ density compared with the density of β$_1$-AR (32) and the ~100-fold smaller sarcolemmal cAMP/PKA signals for 5-HT compared with NE and EPI (Fig. 4) already produce lower functional and biochemical signals in patients with SR (Figs. 4 and 5 and SI Appendix, Fig. S9). In patients with AF, the difference between reduced 5-HT signals and catecholamine signals became more pronounced (Figs. 4 and 5), possibly related to the well-known increase in atrial myocyte size in AF (3, 4). This would increase the diffusion path and dissipation of cAMP from its formation by the cell membrane-bound AC to its intracellular effectors. The especially small amounts of cAMP produced by 5-HT (Fig. 4.4) would be expected to reach these effectors at even lower concentrations in myocytes from patients with AF than in myocytes from patients with SR, thereby reducing the intracellularly relevant phosphorylations catalyzed by PKA.

Our evidence from human right atrium is a model for arrhythmias caused by endogenous catecholamines and 5-HT. Arrhythmias of this sort may trigger extrasystoles that could lead to paroxysmal AF or transient postoperative AF, the latter of which is often averted by treatment with βAR blockers (10). In contrast, βAR blockers neither affect the incidence of chronic AF (33) nor convert AF into SR (34) in patients with advanced heart failure, inconsistent with a contribution of endogenous catecholamines. Our work identifies mechanisms for the vanishing of catecholamine-evoked AF in atrium that has undergone remodeling. CaMKII appears to facilitate agonist-evoked arrhythmias in atrial myocardium of patients with SR; however, once chronic AF is established, agonists fail to elicit arrhythmias, probably related to atrial remodeling, which includes decreases in CaMKII-mediated processes. Because of this pathophysiological adaptation, it appears redundant to use CaMKII inhibitors in AF, as has been proposed by others.

**Materials and Methods**

Right atrial appendages were collected with informed consent from patients undergoing cardiac surgery at the Department of Heart Surgery, Dresden University of Technology. These studies were approved by the Medical Faculty Ethics Committee of Dresden University of Technology (document EK790799). Patient characteristics are detailed in SI Appendix, Table S1. The methodology for experiments with atrial trabeculae, IC$_{50}$, CaTs, and Western blots are described in SI Appendix, Materials and Methods. Data comparisons were made with ANOVA and nonpaired or paired t tests as appropriate, using GraphPad Prism and IBM SPSS 19.0.1.

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