Serotonin increases L-type Ca\(^{2+}\) current and SR Ca\(^{2+}\) content through 5-HT\(_{4}\) receptors in failing rat ventricular cardiomyocytes

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Birkeland JA, Swift F, Tovsrud N, Enger U, Lunde PK, Qvigstad E, Levy FO, Sejersted OM, Sjaastad I. Serotonin increases L-type Ca\(^{2+}\) current and SR Ca\(^{2+}\) content through 5-HT\(_{4}\) receptors in failing rat ventricular cardiomyocytes. Am J Physiol Heart Circ Physiol 293: H2367–H2376, 2007. First published July 27, 2007; doi:10.1152/ajpheart.01375.2006.—Rats with congestive heart failure (CHF) develop ventricular inotropic responsiveness to serotonin (5-HT), mediated through 5-HT\(_{2A}\) and 5-HT\(_{4}\) receptors. Human ventricle is similarly responsive to 5-HT through 5-HT\(_{4}\) receptors. We studied isolated ventricular cardiomyocytes to clarify the effects of 5-HT on intracellular Ca\(^{2+}\) handling. Left-ventricular cardiomyocytes were isolated from male Wistar rats 6 wk after induction of postinfarction CHF. Contractile function and Ca\(^{2+}\) transients were measured in field-stimulated cardiomyocytes, and L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) and sarcoplasmic reticulum (SR) Ca\(^{2+}\) content were measured in voltage-clamped cells. Protein phosphorylation was measured by Western blotting or phosphoprotein gel staining. 5-HT\(_{4}\)- and 5-HT\(_{2A}\)-receptor stimulation induced a positive inotropic response of 33 and 18% (both P < 0.05) and also increased Ca\(^{2+}\) transient (44 and 6%, respectively; both P < 0.05). I\(_{\text{Ca,L}}\) and SR Ca\(^{2+}\) content increased after 5-HT\(_{4}\)-receptor stimulation (57 and 65%; both P < 0.05). Phospholamban serine16 (PLB-Ser16) and troponin I phosphorylation increased by 26 and 13% after 5-HT\(_{4}\)-receptor stimulation (P < 0.05). 5-HT\(_{2A}\)-receptor stimulation increased the action potential duration and did not significantly change the phosphorylation of PLB-Ser16 or troponin I, but it increased myosin light chain 2 (MLC2) phosphorylation. In conclusion, the positive inotropic response to 5-HT\(_{4}\) stimulation results from increased I\(_{\text{Ca,L}}\) and increased phosphorylation of PLB-Ser16, which increases the SR Ca\(^{2+}\) content. 5-HT\(_{4}\) stimulation is thus, like β-adrenoceptor stimulation, possibly energetically unfavorable in CHF. 5-HT\(_{2A}\)-receptor stimulation, previously studied in acute CHF, induces a positive inotropic response also in chronic CHF, probably mediated by MLC2 phosphorylation.

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Serotonin increases L-type Ca\(^{2+}\) current and SR Ca\(^{2+}\) content through 5-HT\(_{4}\) receptors in the chronic phase of CHF, a 5-HT\(_{4}\)-receptor-mediated response appeared more prominent (40). A 5-HT\(_{4}\)-receptor response is also present in ventricular muscle strips from porcine hearts and explanted human CHF hearts (9).

In papillary muscles, the 5-HT\(_{4}\)-receptor induces a PIR with a shortening of the contraction-relaxation cycle (CRC), similar to the β-adrenoceptor-mediated PIR (40). A shortened CRC is considered a hallmark of cAMP-mediated inotropic effects (48) and suggests that the 5-HT\(_{4}\)-receptor, which is Gs coupled (27), induces a PIR mediated through cAMP. This would be consistent with data from human atrial myocytes (34) and with the increased PKA activity observed after 5-HT\(_{4}\)-receptor stimulation in porcine ventricle (9). However, the increase in cAMP found after 5-HT\(_{4}\)-receptor stimulation of failing rat LV myocardium was very small compared with that following β-adrenoceptor stimulation (40). Thus the role of cAMP in 5-HT\(_{4}\)-mediated signaling in the failing LV is still uncertain.

The effect of cAMP and PKA on CRC characteristics is mainly mediated through altered cellular Ca\(^{2+}\) handling and phosphorylation of troponin I (28). This way of increasing cardiac contractility is considered energetically unfavorable and contributing to the deleterious effects of β-adrenoceptor stimulation in heart failure (16, 29).

To test the hypothesis that 5-HT\(_{4}\)-receptor stimulation could be deleterious in CHF, similar to β-adrenoceptor stimulation, we recently treated rats with CHF after myocardial infarction with a 5-HT\(_{4}\)-an antagonist and found effects of treatment consistent with improved cardiac function (6). At the cellular level, studies of 5-HT\(_{4}\)-receptor signaling in cardiac myocytes have been restricted to atrial myocytes of pig and human (18, 34) [for review, see Kaumann and Levy (23)], where 5-HT\(_{4}\)-receptor stimulation has been linked to arrhythmia (21) or to virally transfected ventricular myocytes (11). Ventricular myocytes from failing rat hearts may represent a model system for 5-HT\(_{4}\) receptors in human ventricular cardiomyocytes. We therefore considered it important to determine the effect of 5-HT\(_{4}\)-receptor stimulation on intracellular Ca\(^{2+}\) homeostasis and phosphorylation of proteins involved in the excitation-contraction coupling in ventricular cardiomyocytes isolated from failing rat hearts.

The inotropic response following 5-HT\(_{2A}\)-receptor stimulation has been explored in acute CHF (41) but not in chronic CHF. In acute CHF, the 5-HT\(_{2A}\) receptor exerts its PIR through phosphorylation of myosin light chain 2 (MLC2) (41), which is known to increase myofilament Ca\(^{2+}\) sensitivity (33). Al-
though 5-HT2A stimulation did not have an effect on Ca2⁺-transient magnitude in our previous study (41), other changes in the Ca2⁺ cycling might still occur after 5-HT2A stimulation.

We have now explored inotropic responses to 5-HT in isolated ventricular cardiomyocytes from rats with chronic CHF and found PIRs mediated through both 5-HT2A and 5-HT4 receptors. Selective 5-HT4-receptor stimulation increased peak Ca2⁺ transient by increasing both L-type Ca2⁺ current (ICa,L) and sarcoplasmic reticulum (SR) Ca2⁺ load, whereas the 5-HT2A receptor mainly exerted its action independently of alterations in Ca2⁺ handling.

MATERIALS AND METHODS

CHF model and cell isolation. Animal care was according to the Norwegian Animal Welfare Act, conforming to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996), and all protocols were reviewed and approved by Institutional Animal Care Committee. Two animals per cage were given access to food and water ad libitum in a temperature-regulated room on a 12:12-h day/night cycle.

A myocardial infarction was induced in anesthetized (68% N2O-29% O2-3% Isoflurane; Abbot Scandinavia, Solna, Sweden) male Wistar rats (320 g) by proximal ligation of the left coronary artery as previously described (47). After 6 wk, CHF was verified as previously described (47). The lung weight was 4.1 ± 0.1 g (n = 85), and the LV end-diastolic pressure was 25.7 ± 0.7 mmHg in the rats (n = 85), which secured a safe diagnosis of CHF (47). Cardiomyocytes were isolated as previously described (41).

Contraction measurements. Contractions were recorded in field-stimulated (1 Hz) cardiomyocytes attached to laminin-coated coverslips by using a video edge-detection system (Crescent Electronics, Sandy, UT). The cells were superfused with solution A, containing (in mM) 140 NaCl, 5 HEPES, 5.4 KCl, 1 CaCl₂, 0.5 MgCl₂, 5.5 d-glucose, 0.4 Na₂HPO₄, pH 7.4 (37°C) and also containing blockers of adrenoceptors and muscarinic cholinergic receptors (0.1 μM prazosin, 1 μM timolol, 1 μM atropine). After steady-state contractions were achieved, 5-HT (10 μM) or isoproterenol (Iso; 10 μM) was added to the solution. The concentrations of 5-HT and Iso were chosen to obtain maximal responses (40, 46). Increase in the ratio of fractional shortening (FS) divided by time to peak contraction (TTP) is referred to as a PIR. Cardiomyocyte contraction rate is expressed as the maximum negative derivative of change in cell length [(dL/dt)max] and relaxation rate as the maximum derivative of increase in cell length [(dL/dt)max]. Relaxation time (RT50) was calculated as time from peak contraction to 50% relaxation.

In all experiments, 5-HT4 and 5-HT2A responses were measured by stimulation with 10 μM 5-HT in the presence of 0.1 μM ketanserin or 1.0 μM GR-113808, respectively. Also, timolol was excluded from the solutions during experiments using Iso.

Ca2⁺ imaging. Cardiomyocytes loaded (30 min, 23°C) with 20 μM fluo-4-AM (Molecular Probes, Eugene, OR) were superfused with solution A. Contraction and whole cell Ca2⁺ transients were recorded in field-stimulated cardiomyocytes (1 Hz, 37°C) simultaneously by using visible light and a mercury lamp exciting the cells at 485 nm. Emitted and reflected light were split into a video edge-detection system and a photomultiplier (Photon Technology International, Monmouth Junction, NJ) counting at 525 nm. After steady-state transients were achieved, 5-HT (10 μM) or Iso (10 μM) was added to solution A. Fluorescence data were presented as systolic fluorescence divided by diastolic fluorescence (F/F₀). Decay of the transient is expressed as the maximum negative derivative of F/F₀ [(−dF/dt)max] and the time from peak transient to 90% decay (TTD90).

ICa,L and SR Ca2⁺ load. Voltage-clamp experiments were performed with patch electrodes (3–5 MΩ) using an Axoclamp 2B in discontinuous mode or an Axopatch 200B, and data acquisition and analysis were performed with pCLAMP 9 software (Axon Instruments, Foster City, CA). Myocytes were superfused with solution B containing 20 mM CsCl, 1 mM MgCl₂, 135 mM NaCl, 10 mM HEPES, 10 mM glucose, 4 mM 4-aminopyridine, 1 mM CaCl₂, 0.1 mM prazosin, 1 μM timolol, and 1 μM atropine, pH 7.40 (37°C). The time from patch rupture to recording of ICa,L was standardized to 3 min. During this period, the cells were superfused with solution B or solution supplemented with 5-HT or Iso.

ICa,L was activated by test pulses (200 ms) in 10-nM increments from −50 to 20 mV and in 20-mV increments from 20 to 80 mV. The test pulses were preceded by 10 conditioning pulses (50 ms; 1 Hz) from −80 to 0 mV. ICa,L was normalized to cell capacitance and was presented as current density. Data are presented as current-voltage (I-V) plots. The area under the I-V plot is presented as area under the curve (AUC). To assess the degree of leftward shift of peak ICa,L induced by 5-HT, and β-adrenoceptor stimulation at comparable current magnitudes, a submaximal Iso concentration of 100 nM was used in these experiments.

After 10 conditioning pulses (50 ms, 1 Hz, from −80 to 0 mV), SR Ca2⁺ content was assessed by applying 10 mM caffeine for 10 s, holding at −50 mV. The inward Na⁺/Ca2⁺ exchange current (INa/Ca) was measured and integrated, and the integral was normalized to cell capacitance.

Action potential measurements. Action potentials (APs) were recorded (37°C) under current clamp by using high-resistance micro-electrodes filled with 3 M KCl (25–35 MΩ). Steady-state APs were achieved before 5-HT (10 μM) was added to solution A containing 113808 (1 mM) or ketanserin (0.1 μM). The cells were stimulated for another 3–5 min to obtain maximal effect of 5-HT on the APs. Data are expressed as time from initiation of the AP until 50 and 90% of the initial resting membrane potential was regained (APD50 and APD90, respectively).

Phosphorylation of Ca2⁺-handling proteins. In each cell-culture experiment, freshly isolated cardiomyocytes from three hearts were pooled. These cardiomyocytes were divided into five laminin-coated culture flasks (~90 cm²) containing cell-isolation solution (41). A total of 5–7 experiments were performed with each stimulation protocol. The cardiomyocytes were incubated (37°C) in solution A for 10 min and then field stimulated (1 Hz, 37°C) for 5 min. Thereafter, the cells were field stimulated for 4 min (1 Hz, 37°C) in the presence of one of the following solutions: solution A; A-timolol + 10 μM Iso; solution A + 10 μM 5-HT; solution A + 10 μM 5-HT + 1 μM GR-113808; or solution A + 10 μM 5-HT + 0.1 μM ketanserin. An average of 17,500 cardiomyocytes/cm², yielding ~1.6 × 10⁶ cells from each flask, were harvested in PBS containing (in mM) 137 NaCl, 8 Na₂HPO₄, 2.7 KCl, and 1.5 KH₂PO₄, pH 7.40, and were frozen on liquid N₂.

Cardiomyocytes were homogenized (Polytron 1200, 4°C) in a buffer containing (in mM) 210 sucrose, 2 EGTA, 40 NaCl, 30 HEPES, 5 EDTA, protease inhibitors (Roche 11873580001), and phosphatase inhibitors (Sigma P2850). Protein concentrations were quantified using a micro BCA protein assay kit (Pierce 23231). Homogenates were denatured (37°C for 15 min for phospholamban (PLB) and 98°C for 5 min for troponin I) in sample buffer. Proteins were size fractionated on SDS-PAGE gel (15% for PLB and 12% for tropomin I) and were blotted to 0.45-μM PVDF membranes (Amerham, cat. no. RPNS303F). PVDF membranes were blocked in 5% fat-free dry milk in Tris-buffered saline with Tween overnight and were incubated with primary antibody for 1 h at room temperature, then secondary antibody for 1 h at room temperature. The following primary antibodies were used: anti-phospho-PLB, Ser1⁴ (A010-12, Badrilla, 1:2,500), anti-phospho-PLB, Thr1⁷ (A010-13, Badrilla, 1:5,000), anti-PLB (MA3-922, Affinity Bioreagents, 1:3,000), and anti-phospho-troponin I (cardiac; Ser2³²; no. 4004, Cell Signaling Technology, 1:1,000).
blots were developed by using enhanced chemiluminescence (ECL+; Amersham). Signals were quantified by using ImageQuant software (Amersham).

**Phosphoprotein gel stain.** Myofibrillar proteins were isolated as described by Bozzo et al. (7) and Mounier et al. (31). Cardiomyocytes were homogenized (Polytron 1200) in 500 μL 6.35 mM EDTA, pH 7.0, with protease (Roche 118735800011) and phosphatase inhibitors (Sigma P2850). Homogenate was centrifuged at 13,000 rpm for 10 min (4°C). The pellet was washed twice in 50 mM KCl containing protease and phosphatase inhibitors. Proteins were dissolved in 2% SDS, and protein content was determined by Pierce protein kit (catalog no. 23231).

Separation of myofibrillar proteins (2–5 μg) was done by constant-current (20 mA/2 gels) SDS-glycine-glycerol gels for 190 min (23°C) [modified from O’Connell et al. (32)]. The gels were stained for rat soleus and extensor digitorum longus muscles as standards. Quantification of the MLC bands was done with ImageQuant TL (Amersham). Signals were quantified by using ImageQuant software.

**Statistics.** Results are expressed as means ± SE. Statistical analysis was performed on raw data with t-test or a one-way ANOVA with a post hoc Student-Newman-Keuls test. P < 0.05 was considered statistically significant. Cardiomyocytes from three or more rats were used for all protocols, and n = number of cardiomyocytes.

**RESULTS**

Both 5-HT4- and 5-HT2A-receptor stimulation induce a PIR in cardiomyocytes. To test the 5-HT responsiveness in LV cardiomyocytes from CHF rats, we measured contraction before and after stimulation of the 5-HT receptors. 5-HT4-receptor stimulation induced a PIR of 33% (Fig. 1, A and B; P < 0.05), with no changes in TTP or RT50 (74 ± 5 vs. 77 ± 5 ms and 33 ± 4 vs. 32 ± 3 ms). Consistent with this, both (−dL/dt)max and (dL/dt)max increased (54 ± 10 and 91 ± 17%; P < 0.05). Selective stimulation of the 5-HT2A receptor resulted in a PIR that was smaller than the 5-HT3 receptor-induced PIR (Fig. 1, A and B) and did not significantly change TTP and RT50 (75 ± 3 vs. 76 ± 4 ms and 31 ± 2 vs. 30 ± 1 ms) but induced a symmetrical increase in (−dL/dt)max and (dL/dt)max (28 ± 8 and 33 ± 14%; P < 0.05). Combined stimulation of both receptors gave an almost additive PIR (Fig. 1, A and B), suggesting activation of two separate pathways that can mediate inotropic responses. After the 5-HT4, 5-HT2A, or combined receptor-mediated PIRs were fully developed, Iso (10 μM) was added as a positive control. Iso further increased the PIR by 44 ± 12, 62 ± 7, and 45 ± 9%, respectively (P < 0.05). To explore the signaling pathways for the 5-HT2A and 5-HT4 receptors, their effects on Ca2+ handling were examined.

5-HT3-receptor stimulation increases the Ca2+ transient. The effect of 5-HT on Ca2+ handling was first assessed by measuring Ca2+ transients in cells showing a PIR. 5-HT3-receptor stimulation increased F/F0 by 44% (Fig. 2, A and B; P < 0.05). Moreover, the decay of the Ca2+ transient became faster after stimulation, as shown by the increased (−dF/dt)max and the reduced TTD90 (Fig. 2B; P < 0.05). On the other hand, selective 5-HT2A stimulation elicited a much smaller, although significant, increase in F/F0. The (−dF/dt)max increased (P < 0.05), whereas there was no significant change in TTD90 (Fig. 2, C and D). As a positive control, Iso (10 μM) increased F/F0 and (−dF/dt)max and reduced TTD90 as expected (Fig. 2, E and F; all P < 0.05).

5-HT3-receptor stimulation increases Icalc. To assess whether the increased Ca2+ transients were due to increased Icalc, we performed voltage-clamp experiments. Stimulation of the 5-HT3 receptor increased peak Icalc, as shown in the representative tracings in Fig. 3A. The Icalc was measured over a wide range of membrane potentials, and the I-V plots are presented.
in Fig. 3B. Selective 5-HT4-receptor stimulation increased the AUC by 57% compared with control (P < 0.05; Fig. 3C). This was comparable with the increase in AUC induced by a low, submaximal dose of Iso (100 nM). However, stimulation with Iso resulted in a leftward shift in the peak of the I-V plot from 0 to ~10 mV (data not shown). Stimulation of the 5-HT4 receptor did not shift peak ICa,L to the left (Fig. 3B).

An increase in peak ICa,L is not always synonymous with increased influx of Ca2+, because channel inactivation might be altered. Hence, the ICa,L was integrated and found to be 44% higher after 5-HT4-receptor stimulation (Fig. 3D; P < 0.05). 5-HT2A- and combined receptor stimulation did not significantly alter the ICa,L measured as AUC.

5-HT2A- and 5-HT4-receptor stimulation alters the AP duration. The slight increase in Ca2+ transient induced by 5-HT2A-receptor stimulation might be explained by alterations in AP configuration. APD90 increased 26% in response to 5-HT2A-receptor stimulation and decreased by 8% in response to 5-HT3-receptor stimulation (Fig. 4, P < 0.05). However, APD80 was not significantly changed in response to either 5-HT2A- or 5-HT4-receptor stimulation.

5-HT4-receptor stimulation increases PLB-Ser16 phosphorylation and SR Ca2+ content. In addition to the 5-HT4 receptor-mediated increase in ICa,L, an increase in SR Ca2+ content could also contribute to the increase in Ca2+ transients. Selective 5-HT4 stimulation elicited a 65% increase in the SR Ca2+ content compared with control (P < 0.05), as measured by caffeine-induced (10 mM) ICa,NCX (Fig. 5, A–C). This was comparable with the increase in SR Ca2+ content elicited by a low dose of Iso (100 nM, 63%, P < 0.05), which served as a positive control. 5-HT2A stimulation did not significantly alter SR Ca2+ content (Fig. 5C). The observed increase in SR Ca2+ content after 5-HT4-receptor stimulation could be a result of increased ICa,L but also of phosphorylation of PLB, resulting in increased sarcoplasmic reticulum Ca2+-ATPase 2 (SERCA2A)-mediated Ca2+ uptake. Phosphorylation of PLB-Ser13 increased in response to 5-HT4-receptor stimulation (Fig. 5D; P < 0.05). Stimulation of the 5-HT2A receptor did not induce a significant increase in PLB-Ser13 phosphorylation. PLB-Thr17 phosphorylation was not significantly altered by 5-HT4- or 5-HT2A-receptor stimulation (data not shown). Together, this suggests that the SR Ca2+ content increased after
5-HT4-receptor stimulation because of a higher SERCA2 pumping rate. This coincides with the transient data showing reduced TTD90 in response to 5-HT4-receptor stimulation. The 5-HT2A receptor-mediated PIR, on the other hand, is not associated with a significant effect on SR Ca2+/H11001 handling.

5-HT2A-receptor stimulation alters the contraction to Ca2+/H11001 transient ratio. To assess possible effects of 5-HT on myofilament Ca2+/H11001 sensitivity, we measured Ca2+/H11001 transients and contractions simultaneously. The percent increase in FS per percent increase in F/F0 was significantly higher in response to 5-HT2A-receptor stimulation than 5-HT4-receptor or β-adrenoceptor stimulation (Fig. 6A; P < 0.05). We also found a 5-HT2A-mediated increase in MLC2 phosphorylation (Fig. 6B; P < 0.05). Because MLC2 phosphorylation increases myofilament Ca2+/H11001 sensitivity, this may explain the larger increase in FS-to-F ratio after 5-HT2A-receptor stimulation. β-adrenoceptor stimulation, on the other hand, is known to decrease myofilament sensitivity due to troponin I phosphorylation (49).

Phosphorylation of troponin I increased with 5-HT4 stimulation (Fig. 6C; P < 0.05) but was not significantly changed with 5-HT2A stimulation.

**DISCUSSION**

A PIR to 5-HT is demonstrated in isolated LV cardiomyocytes from rats with chronic postinfarction CHF. The PIR is mediated through activation of both the 5-HT4 and 5-HT2A receptors. 5-HT4-receptor stimulation increases I_{Ca,L}, PLB-Ser16 phosphorylation, troponin I phosphorylation, and SR Ca2+/H11001 content, resulting in increased Ca2+/H11001-transient magnitude, which can explain the PIR. The 5-HT2A-mediated PIR is accompanied by an increase in MLC2 phosphorylation and a minor increase in Ca2+/H11001 transients, probably reflecting an increased myofilament Ca2+/H11001 sensitivity.

The Gs-coupled 5-HT4 receptor is known to induce a PIR in the atria (24). This is due to a robust activation of the cAMP-PKA pathway, which leads to increased I_{Ca,L} (18, 34). Recently, we demonstrated a 5-HT4 receptor-mediated PIR and an induction of 5-HT4-receptor mRNA in LV papillary muscles from rats with chronic postinfarction CHF (40). The EC50 for 5-HT on the 5-HT4 receptors was 0.03 μM, which is 10-fold higher than the estimated plasma concentration of 5-HT in rats (26). This suggests that 5-HT receptors are stimulated in vivo in our model system. A 5-HT4 receptor-mediated PIR has also been demonstrated in ventricular trabecular muscles from failing human hearts (9). This implies that cardiomyocytes from failing rat ventricle can serve as a useful model system for studies of cellular effects of 5-HT4-receptor stimulation in ventricular cardiomyocytes from failing human hearts, which are difficult to obtain. Although the cellular effects of 5-HT4 stimulation have been explored in human atrial cardiomyocytes (18, 20, 23, 34), there is a need for a thorough characterization also in ventricular cardiomyocytes in which excitation-contraction coupling is significantly different from atrial cells.
In the present study, a 5-HT₄-mediated PIR is demonstrated in isolated LV cardiomyocytes. The PIR is characterized by a larger increase in the \( \frac{d \Delta L}{d t} \)max than in the \( \frac{-d \Delta L}{d t} \)max, suggesting a larger effect on the relaxation than on the contraction phase of the CRC. However, the effect on the relaxation was not as prominent as we observed in our previous study (40), because we did not detect any shortening of the CRCs. The lack of CRC shortening in the cardiomyocytes could reflect that unloaded cell shortening represents a different way of assessing contractile performance than isometric force in papillary muscles. Consistent with this, we did not observe any CRC shortening after \( \alpha_1 \)-adrenoceptor stimulation.

However, 5-HT₄-receptor stimulation induced an increased Ca²⁺ transient with a faster decay, consistent with both the 5-HT₄-mediated PIR and the shortening of the CRC that we observed in our previous study (40). 5-HT₄-receptor stimulation also induced phosphorylation of both PLB-Ser₁₆ and troponin I but had no significant effect on PLB-Thr₁₇. Because increased PLB-Ser₁₆ phosphorylation is known to enhance SR Ca²⁺ reuptake and to subsequently increase SR Ca²⁺ content (44), this explains the faster decay of the Ca²⁺ transient and the increased SR Ca²⁺ content seen after 5-HT₄-receptor stimulation.

A shortening of the AP was also observed in response to 5-HT₄-receptor stimulation. The shortened AP could increase Ca²⁺ extrusion through the NCX and result in decreased SR Ca²⁺ load. However, this effect is probably counterbalanced by the increased SR Ca²⁺ reuptake by SERCA₂, because SR Ca²⁺ content increased after 5-HT₄-receptor stimulation.

As shown in Fig. 3, the \( I_{Ca,L} \) increased after 5-HT₄-receptor stimulation, and a higher \( I_{Ca,L} \) is known to induce a larger Ca²⁺ release from the SR (13). Because the SR Ca²⁺ content is also increased, a given \( I_{Ca,L} \) will induce a larger Ca²⁺ release (4). Together, this probably explains the increased Ca²⁺ transient after 5-HT₄-receptor stimulation. However, an effect of 5-HT₄-receptor stimulation on the ryanodine receptor (RyR) cannot be ruled out, but this aspect has not been explored because the mechanisms regulating RyR phosphorylation are under debate (45).

Several 5-HT₄ receptor splice variants are expressed in the rat heart (2, 8), but it is not yet clear which of these splice variants are the main contributors to 5-HT₄-mediated inotropic responses. The findings of Pindon et al. (37) and Castro et al. (11) that 5-HT₄(b) receptors but not 5-HT₄(a) or 5-HT₄(d) receptors show dual coupling to Gₛ and Gᵢ opens the possibility that 5-HT₄-mediated responses in the heart could be modulated by Gi, as has been reported for \( \beta_2 \)-adrenoceptors, at least in some systems. The inhibitory action of Gᵢ was reported to prevent phosphorylation of PLB through \( \beta_2 \)-adrenoceptors in rodents, and thus no lusitropic effect was observed. However, in the presence of Gᵢ inhibition by pertussis toxin, a \( \beta_2 \)-adrenoceptor-mediated increase in PLB phosphorylation was revealed (19, 25). Which 5-HT₄ splice variant is mediating the inotropic effects of 5-HT in failing cardiomyocytes, and whether this effect is modulated by Gᵢ, will remain a question for future studies. However, the similarity between the func-

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**Fig. 4.** 5-HT₂A- and 5-HT₄-receptor stimulation alter action potential (AP) duration. A and C: representative AP tracings before and after 5-HT₂A(A; \( n = 4 \)) or 5-HT₄(C; \( n = 5 \)) receptor stimulation. B and D: mean time from initiation of AP to when 50% and 90% of initial resting membrane potential was regained (APD₅₀ and APD₉₀, respectively) before and after 5-HT₂A(B) or 5-HT₄(D) receptor stimulation. *P < 0.05 vs. control, paired t-test.
tional responses through β1-adrenoceptors and 5-HT4 with respect to PLB and troponin I phosphorylation and to CRC shortening might indicate that 5-HT4 responses are not Gi-coupled in the failing cardiomyocytes we have studied.

Assuming that the 5-HT4 receptor is Gs coupled, intracellular signaling involving a rise in cAMP could be expected. Stimulation of β1-adrenoceptors, which are also Gs coupled, induces a global increase in cAMP. Interestingly, in our previous study we only detected a small, although significant, increase in global cAMP after 5-HT4-receptor stimulation both in perfused hearts and in isolated cardiomyocytes (40). One possibility is that cAMP production is spatially compartmentalized. It is known that different Gs-coupled receptors, which induce cAMP formation, exert various downstream effects, e.g., the β1- and the β2-adrenoceptors (3). Also the prostaglandin E1 receptor increases global cAMP level but has no cardiac chronotropic (22) or inotropic effect (10, 17). Several mechanisms may explain such spatial restriction of the cAMP effect. One possibility is embedding of receptors, G proteins, and adenylyl cyclases into caveolae (35). Also, cAMP diffusion might be restricted by different sets of phosphodiesterases, as suggested by Rochais et al. (43). Finally, PKA phosphorylation might be removed by phosphatases that are known to colocalize with target proteins and kinases in protein complexes. Thus it is possible that the 5-HT4 receptor induces a compartmentalized increase in cAMP that activates PKA-mediated phosphorylation of PLB-Ser16 and troponin I.

Recently, we (41) demonstrated a 5-HT2A-mediated PIR in LV papillary muscles from rats with acute postinfarction CHF and showed that 5-HT2A-receptor stimulation leads to phosphorylation of MLC2, which is known to increase myofilament Ca2+ sensitivity (30). We now expand our previous findings by demonstrating that the 5-HT2A receptor-mediated PIR is induced not only in acute heart failure but also in the remodeled and chronically failing hearts. We also show that the response is present in isolated cardiomyocytes harvested from the septum, an area remote to the infarcted area, not only in the border zone that the papillary muscles represent. Our previous study on chronic CHF did not show a 5-HT2A-mediated PIR in papillary muscles, based on a lack of rightward shift of the normalized concentration-response curve to 5-HT by ketanserin (40). However, given that we now detect a 5-HT2A-mediated inotropic response in cardiomyocytes from the same chronic CHF model, it is likely that papillary muscles studied in the presence of a 5-HT4 antagonist could reveal a 5-HT2A response, as we found in acute CHF (41). Consistent with this hypothesis, ketanserin produced a slight but nonsignificant leftward shift of the normalized concentration-response curve.
to 5-HT in papillary muscles (40), as expected if 5-HT2A receptors with lower affinity to 5-HT than 5-HT4 receptors contributed to the 5-HT response.

The 5-HT2A-mediated PIR is not associated with alterations in CRC characteristics. The 5-HT2A receptor is known to be Gq coupled (39). Other Gq-coupled receptors, such as the α1-adrenoceptor and the endothelin receptors, induce PIRs that have similar CRC characteristics to the response induced by 5-HT2A-receptor stimulation in the present study (12, 36). In the acute CHF model, we did not observe any increase in the Ca2⁺ transients after 5-HT2A-receptor stimulation (41). This is in contrast to the present data from chronic CHF, which show a small but significant increase in the Ca2⁺ transient after 5-HT2A-receptor stimulation. The increased Ca2⁺ transient could be explained by a small 5-HT4 stimulation, but this is unlikely because the presence of 1 μM GR-113808 causes >99.9% receptor occupancy of the 5-HT4 receptor in our system (40). In addition, we have previously shown that 1 μM GR-113808 abolishes the 5-HT4-mediated response in the presence of 10 μM 5-HT (41).

Interestingly, our data coincide with findings on the effect of α1-adrenoceptor stimulation on the Ca2⁺ transient (14). Because we found the Ca2⁺ transient increased, an increase in ICa,L or SR Ca2⁺ content would be expected. However, the
18% numerical difference in peak \( I_{\text{Ca-L}} \) did not reach statistical significance (\( P = 0.06 \)) but could nevertheless reflect a slight increase in \( I_{\text{F/F0}} \), similar to the mechanism behind the \( \alpha_1 \)-adrenocceptor-mediated increase in \( Ca^{2+} \) transient (14). It has also been suggested that \( \alpha_1 \)-adrenocceptor stimulation increases the AP duration by reducing the transient outward \( K^+ \) current (\( I_{\text{o}} \)) (15), favoring \( Ca^{2+} \) influx, which is known to increase the \( Ca^{2+} \) transient (5). Interestingly, 5-HT \(_{2A}\)-receptor stimulation also induced a prolongation of the AP (Fig. 4), which may be explained by a 5-HT \(_{2A}\)-mediated inhibition of the \( I_{\text{o}} \), as shown by Zhao et al. (50). A prolonged AP could increase \( Ca^{2+} \) influx through NCX and thus contribute to the small increase in the \( Ca^{2+} \) transient after 5-HT \(_{2A}\)-receptor stimulation. However, the small increase in the \( Ca^{2+} \) transient cannot fully explain the observed increase in contraction magnitude. Rather, the observed 5-HT \(_{2A}\)-mediated increase in MLC2 phosphorylation and increased contraction-to-fluorescence ratio suggest increased myofilament \( Ca^{2+} \) sensitivity as the primary inotropic mechanism, although myofilament \( Ca^{2+} \) sensitivity has not been directly assessed. This corresponds to our previous finding in acute CHF (41) and to the primary mechanism implied in \( \alpha_1 \)-adrenocceptor mediated PIR (1).

However, \( \alpha_1 \)-adrenocceptor stimulation has also been suggested to increase the intracellular pH by activation of the \( Na^+/H^+ \)-exchanger. Increased pH increases the myofilament \( Ca^{2+} \) sensitivity (5). In our previous study (41), addition of an MLC kinase inhibitor (MLC-9) abolished both the 5-HT \(_{2A}\) receptor-mediated PIR and the subsequent MLC2 phosphorylation. However, we cannot rule out an additional \( Na^+/H^+ \) exchanger effect in the 5-HT \(_{2A}\) receptor-mediated PIR.

We have recently investigated (6) the effect of 5-HT \(_{3}\)-receptor blockade on cardiac function and found reduced cardiac remodeling and signs of improved diastolic and systolic function. One possible explanation of this beneficial effect could be blockade of the 5-HT \(_{3}\)-mediated effect on cellular \( Ca^{2+} \) cycling. Increasing cardiac contractility through increased \( Ca^{2+} \) cycling is considered energetically unfavorable as visualized by the effects of \( \beta \)-adrenocceptor stimulation on myocardial energetics (16). Thus 5-HT \(_{3}\)-receptor blockers, which in parallel with \( \beta \)-adrenocceptor blockers, reduce cardiac energy consumption and thus contribute to restore energy balance in the energetically starved failing heart.

In conclusion, this study shows that the 5-HT \(_{4}\) receptor-mediated PIR in failing ventricular cardiomyocytes results from an increase in \( I_{\text{Ca-L}} \), PLB-Ser\(^{16}\) phosphorylation, SR \( Ca^{2+} \) load, and troponin I phosphorylation, analogous to what is known for the \( \beta_1 \)-adrenocceptor-mediated response. Also, a 5-HT \(_{2A}\)-mediated PIR is present in chronic CHF, and the effect is primarily mediated through MLC2 phosphorylation. 5-HT \(_{2A}\) stimulation induced a minor increase in the \( Ca^{2+} \) transient, possibly explained by a prolongation of the AP.

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