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ORIGINAL PAPER

Carbon monoxide modulates electrical activity of murine myocardium via cGMP-dependent mechanisms

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Abstract Carbon monoxide (CO) is critical in cell signaling, and inhalation of gaseous CO can impact cardiovascular physiology. We have investigated electrophysiological effects of CO and their potential cGMP-dependent mechanism in isolated preparations of murine myocardium. The standard microelectrode technique was used to record myocardial action potentials (APs). Exogenous CO (0.96×10^{-4}–4.8×10^{-4} M) decreased AP duration in atrial and ventricular tissue and accelerated pacemaking activity in sinoatrial node. Inhibitors of heme oxygenases (zinc and tin protoporphyrin IX), which are responsible for endogenous CO production, induced the opposite effects. Inhibitor of soluble guanylate cyclase (sGC), ODQ (10^{-5} M) halved CO-induced AP shortening, while sGC activator azosidnone (10^{-5} M-3×10^{-4} M) and cGMP analog BrcGMP (3×10^{-4} M) induced the same effects as CO. To see if CO effects are attributed to differential regulation of phosphodiesterase 2 (PDE2) and 3 (PDE3), we used inhibitors of these enzymes. Milrinone (2×10^{-6} M), selective inhibitor of cGMP-downregulated PDE3, blocked CO-induced rhythm acceleration. EHNA(2×10^{-6} M), which inhibits cGMP-upregulated PDE2, attenuated CO-induced AP shortening, but failed to induce any positive chronotropic effect. Our findings indicate that PDE2 activity prevails in working myocardium, while PDE3 is more active in sinoatrial node. The results suggest that cardiac effects of CO are at least partly attributed to activation of sGC and subsequent elevation of cGMP intracellular content. In sinoatrial node, this leads to PDE3 inhibition, increased cAMP content, and positive chronotropy, while it also causes PDE2 stimulation in working myocardium, thereby enhancing cAMP degradation and producing AP shortening. Thus, CO induces significant alterations of cardiac electrical activity via cGMP-dependent mechanism and should be considered as a novel regulator of cardiac electrophysiology.

Keywords Carbon monoxide · Heart · Action potential · Guanylate cyclase · cGMP · cAMP

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Abbreviations

CO	Carbon monoxide
HO	Heme oxygenase
NO	Nitric oxide
ROS	Reactive O_2 species
CORMs	CO-releasing molecules
sGC	Soluble guanylate cyclase
PDE	Phosphodiesterase
AP	Action potential
SAN	Sinoatrial node
APD	Action potential duration
CL	Cycle length
ZnPP	Zinc protoporphyrine IX
SnPP	Tin protoporphyrine IX
PKA	Proteinkinase A

Background

For a long time, carbon monoxide (CO) has been considered as a deadly toxic gas, having no smell and color and, therefore, often being called a "silent killer." CO intoxication still remains the most common form of death by poisoning [19]. However, during the last two decades, CO has become recognized as an important endogenous signaling compound regulating a number of physiological functions (for review see [30, 31]). For example, endogenous CO may produce relaxation of different smooth muscle types [31] including the coronary vascular smooth muscle [3]. It has been also considered as a gaseous neural messenger [25], particularly in the neuromuscular junction, where skeletal muscle-derived CO potentiates neurotransmitter release from the motor endplate [21]. CO is produced as a by-product of heme degradation catalyzed by heme oxygenases (HOs). Two isoforms of this enzyme (HO-1 and HO-2) are predominant in mammals. While HO-2 is constitutively expressed in various cell types, expression of HO-1 is induced by cellular stress of different kind including ischemia [5]. Along with nitric oxide (NO) and hydrogen sulfide, CO represents a class of gaseous transmitters.

Cardiotropic effects of CO deserve substantial interest due to putative protective potency of this gasotransmitter. Beneficial effects of preconditioning maintained by exogenous CO, carried by CO-releasing molecules (CORMs), or stimulation of endogenous CO production by HOs are described in numerous studies (for review see [18]). However, the data concerning regulatory cardiotropic effects of CO in normal conditions is still very sparse. Moreover, most of investigators used CORMs as a source of CO in their experiments, although these ruthenium complexes seem to have side effects independent from release of CO per se [10]. There is no doubt that CO affects the myocardial contractility, but, while in the isolated heart models CO induces positive inotropy [16], it suppresses contractile activity in isolated atrial [2] and ventricular tissue preparations [14]. This discrepancy may be explained by CO-induced coronary vasodilation leading to increase in cardiac performance, which masks the direct negative inotropic effect. The beneficial effect of CO in models of ischemia-reperfusion are also much attributed to increase of coronary flow (for review see [18]).

The most important and well-known targets of CO are BK_{Ca} channels and P2X ionotropic receptors [30], but in the myocardium the role of these proteins is quite negligible. However, another important CO target, L-type calcium channels, is widely distributed in the heart. CO inhibits cardiac I_{CaL} indirectly, via binding with cytochrome c oxidase and subsequent increase in reactive O_2 species (ROS) production [20, 23]. Recently, CO was found to modulate cardiac Na^+ current: both CO and CORM2 decreased peak density of I_{Na}, but strongly increased the late component of this current [8], although the latter effect was mediated by stimulation of NO production. Thus, CO may affect some cardiac ionic currents and should be examined as a putative important modulator of cardiac electrical activity.

The present study provides the first complete description of CO-induced changes in electrical activity of murine myocardium. CO-saturated solutions were applied to the preparations instead of CORMs, allowing to exclude the side effects of such compounds. Using the HO inhibitors we demonstrate that endogenously produced CO exerts the same effects as exogenous. This fact supports the physiological significance of CO for the cardiac performance. Finally, we show the crucial role of soluble guanylate cyclase (sGC) stimulation in mediation of CO effects and hypothesize that the opposite direction of CO effects in cardiac pacemaker and working myocardium results from differential patterns of phosphodiesterases (PDEs) activity in these tissues.

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Materials and methods

Ethical approval

The procuration of animals, the husbandry, and the experiments conducted in the present study conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the EU Directive 2010/63/EU for animal experiments. A total of 51 mice were used in this study. All mouse experiments described in this paper were conducted with the approval of the Bioethics Committee of Moscow State University.

Intracellular recordings of electrical activity in cardiac preparations

White outbred male mice weighing 25–30 g were anesthetized by isoflurane inhalation and killed by cervical dislocation. The chest was opened and the heart was rapidly excised and immersed in a physiological solution containing (in mM): NaCl 133.47, KCl 4.69, $NaH_2PO_4 \cdot 2H_2O$ 1.35, $NaHCO_3$ 16.31, $MgSO_4 \cdot 7H_2O$ 1.18, $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 7.7, bubbled with carbogen (95 % O_2, 5 % CO_2), with pH 7.4±0.1. Two types of isolated myocardial preparations were used: right ventricular wall and right atrium containing both the auricle and the intercaval region including the sinoatrial node. During electrophysiological experiments, preparations of right atrium were beating spontaneously, while the ventricular preparations were paced (6 Hz) via silver Teflon-coated electrodes. Preparations were pinned to the bottom of an experimental chamber supplied with a physiological solution at 10 ml/min (38 °C). After 1 h of equilibration, transmembrane potentials were recorded with glass microelectrodes (20–30 MΩ) filled with 3 M KCl and connected to the high-impedance amplifier for intracellular recordings (Neuroprobe Amplifier Model 1600, A-MSystems, Carlsborg, WA, USA). The microelectrodes were manufactured from standard borosilicate glass BF120-60-10 (Sutter Instrument, Novato, CA, USA) using the vertical puller model P-30 (Sutter Instrument). The signal was digitized using ADC E14-140 (L-Card, Moscow, Russia) with digitalization frequency of 10 KHz and analyzed using specific software (DiSoft, Moscow, Russia; Synaptosoft, Fort Lee, NJ, USA). Action potentials (APs) were recorded from the endocardial surface of the preparations. Stable impalements were maintained during the application of CO and different drugs. In preparations of ventricular myocardium changes in the AP duration to 50 % (APD50) and 90 % of repolarization (APD90) were analyzed. In atrial preparations, changes in cycle length (CL) were determined in addition to APD.

RT-PCR

Quantitative mRNA expression for PDE2A, PDE3A, and PDE3B was performed in mouse SAN, the right auricle and right ventricular free wall (see Appendix). All three types of myocardial preparations were obtained from each heart in the same way as the preparations used for electrophysiology. To get the sample of SAN myocardium electrophysiological localization of SAN was performed in each right atrial preparation. The sample of right atrium was placed in the experimental chamber, and rough microelectrode recordings were made immediately to find the position of primary pacemaker. Then, the electrode tip was broken by a drastic movement of manipulator and the small piece of tissue (approximately 1.5 mm in diameter), surrounding the broken tip, was excised. Despite the tiny size of SAN tissue samples, highly effective SV Total RNA Isolation System Z3100 (Promega, Madison, WI, USA) allowed us to get enough RNA for subsequent assays from each sample.

Drugs

HO inhibitor zinc protoporphyrine IX (ZnPP), sGC inhibitor 1H-[1, 2, 4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and PDE3 inhibitor milrinone were purchased from Sigma (St. Louis, MO, USA). HO inhibitor tin protoporphyrine IX (SnPP), membrane-permeable cGMP analog 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (BrcGMP), PDE2 inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) and broad-spectrum PDE inhibitor 3,7-dihydro-1-methyl-3-(2-methylpropyl)-1*H*-purine-2,6-dione (IBMX) were purchased from Tocris Cookson (Bristol, UK). sGC activator azosidnon was a gift from Dr. Marina Artemyeva. All experiments with ZnPP and SnPP were conducted in darkness to avoid their photodegradation.

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Application of CO

CO (99.5 % purity) was purchased from NIIKM gas company (Moscow, Russia). The stock solution of CO was prepared immediately before usage in the experiment by bubbling of physiological solution (50 ml) with CO during 30 min to reach the full saturation. Bubbling was performed in a closed glass cylinder (25 cm in height). Since the water solubility of CO is 26.91 mg/l, the concentration of CO in the stock solution was 0.96 mM [32]. During the experiment the stock solution was used to prepare physiological solutions with different concentrations of CO (4.8×10^{-5}, 0.96×10^{-4}, 2.88×10^{-4}, 4.8×10^{-4} M). These solutions of CO were applied to the experimental chamber immediately after preparation to minimize the evaporation of CO.

Special series of experiments was performed to check the possible effects of hypoxia, which can appear due to the addition of anoxic CO-saturated stock solution. For that purpose, anoxic physiological solution was obtained by bubbling the small portion (50 ml) of normal solution with 99.99 % Ar during 30 min. Anoxic solution was mixed with normal saline in 0.48:0.52 proportion.

Statistical analysis

All results in the text and figures are expressed as mean ±s.e.m. for *n* experiments. The effects of CO, HO inhibitors, and PDE blockers on electrophysiological parameters were compared with respective basal values of these parameters by Wilcoxon test. The effects of 2.88×10^{-4} M CO alone were compared to its effects in the presence of ODQ, BrcGMP, and PDE inhibitors by Mann-Whitney test. The same test was used for comparison of PDE2A and PDE3A relative expression in different types of myocardial samples. $p\leq0.05$ was adopted as the level of significance.

Results

Effects of exogenous CO in working atrial and ventricular myocardium

First, we have investigated the alterations of APD and CL in spontaneously beating right atrium preparations during 8-min application of solution containing CO in different concentrations. Although the preparations of right atria contained sinoatrial node tissue, in these experiments we impaled only typical working cardiomyocytes within the auricle. During the control conditions, APD90, APD50, and CL were 22.1±2.6, 11.7±2.2, and 198±22.5 ms, respectively. The 4.8×10^{-5} M CO did not alter any of these parameters, while the next concentration, 10^{-4} M, significantly decreased APD90. Higher concentrations of CO (2.88×10^{-4} and 4.8×10^{-4} M) produced a marked decrease in APD50 and APD90 accompanied with reduction of CL (Fig. 1). These effects of CO were rapidly developing, reaching the maximum at 4th or 5th minute of superfusion and being at the plateau level or slightly decreasing until the end of CO application. The time course was similar in all subsequent electrophysiological experiments with CO, so only the maximal values of CO effects are presented in the diagrams. All effects of CO were completely reversible, control parameters restored after 5–6 min of washout. No remarkable changes of the resting potential under CO application were observed.

Control experiments with application of hypoxic solution, which imitated the possible hypoxia induced by 4.8×10^{-4} M CO solution, failed to reveal any significant effects of hypoxia on the AP configuration (Fig. 1). Therefore, the described effects of CO solutions are attributed to the action of CO itself, but not the ischemic changes in the myocardium.

In paced preparations of isolated right ventricular wall, control APD90 and APD50 were 24.2±3.4, 12.4±2.8 ms, respectively. CO also induced marked shortening of APs (Fig. 2) that was very similar to the effects registered in spontaneously beating right atria. Similarly to the atrial preparations, hypoxic solution failed to induce any substantial effect (Fig. 2).

Alteration of electrical activity in murine myocardium by HO inhibitors

Thus, exogenous CO applied to myocardial preparations produces substantial effects both in working and pacemaking myocardium. However, the physiological importance of these findings is not evident without demonstration of the role of endogenous CO, which is produced in the heart. HO inhibitors ZnPP and SnPP helped to block CO production in our preparations of murine myocardium. ZnPP (10^{-5} M), which provides a

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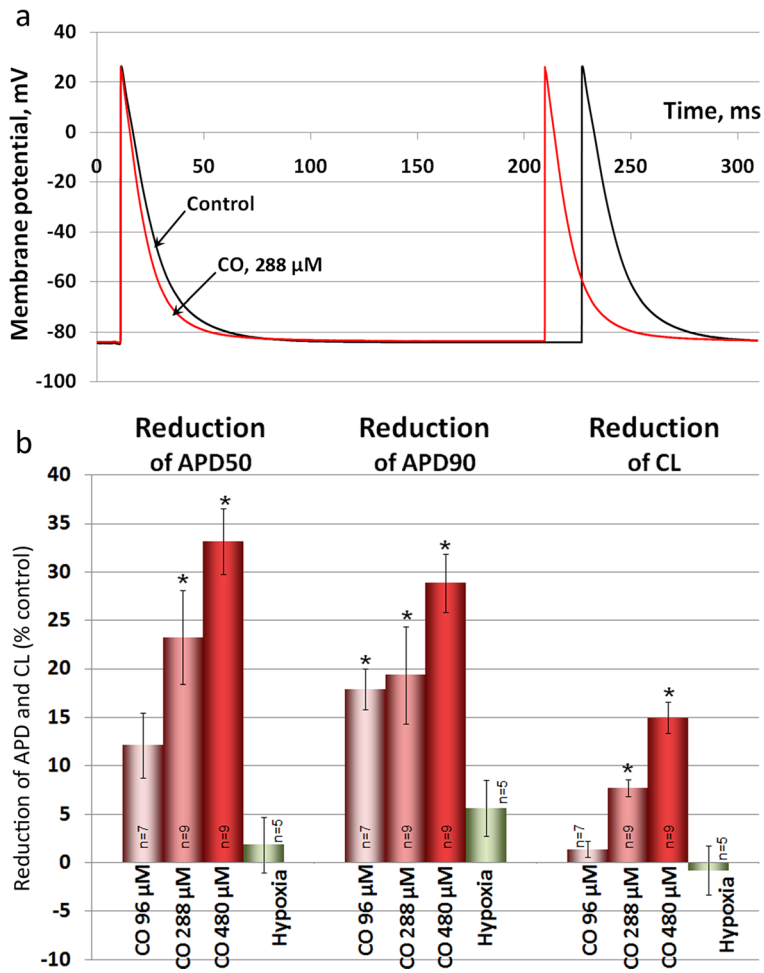

Fig. 1 Effects of exogenous CO in spontaneously beating atrial preparations. **a** Representative original traces of APs recorded in right atrial preparation during control conditions and under 2.88×10^{-4} M CO. **b** Relative intensity of CO (0.96×10^{-4}–4.8×10^{-4} M) effects compared with effects of moderate hypoxia. Data are expressed in % of control values of APD50, APD90, and CL. * = the significance of effect, $p<0.05$, Wilcoxon test

reliable HO inhibition according to the previous studies [22, 29], produced a significant increase in APD50 and APD90 in paced ventricular preparations (Fig. 3b). In spontaneously beating right atria, the same prolongation of APs coincided with rhythm slowing (Fig. 3a, b).

Because some authors point to possible non-selective influence of ZnPP on soluble GC activity [15] and calcium channels [11], we repeated some experimental series using 10^{-5} M SnPP, which is considered to be more specific HO inhibitor. Effects of this compound on APD in ventricular and spontaneously beating atrial preparations and CL in the latter almost reproduced the changes induced by ZnPP (Fig. 3c). In conclusion, inhibitors of myocardial CO production induce effects, which are opposite to the exogenous CO, indicating that endogenous CO acts in the same way as exogenous CO.

Evidence of soluble guanylate cyclase involvement in mediation of CO effects

In the next stage of the study, we have tried to reveal the mechanisms of discovered cardiotropic effects of CO. Spontaneously beating right atrial preparations were selected as test objects for the following experiments, so as to allow the estimation of both shortening of APs in the working myocardium and positive chronotropy of CO.

First, we have checked possible involvement of sGC in mediation of CO effects on myocardial APD and CL. Selective activator of GC azosidnone [4], inhibitor of the same enzyme ODQ and membrane-permeable analog of cGMP, BrcGMP were used in the related experiments.

In general, azosidnone (10^{-5}–3×10^{-4} M) mimicked CO effects, producing marked AP shortening and

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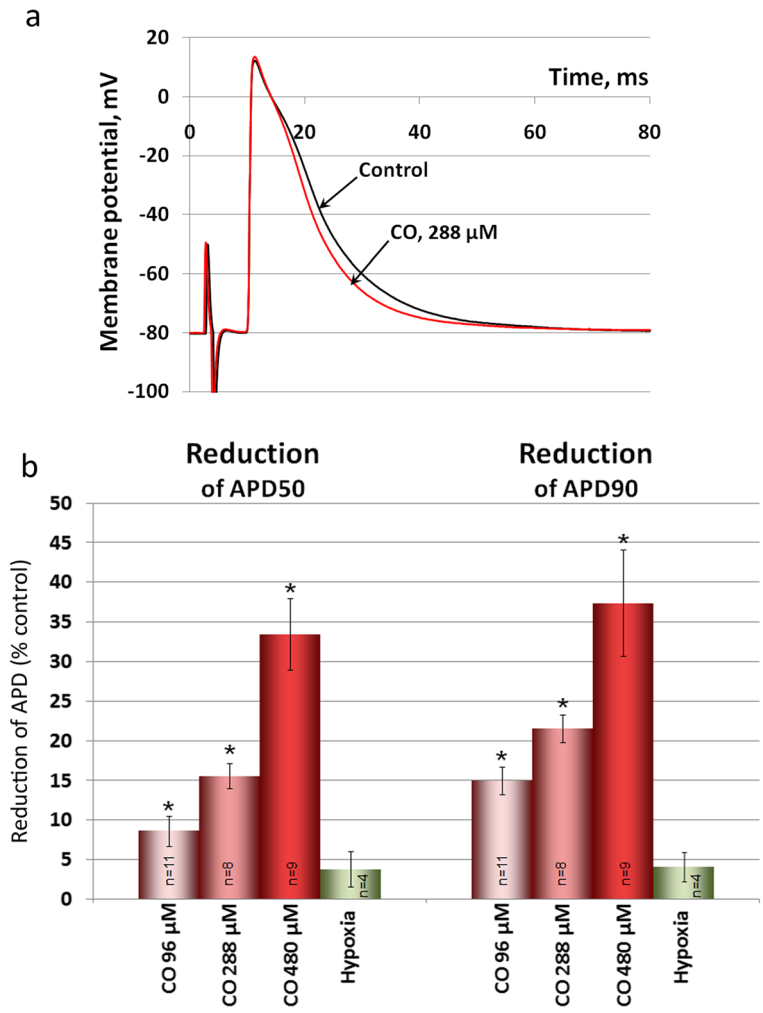

Fig. 2 Effects of exogenous CO in paced ventricular preparations. **a** Representative original traces of APs recorded in right ventricular wall preparation during control conditions and under 2.88×10^{-4} M CO. **b** Relative intensity of CO (0.96×10^{-4}–4.8×10^{-4} M) effects compared with effects of moderate hypoxia in paced ventricular preparations. Data are expressed in % of control values of APD50 and APD90. * = the significance of effect, $p<0.05$, Wilcoxon test

rhythm acceleration (Fig. 4a), although its effects were less prominent (see comparison for 3×10^{-4} M of azosidnone and 2.88×10^{-4} M CO at Fig. 4a). The 10^{-5} M ODQ, which is surely enough for complete inhibition of sGC, failed to alter the sinus rhythm, but provoked a slight increase in APD. Effects of 2.88×10^{-4} M CO in the presence of ODQ were reduced in comparison with normal conditions more than twice (Fig. 4b), although inhibitor did not abolish them completely. Increase in ODQ concentration to 3×10^{-5} M did not lead to a greater suppression of CO effects. Therefore, effects of CO are at least partly attributed to sGC activation, although this mechanism seems to be not the only one present in the myocardium.

This conclusion was further strengthened with the data obtained using BrcGMP. This cGMP analog (3×10^{-4} M) also mimicked CO effects, while in the presence of BrcGMP CO (2.88×10^{-4} M) induced much weaker effects than in normal conditions (Fig. 5).

Modulation of CO effects by inhibitors of PDE2 and PDE3

These results raise the question of the mechanisms, which lead from the elevation of cGMP content to the negative effects on APD in the working atrial and ventricular myocardium, but clear positive chronotropy in the SAN. It has been recently understood that PDEs constitutively downregulate intracellular cAMP content in cardiomyocytes by degradation [27]. In the myocardium, cGMP regulates two PDE isoforms, stimulating PDE2 activity while suppressing PDE3 activity [17]. While the former isoform is stimulated by this

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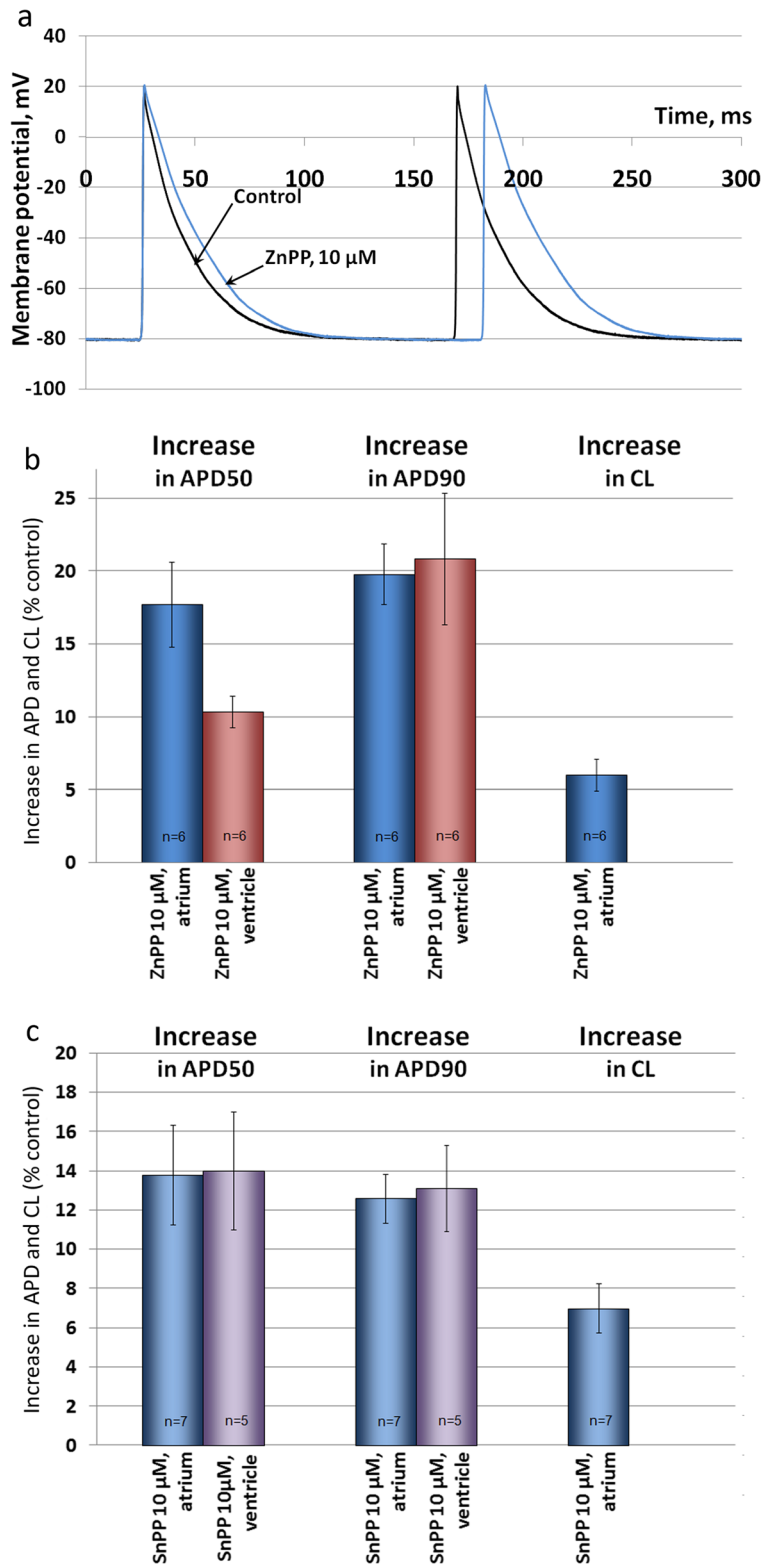

Fig. 3 Effects of HO inhibitors in the preparations of atrial and ventricular working myocardium. **a** Representative original traces of APs recorded in spontaneously beating atrial preparation during control conditions and under 10^{-5} M ZnPP. **b** Relative intensity of ZnPP (10^{-5} M) effects in paced ventricular and spontaneously beating atrial preparations. **c** Intensity of SnPP (10^{-5} M) effects in paced ventricular and spontaneously beating atrial preparations. Data are expressed in % of control values of APD50, APD90, and CL. All effects presented in diagrams are significant, $p<0.05$, Wilcoxon test

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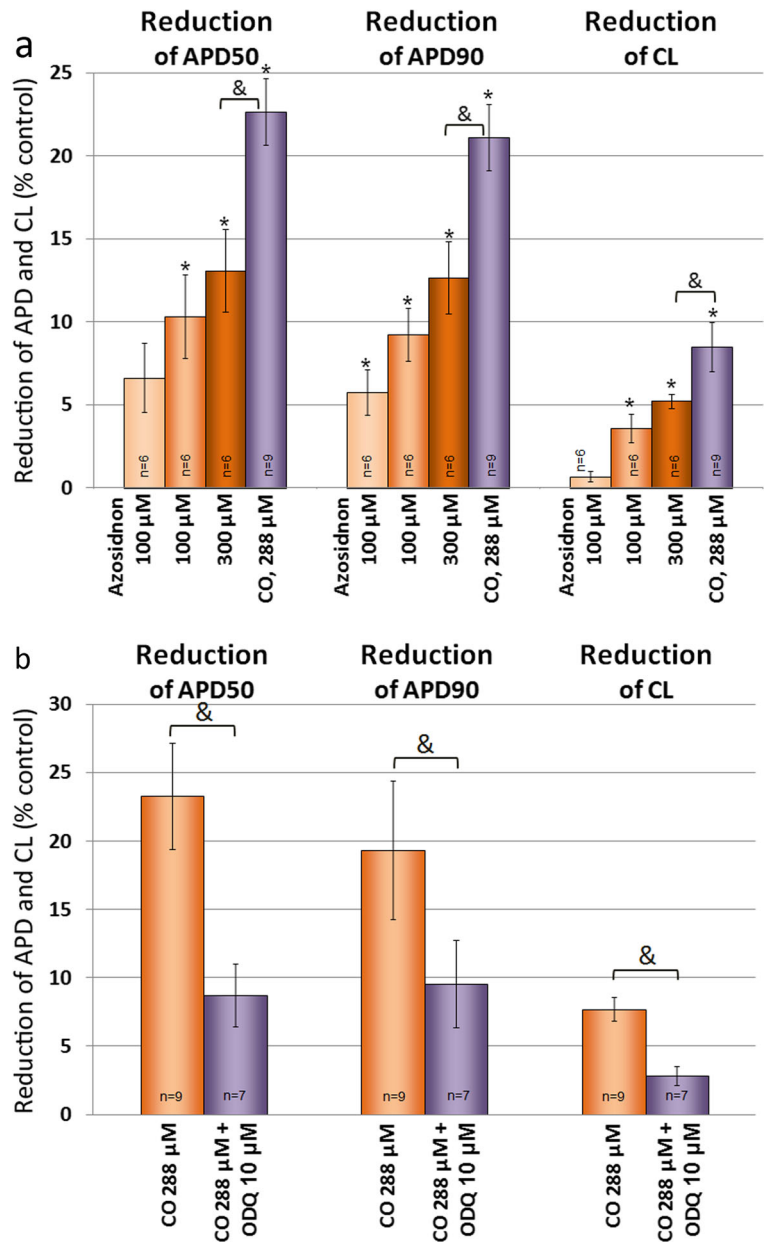

Fig. 4 Relation of CO effects to sGC activity in spontaneously beating atrial preparations. **a** Alteration of electrophysiological parameters by sGC activator azosidnon (10^{-5}–3×10^{-4} M) compared to effects of 2.88×10^{-4} M CO. **b** Relative intensity of CO (2.88×10^{-4} M) effects in normal conditions and in the presence of sGC inhibitor ODQ (10^{-5} M). Data are expressed in % of control values of APD50, APD90, and CL. * = the significance of effect, $p<0.05$, Wilcoxon test. & = significant difference, $p<0.05$, Mann-Whitney test

messenger, PDE3 activity is suppressed. Thus, CO-induced elevation of cGMP level can induce the opposite changes in cAMP content depending on relative basal activity of PDE2 and PDE3. To check this hypothesis, we have used selective inhibitor of PDE2 EHNA, PDE3 inhibitor milrinone, and broad-spectrum PDE blocker IBMX.

Similar to CO, in spontaneously beating right atrial preparations, broad-spectrum IBMX (3×10^{-6} M) and PDE3-specific milrinone (3×10^{-6} M) decreased APD and accelerated sinus rhythm (Fig. 6a), although AP shortening was slighter than under 2.88×10^{-4} M CO. An increase in concentration of IBMX and milrinone to 10^{-5} M caused a drastic acceleration of the sinus rhythm leading to atrial fibrillation (data not shown). PDE2-selective EHNA (3×10^{-6} M) failed to induce any significant alterations of measured parameters.

Milrinone (3×10^{-6} M) or non-specific IBMX (3×10^{-6} M) inhibited the chronotropic effects of CO (2.88×10^{-4} M), while EHNA (3×10^{-6} M) had no effect

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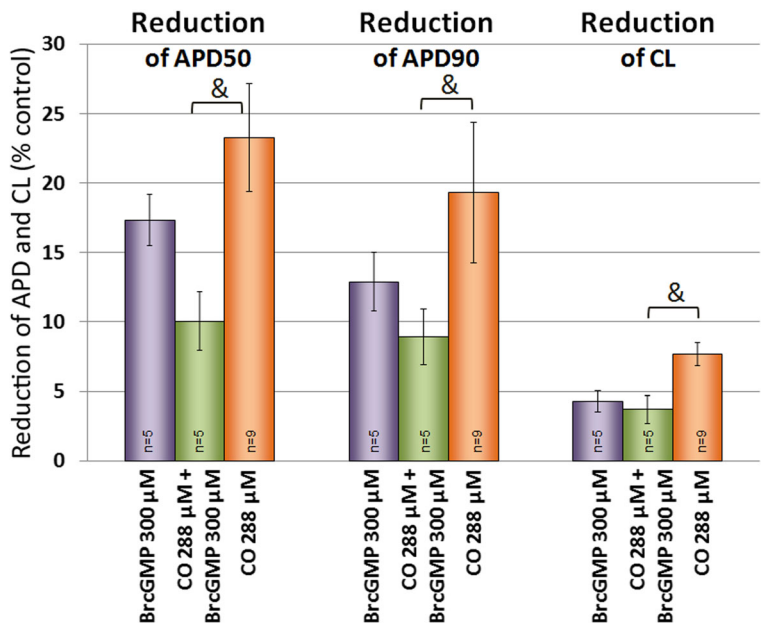

Fig. 5 Alteration of CO-induced effects by BrcGMP. Effects of CO (2.88×10^{-4} M) in the presence of membrane-permeable cGMP analog BrcGMP compared to action of CO in normal conditions. Data are expressed in % of control values of APD50, APD90, and CL. All effects are significant, $p<0.05$, Wilcoxon test. & = significant difference, $p<0.05$, Mann-Whitney test

on CO-induced rhythm acceleration (Fig. 6b). CO-induced reduction of APD was suppressed by all three inhibitors, with IBMX and EHNA being more potent than PDE3-specific milrinone (Fig. 6b). Thus, positive chronotropic effect of CO can be blocked by PDE3 inhibitor, but not PDE2 inhibitor, while negative effects of CO in working atrial myocardium are more sensitive to PDE2 inhibitor and a broad-spectrum inhibitor than PDE3-specific milrinone. These results clearly point to the different activity of PDE2 and PDE3 in the SAN and working atrial myocardium. At the last stage of the study, RT-PCR assays were done to reveal the difference in expression of PDE2 and PDE3 genes underlying this phenomenon.

Difference in expression of PDE2 and PDE3 genes in working myocardium and SAN

According to the results of RT-PCR assays (Fig. 7), the SAN tissue has the lowest level of PDE2A expression, while the highest was detected in the samples of working atrial myocardium. Unexpectedly, PDE3A isoform was also less expressed in the SAN than in right auricle and right ventricular wall. There was no significant difference in PDE3A expression between working atrial and ventricular myocardium.

Discussion

The present study provides the first to our knowledge a detailed investigation of electrophysiological effects of CO on the myocardium of mouse and partially unmasks the molecular mechanisms of these effects. Furthermore, the mouse was chosen as an experimental model instead of the rat (see description of CO effects in rat myocardium in [2]) because of the diverse range of transgenic mice currently available that could be used for careful dissection of more detailed mechanisms in future studies. Experiments with exogenous CO demonstrated two distinct effects: shortening of APs in the working myocardium and acceleration of the sinus rhythm. A positive chronotropic effect of CO observed in these experiments is not surprising, since the similar effect was very recently demonstrated by our group in isolated mouse sinoatrial node preparations [1]. Positive chronotropy correlated with acceleration of slow diastolic depolarization and was also accompanied by reduction of AP amplitude and upstroke velocity.

Control experiments confirmed that both effects are not attributed to moderate hypoxia, which could appear due to the preparation of test CO solutions by dilution of anoxic stock CO solution. Probably, 8 min of application are not enough for development of substantial hypoxic effects.

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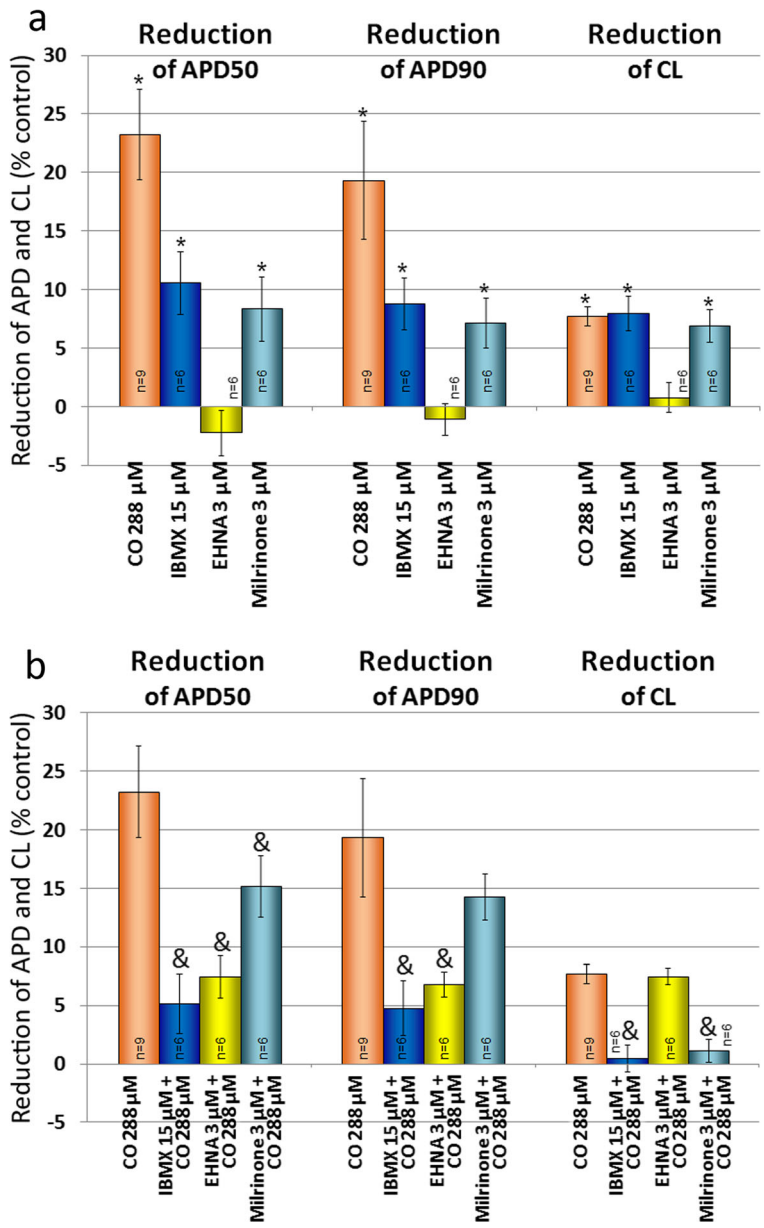

Fig. 6 Relation of CO effects to activity of cGMP-sensitive PDEs in spontaneously beating atrial preparations. **a** Alteration of electrical activity by PDE3 inhibitor milrinone, PDE2 inhibitor EHNA, and broad-spectrum PDE inhibitor IBMX compared to effects of 2.88×10^{-4} M CO. **b** Effects of CO in normal conditions and in the presence of PDEs inhibitors. * = the significance of effect, $p<0.05$, Wilcoxon test. & = significant difference from respective effect of 2.88×10^{-4} M CO, $p<0.05$, Mann-Whitney test

Block of endogenous CO production provoked changes in electrical activity opposite to those induced by exogenous CO, indicating that CO is continuously synthesized by myocardial HO, which provide physiologically relevant concentrations of CO in the heart. According to the previous studies, this function is attributed to HO-2 activity [31] as long as HO-1 is not expressed in normal non-ischemic heart [5].

A natural question about the effective concentrations of CO appears, since the concentrations of CO sufficient for significant effects in our experiments are strikingly high. CO is transported by blood only in the form of carbonmonoxyhemoglobin A due to the strong binding of CO molecules to heme. Estimated physiological concentrations of CO in tissues are rather low, in the nanomolar range if based on normal levels of COHb of 1 to 2 % [19]. However, the correlation between carbonmonoxyhemoglobin and tissue concentrations of CO is not evident, since CO generated in living cells would be immediately scavenged in the cytosol by heme proteins before reaching the bloodstream [31].

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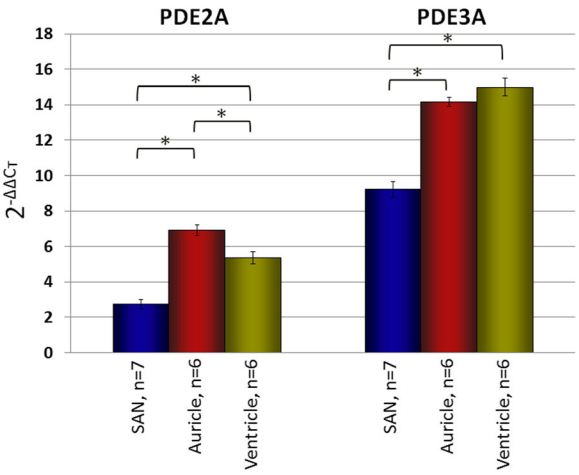

Fig. 7 Quantitative mRNA expression of PDE2A and PDE3A isoforms in murine myocardium. Expression is shown for the samples obtained from the SAN region, working myocardium of right atrium and right ventricular myocardium. Values are normalized to β-actin. Data are means±SEM. * = significant difference, $p<0.05$, Mann-Whitney test

Therefore, local concentrations of CO exactly in the place of its synthesis might be as high as concentrations used in our experiments.

The observed changes in electrical activity patterns induced by CO may be attributed to modulation of various ionic currents. Shortening of AP is usually caused by enhancement of potassium currents, such as I_{KATP} and acetylcholine-induced I_{KACh}, or suppression of L-type calcium current (I_{CaL}). The latter current strongly depends on proteinkinase A (PKA) activity, since PKA increases I_{CaL} amplitude through direct phosphorylation of the L-type calcium channels (see [24] for review). So, decrease in cAMP content is an effective way to reduce AP duration both in atrial and ventricular working fibers.

The rate of sinoatrial node firing is also dependent on cAMP content in pacemaker cells. A few of the leading putative mechanisms by which cAMP may affect SA nodal firing rate include I_f current activation as well as increased Ca^{2+} turnover between the cytosol and sarcoplasmic reticulum. First, I_f current, which has been believed to be the main current responsible for SDD development in sinoatrial cells, is positively regulated by cAMP [6]. Second, cAMP content and PKA activity control the speed of calcium turnover between cytosol and sarcoplasmic reticulum, which is crucial for automaticity of sinoatrial cells [7, 26]. This conception sounds more reliable, as full blockade of I_f current by ivabradine or cesium ions causes just a slight slowing of sinus rhythm [13]. Furthermore, blocking of cAMP production with adenylate cyclase inhibitor MDL or PKA inhibitors leads to cessation of automatic activity in sinoatrial cells [27, 28]. Thus, opposite effects of CO in working myocardium and SAN could be explained if this gas produces contradirectional changes in cAMP content in these tissues.

Since we had no ability to measure cAMP content directly, we have limited our investigation to study of the modulation of CO effects by various substances related to cGMP signaling pathway. As long as CO readily binds to transition metal ions, heme-containing proteins represent the most abundant target for this gas [30]. Among them, sGC is one of the most well-studied and essential for vasodilatory effects of CO. There are multiple crosslinks between cGMP, produced by GC, and cAMP, which are crucial for the regulation of the contractile and electrical properties of myocardium.

Our results suggest that the major portion of the CO effects depends on sGC activity, since sGC inhibitor ODQ suppressed response to CO more than twice and both sGC activator azosidnone and cGMP analog, BrcGMP, produced the same effects as CO. However, a substantial part of CO response persisted under ODQ indicating that observed CO effects are not fully cGMP-mediated. Future studies should reveal the mechanisms of cGMP-independent cardiotropic CO effects. Heme-containing cytochrome c oxidase seems to be the most

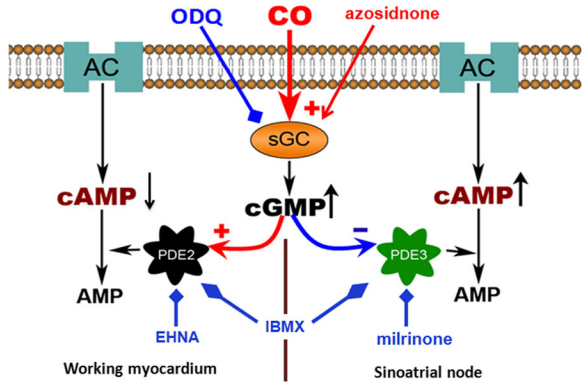

Fig. 8 The putative cGMP-dependent mechanism of CO effects in working and pacemaking myocardium of mouse. CO stimulates cGMP production in cardiomyocytes. cGMP stimulates PDE2, which is more active in working atrial and ventricular myocardium, but inhibits PDE3, which is more active in the SAN. That results in decrease in cAMP content in working myocytes and subsequent AP shortening, but elevation of cAMP level in pacemaking cells, which leads to acceleration of sinus rhythm. "+" = stimulation, "−" = inhibition, *blunt arrow* = blocking effect

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plausible candidate for CO target with potential mediation by ROS. CO-induced increase in ROS was shown to suppress I_{CaL} [20]. Alternatively, a CO-induced decrease in I_{CaL} may diminish sinoatrial APs, since the blocker of I_{CaL} nifedipine produces the same effect in the central part of SAN [12]. If this is true, then I_{CaL} inhibition produced by that cGMP-independent mechanism should be stronger than putative I_{CaL} stimulation caused by increased cAMP content. Thus, the seeming controversy between acceleration of rhythm and decrease in AP amplitude in the sinoatrial tissue can be explained by joint action of two distinct mechanisms of CO effects.

In general, experiments with selective inhibitors of PDE2 and PDE3, which are oppositely regulated by cGMP, confirmed that positive effects of CO in the SAN combine with negative effects in the atrium and ventricle due to the difference in activity of these PDE subtypes in pacemaker and working myocardium. Our results do not completely fit the data of a very recent study by Hua and coauthors [9], where electrophysiological responses to the same inhibitors were studied in the mouse heart. The authors demonstrated marked AP prolongation induced by IBMX in working myocytes and less pronounced increase of APD under action of EHNA, while no changes in AP configuration were caused by milrinone. However, this data was obtained in isolated paced cardiomyocytes, while our experiments were conducted on right atrial preparations beating in sinus rhythm. Shortening of APs induced by IBMX and milrinone in our experiments could be attributed to sinus rhythm acceleration and mimic putative AP prolongation caused by direct action of inhibitors on working myocardium. Heart rate in our experiments was increased by either IBMX and milrinone, but not EHNA, while Hua and coauthors observed a slight positive effect of EHNA, less prominent than effects of other inhibitors. Therefore, PDE3, but not PDE2, takes part in regulation of pacemaker activity.

The results of RT-PCR do not directly support this conclusion, since PDE3 expression in the SAN is lower than in working myocardium in concordance with the data of Hua and coauthors [9]. So, we can just propose following the authors that factors other than expression levels contribute importantly to the different effects of selective PDE inhibitors in different regions of the heart. Moreover, the situation when mRNA level does not correspond to the quantity of related protein and its activity is not infrequent. However, PDE2 is much more prominently expressed in the working myocardium in comparison to SAN. This fact can partly explain the absence of EHNA effects on heart rate.

More important findings concern the modulation of CO effects by PDE inhibitors. Inhibition of PDE2 failed to alter positive chronotropic effect of CO, but strongly attenuated its effects in working myocardium. Contrariwise, milrinone completely abolished rhythm acceleration, but just slightly decreased CO-induced AP shortening. Thus, in the presence of CO, the prevalent role of PDE2 and PDE3 in working myocardium and SAN, respectively, is clear.

The present study demonstrates for the first time the electrophysiological effects of CO in murine myocardium: acceleration of the sinus rhythm and shortening of AP in atrial and ventricular working myocardium. Figure 8 illustrates one of the mechanisms of CO response in working cardiomyocytes and cardiac pacemaker as we can propose it according to our data. Putative cGMP-independent mechanisms are not presented here, since they are beyond the scope of our study.

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Conflict of interest The authors declare that they have no conflict of interests.

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