NO Underlies the Muscarinic Receptor-Mediated Inhibition of I_f in Early Embryonic Heart Cells

*Nora Lang, *Michael Reppel, Juergen Hescheler and ¹Bernd K. Fleischmann

Institute of Neurophysiology, University of Cologne and ¹Institute of Physiology I, Life & Brain Center, University of Bonn, *These authors contributed equally to the manuscript

Key Words

Hyperpolarization-activated cyclic-nucleotide-modulated current (I_f) • Embryonic development • Muscarinic signalling • Nitric oxide

Abstract

Background/Aims: Early embryonic cardiomyocytes beat spontaneously. The hyperpolarization-activated cyclic-nucleotide-modulated current (I_f) appears to be involved in its modulation as it is highly expressed at this stage. The spontaneous beating of early embryonic heart cells is slowed by acetylcholine (ACh), and our earlier studies identified a key role for nitric oxide (NO) in the regulation of the voltage dependent L-type Ca^{2+} current ($I_{\text{Ca,L}}$). The aim of the present study was to clarify whether and via which signalling pathway(s) I_f is regulated upon muscarinic receptor activation in early embryonic (E9.5 to E11.5) cardiomyocytes. **Methods:** The whole-cell patch clamp technique in combination with pharmacology and/or knock out mouse models was used to investigate the regulation of I_f . **Results:** We found that the ACh ana-

logue carbachol (CCh, 10 μmol) led in the majority of cells (68%, $n=50$) to a significant depression of I_f by $16.3 \pm 1.4\%$ ($n=34$, $p<0.01$, voltage steps from -35 mV to -110 mV). This cholinergic inhibition was mediated by the NO/cGMP signalling pathway as it was largely reversed by superfusion with the non selective nitric oxide synthase (NOS) inhibitor N^G-Methyl-L-arginine acetate salt (L-NMMA, 1 mmol), the inhibitor of the soluble guanylyl cyclase (sGC) 1H-[1, 2, 4]Oxadiazolo[4, 3-a]quinoxalin-1-one (ODQ, 100 μmol) and a selective inhibitor of the phosphodiesterase (PDE) type 2 Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 30 μmol). Analysis of the muscarinic signalling in embryonic cardiomyocytes harvested from NOS2 (-/-) and NOS3 (-/-) mice revealed that the NOS3 isoform was entirely responsible for the muscarinic receptor-induced NO production. **Conclusions:** Muscarinic receptor stimulation depresses I_f by generating NO via the NOS3 and the cGMP/PDE type 2 signalling pathway in early embryonic cardiomyocytes. This suggests that NO is a key signalling molecule involved in the regulation of chronotropy of early embryonic heart cells.

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Introduction

The heart beat is initiated by the automatism in specialized cells of the sinoatrial node. Sinusnodal and other cells of the specific conduction system of the heart display the slow diastolic depolarisation, which depolarizes the membrane potential to threshold, thereby evoking an action potential [1]. hyperpolarization-activated cyclic-nucleotide-modulated current (I_f) is thought to be involved in the generation of the diastolic depolarisation [2], particularly its early phase. The channel is activated by hyperpolarization, permeable to Na^+ und K^+ , and displays slow kinetics of activation and deactivation, a linear current voltage relationship and a voltage-dependent block upon extracellular application of Cs^+ . I_f is modulated in adult sinoatrial node cells by hormones of the autonomous nervous system resulting in alterations of the steepness of the diastolic depolarization. This is caused by hormone-induced alterations of the current-voltage (IV) relation of I_f as Isoprenaline (ISO) shifts the $V_{1/2}$ of these channels to more depolarized and acetylcholine (ACh) to more hyperpolarized potentials [4] due to changes of cAMP levels [5, 6].

Several reports have proposed an important role of I_f in the early embryonic mouse heart [7-9]. In both, early embryonic murine [7, 9] and embryonic stem (ES) cell-derived cardiomyocytes [8, 10, 11]. I_f was found to be highly expressed and these cells also displayed spontaneous electrical activity. In contrast to adult pacemaker cells prominent stimulatory and inhibitory effects of hormones of the autonomic nervous system on I_f were seen at strongly hyperpolarized voltages [8]. Beside the mouse, prominent expression of I_f at the embryonic stage was also reported for chick embryonic ventricles [12], ventricular cells from newborn rats [13] and in cells of the cardiac conductive system of different species [14, 15]. At later stages of embryonic development I_f was found to be downregulated and this was accompanied by a loss of the spontaneous electrical activity [7-9]. Muscarinic receptor activation caused, similar to the adult heart, negative chronotropy in early ES cell-and embryonic mouse heart-derived cardiomyocytes. However, this effect was not due to activation of the acetylcholine activated K^+ current [16], suggesting that I_f may play besides $I_{\text{Ca,L}}$ an important role in the regulation. To date the intracellular signalling cascades involved in the muscarinic regulation of I_f are still unknown.

Recent evidence suggests a fundamental role of nitric oxide (NO) during early cardiac development [17].

Our group has previously shown that muscarinic depression of the L-type Ca^{2+} current ($I_{\text{Ca,L}}$) occurs through generation of NO and subsequent activation of the soluble guanylyl cyclase (sGC), cGMP, cGMP-dependent phosphodiesterases (PDE) in both, early embryonic murine and ES cell-derived cardiomyocytes. These findings were corroborated by molecular and immunohistochemical studies where elevated levels of NO synthase (NOS)3, NOS2, sGC and cGMP were detected in the early embryonic heart [18-20] and downregulated during further development. In contrast, in late developmental stage cells muscarinic signalling was found to act via direct inhibition of the adenylyl cyclase (AC) [19, 21].

The aim of the present study was to investigate whether I_f was modulated via muscarinic receptor activation in early embryonic heart cells and whether NO was involved, as reported for $I_{\text{Ca,L}}$. If this was the case, we were also interested to find out which NOS isoform was responsible for the NO generation and have used NOS2 (-/-) and NOS3 (-/-) mice for this purpose.

Material and Methods

Superovulation and preparation of isolated murine cardiomyocytes

Female mice were superovulated for precise staging of embryos [22]. For wild-type mice the outbred strain HIM:OF1 was used. NOS2 (-/-) and NOS3 (-/-) mice were obtained from Jackson Laboratories (Bar Harbor, ME). In the NOS2 mutants the calmodulin binding site was disrupted [23], whereas in the NOS3 mutants a premature translation stop codon was inserted [24].

Isolated murine cardiomyocytes from atria and ventricles of E9.5 to E11.5 embryonic hearts were obtained as reported earlier [25]. Briefly, pregnant mice were killed by cervical dislocation, the uteri were isolated, and whole embryos exposed. The hearts were dissected and separated in atria and ventricles. The dissected atria and ventricles were incubated with 1 mg/ml collagenase B (Boehringer, Mannheim, FRG) for 30 minutes in 37°C and then dissolved in low Ca^{2+} -solution containing (in mM): 85 KCl, 30 K_2HPO_4 , 1 EGTA, 5 Na_2ATP , 5 NaPyruvate, 5 creatine, 20 taurine, 20 glucose, pH 7.2 for 20 minutes at room temperature and thereafter mechanically dissociated by trituration. The isolated cells were plated on collagen-coated glass cover slips and incubated in DMEM with 20% fetal bovine serum and 10 $\mu\text{g/ml}$ gentamycin at 37°C in a humidified CO_2 incubator.

Spontaneously contracting cardiomyocytes could be observed within 12 hours after cell preparation and electrophysiological experiments were performed 36 to 48 hours after plating.

Fig. 1. CCh inhibits I_f in early embryonic cardiomyocytes at strongly hyperpolarized potentials: A, IV curves of I_f were evoked prior (left panel) and in presence of CCh (10 μ mol) in early embryonic murine cardiomyocytes by applying hyperpolarizing voltage steps from -30 to -130 mV in 20 mV increments from a holding potential of -35 mV. Dotted line indicates the zero current level. B, I_f amplitudes of the experiment shown in A demonstrate that CCh application induced maximal inhibition of I_f at strongly hyperpolarized voltage steps. C, Current amplitudes were normalized to I_f density at -130 mV. The values were fitted with a Boltzman function. Asterisks indicate statistical significance ($p < 0.05$).

Electrophysiology

The classic current clamp and voltage clamp mode of the whole cell configuration was applied to record action potentials (APs) and I_f , respectively. Only spontaneously contracting, single cardiomyocytes were used. Patch clamp experiments were performed using an EPC 9 (HEKA, Lambrecht, FRG) amplifier (for detail see also [8]). Data were acquired at a sampling rate of 10 kHz, filtered at 1 kHz, stored on a hard disc and analyzed off-line using the Pulsefit (HEKA, Lambrecht, FRG) analysis software package.

For recording of IV curves, hyperpolarizing voltage steps (2 sec) from -30 to -130 mV in 20 mV intervals were applied (holding potential -35 mV). In most experiments, I_f was recorded by applying 2 sec lasting hyperpolarizing voltage step to -110 mV from a holding potential of -35 mV. Due to the slow activation kinetics of I_f , voltage steps were applied at low frequency (maximum rate 0.1 Hz).

All experiments were performed at 37°C. The pipettes (2–4 M Ω when filled with internal solution) were pulled from Clark (England) borosilicate glass capillaries using a Zeitz puller (DMZ, Munich, FRG). They were filled with internal solution I_1 (containing in mM: KCl 50, MgCl₂ 1, HEPES 10, MgATP 3, EGTA 10, potassium aspartate 80) to measure APs and with internal solution I_2 (containing in mM: NaCl 10, MgCl₂ 1, HEPES 10, MgATP 3, EGTA 10, potassium aspartate 130) to measure I_f . For the measurement of APs the external solution E_1 (containing in mM: NaCl 140, KCl 5.4, MgCl₂ 1, CaCl₂ 1.8, HEPES 10, Glucose 10), for the measurement of I_f the external solution E_2 (containing in mM: NaCl 115, KCl 25, CaCl₂·H₂O 1.8, HEPES 5, Glucose 10, BaCl₂ 1, 4-AP 2, L-Arginine 1) were used. E_2 contained Ba²⁺ to reduce the background K⁺ current and 4-Aminopyridine to minimize the interference from the transient outward K⁺ current. The pH of all solutions was adjusted to 7.4

with KOH and NaOH, respectively at a temperature of 37°C.

Analysis of data

The amplitude of I_f was measured as the difference between the instantaneous current at the beginning and the steady state current at the end of the voltage step [8]. Currents were normalized to cell membrane capacity where indicated. For the IV curves, current amplitudes were normalized and fitted with a Boltzman function. The cellular capacitance and density of I_f (ranging from 2 to 50 pA/pF) showed big variations. Therefore, all effects are reported as percentage of increase or decrease of I_f density. Only cells with a variation of I_f density >5% from baseline were included in the statistics.

Errors are presented as standard error of the mean (S.E.M.). Statistical analysis was performed using paired and unpaired Student's t-test. P-values of < 0.05 were considered as statistically significant.

Reagents

The NO donor 2-(N,N-Diethylamino)-diazene-2-oxide diethylammonium salt (DEA-NONOate) and the NOS inhibitor N^G-Methyl-L-arginine acetate salt (L-NMMA) were purchased from Alexis Biochemicals (Grünberg, Germany); all other substances were purchased from Sigma Aldrich GmbH (Deisenhofen, Germany). 1H-[1, 2, 4]Oxadiazolo[4, 3-a]quinoxalin-1-one (ODQ) and Milrinone were dissolved in dimethylsulfoxid (DMSO; 100 mM, final DMSO concentration 0.01%), the others were dissolved in extracellular solution, and either prepared freshly (DEA-NONOate) or stored frozen at -20°C. Aliquots were thawed immediately before use and diluted in the extracellular solution to the required concentration. Milrinone and ODQ had to be protected from light during the experiments.

Fig. 2. Time course and percentage of cells with CCh induced depression of I_f : A,B, CCh (10 μ M) depressed I_f in a representative early embryonic cardiomyocyte, the effect was partially reversed upon wash out (A). The inhibitory action of CCh is also shown in the time course of the experiment displayed in A (B). I_f was evoked by 2 sec lasting hyperpolarizing voltage steps from -35 mV to -110 mV, the numbers indicate when the currents shown in A were evoked. C,D, Effect of CCh on I_f density in representative cells (C) and on all cells with CCh response (D). E, Percentage of responding (filled bars) and not-responding (shaded bars) cells.

Results

CCh depresses I_f

First we have tested the effect of the muscarinic receptor agonist carbachol (CCh, 10 μ M) on I_f at different step potentials. Figure 1A shows a representative experiment, where 2 sec lasting voltage steps ranging from -30 to -130 mV in 20 mV intervals were applied prior and in the presence of CCh. A prominent inhibitory

Fig. 3. I_f density is modulated by NO and the CCh effect abolished by NOS inhibitors: A: The NO donor DEA-NONOate (400 μ M) depressed I_f in an early embryonic cardiomyocyte (1 control current, 2 in presence of DEA-NONOate) B: Effect of DEA-NONOate (400 μ M) on I_f density in representative early embryonic cardiomyocytes. C,D: Time course of the peak amplitude of basal I_f in response to the unspecific NOS inhibitor L-NMMA (1 mM) (original current traces marked by numbers are shown as inset) (C); I_f density in early embryonic cardiomyocytes prior and in presence of L-NMMA (D). E,F Original current traces (E) and I_f density (F) in presence of CCh (10 μ M) and L-NMMA (1 mM).

effect of CCh was observed at saturating voltage steps to -110 and -130 mV (Fig. 1B) and no strong shift in the IV relation was seen (Fig. 1B). This finding was corroborated in 5 experiments (Fig. 1C) and was in accordance with our earlier data concerning the hormonal regulation of I_f in early stage ES cell-derived cardiomyocytes [8]. This may be related to the high basal levels of cAMP [19, 21] and/or modulation by protein kinase A (PKA)-dependent phosphorylation [8, 15, 26]. In the following

Fig. 4. I_f density is modulated by the sGC and the CCh effect abolished by an inhibitor of sGC in early embryonic cardiomyocytes: A: Time course and original currents (inset) recorded prior and in presence of ODQ (100 μ M, 1 control, 2 ODQ, 3 wash out). B: Effect of ODQ (100 μ M) on basal I_f density. C,D: Effect of ODQ on the CCh-depressed I_f original current traces (C) and the corresponding time course (D) are depicted. E: I_f density in early embryonic cardiomyocytes prior and in presence of CCh (10 μ M) and ODQ (100 μ M).

experiments we have therefore tested the intracellular signalling cascade involved into the muscarinic-mediated depression of I_f by applying hyperpolarizing voltage steps to -110 mV. This is depicted in Figure 2A and B, where representative currents and the corresponding time course of the typical CCh-induced depression of I_f as well as its reversal by wash out are shown. The CCh-induced inhibition of I_f density is illustrated in Figure 2C and amounted overall to $16.4 \pm 1.4\%$ in 34 out of 50 cells (68%, Figure 2E). On average, I_f density was lowered by CCh from 15.9 ± 3.5 pA/pF to 13.6 ± 3.2 pA/pF (Figure 2D;

Fig. 5. I_f density is modulated by PDEs and the CCh effect abolished by a non selective PDE inhibitor in early embryonic cardiomyocytes: A: Time course and original currents (inset) recorded prior and in presence of the non selective PDE inhibitor IBMX (400 μ M, 1 control, 2 IBMX, 3 wash out). B: Effect of IBMX (400 μ M) on basal I_f density. C,D: The effect of IBMX (400 μ M) on CCh-mediated depression of I_f ; original current traces (C) and the corresponding time course (D) are depicted. E: I_f density in early embryonic cardiomyocytes prior and in presence of CCh (10 μ M) and IBMX (400 μ M).

$p=0.00001$). In the remaining 32% of cells we could not detect an effect of CCh on I_f even though the current proved robust in our whole cell recordings. Interestingly, the percentage of cells which proved to be unresponsive to CCh was similar to that reported in our earlier studies on the muscarinic regulation of $I_{Ca,L}$ [19, 21].

Since at E9.5 the atria cannot be cleanly separated from the ventricles, we also performed current clamp measurements to determine the cardiac subtype of individual cells (for detail see also [10]). The CCh-mediated suppression of I_f was found to be similar in pacemaker-

(n=5), atrial- (n=12) and ventricular-like (n=17) cardiomyocytes (data not shown) clearly demonstrating that this response occurred in all cardiac subtypes.

Mediation of the muscarinic CCh effect by the NO/cGMP signalling pathway

We next investigated the signalling pathway(s) involved and determined whether in analogy to the modulation of $I_{Ca,L}$ [19, 21] NO played a role in the muscarinic modulation of I_f . We tested first whether an increase in NO lowered the amplitude of I_f in early embryonic cardiomyocytes. The cells were superfused with the NO donor DEA-NONOate (400 μ M). A prominent irreversible inhibitory effect on I_f density of $28.8 \pm 6.5\%$ (n=10; $p=0.002$) was noticed in all the cells tested (Figure 3A,B).

Since muscarinic receptor activation and NO-donors caused inhibition of I_f we next investigated whether the muscarinic regulation of I_f occurred through a NO/cGMP-dependent pathway in early embryonic cardiomyocytes. First, we tested the effect of the unspecific NOS-inhibitor L-NMMA (1 mmol). In Figure 3C current traces and the corresponding time course show that I_f was enhanced upon extracellular application of L-NMMA by $27.4 \pm 8.1\%$ ($p=0.01$) in 8 out of 11 cells (72.7%); representative cells are displayed in Figure 3D. We also tested the effect of L-NMMA on the CCh-depressed I_f and noticed almost a complete reversal of the CCh effect (Figure 3E,F) to $4.5 \pm 12.5\%$ in all cells tested (Figure 2F; $p=0.00002$). These data clearly suggested that the muscarinic inhibition of I_f was mediated through the generation of NO.

Since NO is known to activate the sGC resulting in an increased production of cGMP we next assessed the influence of ODQ (100 μ M), a selective blocker of the sGC, on basal I_f . Perfusion with ODQ increased I_f (Figure 4A,B) in 8 out of 13 cells (61.5%) by $16.4 \pm 2.4\%$ and this effect could be reversed by wash out (Figure 4A; $p=0.0002$). We further proved the involvement of the sGC in the CCh-mediated depression of I_f by application of ODQ in presence of CCh and found in 4 out of 6 cells (66.7%) an almost complete reversal (reduction of the CCh effect from $21.2 \pm 3.2\%$ to only $5.1 \pm 1.2\%$; $p=0.01$) of the CCh effect (Figure 4C-E).

Since the sGC is known to alter cAMP and related downstream signalling components via PDEs, we next evaluated the effect of non-selective (3-Isobutyl-1-methylxanthine, IBMX) and selective (Erythro-9-(2-hydroxy-3-nonyl)adenine, EHNA; Milrinone) PDE inhibitors on basal and I_f depressed by CCh. IBMX (400 μ M) led to a pronounced stimulation of basal I_f density

Fig. 6. I_f density is modulated by the PDE II and the CCh effect almost reversed by a selective PDE II inhibitor: in early embryonic cardiomyocytes: A: Time course and original currents (inset) recorded prior and in presence of the selective PDE II inhibitor EHNA (30 μ M, 1 control, 2 EHNA, 3 wash out). B: Effect of EHNA (30 μ M) on basal I_f density. C,D: Effect of EHNA on the CCh-mediated depression of I_f ; original current traces (C) and the corresponding time course (D) are depicted. E, I_f density in representative early embryonic cardiomyocytes prior and in presence of CCh (10 μ M) and EHNA (30 μ M).

($23.0 \pm 7.4\%$; $p=0.009$) in 7 out of 10 cardiomyocytes (70%) and this effect could be reversed by wash out (Figure 5A,B). In addition, IBMX completely reverted the CCh effect as the CCh-induced depression of I_f density was reduced from $22.0 \pm 2.5\%$ to $2.9 \pm 2.5\%$ in 4 out of 7 cells (57.1%; Figure 5C-E; $p=0.03$). These data corroborated our earlier findings in regard to a strong intrinsic activity of PDEs in early embryonic cardiomyocytes [8, 25] and also pointed to a CCh-mediated activation of the NO-sGC-PDE signalling cascade.

Fig. 7. CCh-induced depression of I_f is preserved in NOS 2 (-/-) early embryonic cardiomyocytes. A,B CCh (10 μ M) depressed I_f in a representative NOS 2 (-/-) cardiomyocyte and the CCh action was reversed by washout (A) as can also be seen in the corresponding time course (B); the numbers indicate the time points taken of the displayed original current traces. C I_f density under control conditions and in presence of CCh (10 μ M).

Next, we determined the involvement of different PDE subtypes which were found in our earlier work to play an important role in the regulation of $I_{Ca,L}$ in early embryonic cardiomyocytes [21]. The specific PDE II antagonist EHNA (30 μ M) stimulated I_f (Figure 6B) by $13.8 \pm 2.4\%$ in 11 out of 18 cells (61.1%) and this stimulatory effect could be reversed by wash out (Figure 6A; $p=0.0002$). Similar to our earlier findings on the regulation of $I_{Ca,L}$ [19, 21] the selective PDE II blocker EHNA (30 μ M) led to a nearly complete reversal of the CCh (from $11.9 \pm 0.7\%$ to $0.1 \pm 0.6\%$; $p=0.001$) effect in all cardiomyocytes tested ($n=4$, Figure 6C-E). We also performed a set of experiments where we first perfused the cells with EHNA. In none of the cells ($n=5$) an inhibitory effect of CCh could be detected (data not shown) unequivocally demonstrating the involvement of the PDE II in muscarinic depression of I_f . In contrast to the prominent effect of EHNA, the PDE III blocker Milrinone (15 μ M) had almost no influence on basal I_f density as a small stimulation was noticed in only 2 out of 13 cells ($n=13$; 15%).

Fig. 8. CCh stimulates basal I_f in NOS 3 (-/-) early embryonic cardiomyocytes: A,B CCh (10 μ M) was without effect on I_f in a representative NOS 3 (-/-) cardiomyocyte. This can also be seen in the corresponding time course (B); the numbers indicate the time points taken of the original current traces displayed in A. C, I_f density under control conditions and in presence of (10 μ M).

Involvement of the NOS3 isoform in the muscarinic regulation of I_f

Our results clearly demonstrate that NO mediates the CCh effect on I_f . In order to corroborate these findings unequivocally, and to identify the NOS isoform responsible for the muscarinic receptor evoked NO generation, we tested the muscarinic regulation of I_f in NOS2 (-/-) and NOS3 (-/-) mice. We first investigated the muscarinic action of CCh on I_f in early embryonic cardiomyocytes harvested from NOS2 (-/-) mice. As depicted in Figure 7A-C, CCh still suppressed I_f density by $25.0 \pm 7.4\%$ ($p=0.04$) in 4 out of 6 cells and the response was therefore very similar to the results in wild type mice. In contrast, CCh did not depress I_f in early embryonic cardiomyocytes obtained from NOS3 (-/-) mice ($n=7$; Figure 8A,B). In fact, either no response or even a slight stimulatory effect ($n=7$; Figure 8C) of CCh onto I_f was seen in these cells. These data clearly prove the critical role of NO in CCh-mediated inhibition of I_f and the involvement of the NOS3 isoform in the muscarinic signaling cascade of early embryonic heart cells.

Discussion

In the present study we have investigated the muscarinic regulation of I_f in early embryonic murine heart cells and determined in particular the involvement of NO. The study was motivated by earlier work, where the involvement of NO in the muscarinic regulation has been controversially discussed [27-32].

We have identified a novel modulatory pathway of I_f which was corroborated by several pharmacological agents intervening at different sites in the signalling cascade and also by the use of NOS2 and NOS3 (-/-) mouse models. The most important findings of our study are: (i) M2 receptor activation by CCh resulted in depression of I_f by NOS3 isoform-mediated generation of NO. (ii) The muscarinic regulation of I_f was entirely directed through the NO/cGMP/PDE II pathway. (iii) An inhibition of basal I_f density was observed by NO donors and a stimulation of basal I_f density by inhibitors of the NO/cGMP/PDE signal transduction cascade (L-NMMA, ODQ, IBMX and EHNA). Importantly, the inhibitory effect of CCh was fully dependent on the generation of NO because it could be entirely reversed by superfusing the cells with L-NMMA. The critical role of the NOS3 isoform for the muscarinic modulation of I_f was clearly proven by the preserved CCh effect in NOS2 (-/-) mice and the lack of CCh in NOS3 (-/-) mice.

Our data concur with earlier studies in the embryonic heart where a high expression of the NOS3 isoform, as well as of the downstream signalling components were reported [18-20]. In addition, our new findings on the regulation of I_f in early embryonic heart cells are in agreement with data of our group concerning the regulation of $I_{Ca,L}$ in early ES cell-derived [19] and early embryonic cardiomyocytes [21].

Most of the previous work analyzed the modulation of chronotropy and of basal I_f in response to superfusion with NO donors [33, 34]. These studies showed that exogenous NO increased both chronotropy and I_f amplitude. This effect was reported to be mediated via activation of the cGMP/PDE3 signalling pathway [35]. In contrast, reports regarding the modulation of pre-stimulated I_f in response to exogenous NO donors are somewhat contradictory as both, stimulatory and inhibitory effects were observed [34, 36]. This was probably due to the different levels of sGC/cGMP resulting in the inhibition/activation of different PDEs.

The effect of PDEs on I_f amplitude clearly supports our earlier data on elevated AC/cAMP and PDE levels in early embryonic cardiomyocytes. This is similar to adult sinusnodal cells, as an IBMX-induced stimulation of I_f was also detected [34, 37]. Furthermore, the involvement of the NO/cGMP/PDE2 pathway in the muscarinic regulation of $I_{Ca,L}$ in rabbit sinoatrial cells [29] proved similar to our earlier findings in embryonic cardiomyocytes. However, studies on the muscarinic regulation of I_f in adult rabbit sinoatrial cardiomyocytes excluded an involvement of PDEs, clearly pointing to differences in the regulation of I_f between adult and embryonic cardiomyocytes [34, 37].

Early embryonic cardiomyocytes beat spontaneously. While this appears to be evoked by intracellular Ca^{2+} oscillations [38], modulation of the spontaneous beating most likely occurs also via I_f as this current is highly expressed in all cardiac subtypes at the early embryonic stage [7, 9].

Various physiological and pathophysiological functions of NO have been discovered in recent years, whereas only little is known about regulatory pathways during embryonic development. The present and our earlier work points to an important role of NO in general and for muscarinic signalling in particular during early embryonic development in heart muscle cells [19, 21]. Although at this stage the cell biological relevance and genetic mechanisms underlying the differential gene expression of the different NOS isoforms in cardiomyocytes during development are still unclear, the advantages of NO as an elegant signalling molecule are evident because of its high reactivity and very short half time [39]. Since I_f has become an important target for antiarrhythmic treatment [40, 41], investigations concerning the expression and the regulation of this channel during embryonic development appear to be particularly important for a better pathophysiological understanding as pathologically altered cardiomyocytes are known to recapitulate their fetal genetic program [42].

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