Effects of cGMP on L-type calcium current of adult and newborn rabbit ventricular cells

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Abstract

Objective: Cyclic GMP has been shown to be in some respects an inhibitory modulator of heart function. Various studies on the modulation of cardiac L-type calcium current (I_{Ca}) by cGMP in different species show inconsistency and the role of cGMP remains unclear and controversial. The present study was focused on the differences in the modulation of basal I_{Ca} by cGMP in adult and newborn rabbit ventricular cells. Methods: Enzymatically isolated adult and newborn (1-4-day-old) rabbit ventricular myocytes were used to measure I_{Ca} under whole-cell voltage clamp conditions with internal perfusion of isolated cells. Results: We have shown that in adult ventricular cells, the intracellular perfusion of 8BrcGMP did not produce any effect on basal I_{Ca}, while intracellular perfusion of 8BrcGMP or 8CPT-cGMP in newborn ventricular cells significantly and reversibly increased basal I_{Ca} without changing the voltage dependence for activation of I_{Ca}. Both methylene blue and LY-83583 (which inhibit guanylyl cyclase and thus lower cGMP levels), in adult ventricular cells, failed to produce any significant effect on basal I_{Ca}, while in newborn ventricular cells the application of methylene blue or LY-83583 produced irreversible inhibition of basal I_{Ca}. Similarly, KT-5823, an inhibitor of cGMP-dependent protein kinase, also inhibited basal I_{Ca} in newborn ventricular cells but not in adult ventricular cells. However, extracellular application of methylene blue during the intracellular perfusion of 8BrcGMP was unable to inhibit I_{Ca}. Extracellular application of nitrosoglutathione which releases nitric oxide produced a significant increase in I_{Ca} in newborn but not in adult ventricular cells. Extracellular application of a cAMP-dependent protein kinase inhibitor peptide blocked the stimulatory effect of cAMP but not of 8CPT cGMP, while the stimulatory effect of nitrosoglutathione on I_{Ca} was not blocked by the presence of a phosphodiesterase inhibitor (isobutylmethyl-xanthine). Conclusions: We propose that, for newborn rabbit ventricular cells, cGMP plays a crucial role in maintaining basal I_{Ca} by a mechanism mediated via protein-kinase-G-dependent phosphorylation of calcium channels or some associated protein.

Keywords: cGMP; cGMP-dependent protein kinase; Calcium channel, L-type; Development; Rabbit, ventricular myocytes

1. Introduction

The cyclic nucleotide cGMP has been shown to exert a variety of generally inhibitory actions on heart cells, including a negative inotropic effect [5,23] and an inhibition of L-type calcium current density (I_{Ca}, pA/pF) [4,6,11,14,35], particularly in the presence of β-adrenergic stimulation. However, the physiological role of cGMP in regulation of cardiac activity is still controversial, being in some respects antagonistic to that of cAMP [7]. Basal levels of cGMP in heart cells may be of importance in maintaining basal I_{Ca}. cGMP interacts with several different intracellular receptor proteins including protein kinases, cyclic nucleotide phosphodiesterases (PDEs), and ion channels [16]. cGMP activates a specific protein kinase (cGMP-dependent protein kinase, PKG), which can phosphorylate a number of proteins [16,18,36,37]. It is also known to stimulate or inhibit specific PDEs to produce either antagonistic or synergistic effects with cAMP-elevating hormones [3]. Various studies on the effects of cGMP on I_{Ca} in different species have shown dissimilar findings and have failed to observe consistent effects of cGMP on electrical activity of cardiac muscle [10,30]. A number of studies have demonstrated three different path...

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ways by which cGMP may modulate I_{ca}: (i) inhibition of I_{ca} by activation of an endogenous PKG which is presumed to phosphorylate the L-type calcium channel or some regulatory protein [14,19,21]; (ii) inhibition of I_{ca} by activation of cGMP-stimulated PDE and thus indirectly lowering the level of cAMP [10,11,19]; and (iii) stimulation of I_{ca} by inhibition of cGMP-inhibited PDE and thus raising cAMP levels [24]. In mammalian heart, both potentiation and inhibition by cGMP (or cGMP analogs) of β-adrenergic or cAMP effects on I_{ca} were shown in guinea pig ventricular cells by different groups of investigators, but none of these studies showed any significant effects on basal I_{ca}. Levi et al. [14] have shown inhibition of cAMP- or 8Br-cAMP- (a hydrolysis-resistant analog of cAMP) elevated I_{ca} in guinea pig ventricular cells by intracellular application of 5 μM cGMP or extracellular application of 100 μM 8Br-cAMP (a membrane-permeable analog of cGMP). In contrast to these findings, Ono and Trautwein [24] demonstrated an additional increase by 10 μM cGMP of I_{ca} which had been elevated by isoproterenol or forskolin, and no effect of cGMP in the presence of 8Br-cAMP. Intracellular application of PKG in rat ventricular myocytes was also shown to depress I_{ca} [20,21], suggesting a PKG-dependent mechanism for the inhibitory effect of cGMP on I_{ca} in mammalian heart. Recently, in 2-21-day-old rat ventricular myocytes [31], the inhibitory effects of 8Br-cAMP on basal and isoproterenol-stimulated I_{ca} were shown to be present only after the intracellular application of exogenous PKG. However, 8Br-cAMP by itself was unable to produce any effect on basal I_{ca}. These inhibitory effects were proposed to be mediated by a direct phosphorylation of the Ca^{2+}-channel or associated regulatory protein. In adult rabbit ventricular cells, Tohse et al. [33] have shown a small reduction (15-20%) in basal I_{ca} by atrial natriuretic peptide and by extracellular application of 300 μM 8Br-cAMP.

No previous studies on the developmental differences in the effect of cGMP on I_{ca} have appeared. cGMP may be more important in the regulation of basal I_{ca} and β-adrenergic-stimulated I_{ca} in newborn ventricular cells than in adult cells. We have shown that basal levels of cGMP (per mg protein) in isolated ventricular cells were significantly higher in newborn (1-4-day-old) (272 ± 33 fmol/mg protein) compared to adult (55 ± 7 fmol/mg protein) [13] cells. In the present study, we examined the effects of cGMP by elevating its levels either by intracellular application of cGMP analogs by a pipette perfusion system or by stimulating guanylyl cyclase (G-cyclase) by NO using NO donor nitrosoglutathione (GSNO) or lowering its levels by inhibiting G-cyclase by methylene blue and LY-83583 on L-type Ca^{2+} current in adult and newborn rabbit ventricular cells. We have also examined the effects of a cAMP-dependent protein kinase inhibitor (PKI, 5-22), a cGMP-dependent protein kinase inhibitor (KT-5823) and a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, IBMX). Our results suggest a mechanism of action of cGMP in heart cells during early postnatal life altogether different from that in adult heart cells. Another unique aspect of our work is that these developmental differences occur within the same species, suggesting that individual cells, during development, alter their mechanisms of response to cGMP.

2. Methods

2.1. Preparation of isolated cells

New Zealand White adult (1.5-2 kg) and newborn (1-4-day-old) rabbits of either sex were used. Adult rabbits were heparinized (1000 units, i.v.) and anesthetized with sodium pentobarbital (50 mg/kg, i.v.). For newborn rabbits the same drugs were given intraperitoneally. Hearts were rapidly removed via thoracotomy with artificial ventilation and the aorta was cannulated. Single ventricular cells were obtained by enzymatic dissociation as we previously described [25,27]. In brief, a dissected heart was mounted on a Langendorff apparatus and perfused sequentially with oxygenated normal Tyrode solution for 4-5 min, nominally Ca^{2+}-free solution for 5-6 min, the same solution containing 0.08-0.15 mg/ml collagenase (Yakult Co., Japan) and 0.05 mg/ml protease (Type XIV, Sigma) for 5-7 min, and a storage solution for 4-5 min at a rate of 3-4 ml/g/min at 36-37°C. The ventricles were cut into small pieces and triturated in storage solution. Isolated cells were stored at 4°C.

2.2. Recording of whole-cell calcium current

Voltage-clamp experiments were performed as described earlier [25] in the whole-cell configuration of the patch-clamp method by use of an Axopatch-200 patch-clamp amplifier (Axon Instruments, Foster City, CA), using fire-polished borosilicate glass pipettes with resistance of 1-2 MΩ for adult cells and 2-4 MΩ for newborn cells when filled with the normal pipette solution. A tight seal (3-5 GΩ) was established in normal Tyrode solution. After breaking the membrane by applying quick suction to the pipette, the external solution was changed to the test solution. The complete change in the bath solution was obtained within 1 min.

For routine monitoring of I_{ca}, the ventricular cells were depolarized every 10 s from a holding potential of -40 mV to a test potential of +15 mV for 270 ms. No leakage correction was required. This test voltage is based on the peak current of the I-V relationships for both control and isoproterenol stimulated I_{ca} as we previously published [25,26]. We have previously shown that currents elicited with this protocol are stable over long periods of time and are completely blocked by nifedipine [25]. The elicited I_{ca} was filtered at a corner frequency of 2 kHz, digitized at 200-μs intervals, and stored and analyzed on an IBM-com-
plicable computer with pCLAMP6 software (Axon Instruments). $I_{Ca}$ was measured as the peak inward current. To obtain current–voltage relationship, a series of test pulses of 270 ms duration were applied with 10 mV increments from a holding potential of −40 mV. All experiments were performed at room temperature (21–23°C). Membrane capacitance was measured using the calibrated capacity compensation circuit of the Axopatch voltage clamp amplifier using a 5 mV hyperpolarizing pulses. We expressed all the current data as current density (pA/pF) by normalizing the peak $I_{Ca}$ for each cell to the cell capacitance. Cells which showed rundown were excluded from the analysis by only accepting the data from cells in which $I_{Ca}$ was stable in the control condition and reached a stable effect with the tested drugs.

2.3. Solutions and drugs

The compositions of standard solutions used were as follows (mM). Normal Tyrode: NaCl 148.8, KCl 4.0, CaCl$_2$ 1.8, MgCl$_2$ 0.53, Na$_2$PO$_4$ 0.33, HEPES 5.0, glucose 5.0, pH 7.4 with NaOH. Ca$^{2+}$-free solution: NaCl 100, KCl 10, K$_2$PO$_4$ 1.2, MgSO$_4$ 5, taurine 50, glucose 20, HEPES 10, pH 7.1 with NaOH. Storage solution: K glutamate 140, taurine 20, MgCl$_2$ 5, EGTA 1, glucose 10, HEPES 10, pH 7.4 with KOH. $I_{Ca}$ test solution: NaCl 130, CaCl$_2$ 1.8, CsCl 20, MgCl$_2$ 0.53, HEPES 5, glucose 5, pH 7.4 with NaOH. Normal pipette solution: CsOH 110, aspartic acid 90, CsCl 20, tetracethylammonium Cl (TEACl) 10, HEPES 5, EGTA 10, MgATP 5, Na$_2$ creatine phosphate 5, GTP (Tris) 0.4, leupeptin 0.1, pH 7.2 with CsOH. Modifications to these solutions are described below as appropriate for specific protocols. For perfusion of different internal solutions in the patch pipette in whole-cell voltage clamp mode, we use a pipette perfusion system (2PK +, Adams and List, NY), with a quartz capillary which runs from a reservoir to within 400 μm of the tip of the patch pipette. The positive pressure is introduced by a pressure/vacuum generator to flow the solution and is maintained the 8BrcGMP in the internal perfusion solution at the time periods indicated by the horizontal arrows. To wash out 8BrcGMP, cell was perfused with normal internal solution.

2.4. Statistics

All values are presented as mean ± s.e.m. Statistical analyses were performed using SigmaStat for windows with paired t-test. A P-value of less than 0.05 was defined as significant.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 1985).

3. Results

To investigate the effect of increasing levels of cGMP on basal $I_{Ca}$, we applied 8BrcGMP intracellularly by perfusing the patch pipette with a solution containing 8BrcGMP for adult and newborn ventricular cells. We used 8BrcGMP, which is a non-hydrolysable analog of cGMP and known to act more specifically than cGMP on PKG [11,17]. For a newborn ventricular cell the time course of $I_{Ca}$ peak current is shown in Fig. 1A. For this cell, we switched the internal solution to one containing 30 μM 8BrcGMP after achieving a steady-state basal $I_{Ca}$ and this concentration produced an increase in $I_{Ca}$ of 80–100% over basal $I_{Ca}$. Basal $I_{Ca}$ was increased from 2.4 ± 0.2 to 4.3 ± 0.3 pA/pF (P < 0.004, n = 5, 30 μM 8BrcGMP) and to 4.5 ± 0.5 pA/pF (P < 0.002, n = 3, 100 μM 8BrcGMP). In adult ventricular cells 8BrcGMP even at 100 μM, when applied intracellularly by internal perfu-

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**Fig. 1.** (A) Time course of peak value of L-type calcium current (pA/pF) for a newborn rabbit ventricular cell with intracellular (Int.) exposure to 30 μM 8BrcGMP in the internal (Int.) perfusion solution and then the addition of 10 μM methylene blue (MB) to the external (Ext.) solution while maintaining the 8BrcGMP in the internal perfusion solution at the time periods indicated by the horizontal arrows. To wash out 8BrcGMP, cell was perfused with normal internal solution. (B) Time course of peak value of L-type calcium current (pA/pF) for an adult rabbit ventricular cell with intracellular (Int.) exposure to 100 μM 8BrcGMP and then the combination of 100 μM cAMP and 100 μM 8BrcGMP in the internal perfusion solution at the time periods indicated by the horizontal arrows. To wash out cAMP and 8BrcGMP, cell was perfused with normal internal solution.
sion, failed to produce any significant effect. Fig. 1B shows the time course of $I_{\text{Ca}}$ peak current in an adult ventricular cell recorded with a control internal solution, with internal perfusion of 100 $\mu$M 8BrgGMP, with internal perfusion of a solution with 100 $\mu$M 8BrgGMP and 100 $\mu$M cAMP, and then a return to a control internal solution. The internal solution containing 8BrgGMP had no effect on basal $I_{\text{Ca}}$ of this adult ventricular cell. Perfusion with the solution containing both 8BrgGMP and cAMP (100 $\mu$M each) produced a 200% increase over basal $I_{\text{Ca}}$, which is similar to the effects we found for the internal perfusion of 100 $\mu$M cAMP alone for adult ventricular cells (data not shown). The effect of cAMP could easily be washed out by switching the internal solution to the control internal solution. The main problem with the above experiments using internal perfusion to dialyse the testing agents inside the cells was that the time required to produce a steady-state effect was always variable. We tried to minimize this by using low-resistance pipettes, bringing the perfusion capillary as close as possible to the pipette tip and by changing the perfusion pressure depending upon the pipette resistance. In our experiments, the time required to obtain an effect from the agent varied from 2 to 6 min.

To evaluate more directly the involvement of cGMP-dependent protein kinase in the maintenance of basal $I_{\text{Ca}}$, we applied KT-5823, an inhibitor of PKG, extracellularly at a sufficiently low concentration of 0.2 $\mu$M to inhibit PKG much more specifically than PKA. The $K_i$ of KT-5823 for PKG was 0.234 $\mu$M and for PKA was more than 10 $\mu$M [12]. Fig. 2A shows the time course of the effect of KT-5823 on basal $I_{\text{Ca}}$ in a newborn ventricular cell. After a period of control solution, for which the basal $I_{\text{Ca}}$ was 1.6 pA/pF, the application of 0.2 $\mu$M KT-5823 inhibited $I_{\text{Ca}}$ to a level of 0.9 pA/pF. The inhibitory effect of KT-5823 was not reversed after washout of the drug. Average basal $I_{\text{Ca}}$ in newborn ventricular cells was decreased by 0.2 $\mu$M KT-5823 from 2.36 $\pm$ 0.27 to 1.3 $\pm$ 0.17 pA/pF ($P < 0.01$, $n = 5$). In adult ventricular cells, 0.2 $\mu$M KT-5823 had no effect on basal $I_{\text{Ca}}$ (data not shown).

We also used a potent activator of PKG, 8-chlorophenylthio-cGMP (8CPT-cGMP), to stimulate PKG by intracellular perfusion. As with the 8BrgGMP, we perfused the cells with 30 $\mu$M 8CPT-cGMP after achieving a steady-state basal $I_{\text{Ca}}$. In a newborn ventricular cell, the time course of $I_{\text{Ca}}$ peak current is shown in Fig. 2B, showing that the intracellular perfusion of 8CPT-cGMP resulted in an increase of basal $I_{\text{Ca}}$ by about 100%. $I_{\text{Ca}}$ was increased from a basal level of 1.6 to 3.5 pA/pF by 8CPT-cGMP, an increase very similar to that observed in the presence of 8BrgGMP. Fig. 2C shows superimposed current traces recorded from the control period (trace ‘a’) and at the time of maximal effect of 8CPT-cGMP (trace ‘b’). For newborn ventricular cells, average basal $I_{\text{Ca}}$ was increased from 2.2 $\pm$ 0.3 to 4.4 $\pm$ 0.9 pA/pF ($P < 0.05$, $n = 3$) by 30 $\mu$M 8CPT-cGMP. To evaluate a possible shift by 8CPT-cGMP perfusion in the current–voltage relationship, we applied a series of depolarization steps with a 10 mV increment from a holding potential of -40 mV. Fig. 3 shows the current–voltage relationship obtained before and after 8CPT-cGMP perfusion for a newborn cell different from that used in Fig. 2B. As evident from the figure, the voltage dependence for activation of $I_{\text{Ca}}$ was not changed by the presence of 8CPT-cGMP.

In order to determine the effect of lowered cGMP levels on basal $I_{\text{Ca}}$, we used methylene blue or LY-83583, as inhibitors of soluble G-cyclase, to lower intracellular cGMP levels. Methylene blue and LY-83583 have both been used in many preparations as inhibitors of soluble G-cyclase [8,15,22,34]. They inhibit soluble G-cyclase, presumably by oxidizing the ferrous heme group of the enzyme. Methylene blue was applied in the bath at a concentration of 10–100 $\mu$M. Fig. 4A shows the time course of the effect of methylene blue (first at 10 $\mu$M and then at 100 $\mu$M) on $I_{\text{Ca}}$ for an adult rabbit ventricular cell. For this cell, $I_{\text{Ca}}$ density (pA/pF) was not changed (from 5.3 to 5.4 pA/pF).
even at the 100 μM methylene blue concentration. Fig. 4B shows the time course of the effect of methylene blue on basal $I_{\text{Ca}}$ in a newborn rabbit ventricular cell. After a period of control solution, for which the basal $I_{\text{Ca}}$ was 2.8 pA/pF, the application of 10 μM methylene blue inhibited $I_{\text{Ca}}$ over a 5 min period to a level of 0.9 pA/pF. The inhibitory effect of methylene blue was not reversed even after extended washout of the drug. In newborn cells, methylene blue (10–100 μM) applied extracellularly produced a significant inhibitory effect on average basal $I_{\text{Ca}}$ from 2.4 ± 0.2 to 0.85 ± 0.2 pA/pF ($P < 0.005, n = 5, 10$ μM methylene blue) and to 0.55 ± 0.08 pA/pF ($P < 0.003, n = 3, 100$ μM methylene blue). The inhibition produced by the two concentrations of methylene blue (10 and 100 μM) on basal $I_{\text{Ca}}$ was significantly different. In further experiments, we used 10 μM methylene blue to avoid any possible non-specific effects. We also applied methylene blue intracellularly by adding it to the internal solution and found similar results. If methylene blue produces its inhibitory effect on $I_{\text{Ca}}$ by lowering cGMP levels through inhibition of G-cyclase, then under conditions of an excess of cGMP, methylene blue would not be able to produce inhibition. To examine whether the inhibitory effect of methylene blue on $I_{\text{Ca}}$ is mediated by lowering the cGMP levels or if it produces an inhibitory effect non-specifically, we added 10 μM methylene blue to the external solution during the continued internal perfusion of 30 μM 8BrGMP (Fig. 1A). Fig. 1A shows that the addition of methylene blue to the external solution during the continued perfusion of 8BrGMP had no inhibitory effect on $I_{\text{Ca}}$ stimulated by 8BrGMP. Similar results were obtained in two additional cells.

LY-83583 at 10 μM was shown to decrease cGMP levels by 60% in guinea pig ventricular myocardium [28]. We applied LY-83583 in the bath at a concentration of 10 μM. Fig. 4C shows the time course of the effect of LY-83583 on basal $I_{\text{Ca}}$ in a newborn ventricular cell. For this cell, control $I_{\text{Ca}}$ of 3.2 pA/pF was decreased to about 1.8 pA/pF by external application of 10 μM LY-83583 and this effect was not reversible on washout of LY-83583. In newborn ventricular cells, LY-83583 at a concentration of 10 μM produced a 35% inhibition of average basal $I_{\text{Ca}}$ (from 2.39 ± 0.32 to 1.53 ± 0.15 pA/pF, $P < 0.03, n = 4$). The inhibitory effect of LY-83583 on $I_{\text{Ca}}$ was the same whether applied externally or internally. In adult ventricular cells LY-83583 at a concentration of 10 μM produced no significant effect on basal $I_{\text{Ca}}$ (data not shown).

Nitric oxide is well known as a stimulator of soluble G-cyclase and produces its effects by increasing intra-
cellular cGMP concentrations. We used a relatively stable nitric oxide donor (GSNO) which has a half-life of 150 h and does not involve the formation of oxygen free radicals [2]. To determine whether an increase in intracellular cGMP concentration produces effects similar to those of intracellular application of 8Br-cGMP, we applied GSNO at a concentration of 10 μM extracellularly to newborn ventricular cells. In a newborn ventricular cell, the time course of I_{Ca} peak current is shown in Fig. 5A, showing that the extracellular application of GSNO resulted in an increase of basal I_{Ca} by about 50%. I_{Ca} was increased from a basal level of 3.0 to 4.5 pA/pF by GSNO. Fig. 5B shows superimposed current traces recorded from the control period (trace ‘a’) and at the time of maximal effect of GSNO (trace ‘b’). GSNO (10 μM) increased average basal I_{Ca} by about 50% (basal I_{Ca} of 2.52 ± 0.29 pA/pF was increased to 3.76 ± 0.32 pA/pF by GSNO, P < 0.05, n = 5). Application of 10 μM GSNO did not produce any effect in adult ventricular cells. We also used reduced glutathione (GSH) to rule out the possible stimulation of formation of NO from GSNO. GSH (10 μM) did not produce any effect on basal I_{Ca} in newborn ventricular cells (data not shown).

3.1. Does cAMP-dependent protein kinase mediate the effect of cGMP on basal I_{Ca}?

The above results suggested that changes in cGMP levels in NB cells play a crucial role in determining basal I_{Ca} and that the PKG inhibitor KT-5823 inhibits basal I_{Ca}, but it remained unclear whether these effects are mediated by actions of cGMP (or its derivatives) on PKG or resulted from actions of cGMP on cAMP-dependent protein kinase (PKA). To evaluate this possibility, we examined the effects of 8CPT-cGMP under conditions in which PKA had been inhibited. Fig. 6A illustrates results for a newborn ventricular cell for which we used a pipette solution which included 1 μM of PKA inhibitor (5-22, PKI). The presence of PKI produced a gradual decline in the value of basal I_{Ca} to about 40% of the initial value. The subsequent perfusion of internal solution containing 30 μM cAMP and 1 μM PKI failed to produce any increase in the value of I_{Ca} inhibited by PKI. We specifically used 30 μM cAMP to compare the effect of 30 μM cAMP with a 30 μM concentration of the cGMP analog. 30 μM cAMP itself produces about a 100% increase in I_{Ca} when applied intracellularly. Fig. 6B shows a similar experiment for a newborn ventricular cell in which we again included 1 μM PKI in the pipette solution, producing a decline in the value of basal I_{Ca}, but the addition of 30 μM 8CPT-cGMP (to stimulate PKG) to the internal solution with PKI substantially increased the level of I_{Ca} inhibited by PKI. After washout of the 8CPT-cGMP from the internal solution (but retention of PKI in the internal solution) the level of basal I_{Ca} again declined. Intracellular application of 1 μM PKI reduced the average I_{Ca} from 2.16 ± 0.07 to 1.45 ± 0.09 pA/pF (P < 0.05, n = 6), a decrease of 33%. Intracellular application of 10 μM PKI produced a similar decrease of 31% of basal I_{Ca} (from 2.40 ± 0.06 to 1.66 ± 0.05 pA/pF; P < 0.05, n = 7). When we applied 30 μM 8CPT-cGMP intracellularly in the presence of 1 μM PKI, the average I_{Ca} increased from 1.64 ± 0.05 to 2.49 ± 0.06 pA/pF (P < 0.05, n = 3), an increase of 52%. The average increase in I_{Ca} by 8CPT-cGMP was somewhat decreased from 100% under control conditions to 52% under the inhibitory influence of PKI.

3.2. Do phosphodiesterases mediate the stimulatory effect of cGMP?

To exclude the possible involvement of PDE’s in the stimulatory effect of cGMP, we used IBMX, a nonspecific inhibitor of PDE’s near the concentration required for half-maximal stimulation of I_{Ca} (EC_{50} 64 ± 20 μM) in newborn ventricular cells, as we reported earlier [1]. We first exposed newborn ventricular cells to 50 μM IBMX and then added 10 μM GSNO (which increases cGMP levels inside the cell and consistently produces about a 50% increase in basal I_{Ca}) in the presence of 50 μM IBMX. Fig. 7A shows current recordings and time course of I_{Ca} peak current (inset) for a newborn cell before and after application of 50 μM IBMX and then the application of 10 μM GSNO plus 50 μM IBMX after reaching the steady state of I_{Ca} with 50 μM IBMX. I_{Ca} increased from 3.2 to 4.8 pA/pF with the application of 50 μM IBMX
GSNO (10 μM) alone, as we presented above for a separate group of cells, increased basal $I_{\text{Ca}}$ by $51 \pm 5\%$ ($P < 0.05$, $n = 5$). Extracellular application of 50 μM IBMX produced an increase of $49 \pm 7.2\%$ (from $2.83 \pm 0.23$ to $4.2 \pm 0.41$ pA/pF; $P < 0.05$, $n = 4$), which is close to the value we published earlier [1]. When we applied 10 μM GSNO in the presence of 50 μM IBMX, the average $I_{\text{Ca}}$ was further increased from $4.2 \pm 0.41$ to $6.05 \pm 0.66$ pA/pF ($P < 0.05$, $n = 4$), an additional increase of 66% over IBMX alone and an overall increase of $115 \pm 17\%$ over control.

4. Discussion

Cyclic GMP has been shown to be an inhibitory modulator of heart function. It exerts various inhibitory actions on cardiac cells, including a negative inotropic effect [5,23] and an inhibition of calcium current [4,6,11,14,35]. The present study was focused on the differences in the modulation of L-type $I_{\text{Ca}}$ by cGMP in adult and newborn ventricular cells. The salient findings of this study are summarized as follows:

1. In adult ventricular cells, neither inhibitors of G-cyclase (methylene blue and LY-83583 which inhibit G-cyclase and thus lower cGMP levels) nor the internal application of 8BrcGMP produced any significant effect on basal $I_{\text{Ca}}$.

2. In newborn ventricular cells, the application of methylene blue or LY-83583 produced an irreversible inhibition of basal $I_{\text{Ca}}$, while intracellular perfusion of 8BrcGMP or 8CPT-cGMP significantly and reversibly increased basal $I_{\text{Ca}}$.

3. In newborn ventricular cells, extracellular application of methylene blue during the intracellular perfusion of 8BrcGMP or 8CPT-cGMP significantly and reversibly increased basal $I_{\text{Ca}}$.

4. The PKG inhibitor KT-5823 produced an irreversible significant inhibition of basal $I_{\text{Ca}}$ in newborn ventricular cells but not in adult.

5. In newborn ventricular cells, inhibition of cAMP-dependent protein kinase blocked the stimulatory effect of cAMP but not of 8CPT-cGMP.

6. The NO donor GSNO produced a significant and reversible increase in basal $I_{\text{Ca}}$ in newborn ventricular cells but not in adult.
7. The stimulatory effect of GSNO on $I_{Ca}$ was not blocked by the phosphodiesterase inhibitor IBMX, with GSNO producing an additive effect on $I_{Ca}$ stimulated by IBMX.

The lack of any effect of extracellular application of methylene blue or of intracellular perfusion of 8BrcGMP in adult ventricular cells basically supports the earlier findings of several investigators in different species [11,15,22,24], in which they did not observe any effect of cGMP or methylene blue on basal $I_{Ca}$. Ono and Trautwein [24] have shown no effect of intracellular perfusion of cGMP on basal $I_{Ca}$ and the stimulatory effect of intracellular perfusion of cGMP was present only when the $I_{Ca}$ was first stimulated by isoproterenol in guinea pig ventricular cells. In contrast to our findings on adult rabbit ventricular cells, Tohse et al. [33] have shown a relatively small inhibitory effect of extracellular application of 300 μM 8BrCAMP and of atrial natriuretic peptide on basal $I_{Ca}$. A direct negative effect of extracellular application of 8BrCAMP in chick embryonic heart cells was shown on ‘slow response’ action potentials [32] and basal $I_{Ca}$ [35]. Our findings in newborn cells in which intracellular perfusion of 8BrCAMP or 8CPT-cGMP raised basal $I_{Ca}$ are certainly surprising and entirely different from the earlier findings in adult cells or in cultured avian embryonic heart cells. We have also shown that the nitric oxide donor GSNO produced a stimulatory effect similar to 8BrCAMP or 8CPT-cGMP in newborn ventricular cells but no effect in adult ventricular cells. SIN-1, which is a nitric oxide donor and has been shown to increase intracellular cGMP by G-cyclase stimulation, had no effect on basal $I_{Ca}$ and had both inhibitory and stimulatory effects on isoproterenol-stimulated $I_{Ca}$ [34] in guinea pig ventricular cells. In a recent study on 2–21-day-old rat ventricular myocytes, Sumii and Spertelakis [31] have shown that 8BrCAMP itself did not produce any effect on basal $I_{Ca}$, but that exogenous PKG with 8BrCAMP, when applied intracellularly, produced inhibition of basal $I_{Ca}$. This could be because the type of PKG isoform present in newborn rabbit heart may be different from that present in rat heart or there may be species differences in the regulatory sites for kinases modulating the calcium channel. Moreover, in newborn rabbit ventricular cells, we found that the PKG inhibitor KT-5823 inhibits basal $I_{Ca}$, which further strengthens our proposal that PKG is not inhibitory but is stimulatory in newborn rabbit ventricular cells.

We used methylene blue, which has been widely used as an inhibitor of soluble G-cyclase, in our experiments to lower the intracellular cGMP concentration. Methylene blue had no effect in adult cells but significantly inhibited the basal $I_{Ca}$ in newborn cells. A similar inhibitory effect on $I_{Ca}$ was observed with another G-cyclase inhibitor (LY-83583), which confirms the involvement of G-cyclase in the regulation of $I_{Ca}$. Methylene blue mediated inhibition was irreversible on washout of the methylene blue, but inhibition of $I_{Ca}$ by methylene blue could be prevented by the prior internal perfusion of the cell by a cGMP analog. This further confirms that the lowering of cGMP levels in newborn cells inhibits basal $I_{Ca}$ and that increases in cGMP levels increase basal $I_{Ca}$. A recent study of Levi et al. [15] on adult guinea pig ventricular cells showed no effect of methylene blue on basal $I_{Ca}$. In contrast to the results of Levi et al. [15], Wahler and Dollinger [34] used guinea pig ventricular cells and showed an increase in basal $I_{Ca}$, when LY-83583 (at 10 and 100 μM) was added to the pipette solution. They also showed that 0.1 μM KT5823, an inhibitor of PKG, had no effect on basal $I_{Ca}$ [34]. Our results also showed that KT-5823 had no effect in adult rat ventricular cells, but inhibited basal $I_{Ca}$ in newborn rabbit ventricular cells. It is important to note that the inhibitory effects of methylene blue and LY-83583, both of which presumably inhibit PKG by lowering the cGMP concentration, and of PKG inhibitor KT-5823, were irreversible. The lack of reversibility of methylene blue and LY-83583 could be because of irreversible inhibition of G-cyclase due to the oxidation of the ferrous heme moiety of enzyme.

In our studies, the stimulatory effect of 8CPT-cGMP was not blocked by PKI, but was somewhat attenuated. The stimulatory effect of GSNO was present even after $I_{Ca}$ was increased by the inhibition of phosphodiesterases by exposure to IBMX. IBMX is known to inhibit all types of PDE’s equipotently [29] and may result in an increase in cAMP as well as cGMP levels, because all PDE’s except type IV PDE (Ripipram-sensitive) can hydrolyze both cAMP and cGMP. This increase in cAMP levels by IBMX might be much greater than the change in cGMP levels for the following reasons: (a) cGMP-stimulated type II PDE ($K_m$ 30 μM for cAMP and 10 μM for cGMP) and cGMP-inhibited type III PDE ($K_m$ 0.15 μM for cAMP and 0.06 μM for cGMP) purified from bovine heart were shown to have almost the same $K_m$ for both cAMP and cGMP [9], but the basal levels of cAMP are about 100-fold higher than cGMP in both adult and newborn heart cells [13]; and (b) we have shown that cGMP-insensitive type IV PDE is a predominant PDE present in newborn heart cells, and this PDE hydrolyses only cAMP with high affinity and does not hydrolyze cGMP [1]. We assume that IBMX treatment predominantly increases cAMP levels and that changes in cGMP levels could be very small. After exposing the cells to IBMX, cGMP levels were raised further by subsequent exposure to GSNO, which resulted in a further increase in $I_{Ca}$. These findings clearly suggest that the stimulation of $I_{Ca}$ by 8BrCAMP, 8CPT-cGMP, or GSNO is probably not mediated via the activation of cAMP-dependent protein kinase or by inhibition of cGMP inhibited phosphodiesterases. The observed stimulatory effects of 8BrCAMP, 8CPT-cGMP, or GSNO are more likely due to the activation of protein kinase G. The attenuation of the stimulatory effect of 8CPT cGMP in the presence of PKI could presumably be due to the interactions between
regulatory sites of PKA and PKG. It seems that cGMP and cAMP are not antagonistic and that the role of cGMP in the regulation of $I_{Ca}$ stimulated by elevated cAMP may be synergistic or additive in newborn heart cells.

We suggest that cGMP levels in newborn rabbit ventricular cells play a crucial role in determining the basal $I_{Ca}$ density. Our findings on the effect of cGMP on basal $I_{Ca}$ in newborn cells are unique and fundamentally different from the findings by other investigators on the effects of cGMP on basal and cAMP stimulated-$I_{Ca}$ in heart cells of different species. Our data suggest that intracellular cGMP levels may be of great significance in regulating basal $I_{Ca}$ in newborn, but not in adult, rabbit ventricular cells. This difference between adult and newborn may also be due to a different mechanism of action of cGMP in heart cells in early postnatal life. We have shown that basal cGMP levels, which are primarily determined by basal G-cyclase activity, are much higher in newborn than in adult rabbit heart cells [13]. Two general classes of PKG exist in vertebrate cells: type I and type II [17]. Type I exists in two isoforms (α and β) [17]. Differing ratios of these distinct isoforms of PKG in newborn compared to adult cells may be responsible for the different roles of cGMP. We suggest that newborn rabbit ventricular cells may have a phosphorylation site associated with the calcium channel which can be phosphorylated by one or both types of PKG and that this site, when phosphorylated, increases the availability of L-type calcium channels and is involved in maintaining basal $I_{Ca}$.

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