cGMP Inhibits the Activity of Single Calcium Channels in Embryonic Chick Heart Cells

Noritsugu Tohse and Nicholas Sperelakis

Effects of cGMP on the slow (L-type) Ca\(^{2+}\) channels of cultured chick embryonic cardiomyocytes were investigated by a cell-attached patch-clamp method. Superfusion of the single cells with 8-bromo-cGMP, a membrane-permeable derivative of cGMP, inhibited the single-channel activity. The cyclic nucleotide decreased, in a concentration-dependent manner, the ensemble averaged currents obtained from multichannel patches. 8-Bromo-cGMP (1 mM) completely abolished the currents (n=8), whereas 0.1 mM only slightly decreased the currents (n=4). The influence of cGMP on the characteristics of the single Ca\(^{2+}\) channels was examined using 0.3 mM 8-bromo-cGMP. Unit amplitude and slope conductance of the Ca\(^{2+}\) channel was not changed (25 pS in control versus 24 pS in the presence of cGMP). Analysis of single-channel kinetics showed that cGMP prolonged the slow time constant for the closed-time histogram (from 6.7 to 15.4 msec); the other time constants (for the open-time and closed-time histograms) were not affected. cGMP-induced inhibition of the Ca\(^{2+}\) channels may be mediated by cGMP-dependent protein kinase, because 8-bromo-cGMP is a potent activator of this protein kinase and does not stimulate cAMP hydrolysis. The present results suggest that cGMP opposes the effects of cAMP on the L-type Ca\(^{2+}\) channels in myocardial cells. (Circulation Research 1991;69:325–331)

Phosphorylation of ion channels in cardiac sarcolemma is an important process in the regulation of cardiac excitation by several hormones. cAMP-dependent protein kinase (CA-PK) stimulates the activities of the slow (L-type) Ca\(^{2+}\) channels,\(^1,2\) the delayed-rectifier K\(^+\) channels,\(^1,3,4\) and the Cl\(^-\) channels.\(^5\) Ca\(^{2+}\)-activated phospholipid-sensitive protein kinase enhances the activity of the delayed-rectifier K\(^+\) channels.\(^6,7\) Although the effect of cGMP-dependent protein kinase (cG-PK) on cardiac ion channels is not known, the cG-PK enzyme does exist in cardiac tissues.\(^8\)

Various hormones, including acetylcholine,\(^9\) atrial natriuretic peptide,\(^10\) and histamine (H\(_1\)-receptor),\(^11\) increase the intracellular cGMP level in cardiac myocytes. Acetylcholine was reported to produce a negative inotropic effect accompanied by an increase in the cGMP level.\(^9,12\) Some reports\(^13,14\) suggested that cGMP might inhibit the slow Ca\(^{2+}\) current (I\(_{ca}\)) in cardiac muscles. On the other hand, some other reports\(^15,16\) did not agree with this hypothesis.

We showed that application of cGMP, by intracellular injection\(^17,18\) or by using the membrane-permeable derivative 8-bromo-cGMP,\(^18,19\) inhibited the slow action potentials in guinea pig papillary muscles and chick embryonic heart cells. Furthermore, we showed that 8-bromo-cGMP inhibited I\(_{ca}\) in chick embryonic ventricular myocytes and suggested that cGMP-induced inhibition of I\(_{ca}\) was mediated by activation of cG-PK.\(^20\) Fischmeister and Hartzell\(^21\) showed that cGMP could only reverse cAMP enhancement of I\(_{ca}\) in single ventricular cells from frog heart and suggested that cGMP acted by reducing the cAMP level through the stimulation of cAMP-phosphodiesterase.\(^21\) However, they recently suggested that, in guinea pig ventricular cells, the inhibition of the enhanced I\(_{ca}\) might be mediated by activation of cG-PK.\(^22\)

In the present study, to examine the effects of cGMP on single-channel activity of L-type Ca\(^{2+}\) channels, 8-bromo-cGMP was externally applied to 3-day-old chick embryonic heart cells. cGMP inhibited the activity of the Ca\(^{2+}\) slow channels. Thus cGMP has an effect that is antagonistic to cAMP. The present results on single Ca\(^{2+}\) channels provide supporting evidence for the "Yin-Yang hypothesis."\(^23\)

Materials and Methods

Cell Culture Preparation

 Cultures of single cells were prepared from 3-day-old embryonic chick hearts by a method similar to that described previously.\(^24\) In brief, 12 dozen fertilized
white Leghorn chick embryos were incubated for 3 days at 37.5°C. Hearts were sterilely removed and collected in an ice-cold balanced salt solution. Tissue digestion was accomplished by gentle stirring of the hearts in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free Ringer’s solution containing 0.01% trypsin (Sigma Chemical Co., St. Louis). The cell suspensions were harvested at 5-minute intervals, and collected cells were washed three times in culture medium (M199, GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum. The cells were plated on glass cover slips in plastic Petri dishes (35 mm) at a concentration of 10,000–15,000 cells/ml. The cultures were maintained at 37°C and pH 7.4 in a moist air/CO\(_2\) incubator for a period of 1–5 days before use.

**Solutions and Materials**

The composition of the depolarizing solution used to bathe the cell was (mM) KCl 150, glucose 10, EGTA 10, and HEPES-KOH buffer 10 (pH 7.4). The solution used in the recording patch pipette contained (mM) BaCl\(_2\) 50, choline chloride 70, and HEPES-Tris base buffer 10 (pH 7.4). The pipette solution usually also contained 30 µM tetrodotoxin (Sigma) to block fast Na\(^+\) channel activity.\(^{24}\) 8-Bromo-cGMP (sodium salt) was obtained from Sigma. Nifedipine was obtained from Knoll Pharmaceuticals, Whippany, N.J. All experiments were carried out at room temperature (±25°C).

**Recording Techniques**

Single-channel currents were recorded by the cell-attached configuration of the patch-clamp method,\(^{25,26}\) using glass patch pipettes with a resistance of 3–10 MΩ. An intrapipette electrode was connected to the input stage of a patch-clamp amplifier (model EPC-7, List, Darmstadt, FRG). Current signals were filtered at 1 kHz (eight-pole Bessel filter), digitized at 3 kHz, and stored in a personal computer (IBM-compatible computer system). The pulse generation and the storing and analysis of the digitized signals were carried out using PCLAMP software (version 5.03, Axon Instruments, Burlingame, Calif.).

Single-channel currents were evoked by depolarizing voltage pulses to 0 mV, from a holding potential of −80 mV, at a repetition rate of 0.5 Hz and duration of 300 msec. For the current–voltage relation, depolarizing pulses to −40, −30, −20, −10, 0, 10, and 20 mV were applied at an interval of 2 seconds.

All values are presented as mean±SEM. The slope conductance and the open-time and closed-time histograms were analyzed and fitted by the method of least squares. Statistical analyses were performed using Student’s paired t test.

**Results**

The cultured cardiomyocytes, prepared from 3-day-old chick embryonic hearts, were perfused with the external depolarizing solution containing 150 mM K\(^+\). The membrane potential of the cells should be approximately 0 mV in this solution. This allows the potential of the patch membrane to be approximated from the value of the pipette potential.

Depolarizing test pulses to 0 mV from a holding potential of −80 mV (at an interval of 2 seconds) elicited inward unitary currents (Figure 1A). These unitary currents were observed at potentials above −30 mV. They were blocked by the Ca\(^{2+}\) slow channel blocker, nifedipine (3 µM). The ensemble averaged currents from these channel activities showed no inactivation, even at the end of a 300-msec test pulse (Figure 1C). Therefore, these currents are carried through the L-type Ca\(^{2+}\) channels.\(^{27}\)

Addition of 1 mM 8-bromo-cGMP to the external perfusing solution produced a pronounced decrease in the Ca\(^{2+}\) channel activity. The channel activity was completely abolished by 2–3 minutes after exposure to cGMP. Figure 1B illustrates such inhibition by cGMP at 5 minutes. Complete inhibition of the Ca\(^{2+}\) channel activity by 1 mM 8-bromo-cGMP was also observed in seven other cells. The channel activity was difficult to recover after the cyclic nucleotide was washed out; partial recovery was observed in only one cell after 20 minutes of washout.

Figure 2 illustrates the concentration–response relation between 8-bromo-cGMP and the ensemble averaged current. The ensemble averaged currents of the Ca\(^{2+}\) channels were obtained from 29 consecutive tracings, elicited by the depolarizing pulses. Figure 2A shows the ensemble averaged currents from representative experiments. In these experiments, the patches possessed two to four functional channels. Each cell received only one treatment (either 0.1, 0.3, or 1.0 mM 8-bromo-cGMP). 8-Bromo-cGMP decreased the ensemble averaged Ca\(^{2+}\) current in a dose-dependent manner: 0.1 mM hardly affected the current, 0.3 mM markedly decreased it, and 1 mM completely abolished it. Figure 2B graphically summarizes the data from 19 cells. Amplitude of the ensemble current was measured at 90 msec after the start of the 300-msec test pulse, because the current was fully activated (saturated) at this time. 8-Bromo-cGMP (0.3 mM) decreased the Ca\(^{2+}\) current to 56.4±5.0% of control amplitude. The half-maximum response is evoked at approximately 0.3 mM cGMP.

To examine the effects of cGMP on the characteristics of the single Ca\(^{2+}\) channels, a concentration of 0.3 mM was chosen, which produces a moderate inhibition of the Ca\(^{2+}\) channel activity. Figure 3 shows the current–voltage relation of the unitary Ca\(^{2+}\) currents in the absence and presence of 0.3 mM 8-bromo-cGMP. As can be seen in Figure 3A, cGMP produced no change in the unit amplitude at each potential. The slope conductance was 25 and 24 pS, in the absence and presence of cGMP, respectively (Figure 3B). Therefore, cGMP had no effect on the unitary conductance of L-type Ca\(^{2+}\) channels.

Figures 4 and 5 present the analyses of the open–closed kinetics of the Ca\(^{2+}\) channels using pooled data collected from the best three patches, in which only one functioning Ca\(^{2+}\) channel was apparently present in the patch. All data collected...
Kinetics in the Constant Control Time of Myocardial Cells were versus 15.4 msec Experiments Seven recordings (n=29).

Current and Current and both the cGMP, grams of mM) state, open to two components. The inhibition of the time prolongation in the control condition (absence of 8-bromo-cGMP) was virtually unchanged. The effective concentration of 8-bromo-cGMP was >0.1 mM. Nawrath29 showed that 8-bromo-cGMP inhibited contraction of cat papillary muscles at concentrations of 0.01–0.1 mM. In the partially depolarized papillary muscles (guinea pig), 1 μM (0.001 mM) 8-bromo-cGMP produced significant inhibition of contraction and maximum dV/dt of the slow action potentials. However, 1 mM 8-bromo-cGMP was re-

Table 1 presents the unpooled data collected from seven patches, including the three patches shown in Figures 4 and 5. Each value listed is the mean from seven histograms (one for each cell). These data are qualitatively similar to the pooled data from the three patches shown in Figures 4 and 5 but differ somewhat quantitatively with respect to the time constants.

**Discussion**

In the present study, 8-bromo-cGMP, which is a membrane-permeable derivative of cGMP, inhibited the activity of the L-type Ca\(^{2+}\) channels in 3-day-old embryonic chick heart cells. The inhibition of the Ca\(^{2+}\) channel activity by 8-bromo-cGMP is not due to “run down,”28 because we observed that the Ca\(^{2+}\) channel activity was stable for 5–10 minutes after establishment of the gigaseal (n=3). In addition, the Ca\(^{2+}\) channel activity was hardly changed at 5 minutes after the application of 0.1 mM 8-bromo-cGMP, a concentration that is ineffective on the Ca\(^{2+}\) channels (Figure 2).

The effective concentration of 8-bromo-cGMP was >0.1 mM. Nawrath29 showed that 8-bromo-cGMP inhibited contraction of cat papillary muscles at concentrations of 0.01–0.1 mM. In the partially depolarized papillary muscles (guinea pig), 1 μM (0.001 mM) 8-bromo-cGMP produced significant inhibition of contraction and maximum dV/dt of the slow action potentials. However, 1 mM 8-bromo-cGMP was re-

Table 1. Summary of the Time Constants for Open-Closed Kinetics in the Absence and Presence of 8-Bromo-cGMP From All Seven Experiments

<table>
<thead>
<tr>
<th>Time constant</th>
<th>Control</th>
<th>8-Bromo-cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\tau_d) (msec)</td>
<td>0.45±0.05</td>
<td>0.39±0.08</td>
</tr>
<tr>
<td>(\tau_c) (msec)</td>
<td>6.4±2.8</td>
<td>5.0±1.9</td>
</tr>
<tr>
<td>(\tau_a) (msec)</td>
<td>0.51±0.03</td>
<td>0.61±0.14</td>
</tr>
<tr>
<td>(\tau_m) (msec)</td>
<td>7.9±1.4</td>
<td>19.8±5.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=7. \(\tau_d\) and \(\tau_m\) time constants of the open state, fast and slow components, respectively; \(\tau_c\) and \(\tau_a\) time constants of the closed state, fast and slow components, respectively. Each value listed is the mean from seven histograms (one for each cell).

From the three patches were pooled for this analysis to produce one histogram for each time constant. In the control condition (absence of 8-bromo-cGMP), both the open-time and closed-time histograms of the Ca\(^{2+}\) channels were well fitted by two exponential components. 8-Bromo-cGMP (0.3 mM) markedly prolonged the slow time constant in the closed-time histogram: 6.7 msec (control) versus 15.4 msec (cGMP) in Figure 5; the other time constants were virtually unchanged. The prolongation of the slow time constant may explain the inhibition of the Ca\(^{2+}\) channel activity by 8-bromo-cGMP.

Figure 1. Current recordings showing effect of 8-bromo-cGMP (8-Br-cGMP) on the Ca\(^{2+}\) slow channel activity in single myocardial cells isolated from 3-day-old embryonic chick hearts. Cell-attached patch configuration was used. Single-channel currents were evoked by depolarizing voltage pulses to 0 mV from a holding potential of −80 mV, at a repetition rate of 0.5 Hz and duration of 300 msec. Panels A and B: Examples of original current recordings from the same patch, before (panel A) and after (panel B) superfusion with 1.0 mM 8-bromo-cGMP. Panels C and D: Ensemble averaged currents calculated from the original current recordings (n=29). The current tracings were low-pass filtered at 1 kHz and corrected for leakage and capacitive currents. Current and time calibrations are given at the lower right.
required for complete block of the action potentials.\textsuperscript{18} On the other hand, $I_{\text{Ca}}$ was reduced, but not completely blocked, by 1 mM 8-bromo-cGMP in 17-day-old embryonic chick heart cells.\textsuperscript{20} These discordant results may be due to differences in the preparations and experimental conditions. In any case, 8-bromo-cGMP, at concentrations of 0.3–1 mM, is effective in depressing contraction and inhibiting Ca\textsuperscript{2+} channels in cardiac muscles.

8-Bromo-cGMP inhibited the ensemble averaged current of the Ca\textsuperscript{2+} channels. On the other hand, the slope conductance of the Ca\textsuperscript{2+} channels was identical in the absence or presence of 8-bromo-cGMP. The ensemble averaged current, $I(t)$, at each time point (t) during the test pulse using multichannel patches is represented as

$$I(t) = i \cdot N \cdot P_o(t)$$  \hspace{1cm} (1)

where $i$ is the unit amplitude of the channel current, $N$ is the number of functional channels per patch, and $P_o(t)$ is the probability of channels being in the open state at time $t$. Therefore, 8-bromo-cGMP may decrease $N \cdot P_o(t)$, because the unit current amplitude (or slope conductance) was not changed. In the analysis of the open–closed kinetics using single-channel patches ($N=1$), 8-bromo-cGMP prolonged the time constant of the slow closed-time component, without affecting the time constants of the other components. This change may contribute to the decrease in $P_o(t)$. In addition, it is possible that cGMP decreases $N$.

Three possibilities have been proposed\textsuperscript{30} for the mechanism of the inhibition of the Ca\textsuperscript{2+} slow channel activity by cGMP: 1) activation of cG-PK and phosphorylation of the calcium channel or an associated protein, 2) activation of cGMP-stimulated cAMP phosphodiesterase, and 3) activation of cA-PK. Possibility 3 is unlikely because the activation of cA-PK stimulates the Ca\textsuperscript{2+} channel activity in various heart cells\textsuperscript{1,2,31,32} and 8-bromo-cAMP increases the Ca\textsuperscript{2+} channel activity in young embryonic heart cells (data not shown). Possibility 2 was proposed by Fischmeister and Hartzell\textsuperscript{21} for single frog ventricular cells. They showed that the intracellular perfusion with cGMP reduced $I_{\text{Ca}}$ that was previously increased by cAMP but that the basal $I_{\text{Ca}}$ was not affected. The inhibition of $I_{\text{Ca}}$ by cGMP was not observed in the presence of 8-bromo-cAMP, and 8-bromo-cGMP failed to mimic the inhibition. Therefore, they concluded that cGMP reduced the cAMP level through enhancement of cAMP hydrolysis. However, in subsequent experiments on guinea pig ventricular cells, they reported, in agreement with us\textsuperscript{18,20} that cGMP may act to reduce $I_{\text{Ca}}$ by activation of cG-PK and not by reduction of cAMP.\textsuperscript{22} In addition, possibility 2 seems unlikely to be the explanation for the present results, because 1) 8-bromo-cGMP is thought not to appreciably stimulate cGMP-stimulated phosphodiesterase in frog heart\textsuperscript{30,33} 2) 8-bromo-cGMP (1 mM) did not reduce the cAMP level (elevated by forskolin, isoproterenol, and histamine) in guinea pig papillary muscles,\textsuperscript{19} and 3) the increase in cGMP level produced by acetylcholine or nitroprusside in 3-day-old chick embryonic hearts was not accompanied by a decrease in the cAMP level, even though the basal cAMP level is very high.\textsuperscript{34}

Therefore, possibility 1 is the most likely explanation for the mechanism of the cGMP inhibition of the Ca\textsuperscript{2+}...
FIGURE 3. Current–voltage relation of unitary Ca\textsuperscript{2+} currents in absence and presence of 0.3 mM 8-bromo-cGMP (8-Br-cGMP). Single-channel conductance was not affected by cGMP. Panel A: Ca\textsuperscript{2+} channel currents recorded from a patch containing two channels in absence and presence of 8-bromo-cGMP. Four different command potentials (−20, −10, 0, and 10 mV) were applied from a holding potential of −80 mV. Arrow at the left side of each tracing indicates the open level of the first channel. Panel B: Graph showing current–voltage (I–V) relations of the Ca\textsuperscript{2+} channel currents in absence and presence of 8-bromo-cGMP. Data are given as the mean±SEM. Numerals in parentheses give number of patches. The data points were fitted by the method of least squares. As can be seen, there was no difference in the slope conductance: 25 pS (control) vs. 24 pS (cGMP).

slow channel activity. 8-Bromo-cGMP has been reported to be a potent activator of the cG-PK, and Levi et al suggested that cG-PK may participate in the inhibition of I\textsubscript{Ca} by cGMP in guinea pig ventricular cells. Cuppoletti et al. showed that a 50 kDa substrate for cG-PK existed in the sarcolemma of guinea pig ventricular muscles. Therefore, we believe that 8-bromo-cGMP may activate cG-PK and thereby inhibit activity of the L-type Ca\textsuperscript{2+} channels. Evidence supporting this hypothesis has recently appeared using intracellular perfusion of single myocardial cells with an active fragment of cG-PK. However, the possibility that cGMP exerts a direct inhibition on the Ca\textsuperscript{2+} slow channel cannot be excluded.

The effects of 8-bromo-cGMP on the Ca\textsuperscript{2+} channel activity is opposite that of cA-PK activation, which
produces an increase in $N \cdot P_c(t)$ without affecting unit amplitude.\(^{31,32}\) Therefore, this finding implies that cG-PK phosphorylation can antagonize cA-PK phosphorylation of the L-type Ca\(^{2+}\) channels, as we previously suggested.\(^{2,18}\) Two sites of action of cG-PK are possible for this antagonism. One site is on the cA-PK enzyme activity itself. For example, the cG-PK can phosphorylate the regulatory subunit of the type I cA-PK.\(^{38}\) Another site is at a different phosphorylation site on the Ca\(^{2+}\) channel protein (from that phosphorylated by cA-PK), and cG-PK may preferentially phosphorylate this site. However, we emphasize that there is no direct evidence provided in our study that the effect of cGMP was mediated by phosphorylation of the Ca\(^{2+}\) slow channel protein.

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