ATP Directly Affects Junctional Conductance Between Paired Ventricular Myocytes Isolated From Guinea Pig Heart

Hiroki Sugiura, Junji Toyama, Naoya Tsuboi, Kaichiro Kamiya, and Itsuo Kodama

Effects of ATP on junctional conductance (gj) were investigated in paired ventricular myocytes isolated from guinea pig hearts. One cell of the pair was voltage-clamped with a single-patch pipette, and gj was measured after the perforation of the nonjunctional membrane of the partner cell. The current-voltage relation of gj was linear between −30 and +30 mV. The control gj at 5.0 mM ATP in 88 pairs of cells ranged from 100 to 1,055 nS (average, 268 nS). ATP within the range from 0.1 to 5.0 mM increased gj in a dose-dependent manner. The Hill coefficient was 2.6, and the half-maximum effective concentration of ATP was 0.68 mM. Adenyllylimidophosphate (2 mM) caused a transient increase in gj in the presence of 0.5 mM ATP, but forskolin (30 μM), cyclic AMP (50 μM), catalytic subunit of cyclic AMP–dependent protein kinase (1 μM), and ADP (10 mM) had no significant effect on gj. The temperature coefficient of gj in the presence of 5.0 mM ATP was 1.29. These findings suggest that gj in paired ventricular myocytes is directly regulated by ATP probably through a specific ligand-receptor interaction between ATP and gap junctional channel protein. (Circulation Research 1990;66:1095–1102)

Primary effects of ischemia on cardiac muscle include decreases of ATP and creatine phosphate within affected fibers. These decreases result in a depression of active transport systems, which elevates intracellular concentrations of Na⁺, Ca²⁺, and protons.¹ Previous studies²⁻⁴ have shown that electrical coupling was suppressed when energy metabolism was inhibited by various experimental conditions, such as hypoxia or ischemia, or in the presence of metabolic inhibitors. In some cases, uncoupling effects have been shown to be reversed by intracellular injection of ATP² or EDTA³ or by addition of glucose to the extracellular perfusate.⁴

Abundant studies indicate that increases in intracellular concentrations of Na⁺, Ca²⁺, or protons⁵⁻⁷ cause cell decoupling, and it has been assumed that uncoupling effects of ischemia are due to changes in these ions. It has also been reported that in various tissues junctional conductance (gj) is either increased⁸⁻¹⁰ or decreased¹¹⁻¹³ as a consequence of cyclic AMP (cAMP)–mediated phosphorylation of the channel protein. Recent advances in cardiac electrophysiology have proved that some ionic channels in sarcolemmal membrane can be regulated by ligand-receptor interaction between ATP and channel proteins¹⁴ or by phosphorylation of channel proteins.¹⁵

In the present study, we investigated the direct effect of ATP on cardiac gj by using the whole-cell clamp of paired ventricular myocytes, one of which was perforated to allow intracellular diffusion with extracellular solutions.⁷ We report here that gj is strongly affected by ATP from 0.1 to 5.0 mM and that this effect is direct and, thus, analogous to the effect reported on other sarcolemmal channels.

Materials and Methods

Preparations

Enzymatic procedure for the isolation of paired ventricular cells was modified from the method as described by Mitra and Morad.¹⁶ Briefly, guinea pigs weighing 150–250 g were killed by an overdose of pentobarbital sodium. The heart was quickly removed, and the aorta was immediately cannulated for Langendorff perfusion. Subsequently, the coronary artery was perfused backward with Ca²⁺-free Tyrode’s solution for 3–4 minutes. Thereafter, the heart was perfused by 50 ml Ca²⁺-free Tyrode’s solution containing 50 mg collagenase (type I, Sigma Chemical, St. Louis, Missouri) and 10 mg protease
TABLE 1. Composition of Internal Solutions

<table>
<thead>
<tr>
<th>Pipette (mM)</th>
<th>Na$_2$ATP</th>
<th>MgCl$_2$</th>
<th>Aspartic acid</th>
<th>CsOH</th>
<th>EGTA</th>
<th>HEPES</th>
<th>TEA</th>
<th>Na$_2$CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test solution(mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (control)</td>
<td>5.0</td>
<td>3.0</td>
<td>110</td>
<td>110</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>3.0</td>
<td>110</td>
<td>110</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.5</td>
<td>110</td>
<td>110</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>1.0</td>
<td>110</td>
<td>110</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>0.5</td>
<td>110</td>
<td>110</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

TEA, tetraethylammonium chloride; CRP, creatine phosphate. pH of internal solutions was adjusted to 7.2 with CsOH. ATP concentrations in internal test solutions 2–5 are varied to 1.5, 1.0, 0.5, and 0.1 mM, respectively. In each of the test solutions, the concentration of MgCl$_2$ was varied to keep free-Mg$^{2+}$ concentration less than 0.3 mM.

Electrophysiological Experiments

The method for measuring $g_i$ and the intracellular perfusion technique were essentially similar to those as described by Noma and Tsuboi$^7$ except that the whole-cell clamp was carried out by using a single-patch pipette instead of the double-pipette method. In brief, one cell of the pair was voltage-clamped with a giga-sealed patch pipette filled with pipette solution (Table 1). After clamping the membrane potential of a paired cell at approximately 0 mV, bath solution was switched from normal Tyrode’s solution (1.8 mM Ca$^{2+}$) to a Cs$^+$-rich and low-Ca$^{2+}$ internal test solution (test solution 1). Subsequently, the nonjunctional membrane of the partner cell was perforated by crushing the tip of the other pipette against the cell. This procedure allowed access of bath solution to the junctional channel of the perforated cell. Under this condition, current from the clamped cell flows through two pathways. One is through the nonjunctional membrane of the clamped cell to the ground. The other is through the gap junction and the interior of the perforated cell to the ground. The equivalent circuit after perforation of the nonjunctional membrane of the partner cell is illustrated in Figure 1. The value of input conductance ($g_i$) through the electrode after cell membrane perforation was given by

$$g_i = g_{m1} + g_i \times (g_{m2} + g_i)/(g_i + g_{m2} + g_s)$$

where $g_i$, $g_{m1}$, and $g_{m2}$ are the conductances of the clamped cell, the partner cell, and the perforated cell, respectively. Since $g_s$, the conductance of the gap junction, is much less than $g_i$ and $g_{m2}$, the approximate value of $g_i$ was obtained by

$$g_i \approx g_{m1}$$

The value of $g_{m1}$ was approximated as half of the total nonjunctional membrane conductance of a paired cell ($g_{m1} + g_{m2}$), which had been measured before perforation of the nonjunctional membrane of the partner cell. Series resistance of the electrode

(type XIV, Sigma Chemical) for 3–10 minutes. Then, the collagenase solution was washed out by perfusing with high-K$^+$ and low-Cl$^-$ solution (KB medium).$^{17}$ At the end of the above procedure, the ventricle was cut into pieces and was stored in the KB medium at 4°C before use. Cells were dispersed in the recording chamber (0.1–0.2 ml) on the stage of an inverted phase-contrast microscope (Diaphoto-TMD, Nikon, Japan) and were continuously perfused with normal Tyrode’s solution (1.8 mM Ca$^{2+}$) at a rate of 2–3 ml/min. About 50% of the cells were rod-shaped in normal Tyrode’s solution. Paired cells were distinguished from single cells by the presence of contact regions between apposed cells.

Solutions

Normal Tyrode’s solution consisted of (mM) NaCl 136.9, Na$_2$HPO$_4$ 0.33, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 0.5, glucose 5, and HEPES 5; pH was adjusted to 7.4 with NaOH. Internal solutions are listed in Table 1. Ca$^{2+}$-free Tyrode’s solution was prepared by omitting CaCl$_2$ from normal Tyrode’s solution. Pipette solution was essentially the same as the internal test solution (test solution 1) except that creatine phosphate was added as a reservoir of high-energy phosphates.

Internal test solutions 1–5 were used to perfuse the perforated cell interior at various ATP concentrations. To keep free-Mg$^{2+}$ concentration constant in the internal test solution, the amount of MgCl$_2$ was varied between 0.1 and 3.0 mM in proportion to ATP concentrations. Free-Ca$^{2+}$ and Mg$^{2+}$ concentrations in each of internal test solutions, which had been estimated with the stability constants for ATP, Ca$^{2+}$, Mg$^{2+}$, and EGTA at a given pH,$^{18,19}$ were limited to a level less than 1 nM and 0.3 mM, respectively. Noma and Tsuboi$^7$ showed that these Ca$^{2+}$ and Mg$^{2+}$ concentrations had no effects on $g_i$.

Forskolin, cAMP, and adenylylimidodiphosphate were directly dissolved in the internal test solutions. ADP was dissolved in the internal test solution containing 10 μM P$^i$.P$^i$-di(adenosine-5') pentaphosphate (AP$_2$A), a potent inhibitor of adenosine kinase. Catalytic subunit of cAMP-dependent protein kinase (c-subunit) was dissolved in the internal test solution containing 32.4 mM DL-dithiothreitol to prevent the inactivation of c-subunit. In preliminary experiments, AP$_2$A and DL-dithiothreitol had no effect on $g_i$ (results not shown). All experiments were carried out at 34°C–35°C unless otherwise stated.
were considered to be negligible, because the negative pressure of pipette and the input resistance under control condition at 5.0 mM ATP were kept constant. Paired cells having gs of larger than 100 nS were used in our experiments. When membrane leak conductance increased noticeably within 3 minutes after cell membrane perforation, the cells were discarded. Ramp command potential between −30 and +30 mV with a duration of 2.5 seconds was applied to the clamped cell every 6 seconds. gₗ was calculated from the slope conductance of the current output.

Values were presented in mean±SEM unless otherwise stated. Statistical analysis was performed by Student’s paired t test, and values of p<0.05 were considered to indicate a significant difference.

**Results**

### Control gₛ

Figure 2 illustrates changes of gₛ after the perforation of the nonjunctional membrane of the partner cell. Current output in response to the command potential of ±30 mV was increased suddenly after the perforation (Figure 2A). The experiment was carried out at 5.0 mM ATP in the bath solution. Therefore, the average value of gₛ was increased from 47±3 to 292±15 nS (n=88) (Figure 2B). The current-voltage relations before and after the perforation were linear over the range from −30 to +30 mV (Figure 2C), indicating no voltage dependency of junctional as well as nonjunctional membrane conductances under such Cs⁺-rich and low-Ca²⁺ conditions. The control gₛ was calculated to be from 100 to 1,055 nS (average, 268±15 nS).

**Effects of ATP Reduction on gₛ**

We measured gₛ with internal test solutions containing various ATP concentrations (0.1–5.0 mM).

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**Figure 2.** Tracings and graphs showing change of input conductance (gi) after cell membrane perforation. Panel A: Original ramp voltage-clamp pulse between −30 and +30 mV and current output after the perforation. Closed triangle (▲) indicates the time of cell membrane perforation. Panel B: Change of gi after cell membrane perforation. gi was suddenly increased from 14 to 501 nS by the perforation and became stable in 1 minute. Panel C: Current (I)–voltage (V) relation before and after cell membrane perforation. Tracings obtained at the times indicated by 'a' and 'b' in panel A are illustrated. I–V relations obtained before and after the perforation were linear between −30 and +30 mV.
Our preliminary experiments have shown that with potassium channels blocked by cesium and tetraethylammonium chloride, \( g_{\text{m}} \) values of ventricular myocytes were unaffected by similar changes in ATP concentrations of the bath solution (results not shown). Therefore, \( g_i \) was calculated by assuming that the initial \( g_{\text{m}} \) value was kept constant throughout the experiment.

Representative results are shown in Figure 3. When ATP concentration was reduced from control (5.0 mM) to 1.0, 0.5, or 0.1 mM, \( g_i \) decreased in a concentration-dependent manner. Linear current-voltage relation of \( g_i \) indicating no voltage dependency of \( g_i \) was maintained even at the lower ATP concentrations. At the resumption of control ATP (5.0 mM), \( g_i \) returned toward the control level. In 18 of 40 experiments (45%), half or more \( g_i \) recovery was not attained at the resumption of 5.0 mM ATP. Such irreversible data were discarded, and the remaining 22 experiments were used for data analysis. The irreversible decoupling is most likely explained by mechanical breakdown of gap junctional structure due to rigor induced by ATP depletion.

Figure 4 summarizes the results obtained from 22 pairs of myocytes. Values of \( g_i \) measured at each ATP concentration were normalized with reference to the average of those at 5.0 mM ATP before and after the reduction of ATP. The smooth curve is a least-squares fit of the Michaelis-Menten equation to the plotted dose-response data. The Hill coefficient was 2.6, and the half-maximum effective concentration of ATP was 0.68 mM. The calculated Michaelis-Menten curve suggests that \( g_i \) would reach 0 nS at ATP concentrations less than 0.1 mM. However, we were not able to determine the exact ATP level causing complete cell
decoupling because most experiments under such conditions showed irreversible \( g_i \) reduction.

The above-mentioned results have revealed that \( g_i \) is directly regulated by ATP under the condition in which free-Ca\(^{2+}\), -Mg\(^{2+}\), and -proton concentrations are kept constant. The following experiments were designed to clarify possible underlying mechanisms for the regulation.

**Effects of Forskolin, cAMP, and c-Subunit on \( g_i \)**

The ATP-dependent change in \( g_i \) could be explained by phosphorylation of gap junctional channel proteins through activation of cAMP-dependent protein kinase. If such a mechanism is responsible for the opening of gap junctional channels, \( g_i \) would be increased by stimulation of adenylate cyclase in the cell membrane or by an elevation of cAMP or c-subunit in the cytoplasm. This prediction was tested by applying forskolin (30 \( \mu \)M), cAMP (50 \( \mu \)M), or c-subunit (1 \( \mu \)M) to internal test solutions containing 5.0 mM or 0.5 mM ATP.

Application of these three substances for 5 minutes in the medium containing 5.0 mM ATP had no significant effect on \( g_i \) (Table 2). Figure 5 shows effects of forskolin (30 \( \mu \)M) and cAMP (50 \( \mu \)M) applied for 3 minutes in the low ATP (0.5 mM) medium. These substances did not reverse \( g_i \) values, which had been decreased by the ATP reduction (Table 3). These findings may indicate that opening of gap junction by ATP is not mediated by cAMP-dependent phosphorylation of channel proteins.
TABLE 2. Change in Junctional Conductance in the Presence of 5.0 mM ATP

<table>
<thead>
<tr>
<th></th>
<th>Before (nS)</th>
<th>After (nS)</th>
<th>$G_j$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin (n=5)</td>
<td>247±38</td>
<td>233±28</td>
<td>94±5</td>
</tr>
<tr>
<td>Cyclic AMP (n=13)</td>
<td>236±27</td>
<td>225±23</td>
<td>97±2</td>
</tr>
<tr>
<td>c-Subunit (n=7)</td>
<td>334±62</td>
<td>284±44</td>
<td>89±6</td>
</tr>
<tr>
<td>Adenylylimidodiphosphate (n=5)</td>
<td>195±21</td>
<td>192±18</td>
<td>99±5</td>
</tr>
<tr>
<td>ADP (n=5)</td>
<td>168±14</td>
<td>169±17</td>
<td>102±8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. $g_j$ junctional conductance; $G_j$, normalized $g_j$. Subunit, catalytic subunit of cyclic AMP-dependent protein kinase. Values of $G_j$ were normalized by the value of $g_j$ measured just before the addition of each drug in the presence of 5.0 mM ATP.

Effects of Adenylylimidodiphosphate and ADP on $g_j$

Some sarcolemmal channels have been reported to be regulated by the ligand-receptor interaction between ATP and channel proteins. 14 The ATP-dependent change in $g_j$ might be explained by a similar mechanism. We tested this alternative possibility by using nonhydrolyzable ATP analogue (adenylylimidodiphosphate) and ADP. Application of adenylylimidodiphosphate (2 mM) or ADP (10 mM) to the internal bath solution containing 5.0 mM ATP did not affect $g_j$ (Table 3).

Figure 6 shows experiments in the low (0.5 mM) ATP medium. Application of adenylylimidodiphosphate (2 mM) caused a partial and transient reversal of $g_j$ that had been decreased by the ATP reduction from 5.0 to 0.5 mM. Peak $g_j$ values during adenylylimidodiphosphate application were 56% larger than the pretreatment level at 0.5 mM ATP (Table 3). However, the increment of $g_j$ was not maintained and dissipated spontaneously within several minutes (Figure 6A).

Application of ADP (10 mM) at 0.5 mM ATP did not cause such a partial reversal of $g_j$ (Figure 6B, Table 3).

Effects of Temperature on $g_j$

Effects of temperature on $g_j$ were examined in the control (5.0 mM) ATP medium. As the bath temperature was decreased from 35°C to 28°C and 24°C, $g_j$ decreased in steps, and the change was reversible (Figure 7). The temperature coefficient ($Q_{10}$) in 10 experiments was 1.29.

Discussion

In the present experiments, we measured $g_j$ of ventricular cell pairs isolated from adult guinea pig hearts through the use of a single voltage-clamp approach. The current-voltage relation of junctional membrane was linear between -30 mV and +30 mV, indicating that $g_j$ has no voltage dependency under such Cs⁺-rich and low-Ca²⁺ conditions. This finding is in agreement with previous studies on adult ventricular cell pairs of guinea pig or rat. 7,20,21 Recently, Rook et al. 22 have shown that $g_j$ of neonatal rat heart pairs exhibits marked voltage and time dependency when cell coupling is inhibited by a decrease of the number of channels involved. However, the voltage dependency is not observed until the transjunctional potential exceeds ±50 mV. Therefore, our data are

![Graph showing effects of forskolin on junctional conductance (gj). A reduction of ATP from 5.0 to 0.5 mM resulted in a decrease of gj from 452 to 42 nS.](image_url)
also in agreement with Rook et al.\textsuperscript{22} regarding the voltage range examined.

We estimated \( g_i \) values by assuming that nonjunctional membrane conductance of the voltage-clamped cell (\( g_{m1} \)) is one half of the input conductance before perforation of the other cell of the pair (\( g_{m1} + g_{m2} \)). There is no evidence indicating that \( g_{m1} \) is similar to \( g_{m2} \) and that \( g_{m1} \) is kept constant throughout the experiments even after perforation. Accordingly, such an assumption might introduce variable errors in estimating the precise amount of \( g_i \) values. The potential error is considered to be minimal under the control condition in which \( g_{m1} \) is less than 10\% of \( g_i \), but it may increase as \( g_i \) declines. Although this would limit the quantitative accuracy, we nevertheless think that it does not invalidate our conclusion.

Our control \( g_i \) values in ventricular cell pairs (average, 268 nS) are more or less similar to those reported by Kameyama (710 nS in guinea pig)\textsuperscript{23} and those by Weingart and Maurer (250 nS in guinea pig and 590 nS in rat).\textsuperscript{21,24} Noma and Tsuboi\textsuperscript{7} and Wittenberg et al.\textsuperscript{25} showed somewhat higher control values (1,000 nS in guinea pig, 1,250–2,530 nS in rat). Such discrepancy might be attributed to different methods for cell isolation and for \( g_i \) measurement or to different species employed.

In the present experiments, \( g_i \) decreased in a concentration-dependent manner when intracellular ATP concentration was reduced to less than 1.5 mM under the condition where free-Ca\textsuperscript{2+}, -Mg\textsuperscript{2+}, and -proton concentrations were kept constant. This ATP-dependent change in \( g_i \) was reversible. The half-maximum effective concentration of ATP was 0.68 mM.

Intracellular ATP concentration of intact cardiac myocytes under physiological conditions is kept at a level ranging from 3.0 to 7.5 mM.\textsuperscript{26–28} Under some pathological conditions in which energy metabolism is inhibited, a depletion of creatine phosphate is followed by a significant decrease in ATP. Recent studies have shown that intracellular ATP concentration was decreased by 10–90\% from control when...
cardiac muscles were treated by ischemia or hypoxia for 10–25 minutes. 26–28 ATP-dependent active ionic transport through sarcolemma and through membranes of intracellular organelles is inhibited under such conditions leading to an accumulation of Ca2+ and H+ in the cytosol. 24–26 Cell decoupling induced by ischemia or hypoxia has been mainly attributed to an elevation of these intracellular cations. 24–26 The present data suggest that the ATP depletion may also contribute to cell decoupling.

As to the effect of cAMP on cell coupling, there are some discrepancies among previous reports. In horizontal cells of the turtle retina11,12 or fish retina,13 the permeability of gap junction was shown to be inhibited by an increase of intracellular cAMP. In contrast, De Mello9 demonstrated that intracellular injection of cAMP into cardiac myocytes enhanced cell-to-cell coupling. Burt and Spray10 have recently shown the similar improvement of cardiac cell coupling by an elevation of intracellular cAMP under low-Ca2+ conditions. In addition, Wojtczak29 has shown that cAMP had no effects on cell coupling under physiological conditions but inhibited cell coupling during calcium-overload conditions, such as hypoxia.

cAMP is known to affect many aspects of cell functions including energy metabolism, contraction, and intracellular ionic concentrations. An elevation of cAMP in these previous studies may have affected gj through various secondary changes in such cell functions. The present results seem to indicate that cAMP has no direct action on cardiac gj, provided intracellular ATP, Mg2+, Ca2+, and H+ are kept constant.

Recently, Pressler and Hathaway30 showed that the isolated heart gap junctional protein is phosphorylated by cAMP-dependent protein kinase like liver or lens gap junctional protein. 8,31 However, it is not clear whether phosphorylation of gap junctional protein increases or decreases cell coupling. In the present study, gj was not affected by cAMP. This may indicate that the open state of the gap junctional channel is not maintained by phosphorylation of the channel protein.

Adenylylimidodiphosphate caused a partial and transient reversal of gj that had been depressed by the ATP reduction. Such a transient reversal of gj was not induced by ADP. These findings indicate that nonhydrolyzable ATP analogue (adenylylimidodiphosphate), which has a similar molecular structure to ATP, could partially substitute for ATP. Accordingly, we suggest that gj is maintained by ATP through the ligand-receptor interaction between ATP and gap junctional channel protein. However, the partial reversal of gj by adenylylimidodiphosphate was not maintained and dissipated spontaneously within several minutes. Further experimental studies will be required to clarify the underlying mechanism for this phenomenon.

Our data showed that Q10 of the gap junction was 1.29, in agreement with experiments on the junction of earthworm septate median giant axon. 32 This Q10 value is comparable with that of aqueous diffusion of ions (Q10 = 1.3) and is much lower than that of enzyme reactions (Q10 = 3), such as phosphorylation regulated by high-energy phosphates. 33 Such a low Q10 value seems to support our proposal that the regulation of gap junctional channel is mediated by ligand-receptor interaction between ATP and channel proteins, because it would require minimal energy consumption.

In summary, ATP regulates cell coupling between paired ventricular myocytes independently of intracellular free Ca2+, Mg2+, and protons. We suggest that ATP-dependent change in gj is mediated by the ligand-receptor interaction between ATP and gap junctional channel protein.

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