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Enhancement of Intracellular Cl^- Concentrations Induced by Extracellular ATP in Guinea Pig Ventricular Muscle

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ABSTRACT—We investigated effects of extracellular ATP on intracellular chloride activities ($[Cl^-]_i$) and possible contribution of the Cl^--HCO_3^- exchange to this increase in $[Cl^-]_i$ in isolated guinea pig ventricular muscles. The $[Cl^-]_i$ and intracellular pH (pH_i) were recorded in quiescent ventricular muscles using double-barreled ion-selective microelectrode techniques. MgATP at a concentration higher than 0.1 mM, induced an increase in $[Cl^-]_i$, and this increase in $[Cl^-]_i$ was dependent on the concentration of ATP but not on the concentration of magnesium ions present in the perfusion solution. NaADP, but not NaAMP, at a concentration of 0.5 mM induced a similar increase in $[Cl^-]_i$ as that induced by MgATP. However, the NaADP-induced increase in $[Cl^-]_i$ was transient and gradually returned to the control level even though NaADP was continuously present. Furthermore, ATP also triggered a transient acidification of pH_i, and both increases in $[Cl^-]_i$ and intracellular H^+ induced by ATP were prevented when preparations were pretreated with stilbene derivatives, SITS and DIDS, or perfused with a Cl^--free solution. Our findings showed that the increased extracellular ATP concentrations might trigger an increase in $[Cl^-]_i$ in ventricular muscles. In light of previous studies showing that cardiac ischemia induced increases in extracellular nucleotide concentrations and $[Cl^-]_i$ in ventricular muscles, we propose that ischemia-induced accumulation of ATP concentration in the extracellular space may be an important factor to trigger increment of $[Cl^-]_i$ during ischemic conditions.

Keywords: Extracellular ATP, Intracellular chloride activity, Ion-selective microelectrode, Cl^--HCO_3^- exchange

Since the classical observations on extracellular ATP from Drury and Szent-Gyorgyi (1), it is known that extracellular ATP and its analogues exert important electrophysiological effects and induce multiple functional changes at micromolar concentrations in the mammalian heart (see reviews in refs. 2 – 4). With activation of P_2 purinergic receptors, ATP induces a positive inotropic effect, increases the hydrolysis of phosphoinositides and enhances excitability or favors slow conduction in rat ventricular muscles (5 – 7). In addition to their normal physiological roles, it is clear that purinoceptors are active during pathophysiological conditions such as myocardial infarction that involve ischemia or traumatic tissue damage or that cause shock. As a consequence of a release of ATP and other purine nucleotides from various cell types, the local extracellular purine nucleotide concentration increases and can temporarily exceed 100 μM in response to local traumas, including hypoxia or ischemia (3).

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The effects of extracellular ATP on cationic conductances in cardiac, skeletal and smooth muscle cells are described in detail. With patch-clamp and micro-spectrofluorimetry techniques, it is known that activation of P_2 receptors with extracellular application of ATP increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cardiomyocytes (8, 9) in cultured skeletal (10, 11) and smooth muscle cells (12, 13). This effect of ATP has been attributed either to the activation of an inward current that depolarizes the membrane and indirectly activates the L-type Ca^{2+} current (I_{Ca}) (14, 15) or to the increased production of inositol 1,4,5-trisphosphate, which stimulates Ca^{2+} release from intracellular stores (16). However, current studies cannot fully explain the action of ATP facilitating the induction of the delayed after-depolarization and the early after-depolarization (17). In addition to a transient increase in inward currents, ATP also slowly activates a Cl^- conductance in guinea pig atria (18) or in rat (19) and mouse (20) ventricular myocytes. On rapid application, ATP may trigger membrane depolarization by activation of a Cl^- conductance or by inducing an acidification

following Cl^--HCO_3^- exchanger stimulation in isolated single rat cardiac cells (14). Furthermore, adenosine, ADP and AMP are equipotent to ATP to induce Cl^- current activation (4). To demonstrate that extracellular ATP would induce activation of the Cl^--HCO_3^- exchanger in cardiac cells, Pucéat and colleagues measured the intracellular pH (pH_i) using Snarf-1, a pH-sensitive probe, and their findings clearly showed that extracellular ATP induced transient acidification followed by an alkalinization in single rat cardiac cells (21). Obviously, both ATP-activated Cl^- conductances and ATP-stimulated activation of the Cl^--HCO_3^- exchanger would affect the intracellular chloride homeostasis. However, it is not clear whether extracellular ATP induces changes in intracellular chloride activity ($[Cl^-]_i$) in cardiac cells.

In the present study, we investigated the effects of extracellular ATP on $[Cl^-]_i$ in guinea pig ventricular papillary muscles using ion-selective microelectrode techniques. Our findings showed that ATP at concentrations greater than 0.1 mM induced an increase in $[Cl^-]_i$ in quiescent muscles. The ATP-induced elevation of $[Cl^-]_i$ was suppressed by stilbene derivatives and an external Cl^--free condition that were known as potent inhibitors of Cl^--transports in cardiac myocytes and other cells. Our findings suggest that activation of the Cl^--HCO_3^- exchanger is involved in the ATP-induced changes in $[Cl^-]_i$ and pH_i, which may be important factors contributing to the generation of arrhythmias during ischemia.

MATERIALS AND METHODS

Preparation and solutions

Guinea pigs of either sex weighing 250–300 g (Inoue Experimental Animal Center, Kumamoto) were stunned, and their hearts were quickly removed and immersed in cold Tyrode solution. Papillary muscles, 3–5 mm in length and about 0.5 mm in diameter, were excised and mounted in a flow chamber (a bath chamber in a volume of about 2 ml) and superfused continuously at approximately 5 ml/min with $NaHCO_3$-buffered Tyrode solution equilibrated with a 95% O_2 + 5% CO_2 gas-mixture. The Tyrode solution was composed of: 127 mM NaCl, 4 mM KCl, 0.25 mM $MgCl_2$, 1.4 mM $CaCl_2$ and 5.5 mM glucose and was buffered with 20 mM $NaHCO_3$. A Cl^--free solution was made by replacement of Cl^- with equimolar gluconate as described previously (22, 23), and a Na^+-free solution was made by replacement of Na^+ with *N*-methyl-D-glucamine. The pH of the solution was adjusted to 7.4 by adding HCl in HCO_3^--buffered solution, and the pH of HCO_3^--buffered Cl^--free solutions was adjusted by adding acetic acid. The temperature of the solutions was monitored using a thermocouple-type thermoprobe (PTC-201; Unique Medical, Tokyo) placed in the bath chamber and was maintained at 36 ± 0.5°C. The preparations were equilibrated without stimulation for 1 h before the start of the experiments.

Experimental procedure

The $[Cl^-]_i$ and pH_i were measured with double-barreled ion-selective microelectrode techniques as described previously (23, 24). All experiments were done in quiescent guinea pig ventricular muscles obtained from different animals. To see the effects of extracellular ATP on $[Cl^-]_i$, the quiescent preparations obtained from different animals were impaled with double-barreled Cl^--selective microelectrodes (one impalement from one muscle) for 15 min to record control levels of $[Cl^-]_i$ and membrane potentials (V_m). Then, the muscles were perfused with the Tyrode solutions containing MgATP, NaATP or NaADP and NaAMP at the desired concentrations for 15 min followed by washout for 25 min. To observe the effects of SITS or Cl^--free conditions on ATP-induced changes in $[Cl^-]_i$, 7 preparations obtained from 7 muscles were treated with 0.5 mM SITS and 6 other muscles were perfused with a Cl^--free solution, following the same $[Cl^-]_i$ -measure procedures as described above. To investigate effects of extracellular ATP on pH_i, guinea pig ventricular muscles were exposed to MgATP at concentrations of 0.1 or 0.5 mM, and then pH_i-recordings were measured. The effects of ATP on pH_i were also observed in the presence of stilbene derivatives, in the absence of extracellular Cl^- or Na^+.

Double-barreled ion-selective microelectrode recordings

The double-barreled ion-selective microelectrodes were constructed as described previously using liquid-sensor cocktails for Cl^- or H^+ ions, chloride ionophore I-cocktail A (model 24902; Fluka Chemika-Biochemika, Buchs, Switzerland) for Cl^--selective electrodes (23) or hydrogen ionophore I-cocktail B (model 95293, Fluka) for H^+-selective electrodes (24, 25). Two capillaries were designated as tube A for a reference electrode measuring the transmembrane potential and tube B for the ionic sensor. Pure acetone was injected into the open end of tube A to maintain hydrophilic quality. After the entire tip was dipped into a fresh solution of 0.1% silicone oil KF-96 (Shin-etsu Chemical Industry Co., Tokyo) diluted with trichloroethylene, the electrode was baked on a hot plate at 300°C for 30 min. Then, tube B was back-filled with the liquid ion exchanger until the exchanger column reached 70–100 μm in length from the tip. The remaining portion of tube B was filled with 120 mM KCl plus 30 mM NaCl for the Cl^- sensor (26) and a phosphate-buffered solution (40 mM KH_2PO_4, 23 mM NaOH and 15 mM NaCl) (27) for the pH sensor. Reference tube A was back-filled with 0.5 mM KCl.

The Cl^--selective electrodes were calibrated in Tyrode solutions with different concentrations of Cl^- or 142 mM

NaCl solution in which Cl^- was replaced with gluconate to make a set of 4 solutions (23, 28). The concentrations of Cl^- in each solution were 0.5, 5, 50 and 142 mM, respectively and the cationic concentration was adjusted to 142 mM. It shows a sensitivity of more than 55 mV/decade in the range between 0.5 to 142 mM Cl^-. The calibration of H^+-selective electrodes was done as described previously (24, 25).

To record the membrane potentials reflecting ion activities, individual outputs of the double-barreled microelectrodes were connected to each input probe of a high-input impedance amplifier (FD223-D; W-P Instruments, New Haven, CT, USA) through a Teflon tube containing the respective electrolyte solutions for each barrel. The intracellular ionic activity signals were obtained differentially [Cl^- or H^+ output (V_{Cl} or V_H), V_m] with respect to the Ag-AgCl ground electrode. All outputs were monitored on-line using digital voltmeters built into the amplifiers and a digital memory oscilloscope (DMS-6430; Iwatsu, Tokyo). Both Cl^- activity and V_m signals were recorded simultaneously using a pen recorder with 80-Hz frequency response (Recti-Horiz-8K; San-ei, Tokyo) and saved with a data recorder (RD101T; TEAC, Tokyo). The differential potential and V_m were also measured directly using digital voltmeters installed in the amplifiers (FD223-D, W-P Instruments) to a precision of 0.1 mV.

Drugs and statistics

All salts used to prepare solutions and SITS or DIDS were obtained from Sigma (St. Louis, MO, USA). The salts used to prepare solutions were dissolved directly in Tyrode solution and SITS or DIDS was dissolved in the dark with Tyrode solution at the desired concentration. ATP and ADP and AMP were prepared as a stock solution

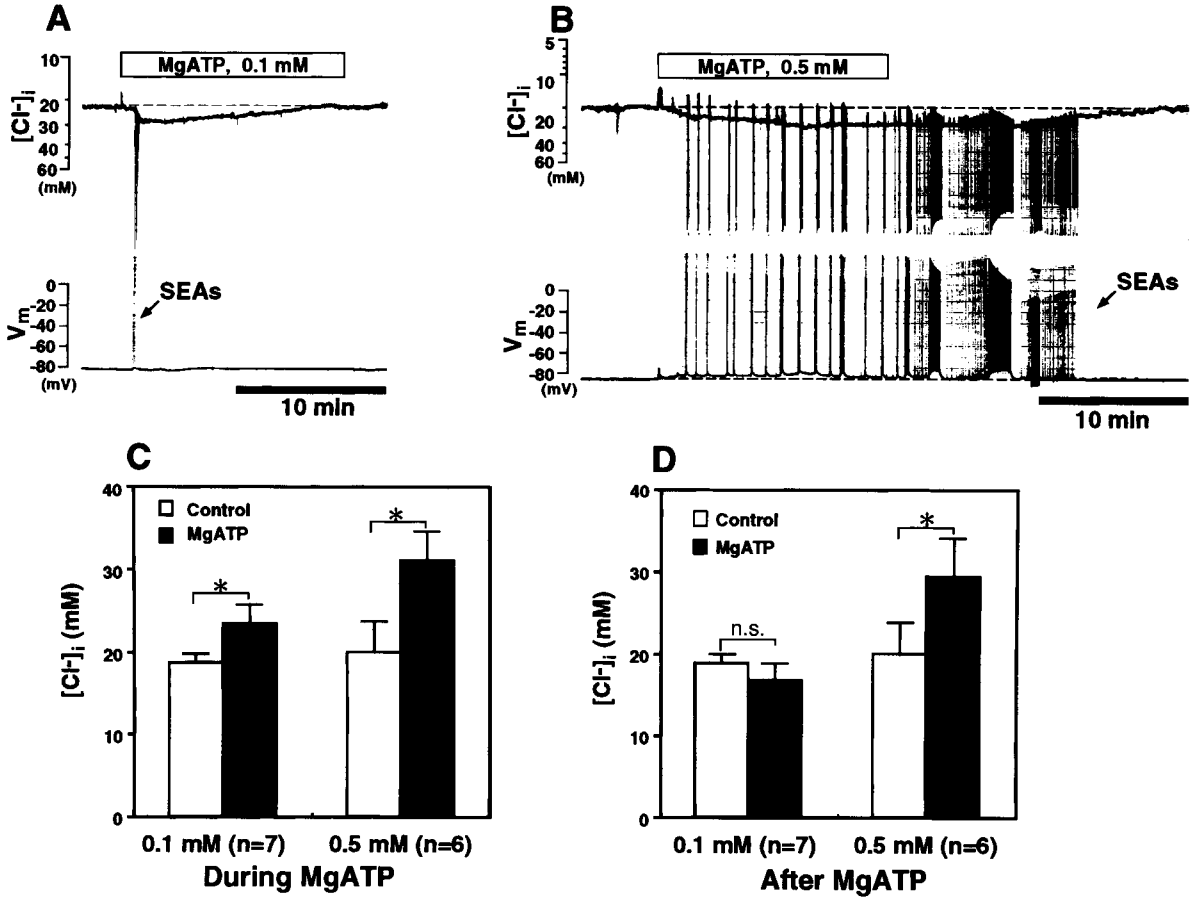

Fig. 1. MgATP-induced increase in intracellular chloride concentrations ($[Cl^-]_i$) in quiescent guinea pig ventricular papillary muscles. A and B: Representative traces of changes in $[Cl^-]_i$ and membrane potentials (V_m) in quiescent muscles after application of MgATP at 0.1 mM and 0.5 mM for 15 min are shown. Arrows indicate that SEAs were triggered in these cells. The frequency of ATP-induced SEAs at 0.5 mM was higher than that at 0.1 mM. C: Means of peak values of $[Cl^-]_i$ during exposure to MgATP at 0.1 mM in 7 cells from 7 muscles or exposure to MgATP at 0.5 mM in 6 cells obtained from 6 muscles. D: The peak $[Cl^-]_i$ values obtained just before washout of ATP. *, Compared with values of $[Cl^-]_i$ obtained from the control, $P<0.05$. n.s.: There are no significant differences. □, Control; ■, MgATP.

before use. Data were analyzed by basic statistical methods including the two-tailed Student's *t*-test (paired and unpaired). Data are expressed as means ± S.E.M. and significance was established at $P<0.05$.

RESULTS

Extracellular application of ATP induces an increase in $[Cl^-]_i$

Effects of MgATP on $[Cl^-]_i$: The $[Cl^-]_i$ and V_m was recorded with double-barreled ion-selective microelectrodes in quiescent muscles. MgATP at concentrations of 1 and 10 μM did not induce any changes in $[Cl^-]_i$ (data not shown). However, MgATP at a concentration of 0.1 mM induced a transient elevation of $[Cl^-]_i$ in 6 impaled cells (obtained from 6 muscles), or a biphasic change of $[Cl^-]_i$ in one cell (from one muscle), as shown as a representative tracing in Fig. 1A. At a high concentration (0.5 mM), MgATP induced an elevation in $[Cl^-]_i$, which was maintained at a higher level after washout (Fig. 1B). Figure 1 C and D show that 0.1 or 0.5 mM MgATP-induced $[Cl^-]_i$ changes during the initial phase (peak values during application of MgATP) and the later phase (data obtained just before onset of washout of MgATP), respectively. Furthermore, during exposure to MgATP, the spontaneous electrical activities (SEAs) occurred at concentrations of both 0.1 (2/7 cells) and 0.5 mM (3/6 cells). The SEAs triggered by application of 0.1 mM ATP were transient (Fig. 1A) but that induced by 0.5 mM ATP showed a complex firing-pattern and the duration of the incidence of SEAs was sustained (Fig. 1B). According to some previous studies, it is known that extracellular application of ATP triggered oscillatory contraction (29) or depolarization followed by oscillatory activity in adult rat ventricular myocytes (14). A generally accepted explanation for this effect of ATP was that ATP induced an increase in $[Ca^{2+}]_i$ (5, 30) by activation of Ca^{2+} influx across the sarcolemma or by triggering Ca^{2+} release from its intracellular stores (5, 29, 30). However, at this moment, it is unclear whether ATP-induced increase in $[Cl^-]_i$ is involved in ATP-triggered SEA in the present study.

Effects of NaATP and NaADP on $[Cl^-]_i$: To clarify whether other ATP derivatives affected $[Cl^-]_i$ in the similar manner as MgATP did in guinea pig ventricular muscle, effects of NaATP, NaADP or NaAMP on $[Cl^-]_i$ were examined. NaATP at 0.1 and 0.5 mM increased $[Cl^-]_i$ and the magnitude of the NaATP-induced elevation in the $[Cl^-]_i$ was similar to that induced by MgATP. However, NaADP at a concentration of 0.1 mM did not induce detectable increase in $[Cl^-]_i$. In contrast, NaADP at a high concentration (0.5 mM) induced an increase in the $[Cl^-]_i$, and the peak of $[Cl^-]_i$ attained a level of almost 30 mM from 21 mM at the control level (Fig. 2A). This NaADP-triggered

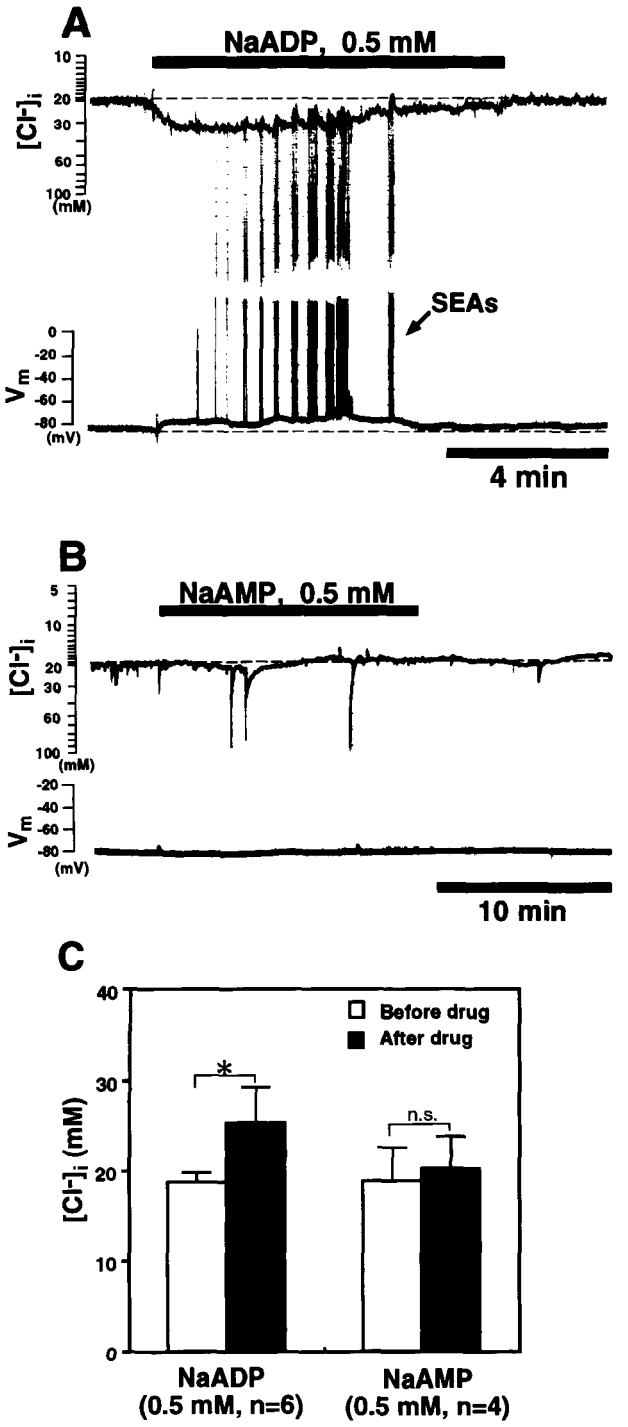

Fig. 2. Effects of NaADP and NaAMP on $[Cl^-]_i$ in ventricular muscles. A: NaADP at 0.5 mM induced an increase in $[Cl^-]_i$ similar to that induced by MgATP. However, the NaADP-induced increase in $[Cl^-]_i$ was not sustained as those induced by MgATP, but gradually returned to the control level even though NaADP was continuously present. B: NaAMP at a concentration of 0.5 mM had no effect on $[Cl^-]_i$. C: Effects of NaADP on $[Cl^-]_i$ in 6 impalements from 6 muscles or NaAMP on $[Cl^-]_i$ in 4 impalements from 4 muscles were summarized. *, Compared with values of $[Cl^-]_i$ obtained from the control, $P<0.05$. n.s.: There are no significant differences. □, Before application of drugs; ■, Peak values of $[Cl^-]_i$ after exposure to NaADP at 0.5 mM or NaAMP at 0.5 mM.

increase in $[Cl^-]_i$ was transient and gradually returned to control level even though NaADP was continuously present. On the other hand, NaAMP, even at a high concentration (0.5 mM), did not induce any detectable changes in $[Cl^-]_i$ (Fig. 2B). Similar results for effects of NaADP obtained from 6 impalements (in 6 muscles) or effects of NaAMP (4 cells from 4 muscles) are summarized in Fig. 2C.

Stilbene derivatives and external Cl^--free conditions inhibited ATP-induced increases in $[Cl^-]_i$

Since it has been shown that extracellular ATP activated the Cl^--HCO_3^- exchange in single rat ventricular myocytes (21), it is possible that ATP-induced changes in $[Cl^-]_i$ would result from an influx of Cl^- via the activation of the Cl^--HCO_3^- exchanger. Therefore, we examined whether stilbene derivatives, SITS and DIDS, or external Cl^--free conditions would have any effects on ATP-induced changes in $[Cl^-]_i$.

Effects of stilbene derivatives and external Cl^--free conditions on $[Cl^-]_i$: We observed effects of SITS, DIDS or external Cl^--free conditions on $[Cl^-]_i$ in guinea pig ventricular muscles, and the data are summarized in Table 1. When papillary muscles were exposed to 0.5 mM SITS for 15 min, $[Cl^-]_i$ gradually decreased to 10.5 ± 0.1 mM from 17.5 ± 0.5 mM at the control level (n = 7 cells from 7 muscles, $P<0.05$). A similar result was obtained when DIDS was applied at 0.1 mM for 15 min (before application of DIDS, $[Cl^-]_i$ was 19.3 ± 0.7 mM; after using DIDS, the $[Cl^-]_i$ became 12.4 ± 1.1 mM; n = 5, $P<0.05$). In a nominally Cl^--free condition in which Cl^- was replaced with equimolar gluconate (22, 23), the $[Cl^-]_i$ decreased gradually until the solution was switched to normal Tyrode solution (in the control, $[Cl^-]_i$ was 20.3 ± 1.0 mM; and in the Cl^--free condition, $[Cl^-]_i$ became 7.3 ± 0.4 mM; n = 6, $P<0.05$).

Both stilbene derivatives and Cl^--free conditions inhibited ATP-induced increases in $[Cl^-]_i$: We then examined whether stilbene derivatives and Cl^--free conditions affect the ATP-triggered increase in $[Cl^-]_i$. Our results showed

Table 1. Effects of stilbene derivatives and extracellular Cl^--free solutions on intracellular Cl^- activities in guinea pig ventricular muscles

Treatment	$[Cl^-]_i$ (mM) before treatment	$[Cl^-]_i$ (mM) 15 min after treatment
Control (n = 7)	18.7 ± 3.5	18.2 ± 1.6
SITS 0.5 mM (n = 7)	17.5 ± 0.5	10.5 ± 0.1*
DIDS 0.1 mM (n = 5)	19.3 ± 0.7	12.4 ± 1.1*
Cl^--free solution (n = 6)	20.3 ± 1.0	7.3 ± 0.4*

Data are means ± S.E.M. n, no. of impaled cells from different animals. Data were obtained before and after exposure to agents and analyzed by the paired *t*-tests. $[Cl^-]_i$, intracellular chloride activity; *$P<0.05$, compared with value in absence of the agent.

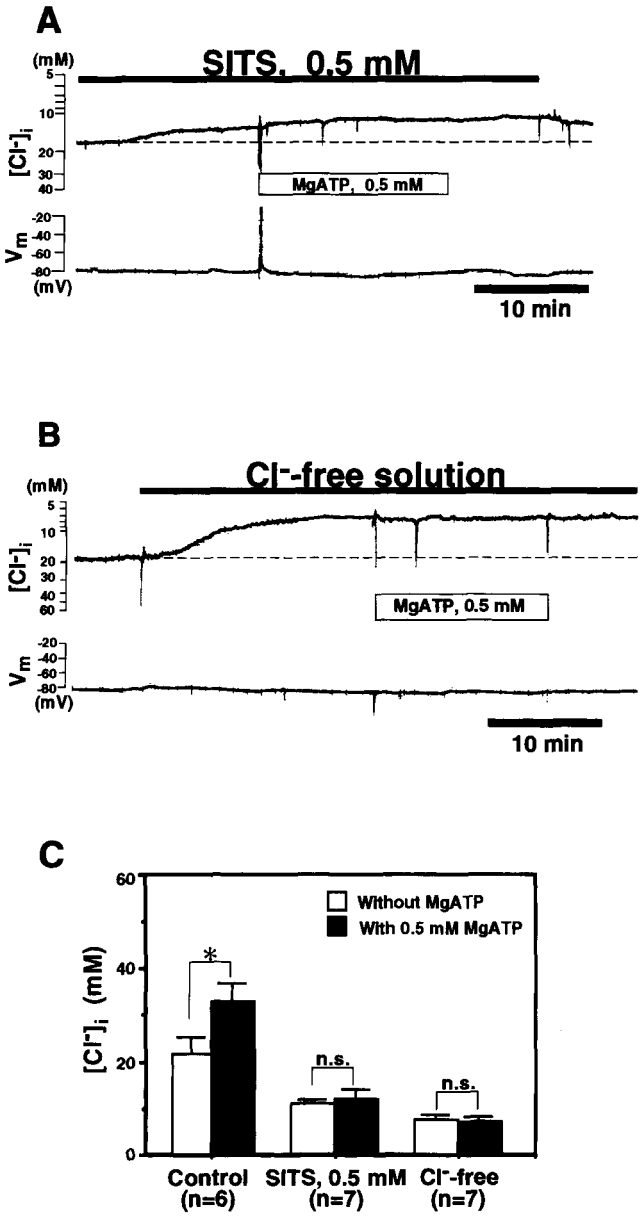

Fig. 3. Effects of SITS or Cl^--free conditions on MgATP-induced increases in $[Cl^-]_i$ in guinea pig ventricular muscles. A: Effects of 0.5 mM MgATP on $[Cl^-]_i$ in a preparation treated with 0.5 mM SITS. B: Representative traces of MgATP on $[Cl^-]_i$ under the Cl^--free condition. Both SITS (0.5 mM) and Cl^--free conditions induced a decrease in $[Cl^-]_i$. However, in the presence of SITS (A) and in the absence of extracellular Cl^- (B), application of MgATP did not induce any increase in $[Cl^-]_i$. C: Similar data obtained from application of MgATP in SITS-pretreated muscles or Cl^--free conditions were summarized. The MgATP-induced elevation of $[Cl^-]_i$ was inhibited by 0.5 mM SITS and suppressed in Cl^--free conditions. Numerals in parentheses indicate numbers of impaled cells from different animals. □, Without application of MgATP; ■, With 0.5 mM MgATP. *, Compared with the data obtained without use of MgATP, there are significant differences ($P<0.05$, paired *t*-test). n.s.: There are no significant differences.

that both SITS and external Cl^--free conditions blocked the ATP-induced increase in $[Cl^-]_i$. As shown in Fig. 3A and B, when the preparations were treated with 0.5 mM SITS or with a Cl^--free solution, application of MgATP did not induced any changes in $[Cl^-]_i$. Figure 3C summarizes the effects of MgATP (0.5 mM) on $[Cl^-]_i$ in the absence or in the presence of SITS or Cl^--free solutions: 1) In the MgATP only group (n = 6), $[Cl^-]_i$ increased from 20.5 ± 2.8 mM of the control level to 32.5 ± 4.6 mM after application of MgATP ($P<0.05$); 2) In the SITS + MgATP group (n = 7), the $[Cl^-]_i$ became 11.5 ± 2.1 mM from 10.5 ± 0.08 mM and was not significantly different from the value obtained before MgATP ($P>0.05$); 3) In a Cl^--free + MgATP group (n = 7), the $[Cl^-]_i$ was 7.25 ± 0.6 mM, and this value was also not significantly different compared with the data of Cl^--free-only (7.3 ± 0.4 mM, $P>0.05$). Furthermore, similar $[Cl^-]_i$-recording results were observed from 3 cells (from 3 muscles) pretreated with 0.1 mM DIDS (the $[Cl^-]_i$ was 12.4 ± 1.1 mM during exposure to DIDS-only (0.1 mM) and was 13.1 ± 0.1 mM after exposure to DIDS + 0.5 mM MgATP ($P>0.05$). These findings indicated that stilbene derivatives and Cl^--free conditions suppressed the MgATP-induced increase in $[Cl^-]_i$.

We examined whether stilbene derivatives also have similar effects on NaATP-induced increase in $[Cl^-]_i$ as that on the MgATP-induced one. Figure 4 shows representative traces of NaATP-induced changes in $[Cl^-]_i$ with or without the presence of 0.5 mM SITS. At a concentration of 0.5 mM, NaATP induced a large increase in $[Cl^-]_i$, and the peak of change in $[Cl^-]_i$ ($\Delta[Cl^-]_i$) was ~15 mM at 5 min after application of NaATP (Fig. 4A). However, the NaATP-induced spikes are dispersive and the elevation in $[Cl^-]_i$ gradually returned to the control level even though NaATP was continuously present. After the preparation was treated with 0.5 mM SITS, the increase in $[Cl^-]_i$ was significantly suppressed and the SEAs did not appear (Fig. 4B). Similar results were obtained from 6 other cells treated with 0.5 mM SITS and summarized in Fig. 4C.

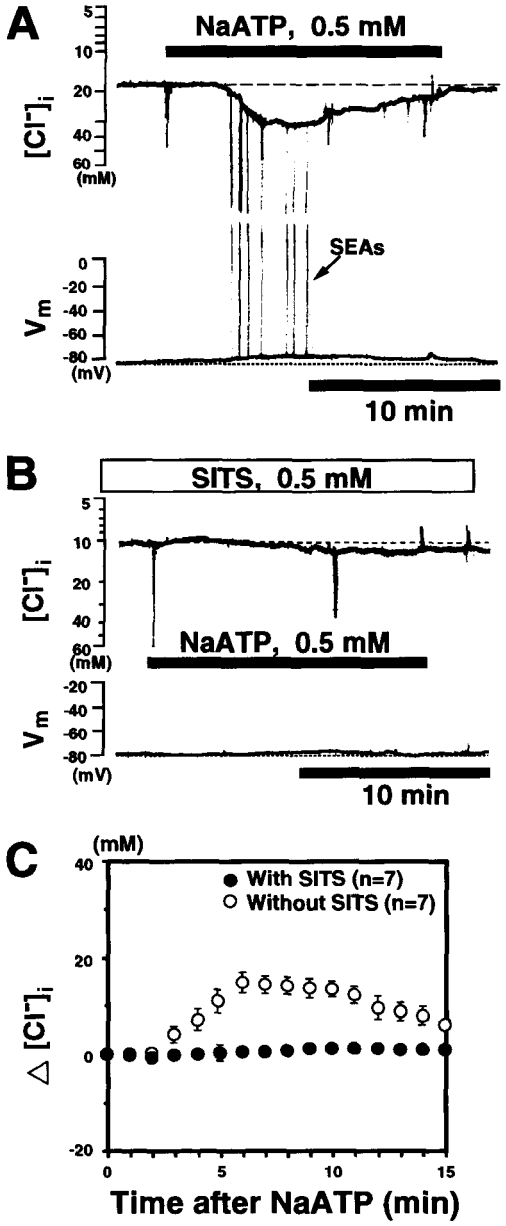

Fig. 4. Effects of SITS on the NaATP-induced increase $[Cl^-]_i$ and SEAs. A: NaATP at a concentration of 0.5 mM increased $[Cl^-]_i$. B: In the cell pretreated with SITS (0.5 mM), the NaATP-induced increase in $[Cl^-]_i$ was suppressed (records obtained from a different preparation from panel A). C: Normalized changes in $[Cl^-]_i$ ($\Delta[Cl^-]_i$) in guinea pig ventricular muscle pretreated with or without 0.5 mM SITS (data obtained from 7 different muscles). ○, effects of NaATP at 0.5 mM without SITS or ●, with 0.5 mM SITS. There are significant differences between untreated and SITS-treated cells at 5 and 10 min after application of NaATP ($P<0.05$, unpaired t-test).

Effects of stilbene derivatives and Cl^--free conditions on MgATP-induced acidification in guinea pig ventricular muscles

MgATP induced a transient acidification followed by an increase in pH_i: To clarify whether the ATP-induced increase in $[Cl^-]_i$ would be due to activation of the Cl^--HCO_3^- exchange, effects of MgATP on pH_i in guinea pig ventricular muscles were also examined using double-barreled pH-selective microelectrodes. As shown in Fig. 5, after exposure to 0.1 mM MgATP, MgATP immediately induced a transient acidification, and then, the pH_i became more alkalized than the control level during 15-min exposure to MgATP (Fig. 5A). The pattern of alteration of pH_i was clearer when preparations were exposed to ATP at 0.5 mM (Fig. 5B). Figure 5, C and D, show pH_i values in the initial phase (data obtained from the peak of the acidification induced by MgATP) and in the later phase (data obtained at the end of MgATP exposure). We also

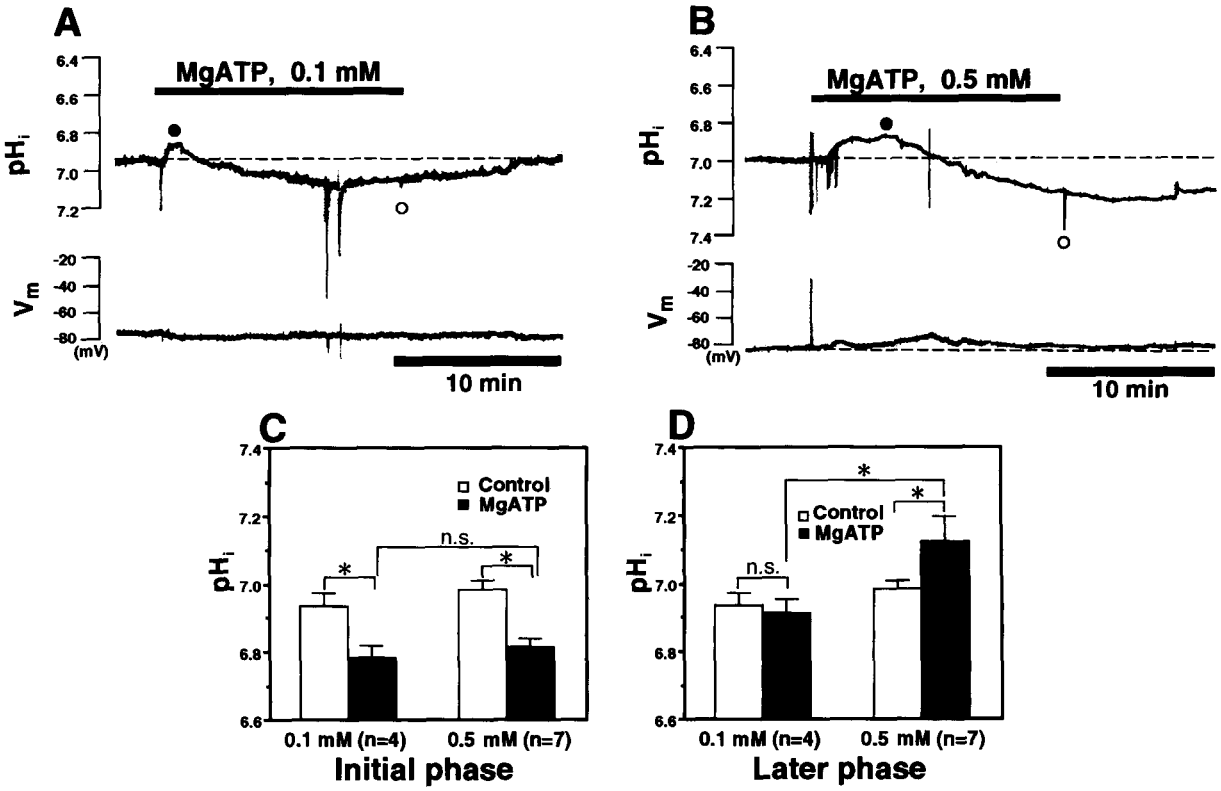

Fig. 5. MgATP-induced changes in pH_i. A transient acidification followed by a sustained increase in pH_i was found after application of MgATP at 0.1 mM (A) and 0.5 mM (B) for 15 min, respectively. C: Peak values of pH_i during the initial phase (as indicated by ● in panels A and B). There was no significant difference for MgATP-induced transient acidification between application of MgATP at 0.1 and 0.5 mM. D: The pH_i values obtained at 15 min after exposure to MgATP (later phase, as indicated by ○ in panels A and B). Note the alkalinization induced by MgATP at 0.5 mM was significantly higher than that at 0.1 mM (*$P<0.05$). n.s.: There are no significant differences. □, Control; ■, MgATP.

investigated the effects of MgATP in 3 cells from 3 muscles perfused with a Cl^--free solution. In the Cl^--free condition, as shown in left traces of Fig. 6A, ATP-induced acidification was not observed. Similarly, ATP also did not trigger any changes in pH_i when preparations were treated with 0.5 mM SITS (n = 5) or 0.1 mM DIDS (n = 4). In the SITS-group, pH_i was 7.030 ± 0.037 at the control period, 7.180 ± 0.035 at the application of 0.5 mM SITS, and 7.174 ± 0.029 after SITS plus MgATP (0.5 mM). In the DIDS-group, pH_i was 7.040 ± 0.08 at the control period, 7.130 ± 0.04 at the application of DIDS, and 7.126 ± 0.02 at the application of DIDS plus MgATP. There was no statistical difference in the presence or in the absence of 0.5 mM MgATP when preparations were treated with stilbene derivatives, including SITS or DIDS ($P>0.05$).

Effects of MgATP on pH_i in the absence of external Na^+: To determine why the time course of ATP-induced acidification is shorter than that of ATP-induced increase in $[Cl^-]_i$, we observed effects of MgATP on pH_i in the presence of amiloride or in the absence of external Na^+, both of which are known to effectively inhibit the Na^+-H^+ exchange. Because some Na^+-dependent H^+ extruding mechanisms such as the Na^+-H^+ exchange and the Na^+-HCO_3^- cotransport can be inhibited in a Na^+-free condition (31), we investigated the effects of MgATP on pH_i in a Na^+-free solution in which Na^+ was replaced with isoosmotic *N*-methyl-D-glucamine (NMDG). In this condition, MgATP induced a sustained acidification, but the alkalization induced by MgATP was not observed. Furthermore, the time course of MgATP-induced acidification is similar to the time course of MgATP-induced increase in $[Cl^-]_i$ (see right traces in Fig. 6A). Similar results were also observed in 4 other impalements from 4 muscles exposed to 0.5 mM MgATP in a Na^+-free condition. The time course of MgATP-induced changes in $[Cl^-]_i$ and pH_i was normalized and shown in Fig. 6B. The ATP-induced acidification was transient when preparations were perfused with a normal Tyrode solution (Fig. 6, B-b). However, it became a sustained acidification when $Na^+$was replaced with *N*-methyl-D-glucamine (Fig. 6, B-c), and the time course of this sustained acidification was similar to that of the ATP-induced increases in $[Cl^-]_i$. Similarly, in 3 cells (from 3

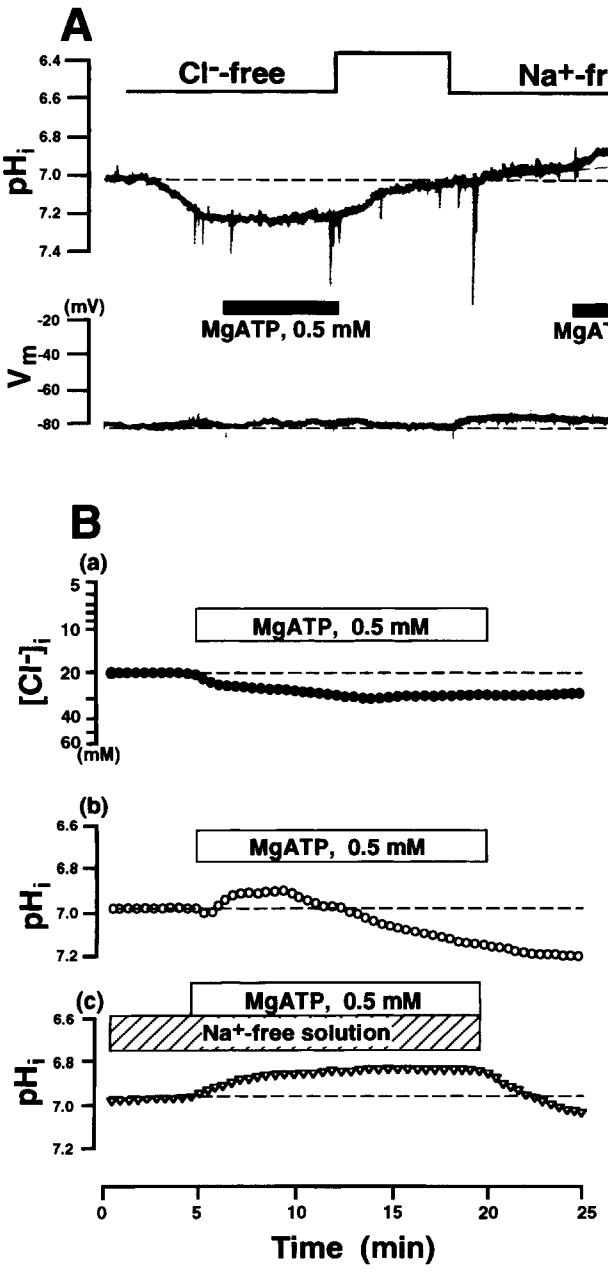

Fig. 6. Time course of MgATP-induced changes in $[Cl^-]i$ and pH_i. A: Effects of MgATP at a concentration of 0.5 mM on pHi in Cl^--free or Na^+-free conditions. As shown in left traces of panel A, removal of extracellular Cl^- completely blocked the transient acidification induced by MgATP. Similar results also were obtained when preparations were treated with 0.1 mM DIDS or 0.5 mM SITS. Note the effects of ATP-induced acidification were abolished in an external Cl^--free condition (left) but sustained in a Na^+-free solution (right). B: Time course of MgATP-induced changes in $[Cl^-]_i$ and pH_i in the presence or in the absence of external Na^+. a: Effects of MgATP at 0.5 mM on $[Cl^-]_i$, and data obtained from Fig. 1B. b: Effects of MgATP on pH_i in the presence of Na^+, the data obtained from Fig. 5B. c: In a Na^+-free solution (Na^+ was replaced with equimolar *N*-methyl-D-glucamine), the Na^+-H^+ exchanger was blocked and MgATP-induced acidification became sustained (data obtained from right traces of panel A). The duration of ATP-induced acidification was similar to those of ATP-triggered increase in $[Cl^-]_i$ as shown in panel a. ○ and ▽, pH_i; ●, $[Cl^-]_i$.

muscles) treated with 1 mM amiloride, pH_i became 6.940 ± 0.032 from 7.091 ± 0.043 at the control period, and was 6.833 ± 0.036 after amiloride + MgATP. The MgATP-induced acidification in pH_i became sustained in the presence of amiloride and was significantly lower than that with amiloride-only ($P<0.05$).

DISCUSSION

The main goal of the present study was to investigate whether extracellular application of ATP induces any change in $[Cl^-]_i$ in guinea pig ventricular muscle. To our knowledge, this study is the first to observe effects of ATP on $[Cl^-]_i$. Our results showed that ATP increased $[Cl^-]_i$ in guinea pig ventricular muscles, and this increase of $[Cl^-]_i$ was suppressed by the extracellular Cl^--free condition and sensitive to stilbene derivatives, SITS and DIDS.

The mechanisms involving the $[Cl^-]_i$ increment in ventricular muscle during exposure to ATP and ADP are complex since the pharmacological effects of stilbene derivatives are known as varied and non-specific. However, the concentrations of SITS and DIDS used in the present study were known to completely inhibit the Cl^--HCO_3^- exchanger in ventricular cells (32–34) and red blood cells (35).

The suppressing effects of SITS and DIDS on ATP-induced $[Cl^-]_i$-elevation, therefore, may be a result of their ability to block the Cl^--HCO_3^- exchanger in ventricular cells. Hence, both increases in $[Cl^-]_i$ and intracellular H^+ induced by ATP in the present study may be explained as extracellular ATP activating a Cl^- influx with a HCO_3^- efflux via activation of the Cl^--HCO_3^- exchange in guinea pig ventricular muscles. This idea is also supported by the observation that effects of ATP on $[Cl^-]_i$ and pH_i were blocked by stilbene derivatives and external Cl^--free solutions. However, comparing the time course of the ATP-induced transient acidosis with that of the elevated $[Cl^-]_i$ induced by ATP, the duration of ATP-induced increase in $[Cl^-]_i$ was longer than that of ATP-induced increases in H^+. This is clear when the concentration of ATP is at 0.5 mM and suggests that changes in $[Cl^-]_i$ are not parallel with the HCO_3^- efflux that resulted from activation of the Cl^--HCO_3^- exchange. However, as reported previously (21), in addition to its activation of the Cl^--HCO_3^- exchange, ATP also strongly stimulated a Na^+-H^+ exchange since ATP-induced acidification would be a factor stimulating the Na^+-H^+ exchange. Thus, if the Na^+-H^+ exchanger is artificially blocked, the duration of ATP-induced increase in $[Cl^-]_i$ would be similar to that of the ATP-induced acidification. Indeed, in the absence of extracellular Na^+ (see Fig. 6) or in the presence of 1 mM amiloride, the MgATP-induced acidification became a sustained one until the perfusion was changed into normal Tyrode solution.

Another possible explanation for ATP-induced elevation of $[Cl^-]_i$ is that ATP activates some Cl^- currents as described in atrial (18, 36, 37), ventricular cells (36, 38, 39) and in epithelial cells (40). Especially, activation of an ATP-activated Cl^- current ($I_{Cl.ATP}$), which may represent a novel and alternative activation of the CFTR Cl^- channel (39), would be involved in the effects of ATP on $[Cl^-]_i$ in the present study because activation of this Cl^- channel would induce some possible Cl^- influxes. However, in the present study, activation of this Cl^- channel may not need to be considered, since in our experiments, the $[Cl^-]_i$ was recorded in quiescent muscles without any electrical stimulation. Under this condition, resting potentials were more negative than the Cl^- reversal potentials, activation of $I_{Cl.ATP}$ would induce an inward current to result in a Cl^--efflux but not Cl^--influx (39, 41). Second, although previous studies showed that ATP induced a large depolarization in rat cardiac myocytes (14), concentrations of ATP used in the present study did not induce detectable depolarization (0.1 mM) or only a slight depolarization of the membrane (0.5 mM).

Once membrane potentials were depolarized to a level more positive than E_{Cl}, which was approximately -33.9 mV when $[Cl^-]_i$ was at 40 mM as previously reported (39) or approximately -50 mV when Cl^- was at approximately 20 mM in the present study, for example, during action potential periods in stimulated muscle, it is possible that activation of $I_{Cl.ATP}$ would induce an outward current which will result in a Cl^- influx via the Cl^- channel. At this point, we have no evidence to show whether ATP induces any change in $[Cl^-]_i$ in stimulated muscles because methodological limitation prevented us to obtain successful impalements for recording $[Cl^-]_i$ in stimulated muscles. However, in our preliminary experiments, we observed effects of MgATP at concentrations from $0.01-1$ mM on action potentials in guinea pig ventricular muscle with a stimulus frequency at 1 Hz. Our results showed neither obvious depolarization of cells like in rat ventricular cells reported previously (14) nor detectable changes in action potentials (data not shown). This result implied that, even though during action potentials, ATP also did not activate $I_{Cl.ATP}$ in our experimental conditions. On the other hand, previous studies showed that only P_1-purinoceptors were present in guinea pig atria, and neither P_1 nor P_2 purinoceptors were present in guinea pig ventricles (42). Thus, activation of the Cl^--HCO_3^- exchange by extracellular ATP in the present study would not be via stimulation of P_1 or P_2 receptors as that in rat or mouse ventricular cells. It is possible that ATP may stimulate a putative P_3 receptor as described recently (21) to activate the Cl^--HCO_3^- exchange and results in an increase in $[Cl^-]_i$ and a transient intracellular acidosis. However, at this moment, we have no evidence to show whether the P_3 receptor is present in guinea pig ventricular muscle.

Many studies suggest that $[Cl^-]_i$ plays an important role in cardiac function during physiological and pathological conditions. In physiological conditions, $[Cl^-]_i$ is approximately 3 times higher than would be expected from a passive distribution at the resting membrane potential (10, 33, 34, 43–46). During ischemia and hypoxia, the $[Cl^-]_i$ was expected to increase (47) and our recent data clearly showed that $[Cl^-]_i$ increased during simulated ischemia made by paraffin oil-covering and perfusion-stopping in guinea pig ventricular muscles (23). Application of Cl^- blockers and substituting of extracellular Cl^- by NO_2 or gluconate can prevent ischemia and reperfusion-induced injury, including arrhythmias (48) or ischemia-induced acidosis (25). Thus, anion manipulation would alter membrane permeability and hence modulate the occurrence of arrhythmias. Since previous studies showed that extracellular ATP was released and increased by many fold in coronary vessels during ischemia (49), at a sufficient concentration to activate the Cl^--HCO_3^- exchange (21), the ATP would be a factor contributing to the increase in $[Cl^-]_i$ during simulated ischemia (23) and other pathological conditions.

The concentrations of ATP used in the present study to induce an increase in $[Cl^-]_i$ were higher than that measured

directly in coronary vessels during ischemia (49). It was also higher than that used in some studies of single myocytes (10 μM, 21), but similar to the concentration used in other single cells (100 μM, 20). One explanation for the difference in the concentration of ATP used in our present study and previous studies is that there is a difference in drug sensitivity for ATP between papillary muscles and isolated single cells. Another possibility is that the impalement sites may affect the concentration of ATP on the impaled cell in the present study. If the electrode is impaled relatively deep, the concentration of ATP may not reach the same concentration as indicated in perfusion solution. On the other hand, a previous report showed that extracellular ATP in the circulation was rapidly metabolized by ectoenzymes (3). If these ectoenzymes were also present in papillary muscle, but not in isolated single cells, it would account for the discrepancy between ATP-sensitivity in papillary muscles and single cells.

In conclusion, our studies showed that extracellular application of ATP and ADP increased the $[Cl^-]_i$ via activation of the Cl^--HCO_3^- exchanger in guinea pig ventricular muscles. This is the first description of the effects of extracellular ATP on intracellular chloride homeostasis using ion-selective microelectrode techniques. To know whether the ATP-induced increase in $[Cl^-]_i$ is important to trigger spontaneous action potentials during hypoxia and ischemia in cardiac muscles, further investigations need to be undertaken.

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