Effects of cyclic AMP on the function of the cardiac gap junction during hypoxia

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BACKGROUND: In the ischemic or hypoxic heart, an impairment of electrical cell-to-cell coupling and a dephosphorylation of the connexins that comprise the gap junction channel were observed. However, it remains to be elucidated whether the dephosphorylation of the connexin during hypoxia is due to alterations in the ionic strength of Ca^{2+} or H^+, and how the activation of protein kinase A (PKA) affects the hypoxia-induced abnormal function of the gap junction.

OBJECTIVES: The effects of hypoxia, intracellular Ca^{2+} overload and intracellular acidosis on the PKA-mediated phosphorylation of connexin 43 (Cx43) were examined in relation to the function of the cardiac gap junction.

METHODS: Hearts isolated from adult, male guinea pigs were used. The intercellular electrical cell-to-cell coupling was evaluated by the longitudinal internal resistance and the conduction velocity observed in in vitro experiments using isolated muscle strip preparations. The phosphorylation of Cx43 was evaluated by an immunoblot (Western blot). The localization of immunoreactive Cx43 at the intercalated disk was detected using confocal laser scan microscopy.

RESULTS: Cyclic AMP or the activation of PKA promotes the intercellular electrical coupling that accompanies an augmentation of the PKA-mediated phosphorylation of Cx43. Electrical cell-to-cell decoupling and reduction of the PKA-mediated phosphorylation of Cx43 were dependent on the progression of hypoxia. These results agree with those observed in the progression of intracellular Ca^{2+} overload or intracellular acidosis. Cyclic AMP or the activation of PKA alleviated the electrical cellular decoupling and the hypoxia-, intracellular Ca^{2+} overload- and intracellular acidosis-induced deteriorated expression of Cx43. These ameliorative effects of cyclic AMP on the function of the gap junction and on the expression of Cx43 weakened as the hypoxia progressed, and as the intracellular ionic strength of Ca^{2+} and H^+ increased.

CONCLUSIONS: In cardiac ventricular muscle cells, cyclic AMP or the activation of PKA promotes electrical cell-to-cell coupling through the gap junction due to an augmentation of the PKA-mediated phosphorylation of Cx43 in the early stage of hypoxia, as well as in normoxia. The suppression of PKA-mediated phosphorylation of Cx43 during hypoxia may be caused by an increase in the intracellular ionic strength of Ca^{2+} and H^+. Thus, the activation of cyclic AMP-dependent PKA may have an antiarrhythmic effect in the early stage of hypoxia.

Key Words: *8-Bromo-cyclic AMP; Cardiac gap junction; Cx43; Hypoxia; PKA-mediated phosphorylation*

In cardiac muscle, the gap junctions greatly contribute to electrical cell-to-cell coupling, which is known to play an important role in intercellular impulse conduction (1,2). Arrhythmogenic factors can impair intercellular conductivity. This physiological function of the gap junction depends on the opening and closing of the channels that comprise the gap junction. In turn, the opening and closing of the channels depends on the phosphorylation of the connexins that comprise the channels because the connexins are phosphoproteins.

It is generally accepted that cyclic AMP increases the electrical conductance of the gap junction and, thus, promotes electrical cell-to-cell coupling in cardiac cells (3,4). These events may result from an increased probability of the channels being open due to the protein kinase A (PKA)-mediated phosphorylation of the connexins (3,5), an augmentation of the synthesis of the connexin protein (6) or an increase in the number of channels with a higher conductance (7). On the other hand, Ca^{2+} (8-14) and H^+ (9,11,14) are known to play a major role in decreasing gap junction conductance and impairing electrical cell-to-cell coupling. A disturbance in the conductivity during hypoxia or ischemia is possibly caused by electrical cell-to-cell decoupling, because a progressive increase in intracellular Ca^{2+} overload and a progressive intracellular acidosis occur during hypoxia or ischemia (15-17). Previously, we reported on experiments on preparations isolated from adult, guinea pig heart, which showed that *D*-sotalol, which elevates the intracellular cyclic AMP level, prevented and restored hypoxia-induced electrical cell-to-cell decoupling (18). However, these ameliorative effects of cyclic AMP were not observed when hypoxia continued for a long time, specifically, for more than 1 h (19). Such evidence leads to the question of whether the phosphorylation of gap junction connexin is affected by the ionic strength of Ca^{2+} or H^+. On the other hand, the dephosphorylation of connexin has also been reported to be induced by ischemia (20,21). However, the relationship between Ca^{2+} or H^+ and the dephosphorylation of connexin,

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as well as the effects of the activation of PKA on the dephosphorylated connexin, still remain to be elucidated.

In the present study, the effects of intracellular Ca^{2+} overload and intracellular acidosis on the PKA-mediated phosphorylation of connexin 43 (Cx43) were examined on the ventricular muscle of adult guinea pig heart in reference to the functions of the gap junction.

Some parts of the present study were previously reported as abstracts from 1997 to 2003 (19,22-27) and were also reviewed in 2004 (28).

METHODS

Animals

Adult male guinea pigs weighing 385±15.5 g (mean ± SEM) were sacrificed by a blow to the head, and the experimental protocol was performed according to the method approved by the Institutional Animal Care and Use Committees of Fukuoka University (Fukuoka, Japan). The animals were heparinized by a intraperitoneal injection of heparin (NOVO Herparin, Novo Nordik A/S, Denmark) at a dose of 1000 U/kg 30 min before they were sacrificed.

Preparations and samples for experiments

Thin endocardial muscle strips (less than 1 mm in thickness, 10 mm to 15 mm in length and 2 mm to 3 mm in width) were isolated from the right ventricular wall after the removal of the heart from the animal, and were used to measure the conduction velocity and the longitudinal internal resistance (ri).

In another series of experiments, the hearts, which were excised quickly from the animal, were mounted on a Langendorff apparatus and were perfused with well-oxygenated standard Krebs solution (130 mM NaCl, 5.4 mM KCl, 11.9 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 5.5 mM glucose, and saturated with a mixture of gases [97% O_2 and 3% CO_2], pH adjusted to 7.4) at a constant pressure (60 mmHg). After 10 min to 15 min of stabilization, the hearts were perfused with the test solutions and Krebs solutions, including reagents. After treatment with either the test solutions or the reagents, ventricular tissue specimens were removed from the Langendorff apparatus and were subjected to Western blotting and histochemistry.

Test solutions

Hypoxic solution: To expose the heart muscle to hypoxia, Krebs solution without glucose, including $Na_2S_2O_4$ (0.5 mM), was perfused while being saturated by a mixture of gases (97% N_2 and 3% CO_2). Under these conditions, the partial pressure of O_2 (PO_2) in the solution was kept at less than 20 mmHg.

Intracellular Ca^{2+} overload: Intracellular Ca^{2+} overload was induced by a reduction of Na^+ in the external perfusing solution substituted with *N*-methyl-*D*-glucamine, while adjusting the pH to 7.4 with HCl. The intracelluar Ca^{2+} level was measured by Fura-2AM (Dojndo, Japan) on ventricular myocytes isolated by collagenase (Figure 1).

Intracellular acidosis: Intracellular acidosis was induced by an increase in the pressure of CO_2. The perfusing solution had a pH of 7.4 (control [normal]), 7.1 to 6.9 (90% O_2 and 10% CO_2, with a PO_2 of 550 mmHg) or 6.1 to 5.9 (50% O_2 and 50% CO_2, with a PO_2 of 400 mmHg), as determined by an alteration in the proportion of O_2 and CO_2, and was checked by a pH meter (i-STAT Portable Clinical Analyser, i-STAT Corperation, USA). Because intracellular acidosis possibly promotes the inflow of Ca^{2+} into the cell through the reverse mode of Na^+-Ca^{2+} exchange following Na^+-H^+ exchange, external Ca^{2+} was excluded in the present study.

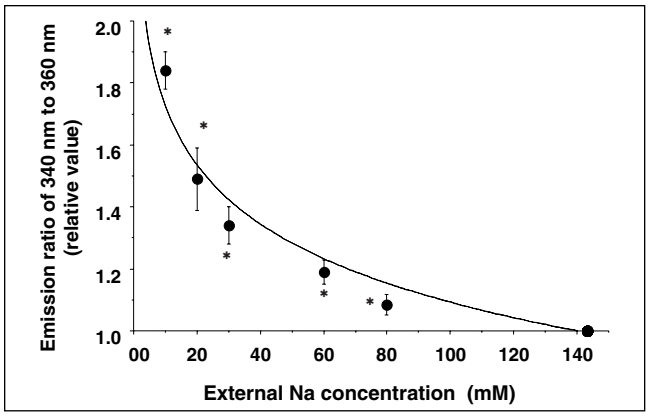

Figure 1) *The intracellular Ca^{2+} level at various external Na^+ concentrations ranging from 10 mM to 143.1 mM (standard) was measured with Fura-2AM (Dojndo, Japan) on ventricular myocytes isolated by collagenase. Each plot and vertical bar shows the relative value of the mean ± SEM. A relative value of 1.0 represents the control value at the standard Na^+ concentration. Based on the experimental results, the intracellular Ca^{2+} concentration was calculated to be 1.04±0.06 μM at an Na^+ concentration of 20 mM. *P<0.001 versus the value at a standard Na^+ concentration*

An evaluation of electrical cell-to-cell coupling

Measurement of the conduction velocity: Preparations of muscle were fixed in a three-compartment chamber separated by thin silicon membranes, which were superfused with well-oxygenated standard Krebs solution at a constant flow (5 mL/min) and temperature (37°C). The middle part of the preparation was 2 mm in distance and 0.1 mL in volume. The preparations were electrically stimulated with a suction electrode at 0.5 Hz on one of two sides. The transmembrane action potential was recorded at each part with a conventional glass microelectrode being kept at a constant distance. The middle part of the muscle was superfused with hypoxic solutions or solutions with reagents. The changes in the conduction velocity at the middle of the muscle were estimated by the distance of the middle part divided by the time difference between the two action potentials from both side parts. At the middle part, the maximum rate of depolarization of the action potential (Vmax) was measured.

Measurement of the ri: The method for measuring ri was based on the principle described by Tuganowski et al (29). The preparations were fixed in a three-compartment chamber separated by thin silicon membranes, which were superfused with well-oxygenated standard Krebs solution at a constant flow (5 mL/min) and temperature (37°C). The middle part of the preparation was 0.8 mm in distance and 0.1 mL in volume. The middle part of the muscle was superfused with reagents and test solutions. The transmembrane action potential was recorded at one of two sides with a conventional glass microelectrode, and the extracellular action potential was recorded with suction electrodes placed on both sides of the preparation. The preparation was electrically stimulated with a suction electrode at 0.5 Hz on one of the two sides. The shunt resistance in the electrical circuit was 50 kΩ. The ri was determined as follows:

$$ri = rs \times Eo\ (1/E2 - 1/E1),$$

where rs is the shunt resistance in the electrical circuit, Eo is the amplitude of the transmembrane action potential, and E1 and E2 are the amplitude of the extracellular action potentials before and after shunting with rs, respectively. A remarkable increase in ri by heptanol, which closes the gap junction channel, and a complete

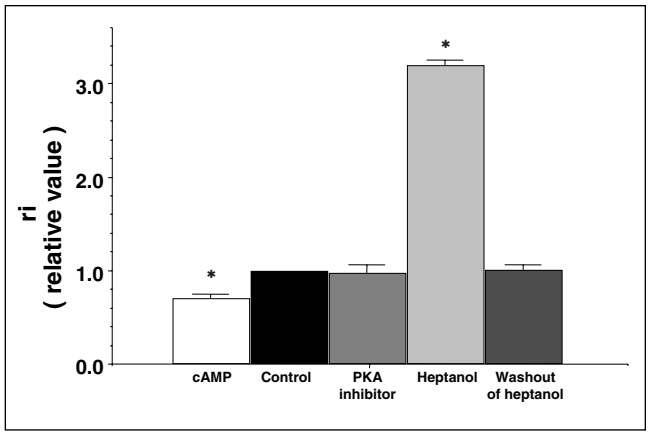

Figure 2) *Effects of cyclic AMP (cAMP), protein kinase A (PKA) inhibitor with cAMP (represented as PKA inhibitor on graph), and heptanol on the longitudinal internal resistance (ri), which is shown as a relative value. A relative value of 1.0 represents the control mean value before treatment of reagents. cAMP (1 μM) and PKA inhibitor (10 μM) were applied for 30 min. Heptanol (1 mM) was applied for 1 min. Each bar corresponds to the mean ± SEM. *P<0.001 versus the control value. Reproduced from reference 38*

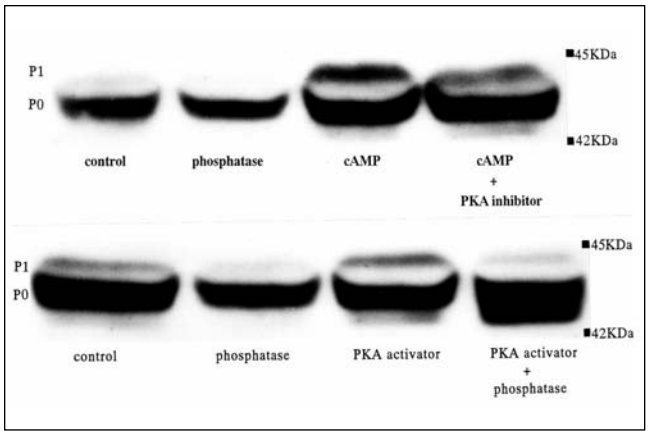

Figure 3) *Representative Western blot findings for connexin 43 showing the effects of cyclic AMP (cAMP), protein kinase A (PKA) activator, cAMP with PKA inhibitor, alkaline phosphatase and PKA activator with phosphatase in normoxia. The control was not treated by any reagents. 8-Bromo-cyclic AMP (1 μM), PKA activator (1 μM) and PKA inhibitor (10 μM) were applied for 60 min on Langendorff perfusion. Alkaline phosphatase (20 U/mL) was treated at the first step of the homogenizing procedure (see Methods). P0 Nonphosphorylated connexin; P1 Phosphorylated connexin*

reversibility by washout of heptanol were demonstrated using this method (Figure 2).

Immunoblot and an evaluation of the phosphoryation of Cx43

Ventricular samples were frozen quickly in liquid nitrogen. Tissue samples were homogenized in Tris buffer (10 mM Tris-HCl and 150 mM NaCl, with a pH of 7.4) that included phenylmethanesulfonyl fluoride at a final concentration of 1 mM on ice and were centrifuged at 200 *g* (1200 rpm) for 15 min at 4°C. The supernatants were mixed with 10% Triton X-100 (Nacalai Tesque, Japan) and were centrifuged again at 100,000 *g* (30,000 rpm) for 30 min and, subsequently, the pellets were subjected to a Western blot analysis. The pellets were solubilized with Laemmli sample dilution buffer (4% sodium dodecyl sulphate [SDS], 125 mM Tris-HCl [pH of 6.8] and 20% glycerol). The protein concentration was measured by a bicinchoninic acid protein assay kit (Pierce, USA). The samples were boiled for 3 min with 2% beta-mercaptoethanol, and 25 μg of total protein was run on a 10% SDS polyacrylamide gel. The separated protein was then electrophoretically transferred to a polyvinylidene fluoride membrane (GE Healthcare Ltd, United Kingdom) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). After the membranes were blocked with 5% skimmed milk in phosphate buffered saline containing 0.1% Tween (Wako, Japan), they were incubated with primary antibody (specific mouse monoclonal anti-Cx43 antibodies, Chemicon International Inc, USA) at a dilution of 1:4000 for 1 h at room temperature. Secondary antibody (antimouse immunoglobulin, GE Healthcare Ltd) was used at a dilution of 1:5000. Cx43 protein-antibody complexes were detected by using chemiluminescence Western blotting detection reagents (ECL Plus [RPN2124], GE Healthcare Ltd), and they were then exposed to chemiluminescence film (Hyperfilm, GE Healthcare Ltd) at room temperature for 5 s to 20 s. The molecular weight was calibrated using a Low Molecular Weight Calibration Kit for SDS electrophoresis (GE Healthcare Ltd).

For the treatment of samples with alkaline phosphatase (from bovine intestinal mucosa, Sigma-Aldrich, USA), the phosphatase was added in the first step of homogenization at a dose of 20 U/mL.

Two isoforms were observed near 43 kDa (Figure 3). The mean density of the higher molecular weight isoform was remarkably reduced by treatment with alkaline phosphatase, and this isoform was determined to be a phosphorylated connexin protein (P1). The lower molecular weight isoform was not affected by alkaline phosphatase and was thought to be the nonphosphorylated connexin (P0). The density ratio of P1 to P0 (P1/P0) was evaluated as the magnitude of the phosphorylation of the connexin.

Immunohistochemistry of Cx43

For the immunohistochemistry of Cx43, the rabbit polyclonal anti-Cx43 antibody (Zymed Lab Inc, USA) was used at dilutions of 1:200 and the goat antirabbit immunoglobulin G (Alexa Fluor 488, Invitrogen, USA) was used at dilutions of 1:200 as the secondary antibody. Immunofluorescence was detected by a confocal laser scan microscopy (LSM-410, Carl Zeiss Inc, Germany).

Densitometry

The mean density of the Cx43 complex isoforms in the immunoblots and the mean fluorescent intensity of immunoreactive particles for Cx43 on the confocal laser scan micrographs were analyzed by NIH Image Software (National Institutes of Health, USA).

Chemicals and reagents

8-Bromo-cyclic AMP (8-bromo-adenosine 3′,5′-cyclic monophosphate sodium salt, Sigma-Aldrich) (used as a cyclic AMP analogue [cyclic AMP mentioned in the figures and the text represents 8-bromo-cyclic AMP]), PKA activator (Sp-adenosine 3′,5′-cyclic monophosphothioate triethylamine salt, Sigma-Aldrich) and PKA inhibitor (Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine salt, Sigma-Aldrich) were used.

Statistical analysis

The data are expressed as means ± SEM. The unpaired Student's *t* test was used to analyze statistical significance (P<0.001) between means.

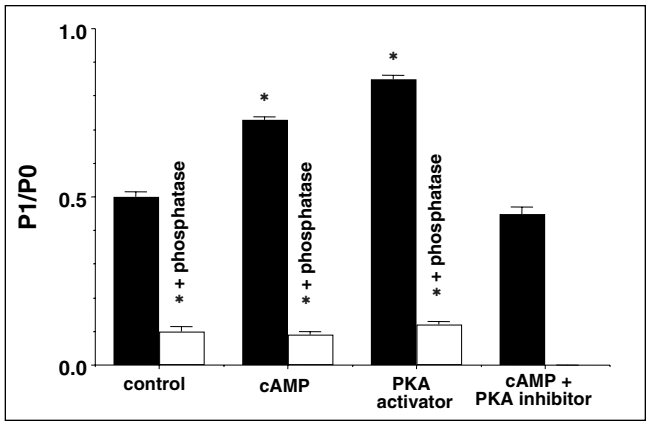

Figure 4) *An analysis of phosphorylated connexin (P1)/nonphosphorylated connexin (P0) from some examples in the same experiment as Figure 3. Shown on the y-axis is the value of the P1/P0 ratio. The control was not treated with any reagents. The white columns represent the value after treatment with alkaline phosphatase. Each bar corresponds to the mean ± SEM. *P<0.001 versus the control value. cAMP Cyclic AMP; PKA Protein kinase A*

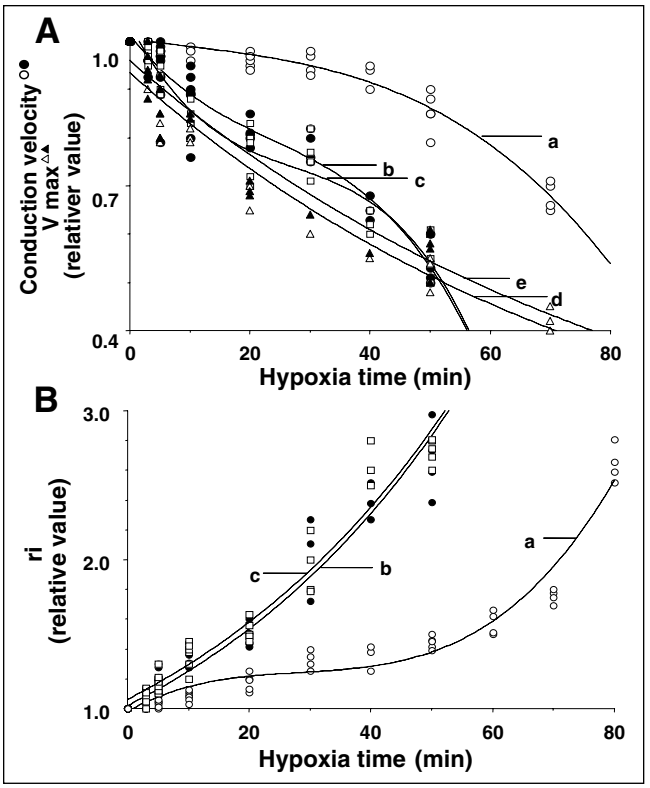

Figure 5) *Time course of changes in the conduction velocity (**A**: **a**, **b** and **c**), the maximum rate of depolarization (Vmax) (**A**: **d** and **e**) and longitudinal internal resistance (ri) (**B**: **a**, **b** and **c**) in hypoxia. Each plot indicates the relative value. A relative value of 1.0 represents the value before hypoxia. Closed circles, open circles and open squares represent values in the absence of cyclic AMP, in the presence of cyclic AMP and in the presence of cyclic AMP with PKA inhibitor, respectively (**A** and **B**). Closed and open triangles represent Vmax in the absence and presence of cyclic AMP, respectively (**A**). The coefficients of correlation are r=0.974 (**A**: **a**), r=0.953 (**A**: **b**), r=0.964 (**A**: **c**), r=0.956 (**A**: **d**), r=0.973 (**A**: **e**), r=0.993 (**B**: **a**), r=0.990 (**B**: **b**) and r=0.978 (**B**: **c**)*

RESULTS

The effects of cyclic AMP on the ri and the phosphorylation of Cx43 in normoxia

In normoxia, cyclic AMP (1 μM) decreased the ri by approximately 30% of the control within 30 min. This effect of cyclic AMP was inhibited or abolished by PKA inhibitor (Figure 2).

The Western blot findings showed that the density of the higher molecular weight isoform of Cx43 (P1) was augmented by cyclic AMP or PKA activator, and it was inhibited by alkaline phosphatase and PKA inhibitor (Figure 3). The P1/P0 ratio was augmented by cyclic AMP and PKA activator, and suppressed by PKA inhibitor and alkaline phosphatase (Figure 4).

The effects of cyclic AMP on the conduction velocity and the ri in hypoxia

The conduction velocity decreased depending on the exposure time to hypoxia. Within 60 min, the conduction velocity decreased to approximately 40% of the control (before hypoxia), and thus, the conductivity was decreased. In the presence of cyclic AMP (1 μM), the changes in the conduction velocity were alleviated and conductivity was maintained for approximately 70 min to 80 min (Figure 5A). Vmax also decreased in a time-dependent manner during hypoxia. In the presence of cyclic AMP, the Vmax did not recover and, instead, was observed to decrease (Figure 5A).

The ri increased depending on the exposure time to hypoxia. Within 60 min, the ri increased to approximately 400% of the control (before hypoxia). In the presence of cyclic AMP (1 μM), a rise in the ri was alleviated during approximately 1 h of hypoxia. However, approximately 90 min later in hypoxia, the ri increased to 400% of the control, even in the presence of cyclic AMP (Figure 5B).

The effects of cyclic AMP on the phosphorylation of Cx43 during hypoxia

A Western blot and densitometry analysis of the immunoblot showed that PKA-mediated phosphorylation decreased as the hypoxia advanced (Figures 6A and 6B). The P1/P0 ratio decreased in a time-dependent manner after exposure to hypoxia (Figure 6B). In the presence of cyclic AMP, the reduction in the P1/P0 ratio was mitigated during approximately 1 h of hypoxia (Figure 6B). The restorative effects of cyclic AMP were not observed at more than 90 min of hypoxia (Figures 6A and 6B). The effects of PKA activation were the same as those of cyclic AMP (not shown in Figure).

The effects of cyclic AMP on the expression of Cx43 at the intercalated disk in normoxia and hypoxia

The effects of cyclic AMP on the expression of Cx43 at the intercalated disk in normoxia and hypoxia are shown on the confocal laser scan micrographs of the immunofluorescence of Cx43 using the polyclonal anti-Cx43 antibody in Figure 7, and analysis of the immunoreactive area at the intercalated disk is shown in Figure 8. At 30 min of hypoxia, the immunoreactivity of the phosphorylated Cx43 was scant and inhomogenous (Figure 7C), and the immunoreactive area was reduced (Figure 8). Cyclic AMP (1 μM) augmented the expression of the immunoreactivity of the phosphorylated Cx43 and the immunoreactive area at the intercalated disk in normoxia, as well as in hypoxia of 30 min (Figures 7B, 7D and 8). Recovery of the expression of Cx43 by cyclic AMP was not observed after more than 90 min of hypoxia (not shown in Figure).

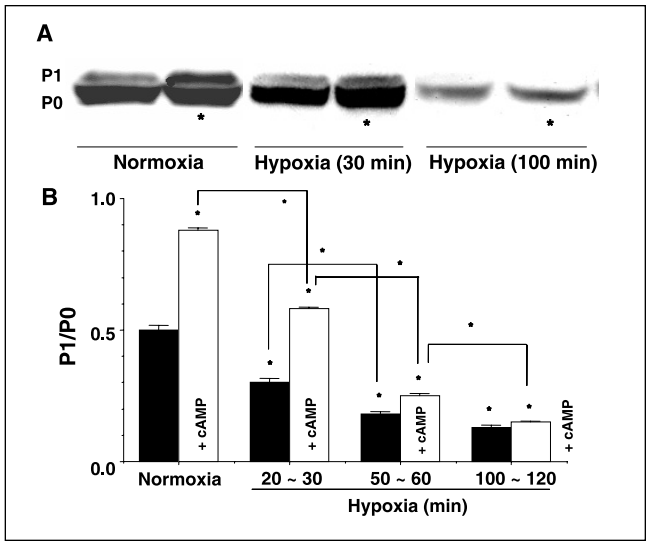

Figure 6) A *Western blot findings for connexin 43 showing the effects of cyclic AMP (cAMP) in normoxia, and at 30 min and 100 min of hypoxia. *The presence of cAMP.* **B** *Analysis of phosphorylated connexin (P1)/nonphosphorylated connexin (P0) from some examples from the same experiment as* **A**. *The y-axis shows the value of the ratio of P1/P0. White columns represent the value in the presence of cAMP (1 μM). Each bar corresponds to the mean ± SEM. *P<0.001 versus the control, and difference between indicated lines*

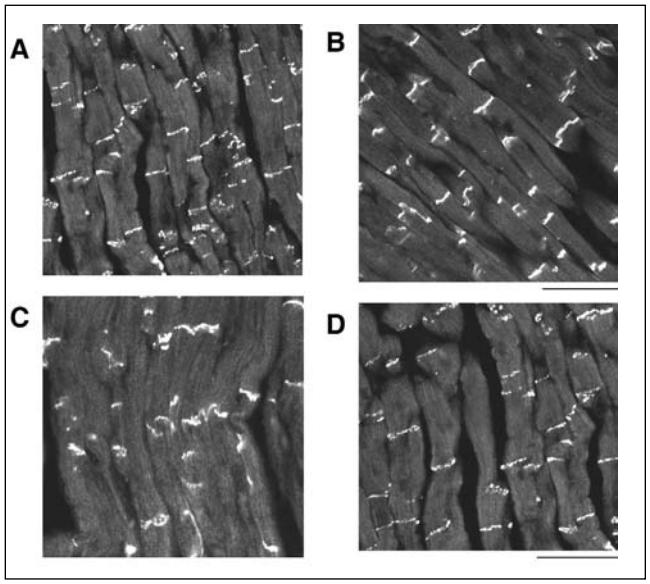

Figure 7) *Confocal laser scan micrographs of the immunofluorescence of connexin 43.* **A** *Normal as control at 30 min normoxia;* **B** *At 30 min after the application of cyclic AMP (1 μM) in normoxia;* **C** *At 30 min of hypoxia;* **D** *At 30 min of hypoxia in the presence of cyclic AMP (1 μM). Scale: 100 μm*

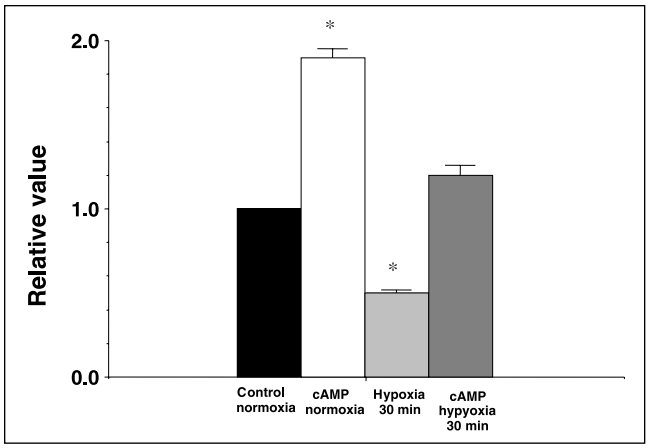

Figure 8) *An analysis of the immunofluorescence findings of connexin 43. A comparison of the area of the immunoreactive particles at the intercalated disk between the control in normoxia, cyclic AMP (cAMP) in normoxia (30 min after application of cAMP [1 μM]), at 30 min of hypoxia and at 30 min of hypoxia in the presence of cAMP (1 μM). The columns represent the relative values (1.0 represents the value of the control in normoxia). Each vertical bar corresponds to the mean ± SEM. *P<0.001 versus the control in normoxia*

The effects of cyclic AMP on the ri in intracellular Ca^{2+} overload

Intracellular Ca^{2+} overload was induced by a reduction in the external Na^+ concentration (see Methods). The ri increased as the external Na^+ concentration was reduced from the normal standard concentration of 143.1 mM to lower concentrations of 78.1 mM and 13.1 mM (Figure 9A). In the presence of cyclic AMP at an Na^+ concentration of 78.1 mM, the increase in the ri was alleviated. The effects of cyclic AMP were abolished by PKA inhibitor (10 μM) (Figure 9A). However, the ameliorative effects of cyclic AMP on the raised ri were no longer observed at an Na^+ concentration of 13.1 mM (Figure 9A).

The effects of cyclic AMP on the phosphorylation of Cx43 in intracellular Ca^{2+} overload

Tissue samples for the Western blot analysis were extracted 60 min after Langendorff perfusion of the low Na^+ concentration solution and the test solutions, including cyclic AMP (1 μM). The P1/P0 ratio decreased as the external Na^+ concentration was reduced (Figures 10A and 11A). Cyclic AMP (1 μM) augmented the P1/P0 ratio at Na^+ concentrations of 143.1 mM and 78.1 mM, but not at 13.1 mM (Figure 10A and 11A).

The effects of cyclic AMP on the ri in intracellular acidosis

The ri increased as the external pH was decreased from the standard concentration (7.4) to lower concentrations (7.1 to 6.9 and 6.1 to 5.9) (Figure 9B). The increase in the ri was alleviated by cyclic AMP (1 μM) at a pH range of 7.1 to 6.9. The effects of cyclic AMP were abolished by PKA inhibitor (10 μM). The restorative effects of cyclic AMP on the raised ri were no longer observed at a pH range of 6.1 to 5.9 (Figure 9B).

The effects of cyclic AMP on the phosphorylation of Cx43 in intracellular acidosis

Tissue samples for a Western blot analysis were extracted 60 min after Langendroff perfusion of the low pH solutions and the test solutions, including cyclic AMP (1 μM) (Figures 10B and 11B). The P1/P0 ratio was compared between each pH level in several examples (Figure 11B). The P1/P0 ratio decreased as the pH was decreased. Cyclic AMP augmented the P1/P0 ratio at the normal standard pH of 7.4 and at the pH range of 7.1 to 6.9, but not at the range of 6.1 to 5.9 (Figures 10B and 11B).

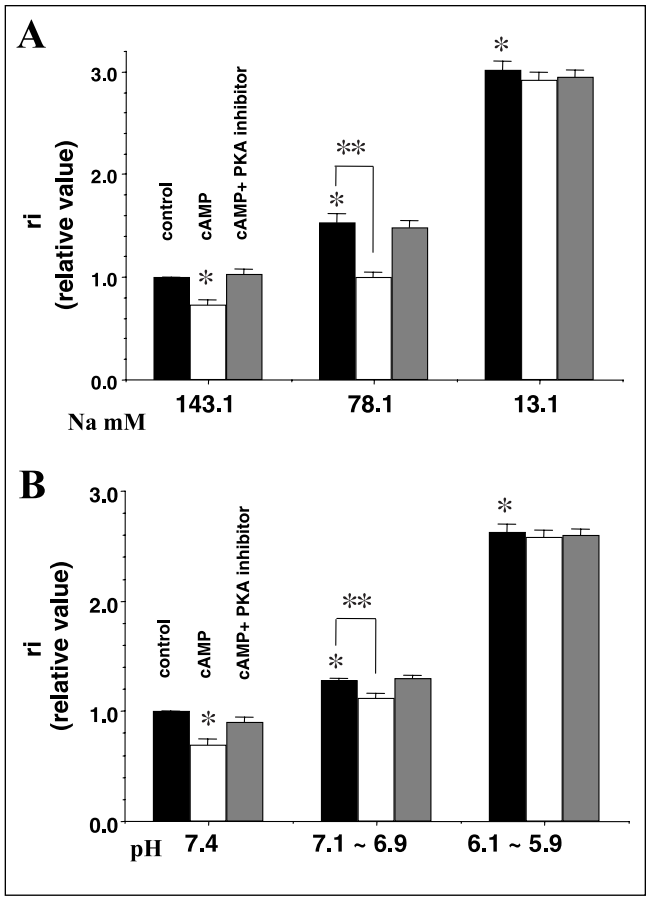

Figure 9) *The effects of cyclic AMP (cAMP) with protein kinase A (PKA) inhibitor on the longitudinal internal resistance (ri) at low concentrations of external Na^+ (**A**) and low pH (**B**). The external Na^+ concentrations varied from standard (143.1 mM) to low concentrations (78.1 mM and 13.1 mM) and pH varied from a standard pH (7.4) to low pH levels (7.1 to 6.9 and 6.1 to 5.9). The test solutions were applied for 60 min on Langendorff perfusion. Concentrations of cAMP and PKA inhibitor were 1 μM and 10 μM, respectively. The y-axis shows the relative value of ri. A relative value of 1.0 corresponds to the value at the standard Na^+ concentration and standard pH without any reagents. The black, white and grey columns represent the control (before treatment of reagents), the presence of cAMP and the presence of cAMP with PKA inhibitor, respectively. Each bar corresponds to the mean ± SEM. *P<0.001 versus the control; **P<0.001. Reproduced from reference 38*

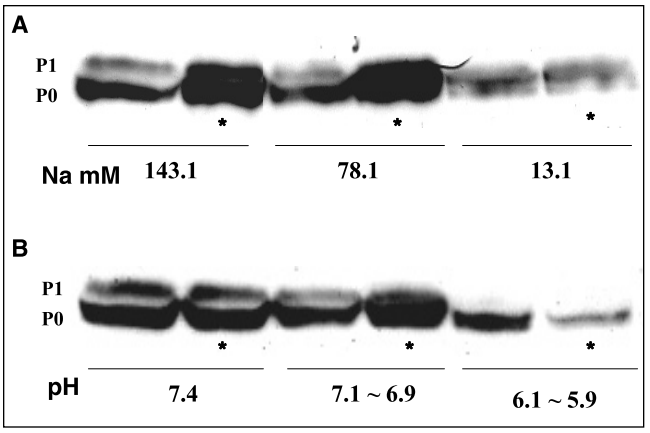

Figure 10) *Representative Western blot findings for connexin 43 showing the effects of cyclic AMP at low concentrations of external Na^+ (**A**) and low pH (**B**). The external Na^+ concentrations varied from standard (143.1 mM) to low concentrations (78.1 mM and 13.1 mM) and the pH varied from a standard pH (7.4) to lower pH levels (7.1 to 6.9 and 6.1 to 5.9). The test solutions were applied for 60 min on Langendorff perfusion. The concentration of cyclic AMP used was 1 μM. *The presence of cyclic AMP. P0 Nonphosphorylated connexin; P1 Phosphorylated connexin*

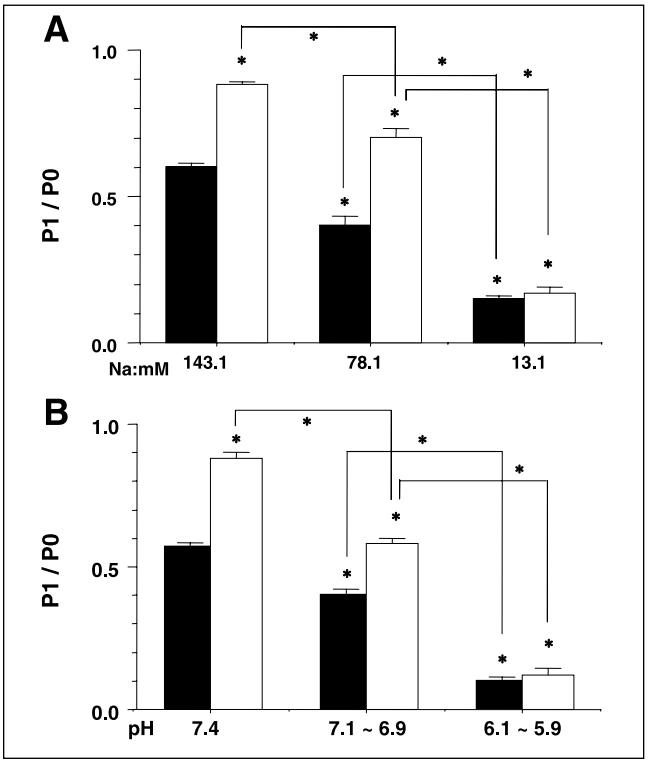

Figure 11) *An analysis of phosphorylated connexin (P1)/nonphosphorylated connexin (P0) from some examples in the same experiment as Figure 10. The y-axis shows the P1/P0 ratio and x-axis shows the external Na^+ concentration (**A**) or the pH (**B**). The white columns represent the value in the presence of cyclic AMP. Each bar corresponds to the mean ± SEM. *P<0.001 versus the control, and difference between indicated lines*

DISCUSSION

In the present study, we examined the influence of cyclic AMP or the activation of PKA on impulse conductivity in normoxia and hypoxia, especially in reference to the function of the gap junction.

Two isoforms of Cx43 were distinctly detected according to the Western blot findings (Figure 3). As described in the Methods section, the ratio of the mean density of the higher molecular weight isoform (P1) to the lower molecular weight isoform (P0), the P1/P0 ratio, was available to evaluate the phosphorylation of Cx43. We used monoclonal anti-Cx43 antibody. It has been reported that in rat heart, the phosphorylated isoform of Cx43 can be detected by polyclonal anti-Cx43 antibody, but not by monoclonal anti-Cx43 antibody (20). In another report (30), it was mentioned that some phosphorylated isoforms of Cx43 near 41 kDa can be detected with the monoclonal antibody. We did not observe a definite difference in the P1/P0 ratio between the monoclonal and polyclonal anti-Cx43 antibodies (data not shown). Thus, it was concluded that monoclonal anti-Cx43 antibody can be used for the

detection of the phosphorylated isoform of Cx43 in the guinea pig heart. P1 was augmented by cyclic AMP or PKA activator and suppressed by PKA inhibitor. As a result, P1 was determined to be an isoform of Cx43 that had undergone PKA-mediated phosphorylation. The P1/P0 ratio can be evaluated as the magnitude of the PKA-mediated phosphorylation of Cx43.

It is generally accepted that cyclic AMP increases the macroscopic electrical conductance of the cardiac gap junction due to the activation of PKA (3-5). The present study showed that cyclic AMP decreased the ri (specifically, it promoted electrical cell-to-cell coupling), and the effects of cyclic AMP were inhibited by the PKA inhibitor. Therefore, it is probable that the action of cyclic AMP on the gap junction is mediated by the activation of PKA. There have been observations that cyclic AMP or the activation of PKA can increase the quantity of Cx43 (6,25-27), increase the area of immunoreactive particles for Cx43 at the intercalated disk (Figures 7B and 8) (25-27) and enhance the gap junction assembly (31). These results may indicate that cyclic AMP or the activation of PKA can increase the number of gap junction channels.

In the present study, it was shown by Western blot analysis that cyclic AMP or PKA activator augmented phosphorylation of Cx43, and this effect was suppressed by PKA inhibitor. Such evidence suggests that the promotive effect of cyclic AMP on the gap junction function is attributed to an augmentation of the PKA-mediated phosphorylation of Cx43.

Thus, it is suggested that an augmentation of the PKA-mediated phosphorylation of Cx43 upregulates the function of the gap junction through an increase in the probability of the gap junction channel being open (3,5), a delay in the proteolytic degradation of the connexin or acceleration of the synthesis of the connexin protein (6).

A deterioration in the conduction velocity and the ri during hypoxia was alleviated by cyclic AMP. The effects of cyclic AMP were inhibited by PKA inhibitor. As a result, it is probable that the effects of cyclic AMP were caused by the activation of PKA. The Vmax tended to decrease in the presence of cyclic AMP during hypoxia. This can be explained by evidence that Vmax was suppressed by the activation of beta-adrenergic receptors in the depolarized membrane (32). Therefore, our findings suggest that the effects of cyclic AMP are caused by an amelioration of the passive property of the membrane, namely, the gap junction. The immunohistochemistry results (Figure 7D) support this idea. However, our results also showed that the ameliorative effects of cyclic AMP on gap junction function were transiently exhibited at the early stage, within 60 min of hypoxia, and later on, they were modified by the ionic strength of Ca^{2+} and H^+. This theory is supported by the fact that intracellular Ca^{2+} overload or acidosis is induced by severe hypoxia (15-17).

In hypoxic cardiac muscle, abnormalities of the intercellular impulse conductivity are often observed. When cardiac muscle is exposed to hypoxia, intracellular Ca^{2+} overload and intracellular acidosis are induced (15-17). Because Ca^{2+} and H^+ are major factors that decrease gap junction conductance (8-14), it is possible to conclude that the hypoxia-induced, cell-to-cell decoupling is explainable by the influence of the intracellularly elevated Ca^{2+} or H^+, or both, on the gap junction.

In the present study, the electrical cell-to-cell coupling was shown to be impaired by intracellular Ca^{2+} overload induced by a reduction in the external Na^+ concentration. When intracellular Ca^{2+} overload was moderate at an external Na^+ concentration of 78.1 mM, the Ca^{2+}-induced cell-to-cell decoupling was recovered by cyclic AMP, and the ameliorative effect of cyclic AMP was suppressed by PKA inhibitor. However, when the Ca^{2+} overload was extremely high, such as that achieved at an external Na^+ concentration of 13.1 mM, the ameliorative effect of cyclic AMP was no longer observed. The phosphorylation of Cx43 by PKA was slightly impaired at an external Na^+ concentration of 78.1 mM and remarkably impaired at 13.1 mM. During intracellular Ca^{2+} overload, the effects of cyclic AMP on electrical cell coupling was consistent with those found with cyclic AMP on the phosphorylation of Cx43. These findings suggest that the PKA-mediated phosphorylation of Cx43 is affected by the ionic strength of Ca^{2+}; specifically, it is suppressed by a higher ionic strength of Ca^{2+}.

Intracellular acidosis (pH) is equivalent to extracellular acidosis induced by an elevation in the partial pressure of CO_2 (33,34). Intracellular acidosis possibly promotes an inflow of Ca^{2+} into the cell through activation of the reverse mode of Na^+-Ca^{2+} exchange following Na^+-H^+ exchange. In our model, the effects of intracellular acidosis were found to depend only on the ionic strength of H^+, because external Ca^{2+} was removed. The increase in the ri at a pH range of 7.1 to 6.9 was alleviated by cyclic AMP, but not at a pH range of 6.1 to 5.9. The phosphorylation of Cx43 by PKA was suppressed depending on the magnitude of acidity. Cyclic AMP augmented the phosphorylation at a pH range of 7.1 to 6.9, but not at the pH range of 6.1 to 5.9. The effect of cyclic AMP on intercellular electrical coupling is consistent with that found on the phosphorylation of Cx43. Such evidence suggests that the PKA-mediated phosphorylation of Cx43 is affected by the ionic strength of H^+. In our experiments, the ameliorative effects of cyclic AMP on the electrical decoupling and the dephosphorylation of Cx43 were not observed at a pH below 6.5.

As mentioned above, the PKA-mediated phosphorylation of Cx43 decreased during hypoxia; specifically, it was possibly dephosphorylated by Ca^{2+} or H^+, or both. The activation of protein phosphatase type 1 may mediate the dephosphorylation of Cx43 (21).

It is frequently mentioned that cyclic AMP or the activation of beta-adrenergic receptors is arrhythmogenic due to the promotion of the Ca^{2+} channels in the active membrane (35,36). On the other hand, it is generally accepted that cyclic AMP promotes intercellular electrical coupling (3-7). As shown in the present study, cyclic AMP prevents disturbances in impulse conductivity during hypoxia due to an augmentation of the PKA-mediated phosphorylation of Cx43. It is understandable that the gap junctional uncoupling is an arrhythmogenic factor in acute ischemia (37). As a result, it is probable that cyclic AMP has an antiarrhythmic effect due to the maintenance or promotion of the gap junction function. However, this antiarrhythmic effect of cyclic AMP may be exhibited at an early stage of hypoxia.

CONCLUSIONS

Cyclic AMP promotes intercellular electrical coupling and gap junctional communication via an augmentation of the PKA-mediated phosphorylation of Cx43. The reduction in the PKA-mediated phosphorylation of Cx43 that accompanies deterioration of the intercellular electrical coupling increased as hypoxia advanced. The same results were also observed with a progressive increase in the intracellular ionic strength of Ca^{2+} and H^+. The activation of cyclic AMP-dependent PKA had protective or ameliorative effects on the hypoxia-,

intracellular Ca^{2+} overload- and intracellular acidosis-induced dephosphorylation of Cx43, as well as on alterations in the function of the gap junction. However, these protective or ameliorative effects were not exhibited in the later stage of hypoxia and in the extreme increase in the ionic strength of Ca^{2+} or H^+. Thus, further studies are required to elucidate the mechanisms of the dephosphorylation of the PKA-mediated phosphorylation of Cx43 induced by Ca^{2+} and H^+.

Regarding the pathophysiological significance of these findings, it is suggested that in the early stage of hypoxia, an increase in sympathetic activity or an activation of cyclic AMP-dependent PKA has antiarrhythmogenic effects due to the prevention or amelioration of gap junctional decoupling, whereas in the later stage of hypoxia, it has an arrhythmogenic effect due to promotive effects on Ca^{2+} channel activity rather than due to protective effects on the gap junction.

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