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Involvement of anion exchange in the hypoxia/reoxygenation-induced changes in pH_i and $[Ca^{2+}]_i$ in cardiac myocyte

Hiroshi Kawasaki [a,b], Hitomi Otani [a], Katsuyuki Mishima [a], Hiroji Imamura [b], Chiyoko Inagaki [a,*]

[a] *Department of Pharmacology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan*
[b] *Department of Thoracic and Cardiovascular Surgery, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan*

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Abstract

The involvement of Cl^-/HCO_3^- exchange in hypoxia/reoxygenation-induced changes in pH_i and Ca^{2+} concentration ($[Ca^{2+}]_i$) was examined in rat ventricular myocytes. During 10-min hypoxia, the initial pH_i (7.21 ± 0.04) fell to below 6.8. Subsequent reperfusion with reoxygenated buffer returned this acidic pH_i to the neutral range with increases in $[Ca^{2+}]_i$. These responses were reduced by the removal of Cl^- or HCO_3^- and by the addition of anion exchange inhibitors, SITS (4-acetamido-4′isothiocyanato-stilbene-2,2′disulfonic acid) and DIDS (4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid), while inhibitors for the Cl^- channel and $Na^+/K^+/2Cl^-$ cotransport were without effects. The hypoxia-induced acidification was attenuated by protein kinase C inhibitors, calphostin C and chelerythrine, but not by a protein kinase A inhibitor, KT5720. Under normoxic condition, protein kinase C activation induced a SITS-sensitive acidification. Furthermore, in electrically driven rat papillary muscle, SITS and DIDS improved the recovery of developed tension during the reoxygenation. These results suggest that the hypoxia-induced decrease in pH_i is mediated at least in part by anion exchange stimulation through protein kinase C activation, and this exchange takes part in the reoxygenation-induced Ca^{2+} overload as well as contractile dysfunction.

Keywords: Cardiomyocyte; Hypoxia/reoxygenation; Anion (Cl^-/HCO_3^-) exchange; pH, intracellular; Ca^{2+}, intracellular

1. Introduction

Intracellular pH (pH_i), an important modulator of cardiac contraction, is regulated by three well-characterized ion transport systems, i.e., the Na^+/H^+ exchange, Na^+/HCO_3^- symport, both of which induce an intracellular alkalinization, and the Na^+-independent Cl^-/HCO_3^- exchange acts as an acidifying mechanism through HCO_3^- extrusion (Dart and Vaughan-Jones, 1992; Fliegel and Fröhlich, 1993; Vaughan-Jones, 1986).

In ischemia/reperfused heart, a large drop in pH_i during ischemia has been known to be recovered through Na^+/H^+ exchange, and stimulation of this exchange activity secondarily activates the Na^+/Ca^{2+} exchanger, leading to intracellular Ca^{2+} ($[Ca^{2+}]_i$) overload which then causes an irreversible contractile dysfunction (Tani and Neely, 1989).

Besides cation exchange, anion-related mechanisms may also be involved in the cardiac ischemia/reperfusion-induced ionic imbalance. Our preliminary study showed that the intracellular acidification during hypoxia was less extensive in Cl^- free hypoxic buffer than in Cl^- containing solution (Kawasaki et al., 1998). Moreover, SITS, which blocks the Cl^-/HCO_3^- exchanger and anion channels, reportedly suppresses ischemia-induced acidification in guinea pig ventricular papillary muscle (Lai et al., 1996; Lai and Nishi, 1998) and reperfusion-induced arrhythmia in perfused rat heart (Ridley and Curtis, 1992). Therefore, we hypothesized that Cl^-/HCO_3^- exchange contributes to reperfusion-induced Ca^{2+} overload through the modification of acidification during hypoxia.

In the present study, we confirmed the involvement of anion exchanger in hypoxia-induced acidification and examined the role of this exchanger on hypoxia/reoxygena-

* Corresponding author. Tel.: +81-6-6993-9427; fax: +81-6-6992-2940.
E-mail address: inagaki@takii.kmu.ac.jp (C. Inagaki).

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tion-induced changes in $[Ca^{2+}]_i$ using rat single ventricular myocytes loaded with fluorescent pH and Ca^{2+} indicators. Further, we examined the protective effect of its blockade on the contractile dysfunction using rat ventricular papillary muscle.

2. Materials and methods

2.1. Preparation of isolated cardiomyocytes

All animals were handled in accordance with "Rules of the Animal Experimentation Committee, Kansai Medical University".

Ventricular myocytes were isolated from adult male Sprague–Dawley rats (250–300 g) anesthetized with pentobarbital (2 mg/kg) and ketamine (10 mg/kg). Hearts were quickly removed and Langendorff-perfused for 10 min with oxygenized normal Tyrode's solution containing (mmol/l): NaCl 120, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 5.0, glucose 10.0 and HEPES 20.0, pH 7.4 at 37°C. A brief perfusion with Ca^{2+}-free Tyrode's solution containing 0.1% bovine serum albumin (Fraction V, Sigma) followed, after which the hearts were treated with 0.06% collagenase (Worthington class 2) in the same solution. After 30-min collagenase treatment, the ventricles were minced and incubated for 20 min with stirring in albumin-free Tyrode's solution containing 0.2 mmol/l Ca^{2+} and 0.001% trypsin (Type 3, Sigma). The cells obtained by pipetting were filtered through 120 μm nylon mesh, centrifuged ($85–100 \times g$ for 3 min), and resuspended in the normal Tyrode's solution. These cells were plated on round glass coverslips precoated with an adhesive material, Cell-Tack (Becton Dickinson Labware), and stored at 10°C until used. All experiments were finished within 24 h.

2.2. Hypoxia / reoxygenation model

Myocytes were perfused initially with normal Tyrode's solution equilibrated with a gas mixture of 95% O_2–5% CO_2 and pH-adjusted to 7.4 at 37°C. To simulate hypoxia/reoxygenation, we switched to modified Tyrode's solution (pH 6.8 at 37°C) without glucose to prevent glycolysis and aerated the cells with a gas mixture of 95% N_2–5% CO_2 before treating them with normal Tyrode's solution. Cl^- or HCO_3^- free solution was prepared by equimolar substitution with gluconate$^-$ or HEPES, respectively. The perfusion rate was fixed at 1.0 ml/min with a peristaltic pump throughout the experiment, and it took approximately 30 s to change the perfusion media in the chamber.

2.3. Loading of BCECF and fluo 3

To measure pH_i and $[Ca^{2+}]_i$, cardiomyocytes were loaded with 4 μmol/l BCECF-AM (3′-O-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester) for 30 min, and 4.4 μmol/l fluo 3-AM (1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymeyhyl ester) for 30 min at room temperature, respectively. Fluo 3-AM, dissolved at 4.4 mmol/l in dimethyl sulfoxide (DMSO), was mixed with 4 μl of 20% (w/v) stock solution of the non-ionic detergent Pluronic F-127 in DMSO immediately before use (Borin and Siffert, 1990), and then diluted into the medium to achieve a final concentration of 4.4 μM. Final concentration of Pluronic F-127 was 0.02%. After loading, the cells were washed twice with normal Tyrode's solution, and cell-attached coverslips were placed inside a perfusion chamber (2-ml filling volume) with inflow and outflow tubes.

2.4. Measurement of pH_i and $[Ca^{2+}]_i$

The perfusion chamber was placed on the temperature controlled (37°C) stage positioned on light path of an inverted microscope (IMT-2 with a 50-W xenon lamp; Olympus, Tokyo, Japan), and then perfused with Tyrode's solution. The entire experiments to measure pH_i and $[Ca^{2+}]_i$ were performed at 37°C. BCECF-loaded myocytes were alternately excited with 440- and 490-nm wavelength lights, by changing their filters in a manual fashion. Excitation of fluo 3 was performed at wavelength of 490 nm. Intensities of emitted light (530 nm for BCECF and fluo 3) from a 25×25-μm area within a single fluorescent cell were detected by a photomultiplier (C 2741; Hamamatsu Photonics, Shizuoka, Japan). Autofluorescence from the cells during hypoxia and reoxygenation was negligible.

In situ calibration of the BCECF ratio signal was performed by the use of a high K^+ calibration medium as described by Borzak et al. (1990). This medium contained (mmol/l): KCl 120, NaCl 25, HEPES 10, EGTA 0.5, nigericin 10, and a K^+–H^+ ionophore, adjusted at 37°C with KOH to various pH values (8.0, 7.5, 7.0, 6.5).

In situ calibration of the fluo 3 fluorescence signal was performed by the use of the fluo 3 fluorescence–pCa calibration curve. The calibration medium contained (mmol/l): KCl 122.5, NaCl 5.4, $MgSO_4$ 1.1, glucose 10, HEPES 20, EGTA 2 and $CaCl_2$ to achieve the desired pCa (5, 6, 7, 8), and was pH-adjusted to 7.4 at 37°C with NaOH. This calibration was done at the end of each experiment and the fluorescence–pCa calibration curve was almost linear in the range of pCa 5 to 8.

2.5. Measurement of the contractility of electrically driven papillary muscle

The left ventricular papillary muscles isolated from male Sprague–Dawley rats (300–350 g) were suspended in the oxygenized normal Tyrode's solution at 37°C and loaded with 500 mg. The muscles were driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 10 ms and a voltage ranging 2–4 V, which was twice

the threshold. After 50 min equilibration, the contractility was measured by a force displacement transducer (UL-2, Shinko-Tsushin, Tokyo, Japan) through an amplifier (DS-601B, Shinko-Tsushin) and recorded on a pen-writing recorder (SS-250F, Seconic, Tokyo, Japan).

2.6. Materials

Fluo 3-AM, BCECF-AM, and HEPES were purchased from Dojindo Laboratories (Kumamoto, Japan). Albumin, nigericin, trypsin and PDB (Phorbol-12,13-dibutylate) were purchased from Sigma (St. Louis, MO, USA), while collagenase was from Worthington Biochemical (Malvern, PA, USA). Cell-Tack adhesive was from Becton Dickinson Labware (Bedford, MA, USA). Pluronic F-127 was from Molecular Probes (Eugene, OR, USA). Calphostine C and KT5720 were from Kyowa Medix (Tokyo, Japan), while chelerythrine chloride was from Calbiochem–Novabiochem (La Jolla, CA). All other chemicals were from Wako (Osaka, Japan) and were of the highest purity available.

2.7. Statistical analysis

Student's *t*-test was used for statistical analysis. The differences between mean values with P values less than 0.05 were considered significant.

3. Results

3.1. Hypoxia / reoxygenation-induced changes in the intracellular pH (pH_i)

Fig. 1A shows the typical changes in pH_i as measured by BCECF in single cardiac myocytes during hypoxia and reoxygenation. The averaged pH_i value of unstimulated cells was 7.21 ± 0.04 ($n = 40$). In normoxically perfused cells, the initial pH_i was maintained for at least 30 min, and the 490/440 nm excitation ratio of BCECF, an index for pH_i, was also unchanged within this period, indicating the stability of the present experimental system. Perfusion of the cells with hypoxic glucose-free medium (pH 6.8) decreased the pH_i to 6.82 ± 0.02 rapidly during the first 10 min, and then slowly during the prolonged hypoxic incubation up to 20 min. Reperfusion with oxygenated normal medium after 10-min hypoxia increased the pH_i rapidly during the first 3 min and then slowly to near the prehypoxic level within 10 min. To confirm the involvement of Na^+/H^+ exchange in the pH_i recovery as observed in previous reports (Tani and Neely, 1989), we used MIBA (5-(*N*-methyl-*N*-isobutyl) amiloride, an inhibitor of Na^+/H^+ and Na^+/Ca^{2+} exchange with a K_i of 14 and 84 μmol/l in cardiac myocytes, respectively (Murata et al., 1995) (Fig. 1B). When applied at a concentration of 30 μmol/l 2 min before reoxygenation, this agent significantly inhibited the pH_i recovery during reoxygenation, suggesting that Na^+/H^+ exchange is mainly involved in this response. Consequently, 10-min hypoxia followed by 10-min reoxygenation was chosen as the experimental protocol to simulate ischemia/reoxygenation.

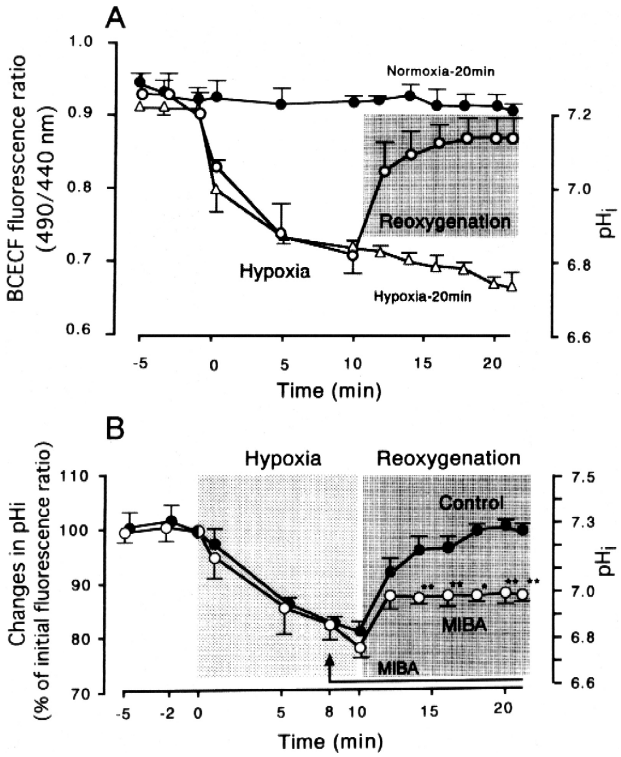

Fig. 1. Hypoxia/reoxygenation-induced changes in pH_i in rat single cardiomyocytes. (A) Changes in pH_i under various conditions. Cells were exposed to normoxic (pH 7.4) or hypoxic (pH 6.8) medium with the following protocols; 20 min normoxia (●), 20 min hypoxia (△), 10 min hypoxia followed by 10 min reoxygenation (○). (B) Effects of MIBA on hypoxia/reoxygenation-induced changes in pH_i. Cells were exposed to hypoxia/reoxygenation medium in the presence (○) or absence (●, control) of an inhibitor of Na^+/H^+ exchange, 30 μmol/l MIBA. MIBA was added 2 min before reoxygenation. The pH_i of a BCECF-loaded myocyte was measured by the fluorescence ratio with 490/440 nm excitation. In (B), the fluorescence ratio of BCECF was expressed as % of that just before hypoxia. The pH_i values on the right ordinate were calibrated as described in Materials and methods. Data are means ± S.E. of 4–8 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control at each time point.

3.2. Effects of Cl^- removal, Cl^- transport inhibitors and HCO_3^- removal on hypoxia / reoxygenation-induced changes in pH_i

The effect of chloride substitution on hypoxia/reoxygenation-induced changes in pH_i was studied in myocytes perfused with Cl^- free solution (Fig. 2A). In the control experiment (normal Cl^-), hypoxic stimulation gradually decreased the pH_i (ΔpH_i; -0.36 ± 0.04 in 10 min), and this response was followed by a recovery during the reperfusion with oxygenated medium. Equimolar sub-

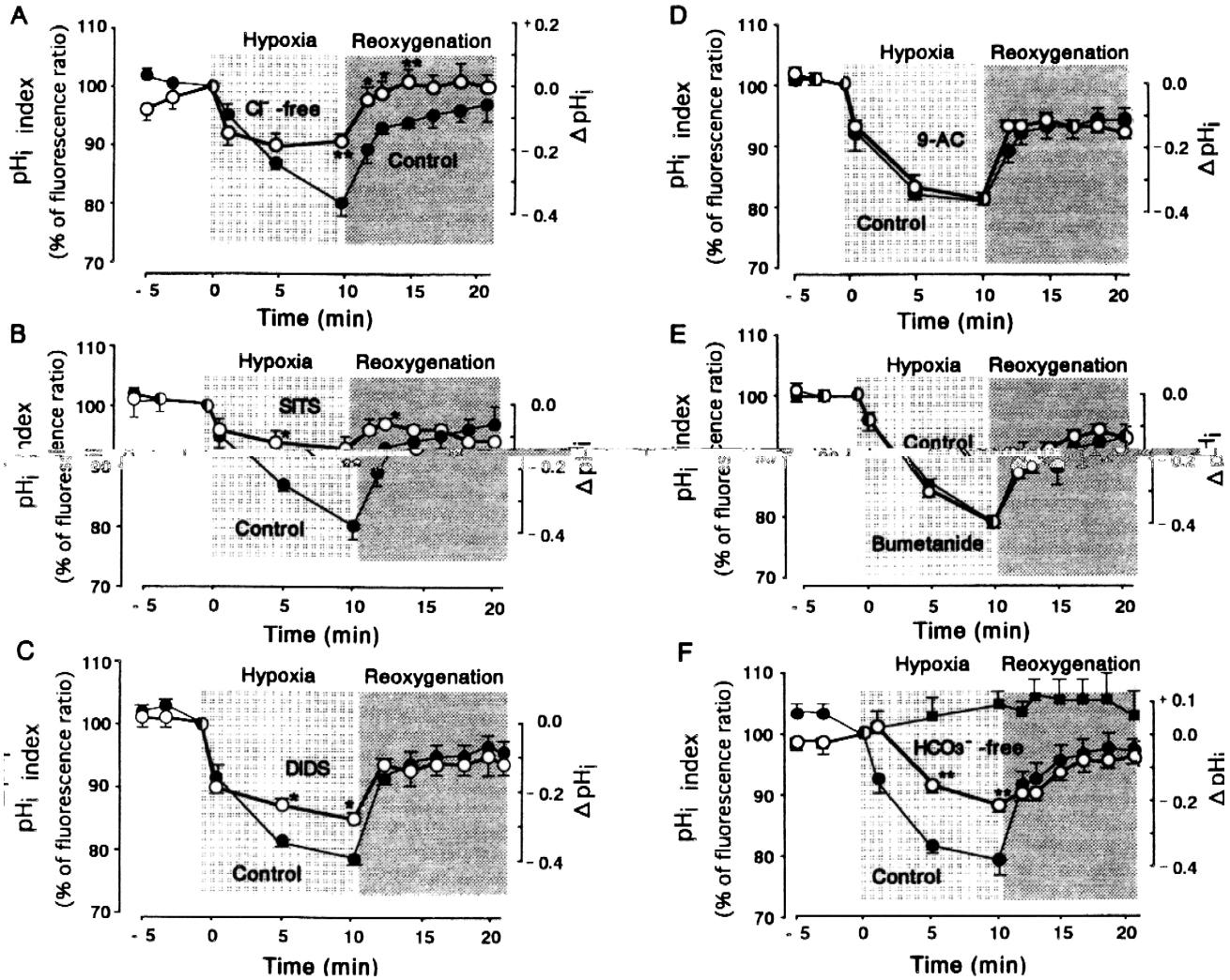

Fig. 2. Effects of anion removal or anion transport inhibitors on the changes in pH_i during hypoxia/reoxygenation. Cells were exposed to control (●) or experimental (○) hypoxia/reoxygenation medium. (A) Cl^- free medium (Cl^- was substituted with gluconate$^-$), (B) SITS (100 μmol/l), (C) DIDS (100 μmol/l), (D) 9-AC (1 mmol/l), (E) bumetanide (30 μmol/l), (F) HCO_3^- free medium (HCO_3^- was substituted with HEPES). Normoxic HCO_3^- free solution for 20 min (■). External HCO_3^- was removed 10 min before hypoxic stimulation. The pH_i index was expressed as % of the fluorescence ratio observed just before hypoxia. The net changes in pH_i (pH_i) from the prehypoxic level were determined by a pH_i–BCECF fluorescence ratio calibration curve. Data are means ± S.E. of 4–8 experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. control at each time point.

stitution of extracellular Cl^- with gluconate$^-$ slightly increased the prehypoxic pH_i to an almost steady level approximately in 5 min. When the myocytes were perfused with Cl^- free hypoxic medium, the decrease in the pH_i (ΔpH_i; -0.16 ± 0.02 in 10 min) was significantly smaller than that in the control, and the reoxygenation-induced recovery of acidic pH_i was much faster than that observed in the presence of Cl^- (Fig. 2A). These results suggest the involvement of Cl^- transport in the hypoxia-induced acidification.

To estimate the Cl^- transporters responsible for acidification in hypoxia, inhibitors of Cl^- transporters such as Cl^-/HCO_3^- exchanger, Cl^- channel or $Na^+/K^+/2Cl^-$ co-transporter, were applied. Pretreatment with Cl^-/HCO_3^- exchange inhibitor SITS (4-acetamido-4′isothiocyanato-stilbene-2,2′disulfonic acid) (100 μmol/l) for 5 min did not affect the prehypoxic level, but it significantly reduced the hypoxia-induced fall of pH_i (Fig. 2B). After reoxygenation, the pH_i was recovered to the neutral range faster than that in the control. A similar inhibitory effect was observed with another Cl^-/HCO_3^- exchange inhibitor, DIDS (4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid) (100 μmol/l) (Fig. 2C). In contrast, a Cl^- channel inhibitor, 9-AC, and a $Na^+/K^+/2Cl^-$ co-transport inhibitor, bumetanide, had no effect on these pH_i changes during hypoxia/reoxygenation at an effective dose of 1 mmol/l and 30 μmol/l (Fig. 2D,E).

Next, the effects of HCO_3^- removal on hypoxia/reoxygenation-induced changes in the pH_i were examined using HCO_3^- free solution (Fig. 2F). Removal of external HCO_3^- alone slightly increased the basal pH_i (ΔpH_i; approximately 0.08 in 10 min) and this level was main-

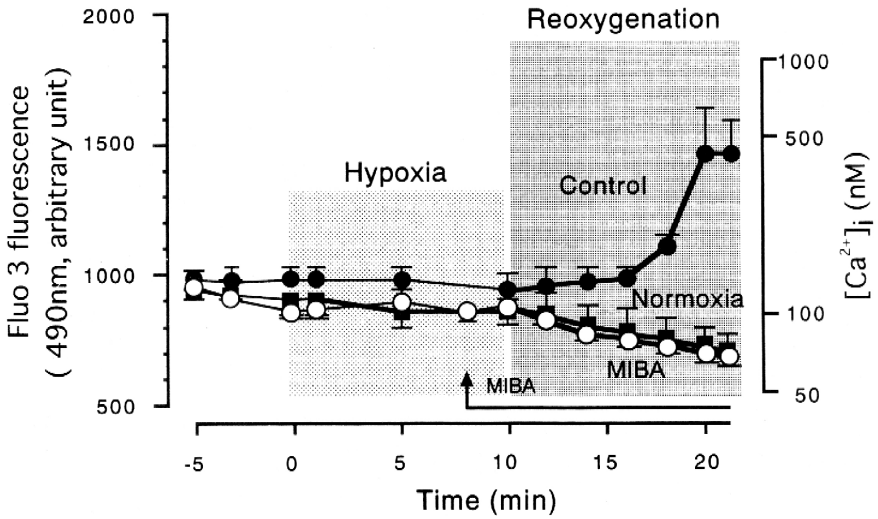

Fig. 3. Hypoxia/reoxygenation-induced changes in $[Ca^{2+}]_i$ in rat single cardiomyocytes. The emitted fluorescence of fluo 3 was measured at 530 nm. Cells were exposed to 30 min normoxia (■) or to 10 min hypoxia followed by 10 min reoxygenation in the presence (○) or absence (●) of 30 μmol/l MIBA. MIBA was added 2 min before reoxygenation. The changes in $[Ca^{2+}]_i$ were determined from a $[Ca^{2+}]_i$–fluo 3 fluorescence calibration curve. Data are means ± S.E. of 3–4 experiments.

tained at least for additional 10 min. A decrease in pH_i during hypoxia was significantly suppressed by the HCO_3^- removal, as observed in Cl^- free or SITS/DIDS-treated condition. These data suggest the involvement of Cl^-/HCO_3^- exchange in the hypoxia-induced acidification.

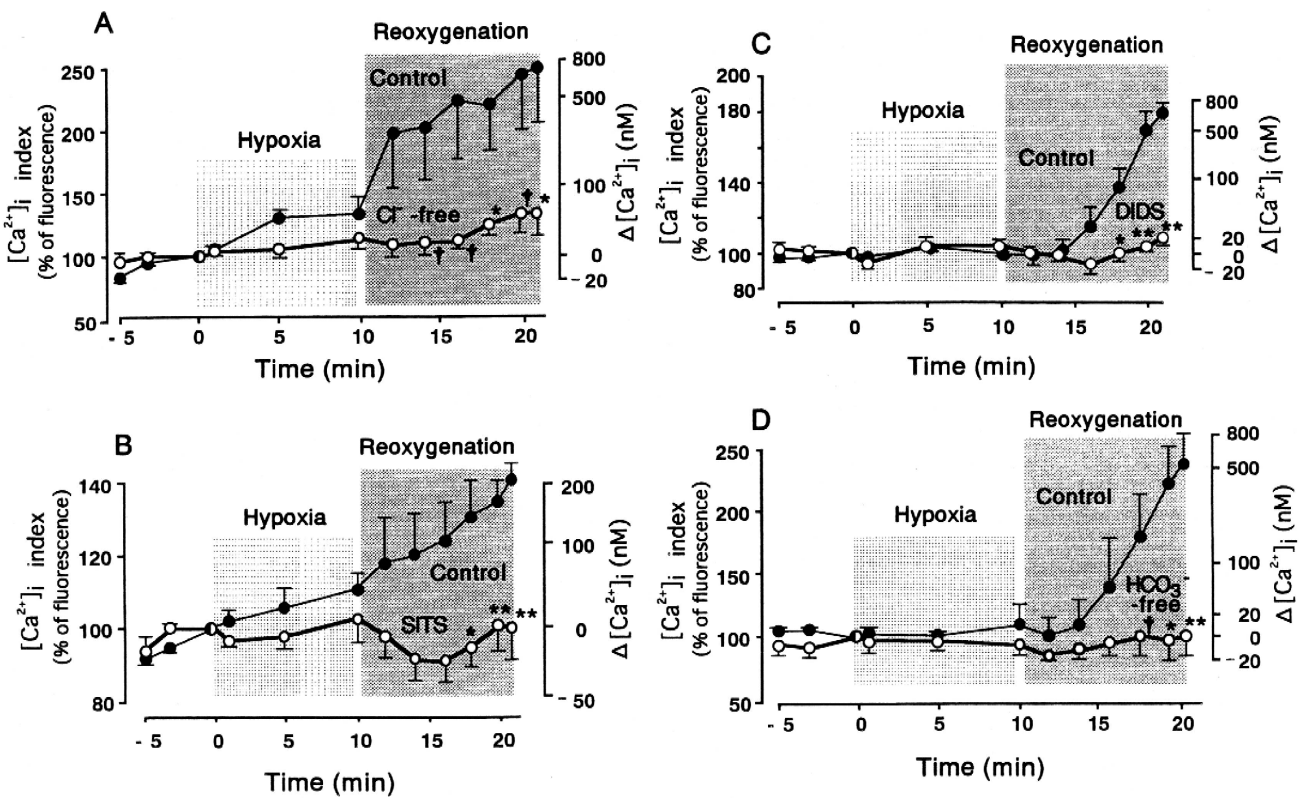

Fig. 4. Effects of anion removal, SITS and DIDS on the changes in $[Ca^{2+}]_i$ during hypoxia/reoxygenation. Cells were exposed to control (●) or experimental (○) hypoxia/reoxygenation medium. (A) Cl^- free medium (Cl^- was substituted with gluconate$^-$), (B) SITS (100 μmol/l), (C) DIDS (100 μmol/l), (D) HCO_3^- free medium (HCO_3^- was substituted with HEPES). External HCO_3^- was removed 10 min before hypoxic stimulation. The $[Ca^{2+}]_i$ index was expressed as % of the fluorescence intensity observed just before hypoxia. The net changes in $[Ca^{2+}]_i$ ($\triangle [Ca^{2+}]_i$) from the prehypoxic level were determined from a $[Ca^{2+}]_i$–fluo 3 fluorescence calibration curve. Data are means ± S.E. of 4–8 experiments. $^{\dagger}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control at each time point.

3.3. Hypoxia / reoxygenation-induced changes in the intracellular Ca^{2+} level

Fig. 3 shows the typical changes in $[Ca^{2+}]_i$ in myocytes during hypoxia/reoxygenation. The intensity of the fluo 3 fluorescence in an unstimulated cell incubated in normal Tyrode's solution was almost stable for at least 30 min. The basal $[Ca^{2+}]_i$ was estimated to be 132 ± 10 nmol/l ($n = 40$) by using the fluo 3 fluorescence–pCa calibration curve, which was almost linear in the range of pCa 5 to 8.

Exposure of myocytes to the hypoxic medium did not induce any significant changes in $[Ca^{2+}]_i$ during 10 min. After reoxygenation, the $[Ca^{2+}]_i$ level was significantly increased to the level around 500 nmol/l in 10 min. Application of 30 μM MIBA at 2 min before reoxygenation completely inhibited this increase in $[Ca^{2+}]_i$, and the same effect was observed with 100 μmol/l MIBA (data not shown). Since the concentration (30 μM) of MIBA is enough to inhibit Na^+/H^+ exchange ($K_i = 14$ μM), but considerably lower than the K_i (84 μM) of Na^+/Ca^{2+} exchange for this drug, the treatment with 30 μM MIBA is thought to inhibit mainly Na^+/H^+ exchange. Next, to examine whether internal Ca^{2+} release was responsible for the increase in $[Ca^{2+}]_i$ during reoxygenation, we carried out the experiment in the presence of ryanodine at 1 μM, known to deplete Ca^{2+} from sarcoplasmic reticulum (SR) (Wang et al., 1997). Compared with untreated groups, pretreatment with ryanodine unaffected the $[Ca^{2+}]_i$ response at 10 min reoxygenation (% of prehypoxic fluorescence intensity of fluo 3; 235 ± 23 and 230 ± 32 with and without ryanodine, respectively, $n = 4$). These results suggest that the reoxygenation-induced increase in $[Ca^{2+}]_i$ involves Na^+/H^+ exchange probably followed by stimulation of Na^+/Ca^{2+} exchange.

3.4. Effects of Cl^- removal, anion exchange inhibitors and HCO_3^- removal on the changes in $[Ca^{2+}]_i$ during hypoxia / reoxygenation

Since Cl^- removal, SITS and DIDS treatments and HCO_3^- removal attenuated the pH_i fall and eventually hastened the subsequent recovery of pH_i (Fig. 2), the effects of these treatments on the hypoxia/reoxygenation-induced changes in $[Ca^{2+}]_i$ were examined. A Cl^- free medium, SITS (100 μmol/l), DIDS (100 μmol/l) or a HCO_3^- free medium almost completely inhibited the reoxygenation-induced increase in $[Ca^{2+}]_i$ (Fig. 4A,B,C,D).

3.5. Effects of protein kinase inhibitors on the hypoxia-induced pH_i decrease

Acute hypoxia stimulates protein kinase C (Goldberg et al., 1997) and adenylyl cyclase activities (Thandroyen et al., 1990). Further, anion exchange activity is reportedly regulated by protein kinase C (Alvaro et al., 1997) and/or protein kinase A (Camilión de Hurtado et al., 1998). Therefore, the effects of inhibitors of these kinases on the hypoxia-induced pH_i decrease were examined. Two structurally different protein kinase C inhibitors, calphostin C (1 μmol/l) and chelerythrine (1 μmol/l) attenuated the pH_i fall at 10 min after hypoxic challenge (Fig. 5A,B). In contrast, a protein kinase A inhibitor, KT5720 (1 μmol/l) unaffected pH_i change during hypoxia (Fig. 5C). These results suggest a role for protein kinase C activation in the acidifying process.

Next, we examined the effects of protein kinase C activation on pH_i to test the possibility that protein kinase

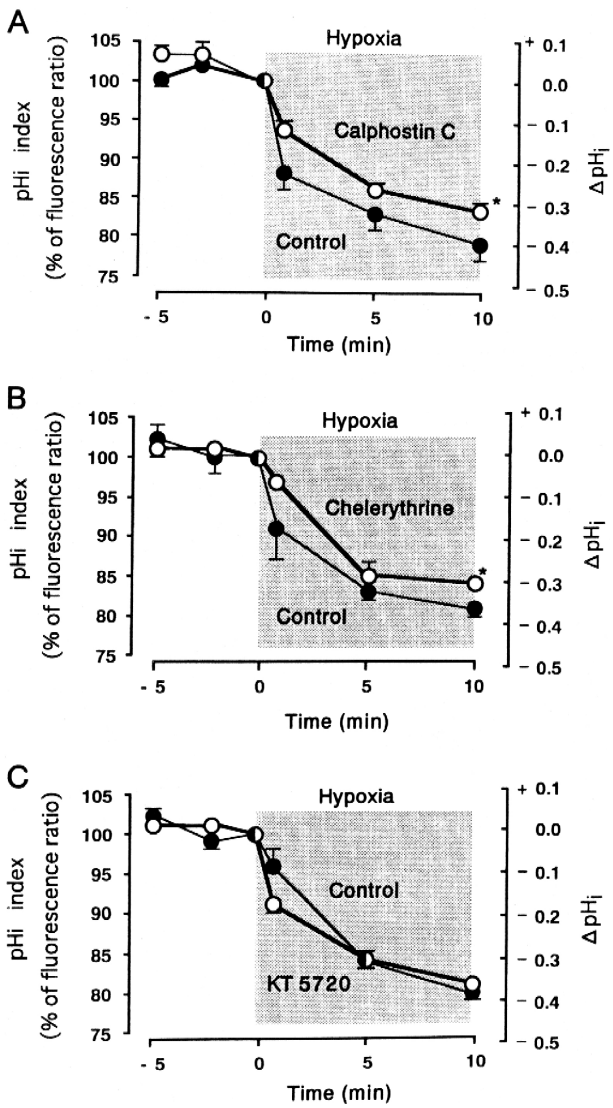

Fig. 5. Effects of protein kinase C inhibitors and a protein kinase A inhibitor on the changes in pH_i during hypoxia. Cells were exposed to hypoxia medium in the presence (○) or absence (●) of 1 μmol/l calphostin C (A), 1 μmol/l chelerythrine (B) or 1 μmol/l KT5720 (C). The pH_i index was expressed as % of the fluorescence ratio observed just before hypoxia. The net changes in pH_i (Δ pH_i) from the prehypoxic level were determined from a pH_i–BCECF fluorescence ratio calibration curve. Data are means ± S.E. of 3–7 experiments. $^*P < 0.05$ vs. control at each time point.

C stimulates Cl^-/HCO_3^- exchange. Under a non-hypoxic condition in the presence of MIBA (30 μmol/l), a protein kinase C activator, phorbol 12,13-dibutylate (PDB, 0.1 μmol/l) decreased the pH_i, and this response was inhibited by SITS (100 μmol/l) (ΔpH_i at 15 min from PDB addition; -0.076 ± 0.022, (control: MIBA alone), $^{*}-0.18 \pm 0.04$ (MIBA + PDB) and $^{\#}-0.044 \pm 0.024$ (MIBA + PDB + SITS), $^{*}P < 0.05$ vs. MIBA alone, $^{\#}P < 0.05$ vs. MIBA + PDB, $n = 4$). 4-Alpha-phorbol, an analog functionally inactive in protein kinase C activation, failed to induce such an acidification (data not shown), excluding the possibility of non-specific action of phorbol ester.

3.6. Effects of SITS and DIDS on hypoxia / reoxygenation-induced changes in contractile responses in rat left ventricular papillary muscle

The last experiments were done to know the effects of anion exchange inhibitors on the contractile responses of rat papillary muscle to hypoxia/reoxygenation. During hypoxia, the contractile force of electrically driven papillary muscle progressively decreased, being almost 10% and 0% of the prehypoxic level by 10 and 20 min after hypoxia, respectively. Reperfusion with oxygenated normal medium for 30 min recovered the depressed contractile force only to 25–40% of the basal value (Fig. 6A,B). SITS (100 μmol/l) and DIDS (100 μmol/l) improved the recovery of contractile force after reoxygenation without any effects on the reduction in contractile force during hypoxia (Fig. 6A,B).

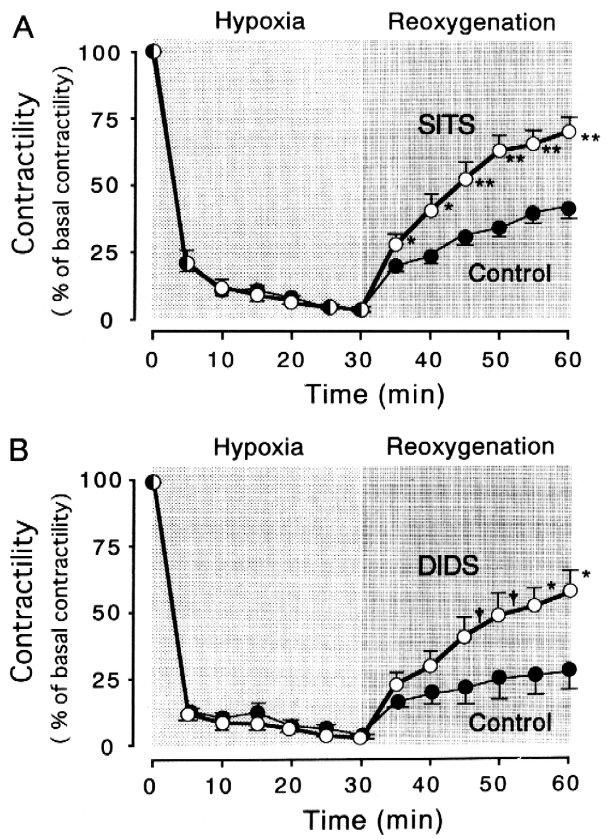

Fig. 6. Effects of SITS and DIDS on the changes in contractility of electrically driven rat papillary muscle during hypoxia/reoxygenation. Rat left ventricular papillary muscles were exposed to hypoxia/reoxygenation medium in the presence (○) or absence (●) of 100 μmol/l SITS (A) or 100 μmol/l DIDS (B). The changes in contractility were expressed as % of that immediately before hypoxia. Data are means ± S.E. of 6–9 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control at each time point.

4. Discussion

The fall in pH_i during hypoxia/ischemia in mammalian cardiac myocytes has been reported to be partly due to mitochondrial ATP hydrolysis, CO_2 retention, and increases in glycogen and triglyceride turnover (Dennis et al., 1991), but the entire profile has not been elucidated. This is the first report to show the relation between anion exchange and reperfusion injury characterized by Ca^{2+} overload at the single cell level.

The acidification of myocytes under simulated hypoxic conditions was weakened by Cl^- substitution with gluconate$^-$ (Fig. 2A), suggesting the participation of Cl^- influx in this response. Among the Cl^- transport inhibitors tested (SITS, DIDS, 9-AC and bumetanide), only SITS and DIDS inhibited the hypoxia-induced acidification (Fig. 2B,C). These results suggest that during hypoxia, the Cl^-/HCO_3^- exchanger in normal mode functions as a pH_i regulator by counter-transporting HCO_3^- out of the cells associated with Cl^- influx. However, in Cl^- free and hypoxic condition, there may be another interpretation for the attenuation of acidification, i.e., augmentation of Cl^-/HCO_3^- exchange in reverse mode (Vaughan-Jones, 1986) through an increase in Cl^- efflux. Further, the weak effect of DIDS compared to SITS may be explained by the reduction of pH_i via inhibition of Na^+/HCO_3^- co-transport, since DIDS has been reported to act on this co-transport (Dart and Vaughan-Jones, 1992) as well as anion exchange. Stilbene derivatives are also known to inhibit Cl^- channels (Tanaka et al., 1996). However, since HCO_3^- removal, but not a more potent Cl^- channel inhibitor, 9-AC, mimicked the effects of SITS/DIDS (Fig. 2), these drugs seemed to act predominantly on Cl^-/HCO_3^- exchanger in our experiments. Supporting this idea, a recent study using the Cl^--selective microelectrode reported a Cl^-/HCO_3^- exchange-mediated elevation of $[Cl^-]_i$ in guinea pig papillary muscle during ischemia (Lai and Nishi, 1998). Other Cl^- dependent mechanism such as Cl^-/OH^- exchange (Sun et al., 1996) may be involved in hypoxia-induced acidification since the inhibition of Cl^-/HCO_3^- exchange did not completely inhibit the acidification.

Upon reoxygenation, Ca^{2+} overload occasionally associated with hypercontracture is mainly induced by an excessive influx of extracellular Ca^{2+} through Na^+/Ca^{2+} exchange, which is stimulated to extrude Na^+ accumulated

within cells (Tani and Neely, 1989). Indeed, in our simulated reoxygenation system, we also observed a marked increase in $[Ca^{2+}]_i$ that was almost completely inhibited by MIBA at the concentration (30 μM), which inhibits mainly Na^+/H^+ exchange, i.e. Na^+ accumulation. This reoxygenation-induced increase in $[Ca^{2+}]_i$ was inhibited by Cl^- removal (Fig. 4A), Cl^-/HCO_3^- exchange inhibitors (Fig. 4B,C) and HCO_3^- removal (Fig. 4D). A possible mechanism for this inhibition is that the Cl^-/HCO_3^- exchange inhibition suppresses the acidification in hypoxia, which then diminishes the Na^+/H^+ exchange activation and subsequent Na^+/Ca^{2+} exchange stimulation during reoxygenation, leading to the inhibition of $[Ca^{2+}]_i$ influx. Stilbene derivatives such as DIDS have also been reported to affect Ca^{2+} release from sarcoplasmic reticulum (Sitsapesan, 1999) and mitochondria (Bernardes et al., 1994). In our experiment, however, these effects on Ca^{2+} homeostasis appeared to be negligible because both SITS and DIDS did not increase the prehypoxic $[Ca^{2+}]_i$. Furthermore, inhibitory effects by these inhibitors were stronger against $[Ca^{2+}]_i$ response than that against pH_i. It may be related to some of their other actions on Ca^{2+} overloading mechanism during reoxygenation.

Recently, the cardiac specific anion exchanger 3 isoform has been shown to contain consensus phosphorylation sites for protein kinase C and protein kinase A (Yannoukakos et al., 1994) and further, protein kinase C–δ activation has been observed in hypoxic myocytes (Goldberg et al., 1997). Therefore, we tested the effects of protein kinase C inhibitors on the drop in pH_i during hypoxia. Calphostin C and chelerythrine were used as specific inhibitors for protein kinase C with respective K_i values of 0.05 and 0.66 μmol/l (Herbert et al., 1990; Kobayashi et al., 1989), and KT5720 was used as a specific inhibitor for protein kinase A with a K_i value of 0.06 μmol/l (Kase et al., 1987). Protein kinase C inhibitors, but not the protein kinase A inhibitor, slightly weakened the acidification, suggesting the participation of protein kinase C in the anion exchange-mediated pH_i modulation during hypoxia. In addition, even under the normoxic- and Na^+/H^+ exchange-suppressed condition, the protein kinase C activator, PDB induced a SITS-sensitive acidification. Thus, hypoxia-induced decrease in pH_i may be mediated by the stimulation of anion exchange partly through protein kinase C activation. Such a protein kinase C action has also been found in Angiotensin II-stimulated cardiac myocytes (Camilión de Hurtado et al., 1998) and Vero cells (Ludt et al., 1991). Further, hypoxia and ischemia have been reported to translocate, i.e. activate protein kinase C in cardiac myocytes (Goldberg et al., 1997; Takeishi et al., 1999). In addition, we considered the involvement of ATP released during hypoxia on the stimulation of anion exchange, since vanadate-sensitive hydrolysis of ATP is reported to stimulate this exchange in the presence of magnesium (Scamps and Vassort, 1990). We examined the effect of vanadate on SITS/DIDS inhibitable pH_i decrease during hypoxia, and found that the degree of acidification at 10-min hypoxia did not differ between vanadate (1 mM) containing- and uncontaining (control) groups (% of prehypoxic fluorescence ratio of BCECF; $77 \pm 1\%$ and $80 \pm 2\%$ with and without vanadate, respectively, $n = 4$). Thus, involvement of extracellular ATP-induced stimulation of Cl^-/HCO_3^- exchange was unlikely under the present experimental condition.

As shown in Fig. 6, reoxygenation induced a contractile dysfunction of papillary muscle. Such a contractile response during reoxygenation is often observed with Ca^{2+} overload. Further, recent reports by Yang and Steele (2000) have demonstrated that reduction of cytosolic ATP increases Ca^{2+} release from SR. From this evidence, ATP level is also thought to take part in the occurrence of such a contractile dysfunction by affecting SR function. As observed in $[Ca^{2+}]_i$ response, protective effects of SITS and DIDS were also observed in reoxygenation-induced decreases in contractility of rat papillary muscle (Fig. 6A,B). In the present study, the effects of SITS/DIDS on SR function in papillary muscle is undefined. However, since Ca^{2+} overload associated with pH_i recovery through Na^+/H^+ exchange is well known to be involved in such a depressed recovery, inhibition of hypoxia-induced acidification by SITS/DIDS could diminish the Na^+/H^+ exchange activity in subsequent reoxygenation, thereby leading to the inhibition of Ca^{2+} overload. This may result in the improved recovery of contractility during reoxygenation period. Thus, Cl^-/HCO_3^- exchange activity during hypoxia appears to play an important role in the development of Ca^{2+} overload and resulting contractile dysfunction after reoxygenation.

In summary, the present study has shown that the hypoxia-induced decrease in pH_i is at least partly mediated by anion exchange stimulation partly through protein kinase C activation, and this exchange therefore takes part in reoxygenation-induced contractile dysfunction as well as Ca^{2+} overload. The modulation of anion transport during hypoxia may be a new strategy to prevent cardiac reoxygenation- or reperfusion-injury.

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References

Alvaro, D., Mennone, A., Boyer, J.L., 1997. Role of kinases and phosphatases in the regulation of fluid secretion and Cl^-/HCO_3^- exchange in cholangiocytes. Am. J. Physiol. 273, G303–G313.

Bernardes, C.F., Meyer-Fernandes, J.R., Basseres, D.S., Castilho, R.F., Vercesi, A.E., 1994. Ca^{2+}-dependent permeabilization of the inner mitochondrial membrane by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Biochem. Biophys. Acta 1188, 93–100.

Borin, M., Siffert, W., 1990. Stimulation by thrombin increases the cytosolic free Na^+ concentration in human platelets. J. Biol. Chem. 265, 19543–19550.

Borzak, S., Kelly, R.A., Krämer, B.K., Matoba, Y., Marsh, J.D., Reers, M., 1990. In situ calibration of fura-2 and BCECF fluorescence in adult rat ventricular myocytes. Am. J. Physiol. 259, H973–H981.

Camilión de Hurtado, M.C., Alvarez, B.V., Pérez, N.G., Ennis, I.L., Cingolani, H.E., 1998. Angiotensin II activates Na^+-independent Cl^-/HCO_3^- exchange in ventricullar myocardinm. Circ. Res. 82, 473–481.

Dart, C., Vaughan-Jones, R.D., 1992. Na^+–HCO_3^- symport in the sheep cardiac Purkinje fibre. J. Physiol. (Lond.) 451, 365–385.

Dennis, S.C., Gevers, W., Opie, L.H., 1991. Protons in ischemia: where do they come from; where do they go to? J. Mol. Cell Cardiol. 23, 1077–1086.

Fliegel, L., Fröhlich, O., 1993. The Na^+/H^+ exchanger: an update on structure, regulation and cardiac physiology. Biochem. J. 296, 273–285.

Goldberg, M., Zhang, H.L., Steinberg, S.F., 1997. Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes. J. Clin. Invest. 99, 55–61.

Herbert, J.M., Augereau, J.M., Gleye, J., Maffrand, J.P., 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 172, 993–999.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., Kaneko, M., 1987. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem. Biophys. Res. Commun. 142, 436–440.

Kawasaki, H., Otani, H., Mishima, K., Inagaki, C., 1998. Involvement of anion exchange in hypoxia/reoxygenation-induced changes in intracellular pH and Ca^{2+} concentration. Arch. Pharm. 358 (Suppl. 2), R636.

Kobayashi, E., Nakano, H., Morimoto, M., Tamaoki, T., 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 159, 548–553.

Lai, Z.F., Nishi, K., 1998. Intracellular chloride activity increases in guinea pig ventricular muscle during simulated ischemia. Am. J. Physiol. 275, H1613–H1619.

Lai, Z.F., Liu, J., Nishi, K., 1996. Effects of stilbene derivatives SITS and DIDS on development of intracellular acidosis during ischemia in isolated guinea pig ventricular papillary muscle in vitro. Jpn. J. Pharmacol. 72, 161–174.

Ludt, J., Tfnnessen, T.I., Sandvig, K., Olsnes, S., 1991. Evidence for involvement of protein kinase C in regulation of intracellular pH by Cl^-/HCO_3^- antiport. J. Membr. Biol. 119, 179–186.

Murata, Y., Harada, K., Nakajima, F., Maruo, J., Morita, T., 1995. Non-selective effects of amiloride and its analogues on ion transport system and their cytotoxicities in cardiac myocytes. Jpn. J. Pharmacol. 68, 279–285.

Ridley, P.D., Curtis, M.J., 1992. Anion manipulation: a new antiarrhythmic approach. Action of substitution of chloride with nitrate on ischemia-and reperfusion-induced ventricular fibrillation and contractile function. Circ. Res. 70, 617–632.

Scamps, F., Vassort, G., 1990. Mechanism of extracellular ATP-induced depolarization in rat isolated ventricular cardiomyocytes. Pflügers Arch. 417, 309–316.

Sitsapesan, R., 1999. Similarities in the effects of DIDS, DBDS and suramin on cardiac ryanodine receptor function. J. Membr. Biol. 168, 159–168.

Sun, B., Leem, C.H., Vaughan-Jones, R.D., 1996. Novel chloride-dependent acid loader in the guinea-pig ventricular myocyte: part of a dual acid-loading mechanism. J. Physiol. (Lond.) 495, 65–82.

Takeishi, Y., Jalili, T., Ball, N.A., Walsh, R.A., 1999. Responses of cardiac protein kinase C isoforms to distinct pathological stimuli are differentially regulated. Circ. Res. 85, 264–271.

Tanaka, H., Matsui, S., Kawanishi, T., Shigenobu, K., 1996. Use of chloride blockers: a novel approach for cardioprotection against ischemia-reperfusion damage. J. Pharmacol. Exp. Ther. 278, 854–861.

Tani, M., Neely, J.R., 1989. Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of Na^+–H^+ and Na^+–Ca^{2+} exchange. Circ. Res. 65, 1045–1056.

Thandroyen, F.T., Muntz, K.H., Buja, L.M., Willerson, J.T., 1990. Alterations in b-adrenergic receptors, adenylate cyclase, and cyclic AMP concentrations during acute myocardial ischemia and reperfusion. Circulation 82 (Suppl. II), II-30–II-37.

Vaughan-Jones, R.D., 1986. An investigation of chloride–bicarbonate exchange in the sheep cardiac Purkinje fibre. J. Physiol. (Lond.) 379, 377–406.

Yang, Z., Steele, D.S., 2000. Effects of cytosolic ATP on spontaneous and triggered Ca^{2+}-induced Ca^{2+} release in permeabilised rat ventricular myocytes. J. Physiol. (Lond.) 523, 29–44.

Yannoukakos, D., Stuart-Tilley, A., Fernandez, H.A., Fey, P., Duyk, G., Alper, S.L., 1994. Molecular cloning, expression, and chromosomal localization of two isoforms of the AE3 anion exchanger from human heart. Circ. Res. 75, 603–614.

Wang, H.X., Lau, S.Y., Huang, S.J., Kwan, C.Y., Wong, T.M., 1997. Cobra venom cardiotoxin induces perturbations of cytosolic calcium homeostasis and hypercontracture in adult rat ventricular myocytes. J. Mol. Cell. Cardiol. 29, 2759–2770.